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Muscarinic Receptors

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Preface

Muscarinic acetylcholine receptors have played a key role in the advancement of knowledge of pharmacology and neurotransmission since the inception of studies in these fields. Indeed, the classical work of Loewi, which led to the identification of acetylcholine as the *Vagusstoff* released by nerve stimulation, thus showing that synaptic transmission was chemical and not electrical, was based on the actions of neurotransmitter at muscarinic receptors. The physiological actions mediated by muscarinic receptors were known and exploited for both therapeutic and nontherapeutic purposes for hundreds of years before the existence of the receptors themselves was recognized. It is remarkable that the study of muscarinic receptors continues to provide new and surprising insights not just to the cholinergic system, but to the broad areas of neurobiology, cell biology, pharmacology, and therapeutics.

Like other members of the G-protein-coupled receptor superfamily, the application of molecular biological approaches to the study of the muscarinic receptors provided dramatically increased knowledge of both their biological complexity and therapeutic potential. The identification in the late 1980s of multiple genes encoding distinct muscarinic receptor subtypes provided the opportunity to develop drugs that would target discrete subsets of muscarinic receptors with decreased global side effects. The more recent demonstration that drugs can act both positively and negatively on the receptors at sites distinct from the acetylcholine binding region has provided even further promise for increasing the therapeutic specificity of muscarinic drugs.

We hope that this volume will provide a broad yet detailed review of current knowledge of muscarinic receptors that will be valuable both to long-time muscarinic investigators and to those new to the field. It describes the detailed insights that have been obtained on the structure, function, and cell biology of muscarinic receptors. This volume also describes physiological analyses of muscarinic receptors and their roles in regulating the function of the brain and of a variety of peripheral tissues. Finally, it demonstrates how the increased knowledge of the

basic biology, pharmacology, and physiology of the muscarinic receptors can be translated into improved therapeutic applications.

We also hope that this book highlights both the excitement of the study of muscarinic receptors and the amazing range of advances that have occurred in recent years. We are sure that the future will continue to yield information on facets of the muscarinic receptors that we have not yet imagined.

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Part I
Muscarinic Receptor Pharmacology and Signaling

Overview of Muscarinic Receptor Subtypes

Richard M. Eglen

Abstract The physiological role of muscarinic receptors is highly complex and, although not completely understood, has become clearer over the last decade. Recent pharmacological evidence with novel compounds, together with data from transgenic mice, suggests that all five subtypes have defined functions in the nervous system as well as mediating the non neuronal, hormonal actions of acetylcholine. Numerous novel agonists, allosteric regulators, and antagonists have now been identified with authentic subtype specificity in vitro and in vivo. These compounds provide additional pharmacological opportunities for selective subtype modulation as well as a new generation of muscarinic receptor-based therapeutics.

Keywords M_1 receptors • M_2 receptors • M_3 receptors • M_4 receptors • M_5 receptors

1 Introduction

Muscarinic receptors, a member of class I, seven transmembrane, G-protein-coupled receptors (GPCRs), comprise five distinct subtypes, denoted as muscarinic M_1, M_2, M_3, M_4, and M_5 receptors (Hammer et al. 1980; Bonner et al. 1987; Caulfield 1993; Caulfield and Birdsall 1998). Acetylcholine exerts physiological control by both hormonal and neuronal mechanisms, via activation of all five muscarinic receptor subtypes. Amongst a wide range of effects, the auto/paracrine actions of acetylcholine include regulation of cell proliferation and cancer, skin cell signaling, and immune responsiveness (Sastry and Sadavongvivad 1978; Eglen 2006; Grando et al. 2007; Wessler and Kirkpatrick 2008; Nirish et al. 2009; Shah et al. 2009). Each muscarinic receptor subtype also has a unique

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distribution throughout the central and peripheral nervous systems, being expressed both pre- and postjunctionally (see Felder et al. 2000 for review). Neuronal muscarinic receptors are involved in several vegetative, sensory, cognitive, and motor functions. Prominent actions of muscarinic receptors in the peripheral nervous system include slowing of the heart rate and stimulation of glandular secretion and smooth muscle contraction (see Eglen et al. 1996; Eglen 2005; Wess et al. 2007 for reviews).

At most stages of development five muscarinic receptors mediate the actions of acetylcholine in almost all tissues, and via hormonal and neuronal effector systems. Given this key physiological role, it is unsurprising that extensive efforts have been made to develop therapeutics that selectively agonize, modulate or antagonize each receptor subtype. Initially, several naturally occurring compounds were found to mimic the actions of the endogenous agonist, acetylcholine, including the agonists, muscarine (a toxin from the mushroom *Aminita muscaria* and from which the receptor family derives its name), pilocarpine (from the *rutaceae* plant family), or antagonists such as atropine or (−)-hyoscine (from the *solanaceae* plant family). These were also used with limited clinical benefits (see Felder et al. 2000; Langmead et al. 2008a for reviews). Over the succeeding decades, many derivatives, while selective for muscarinic receptors over other GPCRs, lacked *intra* subtype selectivity, and exhibited several side effects restricting their therapeutic use. Recently, however, selective compounds, including those that allosterically modulate muscarinic receptors, have been reported that display authentic intra subtype selectivity, and consequently have opened new avenues for therapeutic interventions (Conn et al. 2009a, b).

Early pharmacological studies suggested at least three subtypes (Caulfield and Birdsall 1998) but it was not until the early 1990s, when all five subtypes were cloned, that the diversity in the muscarinic receptor family was fully appreciated (Bonner et al. 1987). Recombinant receptor expression of these subtypes lead subsequently to the unambiguous delineation of muscarinic receptor pharmacology (Dorje et al. 1990; Wang and el-Fakahany 1993). These properties were in good agreement with the pharmacology of endogenously expressed receptors and provided robust tools to characterize agonist and antagonist pharmacology. Over the succeeding decades, concerted medicinal chemistry efforts identified compounds with some degree of selectivity for muscarinic receptor subtypes, resulting in several compounds evaluated clinically. These compounds were augmented by the identification and purification of naturally occurring toxins with exquisite subtype specificity (Karlsson et al. 2000; Servent and Fruchart-Gaillard 2009), collectively providing important pharmacological agents to study the receptor family, in vitro and in vivo. The knowledge generated with these and other compounds was confirmed and extended by phenotypic studies in transgenic mice (generated by homologous recombination methods) lacking muscarinic receptors (see Wess et al. 2003, 2007; Wess 2004 for reviews).

In the last 10 years, a convergence of these new pharmacological tools as well as new insights into GPCR function opened new opportunities for therapeutic intervention. This introductory chapter assesses the current resurgence of interest in

muscarinic receptor physiology and pharmacology, and the development of novel therapeutics. Given the extensive literature on this receptor family, most literature cited is from the last 2 years, with the preceding years being covered in several reviews, each of which has detailed bibliographies (i.e.,Wessler et al. 1998; Felder et al. 2000; Eglen 2005; Birdsall and Lazareno 2005; Langmead et al. 2008b; Wessler and Kirkpatrick 2008; Conn et al. 2009b).

2 Molecular Biology and Biochemistry of Muscarinic Receptors

Muscarinic GPCRs are seven transmembrane, glycoproteins encoded by five distinct genes. All subtypes have been cloned from several species, including human, and exhibit a high degree of species homology. Most muscarinic ligands bind to a highly conserved pocket deep within the transmembrane regions, causing activation via transmembrane domains TM3, TM5, TM6, and TM7 (see Wess 1996; Hulme et al. 2003 for reviews). Acetylcholine binds to amino acid residues on the outer regions of the binding pocket with a critical asparagine (Asp105) residue involved in the binding of the positively charged headgroup. Although Asp105 is conserved in most Class I GPCRs, five additional, key, residues are unique to the muscarinic receptor family, Thr231, Thr234, Tyr148, Tyr506, Tyr529, and Tyr533. This similarity in ligand binding sites across all five subtypes is the principal reason for the difficulties in identifying subtype-selective ligands (Jöhren and Höltje 2002).

However, in addition to the acetylcholine binding site (i.e., the orthosteric site), muscarinic receptor subtypes possess numerous allosteric sites at which compounds can act to modulate agonist function (Mohr et al. 2003; Voigtlander et al. 2003; Wess 2005; Presland 2005; Conn et al. 2009b). Some ligands can occupy both sites, in which case they are designated as diasteric ligands (Mohr et al. 2003, 2010), while others may target one site preferentially. Muscarinic receptors have now proven to be a prototypic class of GPCRs at which the physiology of allosterism has been extensively explored, both in terms of basic research, but also as a means to novel drug candidates (Birdsall and Lazareno 2005; Conn et al. 2009a). The nature of muscarinic allosteric sites differs from the orthosteric binding site and importantly varies markedly between the five subtypes (Conn et al. 2009b). Consequently, allosteric modulators of muscarinic receptors can be highly subtype-selective, in marked contrast to most orthosteric agonists identified to date. This concept, therefore, provides an exciting opportunity for subtype-specific modulation (Voigtlander et al. 2003).

The pharmacology of several "established" compounds is also being reclassified (Tran et al. 2009). For example, an early putative M_1 agonist, McN A 343 (4-hydroxy-2-butynyl trimethylammonium chloride) and a more recent M_2 antagonist, THRX-160209 (4-{*N*-[7-(3-(*S*)-(1-carbamoyl-1,1-diphenylmethyl)pyrrolidin-1-yl)hept-1-yl]-*N*-(*n*-propyl)amino}-1-(2,6-dimethoxybenzyl)piperidine)(Roszkowski 1961; Steinfeld et al. 2007), interact at both the orthosteric and allosteric sites on the muscarinic M_1 and M_2 receptors, respectively, i.e., they are prototypic diasteric M_2 receptor agonists and antagonists (Tran et al. 2009; Mohr et al. 2010). By contrast,

acetylcholine, muscarine and atropine are prototypic of the orthosteric class of muscarinic agonists and antagonists (Mohr et al. 2010). Muscarinic receptors have thus provided important models of GPCR physiology (Lanzafame et al. 2003). Emerging concepts in this area suggest a high degree of complexity – and opportunity – for designing novel muscarinic therapeutics (Kenakin and Miller 2010). For example, muscarinic receptors function as dimers in either homomeric or heterodimeric assemblies (Park and Wells 2003; Novi et al. 2004), although the extent and duration to which this occurs in vivo is probably limited (Hern et al. 2010). Muscarinic receptors also exist in constitutively active states, arising from overexpression of the receptor mutation or from overexpression of the cognate G proteins (guanine nucleotide binding proteins). In the muscarinic receptor field, specifically, the relationship of constitutive activity with human pathology is relatively unexplored, even though the phenomenon clearly influences ligand pharmacology (Jakubík et al. 1995; Spalding and Burstein 2006). Several "silent" antagonists (e.g. atropine) act as inverse agonists in such systems, while partial agonists (e.g., oxotremorine, pilocarpine) express full agonism (Eglen 2005).

Muscarinic receptors signal via heterotrimeric G proteins and mobilize several second messengers. In general, muscarinic M_2 and M_4 receptors preferentially couple to $G_{\alpha i}$, and muscarinic M_1, M_3 and M_5 subtypes to $G_{\alpha q}$ (Peralta et al. 1988; see Lanzafame et al. 2003 for review). The cellular effectors principally depend upon the G_α subunit mobilized. In some cases, the $G_{\beta/\gamma}$ subunits play a key role in cellular signaling and provide a mechanism by which the M_2 receptor activates phospholipase Cβ. Muscarinic M_2 and M_4 receptors inhibit elevated adenylyl cyclase activity, as well as prolong potassium channel or nonselective cation (TRP) channel opening (Ben-Chaim et al. 2003). Muscarinic M_1, M_3 and M_5 receptors mobilize inositol phosphoinositides, notably inositol (1,4,5) trisphosphate (Ins P_3) and 1,2-diacylglycerol, via activation of phosphoinositide-specific phospholipase Cβ, thereby increasing intracellular calcium. These three subtypes also activate other cellular messengers such as nitric oxide or phospholipase A_2; although these effects are ancillary to elevations in intracellular calcium (see Eglen 2005 for review).

There are now numerous examples of a muscarinic receptor subtype coupling to multiple cellular effector pathways (see Antony et al. 2009, and references cited therein). This suggests that muscarinic receptors possess the capacity for "biased agonism," i.e., the agonist receptor complex couples to multiple effector pathways, via multiple G proteins (Kenakin 2007). Signaling through these pathways is therefore dependent upon the agonist (hence the term "biased agonism") and also a property of both the agonist *and* the receptor participating in the agonist/receptor complex (Ehlert 2008; Thomas et al. 2009; Figueroa et al. 2009). Taken together, this complexity raises new possibilities of designing novel muscarinic ligands potentially with tissue-specific pharmacologies.

3 Muscarinic M_1 Receptors

Muscarinic M_1 receptors are abundantly expressed in all major forebrain areas including the cerebral cortex, hippocampus, and striatum (Levey 1993). Consistent with this distribution, activation of muscarinic M_1 receptors is implicated in learning and memory processes (Volpicelli and Levey 2004; Fisher 2008). Enhanced cholinergic receptor activation, either by the use of acetylcholinesterases or muscarinic agonists, ameliorates cognitive decline in many animal models (Doggrell and Evans 2003). Selective M_1 agonism has, indeed, been frequently suggested (see Clader and Wang 2005, for review) as an approach to retard the cognitive decline in dementias, including those seen in Alzheimer's disease, age-associated memory impairment or cognitive impairments associated with schizophrenia (Bartus et al. 1982).

This "cholinergic hypothesis" of dementia is also based on observations that the presynaptic muscarinic M_2 receptor (as well as 5-HT_2 and nicotinic α4–β2 receptors) population selectively declines and the postsynaptic M_1 receptor population is preferentially preserved in Alzheimer's disease (Felder et al. 2000; Fisher et al. 2002, 2003; Fisher 2008). Extensive pharmacological data supporting a role for the M_1 receptor in cognition is now supported by studies with transgenic mice lacking the M_1 receptor, in which memory consolidation processes are impaired (Miyakawa et al. 2001). The effect in this and other animal models is, however, surprisingly modest given the pronounced effect of centrally acting muscarinic antagonists on cognition (Anagnostaras et al. 2003; Wess et al. 2007). These data suggest either a complex role of muscarinic M_1 receptors in cognition or the participation of more than one subtype. Conceivably, muscarinic M_1 receptors are not critical for memory formation, but are important for memory processes involving interactions between the cerebral cortex and hippocampus (Wess et al. 2007). Nonetheless, selective M_1 activation remains a therapeutic approach to Alzheimer's disease, age-associated memory impairment or cognitive impairments associated with schizophrenia, potentially resulting in compounds to improve cognition with few side effects (Bymaster et al. 2002; Fisher et al. 2002, 2003; Langmead et al. 2008a).

The overproduction of amyloid β peptide and its subsequent deposition as insoluble amyloid plaques is a key pathophysiological lesion leading to Alzheimer's disease (Citron 2010). Consequently, reducing the production of this protein may slow disease progression. In isolated tissues, muscarinic M_1 agonism augments the release of the amino terminal form of amyloid precursor protein. Aβ protein promotes activation of protein kinase C and calcium/calmodulin-dependent kinase II: a process counteracted by M_1 receptors. This finding has been subsequently confirmed in Alzheimer's disease patients using the muscarinic agonist, cevimeline, where Aβ levels declined after chronic treatment (see Eglen 2005, for references). Similar observations in Alzheimer's disease patients are seen with the muscarinic M_1 agonists, alvameline, milameline, sabcomeline, RS 86 (2-ethyl-8-methyl-2,8-diazospiro-4,5-decan-1,3-dianhydrobromide), talsaclidine,

and xanomeline, suggesting that M_1 agonists, in general, lower Aβ (particularly Aβ42) levels (Hock et al. 2003). Consistent with this finding is that deletion of M_1 receptors in transgenic mice increases amyloidogenic APP processing (Davis et al. 2010).

Muscarinic M_1 agonism is thus a therapeutic approach to Alzheimer's disease with two potential benefits, i.e., moderate reversal of cognitive impairment and decreased amyloid plaque formation. A common medicinal chemistry goal is therefore to identify centrally acting, potent, and selective agonists for use in this disorder (Jakubík et al. 2008; Fisher 2008). Early clinical studies with muscarinic agonist, such as arecoline, pilocarpine or oxotremorine and RS-86, were disappointing due to their low efficacy and high side effect potential (Felder et al. 2000; Jakubík et al. 2008; Heinrich et al. 2009). Additional compounds, including alvameline, sabcomeline and xanomeline or several spiropiperidines and spiroquinuclidines were identified and clinically evaluated, again with disappointing results and early discontinuation of clinical trials (Jakubík et al. 2008; Langmead et al. 2008a). An exception is cevimeline, a compound with M_1 agonist properties, currently approved for an unrelated autoimmune condition, Sjögren's disease (Fox et al. 2001).

The failure of these compounds in dementia patient trials partly relates to the "receptor reserve" associated with muscarinic M_1 receptor function in Alzheimer pathology (Jakubík et al. 2008). As discussed above, when the M_1 receptor selectivity of an agonist is marginal, and the receptor reserve high, responses at several muscarinic receptor subtypes is frequently seen. This is often the case in many cell phenotypes used in high throughput screening assays aimed at identifying novel muscarinic agonists (Schwarz et al. 1993; Wang and el-Fakahany 1993). Screens such as this are predisposed to identify leads, and numerous secondary screens are required to optimize the efficacy of the leads, and thus their clinical translation (Wood et al. 1999; Eglen et al. 2007). This is most relevant to novel muscarinic agonists of low intrinsic efficacy or mixed allosteric/orthosteric (diasteric) agonists, with which expression of agonism is critically dependent on the prevailing receptor reserve (Mei et al. 1989; Wang and el-Fakahany 1993; Richards and Giersbergen 1995; Heinrich et al. 2009). One recent example is the finding that *N*-desmethylclozapine acts as an antagonist at native human M_1 receptors, but as a partial agonism at the human recombinant M_1 muscarinic receptor (Thomas et al. 2010). It is also likely that several older muscarinic M_1 agonists possess "functional," rather than absolute, receptor subtype selectivity (Eglen 2005). As such, prediction of the degree of agonism in a clinical therapeutic setting can be problematic (Thomas et al. 2010). Nonetheless, novel subtype-selective agonists continue to be developed for the M_1 receptor, a series of 2′ biaryl amides being a recent example (Budzik et al. 2010).

An alternative approach to the design of selective M_1 agonists is to exploit ectopic sites on the muscarinic M_1 receptor that serve to allosterically regulate agonist function (Jakubík et al. 1997; Spalding et al. 2002). This region is not conserved amongst other muscarinic receptor subtypes and highly selective compounds have been synthesized that augment the prevailing cholinergic

activation (Conn et al. 2009b). WIN 62577 (17-β-hydroxy-17-α-ethynyl-Δ-(4)-androstano [3,2-b] pyrimido [1,2-a] benzimidazole), a neurokinin NK_1 receptor antagonist, is an allosteric muscarinic M_3 receptor enhancer with micromolar affinity (Lazareno et al. 2002, 2003), although attempts to modify the compound to produce potent and selective M_1 allosteric enhancers were unsuccessful (Jones et al. 2008).

Recently, muscarinic allosteric agonists of high selectivity (and structural diversity) have been synthesized, including AC-42 (4-*n*-butyl-1-[4-(2-methylphenyl)-4-oxo-1-butyl]-piperidine hydrochloride), 77-LH-28-1 (1-[3-(4-butyl-1-piperidinyl) propyl]-3,4-dihydro-2(1*H*)-quinolinone), VU0090157 (cyclopentyl 1,6-dimethyl-4-(6-nitrobenzo[*d*][1,3]-dioxol-5-yl)-2-oxo-1,2,3,4 tetrahydropyrimidine-5-carboxylate and VU0029767 ((*E*)-2-(4-ethoxyphenylamino)-*N*-((2-hydroxynaphthalen-1-yl) methylene)acetohydrazide (Spalding et al. 2006; Langmead et al. 2006, 2008b; Conn et al. 2009b). Many of these can activate the M1 receptor in the absence of the orthosteric agonist (Conn et al. 2009a). The high selectivity of AC 42 emanates from an ectopic binding site in the upper portions of transmembrane domains TM1 and TM7; a domain of the muscarinic M_1 receptor that markedly diverges within the five subtypes (Spalding et al. 2006). A different, and novel, series of highly selective M_1 receptor allosteric agonists, represented by 1-(1′-2-methylbenzyl)-1,4′-bipiperidin-4-yl)-1*H* benzo[*d*]imidazol-2(3*H*)-one (TBPB) has also been described (Jones et al. 2008). The lead compound in this series also modulates processing of the amyloid precursor protein toward the nonamyloidogenic pathway and decreases Aβ production in vitro (Jones et al. 2008).

VU0090157 and VU0029767 differ from other positive allosteric modulators (PAMs) such as AC-42 in that they do not activate M_1 receptors directly, yet selectively potentiate responses to acetylcholine (Marlo et al. 2009). Comparative data from studies using AC-42, AC-260584 (4-[3-(4-butylpiperidin-1-yl)-propyl]-7-fluoro-4*H*-benzo[1,4]oxazin-3-one), *N*-desmethylclozapine and xanomeline also suggest that allosteric agonists differ from orthosteric ligands – and amongst each other – in their differential ability to modulate cell signaling regulatory pathways (Bradley et al. 2010). Similar conclusions regarding this form of biased ligand signaling (Kenakin 2007) were reported using BQCA (benzyl quinolone carboxylic acid) – also a highly selective allosteric potentiator of M_1 receptors (Ma et al. 2009). Mechanistically, differences were seen between allosteric modulators, in terms of their rank order of potency at signaling via β arrestin recruitment vs. their potency at ameliorating murine cognitive deficits (Shirey et al. 2009; Thomas et al. 2009; Bradley et al. 2010). These data show that the degree of specific ligand bias with respect to β arrestin is important in the design of novel allosteric M_1 ligands.

The muscarinic M_1 receptor plays a role in other disorders including schizophrenia (Bymaster et al. 2002; Scarr 2009). Muscarinic receptors are a compelling target for the treatment of psychosis, since muscarinic antagonists produce symptoms in humans similar to the positive and negative behaviors associated with the disease (Scarr 2009). Genetic polymorphisms of the muscarinic M_1 receptor are also associated with schizophrenia (Liao et al. 2003). Xanomeline exhibits antipsychotic

activity in both preclinical and clinical studies. However, as the compound is a mixed M_1/M_4 agonist, the antidopaminergic effects may also be mediated via the muscarinic M_4 receptor (Shekhar et al. 2008). The antipsychotic drug, clozapine, exhibits limited muscarinic M_1 agonist activity, and these characteristics are more pronounced in a metabolite, *N* des methyl clozapine. Both compounds modulate M_1 receptor activity via an allosteric site that partially overlaps with the orthosteric binding site for acetylcholine (Sur et al. 2003). Furthermore, *N* des methyl clozapine augments hippocampal *N*-methyl-D-aspartate receptor currents, suggesting that agonists possessing both M_1-positive allosteric activity and *N*-methyl-D-aspartate agonism could provide novel antipsychotic therapies (Sur et al. 2003).

A growing literature (see Wessler and Kirkpatrick 2008 for review) suggests that acetylcholine has a hormonal, i.e., extraneuronal, action in order to regulate immune system function. Muscarinic M_1 and M_2 receptors are expressed in human lymphocytes and appear to mediate the autocoid effects of acetylcholine. The immune function of acetylcholine is not extensively established (see Kawashima and Fujii 2004, for review), but emerging data suggest a direct relationship between muscarinic M_1 receptor activation and interleukin 2 production (Nomura et al. 2003). The cholinergic anti-inflammatory pathway has been studied in various models of acute systemic or local inflammation, although it is unclear whether acetylcholine is released from intimately associated vagal nerves or via a local paracrine action (Kawashima and Fujii 2004). Moreover, the precise involvement of the M_1 receptor, or indeed the remaining four subtypes, remains to be definitively characterized (Eglen 2006).

4 Muscarinic M_2 Receptors

Muscarinic M_2 receptors are widely expressed in both central and peripheral nervous systems (Levey 1993). Selective muscarinic M_2 antagonism increases cholinergic overflow by reducing autoreceptor function in both the brain and the periphery. However, studies in mice deficient in both M_2 and M_4 receptors suggest a role for *both* subtypes in modulating hippocampal cholinergic function. Genetic variants in the human M_2 receptor gene also correlate with differences in cognitive performance, as well as bipolar depressive disorders (Cannon et al. 2010). Several workers have suggested (see Sheardown 2002, for references) that either selective M_2 receptor antagonism or compounds with mixed M_2 antagonism and M_1 agonism is a therapeutic approach to increase cholinergic function in Alzheimer's disease, particularly at a stage where cholinergic tone is not completely lost.

The muscarinic M_2 antagonists, SCH 57790 (4-cyclohexyl-α-[4[[4-methoxyphenyl] sulphinyl]-phenyl]-1-piperazine acetonitrile or the pyridobenzodiazepinone, BIBN-99 (5,11-dihydro-8-chloro-11-[[4-[3-[(2,2-dimethyl-1-oxopentyl)-ethylamino] propyl]-1-piperidinyl] acetyl]-6H-pyrido [2,3-b][1,4]benzodiazepin-6-one), improve cognitive performance in preclinical models. Bilateral infusions of muscarinic M_2 antagonists into the dorsolateral striatum of cognitively impaired rats also enhance memory

performance. A series of piperidines are potent and selective M_2 receptor antagonists, with clinical studies in dementia underway using compounds such as SCH 72788 (4-(4-(1(S)-(4-((1,3-benzodioxol-5-yl)sulfonyl)phenyl)ethyl)-3(R)-methyl-1-piperazinyl)-4-methyl-1-(propylsulfonyl)piperidine) (Bohme et al. 2003; Boyle and Lachowicz 2002). These compounds have improved oral bioavailability over SCH 57790, although clinical data have not yet been reported. The potential of muscarinic M_2 receptor antagonism in treatment of cognitive decline has spurred synthesis of other chemical series, notably from the piperidine alkaloids, originally derived from the bark of *Galbulimima baccata*, of which (+) himbacine is a prototypic example. Derivatives include epihimandravine and himbacine analogs containing ring substituted decahydro naphthofurans, hydroisobenzofuran-1 (3H)-ones, benzylidene ketals and dimenthindene derivatives (see Eglen 2005, for references).

In the caudate putamen, muscarinic M_2 receptors act as inhibitory heteroreceptors on dopaminergic terminals. Consequently, selective muscarinic M_2 receptor blockade may provide a therapeutic approach to schizophrenia, a disease associated with excessive dopamine transmission. BuTAC ([5R-(exo)]-6-[4-butylthio-1,2,5-thiadiazol-3-yl]-1-azabicyclo-[3.2.]-octane) is a partial agonist at muscarinic M_2 and M_4 receptors, and an antagonist at muscarinic M_1, M_3 and M_5 receptors (Rasmussen et al. 2001). In rodents, the partial agonist BuTAC exhibits antipsychotic behavior, resembling clozapine and olanzapine, and induces a reduction in dopamine cell firing in the limbic ventral tegmental area, possibly by an M_2 antagonist action (Rasmussen et al. 2001). However, mutated mice lacking the muscarinic M_4 receptor also display supersensivity of dopamine D_1 receptors, indicating that the muscarinic M_4, as opposed to the M_2 receptor is also important in this respect.

Muscarinic M_2 receptors may play a role in adult depressive disorders. Serum cortisol levels are elevated in major depressive disorders, notably in adult women (Cannon et al. 2010). Females possessing a thymidine at nucleotide 1890 in the 3′ untranslated region of the human M_2 receptor gene have an elevated predisposition for major depression. Subsequent work has shown that six single nucleotide polymorphisms (SNPs) have been shown to decrease muscarinic M_2 receptor binding present in the CHRM2 gene associated with bipolar depression. In mice, muscarinic M_2 receptors mediate agonist-induced activation of the hypothalamic–pituitary–adrenocortical axis, as animals deficient in this subtype do not show enhanced release of serum corticosterone in response to muscarinic agonists (Hemrich-Luecke et al. 2002). Moreover, centrally active muscarinic agonists stimulate the hypothalamic–pituitary–adrenocortical axis via the release of corticotrophin-releasing hormone. However, it is unclear from the transgenic mice work where the locus of action of M_2 activation occurs (central vs. peripheral nervous systems). These data, collectively, may implicate activation of muscarinic M_2 receptors in the effects of cortisol-induced depressive disorders, although this concept has yet to be clinically investigated.

In the periphery, the muscarinic M_2 receptor is expressed in the myocardium, and mediates classical negative chronotropic and inotropic effects of acetylcholine.

Accordingly, in mice lacking the M_2 receptor, the bradycardic effects of muscarinic agonists are completely abolished. Recent mRNA and pharmacological data show that other muscarinic subtypes are expressed in this tissue, all acting to modulate ion channel activity and thus heart function. In the myocardium, the muscarinic M_2 receptor is sensitive to changes in membrane voltage, probably at a site in the vicinity of the receptor–G protein interface (Harvey and Belevych 2003). However, the activation of inward rectifying potassium currents in isolated cells is sensitive to prevailing culture conditions, suggesting caution in the interpretation of data relating to muscarinic receptor function and ion channel activation (Himmel et al. 2002).

The relationship of the muscarinic M_2 receptor with myocardial ion channel activation is affected in diseases, such as Chagas' disease; a parasitic infectious diseases associated with long-term cardiac malfunction. Here, circulating M_2 receptor autoantibodies attenuate muscarinic M_2 function, and in idiopathic-dilated cardiomyopathy, muscarinic M_2 autoantibodies are also elevated. In an animal model of cardiomyopathy, cardiac remodeling is also associated with an increase in circulating M_2 receptor autoantibodies, suggesting a similar autoimmune reaction to that seen in the disease. The *Trypanosoma cruzi* antigen, cruzipain, also induces antibodies against the M_2 receptor, directly implicating the receptor in the etiology of Chagas' disease (Hernandez et al. 2003).

In designing compounds to modulate muscarinic M_2 receptor, the role of an allosteric site has been emphasized, with several studies indicating a markedly different structure–activity relationship from the classical agonist binding site (May et al. 2007). The allosteric site modulates agonism in either a negative or positive allosteric fashion. Site-directed mutagenesis studies have shown that two amino acid residues in the muscarinic M_2 receptor entirely account for the allosteric selectivity of compounds, such as curacurine V, alcuronium, gallamine, caracurine V, *bis* (ammonio) alkanes and bisquaternary dimers of strychnine and brucine, as well as their associated derivatives (Gregory et al. 2010). Although exploitation of this site in the design of novel therapeutics acting at this site has not been extensively studied, recent compounds have been explicitly designed to interact as both allosteric and orthosteric M_2 ligands (Steinfeld et al. 2007).

5 Muscarinic M_3 Receptors

The muscarinic M_3 receptor is widely distributed in the CNS, albeit at lower levels than other muscarinic receptor subtypes (Levey 1993). Muscarinic M_3-deficient mice are hypophagic and lean, suggesting a central role for this subtype in regulating food intake. Although this may involve modulation of hypothalamic melanin-concentrating hormone levels, deficits in salivary flow could also contribute to the hypophagic phenotype (Gautam et al. 2008). The M_3 receptor is expressed at relatively high levels in the hypothalamus but is also found in many other brain regions. Currently, little is known about the role of relevance central M_3 receptors.

Brain muscarinic M_3 knockout mice exhibit a dwarf phenotype associated with a pronounced hypoplasia of the anterior pituitary gland and a marked decrease in pituitary and serum growth hormone (GH) and prolactin. These data suggest critical role for central M_3 receptors in promoting longitudinal growth (Gautam et al. 2009). Furthermore, central M_3 receptors may be a pharmacological target to modulate GH release in the treatment of human growth disorders.

Muscarinic M_3 receptors also appear to play a role in regulating bone mass, in that they mediate parasympathetic nervous drive decease in bone resorption, as well as an increase in bone formation. These data, derived from transgenic mice lacking the M3 receptor, appear to suggest a central action (Wess et al. 2007; Shi et al. 2010). Muscarinic M_3 receptors are also present in the hippocampus, where they appear to mediate an increase in learning, via a series of receptor phosphorylation cascade that is independent of the canonical β arrestin/desensitization pathway (Poulin et al. 2010). This suggests that an agonist capable of biased signaling in this pathway may provide a novel approach to increasing learning and memory (Poulin et al. 2010).

Initial indications of muscarinic receptor heterogeneity, developed in the 1960s and 1970s, stemmed from pharmacological studies using isolated myocardium and smooth muscle tissue (Barlow et al. 1976). In these experiments, the muscarinic receptor subtype mediating negative inotropy pharmacologically differed from the subtype mediating smooth muscle contraction (i.e., muscarinic M_2 vs. M_3 receptors, respectively). These differences provided simple bioassays that identified several, structurally diverse antagonists with marked muscarinic M_2 or M_3 selectivity. The most important compounds identified in this way were 4-DAMP (4-diphenyl acetoxy-*N*-methyl piperidine methiodide) and pFHSiD (*para* fluorohexahydrosiliadifenidol), both preferential for the muscarinic M_3 over the M_2 receptor. AF-DX 116 (Otenzepad, 11-[[2-[(diethylamino)methyl]-1-piperidinyl]acetyl]-5,11-dihydro-6*H*-pyrido[2,3-b] [1,4]benzodiazepin-6-one), AF-DX 384 (*N*-[2-[2-[(dipropylamino)methyl]-1-piperidinyl]ethyl]-5,6-dihydro-6-oxo-11*H*-pyrido[2,3-b][1,4] benzodiazepine-11-carboxamide), methoctramine, and tripitramine, conversely, were shown to be preferential for the M_2 over the M_3 receptor (see Eglen et al. 1996, for review). It is now evident that these early bioassays are far more complex in terms of the muscarinic receptors involved. The myocardium, for example, expresses other muscarinic receptors than the M_2 receptor, even though the predominant effect of acetylcholine is mediated by this subtype (Ponicke et al. 2003). In rat-isolated myocytes, as well as in knockout mice M_3 receptors appear to augment inositol phosphate accumulation, resulting in positive inotropic effects (Kitazawa et al. 2009).

Contractile responses in smooth muscle also reflect participation of more than the muscarinic M_3 receptor as most smooth muscle tissues express *both* muscarinic M_2 and M_3 receptors in a ratio of about 4:1 (Michel and Whiting 1987). The pharmacology of the contractile action of muscarinic agonists generally reflects muscarinic M_3 receptors alone. Studies in tissues from several species, including human (Mansfield et al. 2003), show that both receptors are involved in the control of muscle motility and can be revealed under discrete experimental conditions

(Thomas et al. 1993; Ehlert 2003; Kories et al. 2003). These data have implications for drug discovery programs aimed at identifying muscarinic antagonists for treatment smooth muscle dysfunction, specifically in the design of the pharmacological profile required in the compound.

Several lines of data show that muscarinic M_2 receptors play a conditional role in concert with the M_3 receptor to modulate contraction (Unno et al. 2003). Activation of muscarinic M_2 receptors, for example, opposes elevations in smooth muscle myocyte adenylate cyclase activity, thereby abrogating muscle relaxation. Heterologous desensitization of responses to other contractile agents in gastrointestinal smooth muscle also requires activation of both subtypes (Griffin et al. 2004). Muscarinic M_2 receptors open a nonselective cation channel, thereby augmenting entry of extracellular sodium ions – a major mechanism for cholinergic excitation of smooth muscle since cation channel opening may be the predominant mediator of smooth muscle contractile activity (Zholos et al. 2003, 2004). The current model is that muscarinic M_3 receptors exert a permissive role over the M_2-mediated cation current activation via a process that involves elevations in intracellular calcium, possibly independent of Ins P_3 mobilization (Griffin et al. 2009).

Nonetheless, many functional studies suggest a modest role for muscarinic M_2 receptor activation in smooth muscle contraction, unless specialized experimental (or pathophysiological) conditions (e.g., elevated adenylate cyclase levels, heterologous desensitization, aging, elevated insulin levels) exist (see Ehlert et al. 2005; Griffin et al. 2009). Transgenic mice studies show that smooth muscle contraction is not dependent upon muscarinic M_2 receptor and support a major role for M_3 receptor (Matsui et al. 2000; Stengel et al. 2000). Importantly, this phenotype varies between smooth muscles. In the M_3 knockout mice, isolated gastrointestinal smooth muscle motility to muscarinic agonists is impaired by approx. 77% and the residual contraction is mediated by muscarinic M_2 receptors. Qualitatively similar data are seen in isolated urinary detrusor muscle, but the residual M_2 receptor-mediated component is much less than in gastrointestinal tissue (Matsui et al. 2000; Stengel et al. 2000). In vivo data from these mice reveal that gastrointestinal function is unimpaired, arguing that the muscarinic system per se does not control gut function. In contrast, enhanced urinary retention is evident in these animals, suggesting that muscarinic control over the bladder is critical to urinary bladder voiding. Mutant mice lacking *both* M_2 and M_3 receptors exhibit marked distension of the urinary bladder although there are no intestinal complications (Wess et al. 2007).

Collectively, these data suggest that muscarinic M_3 blockade alone is useful for treating urinary tract disorders (Andersson 1993, 2003; Abrams et al. 2006; Fowler et al. 2008). Treatment of gastrointestinal motility disorders may require concurrent blockade of muscarinic M_2 and M_3 receptors. Several muscarinic antagonists have, in fact, been developed as therapeutics for hyperactive smooth muscle disorders, including overactive bladder (OAB), irritable bowel syndrome, and chronic obstructive pulmonary disease (COPD) (Peretto et al. 2009). Of these, tolterodine (Detrol) is a potent muscarinic antagonist developed for the treatment of OAB that possesses equivalent muscarinic M_2 and M_3 receptor affinities but exhibits selective

actions in vivo. Several controlled clinical studies demonstrate a low propensity for dry mouth or alterations in pupillary accommodation at doses of tolterodine that modulate OAB (Raes et al. 2004). However, as the compound in vitro lacks selectivity between muscarinic receptor subtypes its mechanism of selective action in vivo is unclear (Fowler et al. 2008). Fesoterodine (Toviaz) is a closely related compound and possesses a similar profile (Ney et al. 2008) although it shows efficacy in OAB at lower doses (Michel 2008).

Darifenacin (Enablex), in contrast, is a selective muscarinic M_3 antagonist and preliminary data show inhibition of bladder responsiveness at doses that do not affect salivation (Miyamae et al. 2003; Zinner 2007). The compound also reduces OAB; specifically, the time between the first sensation of urgency and urination (Pelman et al. 2008). Solifenacin (Vesicare) is also a compound with selective M_3 antagonist actions with a longer duration of action than darifenacin, but with comparable efficacy to tolterodine. These two compounds, together with tolterodine, are potential frontline therapies for OAB having efficacy at doses accompanied by reduced anticholinergic side effects (Hegde 2006). In some elderly patients, however, with all these agents, dry mouth remains a notable compliance problem. Several other M_3 over M_2-selective compounds are in clinical development for OAB, such as Imidafencin (KRP-197/ONO-8025), Tarafenacin (SVT-40776; Salcedo et al. 2009), the latter possessing enhanced M_3: M_2 selectivity in comparison to darifenacin and solefenacin.

Alternative approaches to reducing anticholinergic side effects in the treatment of OAB have therefore included optimizing the pharmacokinetic profile (Yoshida et al. 2010). For example, Ditropan XL is a transdermal formulation of the nonselective antagonist, oxybutynin (Oxytrol) under advanced clinical evaluation, reportedly with a reduced incidence of side effects and improved compliance, and providing a once-daily treatment for OAB. In a similar fashion, Detrol LA is a formulation of tolterodine that also provides a once-daily treatment regimen. Trospium (Regurin) is also a muscarinic antagonist in clinical evaluation, but has little selectivity between receptors. However, a different formulation of the drug is in development in order to provide once-daily therapy and improved pharmacokinetics. Unlike darifenacin or solifenacin, trospium is a quaternary amine that does not cross the blood–brain barrier and over 3 days of dosing improves OAB. Since it is secreted in the urine unchanged, it may exert a local action in the bladder as it concentrates in the urine. Indeed, an unexplored area of research is the locus of action of muscarinic antagonists at the urothelium; a urinary bladder tissue that expresses muscarinic receptors (Mansfield et al. 2009) and potentially mediate the release of a diffusible factor that induces relaxation of the underlying smooth muscle layer (Chess-Williams 2002; Yoshida et al. 2008; Giglio and Tobin 2009).

Cholinergic constriction of airways involves activation of postjunctional M_3 receptors, as well as prejunctional M_2 receptors. Studies using knockout mice reveal a complex interplay of muscarinic M_2 and M_3 receptors in peripheral airways, with M_1 receptors counteracting cholinergic bronchoconstriction, and neuronal M_2 receptors inhibiting acetylcholine release from parasympathetic nerves (Sarria et al. 2002; Struchmann et al. 2003). The function of these

autoreceptors is selectively abrogated by several agents including parainfluenza infection, double-stranded RNA, ozone exposure, ovalbumin sensitization and vitamin A deficiency, resulting in increased cholinergic overflow and enhanced airway smooth muscle contraction (Moreno et al. 2003). These neuronal effects probably underlie the paradoxical bronchospasm seen with several muscarinic antagonists, such as rapacuronium, in the treatment of asthmatic bronchoconstriction (see Verhein et al. 2009 for review). Localization of eosinophils to airway nerves, via an interaction with specific adhesion molecules, in asthmatics may also attenuate muscarinic M_2 receptor function (Kingham et al. 2003). Collectively, these data indicate that muscarinic antagonism per se is an inappropriate option for the treatment of asthma.

In the treatment of COPD, by contrast, short-acting muscarinic antagonists such as iprotropium and oxitropium have been used as effective bronchodilator therapies, since they reverse airway constriction and reduce bronchial fluid secretion Tiotropium (Spriva) has been suggested as a first-line therapeutic approach with once a day dosing efficacy superior to ipratropium, accompanied by an improved side effect profile (Hansel and Barnes 2002). Tiotropium functionally acts an antagonist preferential for the muscarinic M_1 and M_3 receptors, by virtue of the preferential slow dissociation kinetics from these receptors (Casarosa et al. 2009). Prolonged treatment of COPD patients with the drug does not cause tolerance and is well tolerated, although dry mouth is evident in some patients. A more recent compound with a similar profile in this area is aclidinium, a selective M_3 antagonist presently undergoing clinical evaluation (Maltais et al. 2010). Other compounds, including TD-4208 (Steinfeld et al. 2009), a series of biphenyl piperazines (Jin et al. 2008), are now in development (see Alifano et al. 2010 for review). In severe COPD growing evidence indicates that triple therapy, i.e., a combination of a muscarinic antagonist such as Tiotropium, together with an inhaled corticosteroid and a long-acting β_2 adrenoceptor agonist, provides clinical benefits additional to those associated with each treatment alone (Ray and Alcaraz 2009). To this point, bifunctional compounds, such as TD-5959 (GSK-961081) possessing both muscarinic antagonism and β2 agonism are now in clinical evaluation (Aiyar et al. 2009).

6 Muscarinic M_4 Receptors

In the central nervous system, muscarinic M_4 receptors are distributed in the corpus striatum being co-localized with dopamine receptors on striatal projecting neurons. In the periphery, the subtype is present on various prejunctional nerve endings, where they act to inhibit parasympathetic and sympathetic transmission (Trendelenburg et al. 2003). The muscarinic M_4 receptor may play a role in psychosis, with the mixed M_1/M_4 agonist xanomeline having antipsychotic effects (see above). This compound, even after acute administration, selectively inhibits mesolimbic firing of dopamine cells, suggesting that muscarinic agonists could

have a faster onset of action than current antipsychotics, with fewer side effects (Mirza et al. 2003). Mice lacking the muscarinic M_4 receptor also display an increased sensitivity to the disruptive effect of phencyclidine on prepulse inhibition. This preclinical effect is a model of psychosis and the data support the contention that M_4 receptors are a suitable target for the treatment of schizophrenia (Tzarvara et al. 2003; Chan et al. 2008).

Activation of the muscarinic M_4 receptor is also affected by an allosteric site, and was demonstrated initially with the low potency compound, thiochrome. Later studies showed that the compounds, LY2033298 (3-amino-5-chloro-6-methoxy-4-methylthieno [2,3-b]pyridine-2 carboxylic acid cyclopropylamide) (Chan et al. 2008) and the structurally similar, VU10010 (3-amino-*N*-(4-chlorobenzyl)-4,6-dimethylthieno[2,3-b]pyridine-2-carboxamide); selectively enhanced M_4 agonist function, and were active in animal models predictive of clinical antipsychotic drug efficacy (Nawaratne et al. 2010). These compounds, together with centrally analogues such as VU152099 (3-amino-*N*-(benzo[d][1,3]dioxol-5-ylmethyl)-4,6-dimethylthieno[2,3-*b*]pyridine carboxamide) and VU0152100 (3-amino-*N*-(4-methoxybenzyl)-4,6-dimethylthieno[2,3-*b*]pyridine carboxamide) (Brady et al. 2008) are a new generation of M4 PAMs acting solely via an allosteric site, lacking activity at the orthosteric site (Nawaratne et al. 2010).

Parkinson's disease is a neurodegenerative characterized by slow movements, muscular rigidity, tremor, and balance disturbances. These symptoms arise from the loss of dopaminergic neurons projecting to the striatum, causing an imbalance between the cholinergic and dopaminergic systems, such that the former dominates. Nonselective muscarinic antagonists are effective in treating the disease, although side effects limit their use. Transgenic mice lacking the M_4 receptor show increased locomotor activity and an enhancement of dopamine D_1 receptor-mediated effects (Gomeza et al. 1999). It is likely that the striatal M_4 receptors exert an inhibitory action on dopamine D_1 receptor function. Consequently, selective M_4 antagonists have been developed for the treatment of Parkinson's disease, including benzoxazines such as PD 0298029, the latter of which has a favorable pharmacokinetic profile and good bioavailability in the clinic (Bohme et al. 2002).

Activation of central muscarinic receptors leads to potent antinociception, although the precise nature of the muscarinic receptor subtype(s) mediating the response is unclear. In mice, the analgesic response induced by muscarinic agonists, CMI-936 (2-exo {5-(3-methyl-1,2,4-oxadiazolyl)}-[2.2.1.]-7-azabicycloheptane) or CMI-1145 (2-exo{5-(3-amino-1,2,4-oxadiazolyl)}-[2.2.1.]-7-azabicycloheptane) is pertussis toxin-sensitive; a finding consistent with involvement of either muscarinic M_2 or M_4 receptors (Swedberg et al. 1997). Transgenic mice deficient in muscarinic M_2 receptors also show a striking reduction in muscarinic-dependent antinociceptive responses (Chen et al. 2005). The highly selective muscarinic M_4 antagonist, MT-3 (isolated from the venom of the African green mamba, *Dendroapsis augusticeps*), also antagonizes these responses suggesting that muscarinic M_4 receptors also mediate antinociceptive effects. However, the phenotype of the M_4 deficient mouse indicates no change in the antinociceptive action of muscarinic agonists,

probably due to residual presence of muscarinic M_2 receptors. Indeed, in mice lacking *both* M_2 and M_4 receptors, muscarinic agonists are devoid of analgesic activity (Wess et al. 2003). It is probable that the muscarinic M_2 receptor plays a predominant role in antinociception and the effect of the M_4 receptor is minor by comparison. Selective agonism of the latter will not, however, result in major effects on the cardiovascular system yet may provide a viable approach to analgesia (Wess et al. 2003).

7 Muscarinic M_5 Receptors

The muscarinic M_5 receptor is the only muscarinic subtype expressed by the dopamine-containing neurons of the substantia nigra pars compacta, a structure that provides the principal dopamine innervation to the striatum (Felder et al. 2000). Activation of muscarinic M_5 receptors thus facilitates striatal dopamine release – although it is likely that other muscarinic receptors, including the M_4 receptor, are involved (Vilaro et al. 1990). The muscarinic M_5 receptor is also the predominant subtype expressed in the ventral tegmental area, a tissue that provides major dopamineric innervation to the nucleus accumbens and other limbic areas (Eglen and Nahorski 2000).

These brain areas play a major role in the rewarding effects of several drugs of abuse. In muscarinic M_5-deficient mice stimulation of the laterodorsal tegmental area, which provides major cholinergic input to the ventral tegmental area dopamineric neurons, is markedly disrupted. The potential of selective muscarinic M_5 blockade as an approach to narcotic addiction corroborates to the extensive use of scopolamine and extracted alkaloids in the detoxification of heroin addiction (Basile et al. 2002). Transgenic mice lacking the M_5 receptor do not exhibit increased basal locomotor activity. Several studies indicate that muscarinic M_5 knockout mice are less sensitive to actions of addictive drugs, such as morphine or cocaine (Fink-Jensen et al. 2003). However, these data have recently been disputed in that augmented hyperactivity effects of amphetamine, but not cocaine, were seen, as well as an increase in the release of dopamine from the nucleus accumbens in mice M_5 knockouts (Steidl and Yeomans 2009). Differences in the genetic background between different strains of M_5 knockout mice may contribute to these discrepancies and further studies are clearly required.

Overall, antagonism of muscarinic M_5 receptors may be an important approach to novel therapeutics in both schizophrenia and compound addiction. Although the availability of selective ligands at the muscarinic M_5 receptor is not extensive, amiodarone has recently been reported to be a selective M_5 allosteric modulator. Moreover, it may be the first PAM to be shown to enhance muscarinic agonist efficacy without enhancing potency (Stahl and Ellis 2010). Initial data on more selective compounds including VU0238429 (1-(4-Methoxybenzyl)-5-trifluoromethoxyisatin) are also now emerging (Bridges et al. 2009, 2010), although extensive evaluation of the actions of such compounds in vivo is currently unavailable.

In the periphery, the muscarinic M_5 receptor is expressed at low levels in the iris, esophagus and lymphocytes, although the function in these tissues, if any, is unclear. Brain microvasculature expresses muscarinic receptors, with endothelial cells expressing both muscarinic M_2 and M_5 receptors, and vascular smooth muscle cells expressing all subtypes excepting M_4 receptors (Tayebati et al. 2003). Neuronally released acetylcholine regulates cortical perfusion and blood–brain barrier permeability via changes in local blood flow involving the muscarinic receptor-induced release of nitric oxide. The pharmacological profile of the muscarinic receptor subtype mediating cerebral vascular dilation corresponds best with the M_5 subtype (Elhusseiny et al. 1999, Elhusseiny and Hamel 2000). Indeed, in the rat, muscarinic M_5 receptors have been localized to the circle of Willis and pial arteries. In mice lacking M_5 receptors, cholinergic dilatation of basilar and pail arties is lost, supporting this suggestion. Therapeutically, deficits in cholinergic-induced vasodilatation may be involved in the etiology of Alzheimer's disease, or stroke-induced dementia, suggesting that selective muscarinic M_5 antagonism is a useful approach in these pathologies (Araya et al. 2006).

References

Abrams P, Andersson KE, Buccafusco JJ, Chapple C, de Groat WC, Fryer AD, Kay G, Laties A, Nathanson NM, Pasricha PJ, Wein AJ (2006) Muscarinic receptors: their distribution and function in body systems, and the implications for treating overactive bladder. Br J Pharmacol 148(565–578):2006

Aiyar J, Steinfeld T, Pulido-Rios MT, Chin K, Lee TW, Jasper J, Thomas R, Hegde S, Mammen M (2009) In vitro characterization of TD-5959: a novel bifunctional molecule with muscarinic antagonist and β2-adrenergic agonist activity. Am J Respir Crit Care Med 179:A4552

Alifano A, Cuvelier A, Delage A, Roche N, Lamia B, Molano LC, Couderc L-J, Marquette C-H, Devillier P (2010) Treatment of COPD: from pharmacological to instrumental therapies. Eur Respir Rev 19:7–23

Anagnostaras SG, Murphy GG, Hamilton SE, Mitchell SL, Rahnama NP, Nathanson NM, Silva AJ (2003) Selective cognitive dysfunction in acetylcholine M_1 muscarinic receptor mutant mice. Nat Neurosci 6:51–58

Andersson K-E (1993) Pharmacology of lower urinary tract smooth muscles and penile erectile tissues. Pharmacol Rev 45:253–308

Andersson K-E (2003) Antimuscarinics for treatment of overactive bladder. Lancet 3:46–53

Antony J, Kellershohn K, Mohr-Andra M, Kebig A, Prilla S, Muth M, Heller E, Disingrini T, Dallanoce C, Bertoni S, Schrobang J, Trankle C, Kostenis E, Christopoulos C, Holtje H-D, Barocelli E, De Amici M, Holzgrabe U, Mohr K (2009) Dualsteric GPCR targeting: a novel route to binding and signaling pathway selectivity. FASEB J 23:442–450

Araya R, Noguchi T, Yuhki M, Kitamura N, Higuchi M, Saido TC, Seki K, Itohara S, Kawano M, Tanemura K, Takashima A, Yamada K, Kondoh Y, Kanno I, Wess J, Yamada M (2006) Loss of M5 muscarinic acetylcholine receptors leads to cerebrovascular and neuronal abnormalities and cognitive deficits in mice. Neurobiol Dis 24:334–344

Barlow RB, Berry KJ, Glenton PA, Nilolaou NM, Soh KS (1976) A comparison of affinity constants for muscarine-sensitive acetylcholine receptors in guinea-pig atrial pacemaker cells at 29°C and in ileum at 29°C and 37°C. Br J Pharmacol 58:613–620

Bartus RT, Dean RL, Beer B, Lippa AS (1982) The cholinergic hypothesis of geriatric memory dysfunction. Science 217:408–411

Basile AS, Fedorova I, Zapata A, Liu X, Shippenberg T, Alokesh Duttaroy A, Yamada AM, Wess J (2002) Deletion of the M_5 muscarinic acetylcholine receptor attenuates morphine reinforcement and withdrawal but not morphine analgesia. Proc Natl Acad Sci USA 99:11452–11457

Ben-Chaim Y, Tour O, Dscal N, Parnas I, Parnas H (2003) The M_2 muscarinic G-protein-coupled receptor is voltage-sensitive. J Biol Chem 278:22482–22491

Birdsall NJM, Lazareno S (2005) Allosterism at muscarinic receptors: ligands and mechanisms. Mini Rev Med Chem 5:523–543

Bohme TM, Angelli-Szafran CE, Corinne E, Hallak H, Schwarz RD (2002) Analogs of M4 selective synthetic muscarinic receptor antagonists: synthesis, binding and pharmacokinetic properties. Med Chem Res 11:423–433

Bohme TM, Keim C, Kreutzmann K, Linder M, Dingermann T, Dannhardt G, Mutschler E, Lambrecht G (2003) Structure-activity relationships of dimethindene derivatives as new M_2-selective muscarinic receptor antagonists. J Med Chem 46:856–867

Bonner TI, Buckley NJ, Young AC, Brann MR (1987) Identification of a family of muscarinic acetylcholine receptor genes. Science 237:527–532

Boyle CD, Lachowicz JE (2002) Orally active and selective benzylidene ketal M_2 muscarinic receptor antagonists for the treatment of Alzheimer's disease. Drug Dev Res 56:310–320

Bradley SR, Lameh J, Ohrmund L, Son T, Bajpai A, Nguyen D, Friberg M, Burstein ES, Spalding TA, Ott TR, Schiffer HH, Tabatabaei A, McFarland K, Davis RE, Bonhaus DW (2010) AC-260584, an orally bioavailable M(1) muscarinic receptor allosteric agonist, improves cognitive performance in an animal model. Neuropharmacology 58:365–373

Brady AE, Jones CK, Bridges TM, Kennedy JP, Thompson AD, Heiman JU, Breininger ML, Gentry PR, Yin H, Jadhav SB, Shirey JK, Conn PJ, Lindsley CW (2008) Centrally active allosteric potentiators of the M4 muscarinic acetylcholine receptor reverse amphetamineinduced hyperlocomotor activity in rats. J Pharmacol Exp Ther 327:941–953

Bridges TM, Marlo JE, Niswender CM, Jones CK, Jadhav SB, Gentry PR, Plumley HC, Weaver CD, Conn PJ, Lindsley CW (2009) Discovery of the first highly M5-preferring muscarinic acetylcholine receptor ligand, an M5 positive allosteric modulator derived from a series of 5-trifluoromethoxy *N*-benzyl isatins. J Med Chem 52:3445–3448

Bridges TM, Kennedy JP, Cho HP, Breininger ML, Gentry PR, Hopkins CR, Conn PJ, Lindsley CW (2010) Chemical lead optimization of a pan G(q) mAChR M(1), M(3), M(5) positive allosteric modulator (PAM) lead. Part I: development of the first highly selective M(5) PAM. Bioorg Med Chem Lett 20:558–562

Budzik B, Garzya V, Shi D, Walker G, Lauchart Y, Lucas AJ, Rivero RA, Langmead CJ, Watson J, Wu Z, Forbes IT, Jin J (2010) 2′ Biaryl amides as novel and subtype selective M_1 agonists. Part II: further optimization and profiling. Bioorg Med Chem Lett 20:3545–3549

Bymaster FP, Felder C, Ahmed S, McKinzie D (2002) Muscarinic receptors as a target for drugs treating schizophrenia. Curr Drug Targets CNS Neurol Disord 1:163–181

Cannon DM, Klaver JK, Gandhi SK, Solorio G, Peck SA, Erickson K, Savitz J, Akula N, Eckelman WC, Furey ML, Sahakian BJ, McMahon FJ, Drevets WC (2010) Genetic variation in cholinergic muscarinic-2 receptor gene modulates M_2 receptor binding in vivo and accounts for reduced binding in bipolar disorder. Mol Psychiatry 16(4):407–418

Casarosa P, Bouyssou T, Germeyer S, Schnapp A, Gantner F, Pieper M (2009) Preclinical evaluation of long-acting muscarinic antagonists: comparison of tiotropium and investigational drugs. J Pharmacol Exp Ther 330:660–668

Caulfield MP (1993) Muscarinic receptors – characterization, coupling and function. Pharmacol Ther 58:319–379

Caulfield MP, Birdsall NJM (1998) International union of pharmacology. XVII. Classification of muscarinic acetylcholine receptors. Pharmacol Rev 50:279–290

Chan WY, McKinzie DL, Bose S, Mitchell N, Witkin JM, Thompson RC, Christopoulos A, Lazareno S, Birdsall NJM, Bymaster FP, Felder CC (2008) Allosteric modulation of the

muscarinic M_4 receptor as an approach to treating schizophrenia. Proc Natl Acad Sci USA 105:110978–110983
Chen S-R, Wess J, Pan H-L (2005) Functional activity of the M_2 and M_4 receptor subtypes in the spinal cord studied with muscarinic acetylcholine receptor knockout mice. J Pharmacol Exp Ther 313:765–770
Chess-Williams R (2002) Muscarinic receptors of the urinary bladder: detrusor, urothelial and prejunctional. Auton Autocoid Pharmacol 22:133–145
Citron M (2010) Alzheimer's disease: strategies for disease modification. Nat Rev Drug Discov 9:387–398
Clader JW, Wang Y (2005) Muscarinic agonists and antagonists in the treatment of Alzheimer's disease. Curr Pharm Des 11:3353–3361
Conn PJ, Jones CK, Lindsley CW (2009a) Subtype-selective allosteric modulators of muscarinic receptors for the treatment of CNS disorders. Trends Pharmacol Sci 30:148–155
Conn PJ, Christopoulos C, Lindsley CW (2009b) Allosteric modulators of GPCRs: a novel approach for the treatment of CNS disorders. Nat Rev Drug Discov 8:41–54
Davis AA, Fritz JJ, Wess J, Lah JJ, Levey AI (2010) Deletion of M_1 muscarinic acetylcholine receptors increases amyloid pathology in vitro and in vivo. J Neurosci 30:4190–4196
Doggrell SA, Evans S (2003) Treatment of dementia with neurotransmission modulation. Expet Opin Investig Drugs 12:1633–1654
Dorje F, Wess J, Lambrecht G, Tacke R, Mutschler E, Brann MR (1990) Antagonist binding profiles of five cloned human muscarinic receptor subtypes. J Pharmacol Exp Ther 256:727–733
Eglen RM (2005) Muscarinic receptor subtype pharmacology and physiology. Prog Med Chem 43:105–136
Eglen RM (2006) Muscarinic receptor subtypes in neuronal and non-neuronal cholinergic function. Auton Autacoid Pharmacol 26:219–233
Eglen RM, Nahorski SR (2000) The muscarinic M5 receptor: a silent or emerging subtype? Br J Pharmacol 130:13–21
Eglen RM, Hegde SS, Watson N (1996) Muscarinic receptor subtypes and smooth muscle function. Pharmacol Rev 48:531–565
Eglen RM, Bosse R, Reisine T (2007) Emerging concepts of guanine nucleotide-binding protein-coupled receptor (GPCR) function and implications for high throughput screening. Assay Drug Dev Technol 5:425–452
Ehlert FJ (2003) Pharmacological analysis of the contractile role of M_2 and M_3 muscarinic receptors in smooth muscle. Receptors Channels 9:261–277
Ehlert FJ (2008) On the analysis of ligand-directed signaling at G protein-coupled receptors. Naunyn Schmiedebergs Arch Pharmacol 377:549–577
Ehlert FJ, Griffin MT, Abe DM, Vo TH, Taketo MM, Manabe T, Matsui M (2005) The M_2 muscarinic receptor mediates contraction through indirect mechanisms in mouse urinary bladder. J Pharmacol Exp Ther 313:368–378
Elhusseiny A, Hamel E (2000) Muscarinic – but not nicotinic – receptors mediate acetylcholine dilation of human and bovine intracortical arterioles: involvement of nitric oxide and the m5 receptor subtype. J Cereb Blood Flow Metab 20:298–305
Elhusseiny A, Cohen Z, Olivier A, Stanimirovic D, Hamel E (1999) Functional acetylcholine muscarinic receptor subtypes in human brain microcirculation: identification and cellular localization. J Cereb Blood Flow Metab 19:794–802
Felder CC, Bymaster FP, Ward J, DeLapp N (2000) Therapeutic opportunities for muscarinic receptors in the central nervous system. J Med Chem 43:4333–4353
Figueroa FW, Griffin MT, Ehlert FJ (2009) Selectivity of agonists for the active state of M1 to M4 muscarinic receptor subtypes. J Pharmacol Exp Ther 328:331–342
Fink-Jensen A, Fedorova I, Wortwein G, Woldbye DP, Rasmussen T, Thomsen M, Bolwig TG, Knitowski KM, McKinzie DL, Yamada M, Wess J, Basile A (2003) Role for M5 muscarinic acetylcholine receptors in cocaine addiction. J Neurosci Res 74:91–96

Fisher A (2008) M1 muscarinic agonists target major hallmarks of Alzheimer's disease – the pivotal role of brain M1 receptors. Neurodegener Dis 5:237–240

Fisher A, Brandeis R, Haring R, Bar-Ner N, Kliger-Spatz M, Natan N, Sonego H, Marcovitch I, Pittel Z (2002) Impact of muscarinic agonists for successful therapy of Alzheimer's disease. J Neural Transm Suppl 62:189–202

Fisher A, Pittel Z, Haring R, Bar-Ner N, Kliger-Spatz M, Natan N, Egozi I, Sonego H, Marcovitch I, Brandeis R (2003) M1 muscarinic agonists can modulate some of the hallmarks in Alzheimer's disease: implications in future therapy. J Mol Neurosci 20:349–356

Fowler CJ, Griffiths D, de Groat WC (2008) The neural control of micturition. Nat Rev Neurosci 9:453–466

Fox RI, Konttinen Y, Fisher A (2001) Use of muscarinic agonists in the treatment of Sjögren's syndrome. Clin Immunol 101:249–263

Gautam D, Jeon J, Li JH, Han S-J, Hamdan FF, Cui Y, Lu H, Deng C, Gavrilov O, Wess J (2008) Metabolic roles of the M_3 muscarinic acetylcholine receptor studied with M_3 receptor mutant mice: a review. J Recept Signal Transduct Res 28:93–108

Gautam D, Jeon J, Starost MF, Han S-J, Hamdan FF, Cui Y, Parlow AF, Gavrilova O, Szalayova I, Mezey E, Wess J (2009) Neuronal M3 muscarinic acetylcholine receptors are essential for somatotroph proliferation and normal somatic growth. Proc Natl Acad Sci USA 106:6398–6403

Giglio D, Tobin G (2009) Muscarinic receptor subtypes in the lower urinary tract. Pharmacology 83:259–269

Gomeza J, Zhang L, Kostenis E, Felder C, Bymaster F, Brodkin J, Shannon H, Xia B, Deng D-X, Wess J (1999) Enhancement of D1 dopamine receptor-mediated locomotor stimulation in M_4 muscarinic acetylcholine receptor knockout mice. Proc Natl Acad Sci USA 96:10483–10488

Grando SA, Kawashima K, Kirkpatrick CJ, Wessler I (2007) Recent progress in understanding the non-neuronal cholinergic system in humans. Life Sci 80:2181–2185

Gregory KJ, Hall NE, Tobin AB, Sexton PM, Christopoulos A (2010) Identification of orthosteric and allosteric site mutations in M_2 muscarinic acetylcholine receptors that contribute to ligand-selective signaling bias. J Biol Chem 285:7459–7474

Griffin MT, Matsui M, Shehnaz D, Ansari KZ, Taketo MM, Manabe T, Ehlert FJ (2004) Muscarinic agonist-mediated heterologous desensitization in isolated ileum requires activation of both muscarinic M_2 and M_3 receptors. J Pharmacol Exp Ther 308:339–349

Griffin MT, Matsui M, Ostrom RS, Ehlert FJ (2009) The guinea pig ileum lacks the direct, high-potency, M_2-muscarinic, contractile mechanism characteristic of the mouse ileum. Naunyn Schmiedebergs Arch Pharmacol 380:327–335

Hammer R, Berrie CP, Birdsall NJ, Burgen AS, Hulme EC (1980) Pirenzepine distinguishes between different subclasses of muscarinic receptors. Nature 283:90–92

Hansel TT, Barnes PJ (2002) Tiotropium bromide: a novel once-daily anticholinergic bronchodilator for the treatment of COPD. Drugs Today 38:585–600

Harvey RD, Belevych AE (2003) Muscarinic regulation of cardiac ion channels. Br J Pharmacol 139:1074–1084

Hegde SS (2006) Muscarinic receptors in the bladder: from basic research to therapeutics. Br J Pharmacol 147(Suppl 2):S80–S87

Heinrich JN, Butera JA, Carrick T, Kramer A, Kowal D, Lock T, Marquis KL, Pausch MH, Popiolek M, Sun SC, Tseng E, Uveges AJ, Mayer SC (2009) Pharmacological comparison of muscarinic ligands: historical versus more recent muscarinic M1-preferring receptor agonists. Eur J Pharmacol 605:53–56

Hemrich-Luecke SK, Bymaster FP, Evans DC, Wess J, Felder CC (2002) Muscarinic agonist-mediated increases in serum corticosterone levels are abolished in M_2 muscarinic acetylcholine receptor knockout mice. J Pharmacol Exp Ther 303:99–103

Hern JA, Baig AH, Mashanov GI, Birdsall B, Corrie JET, Lazareno S, Molloy JE, Birdsall NJM (2010) Formation and dissociation of M_1 muscarinic receptor dimers seen by total internal reflection fluorescence imaging of single molecules. Proc Natl Acad Sci USA 107:2693–2698

Hernandez CCQ, Barcellos LC, Gimenez L, Diaz E, Cabarcas RAB, Garcia S, Pedrosa RC, Nascimento JHM, Kurtenbach E, Mmasuda MO, Campos de Carvalho AC (2003) Human chagasic IgGs bind to cardiac muscarinic receptors and impair L-type Ca^{2+} currents. Cardiovasc Res 58:55–65

Himmel HM, Pietsch M, Uwe G, Eva M, Ravens U (2002) Changes in morphology and inward rectifier currents in human atrial myocytes depend on culture conditions. Basic Res Cardiol 97:434–444

Hock C, Maddalena A, Raschig A, Muller-Spahn F, Eschweiler G, Hager K, Heuser I, Hampel H, Muller-Thomsen T, Oertel W, Wienrich M, Signorell A, Gonzalez-Agosti C, Nitsch RM (2003) Treatment with the selective muscarinic m1 agonist talsaclidine decreases cerebrospinal fluid levels of A beta 42 in patients with Alzheimer's disease. Amyloid 10:1–6

Hulme EC, Lu ZL, Saldanha JW, Bee MS (2003) Structure and activation of muscarinic acetylcholine receptors. Biochem Soc Trans 31:29–34

Jakubík J, Bacáková L, el-Fakahany EE, Tucek S (1995) Constitutive activity of the M1-M4 subtypes of muscarinic receptors in transfected CHO cells and of muscarinic receptors in the heart cells revealed by negative antagonists. FEBS Lett 377:275–279

Jakubík J, Bacakova L, El-Fakahany EE, Tucek S (1997) Positive cooperativity of acetylcholine and other agonists with allosteric ligands on muscarinic acetylcholine receptors. Mol Pharmacol 52:172–179

Jakubík J, Michal P, Machová E, Dolezal V (2008) Importance and prospects for design of selective muscarinic agonists. Physiol Res 57(Suppl 3):S39–S47

Jin J, Budzik B, Wang Y, Shi D, Wang F, Xie H, Wan Z, Zhu C, Foley JJ, Webb EF, Berlanga M, Burman M, Sarau HM, Morrow DM, Moore ML, Rivero RA, Palovich M, Salmon M, Belmonte KE, Lain DI (2008) Discovery of biphenyl piperazines as novel and long acting muscarinic acetylcholine receptor antagonists. J Med Chem 51:5915–5918

Jöhren K, Höltje HD (2002) A model of the human M_2 muscarinic acetylcholine receptor. J Comput Aided Mol Des 16:795–801

Jones CK, Brady AE, Davis AA, Xiang Z, Bubser M, Tantawy NM, Kane AS, Bridges TM, Kennedy JP, Bradley SR, Peterson TE, Ansari MS, Baldwin RM, Kessler RM, Deutch AY, Lah JJ, Levey AI, Lindsley CW, Conn JP (2008) Novel selective allosteric activator of the M_1 muscarinic acetylcholine receptor regulates amyloid processing and produces antipsychotic-like activity in rats. J Neurosci 28:10422–10433

Karlsson E, Jolkkonen M, Mulugeta E, Onali P, Adem A (2000) Snake toxins with high selectivity for subtypes of muscarinic acetylcholine receptors. Biochimie 82:793–806

Kawashima K, Fujii T (2004) Expression of non-neuronal acetylcholine in lymphocytes and its contribution to the regulation of immune function. Front Biosci 9:2063–2085

Kenakin T (2007) Functional selectivity through protean and biased agonism: who steers the ship? Mol Pharmacol 72:1393–1401

Kenakin TP, Miller LJ (2010) Seven transmembrane receptors as shapeshifting proteins: the impact of allosteric modulation and functional selectivity on new drug discovery. Pharmacol Rev 62:265–304

Kingham PJ, Costello RW, McLean WG (2003) Eosinophil and airway nerve interactions. Pulm Pharmacol Ther 16:9–13

Kitazawa T, Asakawa K, Nakamura T, Teraoka H, Unno T, Komori S, Yamada M, Wess J (2009) M_3 muscarinic receptors mediate positive inotropic responses in mouse atria: a study with muscarinic receptor knockout mice. J Pharmacol Exp Ther 330:487–493

Kories C, Czyborra C, Fetscher C, Schneider T, Krege S, Michel MC (2003) Gender comparison of muscarinic receptor expression and function in rat and human urinary bladder: differential regulation of M_2 and M_3? Naunyn Schmiedebergs Arch Pharmacol 367:524–531

Langmead CJ, Fry VA, Forbes IT, Branch CL, Christopoulos A, Wood MD, Herdon HJ (2006) Probing the molecular mechanism of interaction between 4-*n*-butyl-1-[4-(2-methylphenyl)-4-oxo-1-butyl]-piperidine (AC-42) and the muscarinic M_1 receptor: direct pharmacological evidence that AC-42 is an allosteric agonist. Mol Pharmacol 2006(69):236–246

Langmead CJ, Watson J, Reavill C (2008a) Muscarinic acetylcholine receptors as CNS drug targets. Pharmacol Ther 117:232–243

Langmead CJ, Austin NE, Branch CL, Brown JT, Buchanan KA, Davies CH, Forbes IT, Fry VA, Hagan JJ, Herdon HJ, Jones GA, Jeggo R, Kew JN, Mazzali A, Melarange R, Patel N, Pardoe J, Randall AD, Roberts C, Roopun A, Starr KR, Teriakidis A, Wood MD, Whittington M, Wu Z, Watson J (2008b) Characterization of a CNS penetrant, selective M_1 muscarinic receptor agonist, 77-LH-28-1. Br J Pharmacol 154:1104–1115

Lanzafame AA, Christopoulos A, Mitchelson F (2003) Cellular signaling mechanisms for muscarinic acetylcholine receptors. Receptors Channels 9:241–260

Lazareno S, Popham A, Birdsall NJM (2002) Analogs of WIN 62,577 define a second allosteric site on muscarinic receptors. Mol Pharmacol 62:1492–1505

Lazareno S, Popham A, Birdsall NJM (2003) Progress toward a high-affinity allosteric enhancer at muscarinic M1 receptors. J Mol Neurosci 20:363–367

Levey AI (1993) Immunological localization of m1–m5 muscarinic acetylcholine receptors in peripheral tissues and brain. Life Sci 52:441–448

Liao D-L, Hong C-J, Chen H-M, Chen Y-E, Lee S-M, Chang C-Y, Chen H, Tsai S-J (2003) Association of muscarinic m1 receptor genetic polymorphisms with psychiatric symptoms and cognitive function in schizophrenic patients. Neuropsychobiology 48:72–76

Ma L, Seagera MA, Wittmanna M, Jacobsonb M, Bickela D, Burnoa M, Jones K, Kuzmick Graufeldsa V, Xua G, Pearsona M, McCampbella A, Gasparc R, Shughruec P, Danzigerc A, Reganc C, Flickb R, Pascarellab D, Garsond S, Dorand S, Kreatsoulase C, Vengc L, Lindsleye CW, Shipee W, Kuduke S, Surf C, Kinneyc G, Seabrooka GR, Ray WJ (2009) Selective activation of the M_1 muscarinic acetylcholine receptor achieved by allosteric potentiation. Proc Natl Acad Sci USA 106:15950–15955

Maltais F, Celli B, Porszasz J, Casaburi R, Gil EG, Caracta C (2010) Aclidinium bromide improves exercise endurance, dyspnea and inspiratory capacity in patients with moderate to severe COPD. Am J Respir Crit Care Med 181:A4428

Mansfield KJ, Mitchelson FJ, Moore KH, Burcher E (2003) Muscarinic receptor subtypes in the human colon: lack of evidence for atypical subtypes. Eur J Pharmacol 482:101–109

Mansfield KJ, Chandran JJ, Vaux KJ, Millard RJ, Christopoulos A, Mitchelson FJ, Burcher E (2009) Comparison of receptor binding characteristics of commonly used muscarinic antagonists in human bladder detrusor and mucosa. J Pharmacol Exp Ther 328:893–899

Marlo JE, Niswender CM, Days EL, Bridges TM, Xiang Y, Rodriguez AL, Shirey JK, Brady AE, Nalywajko T, Luo Q, Austin CA, Williams MB, Kim K, Williams R, Orton D, Brown HA, Lindsley CW, Weaver CD, Conn PJ (2009) Discovery and characterization of novel allosteric potentiators of M_1 muscarinic receptors reveals multiple modes of activity. Mol Pharmacol 75:577–588

Matsui M, Motomura D, Kurarsawa H, Fujikawa T, Jiang J, Yuriko K, Shin-ichi T, Makoto MT (2000) Multiple functional defects in peripheral autonomic organs in mice lacking muscarinic acetylcholine receptor gene for the M_3 subtype. Proc Natl Acad Sci USA 97:9579–9584

May LT, Avlani VA, Langmead CJ, Herdon HJ, Wood MD, Sexton PM, Christopoulos A (2007) Structure-function studies of allosteric agonism at M_2 muscarinic acetylcholine receptors. Mol Pharmacol 72:463–476

Mei L, Lai J, Yamamura HI, Roeske WR (1989) The relationship between agonist states of the M1 muscarinic receptor and the hydrolysis of inositol lipids in transfected murine fibroblast cells (B82) expressing different receptor densities. J Pharmacol Exp Ther 251:90–97

Michel MC (2008) Fesoterodine: a novel muscarinic receptor antagonist for the treatment of overactive bladder syndrome. Expert Opin Pharmacother 9:1787–1796

Michel AD, Whiting RL (1987) Direct binding studies on ileal and cardiac muscarinic receptors. Br J Pharmacol 92:755–767

Mirza NR, Peters D, Sparks RG (2003) Xanomeline and the anti-psychotic potential of muscarinic receptor subtype selective agonists. CNS Drug Rev 9:159–186

Miyakawa T, Yamada M, Duttaroy A, Wess J (2001) Hyperactivity and intact hippocampus-dependent learning in mice lacking the M_1 muscarinic acetylcholine receptor. Proc Natl Acad Sci USA 98:14096–14101

Miyamae K, Yoshida M, Murakami S, Iwashita H, Ohtani M, Masunaga K, Ueda S (2003) Pharmacological effects of darifenacin on human isolated urinary bladder. Pharmacology 69:205–211

Mohr K, Tränkle C, Holzgrabe U (2003) Structure/activity relationships of M_2 muscarinic allosteric modulators. Receptors Channels 9:229–240

Mohr M, Tränkle C, Kostenis E, Barocelli E, De Amici M, Holzgrabe U (2010) Rational design of dualsteric GPCR ligands: quests and promise. Br J Pharmacol 159:997–1008

Moreno L, Jacoby DB, Fryer AD (2003) Dexamethasone prevents virus-induced hyperresponsiveness via multiple mechanisms. Am J Physiol 285:L451–L455

Nawaratne V, Leach K, Felder CC, Sexton PM, Christopoulos C (2010) Structural determinants of allosteric agonism and modulation at the M_4 muscarinic acetylcholine receptor: identification of ligand-specific and global activation mechanisms. J Biol Chem 285(25):19012–19021

Ney P, Pandita RK, Newgreen DT, Breidenbach A, Stöhr T, Andersson K-E (2008) Pharmacological characterization of a novel investigational antimuscarinic drug, fesoterodine, in vitro and in vivo. Invest Urol 101:1036–1042

Nirish SK, Cheng SK, Raufman J-P (2009) Muscarinic receptors and ligands in cancer. Am J Physiol Cell Physiol 296:C221–C232

Nomura J, Hosoi T, Okuma Y, Nomura Y (2003) The presence and functions of muscarinic receptors in human T cells: the involvement in IL-2 and IL-2 receptor system. Life Sci 72:2121–2126

Novi F, Marco S, Corsini GU, Maggio R (2004) Receptor oligomerization and homologous receptor down-regulation. J Biol Chem 279:7476–7486

Park PS-H, Wells JW (2003) Monomers and oligomers of the M_2 muscarinic cholinergic receptor purified from Sf9 cells. Biochemistry 42:12960–12971

Pelman RS, Capo JP Jr, Forero-Schwanhaeuser S (2008) Solifenacin at 3 years: a review of efficacy and safety. Postgrad Med 120:85–91

Peralta EG, Ashkenazi A, Winslow JW, Ramachandran J, Capon DJ (1988) Differential regulation of PI hydrolysis and adenylyl cyclase by muscarinic receptor subtypes. Nature 334:434–437

Peretto I, Petrillo P, Imbimbo BP (2009) Medicinal chemistry and therapeutic potential of muscarinic M_3 antagonists. Med Res Rev 29:867–902

Ponicke K, Heinroth-Hoffmann I, Brodde O-E (2003) Demonstration of functional M3-muscarinic receptors in ventricular cardiomyocytes of adult rats. Br J Pharmacol 138:156–160

Poulin B, Butcher A, McWilliams P, Bourgognon J-M, Pawlak R, Kong KC, Bottrill A, Mistry S, Wess J, Rosethorne EM, Charlton SJ, Tobin AB (2010) The M_3-muscarinic receptor regulates learning and memory in a receptor phosphorylation/arrestin-dependent manner. Proc Natl Acad Sci USA 107:9440–9445

Presland J (2005) Identifying novel modulators of G protein-coupled receptors via interaction at allosteric sites. Curr Opin Drug Discov Devel 8:567–576

Raes A, Hoebeke P, Segaert I, Van Laekck E, Dehoorne J, Vande WJ (2004) Retrospective analysis of efficacy and tolerability of tolterodine in children with overactive bladder. Eur Urol 45:240–244

Rasmussen T, Fink-Jensen A, Sauerberg P, Swedberg MD, Thomsen C, Sheardown MJ, Jeppesen L, Calligaro DO, DeLapp NW, Whitesitt C, Ward JS, Shannon HE, Bymaster FP (2001) The muscarinic receptor agonist BuTAC, a novel potential antipsychotic, does not impair learning and memory in mouse passive avoidance. Schizophr Res 15:193–201

Ray NC, Alcaraz L (2009) Muscarinic antagonist-β-adrenergic agonist dual pharmacology molecules as bronchodilators: a patent review. Expert Opin Ther Pat 19:1–12

Roszkowski AP (1961) An unusual type of sympathetic ganglionic stimulant. J Pharmacol Exp Ther 132:156–170

Salcedo C, Davalillo S, Cabellos J, Lagunas C, Balsa S, Pérez-del-Pulgar D, Ballarín M, Fernández AG (2009) In vivo and in vitro pharmacological characterization of SVT-40776, a novel M_3 muscarinic receptor antagonist, for the treatment of overactive bladder. Br J Pharmacol 156:807–817

Sarria B, Naline E, Zhang Y, Cortijo J, Molimard M, Moreau J, Therond P, Advenier C, Esteban J (2002) Muscarinic M_2 receptors in acetylcholine-isoproterenol functional antagonism in human isolated bronchus. Am J Physiol 283:L1125–L1132

Sastry BV, Sadavongvivad C (1978) Cholinergic systems in non-nervous tissues. Pharmacol Rev 30:65–132

Scarr E (2009) Muscarinic receptors in psychiatric disorders – can we mimic 'health'? Neurosignals 17:298–310

Schwarz RD, Davis RE, Jaen JC, Spencer CJ, Tecle H, Thomas AJ (1993) Characterization of muscarinic agonists in recombinant cell lines. Life Sci 52:465–472

Servent D, Fruchart-Gaillard C (2009) Muscarinic toxins: tools for the study of the pharmacological and functional properties of muscarinic receptors. J Neurochem 109:51193–51202

Shah N, Khurana S, Cheng K, Raufman J-P (2009) Muscarinic receptors and ligands in cancer. Am J Physiol Cell Physiol 296:221–232

Sheardown MJ (2002) Muscarinic M_1 receptor agonists and M_2 receptor antagonists as therapeutic targets in Alzheimer's disease. Expert Opin Ther Pat 12:863–870

Shekhar A, Potter WZ, Lightfoot J, Lienemann J, Dubé S, Mallinckrodt C, Bymaster FP, McKinzie DL, Felder CC (2008) Selective muscarinic receptor agonist xanomeline as a novel treatment approach for schizophrenia. Am J Psychiatry 165:1033–1039

Shi Y, Oury F, Yadav VK, Wess J, Liu XS, Guo XE, Murshed M, Karsenty G (2010) Signaling through the M_3 muscarinic receptor favors bone mass accrual by decreasing sympathetic activity. Cell Metab 11:231–238

Shirey JK, Brady AE, Jones PJ, Davis AA, Bridges TM, Kennedy JP, Jadhav SB, Menon UN, Xiang Z, Watson ML, Christian EP, Doherty JJ, Quirk MC, Snyder DH, Lah JJ, Levey AI, Nicolle MM, Lindsley CW, Conn PJ (2009) A selective allosteric potentiator of the M_1 muscarinic acetylcholine receptor increases activity of medial prefrontal cortical neurons and restores impairments in reversal learning. J Neurosci 229:14271–14286

Spalding TA, Burstein ES (2006) Constitutive activity of muscarinic acetylcholine receptors. J Recept Signal Transduct 26:61–85

Spalding TA, Trotter C, Skjaebaek N, Messier TL, Currier EA, Burstein EA, Burstein ES, Li D, Hacksell U, Brann MR (2002) Discovery of an ectopic activation site on the M_1 muscarinic receptor. Mol Pharmacol 61:1297–1302

Spalding TA, Ma JN, Ott TR, Friberg M, Bajpai A, Bradley SR, Davis RE, Brann MR, Burstein ES (2006) Structural requirements of transmembrane domain 3 for activation by the M_1 muscarinic receptor agonists AC-42, AC-260584, clozapine, and *N*-desmethylclozapine: evidence for three distinct modes of receptor activation. Mol Pharmacol 70:1974–1983

Stahl E, Ellis J (2010) Novel allosteric effects of amiodarone at the muscarinic M_5 receptor. J Pharmacol Exp Ther 334(1):214–222

Steidl S, Yeomans JS (2009) M_5 muscarinic receptor knockout mice show reduced morphine-induced locomotion but increased locomotion after cholinergic antagonism in the ventral tegmental area. J Pharmacol Exp Ther 328:263–275

Steinfeld T, Mammen M, Smith JAM, Wilson RD, Jasper JR (2007) A novel multivalent ligand that bridges the allosteric and orthosteric binding sites of the M_2 muscarinic receptor. Mol Pharmacol 72:291–302

Steinfeld T, Pulido-Rios MT, Chin K, King K, Huang JX, Lee TW, Jasper JR, Ji Y, Hegde S, Mammen M (2009) In vitro characterization of TD-4208, a lung-selective and long-acting muscarinic antagonist bronchodilator. Am J Respir Crit Care Med 179:A4553

Stengel PW, Gomez J, Wess J, Cohen M (2000) M_2 and M_4 receptor knockout mice: muscarinic receptor function in cardiac and smooth muscle in vitro. J Pharmacol Exp Ther 292:877–885

Struchmann N, Wiegand S, Gschnell A, Masashisa K, Kummer W, Wess J, Haberberger RV (2003) Role of muscarinic receptor subtypes in the constriction of peripheral airways, studies on receptor deficient mice. Mol Pharmacol 64:1444–1451

Sur C, Mallorga PJ, Wittman M, Jacobson MA, Pascarella D, Williams JB, Brandish PE, Pettibone DJ, Scolnick EM, Conn JP (2003) *N*-desmethylclozapine, an allosteric agonist at muscarinic 1 receptor, potentiates *N*-methyl-D-aspartate receptor activity. Proc Natl Acad Sci USA 100: 13674–13679

Swedberg MD, Sheardown MJ, Sauerberg P, Olesen PH, Suzdak PD, Hansen KT, Bymaster FP, Ward JS, Mitch CH, Calligaro DO, DeLapp NW, Shannon HE (1997) Butylthio[2.2.2] (NNC11-1053/LY297802): an orally active muscarinic agonist analgesic. J Pharmacol Exp Ther 281:876–883

Tayebati SK, Di Tullio MA, Tomassoni D, Amenta F (2003) Localization of the m5 muscarinic cholinergic receptor in rat circle of Willis and pial arteries. Neuroscience 122:205–211

Thomas EA, Baker SA, Ehlert FJ (1993) Functional role for the M_2 muscarinic receptor in smooth muscle of guinea pig ileum. Mol Pharmacol 44:102–110

Thomas RL, Langmead CJ, Wood MD, Challiss RA (2009) Contrasting effects of allosteric and orthosteric agonists on m1 muscarinic acetylcholine receptor internalization and down-regulation. J Pharmacol Exp Ther 331:1086–1095

Thomas DR, Dada A, Jones GA, Deisz RA, Gigout S, Langmead CJ, Werry TD, Hendry N, Hagan JJ, Davies CH, Watson JM (2010) *N*-desmethylclozapine (NDMC) is an antagonist at the human native muscarinic M(1) receptor. Neuropharmacology 58:1206–1214

Tran JA, Chang A, Matsui M, Ehlert FJ (2009) Estimation of relative microscopic affinity constants of agonists for the active state of the receptor in functional studies on M_2 and M_3 muscarinic receptors. Mol Pharmacol 75:381–396

Trendelenburg A-U, Gomeza J, Klebroff W, Zhou H, Wess J (2003) Heterogeneity of presynaptic muscarinic receptors mediating inhibition of sympathetic transmitter release: a study with M_2- and M_4-receptor-deficient mice. Br J Pharmacol 138:469–480

Tzarvara ET, Bymaster FP, Felder CC, Wade M, Gomeza J, Wess J, McKinzie DL, Nomikos GG (2003) Dysregulated hippocampal acetylcholine neurotransmission and impaired cognition in M_2, M_4 and M_2/M_4 muscarinic receptor knockout mice. Mol Psychiatry 8:673–679

Unno T, Matsuyama H, Komori S (2003) Interaction between the M_2- and M_3-receptor subtypes in muscarinic electrical and mechanical responses of intestinal smooth muscles. Neurophysiology 35:262–273

van Richards MH, Giersbergen PL (1995) Human muscarinic receptors expressed in A9L and CHO cells: activation by full and partial agonists. Br J Pharmacol 114:1241–1249

Verhein KC, Fryer AD, Jacoby DB (2009) Neural control of airway inflammation. Curr Allergy Asthma Rep 9:1534–6315

Vilaro MT, Palacios JM, Mengod G (1990) Localization of m5 muscarinic receptor mRNA in rat brain examined by in situ hybridization histochemistry. Neurosci Lett 114:154–159

Voigtlander U, Johren K, Mohr M, Raasch A, Trankle C, Buller S, Ellis J, Holtje HD, Mohr K (2003) Allosteric site on muscarinic acetylcholine receptors: identification of two amino acids in the muscarinic M_2 receptor that account entirely for the M_2/M_5 subtype selectivities of some structurally diverse allosteric ligands in *N*-methylscopolamine-occupied receptors. Mol Pharmacol 64:21–31

Volpicelli LA, Levey AI (2004) Muscarinic acetylcholine receptor subtypes in cerebral cortex and hippocampus. Prog Brain Res 145:59–66

Wang SZ, el-Fakahany EE (1993) Application of transfected cell lines in studies of functional receptor subtype selectivity of muscarinic agonists. J Pharmacol Exp Ther 266:237–243

Wess J (1996) Molecular biology of muscarinic acetylcholine receptors. Crit Rev Neurobiol 10:69–99

Wess J (2004) Muscarinic acetylcholine receptor knockout mice: novel phenotypes and clinical implications. Annu Rev Pharmacol Toxicol 44:423–450

Wess J (2005) Allosteric binding sites on muscarinic acetylcholine receptors. Mol Pharmacol 6:1506–1509

Wess J, Duttarov A, Gomeza J, Zhang W, Yamada M, Felder CC, Bernardini N, Reeh PW (2003) Muscarinic receptor subtypes mediating central and peripheral antinociception studied with muscarinic receptor knockout mice: a review. Life Sci 72:18–19

Wess J, Eglen RM, Gautam D (2007) Muscarinic acetylcholine receptors: mutant mice provide new insights for drug development. Nat Rev Drug Discov 6:721–733

Wessler I, Kirkpatrick CJ (2008) Acetylcholine beyond neurons: the non-neuronal cholinergic system in humans. Br J Pharmacol 154:1558–1571

Wessler I, Kirkpatrick CJ, Racke K (1998) Non-neuronal acetylcholine, a locally acting molecule, widely distributed in biological systems: expression and function in humans. Pharmacol Ther 77:59–79

Wood MD, Murkitt KL, Ho M, Watson JM, Brown F, Hunter AJ, Middlemiss DN (1999) Functional comparison of muscarinic partial agonists at muscarinic receptor subtypes hM_1, hM_2, hM_3, hM_4 and hM_5 using microphysiometry. Br J Pharmacol 126:1620–1624

Yoshida M, Masunaga K, Satoji Y, Maeda Y, Nagata T, Inadome A (2008) Basic and clinical aspects of non-neuronal acetylcholine: expression of non-neuronal acetylcholine in urothelium and its clinical significance. J Pharmacol Sci 106:193–198

Yoshida M, Masunaga K, Nagata T, Yono M, Homma Y (2010) The forefront for novel therapeutic agents based on the pathophysiology of lower urinary tract dysfunction: pathophysiology and pharmacotherapy of overactive bladder. J Pharmacol Sci 112:128–134

Zholos AV, Tsvilovskyy VV, Bolton TB (2003) Muscarinic cholinergic excitation of smooth muscle: signal transduction and single cationic channel properties. Neurophysiology 35: 283–301

Zholos AV, Tsystsyura YD, Gordienko DV, Tsvilovskyy VV, Bolton TB (2004) Phospholipase C, but not $InsP_3$ or DAG, -dependent activation of the muscarinic receptor-operated cation current in guinea-pig ileal smooth muscle cells. Br J Pharmacol 141:23–36

Zinner N (2007) Darifenacin: a muscarinic M_3-selective receptor antagonist for the treatment of overactive bladder. Expert Opin Pharmacother 8:511–523

Structure–Function Studies of Muscarinic Acetylcholine Receptors

Katie Leach, John Simms, Patrick M. Sexton, and Arthur Christopoulos

Abstract There has been great interest in the structure–function relationships of the muscarinic acetylcholine receptors (mAChRs) because these prototypical Family A/class 1 G protein-coupled receptors (GPCRs) are attractive therapeutic targets for both peripheral and central nervous system disorders. A multitude of drugs that act at the mAChRs have been identified over the years, but many of these show minimal selectivity for any one of the five mAChR subtypes over the others, which has hampered their development into therapeutics due to adverse side effects. The lack of drug specificity is primarily due to high sequence similarity in this family of receptor, especially in the orthosteric binding pocket. Thus, there remains an ongoing need for a molecular understanding of how mAChRs bind their ligands, and how selectivity in binding and activation can be achieved. Unfortunately, there remains a paucity of solved high-resolution structures of GPCRs, including the mAChRs, and thus most of our knowledge of structure–function mechanisms related to this receptor family to date has been obtained indirectly through approaches such as mutagenesis. Nonetheless, such studies have revealed a wealth of information that has led to novel insights and may be used to guide future rational drug design campaigns.

Keywords Allosteric • Molecular modeling • Muscarinic receptor • Mutagenesis • Orthosteric • Structure-function

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1 Introduction

GPCRs comprise the largest family of membrane proteins in mammalian organisms (Fredriksson et al. 2003) and respond to a wide range of endogenous and exogenous ligands. Mammalian GPCRs are divided into three main classes based on similarities in their amino acid sequence (Foord et al. 2005). Family A (or class 1) GPCRs, which include the mAChRs, share sequence similarity to rhodopsin; Family B (class 2) GPCRs to secretin receptors; Family C (class 3) GPCRs to metabotropic glutamate receptors. Less than 10% of these three GPCR families already constitute the targets of approximately 30% of all drugs on the market (Harmar et al. 2009; Hopkins and Groom 2002) and, thus, there is ongoing incentive in understanding how the amino acid sequence of these proteins relates to their function and three-dimensional structure in order to facilitate drug discovery. The characteristic structural feature of all these receptors is the presence of an extracellular N-terminal region, intracellular C-terminal region and seven transmembrane (TM)-spanning α-helical domains connected by three extracellular and three intracellular loops. However, until very recently, detailed three-dimensional structural information on GPCRs has been hampered by difficulties in obtaining high-resolution crystal structures of these receptors. This is because they are highly unstable upon removal from their membranous environment and also because they dynamically isomerize between multiple conformations, both of which hinder the crystallization process (Congreve and Marshall 2010). To date, crystallization efforts have been successful for only a few GPCRs; rhodopsin (Palczewski et al. 2000), the β_1 (Warne et al. 2008) and β_2 (Cherezov et al. 2007; Rasmussen et al. 2007; Rosenbaum et al. 2007) adrenergic receptors (β-ARs), and the A_{2A} adenosine receptor (Jaakola et al. 2008).[1] As a consequence, computational approaches, such as homology modeling and associated methods, are the mainstay of rationalizing structural information derived at other GPCRs, such as the mAChR family. However, molecular models, in and of themselves, have only limited utility if not used in conjunction with molecular and biophysical techniques that can help to refine our structural and functional understanding of a protein. In this regard, the mAChRs remain a prototypical Family A GPCR model system that has been extensively explored by site-directed mutagenesis and related approaches, which is the focus of this chapter.

2 Amino Acids That Are Essential for Stabilization of the Receptor Structure

Substitution of amino acids that are essential for the structural stability and folding of a protein can lead to impairment in its assembly, maturation, and/or trafficking. In the mAChRs, substitutions of certain amino acid residues that are conserved

[1] At the time of writing, the crystal structures of antagonist-bound chemokine CXCR4 and dopamine D_3 receptors have been solved but not published.

across all five mAChR subtypes cause a reduction in receptor expression, in particular mutation of $Asp^{2.50}$, $Leu^{3.43}$, $Asp^{3.49}$, $Tyr^{3.51}$, $Trp^{4.50}$, and $Pro^{7.50}$ (Hulme et al. 2001, 2003a; Lu et al. 1997, 2001; Lu and Hulme 1999) [numbering in superscript corresponds to the Ballesteros–Weinstein system (Ballesteros et al. 1995)]. In fact, these residues are highly conserved throughout the TM domains of Family A GPCRs and thus likely serve an important role in maintaining the overall helical structure of these receptors. For instance, in the rhodopsin X-ray crystal structure, $Asp^{2.50}$ interacts with $Asn^{1.50}$ and $Asn^{7.49}$ in a hydrogen-bonded network of residues mediated by water molecules that may assist in the initial folding of the receptor, whilst at later stages of the receptor lifetime these residues are implicated in signaling cascades. $Asp^{3.49}$ and $Tyr^{3.51}$ are two additional residues that are essential for the function of the majority of Family A GPCRs. In the mAChRs, only His, Asn, or Glu substitutions are tolerated at the position of $Asp^{3.49}$, although even in these instances receptor expression levels are reduced. Substitution with any other amino acid at this position generally results in undetectable levels of radioligand binding (Lu et al. 1997), suggesting that this residue is critical for maintaining a receptor conformation able to bind ligand.

In addition to traditional approaches that have relied on rationally guided systematic mutagenesis of the mAChR, a more recent, higher-throughput, random mutagenesis study identified a number of additional mutations that profoundly affected the expression of the M_3 mAChR (Li et al. 2007a), suggesting that there remains much to be learned about the structural determinants of mAChR stability

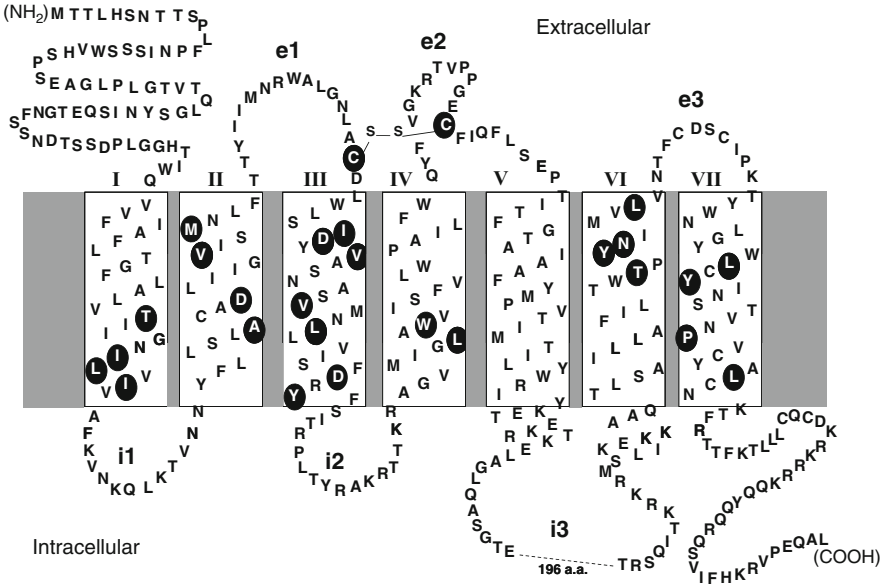

Fig. 1 Snake diagram of the M_3 mAChR. Residues labeled within the *black circles* indicate amino acids that have been implicated in the control of receptor expression

and expression. Many of these substitutions were for proline or charged amino acids, which are likely to disrupt important interactions that are essential for the folding of the receptor, including Thr$^{1.46}$Pro, Ile$^{1.51}$Asn, Leu$^{1.52}$Pro, Ile$^{1.54}$Asn, Ala$^{2.47}$Pro, Val$^{2.55}$Asp, Cys^{140}Gly, Ile$^{3.31}$Asp, Val$^{3.34}$Glu, Leu$^{4.48}$Pro, Cys^{220}Ser, Thr$^{6.49}$Asn, Leu$^{6.56}$Pro, Leu$^{6.56}$Gln, Leu$^{7.41}$Pro, and Leu$^{7.55}$Pro (Fig. 1). Interestingly, mapping these positions onto models of the mAChR revealed that they are adjacent, in either 2D or 3D space, to conserved residues and may affect their local environment. In contrast to the above mutations, another series of positions (Met$^{4.43}$, Leu$^{4.46}$, Leu$^{4.49}$, Ala$^{4.58}$, Phe$^{4.61}$, and Thr$^{7.47}$) have also been shown to contribute to receptor conformation(s) that can result in an increase in the amount of cell surface expression.

3 Elucidation of the mAChR Orthosteric Binding Site

A wide range of structurally diverse ligands bind to the orthosteric site of GPCRs and, as such, a number of different domains may potentially form the ligand binding pocket, depending on the receptor. To date the high-resolution X-ray crystal structures for orthosteric inverse agonist-bound rhodopsin, the β_1- and β_2-ARs, and the A_{2A} adenosine receptor, have all been solved (Palczewski et al. 2000; Cherezov et al. 2007; Rasmussen et al. 2007; Jaakola et al. 2008). Rhodopsin is not a typical GPCR in that its ligand, 11-*cis*-retinal, is covalently bound to Lys$^{7.43}$ via a Schiff base in the inactive form of the receptor. 11-*cis*-retinal also makes a number of additional contacts within the receptor that contribute to a binding pocket that shares similarities with the orthosteric binding site in the β-ARs (Rasmussen et al. 2007), being comprised of residues positioned predominantly in TMIII, TMV, and TMVI. Residues that form the binding crevice in rhodopsin and the β-ARs include 3.28, 3.29, 3.32, 3.33, 3.35, 3.36, 3.37, 5.41, 5.42, 5.43, 5.46, 5.47, 6.44, 6.48, 6.51, 6.52, 6.55, 7.35, 7.39, and 7.40. In contrast, the binding site for the A_{2A} receptor antagonist, ZM241385, is somewhat different and involves residues predominantly located in TMII, TMVI, and TMVII (Jaakola et al. 2008). Mutagenesis data support the hypothesis that the orthosteric binding site in mAChRs closely resembles that of rhodopsin and the β-ARs. Some of the first studies that investigated the location of the ACh binding site in mAChRs involved propylbenzilylcholine and acetylcholine mustards. These alkylating agents were used to highlight an important interaction that occurs between the common ammonium moiety that exists in all biogenic amines and Asp$^{3.32}$ (Curtis et al. 1989; Spalding et al. 1994; Kurtenbach et al. 1990), conserved within TMIII of the biogenic amine receptors. A series of site-directed mutagenic studies have since identified additional amino acids that are equally critical for the binding of ACh to the mAChRs (Lu et al. 2001; Wess et al. 1991; Ward et al. 1999). Generation of a homology model of the M_1 mAChR based on the structure of bovine rhodopsin has predicted residues that most probably form direct contact points for ACh (Fig. 2a), including five residues in particular: Tyr$^{3.33}$, Thr$^{5.39}$, Thr$^{5.42}$, Tyr$^{6.51}$, and Tyr$^{7.39}$

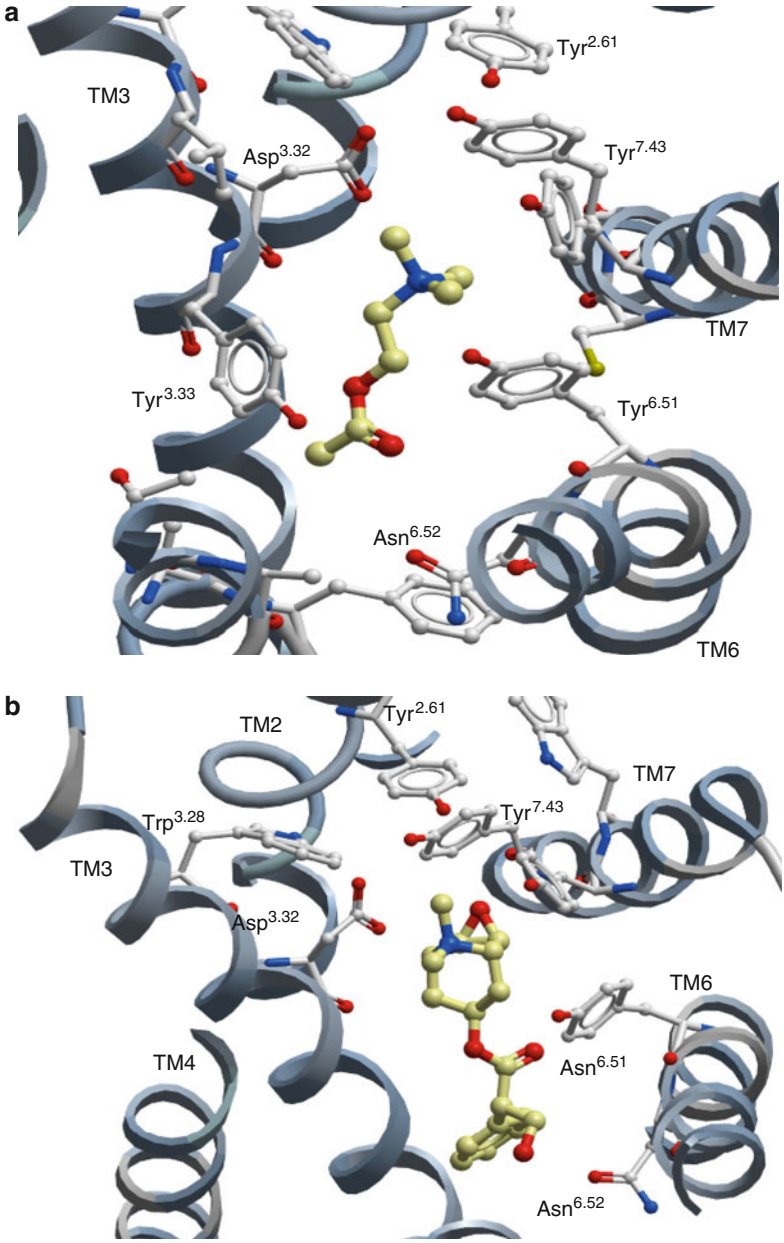

Fig. 2 The orthosteric binding pocket of mAChRs. A homology model of the M_2 mAChR was constructed using the crystal structure of the inactive-state β_2 adrenergic receptor as a template. Docking of (**a**) ACh or (**b**) NMS was performed and the key residues contributing to the respective pockets are also indicated

(Hulme et al. 2003a, b). Further residues that have been implicated in the binding of ACh have also been identified as $Trp^{3.28}$, $Leu^{3.29}$, $Ser^{3.36}$, $Asn^{3.37}$, $Trp^{4.50}$, $Ser^{4.53}$, $Trp^{4.57}$, $Ala^{5.46}$, $Leu^{6.56}$, $Cys^{7.42}$, and $Tyr^{7.43}$ (Hulme et al. 2003a, b; Lu et al. 2001; Hulme and Lu 1998). However, visual inspection of homology models of the mAChR suggests that some of these amino acids lie outside of the orthosteric site and may thus affect the route of entry for the ligand into its main binding site crevice.

Many of the residues that are essential for ACh binding are equally as important for the binding of inverse agonists/antagonists such as *N*-methyl scopolamine (NMS), quinuclidinyl benzilate (QNB) and atropine to the mAChRs (Fig. 2b), although some subtle differences have been observed with regards to amino acids that contribute to the binding of these different ligands. For instance, although $Asn^{6.52}$ is predicted to face into the ligand binding pocket and is important for the binding of atropine and NMS (Ward et al. 1999; Bluml et al. 1994a), it has a lesser role in ACh and QNB binding (Bluml et al. 1994a). Similarly, the binding of QNB is not significantly affected by substitution of $Tyr^{6.51}$ (Ward et al. 1999), $Tyr^{7.39}$, $Cys^{7.42}$, or $Tyr^{7.43}$ (Lu et al. 2001). $Phe^{5.47}$, on the other hand, which does not appear to interact with ACh and QNB, has been predicted to lie in close proximity to NMS and may be positioned at the very bottom of the NMS binding site, which extends deeper into the helical bundle than the ACh binding site (Goodwin et al. 2007). Thus, different ligands clearly form molecular interactions with different amino acid residues.

In addition to the role of the TM domains in binding orthosteric ligands, there is some evidence that the extracellular domains of the mAChRs may contribute structural stability to the orthosteric binding site. Family A GPCRs possess two conserved cysteine residues that form a disulfide bond between the extracellular portion of TMIII and the second extracellular loop of the receptors. In rhodopsin, part of the second extracellular loop folds into the center of the helical bundle, with Glu^{181} (residues that lie outside the TM domains are indicated by their amino acid position) orientated toward 11-*cis*-retinal (Palczewski et al. 2000). Similarly, $Thr^{5.34}$ at the junction of TMV and the second extracellular loop in the β_1-AR is directed toward the ligand binding pocket, suggesting that this extracellular region may form a "cap" to that pocket. Thus, in most mAChR structural models that are based on homology with rhodopsin or the β-ARs, the second extracellular loop of these receptors defines a boundary of the orthosteric binding site that forms a lid-like structure over the top of the crevice. Although substitution of amino acid residues in the second extracellular loop does not significantly alter the binding affinity of prototypical orthosteric ligands, restriction of flexibility of this region in the M_2 mAChR (via engineering of an additional disulfide bond) was shown to substantially hinder the access of ligands such as NMS and ACh to the orthosteric binding site (Avlani et al. 2007). Residues that lie in close proximity to the cysteine residues responsible for the conserved disulfide bond have additionally been implicated in regulating the access of orthosteric ligands into the binding pocket. For instance, substitution of $Asp^{3.26}$ reduces the binding of orthosteric ligands such as ACh, QNB, and NMS (Goodwin et al. 2007). It has been speculated that this

residue may be involved in the initial contact of positively charged orthosteric muscarinic ligands with their receptors before they enter into the main binding site (Lu et al. 2001; Goodwin et al. 2007; Jakubik et al. 2000). Structural predictions of the M_1 mAChR suggest that $Ser^{4.53}$, $Trp^{4.57}$, and $Ile^{4.61}$ lie in close proximity to $Asp^{3.26}$ and may also act to form this "peripheral" binding site (Lu et al. 2001).

The boundary between the top extracellular portion of TMII and the first extracellular loop in the mAChRs has also been implicated in the binding affinity of orthosteric ligands, with substitution of Trp^{99} in the M_1 mAChR for Ala or Phe, or the equivalent Trp^{133} for Gly in the M_3 mAChR, significantly reducing the binding affinity of ACh, NMS, and QNB (Li et al. 2007a; Matsui et al. 1995; Avlani et al. 2010). Similarly, mutation of $Asn^{2.68}$ Ile at the junction of TMII and the first extracellular loop in the M_3 mAChR results in a reduction in the binding of [^{3}H]NMS (Li et al. 2007a). Interestingly, recent in silico studies have suggested that the extracellular loops can have a strong influence on how TM helices pack together and, as such, perturbation of the extracellular loops may have an additional effect on the fine packing in the TM helices; it is thus possible that effects of extracellular loop mutations on orthosteric ligand binding may reflect such indirect perturbations of the orthosteric pocket.

4 Elucidation of mAChR Allosteric Binding Sites

In addition to the orthosteric binding site, it is now well established that GPCRs can possess topographically distinct allosteric sites (May et al. 2007a). Indeed, studies of the phenomenon at the mAChRs represent the earliest known examples in the field, dating back to the late 1960s and early 1970s when investigators described noncompetitive interactions between orthosteric mAChR agonists and the neuromuscular blocking agent, gallamine, or certain alkane-bis-ammonium compounds, exemplified by C_7/3-phth (Clark and Mitchelson 1976; Lullmann et al. 1969). Since that time, the actions of additional allosteric mAChR modulators have been characterized (Stockton et al. 1983; Lazareno and Birdsall 1995; Lazareno et al. 1998). Although beyond the scope of this chapter, it should be noted that there now exists a relatively rich, and expanding, allosteric pharmacology around the mAChRs, including prototypical negative allosteric modulators, such as gallamine and C_7/3-phth, as well as positive modulators of ACh, such as brucine and BQCA at the M_1 mAChR, LY2033298 at the M_4 mAChR, and VU0238429 at the M_5 mAChR (Lazareno et al. 1998; Birdsall et al. 1999; Chan et al. 2008; Gharagozloo et al. 1999; Leach et al. 2010; Ma et al. 2009; May et al. 2007b). In recent years, a number of putative allosteric agonists, which can activate the receptor in their own right, have also been identified (Chan et al. 2008; Leach et al. 2010; Ma et al. 2009; May et al. 2007b; Nawaratne et al. 2008; Jones et al. 2008; Langmead et al. 2006; Spalding et al. 2006; Sur et al. 2003; Thomas et al. 2008; Bridges et al. 2009).

There is compelling pharmacological evidence indicating that there are at least two allosteric binding sites on the mAChRs that can be targeted by small

molecule ligands. The best characterized site, referred to herein as the "prototypical modulator site," recognizes compounds such as gallamine, C_7/3-phth, brucine and alcuronium, whereas the "second" allosteric site binds certain indolocarbazoles and the benzimidazole analogs, WIN 51,708 and WIN 62,577 (Lazareno et al. 2000; Lanzafame et al. 2006). To date, all mutagenesis studies of mAChR allosteric binding sites have focused on the prototypical modulator site, which is believed to comprise epitopes that are more extracellularly located than those within the TM-bound orthosteric pocket. The location of the "second" allosteric site is currently unknown, although a molecular modeling study has suggested an intracellular location (Espinoza-Fonseca and Trujillo-Ferrara 2005, 2006).

Given that many prototypical modulators interact with all five mAChR subtypes, it is likely that some conserved residues may be involved in their actions. An early study at the M_1 mAChR proposed that $Trp^{3.28}$ and $Trp^{7.35}$, which lie at the extracellular end of TMIII and TMVII, respectively, may serve such a role, at least with respect to the binding of gallamine (Matsui et al. 1995). However, another key aspect of allosteric modulator action is that these compounds typically display greater degrees of selectivity across mAChR subtypes than do orthosteric ligands, and thus nonconserved amino acids must also contribute to modulator binding and/or actions. For example, gallamine binds with higher affinity to the M_2 mAChR than to the other mAChR subtypes and this preference has been attributed, in part, to the interaction of gallamine with residues located in the second extracellular loop of the M_2 mAChR, predominantly Tyr^{177} and to a lesser degree ^{172}Glu-Asp-Gly-Glu^{175}, as well as residues at the junction of the third extracellular loop and the top of TMVII, namely $Asn^{7.32}$, $Trp^{7.35}$, and $Thr^{7.36}$ (May et al. 2007b; Voigtlander et al. 2003; Huang et al. 2005; Prilla et al. 2006; Valant et al. 2008). Similarly, in the M_4 mAChR, $Ser^{7.36}$ has been implicated in gallamine binding (Buller et al. 2002), whilst $Glu^{7.32}$ in the M_1 mAChR has been implicated in the transmission of positive cooperativity between brucine and ACh (Stewart et al. 2010).

The binding site for the allosteric modulator/agonist, LY2033298, may also overlap with the prototypical allosteric site, because the interaction between LY2033298 and C_7/3-phth appears competitive (Leach et al. 2010). In support of this hypothesis, alanine substitution of Phe^{186} in the second extracellular loop of the M_4 mAChR, which corresponds to Tyr^{177} in the M_2 mAChR, markedly attenuates the binding of LY2033298 (Nawaratne et al. 2010). Interestingly, the equivalent position in the M_1 and M_3 mAChRs is also an aromatic residue, suggesting that aromaticity is an important characteristic in this region of the second extracellular loop of most mAChRs. Moreover, alanine substitution of the Tyr in this position of the M_1 mAChR extracellular loop greatly diminished the potency of BQCA as an allosteric modulator of ACh (Ma et al. 2009). Also in agreement with prior studies on the M_1 mAChR that focused on gallamine, substitution of the conserved $Trp^{3.28}$ and $Leu^{3.29}$ in the M_4 mAChR with alanine decreased the affinity of both C_7/3-phth and LY2033298 (Leach et al. 2011). However, alanine substitution of $Asp^{7.32}$ in the M_4 mAChR to the corresponding $Asn^{7.32}$ in the M_2 mAChR had no significant effect on the binding affinity of LY2033298 (Chan et al. 2008). Similarly, mutation

of $Ser^{7.36}$ (M_4 mAChR) to the corresponding $Thr^{7.36}$ in the M_2 mAChR did not alter the interaction between LY2033298 and ACh (Chan et al. 2008). Thus, as with orthosteric ligands, it appears that allosteric ligands can recognize a common site but, nonetheless, adopt different poses within that site such that they display differential sensitivity to specific mutations.

More recently, a novel class of ligand has been described that can bridge *both* orthosteric and allosteric sites concomitantly. Such ligands have been termed "bitopic," and it is possible that a number of putative "allosteric agonists" may actually fall into this category (Voigtlander et al. 2003). A good example of this phenomenon has been noted with the functionally selective mAChR agonist, McN-A-343. Although exhibiting many properties commensurate with a competitive (orthosteric) mode of action, there have been provocative examples in the literature to suggest that McN-A-343 can also interact allosterically with the M_2 mAChR (May et al. 2007b; Birdsall et al. 1983; Waelbroeck 1994). A subsequent study revealed that the molecule is actually a hybrid composed of orthosteric (trimethylammonium) and allosteric (3-chorophenylcarbamate) moieties (Lanzafame et al. 2006), thus providing a possible explanation of previous studies; depending on the experimental conditions, McN-A-343 can adopt a binding pose that bridges both orthosteric and allosteric sites (Fig. 3) or a second pose that only interacts allosterically with a prebound orthosteric ligand. Importantly,

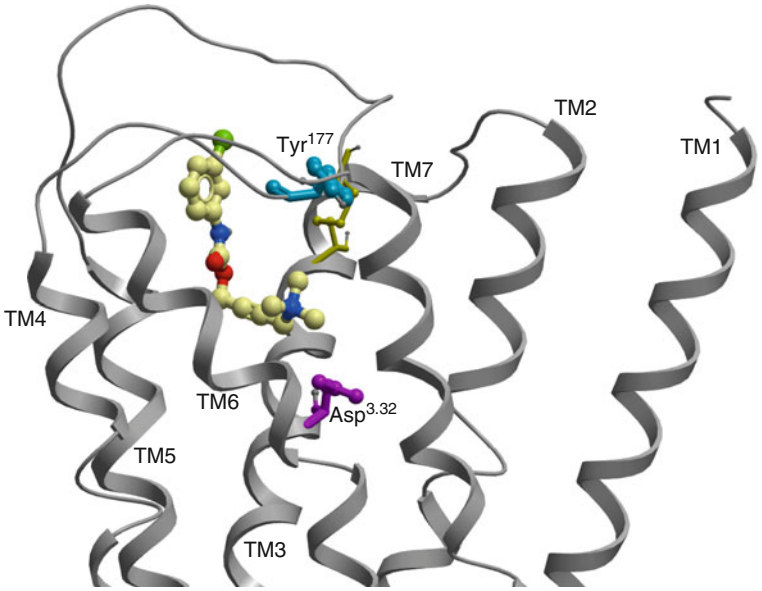

Fig. 3 A possible bitopic binding mode for McN-A-343 at the M_2 mAChR. Two key residues affecting the actions of the agonist in the orthosteric ($Asp^{3.32)}$ and allosteric (Tyr^{177}) pockets are highlighted. Coordinates taken from Valant et al. (2008)

the binding of McN-A-343 is sensitive to mutation of both the key orthosteric site residue, $Asp^{3.32}$, and key allosteric site residue, Tyr^{177}, in the M_2 mAChR (May et al. 2007b; Valant et al. 2008).

Similarly to McN-A-343, there is some evidence that the putative allosteric agonists, AC-42 and its congener 77-LH-28-1, may also bridge both the orthosteric and allosteric binding sites at the M_1 mAChR, and thus are better classed as bitopic, rather than allosteric, agonists. Although the binding of AC-42 is relatively insensitive to orthosteric site mutations at $Tyr^{3.33}$ and $Tyr^{6.51}$ (Spalding et al. 2002, 2006), recent studies have suggested that AC-42 and 77-LH-28-1 could still interact with the key orthosteric site residue, $Asp^{3.32}$, but bind with a significantly different pose to prototypical orthosteric agonists (Lebon et al. 2009). In agreement with this model, the binding affinity of AC-42 is decreased by mutation of $Leu^{3.29}$, whilst that of AC-42 and 77-LH-28-1 is increased by Ala substitution of $Trp^{3.28}$ (Avlani et al. 2010; Spalding et al. 2006; Gregory et al. 2010), indicating that they interact with a region that may border the orthosteric and allosteric binding sites. To accommodate this binding mode, it has been proposed that $Trp^{3.28}$, which would normally face toward the center of the helical bundle, may "flip" outwards and be stabilized by $Phe^{2.56}$ in the M_1 mAChR, which is located on the external side of TMII (Avlani et al. 2010).

Collectively, these recent studies of bitopic ligands highlight a number of important considerations. First, it is possible that ligands previously classed as "functionally selective" may achieve such selectivity as a consequence of a bitopic mechanism of action. Second, caution must be exercised when classifying any novel agonist as "allosteric," unless rigorous pharmacological data are available to suggest that such a compound's agonism indeed arises directly from an interaction with an allosteric site (and not the orthosteric site, as would be expected for a bitopic ligand). Third, it should be possible to rationally design bitopic ligands by purposefully utilizing appropriate orthosteric and allosteric fragments joined together by an optimal linker. A number of recent elegant studies have indeed provided proof of concept for this approach (Disingrini et al. 2006; Steinfeld et al. 2007; Antony et al. 2009).

5 Effects of Mutations on Signaling

The molecular details underlying the activation mechanisms of GPCRs remain largely unknown, and thus represent a major ongoing field of research. Much of the problem lies with the fact that GPCRs are highly dynamic proteins that can adopt multiple active states, each associated with different intracellular interacting partners and functional outcomes. Thus, any interpretation of mutational studies on GPCR signaling must be tempered by the fact that it will be influenced by the choice of functional assay used as a measure of receptor activation.

In general, ACh binding is predicted to initially elicit conformational changes in the mAChRs that result in a reduced pocket volume between key residues,

specifically $Tyr^{3.33}$, $Thr^{5.39}$, $Thr^{5.42}$, $Tyr^{6.51}$, and $Tyr^{7.39}$, that surround the bound ligand (Lu et al. 2002). Not surprisingly therefore, substitutions of these amino acids result in reduced ACh efficacy (Lu et al. 2001; Nawaratne et al. 2010; Gregory et al. 2010; Spalding et al. 1998). However, the efficacy of other agonists need not be affected by mutation of some or all of these residues, as they are likely to adopt differing poses to ACh, depending on their structures. Recent disulfide cross-linking experiments performed on the rat M_3 mAChR also indicated that $Ser^{3.36}$ and $Cys^{7.42}$ lie in close proximity when the receptor is in an active conformation (Han et al. 2005a), consistent with movement of residues that point into the orthosteric binding pocket toward one another. $Ser^{3.36}$ is predicted to face directly into the core of the orthosteric binding pocket thus it may serve as a secondary contact point for ACh when receptor activation is triggered. Indeed, substitution of $Ser^{3.36}$ for Ala in the M_1 (Lu and Hulme 1999), M_2 (Gregory et al. 2010), and M_4 mAChRs (Leach et al. 2011) leads to a large attenuation in the signaling of ACh and other agonists.

The movement of key "inner shell" residues toward ACh causes a reorientation of amino acids located in TMVII, in particular those located in the highly conserved $Asn^{7.49}$-$Pro^{7.50}$-X-$Cys^{7.52}$-$Tyr^{7.53}$ motif, which mediates a large conformational change at the intracellular end of TMVII. Agonist binding triggers movement of $Tyr^{7.53}$ toward $Val^{1.53}$, whilst residues in TMVII that are predicted to face the lipid bilayer move opposite TMI, suggesting a rotational movement of the cytoplasmic end of TMVII (Han et al. 2005b) and concomitant movement of helix VIII away from TMI (Li et al. 2007b). An M_1 model based on homology with rhodopsin predicted that the $Asn^{7.49}$-$Pro^{7.50}$-X-$Cys^{7.52}$-$Tyr^{7.53}$ motif constrains the inactive receptor conformation by forming a network of hydrogen bonds that connect TMVII to TMI, TMII, and TMIII (Lu et al. 2001). In support of this, Ala substitution of $Asn^{7.49}$, $Pro^{7.50}$, and $Tyr^{7.53}$ increases the affinity of ACh for the M_1 mAChR (Lu et al. 2001). However, although $Asn^{7.49}$ interacts with $Thr^{6.43}$ and $Asp^{6.44}$ in the inactive state of rhodopsin, an interaction between $Asn^{7.49}$ and $Asp^{2.50}$ is observed in opsin through crystal waters (Urizar et al. 2005), suggesting that this residue forms new contacts upon receptor activation that are important for the stability of the active receptor state. An identical interaction seems likely in the M_1 mAChR (Bee and Hulme 2007), and the significant reduction in agonist efficacy following mutation of $Asn^{7.49}$ in the mAChRs confirms that this residue is indeed important for stabilizing an active receptor conformation.

In conjunction with the conformational changes associated with TMVII, relocation of the bottom of TMVI away from the helical bundle and toward TMV takes place upon receptor activation. The crystal structures of rhodopsin and opsin show significant differences in the position of TMVI relative to TMIII (Palczewski et al. 2000; Park et al. 2008), particularly within the regions that comprise the retinal binding pocket. The movement of TMVI is driven, in part, by alterations in a region that contains an aromatic cluster of amino acids ($Cys^{6.47}$-$Trp^{6.48}$-$Leu^{6.49}$-$Pro^{6.50}$-$Tyr^{6.51}$-$Ala^{6.52}$ in rhodopsin) located toward the extracellular portion of TMVI (Ruprecht et al. 2004). This leads to the development of a kink at the highly conserved $Pro^{6.50}$, which causes the cytoplasmic end of TMVI to tilt away from

the center of the helical bundle (Park et al. 2008). In the mAChRs, mutations in the Thr$^{6.47}$-Trp$^{6.48}$-Thr/Ala$^{6.49}$-Pro$^{6.50}$-Tyr$^{6.51}$-Asn$^{6.52}$ motif lead to reduced agonist efficacy or nonfunctional receptors (Spalding et al. 1998; Wess et al. 1992), as do substitutions at surrounding residues, including Lys$^{6.29}$, Ala$^{6.34}$ and Ser$^{6.38}$ in the M_3 mAChR and Val$^{6.55}$ and Val$^{6.57}$, Val$^{6.59}$ in the M_3 and M_5 mAChRs (Spalding et al. 1998; Schmidt et al. 2003). In contrast, mutations at other residues in TMVI, including Glu$^{6.30}$ in the M_1 mAChR, Gln$^{6.35}$ in the M_3 mAChR and Ile$^{6.40}$, Ala$^{6.43}$, Phe$^{6.44}$, and Ser$^{6.58}$ in the M_5 mAChR, result in constitutive activity (Spalding et al. 1998; Schmidt et al. 2003; Hogger et al. 1995), suggesting that these residues help to stabilize the ground state of the receptor. Overall, these observations highlight the importance of the cytoplasmic end of TMVI in mAChR activation.

Substitution of conserved amino acids throughout TMIII, TMIV, and TMV, including Asp$^{3.26}$, Asp$^{3.32}$, Ile$^{3.46}$, Trp$^{4.57}$, Pro$^{4.59}$, Thr$^{5.37}$, Ile$^{5.38}$, Ala$^{5.46}$, and Ile$^{5.61}$ can have particularly detrimental effects on agonist efficacy (Lu and Hulme 1999; Nawaratne et al. 2010; Spalding et al. 1998; Schmidt et al. 2003; Lu and Hulme 2000; Page et al. 1995). However, Pro$^{4.59}$ is predicted to face into the lipid bilayer (Wess et al. 1991), thus the effect of mutations at this position may be indirect. In the M_1 mAChR, Ala substitution of Trp$^{3.28}$ also greatly reduces the signaling efficacy of ACh (Lu and Hulme 1999).

More recently, a number of mutations that disrupt the function of the M_3 mAChR were identified in TMI and TMII, including mutations at Thr$^{1.46}$, Ile$^{1.47}$, Asn$^{1.50}$, Val$^{1.53}$, Asn$^{1.60}$, Asn$^{2.38}$, Asn$^{2.39}$, Ser$^{2.45}$, Ala$^{2.49}$, Asp$^{2.50}$, Leu$^{2.51}$, Ser$^{2.57}$ Met$^{2.58}$, Asn$^{2.59}$, Phe$^{2.61}$ Ile$^{2.66}$, and Asn$^{2.68}$ (Li et al. 2007a). Furthermore, residues in the second extracellular loop of the M_3 mAChR are critical for the functional activity of the receptor, including Gln^{207}, Gly^{211}, Arg^{213}, Gly^{218}, Ile^{222}, Phe^{224}, Leu^{225}, and Pro^{228} (Scarselli et al. 2007). This is consistent with observations that conformational changes in the second extracellular loop occur upon activation of rhodopsin, whereby movement of TMV and disruption of the proposed ionic lock between TMIII and TMVI causes rearrangement of the hydrogen bond network that connects the extracellular ends of TMIV, TMV, and TMVI to the second extracellular loop (Ahuja et al. 2009).

In addition to inactivating mutations, amino acid substitutions in TMIII can result in increased constitutive activity of mAChRs, including Leu$^{3.43}$ and Ser$^{3.47}$ in the M_1 mAChR (Lu and Hulme 1999), suggesting a role for these residues in constraining the inactive receptor state. In particular, the highly conserved $^{3.49}$Glu/Asp-Arg-Tyr$^{3.51}$ motif, which is found in approximately 70% of Family A GPCRs, has been implicated in stabilizing the inactive receptor state and enabling a switch to an active receptor conformation. In rhodopsin, the β-ARs and the A_{2A} adenosine receptor, Arg$^{3.50}$ forms a hydrogen bond with the adjacent Glu/Asp$^{3.49}$ (Cohen et al. 1993; Ballesteros et al. 2001; Scheer et al. 1996). In rhodopsin, Arg$^{3.50}$ also forms a key salt bridge with Glu$^{6.30}$ (although this interaction was not present in the β-ARs or A_{2A} adenosine receptor structures) that is broken upon receptor activation as TMVI moves apart from TMIII. In opsin, Arg$^{3.50}$ interacts instead with Tyr$^{5.58}$ and potentially with Tyr$^{7.53}$. Accordingly, mutation of Arg$^{3.50}$ in the M_1 mAChR leads to significant reductions in agonist

efficacy (Jones et al. 1995), suggesting that this residue is essential for stabilizing the active receptor state in the mAChRs. However, and in contrast to observations at other GPCRs, significant effect on the signaling efficacy of ACh is not observed in the M_1 or M_5 mAChRs upon mutation of $Asp^{3.49}$ (Lu et al. 1997; Burstein et al. 1998), suggesting that the postulated hydrogen bond between $Asp^{3.49}$ and $Arg^{3.50}$ does not necessarily exist in the mAChRs.

The bulk of the studies described above have focused on activation mechanisms thought to be "universal" to agonists of the mAChRs. However, recent studies of allosteric and other novel functionally selective agonists of these receptors have begun to identify residues that contribute to receptor activation in a more ligand or pathway-specific manner. For instance, $Phe^{2.56}$ in the M_1 mAChR is essential for the activity of AC-42 and 77-LH-28-1 but not ACh or pilocarpine (Avlani et al. 2010), whilst the efficacy of the allosteric agonist, LY2033298, but not that of ACh or McN-A-343, at the M_4 mAChR is selectively sensitive to mutations of extracellular loop 1 residues Ile^{93} and Lys^{95} (Nawaratne et al. 2010). Conversely, a recent study of the M_2 mAChR identified $Tyr^{3.33}$ as a key residue selectively linking activation of the receptor to the phosphorylation of ERK1/2, irrespective of the nature of the activating ligand (Gregory et al. 2010). These findings indicate that there are likely to be a number of agonist and pathway-specific mechanisms that contribute to receptor signaling, consistent with the hypothesis that mAChRs can adopt multiple active states that are differentially stabilized by various classes of ligand and/or intracellular interacting proteins.

6 The G Protein Binding Interface

The only crystallographic evidence of the interaction between a GPCR and its G protein comes from the structure of opsin and metarhodopsin II in combination with a synthetic peptide composed of the first 11 amino acids of transducin, the cognate Gα subunit for this receptor (Scheerer et al. 2008). This study indicated that the second and third intracellular loops, the cytoplasmic ends of TMIII, TMV, and TMVI and the amino-terminal segment of helix 8 are all involved in G protein binding events. Specifically, an interaction between $Arg^{3.50}$ and a Cys residue in the Gα peptide corresponding to Cys^{347} in transducin was observed, confirming the importance of $Arg^{3.50}$ in receptor signaling and underlining its importance in stabilizing an active receptor state. Likewise, interactions were observed between the transducin peptide and Leu^{226}, Val^{230}, Ala^{233}, Thr^{242}, Thr^{243}, Ala^{246}, and Val^{250} in the third intracellular loop of the receptor.

In agreement with the binding of the transducin peptide to opsin, mutagenic studies suggest an interaction between full-length Gαq and TMV, TMVI and helix 8 of the M_3 mAChR. Residues located at the junctions between the third intracellular loop and TMV and TMVI are particularly important for the recognition by Gαq proteins of the M_3 mAChR, including $Tyr^{5.64}$, $Ala^{6.32}$, $Ala^{6.33}$, $Leu^{6.36}$,

and Ser$^{6.34}$ (Bluml et al. 1994b, c; Blin et al. 1995; Kostenis et al. 1997). Similar observations have been made in the M_5 mAChR (Burstein et al. 1995, 1996; Hill-Eubanks et al. 1996). A recent cysteine cross-linking study additionally identified Leu^{173} and Arg^{176} in the second intracellular loop and Thr^{549}, Thr^{552}, and Thr^{556} in the amino-terminal segment of helix 8 of the M_3 mAChR as residues that directly interact with the carboxy-terminal portion of Gαq (Hu et al. 2010). These interactions were observed in the absence and presence of agonist and even in the presence of the inverse agonist, atropine, suggesting that inactive mAChRs can exist in complex with Gα proteins. However, upon agonist stimulation, an interaction between Ala$^{6.33}$ and the carboxy-terminal region of Gαq was promoted, whilst interactions between Thr^{549} and Thr^{552} and the G protein were enhanced. This is consistent with the concept that agonists trigger movement of the cytoplasmic end of TMVI away from the TM bundle, which enables the carboxy-terminus of Gα to interact with TMV and TMVI. A weaker interaction was detected between Lys^{548} at the junction of TMVII and helix 8 of the M_3 mAChR and Asp^{321} in the carboxy-terminus of Gαq, which was also enhanced by agonist treatment. Interestingly, the same study identified an interaction between Leu^{173} in the second intracellular loop of the M_3 mAChR and Arg^{31} in the amino-terminal region of the Gαq subunit, again in the absence of agonist or inverse agonist, indicating that multiple regions of the G protein are involved in coupling to the receptor.

7 Conclusions

A wealth of mutagenesis-derived information continues to provide insight into the structural and functional role of diverse receptor regions in the mAChRs. Coupled with direct crystallographic information obtained for other Family A GPCRs, we are starting to gain an understanding of the intramolecular interactions that exist in the mAChRs, how mAChR ligands bind to their receptors, and how ligand binding triggers conformational changes in the mAChR structure that ultimately lead to intracellular signaling events. No doubt, given the recent advances in the field of GPCR structural biology, high-resolution structures of ligand-bound mAChRs are a likely outcome in the not-too-distant future. Irrespective of the nature of the experimental paradigm, the most likely gage of success in the area of receptor structure–function analyses is the use of information gained to successfully explain and predict biological events, and to rationally design drugs that can alleviate diseases associated with the mAChRs.

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References

Ahuja S, Hornak V, Yan EC, Syrett N, Goncalves JA, Hirshfeld A, Ziliox M, Sakmar TP, Sheves M, Reeves PJ, Smith SO, Eilers M (2009) Helix movement is coupled to displacement of the second extracellular loop in rhodopsin activation. Nat Struct Mol Biol 16:168–175

Antony J, Kellershohn K, Mohr-Andra M, Kebig A, Prilla S, Muth M, Heller E, Disingrini T, Dallanoce C, Bertoni S, Schrobang J, Trankle C, Kostenis E, Christopoulos A, Holtje HD, Barocelli E, De Amici M, Holzgrabe U, Mohr K (2009) Dualsteric GPCR targeting: a novel route to binding and signaling pathway selectivity. FASEB J 23:442–450

Avlani VA, Gregory KJ, Morton CJ, Parker MW, Sexton PM, Christopoulos A (2007) Critical role for the second extracellular loop in the binding of both orthosteric and allosteric G protein-coupled receptor ligands. J Biol Chem 282:25677–25686

Avlani VA, Langmead CJ, Guida E, Wood MD, Tehan BG, Herdon HJ, Watson JM, Sexton PM, Christopoulos A (2010) Orthosteric and allosteric modes of interaction of novel selective agonists of the M1 muscarinic acetylcholine receptor. Mol Pharmacol 78(1):94–104

Ballesteros JA, Weinstein H, Stuart CS (1995) Integrated methods for the construction of three-dimensional models and computational probing of structure-function relations in G protein-coupled receptors. Methods Neurosci 25:366–428

Ballesteros JA, Jensen AD, Liapakis G, Rasmussen SG, Shi L, Gether U, Javitch JA (2001) Activation of the beta 2-adrenergic receptor involves disruption of an ionic lock between the cytoplasmic ends of transmembrane segments 3 and 6. J Biol Chem 276:29171–29177

Bee MS, Hulme EC (2007) Functional analysis of transmembrane domain 2 of the M1 muscarinic acetylcholine receptor. J Biol Chem 282:32471–32479

Birdsall NJ, Burgen AS, Hulme EC, Stockton JM, Zigmond MJ (1983) The effect of McN-A-343 on muscarinic receptors in the cerebral cortex and heart. Br J Pharmacol 78:257–259

Birdsall NJ, Farries T, Gharagozloo P, Kobayashi S, Lazareno S, Sugimoto M (1999) Subtype-selective positive cooperative interactions between brucine analogs and acetylcholine at muscarinic receptors: functional studies. Mol Pharmacol 55:778–786

Blin N, Yun J, Wess J (1995) Mapping of single amino acid residues required for selective activation of Gq/11 by the m3 muscarinic acetylcholine receptor. J Biol Chem 270:17741–17748

Bluml K, Mutschler E, Wess J (1994a) Functional role in ligand binding and receptor activation of an asparagine residue present in the sixth transmembrane domain of all muscarinic acetylcholine receptors. J Biol Chem 269:18870–18876

Bluml K, Mutschler E, Wess J (1994b) Functional role of a cytoplasmic aromatic amino acid in muscarinic receptor-mediated activation of phospholipase C. J Biol Chem 269:11537–11541

Bluml K, Mutschler E, Wess J (1994c) Identification of an intracellular tyrosine residue critical for muscarinic receptor-mediated stimulation of phosphatidylinositol hydrolysis. J Biol Chem 269:402–405

Bridges TM, Marlo JE, Niswender CM, Jones CK, Jadhav SB, Gentry PR, Plumley HC, Weaver CD, Conn PJ, Lindsley CW (2009) Discovery of the first highly M5-preferring muscarinic acetylcholine receptor ligand, an M5 positive allosteric modulator derived from a series of 5-trifluoromethoxy N-benzyl isatins. J Med Chem 52:3445–3448

Buller S, Zlotos DP, Mohr K, Ellis J (2002) Allosteric site on muscarinic acetylcholine receptors: a single amino acid in transmembrane region 7 is critical to the subtype selectivities of caracurine V derivatives and alkane-bisammonium ligands. Mol Pharmacol 61:160–168

Burstein ES, Spalding TA, Hill-Eubanks D, Brann MR (1995) Structure-function of muscarinic receptor coupling to G proteins. Random saturation mutagenesis identifies a critical determinant of receptor affinity for G proteins. J Biol Chem 270:3141–3146

Burstein ES, Spalding TA, Brann MR (1996) Amino acid side chains that define muscarinic receptor/G-protein coupling. Studies of the third intracellular loop. J Biol Chem 271:2882–2885

Burstein ES, Spalding TA, Brann MR (1998) The second intracellular loop of the m5 muscarinic receptor is the switch which enables G-protein coupling. J Biol Chem 273:24322–24327

Chan WY, McKinzie DL, Bose S, Mitchell SN, Witkin JM, Thompson RC, Christopoulos A, Lazareno S, Birdsall NJM, Bymaster FP, Felder CC (2008) Allosteric modulation of the muscarinic M4 receptor as an approach to treating schizophrenia. Proc Natl Acad Sci USA 105:10978–10983

Cherezov V, Rosenbaum DM, Hanson MA, Rasmussen SG, Thian FS, Kobilka TS, Choi HJ, Kuhn P, Weis WI, Kobilka BK, Stevens RC (2007) High-resolution crystal structure of an engineered human beta2-adrenergic G protein-coupled receptor. Science 318:1258–1265

Clark AL, Mitchelson F (1976) The inhibitory effect of gallamine on muscarinic receptors. Br J Pharmacol 58:323–331

Cohen GB, Yang T, Robinson PR, Oprian DD (1993) Constitutive activation of opsin: influence of charge at position 134 and size at position 296. Biochemistry 32:6111–6115

Congreve M, Marshall F (2010) The impact of GPCR structures on pharmacology and structure-based drug design. Br J Pharmacol 159:986–996

Curtis CA, Wheatley M, Bansal S, Birdsall NJ, Eveleigh P, Pedder EK, Poyner D, Hulme EC (1989) Propylbenzilylcholine mustard labels an acidic residue in transmembrane helix 3 of the muscarinic receptor. J Biol Chem 264:489–495

Disingrini T, Muth M, Dallanoce C, Barocelli E, Bertoni S, Kellershohn K, Mohr K, De Amici M, Holzgrabe U (2006) Design, synthesis, and action of oxotremorine-related hybrid-type allosteric modulators of muscarinic acetylcholine receptors. J Med Chem 49:366–372

Espinoza-Fonseca LM, Trujillo-Ferrara JG (2005) Identification of multiple allosteric sites on the M1 muscarinic acetylcholine receptor. FEBS Lett 579:6726–6732

Espinoza-Fonseca LM, Trujillo-Ferrara JG (2006) The existence of a second allosteric site on the M1 muscarinic acetylcholine receptor and its implications for drug design. Bioorg Med Chem Lett 16:1217–1220

Foord SM, Bonner TI, Neubig RR, Rosser EM, Pin JP, Davenport AP, Spedding M, Harmar AJ (2005) International Union of Pharmacology. XLVI. G protein-coupled receptor list. Pharmacol Rev 57:279–288

Fredriksson R, Lagerstrom MC, Lundin LG, Schioth HB (2003) The G-protein-coupled receptors in the human genome form five main families. Phylogenetic analysis, paralogon groups, and fingerprints. Mol Pharmacol 63:1256–1272

Gharagozloo P, Lazareno S, Popham A, Birdsall NJ (1999) Allosteric interactions of quaternary strychnine and brucine derivatives with muscarinic acetylcholine receptors. J Med Chem 42:438–445

Goodwin JA, Hulme EC, Langmead CJ, Tehan BG (2007) Roof and floor of the muscarinic binding pocket: variations in the binding modes of orthosteric ligands. Mol Pharmacol 72:1484–1496

Gregory KJ, Hall NE, Tobin AB, Sexton PM, Christopoulos A (2010) Identification of orthosteric and allosteric site mutations in M2 muscarinic acetylcholine receptors that contribute to ligand-selective signaling bias. J Biol Chem 285:7459–7474

Han SJ, Hamdan FF, Kim SK, Jacobson KA, Bloodworth LM, Li B, Wess J (2005a) Identification of an agonist-induced conformational change occurring adjacent to the ligand-binding pocket of the M(3) muscarinic acetylcholine receptor. J Biol Chem 280:34849–34858

Han SJ, Hamdan FF, Kim SK, Jacobson KA, Brichta L, Bloodworth LM, Li JH, Wess J (2005b) Pronounced conformational changes following agonist activation of the M(3) muscarinic acetylcholine receptor. J Biol Chem 280:24870–24879

Harmar AJ, Hills RA, Rosser EM, Jones M, Buneman OP, Dunbar DR, Greenhill SD, Hale VA, Sharman JL, Bonner TI, Catterall WA, Davenport AP, Delagrange P, Dollery CT, Foord SM, Gutman GA, Laudet V, Neubig RR, Ohlstein EH, Olsen RW, Peters J, Pin JP, Ruffolo RR, Searls DB, Wright MW, Spedding M (2009) IUPHAR-DB: the IUPHAR database of G protein-coupled receptors and ion channels. Nucleic Acids Res 37:D680–D685

Hill-Eubanks D, Burstein ES, Spalding TA, Brauner-Osborne H, Brann MR (1996) Structure of a G-protein-coupling domain of a muscarinic receptor predicted by random saturation mutagenesis. J Biol Chem 271:3058–3065

Hogger P, Shockley MS, Lameh J, Sadee W (1995) Activating and inactivating mutations in N- and C-terminal i3 loop junctions of muscarinic acetylcholine Hm1 receptors. J Biol Chem 270:7405–7410

Hopkins AL, Groom CR (2002) The druggable genome. Nat Rev Drug Discov 1:727–730

Hu J, Wang Y, Zhang X, Lloyd JR, Li JH, Karpiak J, Costanzi S, Wess J (2010) Structural basis of G protein-coupled receptor-G protein interactions. Nat Chem Biol 6:541–548

Huang XP, Prilla S, Mohr K, Ellis J (2005) Critical amino acid residues of the common allosteric site on the M2 muscarinic acetylcholine receptor: more similarities than differences between the structurally divergent agents gallamine and bis(ammonio)alkane-type hexamethylene-bis-[dimethyl-(3-phthalimidopropyl)ammonium]dibromide. Mol Pharmacol 68:769–778

Hulme EC, Lu ZL (1998) Scanning mutagenesis of transmembrane domain 3 of the M1 muscarinic acetylcholine receptor. J Physiol Paris 92:269–274

Hulme EC, Lu ZL, Bee M, Curtis CA, Saldanha J (2001) The conformational switch in muscarinic acetylcholine receptors. Life Sci 68:2495–2500

Hulme EC, Lu ZL, Saldanha JW, Bee MS (2003a) Structure and activation of muscarinic acetylcholine receptors. Biochem Soc Trans 31:29–34

Hulme EC, Lu ZL, Bee MS (2003b) Scanning mutagenesis studies of the M1 muscarinic acetylcholine receptor. Receptors Channels 9:215–228

Jaakola VP, Griffith MT, Hanson MA, Cherezov V, Chien EY, Lane JR, Ijzerman AP, Stevens RC (2008) The 2.6 angstrom crystal structure of a human A2A adenosine receptor bound to an antagonist. Science 322:1211–1217

Jakubik J, El-Fakahany EE, Tucek S (2000) Evidence for a tandem two-site model of ligand binding to muscarinic acetylcholine receptors. J Biol Chem 275:18836–18844

Jones PG, Curtis CA, Hulme EC (1995) The function of a highly-conserved arginine residue in activation of the muscarinic M1 receptor. Eur J Pharmacol 288:251–257

Jones CK, Brady AE, Davis AA, Xiang Z, Bubser M, Tantawy MN, Kane AS, Bridges TM, Kennedy JP, Bradley SR, Peterson TE, Ansari MS, Baldwin RM, Kessler RM, Deutch AY, Lah JJ, Levey AI, Lindsley CW, Conn PJ (2008) Novel selective allosteric activator of the M1 muscarinic acetylcholine receptor regulates amyloid processing and produces antipsychotic-like activity in rats. J Neurosci 28:10422–10433

Kostenis E, Gomeza J, Lerche C, Wess J (1997) Genetic analysis of receptor-Galphaq coupling selectivity. J Biol Chem 272:23675–23681

Kurtenbach E, Curtis CA, Pedder EK, Aitken A, Harris AC, Hulme EC (1990) Muscarinic acetylcholine receptors. Peptide sequencing identifies residues involved in antagonist binding and disulfide bond formation. J Biol Chem 265:13702–13708

Langmead CJ, Fry VA, Forbes IT, Branch CL, Christopoulos A, Wood MD, Herdon HJ (2006) Probing the molecular mechanism of interaction between 4-n-butyl-1-[4-(2-methylphenyl)-4-oxo-1-butyl]-piperidine (AC-42) and the muscarinic M(1) receptor: direct pharmacological evidence that AC-42 is an allosteric agonist. Mol Pharmacol 69:236–246

Lanzafame AA, Sexton PM, Christopoulos A (2006) Interaction studies of multiple binding sites on m4 muscarinic acetylcholine receptors. Mol Pharmacol 70:736–746

Lazareno S, Birdsall NJ (1995) Detection, quantitation, and verification of allosteric interactions of agents with labeled and unlabeled ligands at G protein-coupled receptors: interactions of strychnine and acetylcholine at muscarinic receptors. Mol Pharmacol 48:362–378

Lazareno S, Gharagozloo P, Kuonen D, Popham A, Birdsall NJ (1998) Subtype-selective positive cooperative interactions between brucine analogues and acetylcholine at muscarinic receptors: radioligand binding studies. Mol Pharmacol 53:573–589

Lazareno S, Popham A, Birdsall NJ (2000) Allosteric interactions of staurosporine and other indolocarbazoles with N-[methyl-(3)H]scopolamine and acetylcholine at muscarinic receptor subtypes: identification of a second allosteric site. Mol Pharmacol 58:194–207

Leach K, Loiacono RE, Felder CC, McKinzie DL, Mogg A, Shaw DB, Sexton PM, Christopoulos A (2010) Molecular mechanisms of action and in vivo validation of an M(4) muscarinic acetylcholine receptor allosteric modulator with potential antipsychotic properties. Neuropsychopharmacology 35:855–869

Leach K, Davey AE, Felder CC, Sexton PM, Christopoulos A (2011) The role of transmembrane domain 3 in the actions of orthosteric, allosteric, and atypical agonists of the M4 muscarinic acetylcholine receptor. Mol. Pharmacol. 79: 855–865

Lebon G, Langmead CJ, Tehan BG, Hulme EC (2009) Mutagenic mapping suggests a novel binding mode for selective agonists of M1 muscarinic acetylcholine receptors. Mol Pharmacol 75:331–341

Li B, Scarselli M, Knudsen CD, Kim SK, Jacobson KA, McMillin SM, Wess J (2007a) Rapid identification of functionally critical amino acids in a G protein-coupled receptor. Nat Methods 4:169–174

Li JH, Han SJ, Hamdan FF, Kim SK, Jacobson KA, Bloodworth LM, Zhang X, Wess J (2007b) Distinct structural changes in a G protein-coupled receptor caused by different classes of agonist ligands. J Biol Chem 282:26284–26293

Lu ZL, Hulme EC (1999) The functional topography of transmembrane domain 3 of the M1 muscarinic acetylcholine receptor, revealed by scanning mutagenesis. J Biol Chem 274:7309–7315

Lu ZL, Hulme EC (2000) A network of conserved intramolecular contacts defines the off-state of the transmembrane switch mechanism in a seven-transmembrane receptor. J Biol Chem 275:5682–5686

Lu ZL, Curtis CA, Jones PG, Pavia J, Hulme EC (1997) The role of the aspartate-arginine-tyrosine triad in the m1 muscarinic receptor: mutations of aspartate 122 and tyrosine 124 decrease receptor expression but do not abolish signaling. Mol Pharmacol 51:234–241

Lu ZL, Saldanha JW, Hulme EC (2001) Transmembrane domains 4 and 7 of the M(1) muscarinic acetylcholine receptor are critical for ligand binding and the receptor activation switch. J Biol Chem 276:34098–34104

Lu ZL, Saldanha JW, Hulme EC (2002) Seven-transmembrane receptors: crystals clarify. Trends Pharmacol Sci 23:140–146

Lullmann H, Ohnesorge FK, Schauwecker GC, Wassermann O (1969) Inhibition of the actions of carbachol and DFP on guinea pig isolated atria by alkane-bis-ammonium compounds. Eur J Pharmacol 6:241–247

Ma L, Seager MA, Wittmann M, Jacobson M, Bickel D, Burno M, Jones K, Graufelds VK, Xu G, Pearson M, McCampbell A, Gaspar R, Shughrue P, Danziger A, Regan C, Flick R, Pascarella D, Garson S, Doran S, Kreatsoulas C, Veng L, Lindsley CW, Shipe W, Kuduk S, Sur C, Kinney G, Seabrook GR, Ray WJ (2009) Selective activation of the M1 muscarinic acetylcholine receptor achieved by allosteric potentiation. Proc Natl Acad Sci USA 106:15950–15955

Matsui H, Lazareno S, Birdsall NJ (1995) Probing of the location of the allosteric site on m1 muscarinic receptors by site-directed mutagenesis. Mol Pharmacol 47:88–98

May LT, Leach K, Sexton PM, Christopoulos A (2007a) Allosteric modulation of G protein-coupled receptors. Annu Rev Pharmacol Toxicol 47:1–51

May LT, Avlani VA, Langmead CJ, Herdon HJ, Wood MD, Sexton PM, Christopoulos A (2007b) Structure-function studies of allosteric agonism at M2 muscarinic acetylcholine receptors. Mol Pharmacol 72:463–476

Nawaratne V, Leach K, Suratman N, Loiacono RE, Felder CC, Armbruster BN, Roth BL, Sexton PM, Christopoulos A (2008) New insights into the function of M4 muscarinic acetylcholine receptors gained using a novel allosteric modulator and a DREADD (designer receptor exclusively activated by a designer drug). Mol Pharmacol 74:1119–1131

Nawaratne V, Leach K, Felder CC, Sexton PM, Christopoulos A (2010) Structural determinants of allosteric agonism and modulation at the M4 muscarinic acetylcholine receptor: identification of ligand-specific and global activation mechanisms. J Biol Chem 285:19012–19021

Page KM, Curtis CA, Jones PG, Hulme EC (1995) The functional role of the binding site aspartate in muscarinic acetylcholine receptors, probed by site-directed mutagenesis. Eur J Pharmacol 289:429–437

Palczewski K, Kumasaka T, Hori T, Behnke CA, Motoshima H, Fox BA, Le Trong I, Teller DC, Okada T, Stenkamp RE, Yamamoto M, Miyano M (2000) Crystal structure of rhodopsin: a G protein-coupled receptor. Science 289:739–745

Park JH, Scheerer P, Hofmann KP, Choe HW, Ernst OP (2008) Crystal structure of the ligand-free G-protein-coupled receptor opsin. Nature 454:183–187

Prilla S, Schrobang J, Ellis J, Holtje HD, Mohr K (2006) Allosteric interactions with muscarinic acetylcholine receptors: complex role of the conserved tryptophan M2422Trp in a critical cluster of amino acids for baseline affinity, subtype selectivity, and cooperativity. Mol Pharmacol 70:181–193

Rasmussen SG, Choi HJ, Rosenbaum DM, Kobilka TS, Thian FS, Edwards PC, Burghammer M, Ratnala VR, Sanishvili R, Fischetti RF, Schertler GF, Weis WI, Kobilka BK (2007) Crystal structure of the human beta2 adrenergic G-protein coupled receptor. Nature 450:383–387

Rosenbaum DM, Cherezov V, Hanson MA, Rasmussen SG, Thian FS, Kobilka TS, Choi HJ, Yao XJ, Weis WI, Stevens RC, Kobilka BK (2007) GPCR engineering yields high-resolution structural insights into beta2-adrenergic receptor function. Science 318:1266–1273

Ruprecht JJ, Mielke T, Vogel R, Villa C, Schertler GF (2004) Electron crystallography reveals the structure of metarhodopsin. EMBO J 23:3609–3620

Scarselli M, Li B, Kim SK, Wess J (2007) Multiple residues in the second extracellular loop are critical for M3 muscarinic acetylcholine receptor activation. J Biol Chem 282:7385–7396

Scheer A, Fanelli F, Costa T, De Benedetti PG, Cotecchia S (1996) Constitutively active mutants of the alpha 1B-adrenergic receptor: role of highly conserved polar amino acids in receptor activation. EMBO J 15:3566–3578

Scheerer P, Park JH, Hildebrand PW, Kim YJ, Krauss N, Choe HW, Hofmann KP, Ernst OP (2008) Crystal structure of opsin in its G-protein-interacting conformation. Nature 455:497–502

Schmidt C, Li B, Bloodworth L, Erlenbach I, Zeng FY, Wess J (2003) Random mutagenesis of the M3 muscarinic acetylcholine receptor expressed in yeast. Identification of point mutations that "silence" a constitutively active mutant M3 receptor and greatly impair receptor/G protein coupling. J Biol Chem 278:30248–30260

Spalding TA, Birdsall NJ, Curtis CA, Hulme EC (1994) Acetylcholine mustard labels the binding site aspartate in muscarinic acetylcholine receptors. J Biol Chem 269:4092–4097

Spalding TA, Burstein ES, Henderson SC, Ducote KR, Brann MR (1998) Identification of a ligand-dependent switch within a muscarinic receptor. J Biol Chem 273:21563–21568

Spalding TA, Trotter C, Skjaerbaek N, Messier TL, Currier EA, Burstein ES, Li D, Hacksell U, Brann MR (2002) Discovery of an ectopic activation site on the M(1) muscarinic receptor. Mol Pharmacol 61:1297–1302

Spalding TA, Ma JN, Ott TR, Friberg M, Bajpai A, Bradley SR, Davis RE, Brann MR, Burstein ES (2006) Structural requirements of transmembrane domain 3 for activation by the M1 muscarinic receptor agonists AC-42, AC-260584, clozapine, and *N*-desmethylclozapine: evidence for three distinct modes of receptor activation. Mol Pharmacol 70:1974–1983

Steinfeld T, Mammen M, Smith JA, Wilson RD, Jasper JR (2007) A novel multivalent ligand that bridges the allosteric and orthosteric binding sites of the M2 muscarinic receptor. Mol Pharmacol 72:291–302

Stewart GD, Sexton PM, Christopoulos A (2010) Prediction of functionally selective allosteric interactions at an M3 muscarinic acetylcholine receptor mutant using Saccharomyces cerevisiae. Mol Pharmacol 78(2):205–214

Stockton JM, Birdsall NJ, Burgen AS, Hulme EC (1983) Modification of the binding properties of muscarinic receptors by gallamine. Mol Pharmacol 23:551–557

Sur C, Mallorga PJ, Wittmann M, Jacobson MA, Pascarella D, Williams JB, Brandish PE, Pettibone DJ, Scolnick EM, Conn PJ (2003) *N*-desmethylclozapine, an allosteric agonist at

muscarinic 1 receptor, potentiates *N*-methyl-*D*-aspartate receptor activity. Proc Natl Acad Sci USA 100:13674–13679

Thomas RL, Mistry R, Langmead CJ, Wood MD, Challiss RA (2008) *G* protein coupling and signaling pathway activation by m1 muscarinic acetylcholine receptor orthosteric and allosteric agonists. J Pharmacol Exp Ther 327:365–374

Urizar E, Claeysen S, Deupi X, Govaerts C, Costagliola S, Vassart G, Pardo L (2005) An activation switch in the rhodopsin family of G protein-coupled receptors: the thyrotropin receptor. J Biol Chem 280:17135–17141

Valant C, Gregory KJ, Hall NE, Scammells PJ, Lew MJ, Sexton PM, Christopoulos A (2008) A novel mechanism of G protein-coupled receptor functional selectivity. Muscarinic partial agonist McN-A-343 as a bitopic orthosteric/allosteric ligand. J Biol Chem 283:29312–29321

Voigtlander U, Johren K, Mohr M, Raasch A, Trankle C, Buller S, Ellis J, Holtje HD, Mohr K (2003) Allosteric site on muscarinic acetylcholine receptors: identification of two amino acids in the muscarinic M2 receptor that account entirely for the M2/M5 subtype selectivities of some structurally diverse allosteric ligands in *N*-methylscopolamine-occupied receptors. Mol Pharmacol 64:21–31

Waelbroeck M (1994) Identification of drugs competing with d-tubocurarine for an allosteric site on cardiac muscarinic receptors. Mol Pharmacol 46:685–692

Ward SD, Curtis CA, Hulme EC (1999) Alanine-scanning mutagenesis of transmembrane domain 6 of the M(1) muscarinic acetylcholine receptor suggests that Tyr381 plays key roles in receptor function. Mol Pharmacol 56:1031–1041

Warne T, Serrano-Vega MJ, Baker JG, Moukhametzianov R, Edwards PC, Henderson R, Leslie AG, Tate CG, Schertler GF (2008) Structure of a beta1-adrenergic G-protein-coupled receptor. Nature 454:486–491

Wess J, Gdula D, Brann MR (1991) Site-directed mutagenesis of the m3 muscarinic receptor: identification of a series of threonine and tyrosine residues involved in agonist but not antagonist binding. EMBO J 10:3729–3734

Wess J, Maggio R, Palmer JR, Vogel Z (1992) Role of conserved threonine and tyrosine residues in acetylcholine binding and muscarinic receptor activation. A study with m3 muscarinic receptor point mutants. J Biol Chem 267:19313–19319

Polymorphisms in Human Muscarinic Receptor Subtype Genes

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Abstract A wide range of polymorphisms have been reported in muscarinic receptor subtype genes, mostly in M_1 and M_2 and, to a lesser extent, M_3 receptors. Most studies linking such genetic variability to phenotype have been performed for brain functions, but a more limited amount of information is also available for cardiac and airway function. Unfortunately, for none of the phenotypes under investigation a robust association with genotype has emerged. Moreover, it remains mostly unclear whether a reported association indicates a causative role of the polymorphism under investigation or merely a role as indicator of other polymorphisms affecting expression and/or function of the receptor. Also, most data on genotype–phenotype associations of muscarinic receptor subtypes are based on cross-sectional samples. Mechanistic studies linking polymorphisms to molecular, cellular, and tissue functions are largely missing. Finally, studies on a possible impact of muscarinic receptor polymorphisms on drug responsiveness are also largely missing. Thus, the field of genomics of muscarinic receptor subtypes is still in an early stage and a considerably greater number of studies will be required to judge the role of muscarinic receptor gene variability in physiology, pathophysiology, and drug treatment.

Keywords Intelligence • Muscarinic receptor • Single nucleotide polymorphism • Tandem repeat polymorphism

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1 Introduction

Most genes exhibit sequence variability in the general population. Such gene variants are of interest as they can change human physiology and/or be associated with disease. They may also affect how other molecules bind to a given protein and hence may change drug responsiveness. The latter type of gene variant is the subject of pharmacogenomics. In an ideal world, the functional consequences of a gene variant are known at the molecular, cellular, tissue, organism, and population level. However, in most cases only information about association of gene variants with phenotypes at the population level is available, which leaves considerable uncertainty for mechanistic interpretation. Thus, for most gene variants, it remains unclear whether reported associations with physiology, disease states or drug responses are causal or merely represent indicators.

Gene variants can be rare and then mostly are referred to as mutations. Such mutations may affect the function of the gene product in a profound way in an individual subject, but typically have only little impact at the population level due to their low prevalence. In contrast, more frequent gene variants, e.g., those occurring in at least 1% of the population, are referred to as polymorphisms and have the potential to be functionally relevant at the population level. Therefore, this chapter will primarily focus on the more frequent polymorphisms.

Various types of gene variants exist. The most frequently observed type is single nucleotide polymorphisms (SNPs), i.e., variants in which a single nucleotide is exchanged. When occurring within the coding region of a gene, this can affect the structure of its product if it results in an amino acid change. Other types of gene variants include the deletion or addition of nucleotides. Within the coding region this can lead to nonsense or truncated proteins. A variation of this theme is seen in variants in which the number of repeats, e.g., of CA nucleotide doublets, varies between subjects. These are mostly found in non-coding gene parts and can affect the activity of the gene promoter and/or mRNA stability. Moreover, any of these gene variants can affect gene splicing. Of note, early detection of gene polymorphisms was based upon differential susceptibility of DNA to various digestion enzymes (Detera-Wadleigh et al. 1989). While such work was important to get the field started, it has provided limited direct information on the underlying molecular gene variant.

Gene variants can affect the function of the corresponding protein in multiple ways. Variants within the coding region resulting in an amino acid change can structurally alter the protein and hence may affect its function and/or stability. Variants outside the coding region may affect the promoter activity, gene splicing, and/or mRNA stability. This may result in an altered expression of structurally unchanged protein.

This chapter will review variants in the five muscarinic receptor subtype genes and their associations with physiology, disease states, and/or drug responses. It is based on a search in the NCBI database of gene variants and a systematic Medline search completed in May 2011 using the key word combination “muscarinic

receptor" and "polymorphism." Additional references were taken from the retrieved articles as well as from congress abstracts. Studies on polymorphisms in non-human muscarinic receptors (Klett and Printz 1995; Du et al. 1996) were not considered.

There are two types of gene variants which can affect muscarinic physiology and/or drug response that will not be dealt with in this chapter. Firstly, variants of genes encoding proteins which interact with muscarinic receptors, e.g., G-proteins or other signal transduction molecules (Rosskopf et al. 2003), may affect the function of the corresponding protein and hence secondarily the function of muscarinic receptors and drug responsiveness. Secondly, specific for the pharmacogenomics of muscarinic receptors, it needs to be considered that gene variants may relate not to the receptors or their signal transduction but rather to the pharmacokinetic properties of the muscarinic receptor ligands. For example, some muscarinic receptor antagonists are metabolized by cytochrome P450 2D6, and the genotype for this enzyme may affect the exposure to such drugs (Brynne et al. 1998; Müller et al. 1993) which can have consequences for their efficacy and/or tolerability (Brynne et al. 1998; Diefenbach et al. 2008).

2 M_1 Receptors

The human M_1 receptor gene (CHRM1) is located on chromosome 11q13 and consists of a single exon containing the entire coding region. The NCBI database contains 82 SNPs for this gene. Several of them are in linkage disequilibrium, indicating the presence of haplotypes (Lucas et al. 2001; Maeda et al. 2006; Lou et al. 2006). While sequencing the exon of the M_1 receptor gene of 245 subjects of various ethnic groups, 15 SNPs were identified of which 9 were in the coding region; only 1 of them was non-synonymous (T1249C, Cys417Arg[1]) but found in only a single subject (Lucas et al. 2001). While most other detected variants were also rare, 43% of subjects exhibited at least one variation. Another study did not detect any non-synonymous SNP of the CHRM1 among 74 subjects, including 48 with dementia (Weiner et al. 2004). Moreover, this study found based on phenotypic screening that the potency of the muscarinic agonist carbachol was similar to that in wild-type cells in all cases. Thus, non-synonymous polymorphisms appear rare within the coding region of the M_1 receptor.

The vast majority of studies on CHRM1 polymorphisms have explored associations with brain functions, but some studies on airway and ocular function have also been reported. With regard to dementia, a small study with receptor sequencing from samples of brains from nine patients with Alzheimer's, six with vascular dementia and three controls, a synonymous SNP was found in two patients

[1] This manuscript refers to specific nucleotides by the single and specific amino acids by the three letter code. Where possible, we provide the rs identification number.

(Ohara et al. 1994). More importantly, a study investigating a single synonymous SNP (rs2067477) in 232 Alzheimer patients and 169 controls found no difference in allele frequency or age of onset of the disease between genotypes (Liu et al. 2005).

Comparing brain samples from 20 schizophrenia patients and controls each, a reduction in M_1 receptor mRNA by about 28% was found, but this could not be linked to the prevalence of four SNPs in the coding region of the receptor gene (Mancama et al. 2003). In another study, 243 schizophrenic patients were not compared to controls but rather patients were assessed according to genotype (rs2067477) (Liao et al. 2003). Genotypes differed in responses in the Wisconsin Card Sorting Test but not in other parameters including age of onset, chlorpromazine equivalents, and Brief Psychiatric Rating Scale.

In 290 patients with acute sciatica, the relationship between 121 SNPs in 14 genes including that for the CHRM1 and pain-related reduction in movement was explored (Mishra et al. 2007). For the CHRM1, this was done for four SNPs (rs17157628, rs544978, rs542269, rs2075748), but after Bonferroni correction for multiple comparisons, none of the SNPs significantly correlated with phenotype (Roland-Morris Disability Questionnaire). A possible relationship between CHRM1 polymorphisms and nicotine dependence has explored a sample of 2,037 European and African American subjects from 602 families (Lou et al. 2006). A haplotype based on 6 SNPs (rs2507821, rs4963323, rs544978, rs542269, rs2075748, rs1938677) was found to be protective against nicotine dependence within the African but not with the European American part of the sample.

Two studies have explored a link between CHRM1 polymorphisms and airway disease. A case-control study of 325 asthma patients and 333 healthy subjects looked at nine SNPs in the M_1 receptor gene (Maeda et al. 2006). Among these, the homozygous T-allele of one SNP upstream of the coding sequence (rs2075748) was associated with a lower risk for asthma (odds ratio 0.29) whereas the homozygous G-allele of another upstream SNP (rs1942499) was associated with a higher risk for asthma (odds ratio 1.86). Taking a third SNP also into consideration, such associations were even stronger at the haplotype level. In vitro experiments using a reporter assay demonstrated that the TA haplotype was associated with lower luciferase activity as compared to the CG haplotype, indicating that these SNPs are not only markers but may actually contribute to the regulation of CHRM1 transcription (Maeda et al. 2006). On the other hand, the odds ratio for responding to the muscarinic antagonist tiotropium was not affected by two other SNPs (rs10897304, rs2067480) in the CHRM1 in a cohort of 138 asthma patients (Park et al. 2009).

A single study of 194 patients with high myopia and 109 control subjects explored four SNPs within the CHRM1, of which two (rs44978 and rs542269) were associated with myopia (Lin et al. 2009) but these results have been questioned on technical grounds (Guggenheim et al. 2010). However, these data are difficult to interpret because homozygosity for the minor allele decreased the odds ratio for myopia, whereas heterozygosity increased it relative to homozygosity for the major allele.

Taken together there are only few if any frequently occurring non-synonymous SNPs within the coding region of the CHRM1. While no robust pattern of genotype/

phenotype relationships is emerging, it is noteworthy that two SNPs upstream of the coding sequence are not only associated with asthma but also with a change in promoter activity. However, this needs to be confirmed in an independent sample.

3 M_2 Receptors

The human M_2 receptor gene (CHRM2) is located on chromosome 7q31-35 and consists of six exons, the last of which contains the entire coding region, as well as a large 5′UTR encoded by multiple exons that can be alternatively spliced (Wang et al. 2004). A splice site is present 46 bp upstream from the start codon (Fenech et al. 2004). The gene has multiple transcription start sites. While an early study with brains from 15 dementia and control patients did not detect gene variants of the CHRM2 (Ohara et al. 1994), later studies including one based on sequencing of 46 control subjects and 46 asthma patients identified SNPs in the coding region and 3′UTR, but the coding SNPs (T1197C and A976C) were synonymous (Fenech et al. 2001). At present the NCBI database contains >500 SNPs for this gene, and some of them are in linkage disequilibrium (Wang et al. 2004; Jones et al. 2004; Hautala et al. 2006; Cohen-Woods et al. 2009). Moreover, a multi-allelic CA tandem repeat polymorphism has been identified 96 bp downstream of the most 5′ transcription start site of the gene (Fenech et al. 2004). This CA repeat, at least in human airway smooth muscle and BEAS-2B cells, can influence transcription of the gene implying functional importance. Most studies on CHRM2 polymorphisms have focused on CNS functions, but several have also explored associations with airway, cardiovascular, and urogenital function.

Most studies regarding brain functions have explored associations with cognition and intelligence. An initial study reported an association of the A1890T SNP in the 3′UTR (rs8191992) with intelligence (Wechsler Adult Intelligence Scale-Revised) based on 828 subjects (Comings et al. 2003). The association exhibited a “gene dose–response” with homozygous T allele carriers having the highest IQ (about four points more than homozygous A allele carriers and explaining about 1% in total variation). This was detected in the overall cohort and in the male and female subgroups. A different group of investigators looking at six different SNPs found that one SNP from intron 5 (rs324650) yielded a similarly strong correlation with IQ in a study of 177 twin pairs as well as 793 members of 316 extended twin families (Gosso et al. 2006). In a follow-up study, these investigators confirmed the relevance of this locus to intelligence using a denser SNP mapping but also reported that genotype at this site did not correlate with CHRM2 mRNA expression in the brain (Gosso et al. 2007). An association of multiple SNPs in the CHRM2 with intelligence was confirmed based on the COGA (Collaborative study of the Genetics of Alcoholism) study of 2,158 individuals from 200 families involving multiple ethnic groups (Dick et al. 2007). In line with these association studies, it has been reported based on 1,337 individuals from 253 families, also derived from the COGA study, that some SNPs in the CHRM2 (rs2350786, rs8191992) associate

with theta band visual-evoked brain oscillations (Jones et al. 2004), which are considered to reflect higher cognitive processing in humans. On the other hand, a study with three cohorts from Australia (1,537 subjects from 730 families), England (758 subjects) and Scotland (2,091 subjects) looking at a range of SNPs in the M_2 receptor gene did not detect an association with cognitive function tests (Lind et al. 2009). Differences between the latter and the previous studies have been attributed to potential bias in subject selection.

An initial study based on 760 subjects reported an association of a A1890T SNP (rs8191992) in the 3′UTR of the CHRM2 with major depression in women but not men (Comings et al. 2002). An association of intronic SNPs from the CHRM2 has also been reported with alcoholism and major depressive syndrome based on the COGA study (2,310 subjects from 262 families) (Wang et al. 2004). While one of these SNPs (rs1824024) was associated with both conditions, several others were associated with one but not the other. However, a later case-control study with 1,420 cases and 1,624 controls failed to confirm an association with depression, including the rs1824024 SNP (Cohen-Woods et al. 2009). Similarly, a study with 474 subjects from 152 families did not detect an association with bipolar disorder with any of 13 SNPs including rs1824024 and rs8191992 (Shi et al. 2007). Within a single study of 406 subjects, rs8191992 did not associate with visual attention or working memory, but significantly modulated the association with an SNP in one of the nicotinic receptor subtypes (Greenwood et al. 2009). Finally, the T-allele of the rs324650 SNP was associated with bipolar but not monopolar depression and accompanied by a significantly increased expression of the receptor at the protein level in at least some brain areas as assessed in PET studies (Cannon et al. 2011). The rs324650SNP was also associated with smoking behavior/nicotine addiction in a study of 5,500 subjects (Mobascher et al. 2009) but only very weakly with alcoholism in a study of 155 subjects (Jung et al. 2011). Within the latter study, however, another SNP, rs1824024, was associated much stronger with alcoholism. Other investigators reported the rs1455858 SNP to be associated adolescent substance abuse (Hendershot et al. 2011).

A smaller number of studies have explored associations between CHRM2 polymorphisms and cardiovascular function. A study of 95 sedentary men reported that heart rate recovery following exercise, known to be under control of M_2 receptors, was associated with rs324640 and exhibited a gene dose–response curve in this regard (Hautala et al. 2006). A similar association was found with rs8191992, as expected based upon the strong linkage between the two SNPs. These authors extended their findings by demonstrating an association between cardiac mortality and these SNPs in a sample of 491 patients with a former myocardial infarction (Hautala et al. 2009).

Although M_3 receptors are the key determinant of airway tone, CHRM2 can play a role in the regulation of airway function by indirectly acting on smooth muscle tone and/or by altering ganglionic transmission. However, studies comparing present and "outgrown" asthma patients (Yamamoto et al. 2002) or asthmatic and control children (Szczepankiewicz et al. 2009) did not detect associations of CHRM2 SNPs with airway disease. Similarly, none of the four SNPs in the M_2

receptor was associated with responder status to the muscarinic antagonist tiotropium (Park et al. 2009), but another study reported a minor association with the degree of response to another muscarinic antagonist, ipratropium (Szczepankiewicz et al. 2009). While mechanistic studies had demonstrated a role for a CA repeat polymorphism in the promoter of the CHRM2 for promoter activity in human airways smooth muscle cells (Fenech et al. 2004), unfortunately no phenotypic in vivo studies have been reported for this polymorphism with regard to airway disease. However, due to a similar role of M_2 receptors in airways and urinary bladder (Michel and Parra 2008), an association between a CA repeat polymorphism in the CHRM2 promoter and lower urinary tract function has been studied (Michel et al. 2009). Within a sample of 1,015 men with voiding dysfunction attributed to benign prostatic hyperplasia, this polymorphism was not associated with any of more than 30 parameters of lower urinary tract function. Finally, an SNP (rs1993068) located close CHRM2 gene demonstrated linkage with body mass index in a genome-wide association study in both linked families and a case-control sample (Laramie et al. 2009).

In conclusion, the non-coding rs324640 from intron 5 and rs8191992 from the 3′UTR are in strong linkage (Hautala et al. 2006) and show the greatest potential to be associated with phenotypes of M_2 receptor function in the CNS and peripheral tissues. The functional role of the CA repeat polymorphism in the CHRM2 promotor also is promising. However, most of the reported associations remain controversial and/or unconfirmed. Moreover, as the two SNPs exist in distinct parts of the gene the question arises which, if any, of them is functionally relevant and which is mainly a genetic marker.

4 M_3 Receptors

The human M_3 receptor gene (CHRM3) is located on chromosome 1q43 and consists of a single exon. The NCBI database contains >1,100 SNPs in this gene, making this the apparently most polymorphic gene among the muscarinic receptors. Moreover, short tandem repeat polymorphisms were detected in the M_3 gene promoter (Donfack et al. 2003).

While screening 46 asthma patients and 46 controls for polymorphisms, no gene variants were detected in the coding region or flanking region of the CHRM3 (Fenech et al. 2001). Similarly, only one rare CHRM3 mutation was found during the sequencing of 102 asthma patients and 70 controls (Yamamoto et al. 2002). Screening 60 Caucasian and African American subjects with and without asthma, four SNPs and two short tandem repeat polymorphisms were detected in the CHRM3 promoter, but none of them was more frequent in a group of 76 asthma patients as compared to 81 controls (Donfack et al. 2003). Another study in 138 asthmatic subjects did not detect associations of five different SNPs in the CHRM3 with responder status to the muscarinic antagonist tiotropium (Park et al. 2009). One study reported that an SNP (rs2165870) located upstream of the promoter of

the CHRM3 gene was associated with the susceptibility for post-operative nausea and vomiting (Janicky et al. 2011). Thus, despite the prominent function of CHRM3 in human physiology, particularly in the airways and the bladder (Michel and Parra 2008) and the large number of polymorphisms reported for this receptor subtype, no consistent evidence for a pathophysiological relevance of these variations has been reported.

5 M_4 Receptors

The human M_4 receptor gene (CHRM4) is located on chromosome 11p12-p11.2 and consists of a single exon. The NCBI database contains only few SNPs of this gene. However, most of those within the coding region are synonymous and for none of them data have been reported linking genetic variability to phenotype.

6 M_5 Receptors

The human M_5 receptor gene (CHRM5) is located on chromosome 15q26 and consists of a single coding exon and three alternatively spliced 5′-UTRs (Anney et al. 2007). The NCBI database contains >200 SNPs of this gene. We have identified only a single study linking a polymorphism of the M_5 gene to phenotype (Anney et al. 2007). In that study, a non-coding C > T SNP (rs7162140) was reported to have a prevalence of 19% and to be associated with the number of cigarettes smoked and cannabis dependence but not with nicotine or alcohol dependence in a sample of 815 Australians of European descent.

7 Conclusions

Major progress has been made in exploring associations between muscarinic receptor subtype gene polymorphisms and physiological and pathophysiological variables. However, in most cases proposed associations remain to be confirmed in independent studies or have proven to be inconsistent. Even in cases where a considerable body of evidence supports an association between genotype and phenotype, in most cases it remains uncertain which specific chain of events connects the gene variant to a given phenotype. Muscarinic receptor polymorphisms with relevance for drug responsiveness have not been elucidated on a large scale. Thus, this research field has promise but apparently is still in its infancy.

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References

Anney RJL, Lotfi-Miri M, Olsson CA et al (2007) Variation in the gene coding for the M5 muscarinic receptor (CHRM5) influences cigarette dose but is not associated with dependence to drugs of addiction: evidence from a prospective population based cohort study of young adults. BMC Genet 8:46

Brynne N, Dalen P, Alvan G et al (1998) Influence of CYP2D6 polymorphism on the pharmacokinetics and pharmacodynamics of tolterodine. Clin Pharmacol Ther 63:529–539

Cannon DM, Klaver JK, Gandhi SK et al (2011) Genetic variation in cholinergic muscarinic-2 receptor gene modulates M_2 receptor binding in vivo and accounts for reduced binding in bipolar disorder. Mol Psychiatry 16:407–418

Cohen-Woods S, Gaysina D, Craddock N et al (2009) Depression case control (DeCC) study fails to support involvement of the muscarinic acetylcholine receptor M_2 (CHRM2) gene in recurrent major depressive disorder. Hum Mol Genet 18:1504–1509

Comings DE, Wu S, Rostamkhani M et al (2002) Association of the muscarinic cholinergic 2 receptor (CHRM2) gene with major depression in women. Am J Med Genet 114:527–529

Comings DE, Wu S, Rostamkhni M et al (2003) Role of the cholinergic muscarinic 2 receptor (CHRM2) gene in cognition. Mol Psychiatry 8:10–13

Detera-Wadleigh SD, Wiesch D, Bonner TI (1989) An SstI polymorphism for the human muscarinic acetylcholine receptor gene, m4 (CHRM 4). Nucleic Acids Res 17:6431

Dick DM, Aliev F, Kramer J et al (2007) Association of CHRM2 with IQ: convering evidence for a gene influencing intelligence. Behav Genet 37:265–272

Diefenbach K, Jaeger K, Wollny A et al (2008) Effect of tolterodine on sleep structure modulated by CYP2D6 genotype. Sleep Med 9:579–582

Donfack J, Kogut P, Forsythe S et al (2003) Sequence variation in the promoter region of the cholinergic receptor muscarinic 3 gene and athma and atopy. J Allergy Clin Immunol 111:527–532

Du Y, Remmers EF, Goldmuntz EA et al (1996) Linkage maps of rat chromosomes 15, 16, 17, 19, and X. Genomics 32:113–116

Fenech AG, Ebejer MJ, Felice AE et al (2001) Mutation screening of the muscarinic M_2 and M_3 receptor genes in normal and asthmatic subjects. Br J Pharmacol 133:43–48

Fenech AG, Billington CK, Swan C et al (2004) Novel polymorphisms influencing transcription of the human CHRM2 gene in airway smooth muscle. Am J Respir Cell Mol Biol 30:678–686

Gosso MF, van Belzen M, de Geus EJC et al (2006) Association between the CHRM2 gene and intelligene in a sample of 304 Dutch families. Genes Brain Behav 5:577–584

Gosso FM, de Geus EJC, Polderman TJC et al (2007) Exploring the functional role of the CHRM2 gene in human cognition: results from a dense genotyping and brain expression study. BMC Med Genet 8:66

Greenwood PM, Lin M-K, Sundararajan R et al (2009) Synergistic effects of genetic variation in nicotinic and muscarinic receptors on visual attention but not working memory. Proc Natl Acad Sci USA 106:3633–3638

Guggenheim JA, Zavats T, Hammond C et al (2010) Lumican and muscarinic acetylcholine receptor 1 gene polymorphisms associated with high myopia. Eye (Lond) 24:1411–1412

Hautala AJ, Rankinen T, Kiviniemi AM et al (2006) Heart rate recovery after maximal exercise is associated with acetylcholine receptor M_2 (CHRM2) gene polymorphism. Am J Physiol 291: H459–H466

Hautala AJ, Tulppo MP, Kiviniemi AM et al (2009) Acetylcholine receptor M_2 gene variants, heart rate recovery, and risk of cardiac death after an acute myocardial infarction. Ann Med 41:197–207

Hendershot CS, Bryan AD, Feldstein Ewing SW et al (2011) Preliminary evidence for associations of CHRM2 with substance use and disinhibition of adolescence. J Abnorm Child Psychol 39:671–681

Janicky PK, Vealey R, Liu J et al (2011) Genome-wide association study using pooled DNA to identify candidate markers mediating susceptibility to postoperative nausea and vomiting. Anesthesiology 115:54–64

Jones KA, Porjesz B, Almasy L et al (2004) Linkage and linkage disequilibrium of evoked EEG oscillations with CHRM2 receptor gene polymorphisms: implications for human brain dynamics and cognition. Int J Psychophysiol 53:75–90

Jung MH, Park BL, Lee BC et al (2011) Association of CHRM2 polymorphisms with severity of alcohol dependence. Genes Brain Behav 10:253–256

Klett CP, Printz MP (1995) Is the M_1-muscarinic recpetor a candidate gene for hypertension in the spontaneously hypertensive rat? Clin Exp Pharmacol Physiol 22(Suppl):S4–S6

Laramie JM, Wilk JB, Williamson SL et al (2009) Multiple genes influence BMI on chromosome 7q31-34: the NHLBI family heart study. Obesity 17:2182–2189

Liao D-L, Hong C-J, Chen H-M et al (2003) Association of muscarinic M_1 receptor genetic polymorphisms with psychiatric symptoms and cognitive function in schizophrenic patients. Neuropsychobiology 48:72–76

Lin H-J, Wan L, Tsai Y et al (2009) Muscarinic acetylcholine receptor 1 gene polymorphisms associated with high myopia. Mol Vis 15:1774–1780

Lind PA, Luciano M, Horan MA et al (2009) No association between cholinergic muscarinic receptor 2 (CHRM2) genetic variation and cognitive abilities in three independent samples. Behav Genet 39:513–523

Liu H-C, Hong C-J, Liu T-Y et al (2005) Association analysis for the muscarinic M_1 receptor genetic polymorphisms and Alzheimer's disease. Dement Geriatr Cogn Disord 19:42–45

Lou X-Y, Ma JZ, Payne TJ et al (2006) Gene-based analysis suggests association of the nicotinic acetylcholine receptor β1 subunit (CHRNB1) and M_1 muscarinic acetylcholine receptor (CHRM1) with vulnerability for nicotine dependence. Hum Genet 120:381–389

Lucas JL, Sadee W, DeYoung JA (2001) Single nucleotide polymorphisms of the human M_1 muscarinic acetylcholine receptor gene. AAPS PharmSci 3:57–61

Maeda Y, Hizawa N, Jinushi E et al (2006) Polymorphisms in the muscarinic receptor 1 gene confer susceptibility to asthma in Japanese subjects. Am J Respir Crit Care Med 174:1119–1124

Mancama D, Arranz MJ, Landau S et al (2003) Reduced expression of the muscarinic 1 receptor cortical subtype in schizophrenia. Am J Med Genet 119B:2–6

Michel MC, Parra S (2008) Similarities and differences in the autonomic control of airway and urinary bladder smooth muscle. Naunyn Schmiedebergs Arch Pharmacol 378:217–224

Michel MC, Pahladsingh R, Teitsma CA et al (2009) Do M_2 muscarinic receptors contribute to human bladder function in vivo? J Urol 181:570–571

Mishra BK, Wu T, Belfer I et al (2007) Do motor control genes contribute to interindividual variability in decreased movement in patients with pain? Mol Pain 3:20

Mobascher A, Rujescu D, Mittelstraß K et al (2010) Association of a variant in the muscarinic acetylcholine receptor 2 gene (CHRM2) with nicotine addiction. Am J Med Genet B Neuropsychiatr Genet 153B:684–690

Müller C, Siegmund W, Huupponen R et al (1993) Kinetics of propiverine as assessed by radioreceptor assay in poor and extensive metabolizers of debrisoquine. Eur J Drug Metab Pharmacokinet 18:265–272

Ohara K, Kondo N, Xie D et al (1994) New sequences of muscarinic acetylcholine receptors (m_1 and m_2) in patients with Alzheimer's disease and vascular dementia. Neurosci Lett 178:23–26

Park H-W, Yang M-S, Park C-S et al (2009) Additive role of tiotropium in severe asthmatics and Arg16Gly in ADRB2 as a potential marker to predict response. Allergy 64:778–783

Rosskopf D, Koch K, Habich C et al (2003) Interaction of Gβ3s, a splice wariant of the G-protein Gβ3, with Gγ- and Gα-proteins. Cell Signal 15:479–488

Shi J, Hattori E, Zou H et al (2007) No evidence for association between 19 cholinergic genes and bipolar disorder. Am J Med Genet 144B:715–723

Szczepankiewicz A, Breborowicz A, Sobkowiak P et al (2009) Association of A/T polymorphism of the CHRM2 gene with bronchodilator response to ipratropium bromide in asthmatic children. Pneumonol Alergol Pol 77:5–10

Wang JC, Hinrichs AL, Stock H et al (2004) Evidence of common and specific genetic effets: association of the muscarinic acetylcholine receptor M_2 (CHRM2) gene with alcohol dependence and major depressive syndrome. Hum Mol Genet 13:1903–1911

Weiner DM, Goodman MW, Colpitts TM et al (2004) Functional screening of drug target genes: m_1 muscarinic acetylcholine receptor phenotypes in denegenerative dementias. Am J Pharmacogenomics 4:119–128

Yamamoto T, Yamashita N, Kuwabara M et al (2002) Mutation screening of the muscarinic m_2 and m_3 receptor genes in asthmatics, outgrouw subjects, and normal controls. Ann Genet 45:109–113

Muscarinic Receptor Trafficking

Cindy Reiner and Neil M. Nathanson

Abstract Knowledge of the mechanisms responsible for the trafficking of neurotransmitter receptors away from the cell surface is of obvious importance in understanding what regulates their expression and function. This chapter will focus on the mechanisms responsible for the internalization and degradation of muscarinic receptors. There are both receptor subtype-specific and cell-type specific differences in muscarinic receptor trafficking. Studies on muscarinic receptor trafficking both in cells in culture and in vivo will be described, and the potential physiological consequences of this trafficking will be discussed.

Keywords Internalization • Sequestration • Downregulation • Membrane protein degradation • Endosome • Recycling • GPCR • Trafficking • Proteomic • Mass spectrometry

1 Introduction

The correct trafficking of neurotransmitter receptors to and from the cell surface is essential both for the maintenance of normal functional activity and for the regulation of cell signaling and function in response to altered physiological inputs. Incorrect or impaired trafficking of proteins to the cell membrane has been implicated in a variety of human diseases. For example, impaired targeting of rhodopsin in the retina and the vasopressin receptor in the kidney can cause retinitis pigmentosa and nephrogenic diabetes insipidus, respectively (Mendes et al. 2005; Bichet 2008). Defects in endocytosis can also cause disease, and pharmacological treatments which result in altered receptor endocytosis and subsequent degradation

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can also have relatively long-term effects on the sensitivity of synaptic transmission (Anderson et al. 1977; Malenka 2003; Peng et al. 2010). In addition, endocytosis can be required for signaling by some cell surface receptors (Daka et al. 1998; Pierce et al. 2000), and different endocytotic pathways can differentially promote signaling or degradation of a given receptor (Di Guglielmo et al. 2003; Miaczynska and Bar-Sagi 2010). Because the trafficking of newly synthesized muscarinic receptors to the membrane has been recently reviewed (Nathanson 2008), this chapter will focus on studies directed at trafficking of muscarinic receptors away from the cell membrane.

2 Initial Studies on mAChR Trafficking

As with the receptors for many neurotransmitters and hormones, the number and function of mAChR expressed in cells or tissues can be altered by continued exposure of agonist leading to a decrease in responsiveness to further stimulation by agonist (Nathanson 1989). There are three distinct modes of regulation which differ both in their mechanism and time-frame: rapid desensitization which results in receptor uncoupling (seconds to minutes), sequestration of receptors away from the cell surface (minutes), and down-regulation of receptors resulting in a decrease in the total number of cellular receptors (hours).

Prolonged agonist treatment was first shown to decrease the total number of mAChR on a variety of cultured cell types, such as neuroblastoma cell lines (Klein et al. 1979; Taylor et al. 1979; Shifrin and Klein 1980), cardiac cells (Galper and Smith 1980), neurons (Siman and Klein 1979; Burgoyne and Pearce 1981), and smooth muscle cells (Takeyasu et al. 1981). This agonist-induced down-regulation of receptor number was suggested to represent a mechanism for the long-term regulation of receptor function in response to varying levels of cholinergic activity. Receptor number decreased over several hours, in contrast to the short-term desensitization of receptor function, which occurs on a time scale of minutes without a decrease in total cellular receptor number (Taylor et al. 1979). The decrease in receptor number was due to an increase in the rate of receptor degradation, and the recovery of receptor number to control levels required de novo protein synthesis (Klein et al. 1979; Taylor et al. 1979). These long-term decreases in mAChR number were accompanied by a decreased physiological sensitivity to cholinergic stimulation. Thus, there were concomitant decreases in neural cells of mAChR-mediated inhibition of adenylyl cyclases activity and mAChR-mediated activation of guanylyl cyclase and phospholipase C (Nathanson et al. 1978; Taylor et al. 1979; Siman and Klein 1981). In cultured heart cells, there were concomitant decreases in mAChR-mediated negative chronotropic responses, inhibition of adenylyl cyclase activity, and stimulation of ^{42}K and ^{86}Rb efflux (Galper et al. 1982a; Hunter and Nathanson 1986).

Similar studies showed that mAChR number could also be regulated in vivo. Agonist-induced downregulation of mAChR number was observed in heart, brain,

and spinal cord (Wise et al. 1980; Halvorsen and Nathanson 1981; Marks et al. 1981; Meyer et al. 1982; Taylor et al. 1982). These decreases in mAChR number also resulted in diminished mAChR-mediated functional responses (Halvorsen and Nathanson 1981; Taylor et al. 1982; Nathanson et al. 1984). The recovery of mAChR to control levels after subsequent blockade of agonist–mAChR interactions was blocked by inhibition of protein synthesis (Hunter and Nathanson 1984), consistent with experiments in cell culture indicating that the receptors which reappear following agonist-induced decreases in receptor number represent newly synthesized receptors.

Administration of acetylcholinesterase inhibitors, which increase endogenous levels of ACh, also decreased mAChR number in brain, ileum, and rat submandibular gland (Gazit et al. 1979; Dawson and Jarrott 1981; Olianas et al. 1984; Costa and Murphy 1985). These decreases in mAChR number also resulted in reduced physiological responsiveness (Olianas et al. 1984; Costa and Murphy 1985). Conversely, administration of muscarinic antagonists increased the number of mAChR found in the brain and heart, presumably because the antagonist prevented endogenously released ACh from activating the mAChR and inducing downregulation (Ben-Barak and Dudai 1980; Wise et al. 1980; Rehavi et al. 1980; Westlind et al. 1981).

The first demonstration of agonist-induced sequestration of mAChR was by Galper et al. (1982b), who determined the binding of the radioligands [^{3}H] quinucidinyl benzilate ([^{3}H])QNB and [^{3}H]N-methylscopolamine ([^{3}H]NMS) to cultured cardiomyocytes after treatment with agonist. Because [^{3}H]NMS is lipophobic, it should only label receptors on the cell surface, while [^{3}H]QNB is lipophilic and should label the total receptor population. Agonists caused a rapid sequestration of the mAChR from the cell surface, so that the receptors were no longer available to bind [^{3}H]NMS but could still bind [^{3}H]QNB; [^{3}H]QNB binding sites were lost from intact cells with a slower time course similar to the loss of binding sites seen in membrane homogenates due to receptor degradation. Agonist treatment also caused a rapid loss of [^{3}H]NMS sites from intact neuronal cells which was temperature-sensitive and rapidly reversible (Maloteaux et al. 1983; Feigenbaum and El-Fakahany 1985). Harden et al. (1985) showed that in 1321N1 astrocytoma cells, the conversion of the mAChR to a form that was not accessible to [^{3}H]NMS was accompanied by an alteration in the apparent subcellular localization of the receptor. After agonist exposure, the receptor no longer was associated with the plasma membrane after centrifugation, but instead sedimented in sucrose gradients with fractions containing light membrane vesicles. The sedimentation of the receptor with this light vesicle fraction was also rapidly reversed following agonist removal. These results provided strong initial evidence for the hypothesis that agonist-induced decreases in mAChR number result from a rapid and initially reversible internalization of the receptor from the cell surface, followed by a slower degradation of the receptor.

3 Receptor Subtype-Specific Differences in Agonist-Induced Internalization of Muscarinic Receptors

While initial work suggested that agonist-induced internalization and agonist-induced downregulation were universal properties of mAChR, molecular and cell biology studies showed that there could be cell type-specific and receptor subtype-specific differences in mAChR sequestration pathways. Scherer and Nathanson (1990) showed that while both the M_1 and M_2 receptors when stably expressed in Y1 adrenal cells exhibited agonist-induced sequestration, expression in Kin-8 cells, a Y1 variant lacking functional cAMP-dependent protein kinase, resulted in increased sensitivity of the M_2 receptor to internalization but did not affect M_1 sequestration. In addition, activation of protein kinase C by treatment with phorbol ester resulted in internalization of the M_1 receptor without affecting M_2 receptor cell surface expression. These results thus indicated that there were differences in the cellular mechanisms involved in the internalization of the two receptors. (Because the Kin-8 cells were derived by mutagenesis of Y1 cells, however, these differences could have been due to the alterations not in cAMP-dependent protein kinase activity but to mutagenesis of some other factor involved in receptor trafficking.)

Goldman et al. (1996) and Schlador et al. (2000) found that, in contrast to the similar levels of agonist-induced sequestration observed in Y1 cells, the M_2 exhibited much greater internalization than the M_1 receptor when expressed in JEG-3 choriocarcinoma cells. These results imply that the M_1 and M_2 receptors utilize different mechanisms or cellular pathways to undergo agonist-induced sequestration and that JEG-3 cells lack some component normally required for maximal internalization of M_1. Koenig and Edwardson (1996) also found that the M_1 and M_3 receptors were much less susceptible than the M_2 and M_4 receptors when expressed in CHO cells. Analysis of the trafficking of chimeric and mutant receptors demonstrated that substitution of five amino acids from M_2 [V385, T386 in the cytoplasmic proximal region of transmembrane domain 6, I389, L390 in the adjacent membrane proximal portion of the third intracellular loop (i3L), and A438 in transmembrane domain 7] was able to confer agonist-sensitive internalization to the M_1 receptor. While these regions in transmembrane domain 6 and the adjacent cytoplasmic domain had been previously implicated in determining the G-protein coupling specificity of the mAChR, the differences in internalization were not related to the differential coupling of M_1 and M_2 to G_q- and G_i-family G-proteins (Goldman et al. 1996; Schlador et al. 2000).

4 Internalization of M_1, M_3, and M_4 via Clathrin-Dependent Pathways

Agonist-induced endocytosis is a complex process, whose mechanism and function vary widely depending on the receptor, agonist treatment, and cell type. The prototypical endocytic pathway is that of the β_2-adrenergic receptor (β_2-AdR).

Agonist activation of the receptor leads to receptor phosphorylation by a member of the G-protein coupled receptor kinase (GRK) family of protein kinases. Receptor phosphorylation allows binding of the non-visual arrestins β-arrestin1 and β-arrestin2, which promotes receptor desensitization by blocking interaction of the β_2-AdR with its G-protein G_s. In addition, β-arrestins can bind the adapter protein AP2 and recruit the receptor into clathrin-coated pits in a dynamin-dependent manner to cause receptor internalization (Bouvier et al. 1988; Lohse et al. 1989; Ferguson et al. 1996). β-arrestins can also serve as adaptors for other proteins and pathways, such as receptor ubiquitination, and coupling the receptor to activation of the MAP kinase cascade (Zhang et al. 1996; Laporte et al. 1999, 2000; Lefkowitz et al. 2006; Schmid and Bohn 2009).

This prototypical internalization pathway involving dynamin, β-arrestin, and clathrin-coated pits is not used by all GPCRs (Zhang et al. 1996). Among the muscarinic receptors, the M_1, M_3 and M_4 subtypes follow the prototypical pathway more closely than the M_2 subtype. For instance, the M_1, M_3 and M_4 subtypes of mAChR internalize through the dynamin-dependent (Lee et al. 1998; Vögler et al. 1998; van Koppen 2001), clathrin-mediated (Vögler et al. 1999a, b; Claing et al. 2000; van Koppen 2001) pathway. The dependence of these three receptors on β-arrestin has been more ambiguous. Overexpression studies of dominant negative β-arrestin constructs in HEK293 cells have shown both a dependence on (Vögler et al. 1999a, b; van Koppen 2001) and an independence from (Lee et al. 1998) β-arrestin. Antisense knockdown of arrestin expression suggests that arrestins can regulate M_3 signaling but not mAChR internalization (Mundell et al. 1999; Luo et al. 2008). In contrast, a phosphorylation-deficient M_3 receptor lost the ability to both bind arrestin (and arrestin-mediated signaling pathways) and to undergo agonist-dependent internalization (Poulin et al. 2010). M_1 receptor activation has been shown to lead to β-arrestin ubiquitination, but the two proteins do not stably co-localize (Mosser et al. 2008). Additionally, this ubiquitination does not affect receptor internalization (Mosser et al. 2008).

5 A Novel Pathway for Internalization of the M_2 Receptor

In contrast to the endocytosis of the other mAChR subtypes which utilizes the relatively common clathrin/dynamin-dependent pathway, agonist-internalization of the M_2 receptor in at least some celltypes utilizes a distinct pathway. In HEK293 cells, M_2 sequestration displays a unique sensitivity to dynamin. Internalization of the receptor is not affected by the dominant negative dynamin, K44A, which has reduced GTP affinity and hydrolysis, although this construct significantly reduces sequestration of M_1, M_3 and M_4 (Vögler et al. 1998; Claing et al. 2000). However, M_2 internalization is inhibited by two other dominant negative dynamin constructs, N272 which lacks its GTP binding domain and K535M, which cannot be activated by PIP_2 (Werbonat et al. 2000). There also appears to be differential dynamin regulation between the M_1 and M_2 receptors as M_1 internalization has been reported

to require dynamin phosphorylation by c-Src while M_2 does not (Werbonat et al. 2000). Early studies indicated that M_2 internalization is not regulated by β-arrestin (Vögler et al. 1999a, b; Mundell and Benovic 2000; Claing et al. 2000) nor are clathrin-coated pits involved (Vögler et al. 1999a, b). More recently, studies using cells derived from β-arrestin knockout mice show that the M_2 receptor is unable to internalize in the absence of β-arrestin (Jones et al. 2006). Since internalization through caveolae is dynamin-dependent and M_2 has been associated with caveolin-rich fractions of cardiac myocytes (Feron et al. 1997), the possibility of caveolar internalization was investigated. Roseberry and Hosey (2001) used immunofluorescence to show that internalized M_2 does not colocalize with caveolin or clathrin, and that nystatin, which disrupts caveolae formation, does not inhibit internalization. This is in contrast to work in cardiac myocytes, where agonist-induced stimulation of M_2 receptors was reported to lead to caveolar sequestration in a dynamin-dependent manner (Dessy et al. 2000).

GIT1 is a protein that was originally isolated as GRK2-interacting protein. GIT1 has multiple binding partners and potential functions. It can bind to endosomes, the focal adhesion adaptor protein paxillin, and PIX proteins, which are guanine nucleotide exchange factors for Rac2 and other small G-proteins. Additionally, it can serve as a GTPase activator protein for ARF small G-proteins and as a scaffold for the MAPK activator MEK1 (Hoefen and Berk 2006). Claing et al. (2000) showed that overexpression of GIT1 inhibited the agonist-induced internalization of the M_1 receptor but did not inhibit the internalization of the M_2 receptor. They also showed that this differential sensitivity to GIT1 was observed with a number of other GPCRs and correlated well the sensitivity of the receptors to endocytosis through a β-arrestin sensitive, clathrin-dependent pathway.

Internalization and desensitization of the β_2-AdR seem to be related, as internalization is a mechanism of receptor resensitization (Yu et al. 1993). However, these processes are independent of each other for the M_2 receptor. A dominant negative GRK2, which cannot transfer phosphate groups from ATP to substrate, decreased the phosphorylation and functional desensitization of M_2 without interfering with internalization (Pals-Rylaarsdam et al. 1995). Additionally, phosphorylation sites on the third intracellular loop of M_2 differentially regulate internalization and desensitization (Pals-Rylaarsdam and Hosey 1997). While overexpression of GRK2 and β-arrestin are able to affect M_2 internalization (Schlador and Nathanson 1997; Tsuga et al. 1998a, b), this appears to not to be the normal pathway for M_2 internalization (Pals-Rylaarsdam and Hosey 1997).

Following internalization, receptors are either recycled back to the cell surface or they are targeted to the lysosome causing downregulation, or permanent loss, of the receptors from the cell. When cells are exposed to carbachol for a short amount of time and then allowed to recover, the M_1, M_3 and M_4, but not the M_2 receptor subtypes are able to recycle back to the cell surface (van Koppen 2001). This difference may be explained in part by the receptor subtypes associations with β-arrestin. Agonist stimulation of both the M_1 and M_2 receptors lead to ubiquitination of β-arrestin (Mosser et al. 2008). However, only the M_2 receptor stably colocalizes with ubiquitinated β-arrestin leading to receptor downregulation (Mosser et al. 2008).

6 The Role of Small G-Proteins in mAChR Trafficking

Members of the Rab and Arf families of small G-proteins are involved in various steps of intracellular transport. Delaney et al. (2002) showed that clathrin- and dynamin-independent internalization of M_2 receptor in HeLa cells was sensitive to inhibition by constitutively active Arf6. Volpicelli et al. (2001) examined the trafficking of the endogenously expressed M_4 receptor in PC12 cells. The receptor was sorted into early endosomes and was Rab5-dependent. Removal of carbachol led to recycling of the M_4 receptor back to the cell surface that was dependent on Rab11a and myosin Vb (Volpicelli et al. 2002). Vögler et al. (1999a, b) tested the role of RhoA on trafficking of mAChR in HEK293 cells. These authors concluded that while overexpression of RhoA could regulate both agonist-induced internalization and trafficking to the cell surface of both M_1 and M_2 receptors, endogenous RhoA did not regulate mAChR trafficking in HEK cells.

Reiner and Nathanson (2008) compared the effects of a number of dominant negative and constitutively active G-proteins on the trafficking of M_2 and M_4 receptors expressed in JEG-3 cells. Consistent with previous observations that these two receptors used different pathways for agonist-induced internalization, M_2 internalization was dependent on Arf6 and Rab22 and independent of Rab5 and Rab11, while internalization of the M_4 receptor was dependent on Rab5 and Rab11 but independent of Arf6 and Rab22. In unstimulated cells, co-expressed M_2 and M_4 receptors exhibited significant co-localization on the cell surface. Following agonist stimulation, internalized M_2 and M_4 receptors were initially localized to different populations of vesicles.

Delaney et al. (2002) showed that the M_2 receptor in HeLa cells initially exhibited agonist-induced internalization that was dynamin- and clathrin-independent, but then was transferred to endosomes from the clathrin-dependent pathway. In accordance with the results of Delaney et al. (2002), Reiner and Nathanson (2008) found that the internalized M_2 and M_4 receptors started to become co-localized to the same vesicles by 30 min after agonist stimulation.

7 Identification of mAChR-Interacting Proteins That Regulate Trafficking

7.1 Regulation of mAChR Trafficking by AGAP1

Bendor et al. (2010) used the yeast two hybrid system to identify the AP-3 adaptor complex regulator AGAP1 as a protein which binds to the i3L of M_5. AGAP1 did not interact with other mAChR subtypes, and M_5 did not interact with other AGAP family members. Both AGAP1 and AP-3 were required for recycling of internalized M_5 in neurons, and blocking this interaction resulted in increased receptor downregulation. Gene-targeted mice expressing a mutated M_5 lacking

the AGAP1 binding domain exhibited decreased M_5-mediated potentiation of dopamine release in the striatum, demonstrating that blocking M_5 recycling in vivo had clear physiological consequences.

7.2 Regulation of mAChR Trafficking by Eukaryotic Elongation Factor 1A2

McClatchy et al. (2002) used a fusion protein consisting of GST and the i3L of the M_4 receptor to identify eukaryotic elongation factor 1A2(eEF1A2) as a M_4 binding partner. eEF1A2 did not interact with the i3Ls of M_1 or M_2, and could be co-immunoprecipitated with M_4 but not M_1 from extracts of PC12 cells. The i3L of M_4 increased guanine nucleotide exchange by eEF1A2, raising the possibility that the M_4–eEF1A2 interaction might regulate protein synthesis. McClatchy et al. (2006) subsequently showed that overexpression of either eEF1A1 or eEF1A2 in PC12 cells inhibited the recovery of M_4 but not M_1 back to the cell surface after agonist-induced internalization. These results suggest that eEF1A2 (and presumably eEF1A1) regulate the recycling of the M_4 receptor in a subtype-specific fashion.

7.3 Regulation of mAChR Trafficking by GASP-1

G-protein-coupled receptor-associated sorting protein-1 (GASP-1) was originally identified as a protein that interacted with the carboxyl tail of the delta opioid receptor, and subsequently shown to interact with the C-tails of many GPCR, including the M_1 and M_2 receptors (Simonin et al. 2004). GASP-1 has been shown to target the sorting of several internalized GPCR to the lysosomal pathway (Moser et al. 2010). While no direct evidence for the regulation of mAChR trafficking by GASP-1 has been presented, a decrease in the number of mAChR in striatum of GASP-1 KO mice has been reported after a cocaine-self-administration paradigm (Boeuf et al. 2009). The mechanism for this decrease and the identity of the mAChR subtypes affected have not been determined.

7.4 Regulation of mAChR Trafficking by RACK1

Reiner et al. (2010) used isotope-coded affinity tagging followed by mass spectroscopy and identified the scaffolding protein RACK1 as a protein enriched in immunoprecipitated M_2-containing complexes from M_2-expressing cells compared to parallel immunoprecipitates from non-M_2 expressing cells. Western blot analysis demonstrated that RACK1 associated with the M_2 receptor in an agonist-regulated fashion, as incubation of cells with carbachol decreased the amount of RACK1

co-immunoprecipitated with the receptor. RACK1 regulated mAChR trafficking in a subtype specific manner. Overexpression of RACK1 decreased both agonist-induced internalization and downregulation of the M_2 receptor but not the M_1 receptor. Decreased RACK1 expression increased the extent of agonist-induced M_2 internalization, but resulted in an unexpected decrease in M_2 downregulation. These results suggest that while the RACK1 may normally inhibit M_2 internalization, it participates in the subsequent trafficking of sequestered receptors to the degradative pathway.

8 Potential Functions of Receptor Internalization

The internalization of receptors from the cell surface has many possible functions. Because internalization is the initial step in long-term downregulation of receptor number, agonist-induced endocytosis represents the beginning of a pathway allowing a cell to change its level of receptor expression and thus its sensitivity to stimulation in response to long-term changes in receptor number. Internalization is also frequently assumed to represent a mechanism for rapidly decreasing receptor signaling (e.g., Tsuga et al. 1998a, b; Shui et al. 2001). However, for receptors which interact with arrestins, it can be difficult to distinguish decreased signaling due to arrestin-dependent internalization from arrestin-mediated functional uncoupling from G-proteins. Furthermore, Pals-Rylaarsdam and Hosey (1997) identified mutations in the M_2 receptor that allowed normal internalization but blocked functional desensitization, showing that internalization could occur without a decrease in receptor function.

Internalization has been implicated in signaling by GPCRs and other receptors to MAPK, G_i, and G_s pathways, although the role of GPCR internalization in MAPK activation may be a reflection of the role of arrestins in both processes (Calebiro et al. 2010; Miaczynska and Bar-Sagi 2010). Budd et al. (1999) showed that the M_3 receptor could activate the Erk1/2 MAPK pathway in an internalization-independent fashion. Montiel et al. (2004) reported that inhibition of agonist-induced mAChR sequestration in Fisher rat thyroid cells by addition of high sucrose inhibited increases in intracellular Ca^{2+} in response to carbachol but not ATP. While these results suggest a role for internalization in mAChR signaling, sucrose also blocked thapsigargin-induced calcium mobilization, raising the possibility that high sucrose had non-specific effects on cell signaling.

Internalization has also been implicated in the regulation of receptor resensitization following desensitization. Inhibition of internalization blocked the resensitization of ß$_2$-adrenergic receptors, suggesting that internalization was required for recovery from desensitization (Yu et al. 1993; Pippig et al. 1995). In contrast, Bogatkewitsch et al. (1996) found that inhibition of internalization of the M_4 receptor expressed in CHO cells greatly increased the recovery of functional activity of desensitized receptors, suggesting that resensitization occured at the plasma membrane and is delayed by internalization.

9 Constitutive Internalization of mAChR

Trafficking of muscarinic receptors away from the plasma membrane can occur both constitutively and in an agonist-dependent fashion. The constitutive endocytic pathways have received little attention compared to the well-studied agonist-induced pathways. Work on the M_2 receptor suggests that these two pathways are similar. Roseberry and Hosey (1999) reported that recovery of both constitutively and agonist-induced internalized receptors to the cell surface of HEK cells had similar time courses and similar partial dependencies on protein synthesis. In contrast, Scarselli and Donaldson (2009) presented evidence that constitutive and agonist-induced internalization of the M_3 receptor utilized different pathways. Constitutive internalization utilized a clathrin-independent pathway, while agonist-induced internalization was clathrin-dependent. Interestingly, a mutant receptor lacking the third cytoplasmic loop exhibited agonist-induced internalization which was clathrin-independent, suggesting that different regions of the receptor mediate these two types of internalization.

10 Receptor Heterodimerization and Trafficking

Receptor dimerization is an important means for regulating receptor activities. Some receptors require homo-dimerization for activation and others form hetero-dimers that result in a change in signal transduction (Kubo and Tateyama 2005). Some dimers are formed in the endoplasmic reticulum and aid in the transport of the receptors to the cells surface; others are formed after the receptors have separately arrived on the cell surface (Devi 2001). Muscarinic receptor dimerization was first proposed based on the presence of appearance of multiple interconvertible polypeptide bands on SDS gels (Avissar et al. 1983) and experiments showing anomalous agonist binding properties (Potter et al. 1991; Hirschberg and Schimerlik 1994). Biochemical evidence for M_3 receptor dimerization was provided by chimeric receptor studies (Maggio et al. 1993) and co-immunoprecipitation of HA- and Flag-tagged mAChRs (Zeng and Wess 1999). Recent work in our lab used bioluminescence resonance energy transfer to show that the M_1, M_2, and M_3 mAChRs are able to form both homo- and hetero-dimers. The subtype-specificity of receptor trafficking could be regulated by heterodimerization: following coexpression of M_3 with M_2 in JEG-3 cells, agonist-induced downregulation of M_3 was more similar to the robust downregulation characteristic of the M_2 receptor than the modest downregulation seen when M_3 is expressed alone. These results suggest that M_2 is able to drive trafficking of a M_2/M_3 heterodimer to the M_2 trafficking pathway (Goin and Nathanson 2006).

Clancy et al. (2007) showed that NGF-differentiated PC12 cells released ACh which could cause internalization of both endogenously expressed M_2 and M_4 and transfected M_2 mAChR. Interesting, the GIRK potassium channels which are

normally activated by the M_2 receptor was also internalized; functional coupling of the mAChR to GIRK was significantly impaired. Cell surface expression of both the M_2 receptor and GIRK channels could be rescued by blocking mAChR activation with atropine. Boyer et al. (2009) found that the M_2 receptor interacted with the $GABA_B$ R2 receptor subunit via residues in the carboxyl-terminal domains of each receptor. This heterodimerization restored cell surface expression and functional responsiveness of M_2 receptors in PC12 cells normally exhibiting receptor sequestration due to release of endogenously produced ACh. Because receptor levels were restored by $GABA_B$ R2 expression when forward trafficking of newly synthesized proteins was blocked by brefeldin A, the authors suggested that GBR2 increased receptor plasma membrane expression by increasing trafficking either from the endoplasmic reticulum or endosomal compartments, perhaps by interfering with receptor downregulation.

11 Regulation of mAChR Trafficking by Allosteric Ligands

In contrast to the actions of classical orthostatic agonists, which bind to the same site on the receptor as ACh, allosteric agonists activate the receptor by binding to a site distinct from the ACh binding site. Conflicting results have been reported on the regulation of mAChR trafficking by allosteric agonists. Davis et al. (2010) found that the M_1 allosteric agonists AC-260584 and TBPB did not induce receptor internalization or downregulation in CHO cells under conditions where they produced functional responses similar in magnitude to orthostatic agonists. Consistent with a role for arrestins in M_1 trafficking, only the orthostatic agonists but not the allosteric agonists could induce β-arrestin2 recruitment to the membrane. Thomas et al. (2008) reported that the M_1 allosteric agonist AC-42 also did not cause M_1 internalization in CHO cells, although the related allosteric agonist 77-LH-28-1 did induce some internalization. In contrast, Davis et al. (2009) found that several M_1 allosteric agonists did induce both β-arrestin1 recruitment and receptor internalization in HEK293 cells, although differences in the extent of receptor recycling was observed with different allosteric agonists. It is not clear if the use of different cell lines was responsible for these discordant results on receptor sequestration.

12 Regulation of mAChR Trafficking by Anti-receptor Antibodies

Patients with Chagas' disease, as well as certain other conditions, have autoantibodies directed against the M_2 mAChR (Nussinovitch and Shoenfeld 2011). These autoantibodies bind to an epitope in the second extracellular loop and can both activate the receptor and induce receptor internalization (Leiros et al. 1997). These antibodies can induce receptor crosslinking (Beltrame et al. 2011),

although it is not known if the internalization is due to receptor crosslinking or if the pathways for agonist-induced and antibody-induced internalization are similar or distinct.

Tolbert and Lameh (1998) found that an antibody raised against the amino terminus of the M_1 receptor could induce receptor sequestration. The antibody did not stimulate receptor signaling and Fab fragments also induced internalization, indicating that receptor crosslinking was not required. Both agonist-induced and antibody-induced sequestration occurred via the clathrin-dependent pathway.

13 Regulation of mAChR Trafficking in AChE Knockout Mice

As noted above, early studies showed that treatment of animals with acetylcholinesterase inhibitors decreased mAChR numbers in multiple tissues, presumably due to the increased downregulation resulting from the resultant increased levels of endogenously released ACh. Knockout mice lacking AChE exhibited decreased numbers of mAChR-binding sites and decreased numbers of M_1, M_2, and M_4 receptors in multiple regions of the brain. This decreased number of mAChR was accompanied by dramatic decreases in a number of functional responses, including mAChR-mediated tremors, salivation, hypothermia, analgesia, seizures, and activation of MAP kinase. There was an increased localization of the remaining M_1, M_2, and M_4 receptors to intracellular puncta in both the hippocampus and striatum (Li et al. 2003; Volpicelli-Daley et al. 2003a, b). Decossas et al. (2003) observed interesting differences in the trafficking of the M_2 receptor on cholinergic neurons of the nucleus basalis magnocellularis from AChE knockout mice compared to wildtype mice acutely treated with AChE inhibitor. M_2 receptors in the somatodendritic domain were decreased at the cell surface, with increased M_2 receptors in endosomes in inhibitor-treated animals and increased M_2 receptors in the endoplasmic reticulum and Golgi in knockout animals. These results suggest different mechanisms of regulation due to increased endocytosis, and decreased transport to the cell surface, respectively. Surprisingly, there was an apparent increase in M_2 receptors at axonal varicosities in the knockout animals and no change in axonal receptor density in inhibitor-treated mice, suggesting that there are also domain-specific difference in receptor trafficking.

14 Conclusions

While much has been learned about the mechanisms responsible for the regulation of mAChR cell surface and total cellular expression since the initial descriptions of agonist-induced sequestration downregulation over 30 years ago, there are many questions which remain about both the molecular components and cellular pathways involved and on the physiological consequences and functions of these

processes. The application of new experimental methods as they are developed will undoubtedly continue to yield new and surprising insights into these regulatory pathways.

References

Anderson RG, Goldstein JL, Brown MS (1977) A mutation that impairs the ability of lipoprotein receptors to localise in coated pits on the cell surface of human fibroblasts. Nature 270:695–699

Avissar S, Amitai G, Sokolovsky M (1983) Oligomeric structure of muscarinic receptors is shown by photoaffinity labeling: subunit assembly may explain high- and low-affinity agonist states. Proc Natl Acad Sci USA 80:156–159

Beltrame SP, Auger SR, Bilder CR et al (2011) Modulation of M(2) muscarinic receptor-receptor interaction by immunoglobulin G antibodies from Chagas' disease patients. Clin Exp Immunol 164:170–179

Ben-Barak J, Dudai Y (1980) Scopolamine induces an increase in muscarinic receptor level in rat hippocampus. Brain Res 193:309–313

Bendor J, Lizardi-Ortiz JE, Westphalen RI et al (2010) AGAP1/AP-3-dependent endocytic recycling of M_5 muscarinic receptors promotes dopamine release. EMBO J 29:2813–2826

Bichet DG (2008) Vasopressin receptor mutations in nephrogenic diabetes insipidus. Semin Nephrol 28:245–251

Boeuf J, Trigo JM, Moreau PH et al (2009) Attenuated behavioural responses to acute and chronic cocaine in GASP-1-deficient mice. Eur J Neurosci 30:860–868

Bogatkewitsch GS, Lenz W, Jakobs KH et al (1996) Receptor internalization delays m_4 muscarinic acetylcholine receptor resensitization at the plasma membrane. Mol Pharmacol 5:424–429

Bouvier M, Hausdorff WP, De Blasi A et al (1988) Removal of phosphorylation sites from the beta 2-adrenergic receptor delays onset of agonist-promoted desensitization. Nature 333:370–373

Boyer SB, Clancy SM, Terunuma M et al (2009) Direct interaction of GABAB receptors with M_2 muscarinic receptors enhances muscarinic signaling. J Neurosci 29:15796–15809

Budd DC, Rae A, Tobin AB (1999) Activation of the mitogen-activated protein kinase pathway by a Gq/11-coupled muscarinic receptor is independent of receptor internalization. J Biol Chem 274:12355–12360

Burgoyne RD, Pearce B (1981) Muscarinic acetylcholine receptors regulation and protein phosphorylation in primary cultures of rat cerebellum. Brain Res 254:55–63

Calebiro D, Nikolaev VO, Persani L et al (2010) Signaling by internalized G-protein-coupled receptors. Trends Pharmacol Sci 31:221–228

Claing A, Perry SJ, Achiriloaie M et al (2000) Multiple endocytic pathways of G protein-coupled receptors delineated by GIT1 sensitivity. Proc Natl Acad Sci USA 97:1119–1124

Clancy SM, Boyer SB, Slesinger PA (2007) Coregulation of natively expressed pertussis toxin-sensitive muscarinic receptors with G-protein-activated potassium channels. J Neurosci 27:6388–6399

Costa LG, Murphy SD (1985) Characterization of muscarinic cholinergic receptors in the submandibular gland of the rat. J Auton Nerv Syst 13:287–301

Daaka Y, Luttrell LM, Ahn S et al (1998) Essential role for G protein-coupled receptor endocytosis in the activation of mitogen-activated protein kinase. J Biol Chem 273:685–688

Davis CN, Bradley SR, Schiffer HH et al (2009) Differential regulation of muscarinic M_1 receptors by orthosteric and allosteric ligands. BMC Pharmacol 9:14

Davis AA, Heilman CJ, Brady AE et al (2010) Differential effects of allosteric M(1) muscarinic acetylcholine receptor agonists on receptor activation, arrestin 3 recruitment, and receptor downregulation. ACS Chem Neurosci 1:542–551

Dawson RM, Jarrott B (1981) Response of muscarinic cholinoceptors of guinea pig brain and ileum to chronic administration of carbamate or organophosphate cholinesterase inhibitors. Biochem Pharmacol 30:2365–2368

Decossas M, Bloch B, Bernard V (2003) Trafficking of the muscarinic m_2 autoreceptor in cholinergic basalocortical neurons in vivo: differential regulation of plasma membrane receptor availability and intraneuronal localization in acetylcholinesterase-deficient and -inhibited mice. J Comp Neurol 462:302–314

Delaney KA, Murph MM, Brown LM et al (2002) Transfer of M_2 muscarinic acetylcholine receptors to clathrin-derived early endosomes following clathrin-independent endocytosis. J Biol Chem 277:33439–33446

Dessy C, Kelly RA, Balligand JL et al (2000) Dynamin mediates caveolar sequestration of muscarinic cholinergic receptors and alteration in NO signaling. EMBO J 19:4272–4280

Devi LA (2001) Heterodimerization of G-protein-coupled receptors: pharmacology, signaling and trafficking. Trends Pharmacol Sci 22:532–537

Di Guglielmo GM, Le Roy C, Goodfellow AF et al (2003) Distinct endocytic pathways regulate TGF-beta receptor signalling and turnover. Nat Cell Biol 5:410–421

Feigenbaum P, El-Fakahany EE (1985) Regulation of muscarinic cholinergic receptor density in neuroblastoma cells by brief exposure to agonist: possible involvement in desensitization of receptor function. J Pharmacol Exp Ther 233:134–140

Ferguson SSG, Downey WE III, Colapietro AM et al (1996) Role of beta-arrestin in mediating agonist-promoted G protein-coupled receptor internalization. Science 271:363–366

Feron O, Smith TW, Michel T et al (1997) Dynamic targeting of the agonist-stimulated m_2 muscarinic acetylcholine receptor to caveolae in cardiac myocytes. J Biol Chem 272:17744–17748

Galper JB, Smith TW (1980) Agonist and guanine nucleotide modulation of muscarinic cholinergic receptors in cultured heart cells. J Biol Chem 255:9571–9579

Galper JB, Dziekan LC, Miura DS et al (1982a) Agonist-induced changes in the modulation of K^+ permeability and beating rate by muscarinic agonists in cultured heart cells. J Gen Physiol 80:231–256

Galper JB, Dziekan LC, O'Hara DS et al (1982b) The biphasic response of muscarinic cholinergic receptors in cultured heart cells to agonists. J Biol Chem 257:10344–10356

Gazit H, Silman I, Dudai Y (1979) Administration of an organophosphate causes a decrease in muscarinic receptor levels in rat brain. Brain Res 174:351–356

Goin JC, Nathanson NM (2006) Quantitative analysis of muscarinic acetylcholine receptor homo- and heterodimerization in live cells: regulation of receptor down-regulation by heterodimerization. J Biol Chem 281:5416–5425

Goldman PS, Schlador ML, Shapiro RA et al (1996) Identification of a region required for subtype-specific agonist-induced sequestration of the m_2 muscarinic acetylcholine receptor. J Biol Chem 271:4215–4222

Halvorsen SW, Nathanson NM (1981) In vivo regulation of muscarinic acetylcholine receptor number and function in embryonic chick heart. J Biol Chem 256:7941–7948

Harden TK, Petch LA, Traynelis SF et al (1985) Agonist-induced alteration in the membrane form of muscarinic cholinergic receptors. J Biol Chem 260:13060–13066

Hirschberg BT, Schimerlik MI (1994) A kinetic model for oxotremorine M binding to recombinant porcine m_2 muscarinic receptors expressed in Chinese hamster ovary cells. J Biol Chem 269:26127–26135

Hoefen RJ, Berk BC (2006) The multifunctional GIT family of proteins. J Cell Sci 119:1469–1475

Hunter DD, Nathanson NM (1984) Decreased physiological sensitivity mediated by newly synthesized muscarinic acetylcholine receptors in the embryonic chick heart. Proc Natl Acad Sci USA 81:3582–3586

Hunter DD, Nathanson NM (1986) Biochemical and physical analyses of newly synthesized muscarinic acetylcholine receptors in cultured embryonic chicken cardiac cells. J Neurosci 6:3739–3748

Jones KT, Echeverry M, Mosser VA et al (2006) Agonist mediated internalization of M_2 mAChR is beta-arrestin-dependent. J Mol Signal 1:7

Klein WL, Nathanson NM, Nirenberg M (1979) Muscarinic acetylcholine receptor regulation by accelerated rate of receptor loss. Biochem Biophys Res Commun 90:506–512

Koenig JA, Edwardson JM (1996) Intracellular trafficking of the muscarinic acetylcholine receptor: importance of subtype and cell type. Mol Pharmacol 49:351–359

Kubo Y, Tateyama M (2005) Towards a view of functioning dimeric metabotropic receptors. Curr Opin Neurobiol 15:289–295

Laporte SA, Oakley RH, Zhang J et al (1999) The beta2-adrenergic receptor/betaarrestin complex recruits the clathrin adaptor AP-2 during endocytosis. Proc Natl Acad Sci USA 96 (7):3712–3717

Laporte SA, Oakley RH, Holt JA et al (2000) The interaction of beta-arrestin with the AP-2 adaptor is required for the clustering of beta 2-adrenergic receptor into clathrin-coated pits. J Biol Chem 275:23120–23126

Lee KB, Pals-Rylaarsdam R, Benovic JL et al (1998) Arrestin-independent internalization of the m_1, m_3, and m_4 subtypes of muscarinic cholinergic receptors. J Biol Chem 273:12967–12972

Lefkowitz RJ, Rajagopal K, Whalen EJ (2006) New roles for beta-arrestins in cell signaling: not just for seven-transmembrane receptors. Mol Cell 24:643–652

Leiros CP, Sterin-Borda L, Borda ES et al (1997) Desensitization and sequestration of human m_2 muscarinic acetylcholine receptors by autoantibodies from patients with Chagas' disease. J Biol Chem 272:12989–12993

Li B, Duysen EG, Volpicelli-Daley LA et al (2003) Regulation of muscarinic acetylcholine receptor function in acetylcholinesterase knockout mice. Pharmacol Biochem Behav 74:977–986

Lohse MJ, Lefkowitz RJ, Caron MG et al (1989) Inhibition of beta-adrenergic receptor kinase prevents rapid homologous desensitization of beta 2-adrenergic receptors. Proc Natl Acad Sci USA 86:3011–3015

Luo J, Busillo JM, Benovic JL (2008) M_3 muscarinic acetylcholine receptor-mediated signaling is regulated by distinct mechanisms. Mol Pharmacol 74:338–347

Maggio R, Vogel Z, Wess J (1993) Reconstitution of functional muscarinic receptors by co-expression of amino- and carboxyl-terminal receptor fragments. FEBS Lett 319(1–2):195–200

Malenka RC (2003) Synaptic plasticity and AMPA receptor trafficking. Ann N Y Acad Sci 1003:1–11

Maloteaux JM, Gossuin A, Pauwels PJ et al (1983) Short-term disappearance of muscarinic cell surface receptors in carbachol-induced desensitization. FEBS Lett 156:103–107

Marks MJ, Artman LD, Patinkin DM et al (1981) Cholinergic adaptations to chronic oxotremorine infusion. J Pharmacol Exp Ther 218:337–343

McClatchy DB, Knudsen CR, Clark BF et al (2002) Novel interaction between the M_4 muscarinic acetylcholine receptor and elongation factor 1A2. J Biol Chem 277:29268–29274

McClatchy DB, Fang G, Levey AI (2006) Elongation factor 1A family regulates the recycling of the M_4 muscarinic acetylcholine receptor. Neurochem Res 31:975–988

Mendes HF, van der Spuy J, Chapple JP et al (2005) Mechanisms of cell death in rhodopsin retinitis pigmentosa: implications for therapy. Trends Mol Med 11:177–185

Meyer MR, Gainer MW, Nathanson NM (1982) In vivo regulation of muscarinic cholinergic receptors in embryonic chick brain. Mol Pharmacol 21:280–286

Miaczynska M, Bar-Sagi D (2010) Signaling endosomes: seeing is believing. Curr Opin Cell Biol 22:535–540

Montiel M, Quesada J, Jimenez E (2004) Activation of second messenger-dependent protein kinases induces muscarinic acetylcholine receptor desensitization in rat thyroid epithelial cells. Mol Cell Endocrinol 223:35–41

Moser E, Kargl J, Whistler JL et al (2010) G protein-coupled receptor-associated sorting protein 1 regulates the postendocytic sorting of seven-transmembrane-spanning G protein-coupled receptors. Pharmacology 86:22–29

Mosser VA, Jones KT, Hoffman KM et al (2008) Differential role of beta-arrestin ubiquitination in agonist-promoted down-regulation of M_1 vs M_2 muscarinic acetylcholine receptors. J Mol Signal 3:20

Mundell SJ, Benovic JL (2000) Selective regulation of endogenous G protein-coupled receptors by arrestins in HEK293 cells. J Biol Chem 275:12900–12908

Mundell SJ, Loudon RP, Benovic JL (1999) Characterization of G protein-coupled receptor regulation in antisense mRNA-expressing cells with reduced arrestin levels. Biochemistry 38:8723–8732

Nathanson NM (1989) Regulation and development of muscarinic acetylcholine number and function. In: Brown JH (ed) The muscarinic receptors. Humana, Clifton, NJ

Nathanson NM (2008) Synthesis, trafficking, and localization of muscarinic acetylcholine receptors. Pharmacol Ther 119:33–43

Nathanson NM, Klein WL, Nirenberg M (1978) Regulation of adenylate cyclase activity mediated by muscarinic acetylcholine receptors. Proc Natl Acad Sci USA 75:1788–1791

Nathanson NM, Holttum J, Hunter DD et al (1984) Partial agonist activity of oxotremorine at muscarinic acetylcholine receptors in the embryonic chick heart. J Pharmacol Exp Ther 229:455–458

Nussinovitch U, Shoenfeld Y (2011) The diagnostic and clinical significance of anti-muscarinic receptor autoantibodies. Clin Rev Allerg Immunol. doi:10.1007/s12016-010-8235-x

Olianas MC, Onali P, Schwartz JP et al (1984) The muscarinic receptor adenylate cyclase complex of rat striatum: desensitization following chronic inhibition of acetylcholinesterase activity. J Neurochem 42:1439–1443

Pals-Rylaarsdam R, Hosey MM (1997) Two homologous phosphorylation domains differentially contribute to desensitization and internalization of the m_2 muscarinic acetylcholine receptor. J Biol Chem 272:14152–14158

Pals-Rylaarsdam R, Xu Y, Witt-Enderby P et al (1995) Desensitization and internalization of the m_2 muscarinic acetylcholine receptor are directed by independent mechanisms. J Biol Chem 270:29004–29011

Peng Y, Zhao J, Gu QH et al (2010) Distinct trafficking and expression mechanisms underlie LTP and LTD of NMDA receptor-mediated synaptic responses. Hippocampus 20:646–658

Pierce KL, Maudsley S, Daaka Y et al (2000) Role of endocytosis in the activation of the extracellular signal-regulated kinase cascade by sequestering and nonsequestering G protein-coupled receptors. Proc Natl Acad Sci USA 97:1489–1494

Pippig S, Andexinger S, Lohse MJ (1995) Sequestration and recycling of beta 2-adrenergic receptors permit receptor resensitization. Mol Pharmacol 47:666–676

Potter LT, Ballesteros LA, Bichajian LH et al (1991) Evidence of paired M_2 muscarinic receptors. Mol Pharmacol 39:211–221

Poulin B, Butcher A, McWilliams P et al (2010) The M_3-muscarinic receptor regulates learning and memory in a receptor phosphorylation/arrestin-dependent manner. Proc Natl Acad Sci USA 107:9440–9445

Rehavi M, Ramot O, Yavetz B et al (1980) Amitripyline: long-term treatment elevates alpha-adrenergic and muscarinic receptor binding in mouse brain. Brain Res 194:443–453

Reiner C, Nathanson NM (2008) The internalization of the M_2 and M_4 muscarinic acetylcholine receptors involves distinct subsets of small G-proteins. Life Sci 82:718–727

Reiner CL, McCullar J, Kow R et al (2010) RACK1 associates with muscarinic receptors and regulates M_2 receptor trafficking. PLoS One 5(10):e13517

Roseberry AG, Hosey MM (2001) Internalization of the M_2 muscarinic acetylcholine receptor proceeds through an atypical pathway in HEK293 cells that is independent of clathrin and caveolae. J Cell Sci 114:739–746

Roseberry, AG, Hosey, MM (1999). Trafficking of M2 muscarinic acetylcholine receptors. J Biol Chem 274:33671–33676.

Scarselli M, Donaldson JG (2009) Constitutive internalization of G protein-coupled receptors and G proteins via clathrin-independent endocytosis. J Biol Chem 284:3577–3585

Scherer NM, Nathanson NM (1990) Differential regulation by agonist and phorbol ester of cloned m_1 and m_2 muscarinic receptors in mouse Y1 adrenal cells and in Y1 cells deficient in cAMP-dependent protein kinase. Biochemistry 29:8475–8483

Schlador ML, Nathanson NM (1997) Synergistic regulation of m_2 muscarinic acetylcholine receptor desensitization and sequestration by G protein-coupled receptor kinase-2 and beta-arrestin-1. J Biol Chem 272:18882–18890

Schlador ML, Grubbs R, Nathanson NM (2000) Multiple topological domains mediate subtype-specific internalization of the M_2 muscarinic acetylcholine receptor. J Biol Chem 275:23295–23302

Schmid CL, Bohn LM (2009) Physiological and pharmacological implications of beta-arrestin regulation. Pharmacol Ther 121:285–293

Shifrin GS, Klein WL (1980) Regulation of muscarinic acetylcholine receptor concentration in cloned neuroblastoma cells. J Neurochem 34:993–999

Shui Z, Yamanushi TT, Boyett MR (2001) Evidence of involvement of GIRK1/GIRK4 in long-term desensitization of cardiac muscarinic K^+ channels. Am J Physiol Heart Circ Physiol 280: H2554–H2562

Siman RG, Klein WL (1979) Cholinergic activity regulates muscarinic receptors in central nervous system cultures. Proc Natl Acad Sci USA 76:4141–4145

Siman RG, Klein WL (1981) Specificity of muscarinic acetylcholine receptor regulation by receptor activity. J Neurochem 37:1099–1108

Simonin F, Karcher P, Boeuf JJ et al (2004) Identification of a novel family of G protein-coupled receptor associated sorting proteins. J Neurochem 89:766–775

Takeyasu K, Uchida S, Lai RT et al (1981) Regulation of muscarinic acetylcholine receptors and contractility of guinea pig vas deferens. Life Sci 28:527–540

Taylor JE, El-Fakahany E, Richelson E (1979) Long-term regulation of muscarinic acetylcholine receptors on cultured nerve cells. Life Sci 25:2181–2187

Taylor JE, Yaksh TL, Richelson E (1982) Agonist regulation of muscarinic acetylcholine receptors in rat spinal cord. J Neurochem 39:521–524

Thomas RL, Mistry R, Langmead CJ et al (2008) G protein coupling and signaling pathway activation by m_1 muscarinic acetylcholine receptor orthosteric and allosteric agonists. J Pharmacol Exp Ther 327:365–374

Tolbert LM, Lameh J (1998) Antibody to epitope tag induces internalization of human muscarinic subtype 1 receptor. J Neurochem 70:113–119

Tsuga H, Kameyama K, Haga T (1998a) Desensitization of human muscarinic acetylcholine receptor m_2 subtypes is caused by their sequestration/internalization. J Biochem 124:863–868

Tsuga H, Kameyama K, Haga T et al (1998b) Internalization and down-regulation of human muscarinic acetylcholine receptor m_2 subtypes. Role of third intracellular m_2 loop and G protein-coupled receptor kinase 2. J Biol Chem 273:5323–5330

van Koppen CJ (2001) Multiple pathways for the dynamin-regulated internalization of muscarinic acetylcholine receptors. Biochem Soc Trans 29:505–508

Vögler O, Bogatkewitsch GS, Wriske C et al (1998) Receptor subtype-specific regulation of muscarinic acetylcholine receptor sequestration by dynamin. Distinct sequestration of m_2 receptors. J Biol Chem 273:12155–12160

Vögler O, Krummenerl P, Schmidt M et al (1999a) RhoA-sensitive trafficking of muscarinic acetylcholine receptors. J Pharmacol Exp Ther 288:36–42

Vögler O, Nolte B, Voss M et al (1999b) Regulation of muscarinic acetylcholine receptor sequestration and function by beta-arrestin. J Biol Chem 274:12333–12338

Volpicelli LA, Lah JJ, Levey AI (2001) Rab5-dependent trafficking of the m_4 muscarinic acetylcholine receptor to the plasma membrane, early endosomes, and multivesicular bodies. J Biol Chem 276:47590–47598

Volpicelli LA, Lah JJ, Fang G et al (2002) Rab11a and myosin Vb regulate recycling of the M_4 muscarinic acetylcholine receptor. J Neurosci 22:9776–9784

Volpicelli-Daley LA, Duysen EG, Lockridge O et al (2003a) Altered hippocampal muscarinic receptors in acetylcholinesterase-deficient mice. Ann Neurol 53:788–796

Volpicelli-Daley LA, Hrabovska A, Duysen EG et al (2003b) Altered striatal function and muscarinic cholinergic receptors in acetylcholinesterase knockout mice. Mol Pharmacol 64:1309–1316

Werbonat Y, Kleutges N, Jakobs KH et al (2000) Essential role of dynamin in internalization of M_2 muscarinic acetylcholine and angiotensin AT1A receptors. J Biol Chem 275:21969–21974

Westlind A, Grynfarh M, Hedlund B et al (1981) Muscarinic supersensitivity induced by septal lesions or chronic atropine treatment. Brain Res 225:131–141

Wise BC, Shoji M, Kuo JF (1980) Decrease or increase in cardiac muscarinic cholinergic receptor number in rats treated with methacholine or atropine. Biochem Biophys Res Commun 92:1136–1142

Yu SS, Lefkowitz RJ, Hausdorff WP (1993) Beta-adrenergic receptor sequestration. A potential mechanism of receptor resensitization. J Biol Chem 268:337–341

Zeng FY, Wess J (1999) Identification and molecular characterization of m_3 muscarinic receptor dimers. J Biol Chem 274:19487–19497

Zhang J, Ferguson SS, Barak LS et al (1996) Dynamin and beta-arrestin reveal distinct mechanisms for G protein-coupled receptor internalization. J Biol Chem 271:18302–18305

Physiological Role of G-Protein Coupled Receptor Phosphorylation

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Abstract It is now well established that G-protein coupled receptors (GPCRs) are hyper-phosphorylated following agonist occupation usually at serine and threonine residues contained on the third intracellular loop and C-terminal tail. After some 2 decades of intensive research, the nature of protein kinases involved in this process together with the signalling consequences of receptor phosphorylation has been firmly established. The major challenge that the field currently faces is placing all this information within a physiological context and determining to what extent does phosphoregulation of GPCRs impact on whole animal responses. In this chapter, we address this issue by describing how GPCR phosphorylation might vary depending on the cell type in which the receptor is expressed and how this might be employed to drive selective regulation of physiological responses.

Keywords G-protein coupled receptor • Phosphorylation • Bar code • G-protein coupled receptor kinase

Abbreviations

GPCR	G-protein coupled receptor
GRK	G-protein coupled receptor kinase

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1 Introduction

It is now well established that nearly all G-protein coupled receptors (GPCRs) are phosphorylated in response to agonist occupation and that this process constitutes the major mechanism of regulation of receptor signalling. The outcome of literally thousands of studies in this area can be distilled into the following general observations.

Agonist occupation is considered to result in a conformational change in the receptor that unmasks phosphorylation sites on the intracellular domains (Kristiansen 2004). This allows for the phosphorylation of these sites by one or more of the family of G-protein coupled receptor (GRK) kinases (Lefkowitz 2004; Pitcher et al. 1998) in a process that promotes the interaction of the activated receptor with the adaptor protein arrestin. The interaction of the phosphorylated receptor with arrestin displaces the G-protein from the receptor by way of steric hindrance and in this way desensitizes receptor/G-protein signalling (Gurevich and Gurevich 2004, 2006a). Arrestin molecules have also been shown to possess a large number of interacting partners (Xiao et al. 2007) the most notable of which are signalling proteins such as components of the MAP kinase pathway (Lefkowitz and Shenoy 2005; Lefkowitz and Whalen 2004), tyrosine kinases (e.g. SRC) (Luttrell and Luttrell 2004) and enzymes such as diacylglycerol kinase (Nelson et al. 2007). It is through the interaction of arrestin with these signalling molecules that arrestin is able to couple the receptor to numerous signalling pathways (Lefkowitz and Whalen 2004). Hence, the phosphorylation-dependent recruitment of arrestin to the receptor not only uncouples the receptor from its cognate G-protein, but through its ability to interact with a plethora of signalling molecules, arrestins are able to mediate a variety of non-G protein signalling pathways (Gurevich and Gurevich 2004, 2006a, b; Lefkowitz and Shenoy 2005; Lefkowitz and Whalen 2004; Whalen et al. 2011).

Whereas this generalized scheme has served us well it is nevertheless a broad brush to paint over the regulatory features of hundreds of non-olfactory GPCRs. It is very likely that the detail of the regulatory mechanisms for each GPCR subtype in its physiologically relevant context will show important differences that will be linked to the physiological role of that particular receptor. The generalized concepts of GPCR regulation, which have largely been described in recombinant systems, are however invaluable in providing a framework, and in many cases the reagents and experimental design, to test the detailed regulatory mechanisms of GPCRs in a physiological context.

In the sections that follow we will examine the evidence that GPCR phosphorylation is a subtle and complex regulatory mechanism that can be tailored to mediate specific signalling outcomes that are associated with desired physiological responses.

2 Rhodopsin Phosphorylation: A Rheostat Control for the Sensitivity of Phototransduction

Rhodopsin is regulated by visual arrestin (arrestin-1) (Gurevich and Gurevich 2006a) that is expressed exclusively in the rod cells (Song et al. 2011) and shows high selectivity for rhodopsin (Vishnivetskiy et al. 2011). The recruitment of arrestin-1 to light-activated rhodopsin uncouples rhodopsin from its G-protein, transduction, and thereby switches off or desensitizes the visual transduction pathway in processes that fits well with the generalized mechanism of GPCR regulation as described earlier.

The ability of rhodopsin to interact with arrestin is determined in a two-component system. The first component is constituted by multiple interactions between the activated receptor and the concave surfaces of the two lobes of arrestin-1 (Gurevich and Gurevich 2006b). The surfaces of arrestin involved in this interaction are collectively described as the activation sensor as they respond to the active conformation of the receptor. The second component, however, is responsible for the high affinity binding between rhodopsin and arrestin-1 and is largely mediated by the interaction of phosphorylated serine residues within the C-terminal tail of the light-activated rhodopsin and the phosphate sensor of arrestin-1 situated in the polar core (Gurevich and Gurevich 2006b; Vishnivetskiy et al. 1999). Depending on the mammalian species there are six or seven potential GRK-1 phosphorylation sites in the C terminus of rhodopsin (Kennedy et al. 2001). Interestingly, it appears that only three of these sites need to be phosphorylated to obtain maximal high affinity binding of arrestin-1 (Mendez et al. 2000). This correlates with the fact that in vivo the maximal phosphorylation status of rhodopsin appears to be relatively low (2–3 sites) (Kennedy et al. 2001; Mendez et al. 2000) and occurs in a sequential manner that correlates with the degree of illumination (Gurevich and Gurevich 2004). Thus, in dark conditions rhodopsin will be largely unphosphorylated but with increasing light intensities the number of phosphorylated sites increases. The functional consequence is that the increasing phosphorylation status of rhodopsin results in a stepwise increase in the affinity of rhodopsin for arrestin-1 (Vishnivetskiy et al. 2007). Thus, under high levels of illumination rhodopsin will be phosphorylated on at least three sites and this results in maximal binding of arrestin and receptor desensitization. In dim light, rhodopsin is phosphorylated on one or two sites and thereby has lower affinity for arrestin-1 and, as a consequence, lower levels of desensitization.

Recent studies have taken this further and described the fact that arrestin-1 may interact with more than one rhodopsin molecule (Sommer et al. 2011). In these studies the ability of arrestin to interact with two molecules of rhodopsin is determined by the level of light activation (Sommer et al. 2011; Vishnivetskiy et al. 2011). Thus, under dim light the receptor is phosphorylated on one of the seven potential serine phosphoacceptor sites on the C terminus and interacts weakly with arrestin-1 which is able to bind to only one molecule of rhodopsin. Under these conditions receptor desensitization will be low and the system is highly sensitive to

light and is described as “dark adapted”. Higher levels of light illumination result in higher levels of receptor phosphorylation and consequently high affinity binding of arrestin-1. Under these conditions arrestin appears able to interact with two rhodopsin molecules via the two lobes of arrestin that appear from X-ray crystal structures to be positioned in a manner that can accommodate two receptors (Hirsch et al. 1999; Sommer et al. 2011). This results in maximal levels of desensitization and for the phototransduction system to be “light adapted”.

Thus, the level of rhodopsin phosphorylation reflects the level of light activation and thus the degree of desensitization of the system can be graded or adapted to correspond to the sensitivity required for different light conditions (Gurevich and Gurevich 2004). Thus, in the phototransduction system the phosphorylation status of rhodopsin appears to act as a rheostat whereby different levels of phosphorylation regulate the level of sensitivity of the phototransduction pathway. Hence, the role played by graded phosphorylation of rhodopsin in the regulation of phototransduction is an excellent example of how by varying the phosphorylation status of a GPCR the physiological response of the receptor can be controlled.

3 Non-visual GPCRs Are Phosphorylated in a Complex Manner

Whereas the visual receptors of rhodopsin and opsin are phosphorylated exclusively by GRK-1 and GRK-2 respectively, the non-visual GPCRs which are considered to be phosphorylated by the other members of the GRK family, namely GRK-3,4,5 and GRK-6. These GRK family members show a ubiquitous tissue expression profile that mirrors that of the non-visual GPCRs. That these GRKs have the potential to phosphorylate some 800 non-visual GPCRs is attributable to the fact that the consensus sequence for the GRKs is rather flexible.

The GRK family are acidotrophic protein kinases of the AGC class of kinases and based on experiments using peptide substrates have a consensus sequence of $D/E_nS/TXXX$ (Onorato et al. 1991). This rather broad consensus sequence together with the widespread expression profile of the GRKs supports the possibility that this kinase family can act on a large number of receptor substrates. This suggestion is upheld by numerous in vitro studies using purified GRKs and receptor substrates (Benovic et al. 1987, 1991; Kwatra et al. 1989, 1993; Pitcher et al. 1992) and more recently by the use of GRK knockdown with siRNA ((Kim et al. 2005; Ren et al. 2005) that confirms the ability of the GRK family to phosphorylate a broad spectrum of receptors.

It has, however, become increasingly clear that GPCRs can be phosphorylated by protein kinases other than the GRKs. This was evident in the very first studies of GPCR regulation where second messenger-regulated protein kinases, such as protein kinase A (PKA), were able to phosphorylate and desensitize the β_2-adrenergic receptor (Hausdorff et al. 1989; Seibold et al. 2000; Tran et al. 2004). Interestingly, the precise mechanism by which the second messenger-regulated protein kinases are able to mediate receptor desensitization has never been satisfactorily explained

particularly since the mechanism does not appear to involve the recruitment of arrestin (Lohse et al. 1992). The investigation of non-GRK-mediated GPCR phosphorylation has now been extended by studies that have determined the role of a number of different protein kinases in the phosphorylation of GPCRs (Tobin 2008; Tobin et al. 2008). These include a role for protein kinase CK2 (Torrecilla et al. 2007), casein kinase 1α (Luo et al. 2008; Tobin et al. 1997), Akt/PKB (Doronin et al. 2002; Gavi et al. 2006; Lee et al. 2001) and tyrosine phosphorylation-mediated phosphorylation by the insulin receptor (Wang et al. 2000) and possibly SRC-like tyrosine kinases (Kramer et al. 2000; Luttrell and Luttrell 2004).

The involvement of a range of protein kinases in the phosphorylation of GPCRs is reflected in the complex nature of the phosphoacceptor sites. Detailed analysis of the M_3-muscarinic receptor by mass spectrometry and tryptic phosphopeptide analysis has determined that there are at least 13 serine and 2 threonine phosphoacceptor sites within the third intracellular loop and C-terminal tail (Butcher et al. 2011; Torrecilla et al. 2007). Anti-phosphosite antibodies to three of these sites demonstrated that on ligand activation most of the phosphorylation sites showed an increase in phosphorylation. However, one site within the third intracellular loop (serine384), which showed high levels of basal phosphorylation, was seen to decrease in phosphorylation following agonist stimulation (Butcher et al. 2011). These studies on the M_3-muscarinic receptor revealed the complex nature of receptor phosphorylation where there are multiple sites of phosphorylation, some of which increase in phosphorylation following agonist stimulation and others that can decrease (Fig. 1).

It appears that similar complex patterns of receptor phosphorylation might be a common occurrence among GPCRs. For example, recent studies from our laboratory have determined that the M_1-muscarinic is phosphorylated at least 11 times within the third intracellular loop and once within the C-tail (unpublished observation). Reconstitution of GRK-2 and casein kinase 1α with the partially purified M_1-muscarinic receptor results in agonist-dependent phosphorylation (Haga et al. 1996; Waugh et al. 1999) indicating that like the M_3-muscarinic receptor this receptor subtype might be a substrate for multiple protein kinases. Furthermore, the multi-site nature of GPCR phosphorylation has also been demonstrated from tryptic phosphosite mapping studies on the bradykinin B_2 receptor (Blaukat et al. 2001), mass spectrometry analysis on the β_2-adrenergic receptor and CXCR4 receptor (Busillo et al. 2010; Trester-Zedlitz et al. 2005) and the use of anti-phospho site antibodies on the somatostatin A2, CXCR4 and β_2-adreneric receptors (Busillo et al. 2010; Ghosh and Schonbrunn 2011; Tran et al. 2004). It appears, therefore, that not only is it the case that nearly all GPCR subtypes are phosphorylated following agonist binding but they are phosphorylated at multiple sites through a mechanism that is likely to involve more than one protein kinase.

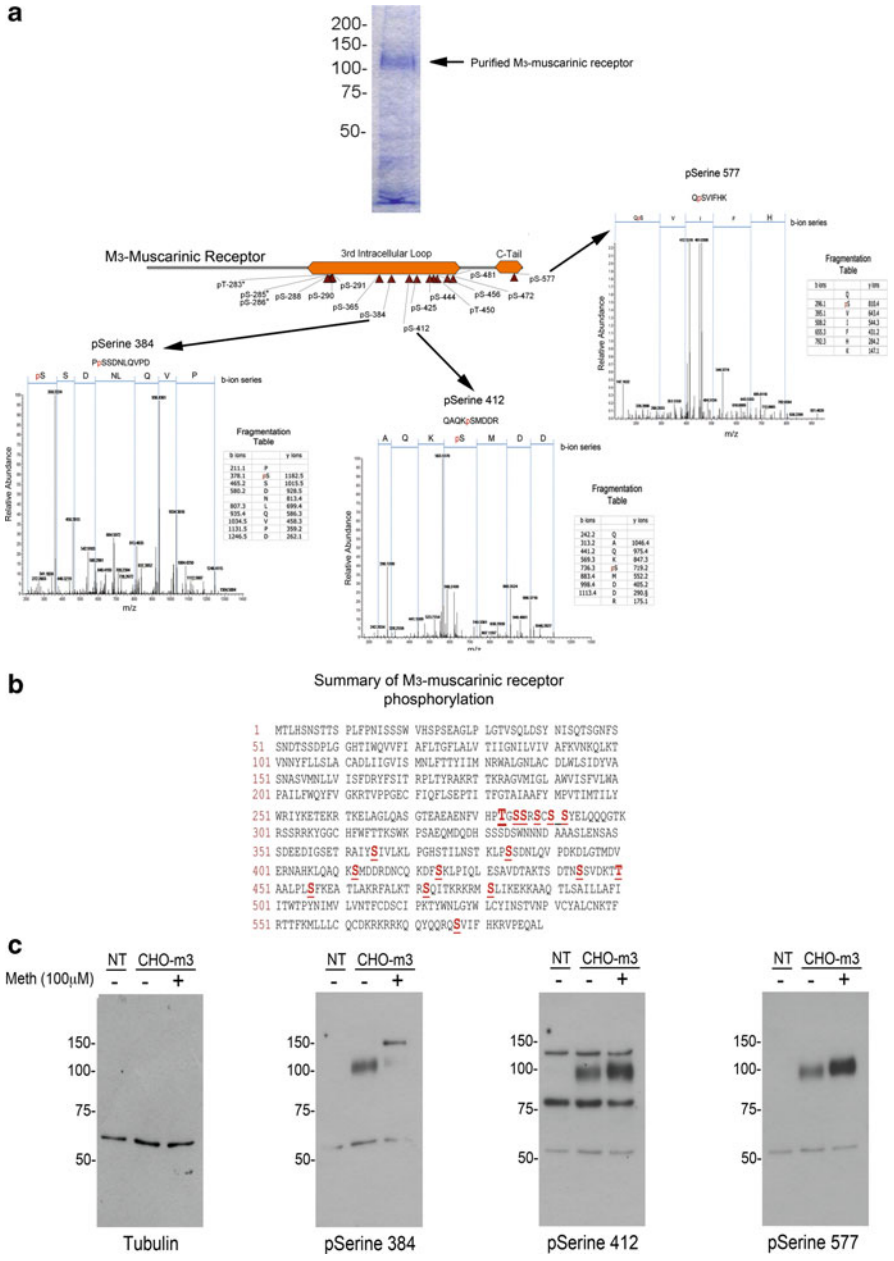

Fig. 1 Mass spectrometry and phosphorylation-specific antibodies reveal sites of phosphorylation on the M3-muscarinic receptor. (**a**) Recombinant mouse M3-muscarinic receptor expressed in CHO cells was stimulated with methacholine (100 μM) for 5 min. Membranes were then prepared from which the receptor was purified. The receptor was subjected to tryptic digestion, and the peptides were analysed by mass spectrometry. Shown is a schematic indicating the phosphoacceptor sites in the third intracellular loop and C-terminal tail. Also shown are typical

4 Site-Specific Phosphorylation Determines GPCR Signalling Properties

One question that arises from the fact that GPCRs are phosphorylated on multiple sites by a range of protein kinases is whether phosphorylation at specific sites has a specific impact on receptor function? If this was the case then this would provide a sophisticated and flexible mechanism to regulate the signalling output of a particular receptor. Studies on the M_3-muscarinic receptor indicated that this may be the case. Work carried out in our laboratory determined that this receptor subtype was phosphorylated by casein kinase 1α (Tobin et al. 1997) and protein kinase CK2 (Torrecilla et al. 2007). These two kinases appeared to regulate different aspects of receptor function with casein kinase 1α impacting on the ability of the receptor to couple to the ERK-1/2 pathway (Budd et al. 1999) and protein kinase CK2 regulating coupling to the JUN-kinase pathway (Torrecilla et al. 2007). In addition, other laboratories have determined that the M_3-muscarinic receptor is phosphorylated by GRK-6 (Willets et al. 2002, 2003) and that this phosphorylation event was the mechanism of receptor desensitization from the phospholipase C/calcium mobilization pathway (Willets et al. 2002, 2003). Furthermore, GRK-mediated phosphorylation also appeared to regulate M_3-muscarinic receptor internalization (Tsuga et al. 1998) whilst casein kinase 1α and protein kinase CK2 did not impact on this receptor process. Thus, in the case of the M_3-muscarinic receptor the three protein kinases that phosphorylated the receptor appeared to phosphorylate distinct sites (Torrecilla et al. 2007) and this resulted in distinct signalling outcomes (Fig. 2).

There is now an accumulating body of evidence from other receptor systems to support specific signalling outcomes from site-specific phosphorylation. The chemokine receptor CXCR4, for example, is regulated by GRK-2, GRK-3, GRK-6 and PKC (Balabanian et al. 2008; Fong et al. 2002; Marchese and Benovic 2001; Orsini et al. 1999; Signoret et al. 1997) phosphorylation. Mass spectrometry determined the precise sites of phosphorylation in the extended C-terminal tail of the receptor from which it was possible to design phospho-specific antibodies (Busillo et al. 2010). Using these antibodies, in conjunction with siRNA knock down of the GRKs, it was possible to not only determine that the GRKs phosphorylated different sites on the receptor but that this had different signalling outcomes (Busillo et al. 2010). Interestingly, the study of CXCR4 phosphorylation also revealed that not only were different sites phosphorylated by different receptor

Fig. 1 (continued) LC-MS/MS traces that identify serines 384, 412 and 577 as phosphoserines. (**b**) Schematic of the full amino acid sequence of the mouse M3-muscarinic receptor indicating the position of the phosphorylated residues in *red* and *underlined*. (**c**) Non-transfected CHO cells (*NT*) or CHO cells expressing the mouse M3-muscarinic receptor (*CHO-m3*) were stimulated with or without methacholine (*Meth*) for 5 min. The cells were then lysed, and the lysate was probed in a Western blot with receptor phospho-specific antibodies. Shown also is a loading control probed for tubulin [Figure adapted from Butcher et al. (2011)]

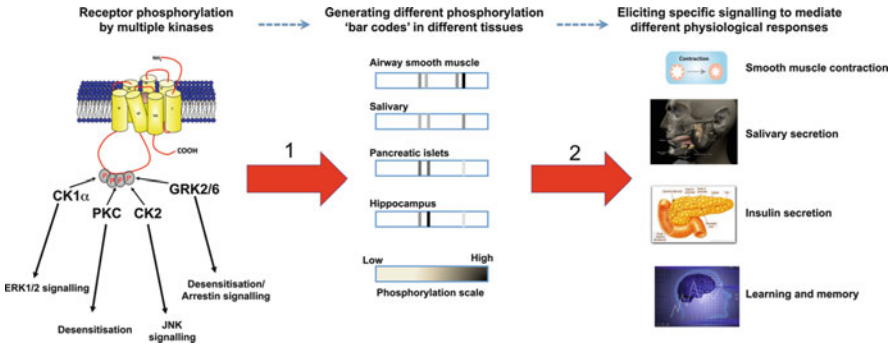

Fig. 2 The phosphorylation bar-code hypothesis. G-protein coupled receptors (GPCRs) are often hyper-phosphorylated in a process that involves more than one receptor kinase. This results in a distinct phosphorylation pattern that can act as a bar code that can direct the signalling outcome of the receptor. Through this bar code specific signalling is mediated to elicit different physiological responses in different tissues. This process is illustrated above using the M_3-muscarinic receptor (M3R) as an example. This receptor can be phosphorylated by a range of protein kinases. The impact on the signalling properties of the receptor made by each protein kinase is different. Thus, by employing these phosphorylation events in a tissue-specific manner the receptor can be differentially phosphorylated in a manner that regulates the signalling properties of the receptor. Through this mechanism the signalling properties of the receptor can be fine tuned to allow the receptor to control different physiological events such as smooth muscle contraction in the airway, salivary secretion in salivary glands, insulin secretion in pancreatic islets and learning and memory in the hippocampus

kinases but that the kinetics of phosphorylation was different for the various sites. Thus, phosphorylation of Serine-330 by GRK-6 was slow compared to the phosphorylation of Serine-324/5 by PKC and GRK-6. We should not be surprised at this finding since it is a common observation that multiply phosphorylated proteins show different rates of phosphorylation at different sites and that this is tightly linked with the outcome of the phospho-signalling pathway (Olsen et al. 2006). It is, however, important to see this played out in the context of GPCR signalling.

This concept has been taken a step further in studies on the somatostatin 2A receptor which is known to be extensively phosphorylated by the GRKs following agonist stimulation on a cluster of serines and a cluster of theronines in the C-terminal tail (Liu et al. 2008). Once again, phospho-specific antibodies have been used to reveal the details of these phosphorylation events where the kinetics of phosphorylation and dephosphorylation were probed (Ghosh and Schonbrunn 2011). These studies revealed that within seconds of agonist stimulation the receptor is phosphorylated firstly on the serine cluster followed by phosphorylation of the theronine cluster. The former taking seconds and the later minutes. Interestingly, dephosphorylation of the serine cluster occurs slowly and only following receptor internalization whereas dephosphorylation of the theronine cluster can occur rapidly at the cell surface as well as slowly on internalized receptors. Thus, the kinetics of phosphorylation and dephosphorylation between different sites is distinct. In addition, the special arrangement of phosphorylated receptor is different so that in

different compartments of the cell (i.e. plasma membrane vs. endosomal) the pattern of phosphorylation on the somatostatin 2A receptor will be different (Ghosh and Schonbrunn 2011). Does this make any functional impact? In the case of the somatostatin 2A receptor, the answer appears to be yes as it has been found that phosphorylation of the theronine cluster was the only important determinant for arrestin recruitment and receptor internalization whilst both phosphorylation of the serine cluster and theronine cluster are necessary for receptor uncoupling from the G-protein (Liu et al. 2008).

The outcome of these studies is that it appears that GPCRs are multiply phosphorylated by a range of protein kinases on sites that have distinct functional impact. Furthermore, these sites can be separated in time and space and in so doing add a further dimension of subtlety of the functional consequence of receptor phosphorylation.

5 Tissue-Specific Patterns of Receptor Phosphorylation

The question we have asked over the last few years is that given the multi-site nature of GPCR phosphorylation is it possible that receptors adopt different phosphorylation patterns in different tissues and through this mechanism direct the signalling outcome of receptors to mediate different physiological responses. This possibility was first investigated by comparing the tryptic phosphopeptide maps of metabolically labelled receptors endogenously expressed in cerebellar granule neurons with that of the receptor expressed as a recombinant protein in CHO cells (Torrecilla et al. 2007). These experiments demonstrated that although there were some phosphopeptides that could be seen to be the same from the receptors extracted from the two cell types there were also phosphopeptides that were only seen from receptors derived from one cell type and not the other (Torrecilla et al. 2007). Hence, this was the first demonstration that a GPCR could be differentially phosphorylated in different cell types.

This has recently been taken further using phospho-specific antibodies to phosphorylation sites on the M_3-muscarinic receptor. Experiments performed in our laboratory have determined that the M_3-muscarinic receptor is differentially phosphorylated in the cortex, hippocampus, pancreas and salivary glands. This raises the prospect that differential tissue specific phosphorylation represents a bar code that encodes the signalling properties of the receptor. By employing different phosphorylation bar codes, the signalling outcome of a particular receptor subtype can be tailored to meet the physiological requirements of that receptor (Fig. 2). In this way, the same receptor subtype can be expressed in different tissue types and mediate an array of physiological functions, in part, by adopting different patterns of phosphorylation and thereby different signalling profiles (Tobin et al. 2008).

6 The Bar Code Hypothesis: A Mechanism of Regulating Physiological Function

The concept that receptor phosphorylation represents a bar code that encodes for the signalling outcome of GPCRs was first suggested from studies on the V2 vasopressin receptor and angiotensin II type 1A receptor (Kim et al. 2005; Ren et al. 2005). The functional effect of downregulation of the GRKs on the coupling of these receptors to the MAP kinase pathway was seen to be different for the various GRKs (Kim et al. 2005; Ren et al. 2005). This was interpreted as meaning that each member of the GRK family phosphorylated different sites on the receptor and this resulted in a different pattern or code that in turn mediated different signalling outcomes (Kim et al. 2005; Ren et al. 2005). This notion was supported by fluorescent resonance energy transfer (FRET) studies that investigated the kinetics of arrestin interaction with the β_2-adrenergic receptor (Violin et al. 2006). In these experiments, phosphorylation of this receptor subtype by different GRKs was seen to result in differences in the temporal profile of arrestin recruitment (Violin et al. 2006). That this was due to a difference in the phosphorylation sites on the β_2-adrenergic receptor was supported by the fact that only GRK-6 and none of the other GRK isotypes phosphorylated residues 355 and 356 in the C-terminal tail of the receptor (Violin et al. 2006). Thus, there appeared to be differential phosphorylation of the β_2-adrenergic receptor by the GRKs and that the functional consequence of these differences was different rates of arrestin recruitment (Violin et al. 2006).

There is therefore a link between the phosphorylation bar code and the signalling outcome of receptors. How the phosphorylation bar code contributes to physiological GPCR responses has yet to be fully defined and is the central challenge facing a full understanding of the bar code hypothesis. Progress towards addressing this question has recently been made in studies using transgenic animals where the phosphorylation sites on the M_3-muscarinic receptor have been mutated thereby providing our first insights into the physiological role played by receptor phosphorylation.

In these studies, our laboratory has generated a transgenic knock-in mouse strain where the serine phosphoacceptor sites in the third intracellular loop of the M_3-muscarinic receptor have been mutated to alanine. Analysis of the phosphorylation status of the receptor in cerebellar granule cells determined that the mutated receptor was significantly reduced in its ability to undergo agonist-dependent phosphorylation (Kong et al. 2010; Poulin et al. 2010). Importantly, functional analysis of the phosphorylation-deficient M_3-muscarinic receptor demonstrated that it was able to couple to the phospholipase C/calcium mobilization pathway normally but was uncoupled from receptor phosphorylation-dependent pathways such as receptor internalization and arrestin recruitment (Kong et al. 2010; Poulin et al. 2010). In this respect, this receptor mutant could be considered as being biased in that it was only able to signal via heterotrimeric G-proteins.

Detailed phenotypic analysis revealed that the mice expressing the phosphorylation-deficient muscarinic receptor had two major physiological defects. The first was related to the fact that earlier studies from the Wess laboratory had determined that the M_3-muscarinic receptor expressed on pancreatic islets was the receptor subtype responsible for cholinergic augmentation of glucose-dependent insulin release (Gautam et al. 2006). It was previously thought that the mechanism by which the cholinergic receptors mediated insulin release was related to the ability of these receptors to signal via phospholipase C and generate inositol 1,4,5 trisphosphate and diacylglycerol with subsequent increases in intracellular calcium and the activation of PKC, respectively (Gilon and Henquin 2001; Gromada and Hughes 2006). These earlier studies placed a central importance of muscarinic receptor coupling to heterotrimeric $G_{q/11}$-protein in cholinergic insulin release. This was highly significant in the interpretation of our studies on the mutant mice expressing a phosphorylation-deficient M_3-muscarinic receptor since despite the fact that this mutant receptor coupled normally to the phospholipase C/calcium mobilization pathway, the mutant mice were glucose intolerance (Kong et al. 2010). Detailed analysis confirmed that the pancreatic islets from the phosphorylation-deficient mutant mice were deficient in muscarinic receptor augmentation of insulin release (Kong et al. 2010). As expected, the mutant receptor was able to couple normally to $G_{q/11}$ protein in pancreatic islets. It was, therefore, concluded that receptor phosphorylation-dependent signalling played a key role in the muscarinic insulin release response. Further experimentation determined that receptor phosphorylation and subsequent arrestin recruitment mediated activation of the protein kinase PKD1 and that this kinase was responsible for stimulating insulin release (Kong et al. 2010). Thus, by using a mutant M_3-muscarinic receptor that was biased towards heterotrimeric G-protein signalling we have established the importance of receptor phosphorylation in coupling the M_3-muscarinic receptor to insulin release.

Using the same transgenic animals we also established that this mutant mouse strain was deficit in learning and memory (Poulin et al. 2010). This defect was centred on hippocampal learning and memory and not only established for the first time the importance of the M_3-muscarinic receptor in hippocampal function but also implicated the importance of receptor phosphorylation/arrestin-dependent signalling in learning and memory (Poulin et al. 2010). This may have important implications for drug discovery since it might be desirable to not only generate M_3-muscarinic receptor agonists for the treatment of cognitive deficit in neurodegenerative disease such as Alzheimer's but to extend this and generate M_3-muscarinic receptor agonists that are biased towards receptor phosphorylation/arrestin-dependent signalling. In this case it is of interest to note that there is a growing interest in biased GPCR ligands (Violin and Lefkowitz 2007; Wisler et al. 2007) which hold out the promise of more specificity in targeting the signalling pathways that are most likely to bring therapeutic benefit (Whalen et al. 2011).

The studies discussed earlier are beginning to determine the important physiological (and potentially pathophysiological) processes that are regulated by GPCR phosphorylation. The challenge is to further describe the role of receptor

phosphorylation but also to link this to the nature of the phosphorylated receptor. The most likely way to accomplish this is via selective pharmacological manipulation using biased ligands. The field is identifying greater numbers of biased ligands that are able to direct signalling preferentially down one particular signalling arm (Violin and Lefkowitz 2007; Whalen et al. 2011). These agents will prove to be invaluable tools in dissecting the relative importance between heterotrimeric G-protein-dependent signalling and receptor phosphorylation-dependent signalling in physiological responses mediated by GPCRs.

7 Conclusions

Although the field has been actively studying the molecular mechanisms of GPCR regulation for many years, how these mechanisms are employed to regulate physiological processes is still in its infancy. Since it is clear that the multi-site nature of GPCR phosphorylation leads to the prospect that the signalling outcome of GPCRs can be regulated, at least in part, by the pattern of phosphorylation, it will clearly be important to relate this to the mechanism by which GPCRs regulate physiological responses. This is particularly important since it is now possible to regulate the phosphorylation status of receptors by biased ligands and thereby through this process it can be envisaged that pharmacological intervention in human disease might be mediated by drugs that control the phosphorylation status of GPCRs.

The importance, therefore, of testing the phosphorylation bar code hypothesis in vivo is highly desirable. The problem lies in the huge technical challenge of monitoring the phosphorylation status of GPCRs in vivo and linking changes in the phosphorylation profile of receptors to specific physiological responses. These challenges are most likely to be overcome by employing ever increasingly sophisticated mouse models where phosphoacceptor sites on GPCRs are mutant, together, with discovering further biased ligands that direct signalling of receptors. Using these tools in conjunction with mass spectrometry phosphoproteomics and phospho-specific antibodies we may start to define the importance of site-specific phosphorylation in the regulation of physiological and pathophysiological responses.

References

Balabanian K, Levoye A, Klemm L, Lagane B, Hermine O, Harriague J, Baleux F, Arenzana-Seisdedos F, Bachelerie F (2008) Leukocyte analysis from WHIM syndrome patients reveals a pivotal role for GRK3 in CXCR4 signaling. J Clin Invest 118:1074–1084

Benovic JL, Regan JW, Matsui H, Mayor F Jr, Cotecchia S, Leeb-Lundberg LM, Caron MG, Lefkowitz RJ (1987) Agonist-dependent phosphorylation of the alpha 2-adrenergic receptor by the beta-adrenergic receptor kinase. J Biol Chem 262:17251–17253

Benovic JL, Onorato JJ, Arriza JL, Stone WC, Lohse M, Jenkins NA, Gilbert DJ, Copeland NG, Caron MG, Lefkowitz RJ (1991) Cloning, expression, and chromosomal localization of beta-adrenergic receptor kinase 2. A new member of the receptor kinase family. J Biol Chem 266:14939–14946

Blaukat A, Pizard A, Breit A, Wernstedt C, Alhenc-Gelas F, Muller-Esterl W, Dikic I (2001) Determination of bradykinin B2 receptor in vivo phosphorylation sites and their role in receptor function. J Biol Chem 276:40431–40440

Budd DC, Rae A, Tobin AB (1999) Activation of the mitogen-activated protein kinase pathway by a Gq/11-coupled muscarinic receptor is independent of receptor internalization. J Biol Chem 274:12355–12360

Busillo JM, Armando S, Sengupta R, Meucci O, Bouvier M, Benovic JL (2010) Site-specific phosphorylation of CXCR4 is dynamically regulated by multiple kinases and results in differential modulation of CXCR4 signaling. J Biol Chem 285(10):7805–7817

Butcher AJ, Prihandoko R, Kong KC, McWilliams P, Edwards JM, Bottrill A, Mistry S, Tobin AB (2011) Differential G-protein-coupled receptor phosphorylation provides evidence for a signaling bar code. J Biol Chem 286:11506–11518

Doronin S, Shumay E, Wang HY, Malbon CC (2002) Akt mediates sequestration of the beta(2)-adrenergic receptor in response to insulin. J Biol Chem 277:15124–15131

Fong AM, Premont RT, Richardson RM, Yu YR, Lefkowitz RJ, Patel DD (2002) Defective lymphocyte chemotaxis in beta-arrestin2- and GRK6-deficient mice. Proc Natl Acad Sci U S A 99:7478–7483

Gautam D, Han SJ, Hamdan FF, Jeon J, Li B, Li JH, Cui Y, Mears D, Lu H, Deng C, Heard T, Wess J (2006) A critical role for beta cell M3 muscarinic acetylcholine receptors in regulating insulin release and blood glucose homeostasis in vivo. Cell Metab 3:449–461

Gavi S, Shumay E, Wang HY, Malbon CC (2006) G-protein-coupled receptors and tyrosine kinases: crossroads in cell signaling and regulation. Trends Endocrinol Metab 17:48–54

Ghosh M, Schonbrunn A (2011) Differential temporal and spatial regulation of somatostatin receptor phosphorylation and dephosphorylation. J Biol Chem 286:13561–13573

Gilon P, Henquin JC (2001) Mechanisms and physiological significance of the cholinergic control of pancreatic beta-cell function. Endocr Rev 22:565–604

Gromada J, Hughes TE (2006) Ringing the dinner bell for insulin: muscarinic M3 receptor activity in the control of pancreatic beta cell function. Cell Metab 3:390–392

Gurevich VV, Gurevich EV (2004) The molecular acrobatics of arrestin activation. Trends Pharmacol Sci 25:105–111

Gurevich EV, Gurevich VV (2006a) Arrestins: ubiquitous regulators of cellular signaling pathways. Genome Biol 7:236

Gurevich VV, Gurevich EV (2006b) The structural basis of arrestin-mediated regulation of G-protein-coupled receptors. Pharmacol Ther 110:465–502

Haga K, Kameyama K, Haga T, Kikkawa U, Shiozaki K, Uchiyama H (1996) Phosphorylation of human m1 muscarinic acetylcholine receptors by G protein-coupled receptor kinase 2 and protein kinase C. J Biol Chem 271:2776–2782

Hausdorff WP, Bouvier M, O'Dowd BF, Irons GP, Caron MG, Lefkowitz RJ (1989) Phosphorylation sites on two domains of the beta 2-adrenergic receptor are involved in distinct pathways of receptor desensitization. J Biol Chem 264:12657–12665

Hirsch JA, Schubert C, Gurevich VV, Sigler PB (1999) The 2.8 A crystal structure of visual arrestin: a model for arrestin's regulation. Cell 97:257–269

Kennedy MJ, Lee KA, Niemi GA, Craven KB, Garwin GG, Saari JC, Hurley JB (2001) Multiple phosphorylation of rhodopsin and the in vivo chemistry underlying rod photoreceptor dark adaptation. Neuron 31:87–101

Kim J, Ahn S, Ren XR, Whalen EJ, Reiter E, Wei H, Lefkowitz RJ (2005) Functional antagonism of different G protein-coupled receptor kinases for beta-arrestin-mediated angiotensin II receptor signaling. Proc Natl Acad Sci U S A 102:1442–1447

Kong KC, Butcher AJ, McWilliams P, Jones D, Wess J, Hamdan FF, Werry T, Rosethorne EM, Charlton SJ, Munson SE, Cragg HA, Smart AD, Tobin AB (2010) M3-muscarinic receptor promotes insulin release via receptor phosphorylation/arrestin-dependent activation of protein kinase D1. Proc Natl Acad Sci U S A 107:21181–21186

Kramer HK, Andria ML, Esposito DH, Simon EJ (2000) Tyrosine phosphorylation of the delta-opioid receptor. Evidence for its role in mitogen-activated protein kinase activation and receptor internalization*. Biochem Pharmacol 60:781–792

Kristiansen K (2004) Molecular mechanisms of ligand binding, signaling, and regulation within the superfamily of G-protein-coupled receptors: molecular modeling and mutagenesis approaches to receptor structure and function. Pharmacol Ther 103:21–80

Kwatra MM, Benovic JL, Caron MG, Lefkowitz RJ, Hosey MM (1989) Phosphorylation of chick heart muscarinic cholinergic receptors by the beta-adrenergic receptor kinase. Biochemistry 28:4543–4547

Kwatra MM, Schwinn DA, Schreurs J, Blank JL, Kim CM, Benovic JL, Krause JE, Caron MG, Lefkowitz RJ (1993) The substance P receptor, which couples to Gq/11, is a substrate of beta-adrenergic receptor kinase 1 and 2. J Biol Chem 268:9161–9164

Lee MJ, Thangada S, Paik JH, Sapkota GP, Ancellin N, Chae SS, Wu M, Morales-Ruiz M, Sessa WC, Alessi DR, Hla T (2001) Akt-mediated phosphorylation of the G protein-coupled receptor EDG-1 is required for endothelial cell chemotaxis. Mol Cell 8:693–704

Lefkowitz RJ (2004) Historical review: a brief history and personal retrospective of seven-transmembrane receptors. Trends Pharmacol Sci 25:413–422

Lefkowitz RJ, Shenoy SK (2005) Transduction of receptor signals by beta-arrestins. Science 308:512–517

Lefkowitz RJ, Whalen EJ (2004) beta-arrestins: traffic cops of cell signaling. Curr Opin Cell Biol 16:162–168

Liu Q, Dewi DA, Liu W, Bee MS, Schonbrunn A (2008) Distinct phosphorylation sites in the SST2A somatostatin receptor control internalization, desensitization, and arrestin binding. Mol Pharmacol 73:292–304

Lohse MJ, Andexinger S, Pitcher J, Trukawinski S, Codina J, Faure JP, Caron MG, Lefkowitz RJ (1992) Receptor-specific desensitization with purified proteins. Kinase dependence and receptor specificity of beta-arrestin and arrestin in the beta 2-adrenergic receptor and rhodopsin systems. J Biol Chem 267:8558–8564

Luo J, Busillo JM, Benovic JL (2008) M3 muscarinic acetylcholine receptor-mediated signaling is regulated by distinct mechanisms. Mol Pharmacol 74:338–347

Luttrell DK, Luttrell LM (2004) Not so strange bedfellows: G-protein-coupled receptors and Src family kinases. Oncogene 23:7969–7978

Marchese A, Benovic JL (2001) Agonist-promoted ubiquitination of the G protein-coupled receptor CXCR4 mediates lysosomal sorting. J Biol Chem 276:45509–45512

Mendez A, Burns ME, Roca A, Lem J, Wu LW, Simon MI, Baylor DA, Chen J (2000) Rapid and reproducible deactivation of rhodopsin requires multiple phosphorylation sites. Neuron 28:153–164

Nelson CD, Perry SJ, Regier DS, Prescott SM, Topham MK, Lefkowitz RJ (2007) Targeting of diacylglycerol degradation to M1 muscarinic receptors by beta-arrestins. Science 315:663–666

Olsen JV, Blagoev B, Gnad F, Macek B, Kumar C, Mortensen P, Mann M (2006) Global, in vivo, and site-specific phosphorylation dynamics in signaling networks. Cell 127:635–648

Onorato JJ, Palczewski K, Regan JW, Caron MG, Lefkowitz RJ, Benovic JL (1991) Role of acidic amino acids in peptide substrates of the beta-adrenergic receptor kinase and rhodopsin kinase. Biochemistry 30:5118–5125

Orsini MJ, Parent JL, Mundell SJ, Marchese A, Benovic JL (1999) Trafficking of the HIV coreceptor CXCR4. Role of arrestins and identification of residues in the c-terminal tail that mediate receptor internalization. J Biol Chem 274:31076–31086

Pitcher JA, Inglese J, Higgins JB, Arriza JL, Casey PJ, Kim C, Benovic JL, Kwatra MM, Caron MG, Lefkowitz RJ (1992) Role of beta gamma subunits of G proteins in targeting the beta-adrenergic receptor kinase to membrane-bound receptors. Science 257:1264–1267

Pitcher JA, Freedman NJ, Lefkowitz RJ (1998) G protein-coupled receptor kinases. Annu Rev Biochem 67:653–692

Poulin B, Butcher A, McWilliams P, Bourgognon JM, Pawlak R, Kong KC, Bottrill A, Mistry S, Wess J, Rosethorne EM, Charlton SJ, Tobin AB (2010) The M3-muscarinic receptor regulates learning and memory in a receptor phosphorylation/arrestin-dependent manner. Proc Natl Acad Sci U S A 107:9440–9445

Ren XR, Reiter E, Ahn S, Kim J, Chen W, Lefkowitz RJ (2005) Different G protein-coupled receptor kinases govern G protein and beta-arrestin-mediated signaling of V2 vasopressin receptor. Proc Natl Acad Sci U S A 102:1448–1453

Seibold A, Williams B, Huang ZF, Friedman J, Moore RH, Knoll BJ, Clark RB (2000) Localization of the sites mediating desensitization of the beta(2)-adrenergic receptor by the GRK pathway. Mol Pharmacol 58:1162–1173

Signoret N, Oldridge J, Pelchen-Matthews A, Klasse PJ, Tran T, Brass LF, Rosenkilde MM, Schwartz TW, Holmes W, Dallas W, Luther MA, Wells TN, Hoxie JA, Marsh M (1997) Phorbol esters and SDF-1 induce rapid endocytosis and down modulation of the chemokine receptor CXCR4. J Cell Biol 139:651–664

Sommer ME, Hofmann KP, Heck M (2011) Arrestin-rhodopsin binding stoichiometry in isolated rod outer segment membranes depends on the percentage of activated receptors. J Biol Chem 286:7359–7369

Song X, Vishnivetskiy SA, Seo J, Chen J, Gurevich EV, Gurevich VV (2011) Arrestin-1 expression level in rods: balancing functional performance and photoreceptor health. Neuroscience 174:37–49

Tobin AB (2008) G-protein-coupled receptor phosphorylation: where, when and by whom. Br J Pharmacol 153(Suppl 1):S167–S176

Tobin AB, Totty NF, Sterlin AE, Nahorski SR (1997) Stimulus-dependent phosphorylation of G-protein-coupled receptors by casein kinase 1alpha. J Biol Chem 272:20844–20849

Tobin AB, Butcher AJ, Kong KC (2008) Location, location, location. . .site-specific GPCR phosphorylation offers a mechanism for cell-type-specific signalling. Trends Pharmacol Sci 29:413–420

Torrecilla I, Spragg EJ, Poulin B, McWilliams PJ, Mistry SC, Blaukat A, Tobin AB (2007) Phosphorylation and regulation of a G protein-coupled receptor by protein kinase CK2. J Cell Biol 177:127–137

Tran TM, Friedman J, Qunaibi E, Baameur F, Moore RH, Clark RB (2004) Characterization of agonist stimulation of cAMP-dependent protein kinase and G protein-coupled receptor kinase phosphorylation of the beta2-adrenergic receptor using phosphoserine-specific antibodies. Mol Pharmacol 65:196–206

Trester-Zedlitz M, Burlingame A, Kobilka B, von Zastrow M (2005) Mass spectrometric analysis of agonist effects on posttranslational modifications of the beta-2 adrenoceptor in mammalian cells. Biochemistry 44:6133–6143

Tsuga H, Okuno E, Kameyama K, Haga T (1998) Sequestration of human muscarinic acetylcholine receptor hm1-hm5 subtypes: effect of G protein-coupled receptor kinases GRK2, GRK4, GRK5 and GRK6. J Pharmacol Exp Ther 284:1218–1226

Violin JD, Lefkowitz RJ (2007) Beta-arrestin-biased ligands at seven-transmembrane receptors. Trends Pharmacol Sci 28:416–422

Violin JD, Ren XR, Lefkowitz RJ (2006) G-protein-coupled receptor kinase specificity for beta-arrestin recruitment to the beta2-adrenergic receptor revealed by fluorescence resonance energy transfer. J Biol Chem 281:20577–20588

Vishnivetskiy SA, Paz CL, Schubert C, Hirsch JA, Sigler PB, Gurevich VV (1999) How does arrestin respond to the phosphorylated state of rhodopsin? J Biol Chem 274:11451–11454

Vishnivetskiy SA, Raman D, Wei J, Kennedy MJ, Hurley JB, Gurevich VV (2007) Regulation of arrestin binding by rhodopsin phosphorylation level. J Biol Chem 282:32075–32083

Vishnivetskiy SA, Gimenez LE, Francis DJ, Hunson SM, Hubbell WL, Klug CS, Gurevich VV (2011) Few residues within an extensive binding interface drive receptor interaction and determine the specificity of arrestin proteins. J Biol Chem 286(27):24288–24299

Wang H, Doronin S, Malbon CC (2000) Insulin activation of mitogen-activated protein kinases Erk1,2 is amplified via beta-adrenergic receptor expression and requires the integrity of the Tyr350 of the receptor. J Biol Chem 275:36086–36093

Waugh MG, Challiss RA, Berstein G, Nahorski SR, Tobin AB (1999) Agonist-induced desensitization and phosphorylation of m1-muscarinic receptors. Biochem J 338(Pt 1):175–183

Whalen EJ, Rajagopal S, Lefkowitz RJ (2011) Therapeutic potential of beta-arrestin- and G protein-biased agonists. Trends Mol Med 17:126–139

Willets JM, Challiss RA, Nahorski SR (2002) Endogenous G protein-coupled receptor kinase 6 Regulates M3 muscarinic acetylcholine receptor phosphorylation and desensitization in human SH-SY5Y neuroblastoma cells. J Biol Chem 277:15523–15529

Willets JM, Mistry R, Nahorski SR, Challiss RA (2003) Specificity of g protein-coupled receptor kinase 6-mediated phosphorylation and regulation of single-cell m3 muscarinic acetylcholine receptor signaling. Mol Pharmacol 64:1059–1068

Wisler JW, DeWire SM, Whalen EJ, Violin JD, Drake MT, Ahn S, Shenoy SK, Lefkowitz RJ (2007) A unique mechanism of beta-blocker action: carvedilol stimulates beta-arrestin signaling. Proc Natl Acad Sci U S A 104:16657–16662

Xiao K, McClatchy DB, Shukla AK, Zhao Y, Chen M, Shenoy SK, Yates JR 3rd, Lefkowitz RJ (2007) Functional specialization of beta-arrestin interactions revealed by proteomic analysis. Proc Natl Acad Sci U S A 104:12011–12016

Novel Muscarinic Receptor Mutant Mouse Models

Jürgen Wess

Abstract Muscarinic acetylcholine (ACh) receptors (mAChRs; M_1–M_5) regulate the activity of an extraordinarily large number of important physiological processes. During the past 10–15 years, studies with whole-body M_1–M_5 mAChR knockout mice have provided many new insights into the physiological and pathophysiological roles of the individual mAChR subtypes. This review will focus on the characterization of a novel generation of mAChR mutant mice, including mice in which distinct mAChR genes have been excised in a tissue- or cell type-specific fashion, various transgenic mouse lines that overexpress wild-type or different mutant M_3 mAChRs in certain tissues or cells only, as well as a novel M_3 mAChR knockin mouse strain deficient in agonist-induced M_3 mAChR phosphorylation. Phenotypic analysis of these new animal models has greatly advanced our understanding of the physiological roles of the various mAChR subtypes and has identified potential targets for the treatment of type 2 diabetes, schizophrenia, Parkinson's disease, drug addiction, cognitive disorders, and several other pathophysiological conditions.

Keywords Beta-cells • Bone mass • Cognition • Cre/loxP technology • Glucose homeostasis • Longitudinal growth • Muscarinic receptor knockin mice • Muscarinic receptor knockout mice • Seizure activity • Transgenic mice

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Abbreviations

ACh	Acetylcholine
CNO	Clozapine-N-oxide
DHPG	((S)-3,5-dihydroxyphenylglycine
DREADD	Designer receptor exclusively activated by designer drug
GH	Growth hormone
GHRH	Growth hormone-releasing hormone
GPCR	G-protein-coupled receptor
i3 loop	Third intracellular loop
IGF-1	Insulin-like growth factor
KI	Knockin
KO	Knockout
LDP	Long-term depression
LFP	Local field potential
LTP	Long-term potentiation
mAChR	Muscarinic acetylcholine receptor
mGluR	Metabotropic glutamate receptor
Oxo-M	Oxotremorine M
PI	Phosphatidylinositol
RASSL	Receptor activated solely by synthetic ligand
SNS	Sympathetic nervous system
T2D	Type 2 diabetes
tTA	tet Transactivator
WT	Wild-type

1 Introduction

Many of the important physiological functions of acetylcholine (ACh) are mediated by a family of G-protein-coupled receptors (GPCRs) referred to as muscarinic ACh receptors (M_1–M_5 mAChRs). At the molecular level, the M_1, M_3, and M_5 receptors selectively couple to G proteins of the G_q/G_{11} family, whereas the M_2 and M_4 receptors preferentially activate G_i-type G proteins (Wess 1996; Caulfield and Birdsall 1998). Each of the five mAChR subtypes shows a distinct pattern of distribution, being expressed in many regions of the CNS (in both neurons and glial cells) and in various peripheral tissues (Wess 1996; Caulfield and Birdsall 1998; Volpicelli and Levey 2004; Abrams et al. 2006). Typically, most tissues and cell types express at least two or more mAChR subtypes. Until very recently, small molecule ligands that can activate or inhibit specific mAChR subtypes with a high degree of selectivity have not been available. For these reasons, classical pharmacological studies aimed at identifying the molecular nature of the mAChR subtype (s) mediating a specific physiological response have often yielded conflicting results.

To overcome the difficulties associated with the use of pharmacological tools of limited mAChR subtype selectivity, several investigators used gene targeting technology to disrupt the function of distinct mAChR genes in embryonic stem cells (via homologous recombination). This approach eventually yielded mutant mouse strains that lacked M_1, M_2, M_3, M_4, or M_5 receptors throughout the body (whole-body mAChR knockout [KO] mice; Wess 2004; Matsui et al. 2004; Wess et al. 2007). The mAChR single KO mice were then intermated to generate several mAChR double KO mouse strains (Wess et al. 2007).

During the past 10–15 years, the various whole-body mAChR mutant mouse strains have been subjected to systematic phenotyping studies. These studies revealed that disruption of the individual mAChR genes leads to distinct pharmacological, behavioral, biochemical, neurochemical, and electrophysiological deficits or changes (Wess 2004; Matsui et al. 2004; Wess et al. 2007). Clearly, these new findings have greatly improved our understanding of the physiological roles of the individual mAChR subtypes. Moreover, the observed phenotypes suggested many new avenues for the development of subtype-selective, clinically useful muscarinic agonists or antagonists. The phenotypic changes characteristic for the different whole-body mAChR KO mouse strains have been the subject of several recent reviews (Wess 2004; Matsui et al. 2004; Wess et al. 2007). In this chapter, I will focus on the phenotypic analysis of novel mAChR mutant mouse strains in which distinct mAChR genes have been excised in a tissue- or cell type-specific fashion (Table 1). I will also briefly summarize the outcome of a behavioral study carried out with a knockin mouse strain in which the wild-type (WT) M_3 mAChR coding sequence was replaced with a phosphorylation-deficient version of the M_3 receptor (Poulin et al. 2010). Finally, I will review the phenotypes of newly generated mAChR mutant mice (transgenic mice) that overexpress the M_3 receptor or certain M_3 receptor-derived mutant receptors in distinct tissues or cells only (Table 1).

Table 1 Summary of new mAChR mutant mouse models reviewed in this chapter

Mutant mouse strain	References
mAChR KO mice selectively lacking	
M_3 receptors in pancreatic β cells	Gautam et al. (2006b)
M_3 receptors in neurons/glial cells	Gautam et al. (2009) and Shi et al. (2010)
M_3 receptors in osteoblasts	Shi et al. (2010)
M_3 receptors in hepatocytes	Li et al. (2009)
M_1 receptors in excitatory neurons of the forebrain	Kamsler et al. (2010)
M_1 receptors in hippocampal CA3 pyramidal cells	Kamsler et al. (2010)
M_4 receptors in D_1 dopamine receptor-expressing neurons	Jeon et al. (2010)
Transgenic mice selectively overexpressing	
M_3 receptors in pancreatic β cells	Gautam et al. (2006b)
M_3 receptors in hepatocytes	Li et al. (2009)
Transgenic mice selectively overexpressing M_3 receptor-based RASSLs	
In pancreatic β cells	Guettier et al. (2009)
In principal neurons of the forebrain	Alexander et al. (2009)

2 M_3 mAChRs Expressed by Pancreatic β Cells Are Critical for Maintaining Normal Blood Glucose Levels

2.1 Analysis of Mutant Mice Selectively Lacking M_3 mAChRs in Pancreatic β Cells

Type 2 diabetes (T2D) has emerged as one of the major threats to human health world-wide (Zimmet et al. 2001). A pathophysiological hallmark of T2D is that pancreatic β cells fail to release sufficient amounts of insulin in order to maintain normal blood glucose levels (β cell dysfunction). Drugs that can promote insulin release from pancreatic β cells are therefore considered useful for the treatment of T2D (Kahn 1994).

Like most other cell types, pancreatic β cells express a large number of GPCRs including the M_3 mAChR (Regard et al. 2007; Ahrén 2009). Consistent with the expression of the M_3 mAChR in pancreatic β cells, pancreatic islets are richly innervated by parasympathetic (cholinergic) nerves (Ahren 2000; Gilon and Henquin 2001). Studies with isolated pancreatic islets prepared from whole-body M_3 mAChR KO mice demonstrated that the M_3 receptor subtype mediates the ability of ACh to enhance glucose-induced insulin secretion (Duttaroy et al. 2004; Zawalich et al. 2004). Activation of β-cell M_3 mAChRs has been shown to trigger increases in intracellular calcium levels and PKC activity, two responses that are considered critical for ACh-mediated enhancement of insulin release (Ahren 2000; Gilon and Henquin 2001).

In order to study the importance of β-cell M_3 mAChRs in maintaining normal blood glucose levels in vivo, we employed Cre/loxP technology to generate mutant mice lacking M_3 receptors in pancreatic β cells only (β-M_3-KO mice; Gautam et al. 2006b). Studies with isolated islets showed that muscarinic agonist-induced phosphatidylinositol (PI) hydrolysis was greatly reduced in islets prepared from β-M_3-KO mice, as compared to islets obtained from control littermates (Gautam et al. 2006b). Consistent with this observation, the ability of the muscarinic agonist, oxotremorine M (Oxo-M), to enhance insulin release in the presence of a stimulatory concentration of glucose (16.7 mM) was greatly diminished in islets prepared from β-M_3-KO mice (Gautam et al. 2006b).

In vivo studies showed that β-M_3-KO mice displayed significantly impaired glucose tolerance and blunted increases in serum insulin levels after oral or intraperitoneal (i.p.) administration of glucose (Gautam et al. 2006b). These observations support the concept that the lack of β-cell M_3 receptors leads to reduced glucose-dependent insulin release in vivo and impaired glucose tolerance, highlighting the critical role of β-cell M_3 receptors in maintaining normal blood glucose levels in vivo.

2.2 Studies with Transgenic Mice Overexpressing M_3 mAChRs in Pancreatic β Cells Only

To test the hypothesis that enhanced signaling through β-cell M_3 receptors might promote glucose-dependent insulin release, Gautam et al. (2006b) generated and analyzed transgenic mice that overexpressed the M_3 receptor selectively in their pancreatic β cells (β-M_3-Tg mice). These initial studies were carried out using a transgene construct coding for a modified version of the M_3 receptor that lacked most of the third intracellular loop (i3 loop). However, transgenic mice that overexpressed the full-length M_3 mAChR in a β-cell-selective fashion showed metabolic changes similar to those described below for the β-M_3-Tg mice (D. Gautam and J. Wess, unpublished results).

In vitro studies demonstrated that muscarinic agonist-stimulated PI hydrolysis was greatly enhanced in islets obtained from β-M_3-Tg mice, as compared to those prepared from WT littermates (Gautam et al. 2006b). In keeping with this finding, Oxo-M-induced stimulation of glucose-dependent insulin secretion was significantly greater in islets obtained from β-M_3-Tg mice (Gautam et al. 2006b). In vivo studies showed that β-M_3-Tg mice displayed significantly reduced blood glucose levels (by ~30–40%), associated with a ~3-fold increase in serum insulin levels. Moreover, the transgenic mice exhibited greatly improved glucose tolerance, most likely due to enhanced glucose-induced insulin release in vivo (Gautam et al. 2006b). Finally, Gautam et al. (2006b) demonstrated that β-M_3-Tg mice were protected against the detrimental metabolic effects associated with the chronic consumption of an energy-rich, high-fat diet, such as hyperglycemia and glucose intolerance (Fig. 1).

Taken together, these observations strongly support the concept that strategies aimed at increasing the activity of β-cell M_3 mAChRs should prove useful to promote insulin release and improve glucose tolerance. As a result, β-cell M_3 mAChRs or components of downstream signaling pathways may represent novel targets for the treatment of T2D.

2.3 RGS4 as a Potent Negative Regulator of M_3 Receptor-Mediated Insulin Secretion

Selective M_3 receptor agonists are not available at present. Moreover, the potential therapeutic use of such drugs (assuming that such compounds can be developed) may cause significant side effects, such as M_3 receptor-mediated smooth muscle contraction or glandular secretion (Caulfield and Birdsall 1998; Eglen 2005; Wess et al. 2007). Thus, it should be of interest to identify M_3 receptor-associated proteins that modulate signaling through β-cell M_3 receptors and, hopefully, show a more restricted pattern of expression. To identify such proteins, Ruiz de Azua et al. (2010) initially used MIN6 mouse insulinoma cells as an in vitro model system. MIN6 cells almost exclusively express the M_3 receptor subtype, and

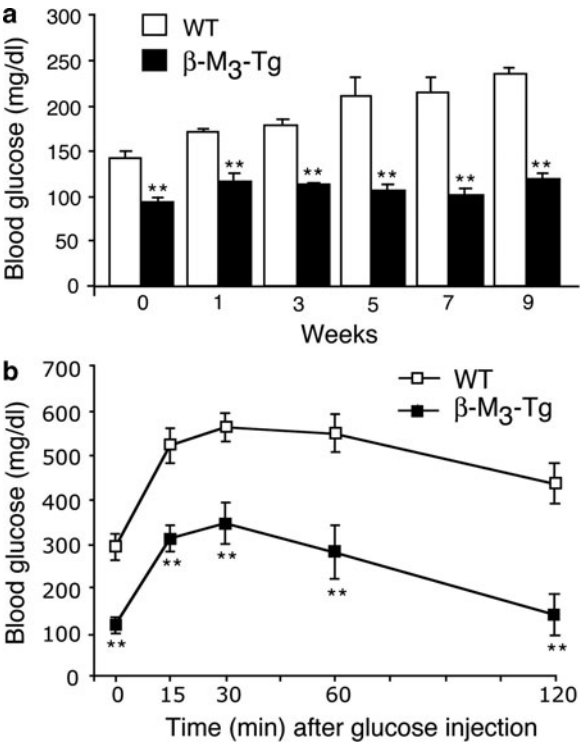

Fig. 1 Improved glucose homeostasis in transgenic mice selectively overexpressing M_3 mAChRs in pancreatic β cells (β-M_3-Tg mice). (**a**) Blood glucose levels of mice maintained on a high-fat diet. β-M_3-Tg mice and WT littermates were maintained on a high-fat diet for 8 weeks. Blood glucose levels were measured at the indicated time points in freely fed mice. (**b**) Glucose tolerance test using mice maintained on a high-fat diet for 8 weeks. Blood glucose levels were measured at the indicated time points following i.p. administration of glucose (2 mg/g). For all experiments, male mice were used ($n = 7$–9). Data are expressed as means ± SEM. $^{**}p < 0.01$, as compared to the corresponding WT value. Data were taken from Gautam et al. (2006b)

incubation of these cells with Oxo-M causes a robust increase in insulin release (Ruiz de Azua et al. 2010).

The lifetime of GPCR-activated G proteins is greatly reduced by the action of RGS proteins, which catalyze the hydrolysis of GTP that is bound to the activated Gα subunit (Ross and Wilkie 2000; Hollinger and Hepler 2002). RGS proteins represent a large protein family consisting of more than 30 different members in mammals (Ross and Wilkie 2000; Hollinger and Hepler 2002). Real-time qRT-PCR studies showed that RGS4 mRNA was by far the most abundant RGS transcript that could be detected in MIN6 cells (Ruiz de Azua et al. 2010). RGS4 was also found to be highly expressed in mouse islets. Interestingly, siRNA-mediated knockdown of RGS4 expression in MIN6 cells led to robust increases in Oxo-M-stimulated elevations in $[Ca^{2+}]_i$ and Oxo-M-induced insulin secretion (Ruiz de Azua et al. 2010), indicating that RGS4 represents a potent negative regulator of M_3 receptor function in this insulinoma cell line.

To study the role of RGS4 in regulating M_3 receptor-induced augmentation of insulin release in a more physiological setting, Ruiz de Azua et al. (2010) carried out insulin secretion studies using isolated islets prepared from RGS4-deficient mice (RGS4 KO mice). In agreement with the data obtained with cultured MIN6 cells, these studies showed that Oxo-M treatment of islets lacking RGS4 led to significantly enhanced increases in glucose-dependent insulin secretion, as compared to WT control islets.

Interestingly, studies with MIN6 cells as well as islets prepared from RGS4 KO mice demonstrated that RGS4 deficiency had little or no effect on the insulin responses observed after activation of other β-cell G_q- or G_s-coupled receptors (Ruiz de Azua et al. 2010), indicating that RGS4 selectively interferes with M_3 receptor function in insulin-containing cells. Accumulating evidence suggests the existence of GPCR/RGS signaling complexes containing additional signaling or scaffolding proteins, including spinophilin, 14-3-3 proteins, or Ca^{2+}/calmodulin (Abramow-Newerly et al. 2006; Bansal et al. 2007). The observed selectivity of RGS4 in regulating M_3 receptor-mediated signaling pathways in pancreatic β cells may therefore depend on the selective interaction of the M_3 receptor with specific components of the RGS4 signaling complex including RGS4 itself.

Since RGS4 is not selectively expressed by pancreatic β cells (it is also found in several other peripheral and central tissues), Ruiz de Azua et al. (2010) used Cre/loxP technology to generate mutant mice that selectively lacked RGS4 in pancreatic β cells (β-RGS4-KO mice). Under basal conditions, these mutant mice did not show any obvious metabolic phenotype. However, following injection of bethanechol, a peripherally acting muscarinic agonist, the β-RGS4-KO mice displayed significantly enhanced increases in insulin secretion and more robust reductions in blood glucose levels, as compared with control littermates (Ruiz de Azua et al. 2010). Studies with β-M_3-KO mice demonstrated that the bethanechol-induced changes in blood glucose and insulin levels require the presence of β-cell M_3 receptors.

These findings indicate that RGS4 acts as a potent negative regulator of M_3 receptor-mediated insulin secretion, raising the possibility that the potential therapeutic use of peripherally acting RGS4 inhibitors may prove useful for the treatment of T2D by enhancing signaling through β-cell M_3 receptors.

3 Neuronal M_3 mAChRs Are Critical for the Proper Development of the Anterior Pituitary Gland and for Normal Longitudinal Growth

The M_3 mAChR is widely expressed throughout the brain (Levey et al. 1994; Oki et al. 2005). To shed light onto the roles of central M_3 mAChRs, Gautam et al. (2009) used Cre/loxP technology to generate mutant mice that lacked M_3 receptors specifically in neurons and glial cells (brain-M3-KO or Br-M3-KO mice). These mice were obtained by crossing a Cre transgene driven by the nestin promoter into

mice that were homozygous for a floxed version of the M_3 receptor gene. In contrast to findings obtained with the whole-body M_3 receptor KO mice (Gautam et al. 2006a), the Br-M3-KO mice did not display any significant changes in food intake, metabolic rate, locomotor activity, body temperature, body fat content, blood glucose and insulin levels, glucose tolerance, or insulin sensitivity (Gautam et al. 2009). These observations suggest that central M_3 receptors do not play a significant role in regulating these processes.

Interestingly, however, the brain-M3-KO mice displayed a dwarf-like appearance (adult mutant mice were ~10% shorter than control littermates; Gautam et al. 2009). This phenotype was associated with a significant reduction in the serum levels of growth hormone (GH) and insulin-like growth factor I (IGF-1; Fig. 2a).

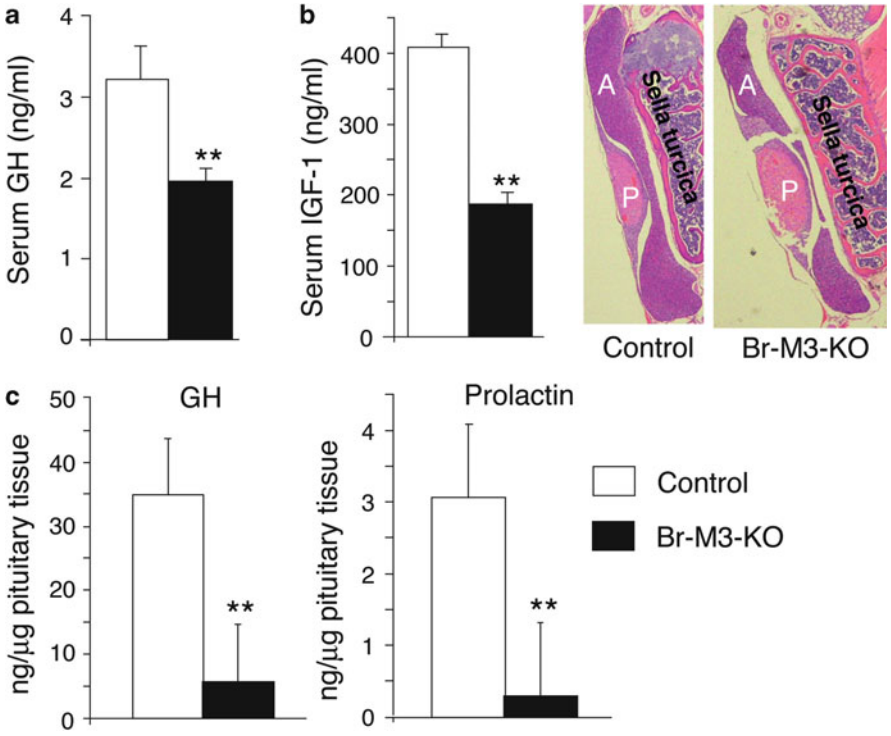

Fig. 2 Brain (Br)-M3-KO mice show reduced levels of hormones critical for somatic growth and hypoplasia of the anterior pituitary gland. (**a**) Reduction in serum GH and IGF-1 levels in Br-M3-KO mice, as compared to control littermates. (**b**) Selective reduction in the size of the anterior pituitary in Br-M3-KO mice. Pituitary glands from Br-M3-KO mice and control littermates were sectioned and stained with H&E. *A* anterior pituitary; *P* posterior pituitary. (**c**) Pronounced decrease in pituitary content of GH and prolactin in Br-M3-KO mice. Pituitary extracts were prepared from Br-M3-KO and control mice, and hormone levels were determined by standard techniques. All experiments were carried out with adult male mice ($n = 6$–10 per group). Data are given as means ± SEM. $^{**}p < 0.01$, as compared to the corresponding control group. Data were taken from Gautam et al. (2009)

Current evidence suggests that IGF-1, following its GH-dependent release from the liver, is the major factor mediating the stimulatory effect of GH on longitudinal growth. It is therefore likely that the reduction in body length displayed by the Br-M3-KO mice is the direct consequence of decreased GH and IGF-1 levels.

Whereas total brain weight was similar in control and Br-M3-KO mice, the weight (size) of the pituitary gland was significantly smaller (by ~75%) in the mutant mice (Gautam et al. 2009). Immunohistochemical studies showed that the Br-M3-KO mice displayed a pronounced hypoplasia of the anterior pituitary gland, associated with greatly reduced pituitary GH and prolactin levels (Gautam et al. 2009; Fig. 2b, c).

Interestingly, selective ablation of hypothalamic growth hormone-releasing hormone (GHRH) neurons (the primary site of GHRH synthesis and storage) in transgenic mice results in phenotypic changes very similar to those observed with Br-M3-KO mice, including a selective reduction in pituitary levels of GH and prolactin (Le Tissier et al. 2005). Since the anterior pituitary is not of neuronal origin, the nestin-Cre transgene is not expressed in this part of the pituitary (Tronche et al. 1999; Wettschureck et al. 2005). Gautam et al. (2009) therefore speculated that the primary defect leading to the hypoplasia of the anterior pituitary in the Br-M3-KO mice resides outside of the pituitary itself. Consistent with this notion, the authors found that hypothalamic GHRH neurons express M_3 mAChRs and that hypothalamic GHRH levels were greatly reduced in Br-M3-KO mice. Interestingly, treatment of Br-M3-KO mice with CJC-1295, a synthetic GHRH analog (Jetté et al. 2005), restored normal pituitary size and serum GH and IGF-1 levels, and normal longitudinal growth (Gautam et al. 2009). Since GHRH is known to play a key role in stimulating the proliferation of pituitary somatotroph cells (Giustina and Veldhuis 1998; Frohman and Kineman 2002), these findings are consistent with a model in which the activity of M_3 mAChRs located on hypothalamic GHRH neurons stimulates GHRH synthesis and/or release.

In conclusion, detailed analysis of Br-M3-KO mice revealed an unexpected and critical role of neuronal M_3 receptors in the proliferation of the anterior pituitary and the stimulation of longitudinal growth. Central M_3 receptors may therefore represent a novel target for the development of drugs useful for the treatment of certain forms of human growth disorders.

4 Neuronal M_3 mAChRs Promote the Accrual of Bone Mass

Shi et al. (2010) recently reported that whole-body M_3 receptor KO mice show a decrease in bone mass, due to decreased bone formation and increased bone resorption. This phenotype was not observed with M_1, M_2, or M_4 receptor-deficient mice. Gene expression studies showed that M_3 mAChR expression in osteoblasts is barely above the detection limit of qRT-PCR. Moreover, mutant mice in which the M_3 mAChR gene had been deleted selectively in osteoblasts did not display any changes in bone mass, bone formation, or bone resorption (Shi et al. 2010),

suggesting that M_3 mAChRs do not regulate bone mass via direct regulation of osteoblast function.

Shi et al. (2010) next demonstrated that the M_3 mAChR is expressed in regions of the brain stem, including the locus coeruleus, which are known to be critically involved in the regulation of bone mass accrual (Takeda et al. 2002; Yadav et al. 2009). To test the potential involvement of central M_3 mAChRs in the regulation of bone mass, the authors generated "neuron-specific" M_3 mAChR KO mice (Br-M3-KO mice) using an approach identical to that described by Gautam et al. (2009). Like the whole-body M_3 receptor KO mice, the Br-M3-KO mice showed a significant reduction in bone mass, due to decreased bone formation and increased bone resorption (Shi et al. 2010). Moreover, similar to whole-body M_3 receptor KO mice (Gautam et al. 2006a), the Br-M3-KO mice displayed an increase in the tone of the sympathetic nervous system (SNS). As mentioned above, M_3 mAChRs are expressed by noradrenergic neurons of the locus coeruleus, activation of which is known to result in an increase in SNS activity. Previous studies have shown that activation of the SNS inhibits bone mass accrual via stimulation of β_2-adrenergic receptors expressed by osteoblasts (Takeda et al. 2002; Elefteriou et al. 2005; Fu et al. 2005). The study by Shi et al. (2010) therefore supports a model in which activation of brain stem M_3 mAChRs results in reduced sympathetic outflow, thus promoting bone mass accrual. These findings may lead to new therapeutic strategies for the treatment of pathophysiological conditions characterized by reduced bone mass.

5 Hepatocyte M_3 mAChRs Are Not Critical for Maintaining Normal Blood Glucose Levels

Accumulating evidence suggests that the activity of efferent hepatic vagal nerves is critical for maintaining normal blood glucose homeostasis (Pocai et al. 2005a, b; Lam et al. 2005; Wang et al. 2008). Li et al. (2009) therefore speculated that the metabolic effects observed after stimulation of efferent hepatic vagal nerves might be mediated by activation of mAChRs expressed by liver hepatocytes. The authors first demonstrated that the M_3 mAChR is the only mAChR subtype expressed by mouse hepatocytes, consistent with data obtained with rat hepatocytes (Vatamaniuk et al. 2003). To examine the potential metabolic importance of this subpopulation of M_3 mAChRs, Li et al. (2009) used Cre/loxP technology to generate mutant mice that lacked M_3 receptors only in hepatocytes (Hep-M3-KO mice). In addition, the authors also created transgenic mice that overexpressed M_3 mAChRs selectively in hepatocytes (Hep-M3-Tg mice). Somewhat surprisingly, detailed phenotypic analysis of these mutant animals did not reveal any significant changes in liver glucose fluxes, hepatic gene expression patterns, or various other metabolic parameters between Hep-M3-KO (or Hep-M3-Tg) mice and their control littermates (Li et al. 2009).

These findings indicate that hepatocyte M_3 mAChRs do not play a critical role in maintaining proper blood glucose homeostasis in vivo. It is therefore possible that other neurotransmitters or neuromodulators, including various neuropeptides, which are co-released with ACh following vagal stimulation, are responsible for the vagus-mediated effects on hepatic glucose fluxes. Identification of these signaling molecules may facilitate the development of novel drugs that are able to modulate hepatic glucose fluxes for therapeutic purposes.

6 Brain Region-Specific M_1 Receptor KO Mice Show Deficits in Hippocampal Long-Term Depression

In a recent study, Kamsler et al. (2010) used Cre/loxP technology to generate two new M_1 mAChR receptor mutant mouse lines. One of the two lines selectively lacked M_1 receptors in excitatory neurons of the forebrain, including the hippocampus, but not in the striatum (FB-M_1-KO mice; Iwasato et al. 2004). In the other line, the M_1 receptor gene was deleted only in hippocampal CA3 pyramidal cells (CA3-M_1-KO mice).

Previous studies have shown that whole-body M_1 receptor KO mice exhibit increased locomotor activity (Miyakawa et al. 2001; Gerber et al. 2001). Interestingly, FB-M_1-KO mice did not show this phenotype (Kamsler et al. 2010), consistent with the hypothesis that the lack of M_1 receptors on inhibitory striatal interneurons is responsible for the hyperlocomotor activity observed with the whole-body M_1 receptor KO mice (Gerber et al. 2001). The first behavioral analysis of whole-body M_1 receptor KO mice failed to detect any significant cognitive deficits that could be clearly dissociated from the observed changes in locomotor activity (Miyakawa et al. 2001). On the other hand, Anagnostaras et al. (2003) reported that whole-body M_1 receptor KO mice acquired contextual fear memory faster than WT control mice but that this type of memory was extinguished more rapidly when the mutant mice were monitored several weeks after fear conditioning. In contrast, Kamsler et al. (2010) found that FB-M_1-KO mice did not display any significant deficits in a similar experimental setup. The authors of the latter study therefore concluded that exaggerated motor responses, rather than changes in learning and memory per se, may be responsible for the behavioral changes observed with whole-body M_1 receptor KO mice during contextual fear memory testing.

Since M_1 receptors are highly expressed in hippocampal pyramidal cells (Volpicelli and Levey 2004) and muscarinic drugs modulate long-term potentiation (LTP) and long-term depression (LDP) in the hippocampus (see, for example, Auerbach and Segal 1996; McCutchen et al. 2006), Kamsler et al. (2010) used FB-M_1-KO mice to examine the potential involvement of M_1 receptors in modulating hippocampal synaptic plasticity. This analysis showed that stimulation of hippocampal slices derived from FB-M_1-KO mice and control littermates resulted in similar excitatory postsynaptic potentials and LTP. In contrast, (S)-

3,5-dihydroxyphenylglycine (DHPG), an agonist of group I metabotropic glutamate receptors (mGluRs), was able to induce LDP in hippocampal slices from control but not from FB-M_1-KO mice. Additional studies indicated that M_1 receptors are required for maintaining normal synaptic release in Schaffer collaterals and a certain basal level of PKC activity in the CA3 region of the hippocampus which contains the cell bodies of the Schaffer collaterals (Kamsler et al. 2010). Interestingly, mGluR-mediated LTD could be rescued in hippocampal slices from CA3-M_1-KO mice following preincubation with a PKC activator, indicative of a presynaptic location of mGluR-mediated induction of LTD. These results suggest that the lack of M_1 receptors in the CA3 region of the hippocampus results in reduced PKC activity, which in turn triggers an increase in the probability of glutamate release from hippocampal synapses.

It should be noted in this context that *Fmr1*-deficient mice, an animal model of human mental retardation syndrome "fragile X," exhibit enhanced mGluR-mediated LTD (Bear et al. 2004) (note that FB-M_1-KO mice display the opposite phenotype). This observation raises the possibility that dampening mGluR-mediated LTD via blockade of central M_1 receptors may prove beneficial in the treatment of humans with fragile X, the most frequent inherited cause of mental retardation.

7 A New Knockin Mouse Strain Expressing a Phosphorylation-Deficient Mutant M_3 mAChR Shows Distinct Cognitive Deficits

The M_3 mAChR, like many other GPCRs, is subject to phosphorylation by various protein kinases at serine residues located within the i3 loop (Budd et al. 2000; Torrecilla et al. 2007). To test the possibility that M_3 mAChR phosphorylation plays a role in modulating M_3 mAChR signaling in vivo, Poulin et al. (2010) generated knockin mice (KI mice) in which the WT M_3 mAChR coding sequence had been replaced (via homologous recombination) with a mutant version of the receptor containing 15 point mutations in serine phospho-acceptor sites within the i3 loop of the receptor. Radioligand binding studies showed that the mutant receptor was expressed in different brain regions at levels similar to those observed with the WT receptor. In addition, biochemical assays demonstrated that the mutant M_3 receptor was able to activate G proteins of the G_q family in a fashion similar to the WT M_3 mAChR. However, studies with cerebellar granule cell neurons indicated that the mutant M_3 receptor showed a pronounced reduction in agonist-induced phosphorylation, as compared with the WT receptor (Poulin et al. 2010).

In most cases, GPCR internalization requires receptor phosphorylation and subsequent recruitment of arrestin(s) (Lefkowitz et al. 2006). Consistent with this notion, agonist-induced internalization of the mutant M_3 receptor was significantly impaired in neurons derived from M_3-KI mice (Poulin et al. 2010). Moreover, the

mutant M_3 mAChR exhibited a significant impairment in agonist-induced arrestin recruitment. These data suggest that the phosphorylation-deficient mutant M_3 mAChR is specifically impaired in initiating arrestin-dependent signaling pathways.

Behavioral studies demonstrated that the M_3-KI mutant mice displayed a selective deficit in fear conditioning learning and memory (a hippocampus-dependent cognitive task), similar to whole-body M_3 receptor KO mice (Poulin et al. 2010). In WT mice, but not in M_3-KI mutant mice, M_3 receptor phosphorylation was upregulated in the hippocampus after fear conditioning. Moreover, following fear conditioning, hippocampal neurons expressing the mutant M_3 receptor showed reduced stimulation of c-Fos expression, a marker of neuronal activity, most likely due to disruption of arrestin-dependent signaling pathways in M_3-KI mice (Poulin et al. 2010). Taken together, these data suggest that the cognitive deficit displayed by the M_3-KI mutant mice is due to impaired signaling of the phosphorylation-deficient mutant M_3 receptor through arrestin-dependent pathways. This finding raises the possibility that "biased" M_3 receptor ligands that are able to selectively promote signaling through phosphorylation-/arrestin-dependent pathways may become useful in the treatment of certain cognitive disorders.

8 Mutant Mice Lacking M_4 mAChRs in a Subpopulation of Striatal Projection Neurons Show Pronounced Behavioral Changes

Phenotypic analysis of whole-body M_4 receptor KO mice suggested that M_4 mAChRs play an important role in regulating dopamine-dependent behaviors and inhibiting dopaminergic neurotransmission in higher brain regions (Gomeza et al. 1999; Felder et al. 2001; Zhang et al. 2002; Tzavara et al. 2004). M_4 receptors are widely expressed throughout the CNS, predominantly in different regions of the forebrain (Levey et al. 1991; Vilaro et al. 1993; Volpicelli and Levey 2004). Interestingly, M_4 receptors are coexpressed with D_1 dopamine receptors in a specific subset of striatal medium spiny neurons which contain GABA as the major neurotransmitter and give rise to the so-called striato-nigral pathway (Bernard et al. 1992; Di Chiara et al. 1994; Ince et al. 1997). To study the physiological relevance of this subpopulation of M_4 receptors, Jeon et al. (2010) generated mutant mice that lacked M_4 receptors only in D_1 dopamine receptor-expressing cells. To create these mutant mice (D1-M4-KO mice), Jeon et al. (2010) crossed mice in which the M_4 receptor coding sequence had been flanked with loxP sites with transgenic mice that expressed Cre recombinase under the control of the D_1 dopamine receptor promoter (Lemberger et al. 2007).

Treatment of striatal membranes prepared from control mice with the D_1 receptor agonist, SKF82958, triggered concentration-dependent increases in cAMP production, as expected (note that the D_1 receptor is selectively coupled to G_s/

G_{olf}). This response was abolished in the simultaneous presence of carbachol. Strikingly, this inhibitory effect of carbachol was no longer observed in striatal membranes prepared from D1-M4-KO mice (Jeon et al. 2010), clearly indicating that M_4 receptor activation inhibits D_1 receptor-mediated cAMP production in the striatum, most likely via inhibition of adenylyl cyclase via G_i-type G proteins.

Jeon et al. (2010) next subjected D1-M4-KO and control mice to a series of behavioral tests that involve the central dopaminergic system. Initially, the authors recorded locomotor responses in mice that had been injected with the D_1 receptor agonist, SKF82958, or the psychostimulants amphetamine or cocaine, both of which increase synaptic dopamine levels. All three drugs caused increased locomotor stimulation in the M_4 receptor mutant mice, particularly at the highest dose used (Jeon et al. 2010), indicating that activation of striatal M_4 receptors counteracts drug-induced hyperlocomotor activity in control mice. The ability of drugs to inhibit amphetamine-induced locomotor activity is frequently used as an animal model to identify compounds with antipsychotic activity. The data described above therefore support the concept that centrally acting M_4 receptor agonists may be useful in the treatment of schizophrenia, consistent with the outcome of a series of recent pharmacological/behavioral studies (Chan et al. 2008; Brady et al. 2008; Shekhar et al. 2008; Woolley et al. 2009).

Haloperidol-induced catalepsy serves as an animal model that mimics the motor side effects of antipsychotic drugs. These side effects are thought to involve changes in the balance between dopaminergic and muscarinic cholinergic neurotransmission in the striatum (Di Chiara et al. 1994). Jeon et al. (2010) found that treatment of D1-M4-KO mice with haloperidol resulted in significantly reduced cataleptic responses, as compared to control littermates. The authors obtained very similar results using risperidone, a so-called second-generation antipsychotic drug. These observations suggest that centrally acting M_4 receptor antagonists may prove beneficial in treating the locomotor side effects associated with the use of antipsychotic drugs.

Repeated treatment of rodents with amphetamine or other psychostimulants leads to enhanced locomotor activity over time, a phenomenon referred to as behavioral sensitization.

Importantly, this effect persists for an extended period of time (weeks or months) after the last drug administration, thus mimicking the long-term sensitivity to drugs observed in human addicts. In both D1-M4-KO and control mice, daily treatment with amphetamine (2 mg/kg, s.c.) for a 6-day period caused time-dependent increases in locomotor activity (Jeon et al. 2010). However, this effect was significantly more pronounced in the M_4 receptor mutant mice. Following the initial 6-day injection period, mice were kept drug-free for 2 weeks and then re-injected with a single dose of amphetamine (2 mg/kg, s.c.). Strikingly, in this test, the amphetamine-pretreated mutant mice showed a significantly more robust hyperlocomotor effect than the amphetamine-pretreated control mice (Jeon et al. 2010). Taken together, these data strongly support the notion that M_4 receptors present on D_1 receptor neurons function to counteract amphetamine-induced behavioral sensitization.

Numerous studies have shown that the rewarding effects of essentially all major drugs of abuse involve the release of dopamine in the nucleus accumbens

(Wise 1996; Koob et al. 1998). The nucleus accumbens is a major component of the ventral striatum that shows a similar cellular architecture as the dorsal striatum and also contains neurons that coexpress M_4 muscarinic and D_1 dopamine receptors (McGinty 1999). In vivo microdialysis studies demonstrated that basal dopamine efflux was increased two- to threefold in the nucleus accumbens of D1-M4-KO mice, as compared to control littermates (Jeon et al. 2010). A similar pattern was observed with amphetamine-treated mice.

The observations that D1-M4-KO mice displayed enhanced behavioral sensitization following amphetamine treatment and increased dopamine efflux in the nucleus accumbens strongly suggest that striatal M_4 receptor activity inhibits the central dopaminergic reward system in control mice. It is therefore conceivable that compounds that can stimulate central (striatal) M_4 receptors may prove useful in the treatment of drug addiction.

9 Transgenic Mice Expressing M_3 Receptor-Based RASSLs Selectively in Pancreatic β Cells Show Striking Metabolic Phenotypes

Several years ago, a yeast genetic screen led to the identification of a mutant human M_3 mAChR that is unable to bind ACh, the physiological agonist, but can be activated efficiently by clozapine-N-oxide (CNO), a pharmacologically inert metabolite of clozapine (Armbruster et al. 2007). This mutant receptor contained two point mutations (Y149C and A239G) within transmembrane domains 3 and 5, respectively (positions 3.33 and 5.46 according to the Ballesteros–Weinstein numbering system). Armbruster et al. (2007) also demonstrated that introduction of the corresponding point mutations into the remaining four mAChR subtypes yielded mutant receptors with similar pharmacological properties. These mutant mAChRs therefore represent a new generation of RASSLs (*r*eceptors *a*ctivated *s*olely by *s*ynthetic *l*igands), a term first coined by Conklin and colleagues (Scearce-Levie et al. 2001; Conklin et al. 2008). These M_3 receptor-based RASSLs are also referred to as DREADDS (*d*esigner *r*eceptors *e*xclusively *a*ctivated by *d*esigner *d*rugs; Armbruster et al. 2007; Alexander et al. 2009; Dong et al. 2010). Using the M_3 receptor-based G_q-RASSL as a template, Guettier et al. (2009) generated two mutant versions of this construct in which distinct intracellular domains of the M_3 mAChR were replaced with the corresponding β_1-adrenergic receptor sequences. The resulting CNO-sensitive mutant M_3 receptors (RASSLs) showed novel G-protein-binding properties. One of the constructs was able to selectively activate G_s (G_s-RASSL), whereas the other one displayed promiscuous coupling properties, being able to simulate both G_s and G_q-type G proteins (G_q/G_s-RASSL).

To explore the in vivo effects of activating distinct G protein signaling pathways in pancreatic β cells, Guettier et al. (2009) generated two strains of transgenic mice that expressed the M_3 receptor-based G_q- or G_s-RASSL in β cells only. For the sake

of simplicity, I will refer to these mutant mice as β-R-q Tg and β-R-s Tg mice in the following. The data obtained with the β-R-s Tg mice largely confirmed previous results highlighting the role of β-cell G_s signaling in augmenting glucose-induced insulin release, maintaining normal blood glucose levels, and promoting an increase in β-cell mass (Doyle and Egan 2007; Baggio and Drucker 2007; Ahrén 2009). On the other hand, phenotypic analysis of the β-R-q Tg mice yielded new insights into the roles of β-cell G_q signaling in β-cell function and whole-body glucose homeostasis.

Strikingly, CNO treatment of β-R-q mice resulted in dose-dependent decreases in blood glucose levels (Fig. 3a), associated with dose-dependent increases in

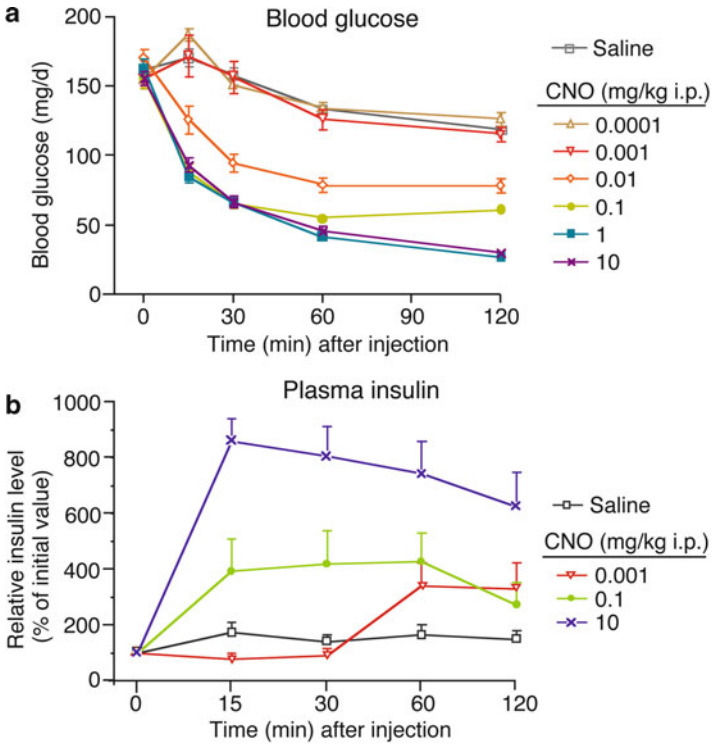

Fig. 3 CNO-induced changes in blood glucose and plasma insulin levels in transgenic mice expressing an M_3 mAChR-based G_q-RASSL in pancreatic β cells only (β-R-q Tg mice). (**a**) CNO-induced decreases in blood glucose levels. β-R-q Tg mice received a single i.p. injection of increasing doses of CNO or vehicle (saline), and blood glucose levels were measured at the indicated time points. (**b**) CNO-induced increases in plasma insulin levels. β-R-q Tg mice received a single i.p. injection of increasing doses of CNO or vehicle (saline), and plasma insulin levels were measured at the indicated time points. Data are expressed as % increase in plasma insulin levels relative to pre-injection values (=100%). Absolute basal insulin levels (prior to injection of saline or CNO) were 1.89 ± 0.34 ng/ml ($n = 24$). All experiments were carried out with adult female mice that had free access to food ($n = 4$–8 per dose and/or group). Data presented as means ± SEM. Data were taken from Guettier et al. (2009)

plasma insulin concentrations (Fig. 3b), indicating that the degree of β−cell G protein signaling could be titrated according to the CNO dose administered (Guettier et al. 2009). CNO-dependent activation of β-cell G_q signaling in β-R-q Tg mice also triggered a pronounced increase in first-phase insulin release in vivo, followed by a more prominent long-lasting second phase of insulin secretion (as compared to WT mice). First-phase insulin release is critical for postprandial glucose homeostasis and a decrease of this activity is a characteristic marker of β-cell dysfunction in the early stages of T2D (Del Prato et al. 2002; Nesher and Cerasi 2002). The observation that acute activation of β-cell G_q signaling strongly stimulates first-phase insulin release is therefore of considerable clinical relevance. Guettier et al. (2009) also demonstrated that CNO-dependent activation of β-cell G_q signaling in β-R-q Tg mice in vivo resulted in a pronounced improvement in glucose tolerance. This effect was observed with mice maintained on regular mouse chow as well as with mice raised on a high-fat diet.

Interestingly, chronic treatment of β-R-q Tg mice with CNO led a significant increase in β-cell mass, associated with an increase in mean islet size and β-cell hypertrophy (Guettier et al. 2009), indicating that chronic activation of β-cell G_q signaling has a stimulatory effect on β-cell mass. To explore the molecular mechanisms underlying the CNO-induced increase in β-cell mass, Guettier et al. (2009) used real-time qRT-PCR analysis to examine the expression levels of many genes important for β-cell function and growth. These studies showed that CNO treatment of islets prepared from β-R-q Tg mice led to a pronounced increase in insulin receptor substrate 2 (IRS-2) mRNA expression, most likely due to G_q-mediated increases in intracellular Ca^{2+} levels (Gilon and Henquin 2001; Lingohr et al. 2006). Previous studies have shown that IRS-2 plays a central role in maintaining β-cell function and β-cell mass (Niessen 2006; White 2006). It is therefore likely that IRS-2 represents a key component of the pathway that links activation of β-cell G_q to increased β-cell mass. Interestingly, CNO-mediated stimulation of β-cell G_q signaling in islets prepared from β-R-q Tg mice also led to small but significant increases in preproinsulin (*Ins2*) and proprotein convertase 1 and 2 transcript levels (the two latter genes code for enzymes that are involved in the conversion of preproinsulin to insulin). This observation therefore suggests that activation of β-cell G_q signaling promotes insulin synthesis.

In general, CNO induced more pronounced metabolic effects in β-R-q Tg than in β-R-s Tg mice. However, since the M_3 receptor-based G_s-RASSL showed some degree of CNO-independent signaling in β-R-s Tg mice (which may have triggered counter-regulatory responses), a direct comparison between the effects observed with the two different mutant mouse strains may not be meaningful (Guettier et al. 2009). However, independent of this issue, CNO-induced activation of β-cell G_q signaling in β-R-q Tg mice had several beneficial effects on glucose homeostasis and β-cell function, strongly suggesting that drugs that can enhance signaling through β-cell G_q-coupled receptors have significant potential for the treatment of T2D and glucose intolerance.

10 Transgenic Mice Expressing an M_3 Receptor-Based G_q-RASSL in Forebrain Principal Cells Display Distinct Electrophysiological and Behavioral Changes

Alexander et al. (2009) recently demonstrated that transgenic mice expressing the M_3 receptor-based G_q-RASSL (R-q) in a cell (region)-specific fashion also *represent* a powerful tool to study the relevance of G_q-mediated signaling pathways in the CNS. The authors first generated transgenic mice (TRE-R-q mice) that expressed R-q under the transcriptional control of the Tet-off system (i.e., R-q expression is repressed by tetracycline or its analog, doxycycline). The TRE-R-q mice were then crossed with CaMKIIαtTA transgenic mice in which the expression of the tet transactivator (tTA) is restricted to principal neurons mainly in the cortex, hippocampus, and striatum (Mayford et al. 1996). The resulting double transgenic mice (tTA-TRE-R-q Tg mice) were then subjected to a series of behavioral and electrophysiological tests.

Alexander et al. (2009) initially carried out whole-cell recordings from CA1 pyramidal neurons of hippocampal slices prepared from tTA-TRE-R-q Tg mice and control littermates. These studies showed that bath application of CNO depolarized CA1 pyramidal cells and increased the firing rate of these neurons only in the transgenic mice. The CNO-mediated electrophysiological responses observed with the transgenic mice could be completely blocked by the PLC inhibitor, U73122, indicating that these effects involved a PLC-dependent pathway.

Behavioral studies showed that peripheral administration of relatively low doses of CNO (0.1 or 0.3 mg/kg) led to significant increases in locomotor activity in the tTA-TRE-R-q Tg mice, but not in WT littermates (Alexander et al. 2009). Interestingly, treatment of the transgenic mice with a somewhat higher dose of CNO (0.5 mg/kg) reproducibly triggered limbic seizures of behavioral class 1. When administered at even higher doses (1 or 5 mg/kg), CNO induced continuous seizure activity (status epilepticus) and death in the tTA-TRE-R-q Tg mice. As expected, CNO was devoid of seizure-inducing activity in control animals at any of the doses tested (Alexander et al. 2009).

It should be noted in this context that systemic injection of pilocarpine, a nonsubtype-specific partial muscarinic agonist, is known to cause seizures in mice (Hamilton et al. 1997). Interestingly, studies with whole-body M_1 mAChR KO mice showed that M_1 receptors, which are also coupled to G_q-type G proteins, are required for the seizure-inducing effects of pilocarpine (Hamilton et al. 1997). It is therefore tempting to speculate that pilocarpine-evoked seizure activity is triggered by a similar cellular/molecular mechanism as that observed with CNO-treated tTA-TRE-R-q Tg mice.

In an attempt to correlate the behavioral effects resulting from CNO treatment of tTA-TRE-R-q Tg mice with specific electrophysiological changes in vivo, Alexander et al. (2009) implanted control and transgenic mice with multielectrode arrays to monitor both local field potentials (LFPs) and spike activity of multiple individual neurons in the hippocampus. In the transgenic mice, CNO evoked dose-dependent

increases in gamma power as detected by spectral analyses of LFP recordings, associated with an increase in the firing rate of hippocampal interneurons. No such electrophysiological changes were observed after CNO treatment of control mice. On the basis of these observations, in conjunction with other lines of evidence, Alexander et al. (2009) speculated that activation of R-q modifies local hippocampal circuit activity via stimulation of hippocampal principal cells, which in turn synaptically activate the firing of hippocampal interneurons, thus triggering gamma oscillations. Moreover, the data reported by Alexander et al. (2009) indicate that CNO treatment of the transgenic mice stimulates G_q-dependent cellular pathways that result in the activation of excitatory pyramidal neurons. It is likely that the G_q-mediated closure of KCNQ potassium channels (M current inhibition) represents a key mechanism through which CNO induces its various effects in the tTA-TRE-R-q Tg mice (Brown and Yu 2000; Zhang et al. 2003).

These findings clearly illustrate that M_3 receptor-based RASSLs represent powerful new tools to control the activity of distinct neuronal subpopulations in a conditional fashion in vivo. The generation and analysis of transgenic mice expressing M_3 receptor-based RASSLs endowed with different G-protein-coupling properties should enable neuroscientists to study GPCR-regulated neuronal pathways and the associated behavioral consequences in unprecedented cellular detail. Clearly, such studies are likely to have a strong impact on the development of novel GPCR-based therapies for many major psychiatric diseases.

11 Concluding Remarks

The phenotypic analysis of whole-body M_1–M_5 mAChR KO mice has greatly advanced our knowledge about the physiological roles of the individual mAChR subtypes, which has been a major driving force behind the resurgent interest in mAChR pharmacology and the development of novel drugs targeting these receptors. As reviewed in this chapter, several new mAChR mutant mouse models, including the first mAChR knockin strain and several conditional KO mice in which specific mAChR subtypes can be inactivated at a certain point during development and/or in a cell type- or tissue-specific fashion, have been developed recently. These new animal models represent even more sophisticated tools that should continue to stimulate research in the mAChR field. Detailed phenotypic analysis of these newly developed mutant mouse strains offers the unique opportunity to dissect the physiological roles of the individual mAChR subtypes in unprecedented cellular detail. It is likely that these studies will greatly stimulate the development of subtype-selective muscarinic ligands for the therapy of a wide range of pathophysiological conditions.

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References

Abramow-Newerly M, Roy AA, Nunn C, Chidiac P (2006) RGS proteins have a signalling complex: interactions between RGS proteins and GPCRs, effectors, and auxiliary proteins. Cell Signal 18:579–591

Abrams P, Andersson KE, Buccafusco JJ et al (2006) Muscarinic receptors: their distribution and function in body systems, and the implications for treating overactive bladder. Br J Pharmacol 148:565–578

Ahren B (2000) Autonomic regulation of islet hormone secretion – implications for health and disease. Diabetologia 2000(43):393–410

Ahrén B (2009) Islet G protein-coupled receptors as potential targets for treatment of type 2 diabetes. Nat Rev Drug Discov 8:369–385

Alexander GM, Rogan SC, Abbas AI et al (2009) Remote control of neuronal activity in transgenic mice expressing evolved G protein-coupled receptors. Neuron 63:27–39

Anagnostaras SG, Murphy GG, Hamilton SE et al (2003) Selective cognitive dysfunction in acetylcholine M_1 muscarinic receptor mutant mice. Nat Neurosci 6:51–58

Armbruster BN, Li X, Pausch MH et al (2007) Evolving the lock to fit the key to create a family of G protein-coupled receptors potently activated by an inert ligand. Proc Natl Acad Sci U S A 104:5163–5168

Auerbach JM, Segal M (1996) Muscarinic receptors mediating depression and long-term potentiation in rat hippocampus. J Physiol 492(Pt 2):479–493

Baggio LL, Drucker DJ (2007) Biology of incretins: GLP-1 and GIP. Gastroenterology 132:2131–2157

Bansal G, Druey KM, Xie Z (2007) R4 RGS proteins: regulation of G-protein signaling and beyond. Pharmacol Ther 116:473–495

Bear MF, Huber KM, Warren ST (2004) The mGluR theory of fragile X mental retardation. Trends Neurosci 27:370–377

Bernard V, Normand E, Bloch B (1992) Phenotypical characterization of the rat striatal neurons expressing muscarinic receptor genes. J Neurosci 12:3591–3600

Brady AE, Jones CK, Bridges TM et al (2008) Centrally active allosteric potentiators of the M_4 muscarinic acetylcholine receptor reverse amphetamine-induced hyperlocomotor activity in rats. J Pharmacol Exp Ther 327:941–953

Brown BS, Yu SP (2000) Modulation and genetic identification of the M channel. Prog Biophys Mol Biol 73:135–166

Budd DC, McDonald JE, Tobin AB (2000) Phosphorylation and regulation of a $G_{q/11}$-coupled receptor by casein kinase 1a. J Biol Chem 275:19667–19675

Caulfield MP, Birdsall NJM (1998) International Union of Pharmacology. XVII. Classification of muscarinic acetylcholine receptors. Pharmacol Rev 50:279–290

Chan WY, McKinzie DL, Bose S et al (2008) Allosteric modulation of the muscarinic M_4 receptor as an approach to treating schizophrenia. Proc Natl Acad Sci U S A 105:10978–10983

Conklin BR, Hsiao EC, Claeysen S et al (2008) Engineering GPCR signaling pathways with RASSLs. Nat Methods 5:673–678

Del Prato S, Marchetti P, Bonadonna RC (2002) Phasic insulin release and metabolic regulation in type 2 diabetes. Diabetes 51(Suppl 1):S109–S116

Di Chiara G, Morelli M, Consolo S (1994) Modulatory functions of neurotransmitters in the striatum: ACh/dopamine/NMDA interactions. Trends Neurosci 17:228–233

Dong S, Rogan SC, Roth BL (2010) Directed molecular evolution of DREADDs: a generic approach to creating next-generation RASSLs. Nat Protoc 5:561–573

Doyle ME, Egan JM (2007) Mechanisms of action of glucagon-like peptide 1 in the pancreas. Pharmacol Ther 113:546–593

Duttaroy A, Zimliki CL, Gautam D et al (2004) Muscarinic stimulation of pancreatic insulin and glucagon release is abolished in M_3 muscarinic acetylcholine receptor-deficient mice. Diabetes 53:1714–1720

Eglen RM (2005) Muscarinic receptor subtype pharmacology and physiology. Prog Med Chem 43:105–136

Elefteriou F, Ahn JD, Takeda S et al (2005) Leptin regulation of bone resorption by the sympathetic nervous system and CART. Nature 434:514–520

Felder CC, Porter AC, Skillman TL et al (2001) Elucidating the role of muscarinic receptors in psychosis. Life Sci 68:2605–2613

Frohman LA, Kineman RD (2002) Growth hormone-releasing hormone and pituitary development, hyperplasia and tumorigenesis. Trends Endocrinol Metab 13:299–303

Fu L, Patel MS, Bradley A et al (2005) The molecular clock mediates leptin-regulated bone formation. Cell 122:803–815

Gautam D, Gavrilova O, Jeon J et al (2006a) Beneficial metabolic effects of M_3 muscarinic acetylcholine receptor deficiency. Cell Metab 4:363–375

Gautam D, Han SJ, Hamdan FF et al (2006b) A critical role for β cell M_3 muscarinic acetylcholine receptors in regulating insulin release and blood glucose homeostasis in vivo. Cell Metab 3:449–461

Gautam D, Jeon J, Starost MF et al (2009) Neuronal M_3 muscarinic acetylcholine receptors are essential for somatotroph proliferation and normal somatic growth. Proc Natl Acad Sci U S A 106:6398–6403

Gerber DJ, Sotnikova TD, Gainetdinov RR et al (2001) Hyperactivity, elevated dopaminergic transmission, and response to amphetamine in M1 muscarinic acetylcholine receptor-deficient mice. Proc Natl Acad Sci U S A 98:15312–15317

Gilon P, Henquin JC (2001) Mechanisms and physiological significance of the cholinergic control of pancreatic β-cell function. Endocr Rev 22:565–604

Giustina A, Veldhuis JD (1998) Pathophysiology of the neuroregulation of growth hormone secretion in experimental animals and the human. Endocr Rev 19:717–797

Gomeza J, Zhang L, Kostenis E et al (1999) Enhancement of D1 dopamine receptor-mediated locomotor stimulation in M_4 muscarinic acetylcholine receptor knockout mice. Proc Natl Acad Sci U S A 96:10483–10488

Guettier JM, Gautam D, Scarselli M et al (2009) A chemical-genetic approach to study G protein regulation of β cell function in vivo. Proc Natl Acad Sci U S A 106:19197–19202

Hamilton SE, Loose MD, Qi M et al (1997) Disruption of the m1 receptor gene ablates muscarinic receptor-dependent M current regulation and seizure activity in mice. Proc Natl Acad Sci U S A 94:13311–13316

Hollinger S, Hepler JR (2002) Cellular regulation of RGS proteins: modulators and integrators of G protein signaling. Pharmacol Rev 54:527–559

Ince E, Ciliax BJ, Levey AI (1997) Differential expression of D1 and D2 dopamine and m4 muscarinic acetylcholine receptor proteins in identified striatonigral neurons. Synapse 27:357–366

Iwasato T, Nomura R, Ando R et al (2004) Dorsal telencephalon-specific expression of Cre recombinase in PAC transgenic mice. Genesis 38:130–138

Jeon J, Dencker D, Wörtwein G et al (2010) A subpopulation of neuronal M_4 muscarinic acetylcholine receptors plays a critical role in modulating dopamine-dependent behaviors. J Neurosci 30:2396–2405

Jetté L, Léger R, Thibaudeau K et al (2005) Human growth hormone-releasing factor (hGRF)1-29-albumin bioconjugates activate the GRF receptor on the anterior pituitary in rats: identification of CJC-1295 as a long-lasting GRF analog. Endocrinology 146:3052–3058

Kahn CR (1994) Insulin action, diabetogenes, and the cause of type II diabetes (Banting Lecture). Diabetes 43:1066–1084

Kamsler A, McHugh TJ, Gerber D et al (2010) Presynaptic m1 muscarinic receptors are necessary for mGluR long-term depression in the hippocampus. Proc Natl Acad Sci U S A 107:1618–1623

Koob GF, Sanna PP, Bloom FE (1998) Neuroscience of addiction. Neuron 21:467–476

Lam TK, Pocai A, Gutierrez-Juarez R et al (2005) Hypothalamic sensing of circulating fatty acids is required for glucose homeostasis. Nat Med 11:320–327

Le Tissier PR, Carmignac DF, Lilley S et al (2005) Hypothalamic growth hormone-releasing hormone (GHRH) deficiency: targeted ablation of GHRH neurons in mice using a viral ion channel transgene. Mol Endocrinol 19:1251–1262

Lefkowitz RJ, Rajagopal K, Whalen EJ (2006) New roles for β-arrestins in cell signaling: not just for seven-transmembrane receptors. Mol Cell 24:643–652

Lemberger T, Parlato R, Dassesse D et al (2007) Expression of Cre recombinase in dopaminoceptive neurons. BMC Neurosci 8:4

Levey AI, Kitt CA, Simonds WF et al (1991) Identification and localization of muscarinic acetylcholine receptor proteins in brain with subtype-specific antibodies. J Neurosci 11:3218–3226

Levey AI, Edmunds SM, Heilman CJ et al (1994) Localization of muscarinic m3 receptor protein and M3 receptor binding in rat brain. Neuroscience 63:207–221

Li JH, Gautam D, Han SJ et al (2009) Hepatic muscarinic acetylcholine receptors are not critically involved in maintaining glucose homeostasis in mice. Diabetes 58:2776–2787

Lingohr MK, Briaud I, Dickson LM et al (2006) Specific regulation of IRS-2 expression by glucose in rat primary pancreatic islet β-cells. J Biol Chem 281:15884–15892

Matsui M, Yamada S, Oki T et al (2004) Functional analysis of muscarinic acetylcholine receptors using knockout mice. Life Sci 75:2971–2981

Mayford M, Bach ME, Huang YY et al (1996) Control of memory formation through regulated expression of a CaMKII transgene. Science 274:1678–1683

McCutchen E, Scheiderer CL, Dobrunz LE, McMahon LL (2006) Coexistence of muscarinic long-term depression with electrically induced long-term potentiation and depression at CA3-CA1 synapses. J Neurophysiol 96:3114–3121

McGinty JF (1999) Regulation of neurotransmitter interactions in the ventral striatum. Ann N Y Acad Sci 877:129–139

Miyakawa T, Yamada M, Duttaroy A, Wess J (2001) Hyperactivity and intact hippocampus-dependent learning in mice lacking the M_1 muscarinic acetylcholine receptor. J Neurosci 21:5239–5250

Nesher R, Cerasi E (2002) Modeling phasic insulin release: immediate and time-dependent effects of glucose. Diabetes 51(Suppl 1):S53–S59

Niessen M (2006) On the role of IRS2 in the regulation of functional β-cell mass. Arch Physiol Biochem 112:65–73

Oki T, Takagi Y, Inagaki S et al (2005) Quantitative analysis of binding parameters of [^{3}H]N-methylscopolamine in central nervous system of muscarinic acetylcholine receptor knockout mice. Brain Res Mol Brain Res 133:6–11

Pocai A, Lam TK, Gutierrez-Juarez R et al (2005a) Hypothalamic K_{ATP} channels control hepatic glucose production. Nature 434:1026–1031

Pocai A, Obici S, Schwartz GJ, Rossetti L (2005b) A brain-liver circuit regulates glucose homeostasis. Cell Metab 1:53–61

Poulin B, Butcher A, McWilliams P et al (2010) The M_3-muscarinic receptor regulates learning and memory in a receptor phosphorylation/arrestin-dependent manner. Proc Natl Acad Sci U S A 107:9440–9445

Regard JB, Kataoka H, Cano DA et al (2007) Probing cell type-specific functions of G_i in vivo identifies GPCR regulators of insulin secretion. J Clin Invest 117:4034–4043

Ross EM, Wilkie TM (2000) GTPase-activating proteins for heterotrimeric G proteins: regulators of G protein signaling (RGS) and RGS-like proteins. Annu Rev Biochem 69:795–827

Ruiz de Azua I, Scarselli M, Rosemond E et al (2010) RGS4 is a negative regulator of insulin release from pancreatic β-cells in vitro and in vivo. Proc Natl Acad Sci U S A 107:7999–8004
Scearce-Levie K, Coward P, Redfern CH, Conklin BR (2001) Engineering receptors activated solely by synthetic ligands (RASSLs). Trends Pharmacol Sci 22:414–420
Shekhar A, Potter WZ, Lightfoot J et al (2008) Selective muscarinic receptor agonist xanomeline as a novel treatment approach for schizophrenia. Am J Psychiatry 165:1033–1039
Shi Y, Oury F, Yadav VK et al (2010) Signaling through the M_3 muscarinic receptor favors bone mass accrual by decreasing sympathetic activity. Cell Metab 11:231–238
Takeda S, Elefteriou F, Levasseur R et al (2002) Leptin regulates bone formation via the sympathetic nervous system. Cell 111:305–317
Torrecilla I, Spragg EJ, Poulin B et al (2007) Phosphorylation and regulation of a G protein-coupled receptor by protein kinase CK2. J Cell Biol 177:127–137
Tronche F, Kellendonk C, Kretz O et al (1999) Disruption of the glucocorticoid receptor gene in the nervous system results in reduced anxiety. Nat Genet 23:99–103
Tzavara ET, Bymaster FP, Davis RJ et al (2004) M_4 muscarinic receptors regulate the dynamics of cholinergic and dopaminergic neurotransmission: relevance to the pathophysiology and treatment of related CNS pathologies. FASEB J 18:1410–1412
Vatamaniuk MZ, Horyn OV, Vatamaniuk OK, Doliba NM (2003) Acetylcholine affects rat liver metabolism via type 3 muscarinic receptors in hepatocytes. Life Sci 72:1871–1882
Vilaro MT, Mengod G, Palacios JM (1993) Advances and limitations of the molecular neuroanatomy of cholinergic receptors: the example of multiple muscarinic receptors. Prog Brain Res 98:95–101
Volpicelli LA, Levey AI (2004) Muscarinic acetylcholine receptor subtypes in cerebral cortex and hippocampus. Prog Brain Res 145:59–66
Wang PY, Caspi L, Lam CK et al (2008) Upper intestinal lipids trigger a gut-brain-liver axis to regulate glucose production. Nature 452:1012–1016
Wess J (1996) Molecular biology of muscarinic acetylcholine receptors. Crit Rev Neurobiol 10:69–99
Wess J (2004) Muscarinic acetylcholine receptor knockout mice: novel phenotypes and clinical implications. Annu Rev Pharmacol Toxicol 44:423–450
Wess J, Eglen RM, Gautam D (2007) Muscarinic acetylcholine receptors: mutant mice provide new insights for drug development. Nat Rev Drug Discov 6:721–733
Wettschureck N, Moers A, Wallenwein B et al (2005) Loss of $G_{q/11}$ family G proteins in the nervous system causes pituitary somatotroph hypoplasia and dwarfism in mice. Mol Cell Biol 25:1942–1948
White MF (2006) Regulating insulin signaling and β-cell function through IRS proteins. Can J Physiol Pharmacol 84:725–737
Wise RA (1996) Neurobiology of addiction. Curr Opin Neurobiol 6:243–251
Woolley ML, Carter HJ, Gartlon JE et al (2009) Attenuation of amphetamine-induced activity by the non-selective muscarinic receptor agonist, xanomeline, is absent in muscarinic M_4 receptor knockout mice and attenuated in muscarinic M_1 receptor knockout mice. Eur J Pharmacol 603:147–149
Yadav VK, Oury F, Suda N et al (2009) A serotonin-dependent mechanism explains the leptin regulation of bone mass, appetite, and energy expenditure. Cell 138:976–989
Zawalich WS, Zawalich KC, Tesz GJ et al (2004) Effects of muscarinic receptor type 3 knockout on mouse islet secretory responses. Biochem Biophys Res Commun 315:872–876
Zhang W, Yamada M, Gomeza J et al (2002) Multiple muscarinic acetylcholine receptor subtypes modulate striatal dopamine release, as studied with M_1-M_5 muscarinic receptor knock-out mice. J Neurosci 22:6347–6352
Zhang H, Craciun LC, Mirshahi T et al (2003) PIP_2 activates KCNQ channels, and its hydrolysis underlies receptor-mediated inhibition of M currents. Neuron 37:963–975
Zimmet P, Alberti KG, Shaw J (2001) Global and societal implications of the diabetes epidemic. Nature 414:782–787

Part II
Muscarinic Receptors in the CNS

Muscarinic Receptor Pharmacology and Circuitry for the Modulation of Cognition

Michael Bubser, Nellie Byun, Michael R. Wood, and Carrie K. Jones

Abstract The muscarinic cholinergic system constitutes an important part of the neuronal circuitry that modulates normal cognition. Muscarinic receptor antagonists are well known to produce or exacerbate impairments in attention, learning, and memory. Conversely, both direct-acting muscarinic receptor agonists and indirect-acting muscarinic cholinergic agonists, such as acetylcholinesterase inhibitors, have shown cognition-enhancing properties, including improvements in normal cognitive function, reversal of cognitive deficits induced by muscarinic receptor antagonists, and attenuation of cognitive deficits in psychiatric and neurological disorders, such as Alzheimer's disease and schizophrenia. However, until recently, the lack of small molecule ligands that antagonize or activate specific muscarinic acetylcholine receptor (mAChR) subtypes with high selectivity has been a major obstacle in defining the relative contributions of individual mAChRs to different aspects of cognitive function and for the development of novel therapeutic agents. These limitations may be potentially overcome by the recent discovery of novel mAChR subtype-selective compounds, notably allosteric agonists and positive allosteric modulators, which exhibit greater selectivity for individual mAChR subtypes than previous mAChR orthosteric agonists. In preclinical studies, these novel ligands have shown promising efficacy in several models for the enhancement of cognition. In this chapter, we will review the muscarinic

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cholinergic circuitry and pharmacology of mAChR agonists and antagonists relevant to the modulation of different aspects of cognition in animals and clinical populations.

Keywords Acetylcholine • Muscarinic acetylcholine receptors • Allosteric agonists • Positive allosteric modulators • Cognition • Learning • Memory • Alzheimer's disease • Schizophrenia • Cortex • Hippocampus

Abbreviations

AC	Adenylyl cyclase
ACh	Acetylcholine
AChEIs	Acetylcholinesterase inhibitors
AD	Alzheimer's disease
ADAS-cog	Alzheimer's Disease assessment scale-cognitive
AMG	Amygdala
BQCA	Benzylquinolone carboxylic acid
cAMP	Cyclic adenosine monophosphate
cc	Corpus callosum
CGI	Clinical Global Impression scale
CNS	Central nervous system
CP	Caudate-putamen
CSF	Cerebrospinal fluid
DA	Dopamine
DBB	Diagonal band of Broca
EC	Entorhinal cortex
EEG	Electrocephalogram
EPSC	Excitatory postsynaptic current
GABA	γ-aminobutyric acid
HPC	Hippocampus
IP3	Inositol triphosphate
KO	Knockout
LDTg	Laterodorsal tegmental nucleus
M1–M5	Muscarinic receptor subtypes M1 through M5
mAChRs	Muscarinic acetylcholine receptors
(m)PFC	(Medial) prefrontal cortex
NAM	Negative allosteric modulator
NAS	Nucleus accumbens
NBM	Nucleus basalis of Meynert
NMDA	*N*-methyl-D-aspartate
OB	Olfactory bulb
PAM	Positive allosteric modulator
PANSS	Positive and negative syndrome scale

PLC	Phospholipase C
PPI	Prepulse inhibition
PPTg	Pedunculopontine tegmental nucleus
SN	Substantia nigra
TBPB	1-(1'-2-methylbenzyl)-1,4'-bipiperidin-4-yl)-1H-benzo[d] imidazol-2(3H)-one
THAL	Thalamus
VTA	Ventral tegmental area
WT	Wildtype

1 Introduction

Normal cognition requires the coordination of numerous complex processes, including sensory information processing, sustained and divided attention, short- and long-term memory, and executive functions. Many neurologic and psychiatric disorders, including senile dementia, Alzheimer's disease (AD), and schizophrenia, are associated with severe impairments in cognitive functions that are directly correlated with poor social and functional outcomes (Green 1996; Green et al. 2004; Farlow and Cummings 2007).

There is now accumulating evidence that modulation of the muscarinic cholinergic system is involved in normal cognitive processes and that imbalances in the neurotransmission of this system may account, at least in part, for the cognitive deficits associated with AD and schizophrenia. For example, nonselective muscarinic acetylcholine receptor (mAChR) antagonists produce or exacerbate impairments in cognition in animals and in healthy control, normal aging and AD populations (Domer and Schueler 1960; Pazzagli and Pepeu 1965; Drachman and Leavitt 1974; Bartus et al. 1982; Sunderland et al. 1986; Newhouse et al. 1988; Rusted and Warburton 1988). In addition, mAChR antagonists can also induce psychotomimetic-like symptoms in healthy humans and/or aggravate existing behavioral disturbances in patients with dementia or psychosis (Osterholm and Camoriano 1982; Agnoli et al. 1983; Hamborg-Petersen et al. 1984; Strauss et al. 1990). Conversely, indirect-acting mAChR agonists, such as acetylcholinesterase inhibitors (AChEIs), and direct-acting mAChR agonists can improve aspects of normal cognitive function and/or improve cognitive impairments in AD patients, and in animals, they reverse deficits induced by mAChR antagonism or lesions of cholinergic basal forebrain circuitry (Aigner and Mishkin 1986; Robbins et al. 1989a, b; Rupniak et al. 1989, 1991; Matsuoka et al. 1991; Bodick et al. 1997a, b; Cummings 2003; Shekhar et al 2008). Nonselective mAChR agonists and AChEIs have also enhanced cognitive performance, particularly in the domains of attention and memory, in schizophrenic patients (see review in Chouinard et al. 2007; Edelstein et al. 1981; Shekhar et al. 2008). Taken together, these observations have led to the hypothesis that selective activators of mAChRs may provide an important alternative approach for the treatment of the cognitive impairments associated with neurologic and psychiatric disorders, such as AD and schizophrenia.

However, while AChEIs are clinically approved for the treatment of mild-to-moderate cognitive dementia associated with AD, the effects of these compounds on deficits in memory and other cognitive functions remain modest (Amenta et al. 2001). Unfortunately, early clinical studies using direct-acting mAChR agonists for AD and schizophrenia have ultimately failed in clinical development due to a lack of true subtype selectivity that resulted in a number of dose-limiting adverse effects from nonselective activation of peripheral mAChRs (Bruno et al. 1986; Bodick et al. 1997a, b; Shekhar et al. 2008). The high conservation of the acetylcholine (ACh) binding site across the five mAChR subtypes has presented a major impediment to the development of highly selective mAChR orthosteric-site ligands. The lack of subtype-selective mAChR ligands has also limited insights into the relative roles of the mAChR subtypes in the different aspects of cognition and the clinical efficacy observed with the AChEIs and nonselective muscarinic mAChR agonists.

Using an alternative strategy, our group and others have recently identified ligands for mAChRs that activate a specific receptor subtype through action at sites that are less highly conserved and topographically distinct relative to the orthosteric binding site of ACh, termed allosteric sites. Allosteric agonists activate the receptor subtype directly in the absence of the endogenous ligand ACh, while positive allosteric modulators (PAMs) bind to an allosteric site and potentiate the effects of ACh, but have no intrinsic activity. Because mAChR PAMs can only exert their effects in the presence of ACh at a given synapse, these ligands may maintain normal temporal and spatial components of endogenous ACh neurotransmission. This latter feature may provide an important advantage in the treatment of cognitive impairments in early stage dementia or schizophrenia, as recent findings suggest that optimal levels of ACh transmission for cognition are dynamic and task dependent (Kozak et al. 2006; Hasselmo and Sarter 2011). To date, these novel allosteric activators of the different mAChR subtypes have shown efficacy in preclinical models for the enhancement of cognition, and possess suitable physiochemical properties for optimization as potential clinical candidates.

In this chapter, we will provide a brief overview of cholinergic circuitry and mAChR distribution and function in the central nervous system (CNS). We will next review the effects of different mAChR antagonists and agonists in preclinical models of cognition and in clinical populations. Finally, we will highlight recent developments with novel subtype-selective allosteric agonists and PAMs of M1 and M4 mAChRs in preclinical models for the enhancement of cognition.

2 Anatomy of the Cholinergic System

2.1 Cholinergic Cell Groups and Their Target Regions

Within the CNS, cholinergic projection neurons are organized into relatively discrete cell groups in the basal forebrain and the caudal mesencephalon. As described in the seminal work of Mesulam and colleagues (Mesulam et al. 1983), six groups of

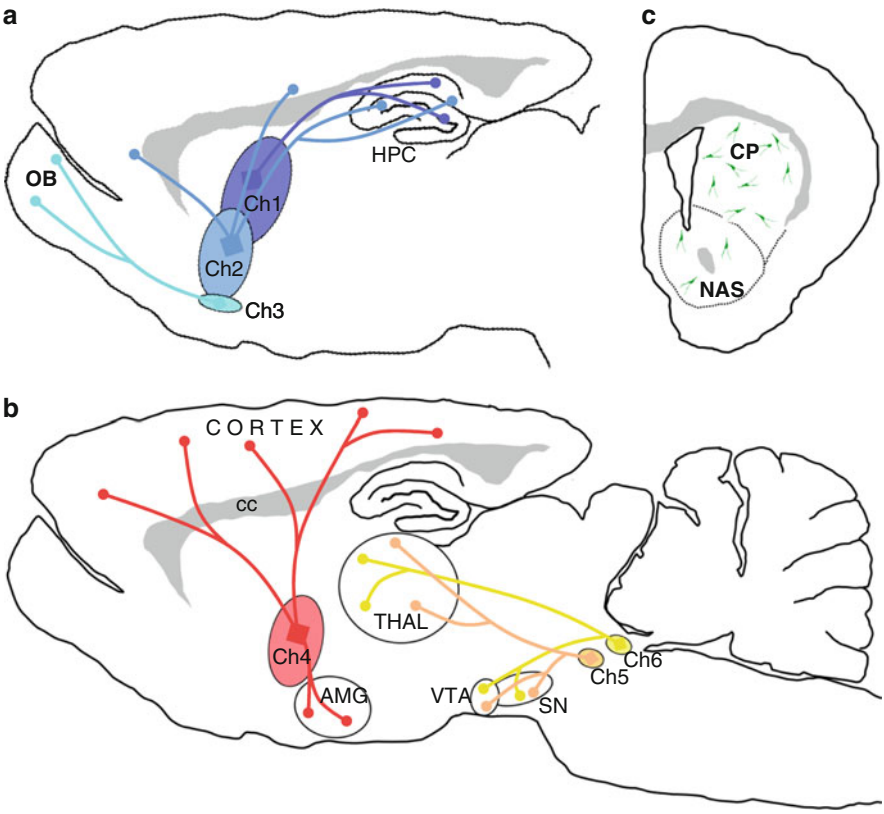

Fig. 1 Schematic diagram illustrating the location of the cholinergic cell groups of the rat brain and their projections. (**a**) Sagittal view showing Ch1 (medial septum), Ch2 (vertical limb of the diagonal band of Broca [DBB]), and Ch3 (horizontal limb of the DBB) and their projections to the hippocampal formation, cerebral cortex, and olfactory bulb. (**b**) Sagittal view depicting Ch4 (nucleus basalis magnocellularis) and its projections throughout the cortex and amygdala, as well as Ch5 (pedunculopontine tegmental nucleus) and Ch6 (laterodorsal tegmental nucleus) innervating the thalamus, substantia nigra, and ventral tegmental area. (**c**) Coronal section through the striatal complex showing large cholinergic interneurons in the dorsal striatum and nucleus accumbens. Drawings are based on the work of Kimura et al. (1980), Mesulam et al. (1983), Eckenstein et al. (1988), and Gould et al. (1989). *Ch1–Ch6* cholinergic cell groups; *AMG* amygdala; *cc* corpus callosum; *CP* caudate-putamen (striatum); *HPC* hippocampus; *NAS* nucleus accumbens; *OB* olfactory bulb; *THAL* thalamus; *SN* substantia nigra; *VTA* ventral tegmental area

cholinergic projection neurons, termed Ch1–Ch6, can be distinguished based on their localization and projection pattern (Fig. 1). Cell groups Ch1–Ch4, located in the basal forebrain of the rat, are thought to be involved in attention, learning, and memory functions (Everitt and Robbins 1997). The cholinergic neurons of the nucleus basalis magnocellularis (Ch4), which in primates is known as the nucleus basalis of Meynert (NBM), provide wide-spread cholinergic projections throughout most of the cerebral cortex, and degeneration of these neurons is a hallmark of AD (McGeer et al. 1986). In addition, the Ch4 cells innervate the amygdaloid complex

(Mesulam et al. 1983; Price and Stern 1983). Cholinergic neurons in the medial septum (Ch1) and the vertical limb of the diagonal band of Broca (Ch2) send projections to the hippocampal formation and to the medial aspects of the cortex, such as the cingulate and retrosplenial cortices (Eckenstein et al. 1988). The olfactory bulb is the recipient of cholinergic projections from the Ch3 cell group, located in the horizontal limb of the diagonal band of Broca. The cholinergic projection neurons of the caudal midbrain, which are involved in arousal, sleep, and the regulation of dopaminergic cell groups (Datta and Siwek 1997), are located in the pedunculopontine tegmental nucleus (PPTg, Ch5) and the laterodorsal tegmental nucleus (LDTg, Ch6), from where they project to the thalamus , the pontine reticular formation, and areas of the ventral midbrain (Mesulam et al. 1983; Satoh and Fibiger 1986; Clarke et al. 1987; Hallanger et al. 1987; Semba et al. 1990). The parcellation scheme developed by Mesulam and colleagues (1983) has proven to be invaluable for conceptualizing the various aspects of cholinergic function. However, the analysis of forebrain cholinergic function is complicated by the fact that non-cholinergic projection neurons are embedded in the cholinergic cell groups (Woolf et al. 1986). Therefore, results from lesion studies targeting the cholinergic basal forebrain need to be interpreted carefully (see Robbins et al. 1989a, b).

2.2 Regional Distribution of Cholinergic Axons

Dense cholinergic fiber plexus originating from the basal forebrain are seen throughout neo- and allocortical areas. The laminar distribution of cholinergic fibers varies slightly across cortical areas, but layer V generally receives the most dense cholinergic fiber innervation (Eckenstein et al. 1988; Mechawar et al. 2000). The cholinergic innervation of the hippocampus is most prolific at the border between stratum oriens and pyramidal layer and in the molecular layer, while the densely packed pyramidal and granule cell layers themselves receive very little cholinergic input (Ichikawa and Hirata 1986; Schäfer et al. 1998). Cholinergic fiber density varies across the nuclei of the amydaloid complex; the most densely innervated area is the basolateral nucleus (Hellendall et al. 1986). In subcortical areas, moderate cholinergic innervations are seen in select thalamic nuclei, including the anteroventral, centromedial, and reticular nuclei (Gonzalo-Ruiz et al. 1995; Schäfer et al. 1998), and in the midbrain dopamine cell groups (Gould et al. 1989; Oakman et al. 1995; Omelchenko and Sesack 2006).

2.3 Striatal Cholinergic Interneurons

The striatal complex, including the nucleus accumbens, does not receive any extrinsic cholinergic innervation, but instead contains cholinergic interneurons as the sole source of ACh. These cholinergic interneurons are scattered throughout the striatal matrix compartment, but are largely absent from striatal patches (Gerfen and Bolam 2010). Although large cholinergic interneurons make up less than five

percent of striatal neurons, their wide dendritic arbors enable them to exert control over a large striatal area (Kimura et al. 1980; Bolam et al. 1984; Phelps et al. 1985).

3 Muscarinic Receptor Distribution

For the purpose of this chapter, we will focus on the well-established distribution of the five mAChR subtypes in the rodent brain. Our description of the distribution of mAChRs will be limited to select brain regions that are thought to be involved in cognition and that either contain cholinergic neurons or receive cholinergic innervations. These areas include the cerebral cortex, hippocampus, thalamus, the basal ganglia, and basal forebrain and caudal midbrain cholinergic cell groups.

3.1 Expression of Muscarinic Receptor Message

Distribution maps of M1–M5 mAChR mRNA, obtained by in situ hybridization histochemistry, show that mAChRs are expressed throughout the rodent brain, albeit not uniformly (Fig. 2). There are pronounced differences in the overall expression levels of the five muscarinic receptors, with M1 and M5 receptors being the most and least abundant receptor subtype, respectively. Moreover, each muscarinic receptor exhibits a regional expression pattern that is strikingly different from other members of the muscarinic receptor family (Brann et al. 1988).

The M1 receptor is not only most prominently expressed in the hippocampus, but is also abundant throughout all layers of the cortex, where the superficial layers stand out by being more intensely labeled than the remaining layers. Striatal medium spiny neurons as well as interneurons also express high levels of M1 message (Bernard et al. 1992); caudal to the striatum, subcortical M1 expression decreases along a rostro-caudal gradient from the diencephalon to the midbrain. Moderately high M2 receptor expression is found mainly in the brain regions containing cholinergic cell bodies (Vilaró et al. 1992) as well as in some thalamic nuclei including the midline, parafascicular, and reticular nuclei. In the hippocampus and cortex, M2 message is sparse; in cortical layer IV, it is completely absent. The M3 receptor is mainly expressed in the hippocampus and in the cortex, except for layers III and IV which are mostly devoid of M3 message. Very low levels of M3 mRNA are seen in the striatum and basal forebrain (Brann et al. 1988). The highest density of M4 receptors is found in the striatal complex (Vilaró et al. 1991), followed by allocortical areas, such as the hippocampus and amygdala. Expression of M4 message is relatively high in all layers of the neocortex; like M2, M4 receptor message is prominently expressed in central cholinergic neurons (Sugaya et al. 1997). The muscarinic receptor with the most restricted expression is M5. It is found in low abundance in the ventral tegmental area and the pars compacta of the substantia nigra (Vilaró et al. 1990).

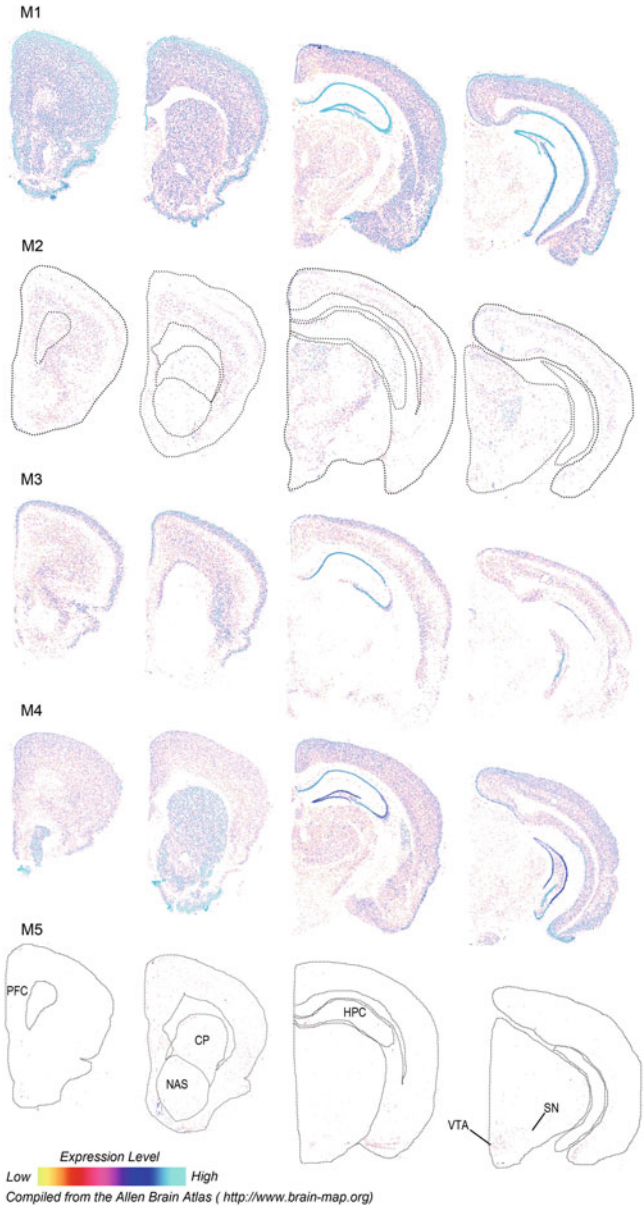

Fig. 2 Distribution of M1–M5 muscarinic receptor mRNA in the mouse brain. This is a composite of images obtained from the *Allen Mouse Brain Atlas* (2009) developed by the Allen Institute for Brain Science (Lein et al. 2007) and available online at http://mouse.brain-map.org. *CP* caudate-putamen; *HPC* hippocampus; *NAS* nucleus accumbens; *PFC* prefrontal cortex; *SN* substantia nigra; *VTA* ventral tegmental area

3.2 Muscarinic Receptor Protein Expression

The global distribution of muscarinic receptor protein was initially assessed using a monoclonal (M35) pan-muscarinic antibody (van der Zee et al. 1989; for review, see van der Zee and Keijser 2011). With the development of subtype-selective muscarinic receptor antibodies, it became feasible to quantitate levels of receptor protein in microdissected brain regions (Li et al. 1991; Wall et al. 1991; Yasuda et al. 1993) and to determine both the cell types expressing certain mAChR subtypes and the (sub)cellular localization of mAChRs at the light and electron microscopic level (Levey et al. 1991; Hersch et al. 1994; Hersch and Levey 1995). Immunohistochemical studies demonstrated that M1–M5 protein distribution corresponds to a large degree with the mRNA expression maps indicating receptor expression at the soma and dendritic level. Furthermore, they revealed that muscarinic receptor proteins were prominently expressed presynaptically as both autoreceptors and heteroceptors (Table 1).

3.2.1 Cortex

M1, M2, and M4 are the most abundant muscarinic receptor proteins in the cortex (Levey et al. 1991). M1 protein, expressed in pyramidal cells, is enriched in layers II/III and VI, whereas M4 is localized in somata of layer II–IV cells. Terminals located in layer IV and at the border between layers V and VI exhibit strong M2 labeling, which is in agreement with the dense cholinergic innervation of these cortical layers and the role of M2 as autoreceptor (Eckenstein et al. 1988; Mechawar et al. 2000).

3.2.2 Hippocampus

The complexity of hippocampal cholinergic circuitry is illuminated by the diverse pre- and postsynaptic distribution of mAChRs, suggesting an intricate muscarinic regulation of hippocampal function. Both intrinsic neurons (pyramidal neurons, granule cells, and interneurons) and terminals originating from basal forebrain and entorhinal cortex prominently express M1–M4 receptors (see Table 1) (Levey et al. 1995b; Rouse and Levey 1996, 1997, 1998; Rouse et al. 1999, 2000).

3.2.3 Amygdala

Pyramidal neurons in the basolateral amygdala, a limbic region involved in learning and expression of fear conditioning, prominently express M1 protein (McDonald and Mascagni 2010).

Table 1 Distribution of muscarinic receptor protein in brain circuits involved in cognition

Subtype	Pre-/post-synaptic	Cerebral cortex	Hippo-campus/entorhinal cortex	Amygdala	Striatum/nucleus accumbens	Basal forebrain	Thalamus
M1	Pre				Excitatory afferents[4]		
	Post	Pyramidal neurons[7,19]	Granule cells, hilar cells, pyramidal cells[10,16]	Pyramidal neurons[11]	Majority of medium spiny neurons[4]	GABAergic cells[2]	
M2	Pre		EC projections to granule cells (medial preforant path)[10]; septohippocampal cholinergic afferents[10,18]; non-cholinergic afferents[10,18]		Asymmetric synapses (excitatory afferents)[4,5] cholinergic and non-cholinergic terminals[4,5]	Unidentified afferents[9]	Unidentified afferents[12]
	Post		Parvalbumin cells[1] Cajal-Retzius cells in EC[1] interneurons[3] and non-pyramidal hippocampal neurons[10]		Aspiny (cholinergic) interneurons[4,5]	GABAergic[2], cholinergic[2,9] and septohippocampal neurons[15]	Antero-dorsal/ventral[12]; parvalbumin neurons of reticular nucleus[13,14]
M3	Pre		EC projections to granule cells (medial and lateral perforant path)[17]		Asymmetric synapses (excitatory afferents)[4,5]		
	Post	Throughout cortex, mainly limbic cortex[8]	Granule, hilar, and pyramidal cells[10,16] interneurons[8]		Some medium spiny neurons[4]	Basal forebrain cells[15]	Antero-dorsal/ventral[8,12]; parvalbumin neurons of reticular nucleus[13]
M4	Pre		GABA afferents (basal forebrain)[10]; EC projections to granule cells (medial perforant path)[17] hilar cell projections[17]		Excitatory striatal afferents[4]		
	Post	Layer IV[7]			Medium spiny D1 dopamine receptor neurons[4,6]		

EC entorhinal cortex; *GABA* γ-aminobutyric acid

[1]Chaudhuri et al. (2005); [2]González et al. (2007); [3]Hájos et al. (1998); [4]Hersch et al. (1994); [5]Hersch and Levey (1995); [6]Ince et al. (1997); [7]Levey et al. (1991); [8]Levey et al. (1994); [9]Levey et al. (1995a); [10]Levey et al. (1995b); [11]McDonald and Mascagni (2010); [12]Oda et al. (2001); [13]Oda et al. (2007); [14]Plummer et al. (1999); [15]Rouse and Levey (1996); [16]Rouse and Levey (1997); [17]Rouse et al. (1999); [18]Rouse et al. (2000); [19]Yamasaki et al. (2010)

3.2.4 Striatum

Approximately eighty percent and close to half of medium spiny neurons, the principal cell type in the striatum, express M1 and M4 receptor proteins, respectively (Hersch et al. 1994). Interestingly, the M4 receptor is mainly localized to the medium spiny neurons projecting to the substantia nigra reticulata (Ince et al. 1997), making M4 an interesting target to alter striatal output pathways differentially. In contrast, M2 protein is mainly expressed in striatal cholinergic interneurons, where the M2 receptor subserves the function of an autoreceptor (Hersch et al. 1994; Hersch and Levey 1995). Presynaptically located M1–M3 receptor proteins are thought to be localized to corticostriatal (M1/M3) and thalamostriatal (M2/M3) terminals (Hersch et al. 1994). Overall, the high expression of mAChRs in the striatum suggests that muscarinic ligand may be useful for modifying striatum-mediated learning processes, in particular procedural learning (Saint-Cyr et al. 1988; Cayzac et al. 2011).

3.2.5 Thalamus

Expression of mAChR proteins in the thalamus is restricted to M1 and M3 in the anterodorsal and -ventral nuclei and to M2 in the reticular nucleus (Oda et al. 2001, 2007). The thalamus as an important relay station to the cortex and striatal complex may, therefore, be subject to muscarinic regulation via M1 and/or M3 mechanisms. The presence of M2 in the reticular nucleus, whose GABAergic projections inhibit thalamic relay nuclei, suggests that M2 may play a role in global control of thalamic output (Cox et al. 1997; Pinault and Deschênes 1998).

3.2.6 Cholinergic Neurons

In the basal forebrain and other cholinergic cell groups, the principal mucarinic receptor protein is M2, which is located both in cholinergic cell bodies and in unidentified axon terminals (Levey et al. 1995a).

4 Role of Muscarinic Receptor Subtypes in Cognition

4.1 Findings with mAChR Antagonists and KO Mice

Based on an extensive literature, nonselective mAChR antagonists, such as scopolamine, disrupt multiple domains of cognitive function, from sensory information gating, attention, and memory to higher problem-solving skills in rodents, monkeys, and humans, as shown in Table 3; also see chemical structures of

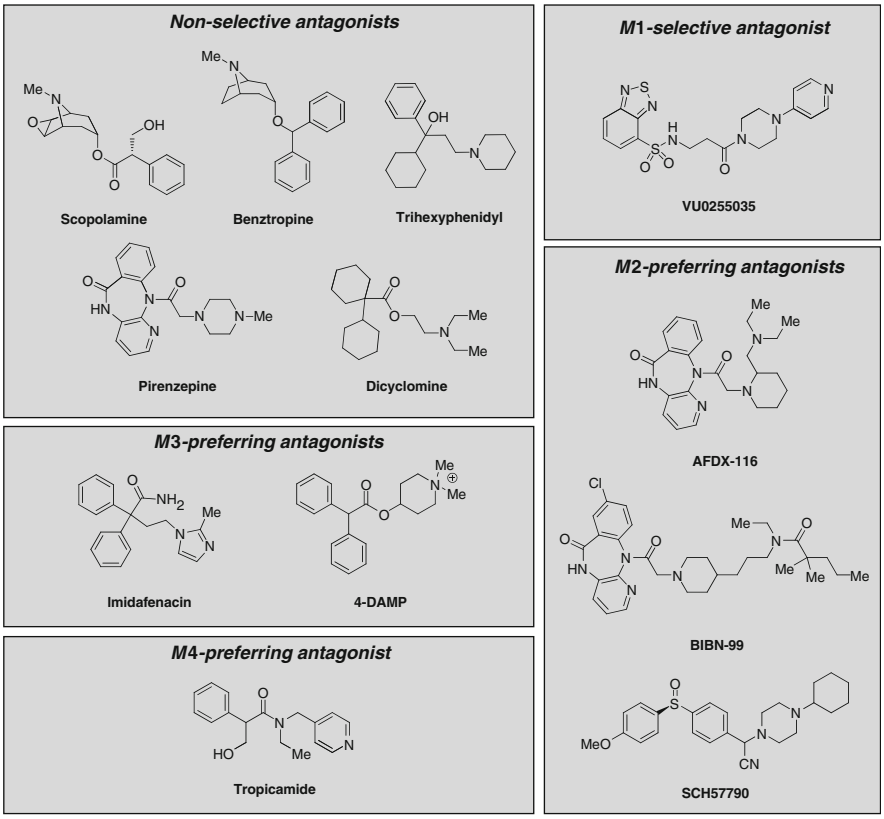

Fig. 3 Chemical structures of representative muscarinic receptor antagonists

representative mAChR antagonists and their in vitro affinities for the different mAChR subtypes in Fig. 3 and Table 2, respectively (see Terry et al. 2006; Barak 2009; Klinkenberg and Blokland 2010 for complete reviews). For example, scopolamine, trihexyphenidyl, and benztropine produced robust dose-dependent disruptions of prepulse inhibition (PPI) of the acoustic startle reflex, a model of sensory information processing, at doses that had no effects on startle response (Jones and Shannon 2000). Scopolamine markedly decreased accuracy and/or response rates in the 5-choice serial reaction time task, a preclinical model of attentional functions used to test rats and monkeys (Jäkälä et al. 1992; Callahan et al. 1993; Jones and Higgins 1995; Higgs et al. 2000; Mirza and Stolerman 2000; Shannon and Love 2005, 2006; Shannon and Eberle 2006; Spinelli et al. 2006). In addition, scopolamine induced impairments in attention in humans, including in the attentional components of the CogState Early Phase Battery and in the digit vigilance test (Ellis et al. 2006; Fredrickson et al. 2008). With regard to learning and memory, muscarinic antagonism with scopolamine produced robust deficits in performance accuracy in numerous memory-related behavioral tasks in rodents and

Table 2 Receptor affinities [nM]of orthosteric muscarinic receptor antagonists

Drug	Receptor					Ligand	Species	References
	M1	*M2*	*M3*	*M4*	*M5*			
Non-selective								
Scopolamine	1.1	2	0.4	0.80	2.07	[^{3}H]-QNB	Human[a]	Bolden et al. (1992)
Benztropine	0.2	1.4	1.1	1.10	2.8	[^{3}H]-QNB	Human[a]	Bolden et al. (1992)
	–	244.0	415.0	97.00	53	[^{3}H]-NMS	Human[a]	
Dicyclomine	57.0	–	–	–	–	[^{3}H]-NMS	Rat[a]	Buckley et al. (1989)
	–	244.0	415.0	97.00	53	[^{3}H]-NMS	Human[a]	
Pirenzepine	8.0	270.0	150.0	28.00	170	[^{3}H]-NMS	Human[a]	Bolden et al. (1992)
Trihexyphenidyl	1.6	7	6.4	2.60	15.9	[^{3}H]-NMS	Human[a]	Bolden et al. (1992)
M1-selective								
VU0255035	–	661.0	876.9	–	2362.3	[^{3}H]-NMS	Human[a]	Sheffler et al. (2009)
	14.9	–	–	1177.7	–	[^{3}H]-NMS	Rat[a]	Sheffler et al. (2009)
M2-preferring								
AFDX-116	776	105.0	1,660	447.0	4,571	[^{3}H]-NMS	Human[a]	Doods et al. (1993)
BIBN-99	1,072	30.0	776.0	174.00	1,445	[^{3}H]-NMS	Human[a]	Doods et al. (1993)
SCH57790	112	2.8	29.0	14.00	309	[^{3}H]-QNB	Human[a]	Lachowicz et al. (1999)
M3-preferring								
Imidafenacin[b,c]	–	4.1[d]	0.3[e]	–	–	–	gp	Miyachi et al. (1999)
4-DAMP	0.6	3.8	0.5	1.17	1.05	[^{3}H]-NMS	Human[e]	Dörje et al. (1991)
M4-preferring								
Tropicamide	66.0	50.0[d]	38.0[f]	–	–	[^{3}H]-NMS	Rat	Lazareno et al. (1990)
	–	–	–	14.00[g]	–	[^{3}H]-PIR	Rabbit	Lazareno et al. (1990)

gp guinea pig; [^{3}H]-NMS, [^{3}H]-*N*-methylscopolamine; [^{3}H]-PIR, [^{3}H]-pirenzepine; [^{3}H]-QNB, [^{3}H]-quinuclinidyl benzylate

[b]KRP 197; ONO 8025

[c]EC_{50} for inhibiting agonist-induced effects on target organ

[d]Heart

[e]Gut

[f]Submandibular gland

[g]Lung

monkeys, including spatial memory tasks such as the Morris water maze and radial arm maze, classic Pavlovian conditioned responding, delayed non-matching to sample, and object recognition tasks (Buresová et al. 1986; Riekkinen et al. 1990; Dennes and Barnes 1993; Anagnostaras et al. 1995, 1999; Rudy 1996; Mishima et al. 2000; Feiro and Gould 2005; Betz et al. 2007; Sheffler et al. 2009; Dietrich and Jenck 2010). In humans, scopolamine decreased performance accuracy in measures of visual and verbal learning and item recognition memory tasks (Sherman et al. 2003; Green et al. 2005; Fredrickson et al. 2008; Thienel et al. 2009). Scopolamine has also been reported to produce impairments in executive functions, including attentional set-shifting in rats and Groton maze learning in humans (Chen et al. 2004; Fredrickson et al. 2008). In review of the dose-related disrupting effects of scopolamine and other nonselective mAChR antagonists, the interpretation of these effects are clearest in measures of sensory discrimination and

attentional function, in which deficits are observed within a dose range that does not produce confounding effects on general motor output and/or levels of arousal as observed in models of learning and memory.

Recent findings from studies using either mAChR KO mice or antagonists are providing more defined roles for each of the mAChR subtypes in the modulation of cognition. In the case of M1 mAChRs, this particular subtype regulates a variety of physiologic effects in hippocampal and cortical brain regions, most notably enhancement of glutamatergic signaling through potentiation of *N*-methyl-D-aspartate (NMDA) receptor function (Marino et al. 1998). Modulation of NMDA receptor neurotransmission is key for the acquisition and consolidation of new learning and memories; and its disruption is speculated to account, at least in part, for the cognitive impairments observed in many neurological and psychiatric disorders (Marino and Conn 2002; Tsai and Coyle, 2002). Consistent with a role of M1 in learning and memory, the M1-preferring mAChR antagonist pirenzepine impaired accuracy and/or acquisition in tasks of passive avoidance, Morris water maze, and visual discrimination in rats (Fig. 3, Tables 2 and 3) (Hunter and Roberts 1988; Drinkenburg et al. 1995). Moreover, M1 mAChR KO mice have reduced long-term potentiation in response to theta burst stimulation, a physiologic endpoint thought to be procognitive in nature (Anagnostaras et al. 2003). In contrast to the effects of nonselective mAChR antagonists, M1 KO mice have shown normal performance in hippocampus-mediated tasks, including in the Morris water maze task with or without scopolamine challenge (Miyakawa et al. 2001), but distinct impairments in behavioral tasks that require medial prefrontal cortex (mPFC) function (Anagnostaras et al. 2003). For example, M1 KO mice relative to wild-type (WT) controls showed pronounced performance deficits in non-matching-to-sample tasks, including win-shift radial arm maze learning and social discrimination tests (Anagnostaras et al. 2003). Despite significant enhancement in the acquisition of contextual fear conditioning, M1 KO mice performed poorly after a time period when the task becomes independent of hippocampal function (Anagnostaras et al. 2003). In support of these findings, the highly selective M1 mAChR antagonist VU0255035 (see Fig. 3, Tables 2 and 3) had no effect on acquisition of contextual fear conditioning, a hippocampus mediated memory task (Sheffler et al. 2009). Taken together, these studies indicate a consistent role for M1 mAChR in the modulation of mPFC-mediated tasks, but future studies using the selective M1 mAChR antagonist VU0255035 are needed to further evaluate the effects of selective disruption of M1 activity in other cognitive functions.

For the role of M2 in cognition, previous studies have postulated that selective M2 mAChR antagonists may provide improvements in the cognitive deficits observed in dementia patients by increasing cholinergic signaling through antagonism of M2 mAChRs on presynaptic cholinergic terminals (Rouse et al. 2000; Zhang et al. 2002; Tzavara et al. 2003). Consistent with this hypothesis, the selective M2 mAChR antagonists, BIBN-99 and SCH57790 (see Fig. 3, Tables 2 and 3) improved performance in the passive avoidance and Morris water maze tasks in normal and aged rats, and in fixed ratio discrimination in monkeys (Table 3) (Quirion et al. 1995; Carey et al. 2001; Rowe et al. 2003). However, M2 mAChRs

Table 3 Effects of muscarinic receptor antagonists on cognition

Domain	Model	Compound	Mechanism	Dose/route (mg/kg)	Species	Effect	References
Gating	Prepulse inhibition of the acoustic startle reflex	Scopolamine	Non-selective	0.3–1.0 S.C.	Rat	Decreased (impaired)	Wu et al. (1993); Jones and Shannon (2000); Sipos et al. (2001); Ukai et al. (2004)
		Scopolamine	Non-selective	0.32–1.8 I.P.	Mouse		Thomsen et al. (2010)
		Trihexyphenidyl	Non-selective	0.3–10 S.C.	Rat		Jones and Shannon (2000)
		Benztropine	Non-selective	0.03–10 S.C.	Rat		Jones and Shannon (2000)
		4-DAMP	M3-preferring	0.03 I.C.V.	Mouse		Ukai et al. (2004)
		Tropicamide	M4-preferring	0.0001 I.C.V.	Mouse		Ukai et al. (2004)
		Pirenzepine	Non-selective	0.0001–0.01 I.C.V.	Mouse	No effect	Ukai et al. (2004)
		AFDX-116	M2-preferring	0.0001–0.01 I.C.V.	Mouse		Ukai et al. (2004)
Attention	Five-choice serial reaction time task	Scopolamine	Non-selective	0.003–0.3 S.C., I.P.	Rat	Impaired performance (decreased accuracy)	Jäkälä et al. (1992); Jones and Higgins (1995); Higgs et al. (2000); Mirza and Stolerman (2000); Shannon and Love (2005); Shannon and Eberle (2006)
		Scopolamine	Non-selective	0.000004–0.04/h I.C.V.	Monkey	Decreased number of responses with no effect on accuracy	Callahan et al. (1993)
		Scopolamine	Non-selective	0.01–0.02 S.C.	Monkey		Spinelli et al. (2006)
	Sustained attention	Scopolamine	Non-selective	0.02–0.2 S.C.	Rat	Impaired performance	Skjoldager and Fowler (1991); Bushnell et al. (1997)
		Scopolamine	Non-selective	0.05–0.2 S.C.	Mouse		Dillon et al. (2009)
		Scopolamine	Non-selective	0.1–0.2 I.P.	Rat	Ameliorated haloperidol-induced reaction time slowing	Brockel and Fowler (1995)
		Benztropine	Non-selective	1.0–6.0 I.P.	Rat		Brockel and Fowler (1995)
	Attention (CogState Early Phase battery)	Scopolamine	Non-selective	0.2–0.6 S.C.	Human	Impaired performance	Fredrickson et al. (2008)
	Sustained visual attention	Scopolamine	Non-selective	0.4 I.M.	Human		Ellis et al. (2006)

(continued)

Table 3 (continued)

Domain	Model	Compound	Mechanism	Dose/route (mg/kg)	Species	Effect	References
	(digit vigilance test)						
Learning and memory	Visuopatial delayed non-matching to sample	Tropicamide	M4-preferring	1.25–20 I.P.	Rat	Decreased accuracy	Betz et al. (2007)
	Radial arm maze	Scopolamine	Non-selective	0.5 I.P., 0.02 I.C.V.	Rat	Impaired performance	Mishima et al. (2000)
		Scopolamine	Non-selective	0.03–0.1 S.C.	Rat		Dennes and Barnes (1993)
	Spontaneous alternation	Scopolamine	Non-selective	0.1–1.0 I.P.	Rat	Decreased alternation	Squire (1969)
	Spatial alternation	Scopolamine	Non-selective	0.01–2.0 S.C.	Rat		Bymaster et al. (1993); Means et al. (1996)
		Pirenzepine	Non-selective	0.3–3.0 S.C.	Rat		Bymaster et al. (1993)
		Trihexyphenidyl	Non-selective	3–30 S.C.	Rat		Bymaster et al. (1993)
	Delayed spatial alternation	Scopolamine	Non-selective	0.01–1.0 I.P., S.C.	Rat		Dudchenko and Sarter (1992); Baron et al. (1998)
	Spatial delayed response	Scopolamine	Non-selective	0.01–0.03 I.M.	Monkey	Reduced accuracy	Rupniak et al. (1991)
	Morris water maze	Scopolamine	Non-selective	0.1–1.0 I.P., S.C.	Rat	Impaired acquisition and retention	Buresová et al. (1986); Riekkinen et al. (1990); Dietrich and Jenck (2010)
		Pirenzepine	Non-selective	0.01–0.03 I.C.V.	Rat	Impaired acquisition	Hunter and Roberts (1988)
		BIBN-99	M2-preferring	0.5 S.C	Rat	Improved performance in aged rats	Quirion et al. (1995); Rowe et al. (2003)
		Imidafenacin	M3-preferring	1.0–10		No effect	Kobayashi et al. (2007)
	Passive avoidance	Pirenzepine	Non-selective	0.001 I.C.V.	Rat	Impaired performance	Suzuki et al. (1995)
		Scopolamine	Non-selective	0.75 S.C.	Rat		Pitsikas et al. (2001)
		SCH57790	M2-preferring	0.003–3.0 P.O.	Rat	Improved performance (increased latency)	Carey et al. (2001)
	Novel object recognition test	Scopolamine	Non-selective	0.1–0.75 I.P., S.C.	Rat	Impaired performance (decreased recognition)	Ennaceur and Meliani (1992); Besheer et al. (2001); Warburton et al. (2003); Myhrer et al. (2004)
		Scopolamine	Non-selective	0.3–3.0 S.C.	Mouse		Dodart et al. (1997)
		Scopolamine	Non-selective	0.1–100 I.P., S.C.	Rat	Impaired	

	Contextual fear conditioning						Anagnostaras et al. (1995, 1999); Rudy (1996); Sheffler et al. (2009)
		Dicyclomine	M1 > M2-M5	2.0–64.0 I.P.	Rat		Fornari et al. (2000)
		Scopolamine	Non-selective	0.1–1.0 I.P.	Mouse		Feiro and Gould 2005); Ma et al. (2009)
		VU0255035	M1	3.0–10.0 I.P.	Rat	No effect	Sheffler et al. (2009)
	Cue fear conditioning	Scopolamine	Non-selective	0.1–100 I.P., S.C.	Rat	Impaired	Rudy (1996); Anagnostaras et al. (1999)
		Scopolamine	Non-selective	0.1–1.0 I.P.	Mouse		Feiro and Gould (2005)
		Dicyclomine	M1> M2-M5	2.0–64.0 I.P.	Rat	No effect	Fornari et al. (2000)
	Visual		discrimination	Scopolamine	Non-selective	0.125–0.8 S.C., I.P.	Rat
		Decreased accuracy, increased response latency and omissions	Leaton and Kreindler (1972); Drinkenburg et al. (1995)				
			Pirenzepine	Non-selective	0.0032–0.032 I.C.V.	Rat	Drinkenburg et al. (1995)
			AFDX 116	M2-pref	0.0032–0.032 I.C.V.	Rat	Drinkenburg et al. (1995)
	Scopolamine	Non-selective	0.2–0.625 I.P.	Rat	Impaired responding, but not accuracy	Andrews et al. (1992)	
	Delayed visual		discrimination	Scopolamine	Non-selective	0.02–1.0 I.P.	Rat
	Decreased accuracy	Andrews et al. (1992)					
		Visual learning (CogState Early Phase battery)	Scopolamine	Non-selective	0.2–0.6 S.C.	Human	Fredrickson et al. (2008)
		Fixed ratio	discrimination	Scopolamine	Non-selective	0.001–0.18 I.M. Moerschbaecher (1993)	Monkey
Pakarinen and SCH57790	M2-pref	0.01–0.03 P.O.	Monkey				

(continued)

Table 3 (continued)

Domain	Model	Compound	Mechanism	Dose/route (mg/kg)	Species	Effect	References
				Improved performance	Carey et al. (2001)		
Conditional discrimination				Scopolamine	Non-selective	0.01–1.0 I.P.	Monkey
Decreased response rate and accuracy	Savage et al. (1996)						
	Operant conditioning: differential reinforcement of low rates	Scopolamine	Non-selective	0.01–0.056 I.M.	Monkey	Decreased responses and earned rewards	McDonough (1982)
	Verbal learning memory	Scopolamine	Non-selective	0.4 I.V.	Human	Impaired	Thienel et al. (2009)
	Item recognition memory	Scopolamine	Non-selective	0.4 I.V.	Human	Impaired	Sherman et al. (2003)
	Spatial and object n-back tests	Scopolamine	Non-selective	0.4 I.M.	Human	Impaired	Green et al. (2005)
Executive function	Set-shifting	Scopolamine	Non-selective	0.10–0.25 I.P.	Rat	Impaired reversal of intradimensional shift	Chen et al. (2004)
	Groton maze learning test	Scopolamine	Non-selective	0.2–0.6 S.C.	Human	Reduced accuracy	Fredrickson et al. (2008)

also function as heteroceptors localized on the axon terminals of non-cholinergic neurons that mediate presynaptic regulation of release of other neurotransmitters (Rouse et al. 2000). Not surprisingly, M2 mAChR KO mice have shown deficits in tasks of working memory and cognitive flexibility, as well as hippocampal long-term potentiation, suggesting that blockade of M2 mAChRs on both cholinergic and non-cholinergic nerve terminals may disrupt, not enhance, overall cognitive function (Tzavara et al. 2003; Seeger et al. 2004). Consistent with the M2 KO mouse cognitive phenotype AFDX116, another selective M2 mAChR antagonist decreased accuracy and increased response latencies and omissions in a rodent visual discrimination task (see Fig. 3, Tables 2 and 3) (Drinkenburg et al. 1995). Thus, more detailed studies with M2 mAChR antagonists are needed to further understand the full therapeutic potential of M2 mAChR antagonists for the treatment of clinical populations with varying levels of cholinergic tone.

To date, the relative importance of the M3 mAChR in modulating different aspects of cognitive function remains undefined. M3 mAChR KO mice have shown robust impairments in contextual fear conditioning, a classic hippocampus-mediated memory task (Poulin et al. 2010). However, there are currently no selective M3 mAChR antagonists reported in the literature, and the M3-preferring antagonist imidafenacin had no effect on performance in the Morris water maze, another hippocampus-mediated memory task (Kobayashi et al. 2007) (see Fig. 3, Tables 2 and 3). Whether selective M3 mAChR activators may have procognitive properties remains unclear as does the issue whether a viable therapeutic index could be achieved between activation of central and peripheral M3 mAChRs.

The significance of M4 mAChRs in cognitive functions remains unclear because of the pre- and postsynaptic localization of M4 mAChRs within the CNS (Levey et al. 1991; Zang and Creese 1997; Zhang et al. 2002; Tzavara et al. 2004). Previous in vivo microdialysis studies have shown significant increases in basal midbrain extracellular ACh concentrations in M4, but not M2 mAChR KO mice (Tzavara et al. 2004). Moreover, scopolamine-induced increases in midbrain extracellular ACh concentrations were dampened in the M4 mAChR KO mice (Tzavara et al. 2004). M4 mAChR KO mice also displayed increased DA efflux in response to psychotomimetics (Tzavara et al. 2004). These findings suggest that activation of M4 mAChRs may provide feedback control on basal and evoked DA release in the striatum. The tight regulation of striatal DA and ACh neurotransmission by M4 mAChRs may be critical for cognitive functions, such as procedural learning and effort-based decision making, tasks that require striatal involvement. Interestingly, the M4-preferring mAChR antagonist tropicamide disrupted PPI of the acoustic startle reflex, a task that is dependent on proper mesolimbic DA neurotransmission (Ukai et al. 2004) (Fig. 3, Tables 2 and 3). Tropicamide administration also resulted in decreased accuracy in a visuospatial delayed non-matching-to-sample task in rats (Betz et al. 2007). Studies using selective M4 mAChR agonists and antagonists need to further dissect the role of M4 mAChRs in other aspects of cognition, as will be discussed in the allosteric modulator section of this chapter.

With the expression of M5 mAChRs limited to the VTA and substantia nigra pars compacta, it is not surprising that preliminary studies with M5 mAChR KO

mice have reported disruptions in the proper regulation of dopaminec-mediated behavioral tasks (Vilaró et al. 1990; Weiner et al. 1990). In particular, M5 mAChR KO mice have impaired PPI (Thomsen et al. 2007) and reduced sensitivity to the effects of different drugs of abuse (Basile et al. 2002; Fink-Jensen et al. 2003; Yamada et al. 2003; Thomsen et al. 2005; Steidl and Yeomans 2009). While there are currently no available selective M5 mAChR antagonists, the studies with M5 mAChR KO mice suggest that selective blockade of M5 mAChRs might be useful for regulating the hyperactivation of mesolimbic dopaminergic circuitry in patients with schizophrenia. Moreover, the proper function of nonneuronal M5 mAChRs expressed in the cerebrovasculature that control cerebrovasodilation and blood flow may also indirectly impact cognitive functions (Yamada et al. 2001; Araya et al. 2006). Vascular pathology has been implicated in AD, and dysfunction in cholinergic control of cerebral blood vessel dilation may contribute, in part, to the pathophysiology of this disease. Cerebrovascular deficits in M5 mAChR KO mice are associated with neuronal atrophy and deficits in performance of the novel object recognition task (Araya et al. 2006), which further support the role of M5 mAChRs in the modulation of cognitive function through nonneuronal mechanisms.

4.2 Findings with mAChR Orthosteric Agonists

Over the last 2 decades, the drive to improve cognitive impairments in patient populations with AD and other dementias has resulted in the development of two major pharmacologic approaches that modulate mACh neurotransmission, specifically indirect modulation through the enhancement of general cholinergic tone with AChEIs and direct modulation by mAChR orthosteric agonists. To date, only the AChEIs tacrine, donepezil, galantamine, and rivastigmine are clinically approved for the treatment of cognitive impairments associated with mild-to-moderate AD. While AChEIs can improve cognitive deficits in dementia patients, their therapeutic benefits are limited by a short duration of action, dose-limiting side effects, relatively modest efficacy on memory deficits, and a large population of non-responders (Pepeu and Giovannini 2010; Birks 2006; Birks and Flicker 2006; Persson et al. 2009; Hasselmo 2006; Barten and Albright 2008).

As an alternative to the limited clinical utility of AChEIs, considerable efforts have been focused on the development of highly selective mAChR orthosteric agonists for the treatment of cognitive impairments in AD; representative chemical structures for each compound are depicted in Fig. 4 with their in vitro binding affinities at each mAChR subtype described in Table 4 and highlighted efficacy in different cognitive tasks shown in Table 5. All of the mAChR agonists presented in Table 4, including the reported M1-preferring agonist WAY-132983 and the M1/M4-preferring mAChR agonist xanomeline, exhibit relatively nonselective profiles of binding affinities across the different mAChR subtypes, underscoring the drawback of designing orthosteric site ligands that target the highly conserved ACh

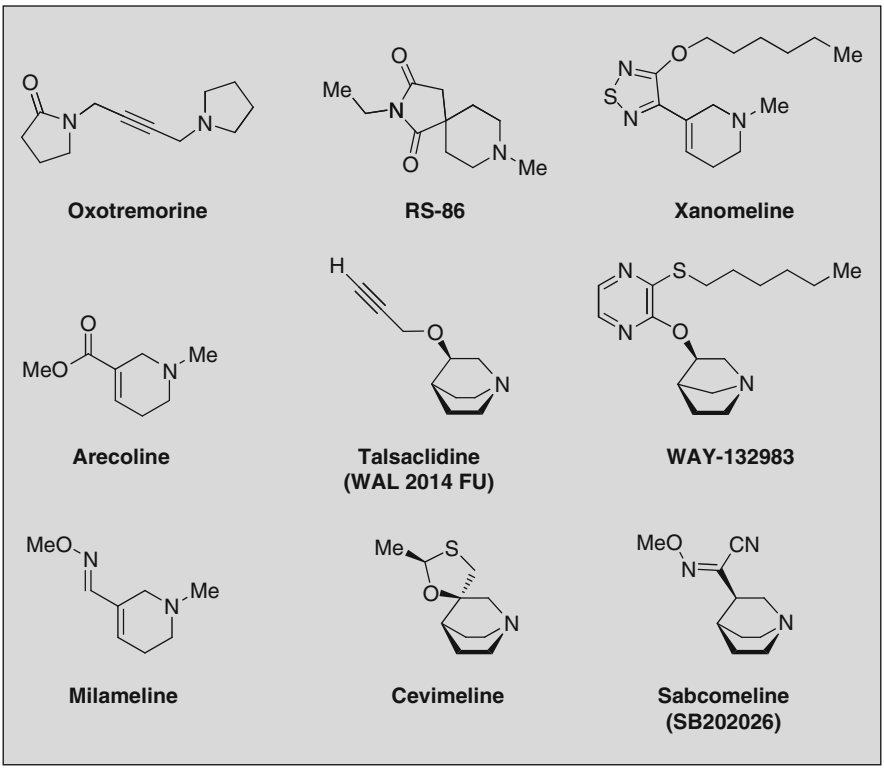

Fig. 4 Chemical structures of representative orthosteric muscarinic agonists

Table 4 Receptor affinities [nM]of orthosteric muscarinic receptor agonists

Drug	Receptor					Ligand	Species	References
	M1	M2	M3	M4	M5			
Arecoline	29	2.4	43	60	56	[^{3}H]-QNB	Human	Kim et al. (2003)
Cevimeline[a]	4,850	854	2,575	1,012		[^{3}H]-QNB	Human	Loudon et al. (1997)
Milameline[b]	2,300	2,400	3,600	3,900	4,300	[^{3}H]-NMS	Human	Sedman et al. (1995)
Oxotremorine	923	70	881	454	–	[^{3}H]-QNB	Human	Loudon et al. (1997)
RS-86	22,900[c]	39,200[d]	–	–	–	[^{3}H]-QNB	Rat	Palacios et al. (1986)
Sabcomeline[e]	230	204	120	267	–	[^{3}H]-QNB	Human	Loudon et al. (1997)
Talsaclidine[f]	25,500	7,100	34,000	–	–	–	Human	Wienrich et al. (2002)
WAY-132983	17.8	9.4	29.0	10.6	20.0	[^{3}H]-NMS	Human	Sullivan et al. (2007)
Xanomeline	79.4	125.9	39.8	20.0	39.8	[^{3}H]-QNB	Human	Watson et al. (1998)

[^{3}H]-QNB, [^{3}H]-quinuclinidyl benzylate
[a]AF102B
[b]CL-979, PD-129,409, Ru-35926
[c]Cortex
[d]Brain stem;
[e]SB202026
[f]WAL2014FU

binding site of the five mAChR subtypes. Due to the relatively nonselective in vitro binding profiles for each of these mAChR orthosteric agonists, the role(s) of the different mAChR subtypes in the observed in vivo effects of these compounds remain unclear. However, as shown in Table 5, the majority of mAChR orthosteric agonists produced robust reversals of pharmacologic and/or lesion-induced deficits in different cognitive domains, including sensory information processing, attention, and various aspects of learning and memory. For example, oxotremorine and xanomeline reversed deficits in PPI induced by the non-selective mAChR antagonist scopolamine and the D1/D2 dopamine receptor agonist apomorphine (Jones and Shannon 2000; Stanhope et al. 2001; Jones et al. 2005) (Table 5). Cevimeline improved performance in divided or visuospatial attentional tasks in monkeys (O'Neill et al. 1999; 2003) (Table 5). In models of learning and memory, the mAChR agonists milameline, xanomeline, WAY-132983, and cevimeline enhanced performance in spatial and delayed nonmatching to sample radial arm maze tasks in scopolamine-impaired, cholinergic-lesioned, and aged rats (M'Harzi et al. 1995; Brandeis et al. 1990; Hodges et al. 1999; Bartolomeo et al. 2000) (Table 5). In addition, oxotremorine and RS-86 reversed disruptions in Morris water maze tasks induced by hemicholinium-3 (Hagan et al. 1989). Notable nonhuman primate studies include improved reversal learning in delayed non-matching-to-sample tasks after administration of mAChR agonists arecoline and RS-86 (Rupniak et al. 1989, 1992) (Table 5). Moreover, milameline also had effects on cortical EEG parameters consistent with enhanced arousal in monkeys (Schwarz et al. 1999), while sabcolemine and arecoline induced hippocampal rhythmical slow wave activity, a procognitive biomarker, in anesthetized rats (Loudon et al. 1997) (Table 5). Finally, a potential disease-modifying effect of mAChR agonists in AD has been revealed by clinical studies with sabcomeline and talsaclidine in which treated AD patients showed decreases in cerebrospinal fluid (CSF) levels of total Aβ or $A\beta_{40}$ and $A\beta_{42}$, indicative of a reduction in the pro-amyloidogenic processing of the amyloid precursor protein (Hock et al. 2000, 2003). These data are consistent with earlier studies using another mAChR agonist, AF102B (Fisher 2007). However, other studies have shown that decreased CSF $A\beta^{42}$ may predict cognitive decline in AD (Motter et al. 1995; Galasko et al. 1998; Sunderland et al. 2003; Fagan et al. 2006) and, thus raise the question which amyloid fraction in CSF may be the most suitable biomarker for predicting, predicting pro-amyloidogenic processing of amyloid precursor protein in brain tissue (Motter et al. 1995; Galasko et al. 1998; Sunderland et al. 2003; Fagan et al. 2006). Future studies are needed to clarify these important issues in the AD literature. Taken together, there is a robust preclinical, and in some cases clinical, profile for the efficacy of mAChR agonists in the enhancement of different aspects of cognition. However, as discussed in the introduction, all of the mAChR orthosteric agonists described in Table 5 have failed to advance into further clinical development due to a lack of true subtype selectivity.

Despite the overall clinical failure of mAChR orthosteric agonists, two clinical studies with the M1/M4-preferring mAChR agonist xanomeline have provided critical proof-of-concept efficacy for the reversal of cognitive impairments and behavioral disturbances observed in AD and schizophrenia patients. In a clinical

Table 5 Effects of orthosteric muscarinic agonists on cognition

Domain	Model	Compound	Mechanism	Dose/route (mg/kg)	Species	Effect	References
Gating	Prepulse inhibition of the acoustic startle reflex	Oxotremorine	Full non-selective	0.3–5.6 S.C.	Rat	Reversed of scopolamine-induced disruption	Jones and Shannon (2000)
		Oxotremorine	Full non-selective	0.03–0.30 S.C.	Rat	Attenuated apomorphine-induced disruption	Jones et al. (2005)
		RS-86	M1 > M2–M5	0.3–3.0 S.C.	Rat		Jones et al. (2005)
		Xanomeline	M1/M4 pref	1–30 S.C.	Rat	Reversed apomorphine-induced disruption	Stanhope et al. (2001); Jones et al. (2005)
		Milameline	Partial non-selective	0.3–3.0 S.C.	Rat		Jones et al. (2005)
		Sabcomeline	Partial: M1 > M2–M5	0.3–3.0 S.C.	Rat		Jones et al. (2005)
Attention	Divided attention task	Cevimeline	M1 > M2–M5	0.1–2.1 I.M	Monkey	Increased accuracy	O'Neill et al. (1999)
	Visuospatial attention task	Cevimeline	M1 > M2–M5	0.1–2.1 I.M	Monkey		O'Neill et al. (2003)
Learning and memory	Radial arm maze: delayed non-matching to sample (DNMTS)	Milameline	Partial non -selective	0.02–0.5 P.O.	Rat	Improved scopolamine-induced deficits	M'Harzi et al. (1995)
		Xanomeline	M1/M4 pref	0.1–5.4 I.P.	Rat		Bartolomeo et al. (2000)
		WAY-132983	M1 pref	0.1– 3.0 I.P.	Rat		Bartolomeo et al. (2000)
		Cevimeline	M1 > M2–M5	1.0 I.P.	Rat	Improved choice accuracy in aged rats	Brandeis et al. (1990)
		Cevimeline	M1 > M2–M5	1.0 I.P.	Rat	Decreased post-delay errors in AF64A-lesioned rats	Brandeis et al. (1990)
		WAY-132983	M1 pref	0.03/d S.C.	Rat		Bartolomeo et al. (2000)
		Xanomeline	M1/M4 pref	0.3/d S.C.	Rat		Bartolomeo et al. (2000)
		Arecoline	Partial non-selective	1.0/d S.C.	Rat		Bartolomeo et al. (2000)
	Visuospatial DNMTS	Arecoline	Partial non-selective	0.1–1.8 I.M.	Monkey	No effect on scoplomaine-induced deficit	Rupniak et al. (1989)
		RS-86	M1 > M2–M5	1.5–2.25 I.M.	Monkey	Attenuated scopolamine-induced deficit	Rupniak et al. (1992)
	Radial maze: spatial and cue	Sabcomeline	Partial: M1 > M2–M5	0.01–0.156 P.O.	Rat	Reduced reference and working memory	Hodges et al. (1999)
		RS-86	M1 > M2–M5	0.05–0.781 P.O.	Rat		Hodges et al. (1999)

(continued)

Table 5 (continued)

Domain	Model	Compound	Mechanism	Dose/route (mg/kg)	Species	Effect	References
						errors in basal forebrain lesioned rats	
	Morris water maze	Arecoline	Partial non-selective	0.046–1.0 S.C.	Rat	Reversed spatial learning deficit induced by hemicholinium-3	Hagan et al. (1989)
		Oxotremorine	Full non-selective	0.03–0.10 S.C.	Rat		Hagan et al. (1989)
		RS-86	M1 > M2–M5	0.46–1.0 S.C.	Rat		Hagan et al. (1989)
	T-maze	Cevimeline	M1 > M2–M5	5/d I.P.	Rat	Improved performance in AF64A-lesioned rats	Nakahara et al. (1989)
		Sabcomeline	Partial: M1 > M2–M5	0.001–1.0 I.P.	Rat	Reversed delay-induced deficits	Hatcher et al. (1998)
		RS-86	M1 > M2–M5	0.2–3.0 I.P.	Rat		Hatcher et al. (1998)
	Passive avoidance	Cevimeline	M1 > M2–M5	1.0 I.P., S.C.	Rat	Improved performance in AF64A-lesioned animals	Fisher et al. (1991)
		Arecoline	Partial non-selective	0.01–1.0 S.C.	Rat	Reduced performance deficit in young rats	Smith et al. (1996)
		Oxotremorine	Full non-selective	0.01–1.0 S.C.	Rat		Smith et al. (1996)
	Conditioned suppression of drinking reference memory task	Cevimeline	M1 > M2–M5	5.0 I.P.	Rat	Reversed scopolamine-induced deficit	Dawson et al. (1994)
	Delayed matching to sample task	Cevimeline	M1 > M2–M5	0.1–2.1 I.M.	Monkey	Improved performance in aged monkeys	O'Neill et al. (1998)
		WAY-132983	M1 pref	0.01–0.1 P.O.	Monkey		Bartolomeo et al. (2000)
		Talsaclidine	Full: M1, Partial: M2/M3	0.6–2.4 P.O.	Monkey		Terry et al. (2002)
	Visual object discrimination	Sabcomeline	Partial: M1 > M2–M5	0.03 P.O.	Monkey	Improved reversal learning	Harries et al. (1998)
		Cevimeline	M1 > M2–M5	0.1 P.O.	Monkey	No effect on scopolamine-induced deficits	Harries et al. (1998)
	Visuospatial recognition memory	Arecoline	Partial non-selective	0.05– 0.1 I.M.	Monkey	Improved performance	Rupniak et al. (1989)
		Cevimeline	M1 > M2–M5	3.0–6.0 I.M.	Monkey	No effect	Rupniak et al. (1992)
	Verbal learning	Xanomeline	M1/M4 pref	25.0–50.0 T.I.D. P.O.	Human	Improved in schizophrenia patients	Shekhar et al. (2008)
	Short term memory	Xanomeline	M1/M4 pref	25.0–50.0 T.I.D. P.O.	Human		Shekhar et al. (2008)
	Spoken language[a]	Xanomeline	M1/M4 pref	20.0–75.0 P.O.	Human	Improved in AD patients	Bodick et al. (1997a)

	Word finding difficulty[a]	Xanomeline	M1/M4 pref	20.0–75.0 P.O.	Human		Bodick et al. (1997a)
	Constructional praxis[a]	Xanomeline	M1/M4 pref	20.0–75.0 P.O.	Human		Bodick et al. (1997a)
	Delayed word recall (CNTB)	Xanomeline	M1/M4 pref	20.0–75.0 P.O.	Human		Veroff et al. (1998)
	Word recall	Xanomeline	M1/M4 pref	20.0–75.0 P.O.	Human	No improvement in AD	Bodick et al. (1997a)
Pro-cognitive effects	Cortical EEG	Milameline	Partial non-selective	0.01–0.032 I.M.	Monkey	Decreased power (arousal)	Schwarz et al. (1999)
	Hippocamapal EEG	Sabcomeline	Partial: M1 > M2–M5	0.018 I.V.	Rat	Increased rhythmical slow wave activity	Loudon et al. (1997)
		Arecoline	Partial non-selective	0.32 I.V.	Rat		Loudon et al. (1997)
	CSF amyloid levels	Talsaclidine	Full: M1 Partial: M2/M3	0.6–4.7 P.O.	Human	Decreasd amyloid-β and amyloid-β 42	Hock et al. (2000, 2003)
		Sabcomeline	Partial: M1 > M2–M5	0.025-0.075 B.D.	Human		Hock et al. (2000, 2003)
	Cortical blood flow	Milameline	Partial non-selective	0.1–0.10 S.C.	Rat	Increased	Schwarz et al. (1999)

CSF cerebrospinal fluid; *CNTB* computerized neuropsychological test battery; *DNMTS* delayed nonmatching to sample

[a]Alzheimer's disease assessment scale cognition battery

trial with mild-to-moderate AD patients, xanomeline improved aspects of cognitive performance as measured by the Alzheimer's disease assessment scale cognitive (ADAS-cog) battery, including spoken language ability, word-finding difficulty in spontaneous speech, and constructional praxis (i.e., three-dimensional motor planning and execution) (Bodick et al. 1997a, b). Xanomeline also significantly improved a number of behavioral disturbances, including agitation, vocal outbursts, and hallucinations, observed in AD patients (Bodick et al. 1997a, b). In a separate clinical trial conducted in a small group of treatment refractory schizophrenic patients, xanomeline produced a significant enhancement in verbal learning and short-term memory functions, as well as decreased positive symptoms (Shekhar et al. 2008). The dose-limiting adverse effects observed in the xanomeline treatment groups in both clinical studies, due to the nonselective activation of peripheral mAChRs, halted further development of this compound.

4.3 Allosteric Agonists and Positive Allosteric Modulators

In recent years, several groups in both academia and industry have pursued a novel strategy for the discovery of mAChR ligands that stimulate a specific receptor subtype by targeting sites that are less highly conserved than the orthosteric ACh binding site, termed allosteric sites (Fig. 5a). As discussed in the following sections, allosteric activators of mAChRs exhibit high subtype selectivity and different mechanisms of action in comparison with orthosteric mAChR agonists. For example, PAMs of mAChRs exhibit no intrinsic activity at the receptor (Fig. 5b), but can bind to an allosteric site and potentiate the effects of the endogenous ligand ACh through enhancement of the affinity of ACh for the orthosteric site and/or increased coupling efficiency to the G-proteins (Fig. 5c). In contrast, allosteric mAChR agonists bind to an allosteric site on the receptor and can directly activate the receptor in the absence of ACh (Christopoulos 2002; Waelbroeck 2003; Conn et al. 2009). Discovery of these novel allosteric mAChR activators is providing exciting tools for further characterization of the roles of different mAChRs on cognition.

4.3.1 M1 Allosteric Modulators

As shown in Fig. 6, there has been excellent progress in the identification of several M1 allosteric activators for critical proof-of-concept studies in preclinical models (see representative chemical structures for the M1 allosteric agonists and PAMs in Fig. 6 with the in vitro functional potencies at each subtype, if available, described in Table 6 and highlighted efficacy in different preclinical cognitive tasks shown in Table 7.

AC-260584 is an analog of the first-generation M1 allosteric mAChR agonist AC-42 that was shown to have activity through binding at an allosteric site on the M1 mAChR (Heinrich et al. 2009; Spalding et al. 2002; Langmead et al. 2006).

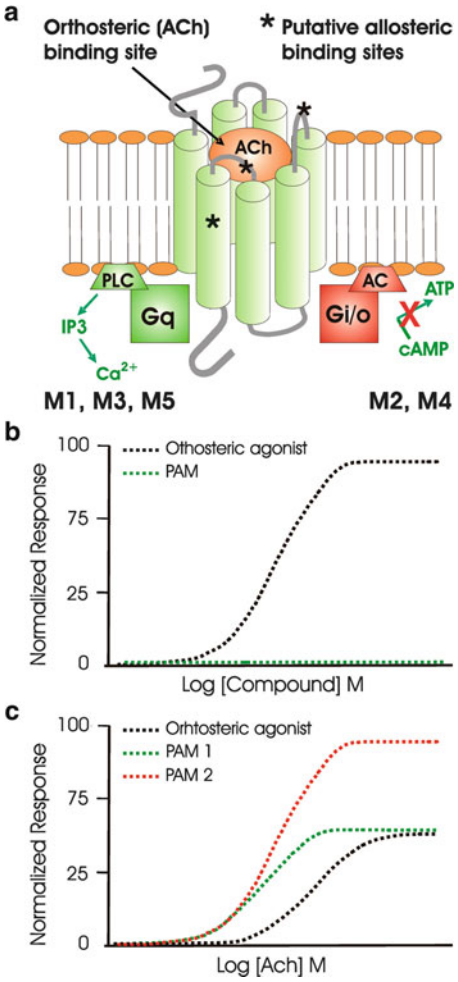

Fig. 5 Schematic representation of a muscarinic acetylcholine receptor showing orthosteric and putative allosteric binding sites and effector mechanisms (**a**). Each of the five mAChR subtypes is a seven-transmembrane protein. Allosteric activators bind to sites other than the orthosteric Ach binding site to activate or potentiate the receptor. Muscarinic receptors are divided into two functional classes based on G-protein-coupled receptor coupling. M1, M3, and M5 mAChRs couple to Gq/G11, which results in increased intracellular calcium levels via phospholipase C activation. M2 and M4 mAChRs couple to Gi/o, resulting in the inhibition of adenylyl cyclase and ion channels. Unlike orthosteric agonists, PAMs have no intrinsic activity (**b**). The graph in (**c**) illustrates two potential modes of action of PAMs in a cell-based system: affinity modulation (PAM1) with a resulting leftward shift of the concentration–response curve and efficacy modulation (PAM2) leading to an increase in maximal response. *AC* adenylyl cyclase; *ACh* acetylcholine; *cAMP* cyclic AMP; *IP3* inositol triphosphate; *M1–M5* muscarinic cholinergic receptor subtypes 1–5; *PAM* positive allosteric modulator; *PLC* phospholipase C

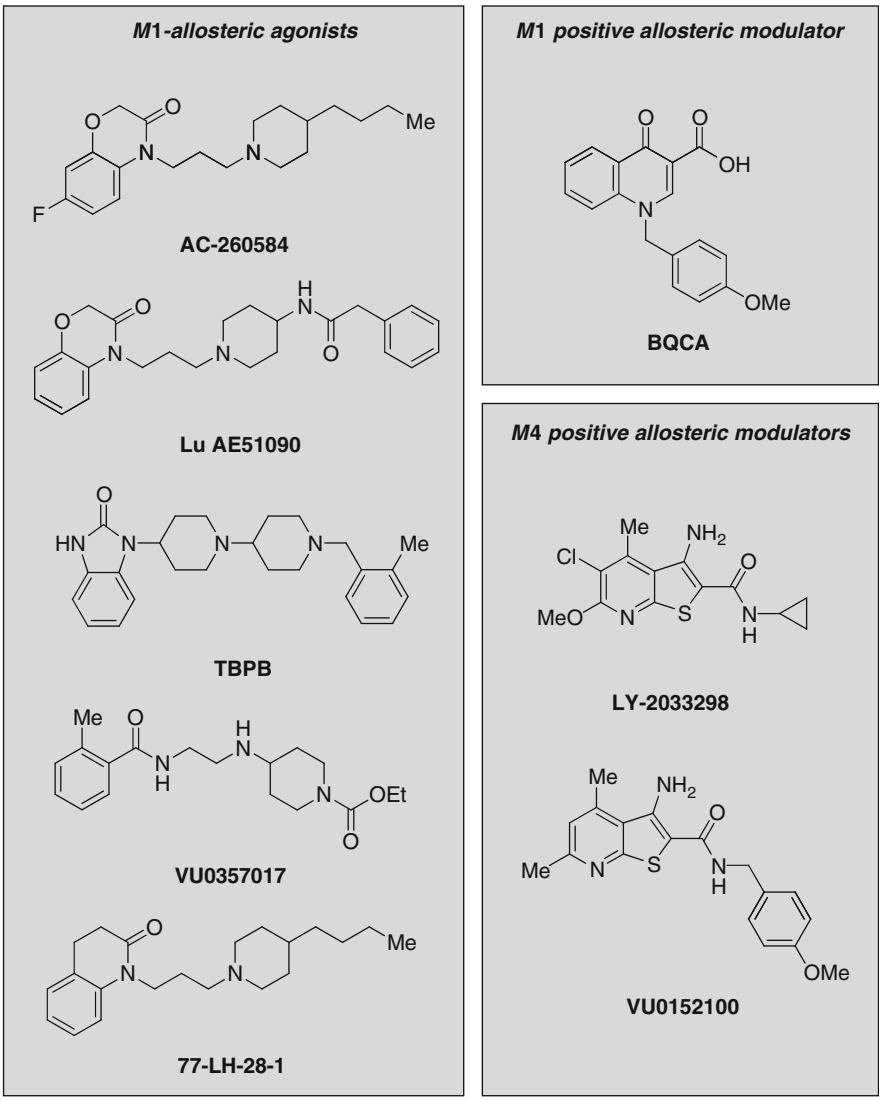

Fig. 6 Chemical structures of representative muscarinic receptor allosteric agonists and positive allosteric modulators

AC-260584 has been reported to enhance memory functions as assessed in the novel object recognition and Morris water maze tasks in mice, as well as produce effects in preclinical models predictive of antipsychotic-like effects (Bradley et al. 2010; Vanover et al. 2008) (Table 7). Unfortunately, interpretation of the in vivo efficacy of AC-260584 is confounded by off-target effects at dopamine D2, adrenergic α1A, and serotonin 5-HT^{2A} receptors (Heinrich et al. 2009). The M1 allosteric agonist, 77-LH-28-1, is another systemically active AC-42 analog (Langmead et al.

Table 6 Functional responses of allosteric muscarinic receptor modulators at cloned muscarinic receptors

Drug	EC_{50} (nM)					Assay	Source	References
	M1	M2	M3	M4	M5		Cloned	
Allosteric M1 agonist								
AC-260584	2	470	415	>10,000	189	Ca^{2+}/cAMP	Human	Heinrich et al. (2009)
Lu AE51090	61	>10,000	>10,000	>10,000	>10,000	Ca^{2+}	Human	Sams et al. (2010)
TBPB		>30,000	>30,000		>30,000	Ca^{2+}	Human	Jones et al. (2008)
	158			>30,000		Ca^{2+}	Rat	
VU0357017		>30,000	>30,000		>30,000	Ca^{2+}	Human	Lebois et al. (2010)
	198			>30,000		Ca^{2+}	Rat	
77-LH-28-1	8	>10,000	2,512	>10,000	>10,000	Ca^{2+}	Human	Langmead (2008)
	2	***765***	159	***>10,000***	206	Ca^{2+}***/cAMP***	Human	Heinrich et al. (2009)
Allosteric M1 PAM								
BQCA	845	>100,000	>100,000	>100,000	>100,000	Ca^{2+}	Human	Ma et al. (2009)
Allosteric M4 PAM								
LY-2033298	N/A	N/A	N/A	8	N/A	Ca^{2+}	Human	Chan et al. 2008; Leach et al. (2010)
VU0152100		>30,000	>30,000		>30,000	Ca^{2+}	Human	Brady et al. (2008)
	>30,000			380		Ca^{2+}	Rat	

Ca^{2+} calcium mobilization assay; *cAMP* inhibition of forskolin-induced cAMP accumulation; *N/A* not available

Table 7 Effects of allosteric muscarinic receptor agonists on cognition

Domain	Model	Compound	Mechanism	Dose/Route (mg/kg)	Species	Effect	References
Gating	Prepulse inhibition of the acoustic startle reflex	LY-2033298	M4 PAM	10.0–30.0 S.C.	Rat	Reversed of apomorphine-induced disruption	Chan et al. (2008)
		TBPB	M1 agonist	10.0–100 S.C.	Rat		Kane (2008)
Learning and memory	Novel object recognition	AC-260584	M1 agonist	10.0 S.C.	Mouse	Increased interaction	Bradley et al. (2010)
	Morris water maze	AC-260584	M1 agonist	1.0 I.P.	Mouse	Increased retention of platform location	Vanover et al. (2008)
	Contextual fear conditioning	BQCA	M1 PAM	5.0–20.0 I.P.	Mouse	Reversed scopolamine-induced disruption	Ma et al. (2009)
		VU0357017	M1 agonist	1.0–1.0 I.P.	Rat		Lebois et al. (2010)
		TBPB	M1 agonist	1.0–30.0 I.P.	Rat		Kane (2008)
		VU0152100	M4 PAM	10 – 56.6 S.C.	Rat	Reversed amphetamine-induced disruption	Byun et al. (2011)
	Cue fear conditioning	TBPB	M1 agonist	1.0–30.0 I.P	Rat		Kane (2008)
	Y-maze delayed alternation	Lu AE51090	M1 agonist	0.31–20	Mouse	Reversed delay-dependent memory decay	Sams et al. (2010)
Executive function	Reversal learning	BQCA	M1 PAM	30.0 S.C.	Mouse	Ameliorated deficits in Tg2576 mice (AD model)	Shirey et al. 2009
Procognitive effects	EEG (sleep)	BQCA	M1 PAM	10.0 I.P.	Rat	Increased wakefulness; inhibited delta sleep	Ma et al. (2009)
	Electrophysiology (in vivo)	77-LH-28-1	M4 PAM	3.0 S.C.	Rat	Increased hippocampal cell firing	Langmead et al. (2008)
		BQCA	M1 PAM	20.0 I.P.	Rat	Increased mPFC neuron firing rate	Shirey et al. (2009)
	Cerebral blood flow	BQCA	M1 PAM	1–10.0 I.P.	Rat	Enhanced	Ma et al. (2009)

2008) with high selectivity for M1 and some weak M3 agonist activity (Heinrich et al. 2009) (see Fig. 6, Tables 6 and 7). Functional and site-directed mutagenesis studies have established that 77-LH-28-1not only acts as a "bi-topic" agonist that binds to a site that overlaps with the orthosteric site, but also includes an allosteric site that modulates affinity of the ACh site (Avlani et al. 2010). Several physiologic effects thought to potentiate cognition, including increased hippocampal CA1 pyramidal cell firing in vitro and in vivo and induction of synchronous network activity through increased CA3 hippocampal γ oscillations, are increased with 77-LH-28-1 treatment (Langmead et al. 2008; Buchanan et al. 2010; Jo et al. 2010; Spencer et al. 2010). Another highly selective AC-42-based compound, Lu AE51090, reversed delay-dependent memory decay in a Y-maze delayed alternation paradigm (Sams et al. 2010) (Fig. 6, Tables 6 and 7).

There are now additional second-generation, systemically active and highly selective M1 allosteric agonists and PAMs that are serving as important tools for determining the role of selective activation of M1 mAChRs in native tissue preparations and in animal models of cognition, including the M1 allosteric agonists TBPB, which is a selective and potent M1 allosteric agonist in recombinant systems (Jones et al. 2008) (Fig. 6, Tables 6 and 7). Site-directed mutagenesis studies have revealed that point mutations in the ACh binding site that reduce the activity of orthosteric mAChR agonists at M1 produce no change in the response to TBPB. A Schild analysis for the blockade of TBPB effects with the orthosteric mAChR antagonist atropine showed that TBPB interacts with the orthosteric site in a noncompetitive manner (Jones et al. 2008). Based on an allosteric ternary complex model for the actions of two molecules that interact with distinct sites on a receptor, these results collectively suggest that TBPB may act as an allosteric M1 agonist (Christopoulos and Mitchelson 1997; Jacobson et al. 2010). However, further studies are warranted as it cannot be ruled out that TBPB may act as a bi-topic agonist, similar to 77-LH-28-1 (Avlani et al. 2010). In native tissue preparations, TBPB potentiated NMDA receptor currents in CA1 hippocampal pyramindal cells, a function that is thought to contribute to the procognitive effects of mAChR agonists, as described earlier (Jones et al. 2008). In several preclinical models predictive of antipsychotic-like activity, TBPB produced efficacy at doses that do not induce the side effects associated with nonselective stimulation of peripheral mAChRs. More importantly, TBPB reversed apomorphine-induced deficits in PPI of the acoustic startle reflex and scopolamine-induced impairments in the acquisition of a hippocampal working memory task, contextual fear conditioning (Kane 2008). In addition, selective activation of M1 by TBPB increased the non-amyloidogenic processing of the amyloid precursor protein and reduced Aβ formation in vitro, as previously reported with other nonselective mAChR agonists. These data are consistent with the hypothesis that selective activation of M1 mAChRs may provide both enhancement of cognitive functions and potential disease-modifying activity for the treatment of symptoms associated with AD.

Finally, VU0357017 represents a highly potent, selective, and systemically active third-generation M1 allosteric agonist (Lebois et al. 2010) (Fig. 6, Tables 6 and 7). Unlike the other allosteric M1 agonists, VU0357017 activates the M1

mAChR at a novel allosteric site on the third extracellular loop, instead of within the seven transmembrane domain (Lebois et al. 2010). This compound potentiated NMDA receptor currents in slice electrophysiology experiments and blocked scopolamine-induced deficits in contextual fear conditioning (Lebois et al. 2010).

4.3.2 M1 Positive Allosteric Modulators

A major advance in the development of systemically active and selective M1 PAMs was the identification and characterization of benzylquinolone carboxylic acid (BQCA) (Fig. 6). In cell-based systems, BQCA is a potent PAM with a 129-fold leftward shift of the ACh concentration–response curve with high M1 selectivity that lacks agonist, potentiator, or antagonist activity at M2–M5 up to 100 μM (Ma et al. 2009) (Table 6). In addition, BQCA increases the affinity of the M1 mAChR for ACh, but does not bind at the orthosteric ACh binding site. In native tissue, BQCA increased mPFC spontaneous excitatory postsynaptic currents (sEPSCs) and potentiated carbachol-induced effects on sEPSCs frequency, and these effects were absent in M1 mAChR KO mice (Shirey et al. 2009). With in vivo electrophysiological techniques, BQCA was also shown to enhance firing rates of mPFC neurons after systemic administration (Shirey et al. 2009) (Table 7). In animal studies, BQCA reversed scopolamine-induced disruptions of the hippocampus-mediated memory task of contextual fear conditioning, increased wakefulness, decreased delta sleep, and restored deficits in mPFC-dependent discrimination reversal learning in a transgenic mouse that overexpresses a familial AD mutant form of the amyloid precursor protein (Tg2576 mice) (Ma et al. 2009; Shirey et al. 2009) (Table 7). Interestingly, BQCA also increased cortical blood flow, a process previously attributed to M5 mAChR activation based on KO studies (Yamada et al. 2001, 2003). Taken together, studies with M1 allosteric agonists and PAMs have demonstrated that selective activation of M1 produces efficacy in preclinical models of cognitive enhancement similar to the effects observed with other nonselective mAChR agonists, and indicate an important role for M1 activation in prefrontal cortex-dependent synaptic plasticity and learning.

4.3.3 M4 Positive Allosteric Modulators

There have also been recent developments in the identification of systemically active M4 PAMs, including LY2033298 and VU0152100 (Chan et al. 2008; Brady et al. 2008) (see Fig. 6 for chemical structures, and Tables 6 and 7 for in vitro properties and functional effects, respectively). LY2033298 represents a highly selective M4 PAM that robustly potentiates the response of ACh through binding at residue F186 in the third extracellular loop (o3) of the receptor (Nawaratne et al. 2010), but does not directly activate M4 mAChRs. Using rat M4 AChRs (rM4) membranes in cell-based studies, the in vitro potency of LY2033298 for potentiation of [3H]-oxotremorine-M was decreased by fivefold to sixfold in comparison with studies using human M4 AChR (hM4) membranes (hM4 EC50 = 8 nM; see

Table 6). Across all in vivo models tested to date, LY2033298 had no effects when administered alone, but potentiated the effects of a subthreshold dose of the nonselective mAChR agonist oxotremorine in the inhibition of conditioned avoidance responding and reversal of apomorphine-induced disruption of the PPI (Chan et al. 2008; Leach et al. 2010; Suratman et al. 2011). The observed lower potency of LY2033298 at the rat M4 mAChR has been postulated to account for the lack of efficacy observed in animal models with the LY2033298 alone.

More recently, another highly selective, systemically active M4 mAChR PAM, VU0152100, with a 30- to 70-fold leftward shift in the ACh response was discovered (Brady et al. 2008) (Fig. 6). VU0152100 exhibits high mAChR subtype selectivity for M4 (see Table 6) relative to the other mAChRs and 15 other GPCRs that are highly expressed in the brain (Brady et al. 2008), and increases M4 mAChR receptor affinity for ACh without competing for the orthosteric ACh binding site (Brady et al. 2008). In preclinical studies, VU0152100 reversed amphetamine-induced hyperlocomotion and disruptions in the acquisition of contextual fear conditioning (Byun et al. 2011). Interestingly, these findings suggest that there is sufficient endogenous ACh tone to potentiate cholinergic responses when VU0152100 is administered alone. Although preliminary, these studies using selective M4 mAChR PAMs indicate that selective activation of M4 mAChRs produces efficacy in preclinical models predictive of antipsychosis-like activity comparable to the effects observed with xanomeline and other mAChR agonists and hint at some potential cognition enhancing effects.

5 Summary

Converging findings with subtype-selective mAChR activators and mAChR antagonists and KO mice are providing important validation for the role of the muscarinic cholinergic system in the modulation of normal cognitive functions and in the potential reversal of cognitive deficits observed in neurologic and psychiatric disorders, including AD and schizophrenia. Discovery of the novel subtype-selective mAChR ligands is also providing critical tools to better understand the relative roles of the mAChR subtypes in the different aspects of cognition and in the observed efficacy with AChEIs and orthosteric mAChR agonists. To date, selective M1 and M4 allosteric agonists and/or PAMs are providing the most promising preclinical data for the potential treatment of cognitive impairments and behavioral disturbance associated with dementia or schizophrenia.

References

Agnoli A, Martucci N, Manna V, Conti L, Fioravanti M (1983) Effect of cholinergic and anticholinergic drugs on short-term memory in Alzheimer's dementia: a neuropsychological and computerized electroencephalographic study. Clin Neuropharmacol 6:311–323

Aigner TG, Mishkin M (1986) The effects of physostigmine and scopolamine on recognition memory in monkeys. Behav Neural Biol 45:81–87

Anagnostaras SG, Maren S, Fanselow MS (1995) Scopolamine selectively disrupts the acquisition of contextual fear conditioning in rats. Neurobiol Learn Mem 64:191–194

Anagnostaras SG, Murphy GG, Hamilton SE, Mitchell SL, Rahnama NP, Nathanson NM, Silva AJ (2003) Selective cognitive dysfunction in acetylcholine M1 muscarinic receptor mutant mice. Nat Neurosci 6:51–58

Anagnostaras SG, Maren S, Sage JR, Goodrich S, Fanselow MS (1999) Scopolamine and Pavlovian fear conditioning in rats: dose-effect analysis. Neuropsychopharmacology 21 (6):731–744

Amenta F, Parnetti L, Gallai V, Wallin A (2001) Treatment of cognitive dysfunction associated with Alzheimer's disease with cholinergic precursors. Ineffective treatments or inappropriate approaches? Mech Ageing Dev 122:2025–2040

Andrews JS, Grützner M, Stephens DN (1992) Effects of cholinergic and non-cholinergic drugs on visual discrimination and delayed visual discrimination performance in rats. Psychopharmacology (Berl) 106:523–530

Araya R, Noguchi T, Yuhki M, Kitamura N, Higuchi M, Saido TC, Seki K, Itohara S, Kawano M, Tanemura K, Takashima A, Yamada K, Kondoh Y, Kanno I, Wess J, Yamada M (2006) Loss of M5 muscarinic acetylcholine receptors leads to cerebrovascular and neuronal abnormalities and cognitive deficits in mice. Neurobiol Dis 24:334–344

Avlani VA, Langmead CJ, Guida E, Wood MD, Tehan BG, Herdon HJ, Watson JM, Sexton PM, Christopoulos A (2010) Orthosteric and allosteric modes of interaction of novel selective agonists of the M1 muscarinic acetylcholine receptor. Mol Pharmacol 78(1):94–104

Barak S (2009) Modeling cholinergic aspects of schizophrenia: focus on the antimuscarinic syndrome. Behav Brain Res 204:335–351

Baron SP, Wright D, Wenger GR (1998) Effects of drugs of abuse and scopolamine on memory in rats: delayed spatial alternation and matching to position. Psychopharmacology 137:7–14

Barten DM, Albright CF (2008) Therapeutic strategies for Alzheimer's disease. Mol Neurobiol 37:171–186

Bartolomeo AC, Morris H, Buccafusco JJ, Kille N, Rosenzweig-Lipson S, Husbands MG, Sabb AL, Abou-Gharbia M, Moyer JA, Boast CA (2000) The preclinical pharmacological profile of WAY-132983, a potent M1 preferring agonist. J Pharmacol Exp Ther 292:584–596

Bartus RT, Dean RL III, Beer B, Lippa AS (1982) The cholinergic hypothesis of geriatric memory dysfunction. Science 217:408–417

Basile AS, Fedorova I, Zapata A, Liu XG, Shippenberg T, Duttaroy A, Yamada M, Wess J (2002) Deletion of the M5 muscarinic acetylcholine receptor attenuates morphine reinforcement and withdrawal but not morphine analgesia. Proc Natl Acad Sci U S A 99:11452–11457

Bernard V, Normand E, Bloch B (1992) Phenotypical characterization of the rat striatal neurons expressing muscarinic receptor genes. J Neurosci 12:3591–3600

Besheer J, Short KR, Bevins RA (2001) Dopaminergic and cholinergic antagonism in a novel-object detection task with rats. Behav Brain Res 126:211–217

Betz AJ, McLaughlin PJ, Burgos M, Weber SM, Salamone JD (2007) The muscarinic receptor antagonist tropicamide suppresses tremulous jaw movements in a rodent model of parkinsonian tremor: possible role of M4 receptors. Psychopharmacology 194:347–359

Birks J (2006) Cholinesterase inhibitors for Alzheimer's disease. Cochrane Database Syst Rev 25 (1):CD005593

Birks J, Flicker L (2006) Donepezil for mild cognitive impairment. Cochrane Database Syst Rev 3: CD006104

Bodick NC, Offen WW, Levey AI et al (1997a) Effects of xanomeline, a selective muscarinic receptor agonist, on cognitive function and behavioral symptoms in Alzheimer disease. Arch Neurol 54:465–473

Bodick NC, Offen WW, Shannon HE et al (1997b) The selective muscarinic agonist xanomeline improves both the cognitive deficits and behavioral symptoms of Alzheimer disease. Alzheimer Dis Assoc Disord 11(Suppl 4):S16–S22

Bolam JP, Wainer BH, Smith AD (1984) Characterization of cholinergic neurons in the rat neostriatum: a combination of choline acetyltransferase immunocytochemistry, Golgi-impregnation and electron microscopy. Neuroscience 12:711–718

Bolden C, Cusack B, Richelson E (1992) Antagonism by antimuscarinic and neuroleptic compounds at the five cloned human muscarinic cholinergic receptors expressed in Chinese hamster ovary cells. J Pharmacol Exp Ther 260:576–580

Bradley SR, Lameh J, Ohrmund L et al (2010) AC-260584, an orally bioavailable M(1) muscarinic receptor allosteric agonist, improves cognitive performance in an animal model. Neuropharmacology 58:365–373

Brandeis R, Dachir S, Sapir M, Levy A, Fisher A (1990) Reversal of age-related cognitive impairments by an M1 cholinergic agonist, AF102B. Pharmacol Biochem Behav 36:89–95

Brady AE, Jones CK, Bridges TM, Kennedy JP, Thompson AD, Heiman JU, Breininger ML, Gentry PR, Yin H, Jadhav SB, Shirey JK, Conn PJ, Lindsley CW (2008) Centrally active allosteric potentiators of the M4 muscarinic acetylcholine receptor reverse amphetamine-induced hyperlocomotor activity in rats. J Pharmacol Exp Ther 327:941–953

Brann MR, Buckley NJ, Bonner TI (1988) The striatum and cerebral cortex express different muscarinic receptor mRNAs. FEBS Lett 28:90–94

Brockel BJ, Fowler SC (1995) Effects of chronic haloperidol on reaction time and errors in a sustained attention task: partial reversal by anticholinergics and by amphetamine. J Pharmacol Exp Ther 275:1090–1098

Bruno G, Mohr E, Gillespie M, Fedio P, Chase TN (1986) Muscarinic agonist therapy of Alzheimer's disease. A clinical trial of RS-86. Arch Neurol 43:659–661

Buchanan KA, Petrovic MM, Chamberlain SE, Marrion NV, Mellor JR (2010) Facilitation of long-term potentiation by muscarinic M(1) receptors is mediated by inhibition of SK channels. Neuron 68:948–963

Buckley NJ, Bonner TI, Buckley CM, Brann MR (1989) Antagonist binding properties of five cloned muscarinic receptors expressed in CHO-K1 cells. Mol Pharmacol 35:469–476

Buresová O, Bolhuis JJ, Bures J (1986) Differential effects of cholinergic blockade on performance of rats in the water tank navigation task and in a radial water maze. Behav Neurosci 100:476–482

Bushnell PJ, Oshiro WM, Padnos BK (1997) Detection of visual signals by rats: effects of chlordiazepoxide and cholinergic and adrenergic drugs on sustained attention. Psychopharmacology (Berl) 134:230–241

Bymaster FP, Heath I, Hendrix JC, Shannon HE (1993) Comparative behavioral and neurochemical activities of cholinergic antagonists in rats. J Pharmacol Exp Ther 267:16–24

Byun N, Lawson K, Gore JC, Conn PJ, Jones CK (2011) Antipsychotic-like profile and reversal of cognitive impairment with the positive allosteric modulator of the M4 muscarinic acetylcholine receptor VU0152100. Abstracts of the 13th International Congress on Schizophrenia Research (ICOSR). Schizophr Bull Suppl 1:1–342

Callahan MJ, Kinsora JJ, Harbaugh RE, Reeder TM, Davis RE (1993) Continuous ICV infusion of scopolamine impairs sustained attention of rhesus monkeys. Neurobiol Aging 14:147–151

Carey GJ, Billard W, Binch H 3rd, Cohen-Williams M, Crosby G, Grzelak M, Guzik H, Kozlowski JA, Lowe DB, Pond AJ, Tedesco RP, Watkins RW, Coffin VL (2001) SCH 57790, a selective muscarinic M(2) receptor antagonist, releases acetylcholine and produces cognitive enhancement in laboratory animals. Eur J Pharmacol 431:189–200

Cayzac S, Delcasso S, Paz V, Jeantet Y, Cho YH (2011) Changes in striatal procedural memory coding correlate with learning deficits in a mouse model of Huntington disease. Proc Natl Acad Sci U S A 108:9280–9285

Chan WY, McKinzie DL, Bose S, Mitchell SN, Witkin JM, Thompson RC, Christopoulos A, Lazareno S, Birdsall NJ, Bymaster FP, Felder CC (2008) Allosteric modulation of the muscarinic M4 receptor as an approach to treating schizophrenia. Proc Natl Acad Sci U S A 105:10978–10983

Chaudhuri JD, Hiltunen M, Nykänen M, Ylä-Herttuala S, Soininen H, Miettinen R (2005) Localization of M2 muscarinic receptor protein in parvalbumin and calretinin containing cells of the adult rat entorhinal cortex using two complementary methods. Neuroscience 131:557–566

Chen KC, Baxter MG, Rodefer JS (2004) Central blockade of muscarinic cholinergic receptors disrupts affective and attentional set-shifting. Eur J Neurosci 20:1081–1088

Chouinard S, Sepehry AA, Stip E (2007) Oral cholinesterase inhibitor add-on therapy for cognitive enhancement in schizophrenia: a quantitative systematic re-view, part I. Clin Neuropharmacol 30:169–182

Christopoulos A (2002) Allosteric binding sites on cell-surface receptors: novel targets for drug discovery. Nat Rev Drug Discov 1:198–210

Christopoulos A, Mitchelson F (1997) Application of an allosteric ternary complex model to the technique of pharmacological resultant analysis. J Pharm Pharmacol 49:781–786

Clarke PB, Hommer DW, Pert A, Skirboll LR (1987) Innervation of substantia nigra neurons by cholinergic afferents from pedunculopontine nucleus in the rat: neuroanatomical and electrophysiological evidence. Neuroscience 23:1011–1019

Conn PJ, Jones CK, Lindsley CW (2009) Subtype-selective allosteric modulators of muscarinic receptors for the treatment of CNS disorders. Trends Pharmacol Sci 30:148–155

Cox CL, Huguenard JR, Prince DA (1997) Nucleus reticularis neurons mediate diverse inhibitory effects in thalamus. Proc Natl Acad Sci U S A 94:8854–8859

Cummings JL (2003) Use of cholinesterase inhibitors in clinical practice: evidence-based recommendations. Am J Geriatr Psychiatry 11:131–145

Datta S, Siwek DF (1997) Excitation of the brain stem pedunculopontine tegmentum cholinergic cells induces wakefulness and REM sleep. J Neurophysiol 77(6):2975–2988

Dawson GR, Bayley P, Channell S, Iversen SD (1994) A comparison of the effects of the novel muscarinic receptor agonists L-689,660 and AF102B in tests of reference and working memory. Psychopharmacology 113(3–4):361–368

Dennes RP, Barnes JC (1993) Attenuation of scopolamine-induced spatial memory deficits in the rat by cholinomimetic and non-cholinomimetic drugs using a novel task in the 12-arm radial maze. Psychopharmacology (Berl) 111:435–441

Dietrich H, Jenck F (2010) Intact learning and memory in rats following treat-ment with the dual orexin receptor antagonist almorexant. Psychopharmacology 212:145–154

Dillon GM, Shelton D, McKinney AP, Caniga M, Marcus JN, Ferguson MT, Kornecook TJ, Dodart JC (2009) Prefrontal cortex lesions and scopolamine impair attention performance of C57BL/6 mice in a novel 2-choice visual discrimination task. Behav Brain Res 204:67–76

Dodart JC, Mathis C, Ungerer A (1997) Scopolamine-induced deficits in a two-trial object recognition task in mice. Neuroreport 24:1173–1178

Dörje F, Wess J, Lambrecht G, Tacke R, Mutschler E, Brann MR (1991) Antagonist binding profiles of five cloned human muscarinic receptor subtypes. J Pharmacol Exp Ther 256:727–733

Domer FR, Schueler FW (1960) Investigations of the amnesic properties of scopolamine and related compounds. Arch Int Pharmacodyn Ther 127:449–458

Doods H, Entzeroth M, Ziegler H, Schiavi G, Engel W, Mihm G, Rudolf K, Eberlein W (1993) Characterization of BIBN 99: a lipophilic and selective muscarinic M2 receptor antagonist. Eur J Pharmacol 242:23–30

Drinkenburg WH, Sondag HN, Coenders CJ, Andrews JS, Vossen JM (1995) Effects of selective antagonism or depletion of the cholinergic system on visual discrimination performance in rats. Behav Pharmacol 6:695–702

Drachman D, Leavitt J (1974) Human memory and the cholinergic system. A relationship to aging? Arch Neurol 30:113–121

Dudchenko P, Sarter M (1992) Behavioral microanalysis of spatial delayed alternation performance: rehearsal through overt behavior, and effects of scopolamine and chlordiazepoxide. Psychopharmacology (Berl) 107:263–270

Eckenstein FP, Baughman RW, Quinn J (1988) An anatomical study of cholinergic innervation in rat cerebral cortex. Neuroscience 25:457–474

Edelstein P, Schultz JR, Hirschowitz J et al (1981) Physostigmine and lithium in the schizophrenias. Am J Psychiatry 138:1078–1081

Ellis JR, Ellis KA, Bartholomeusz CF, Harrison BJ, Wesnes KA, Erskine FF, Vitetta L, Nathan PJ (2006) Muscarinic and nicotinic receptors synergistically modulate working memory and attention in humans. Int J Neuropsychopharmacol 9:175–189

Ennaceur A, Meliani K (1992) Effects of physostigmine and scopolamine on rats' performances in object-recognition and radial-maze tests. Psychopharmacology (Berl) 109:321–330

Everitt BJ, Robbins TW (1997) Central cholinergic systems and cognition. Annu Rev Psychol 48:649–684

Fagan AM, Mintun MA, Mach RH, Lee SY, Dence CS, Shah AR, LaRossa GN, Spinner ML, Klunk WE, Mathis CA, DeKosky ST, Morris JC, Holtzman DM (2006) Inverse relation between in vivo amyloid imaging load and cerebrospinal fluid Abeta42 in humans. Ann Neurol 59:512–519

Farlow MR, Cummings JL (2007) Effective pharmacologic management of Alzheimer's disease. Am J Med 120:388–397

Feiro O, Gould TJ (2005) The interactive effects of nicotinic and muscarinic cholinergic receptor inhibition on fear conditioning in young and aged C57BL/6 mice. Pharmacol Biochem Behav 80:251–262

Fink-Jensen A, Fedorova I, Wörtwein G et al (2003) Role for M5 muscarinic acetylcholine receptors in cocaine addiction. J Neurosci Res 74:91–96

Fisher A (2007) M1 muscarinic agonists target major hallmarks of Alzheimer's disease – an update. Curr Alzheimer Res 4:577–580

Fisher A, Brandeis R, Karton I, Pittel Z, Gurwitz D, Haring R, Sapir M, Levy A, Heldman E (1991) (+−)-cis-2-methyl-spiro(1,3-oxathiolane-5,3′)quinuclidine, an M1 selective cholinergic agonist, attenuates cognitive dysfunctions in an animal model of Alzheimer's disease. J Pharmacol Exp Ther 257:392–403

Fornari RV, Moreira KM, Oliveira MG (2000) Effects of the selective M1 muscarinic receptor antagonist dicyclomine on emotional memory. Learn Mem 7:287–292

Fredrickson A, Snyder PJ, Cromer J, Thomas E, Lewis M, Maruff P (2008) The use of effect sizes to characterize the nature of cognitive change in psychopharmacological studies: an example with scopolamine. Hum Psychopharmacol Clin Exp 23:425–436

Galasko D, Chang L, Motter R, Clark CM, Kaye J, Knopman D, Thomas R, Kholodenko D, Schenk D, Lieberburg I, Miller B, Green R, Basherad R, Kertiles L, Boss MA, Seubert P (1998) High cerebrospinal fluid tau and low amyloid beta42 levels in the clinical diagnosis of Alzheimer disease and relation to apolipoprotein E genotype. Arch Neurol 55:937–945

Gerfen CR, Bolam JP (2010) The neuroanatomical organization of the basal ganglia. In: Steiner H, Tseng KY (eds) Handbook of basal ganglia structure and function. Academic Press, London

González I, Arévalo-Serrano J, Sanz-Anquela JM, Gonzalo-Ruiz A (2007) Effects of beta-amyloid protein on M1 and M2 subtypes of muscarinic acetylcholine receptors in the medial septum-diagonal band complex of the rat: relationship with cholinergic, GABAergic, and calcium-binding protein perikarya. Acta Neuropathol 113:637–651

Gonzalo-Ruiz A, Sanz-Anquela MJ, Lieberman AR (1995) Cholinergic projections to the anterior thalamic nuclei in the rat: a combined retrograde tracing and choline acetyl transferase immunohistochemical study. Anat Embryol (Berl) 192(4):335–49

Gould E, Woolf NJ, Butcher LL (1989) Cholinergic projections to the substantia nigra from the pedunculopontine and laterodorsal tegmental nuclei. Neuroscience 28:611–623

Green MF (1996) What are the functional consequences of neurocognitive deficits in schizophrenia? Am J Psychiatry 153:321–330

Green A, Ellis KA, Ellis J, Bartholomeusz CF, Ilic S, Croft RJ, Phan KL, Nathan PJ (2005) Muscarinic and nicotinic receptor modulation of object and spatial n-back working memory in humans. Pharmacol Biochem Behav 81:575–584

Green MF, Kern RS, Heaton RK (2004) Longitudinal studies of cognition and functional outcome in schizophrenia: implications for MATRICS. Schizophr Res 72:41–51
Hagan JJ, Jansen JH, Broekkamp CL (1989) Hemicholinium-3 impairs spatial learning and the deficit is reversed by cholinomimetics. Psychopharmacology 98:347–356
Hájos N, Papp EC, Acsády L, Levey AI, Freund TF (1998) Distinct interneuron types express m2 muscarinic receptor immunoreactivity on their dendrites or axon terminals in the hippocampus. Neuroscience 82:355–376
Hallanger AE, Levey AI, Lee HJ, Rye DB, Wainer BH (1987) The origins of cholinergic and other subcortical afferents to the thalamus in the rat. J Comp Neurol 262:105–124
Hamborg-Petersen B, Nielsen MM, Thordal C (1984) Toxic effect of scopolamine eye drops in children. Acta Ophthalmol 62:485–488
Harries MH, Samson NA, Cilia J, Hunter AJ (1998) The profile of sabcomeline (SB-202026), a functionally selective M1 receptor partial agonist, in the marmoset. Br J Pharmacol 124:409–415
Hasselmo ME (2006) The role of acetylcholine in learning and memory. Curr Opin Neurobiol 16 (6):710–715
Hasselmo ME, Sarter M (2011) Modes and models of forebrain cholinergic neuromodulation of cognition. Neuropsychopharmacology 36(1):52–73
Hatcher JP, Loudon JM, Hagan JJ, Clark MS (1998) Sabcomeline (SB-202026), a functionally selective M1 receptor partial agonist, reverses delay-induced deficits in the T-maze. Psychopharmacology 138:275–282
Heinrich JN, Butera JA, Carrick T, Kramer A, Kowal D, Lock T, Marquis KL, Pausch MH, Popiolek M, Sun SC, Tseng E, Uveges AJ, Mayer SC (2009) Pharmacological comparison of muscarinic ligands: historical versus more recent muscarinic M1-preferring receptor agonists. Eur J Pharmacol 605:53–56
Hellendall RP, Godfrey DA, Ross CD, Armstrong DM, Price JL (1986) The distribution of choline acetyltransferase in the rat amygdaloid complex and adjacent cortical areas, as determined by quantitative micro-assay and immunohistochemistry. J Comp Neurol 249:486–498
Hersch SM, Levey AI (1995) Diverse pre- and post-synaptic expression of m1-m4 muscarinic receptor proteins in neurons and afferents in the rat neostriatum. Life Sci 56:931–938
Hersch SM, Gutekunst CA, Rees HD, Heilman CJ, Levey AI (1994) Distribution of m1-m4 muscarinic receptor proteins in the rat striatum: light and electron microscopic immunocytochemistry using subtype-specific antibodies. J Neurosci 14:3351–3363
Higgs S, Deacon RMJ, Rawlins JNP (2000) Effects of scopolamine on a novel choice serial reaction time task. Eur J Neurosci 12:1781–1788
Hock C, Maddalena A, Heuser I, Naber D, Oertel W, von der Kammer H, Wienrich M, Raschig A, Deng M, Growdon JH, Nitsch RM (2000) Treatment with the selective muscarinic agonist talsaclidine decreases cerebrospinal fluid levels of total amyloid beta-peptide in patients with Alzheimer's disease. Ann N Y Acad Sci 920:285–291
Hock C, Maddalena A, Raschig A, Müller-Spahn F, Eschweiler G, Hager K, Heuser I, Hampel H, Müller-Thomsen T, Oertel W, Wienrich M, Signorell A, Gonzalez-Agosti C, Nitsch RM (2003) Treatment with the selective muscarinic m1 agonist talsaclidine decreases cerebrospinal fluid levels of A beta 42 in patients with Alzheimer's disease. Amyloid 10:1–6
Hodges H, Peters S, Gray JA, Hunter AJ (1999) Counteractive effects of a partial (sabcomeline) and a full (RS86) muscarinic receptor agonist on deficits in radial maze performance induced by S-AMPA lesions of the basal forebrain and medial septal area. Behav Brain Res 99:81–92
Hunter AJ, Roberts FF (1988) The effect of pirenzepine on spatial learning in the Morris Water Maze. Pharmacol Biochem Behav 30:519–523
Ichikawa T, Hirata Y (1986) Organization of choline acetyltransferase-containing structures in the forebrain of the rat. J Neurosci 6:281–292
Ince E, Ciliax BJ, Levey AI (1997) Differential expression of D1 and D2 dopamine and m4 muscarinic acetylcholine receptor proteins in identified striatonigral neurons. Synapse 27:357–366
Jacobson MA, Kreatsoulas C, Pascarella DM, O'Brien JA, Sur C (2010) The M1 muscarinic receptor allosteric agonists AC-42 and 1-[1′-(2-methylbenzyl)-1,4′-bipiperidin-4-yl]-1,3-dihydro-2H-

benzimidazol-2-one bind to a unique site distinct from the acetylcholine orthosteric site. Mol Pharmacol 78:648–657

Jäkälä P, Sirviö J, Jolkkonen J, Riekkinen P Jr, Acsady L, Riekkinen P (1992) The effects of p-chlorophenylalanine-induced serotonin synthesis inhibition and muscarinic blockade on the performance of rats in a 5-choice serial reaction time task. Behav Brain Res 51:29–40

Jo J, Son GH, Winters BL, Kim MJ, Whitcomb DJ, Dickinson BA, Lee YB, Futai K, Amici M, Sheng M, Collingridge GL, Cho K (2010) Muscarinic receptors induce LTD of NMDAR EPSCs via a mechanism involving hippocalcin, AP2 and PSD-95. Nat Neurosci 13 (10):1216–24

Jones CK, Shannon HE (2000) Muscarinic cholinergic modulation of prepulse inhibition of the acoustic startle reflex. J Pharmacol Exp Ther 294:1017–1023

Jones CK, Eberle EL, Shaw DB, McKinzie DL, Shannon HE (2005) Pharmacologic interactions between the muscarinic cholinergic and dopaminergic systems in the modulation of prepulse inhibition in rats. J Pharmacol Exp Ther 312:1055–1063

Jones CK, Brady AE, Davis AA, Xiang Z, Bubser M, Tantawy MN, Kane AS, Bridges TM, Kennedy JP, Bradley SR, Peterson TE, Ansari MS, Baldwin RM, Kessler RM, Deutch AY, Lah JJ, Levey AI, Lindsley CW, Conn PJ (2008) Novel selective allosteric activator of the M1 muscarinic acetylcholine receptor regulates amyloid processing and produces antipsychotic-like activity in rats. J Neurosci 28:10422–10433

Jones DN, Higgins GA (1995) Effect of scopolamine on visual attention in rats. Psychopharmacology (Berl) 120:142–149

Kane A (2008) The in vivo characterization of TBPB, a novel allosteric agonist of M1 muscarinic receptors: implications for the role of the M1 muscarinic receptor in treatment of schizophrenia. S08 NSC 296 Thesis Defense Vanderbilt University

Kim MG, Bodor ET, Wang C, Harden TK, Kohn H (2003) C(8) substituted 1-azabicyclo[3.3.1] non-3-enes and C(8) substituted 1-azabicyclo[3.3.1]nonan-4-ones: novel muscarinic receptor antagonists. J Med Chem 46:2216–2226

Kimura H, McGeer PL, Peng F, McGeer EG (1980) Choline acetyltransferase-containing neurons in rodent brain demonstrated by immunohistochemistry. Science 208:1057–1059

Klinkenberg I, Blokland A (2010) The validity of scopolamine as a pharmacological model for cognitive impairment. Neurosci Biobehav Rev 34:1307–1350

Kobayashi F, Yageta Y, Yamazaki T, Wakabayashi E, Inoue M, Segawa M, Matsuzawa S (2007) Pharmacological effects of imidafenacin (KRP-197/ONO-8025), a new bladder selective anticholinergic agent, in rats. Comparison of effects on urinary bladder capacity and contraction, salivary secretion and performance in the Morris water maze task. Arzneimittelforschung 57:147–154

Kozak R, Bruno JP, Sarter M (2006) Augmented prefrontal acetylcholine release during challenged attentional performance. Cereb Cortex 16:9–17

Lachowicz JE, Lowe D, Duffy RA, Ruperto V, Taylor LA, Guzik H, Brown J, Berger JG, Tice M, McQuade R, Kozlowski J, Clader J, Strader CD, Murgolo N (1999) SCH 57790: a novel M2 receptor selective antagonist. Life Sci 64:535–539

Langmead CJ, Austin NE, Branch CL, Brown JT, Buchanan KA, Davies CH, Forbes IT, Fry VA, Hagan JJ, Herdon HJ, Jones GA, Jeggo R, Kew JN, Mazzali A, Melarange R, Patel N, Pardoe J, Randall AD, Roberts C, Roopun A, Starr KR, Teriakidis A, Wood MD, Whittington M, Wu Z, Watson J (2008) Characterization of a CNS penetrant, selective M1 muscarinic receptor agonist, 77-LH-28-1. Br J Pharmacol 54:1104–1115

Langmead CJ, Fry VA, Forbes IT, Branch CL, Christopoulos A, Wood MD, Herdon HJ (2006) Probing the molecular mechanism of interaction between 4-n-butyl-1-[4-(2-methylphenyl)-4-oxo-1-butyl]-piperidine (AC-42) and the muscarinic M(1) receptor: direct pharmacological evidence that AC-42 is an allosteric agonist. Mol Pharmacol 69(1):236–46.

Lazareno S, Buckley NJ, Roberts FF (1990) Characterization of muscarinic M4 binding sites in rabbit lung, chicken heart, and NG108-15 cells. Mol Pharmacol 38:805–815

Leach K, Loiacono RE, Felder CC et al (2010) Molecular mechanisms of action and in vivo validation of an M4 muscarinic acetylcholine receptor allosteric modulator with potential antipsychotic properties. Neuropsychopharmacology 35:855–869

Leaton RN, Kreindler M (1972) Effects of physostigmine and scopolamine on operant brightness discrimination in the rat. Physiol Behav 9:121–123

Lebois EP, Bridges TM, Lewis LM, Dawson ES, Kane AS, Xiang Z, Jadhav SB, Yin H, Kennedy JP, Meiler J, Niswender CM, Jones CK, Conn PJ, Weaver CD, Lindsley CW (2010) Discovery and characterization of novel subtype-selective allosteric agonists for the investigation of M1 receptor function in the central nervous system. ACS Chem Neurosci 1:104–121

Lein ES, Hawrylycz MJ, Ao N, Ayres M, Bensinger A, Bernard A, Boe AF, Boguski MS, Brockway KS, Byrnes EJ, Chen L, Chen L, Chen TM, Chin MC, Chong J, Crook BE, Czaplinska A, Dang CN, Datta S, Dee NR, Desaki AL, Desta T, Diep E, Dolbeare TA, Donelan MJ, Dong HW, Dougherty JG, Duncan BJ, Ebbert AJ, Eichele G, Estin LK, Faber C, Facer BA, Fields R, Fischer SR, Fliss TP, Frensley C, Gates SN, Glattfelder KJ, Halverson KR, Hart MR, Hohmann JG, Howell MP, Jeung DP, Johnson RA, Karr PT, Kawal R, Kidney JM, Knapik RH, Kuan CL, Lake JH, Laramee AR, Larsen KD, Lau C, Lemon TA, Liang AJ, Liu Y, Luong LT, Michaels J, Morgan JJ, Morgan RJ, Mortrud MT, Mosqueda NF, Ng LL, Ng R, Orta GJ, Overly CC, Pak TH, Parry SE, Pathak SD, Pearson OC, Puchalski RB, Riley ZL, Rockett HR, Rowland SA, Royall JJ, Ruiz MJ, Sarno NR, Schaffnit K, Shapovalova NV, Sivisay T, Slaughterbeck CR, Smith SC, Smith KA, Smith BI, Sodt AJ, Stewart NN, Stumpf KR, Sunkin SM, Sutram M, Tam A, Teemer CD, Thaller C, Thompson CL, Varnam LR, Visel A, Whitlock RM, Wohnoutka PE, Wolkey CK, Wong VY, Wood M, Yaylaoglu MB, Young RC, Youngstrom BL, Yuan XF, Zhang B, Zwingman TA, Jones AR (2007) Genome-wide atlas of gene expression in the adult mouse brain. Nature 445(7124):168–76

Levey AI, Kitt CA, Simonds WF, Price DL, Brann MR (1991) Identification and localization of muscarinic acetylcholine receptor proteins in brain with subtype-specific antibodies. J Neurosci 11:3218–3226

Levey AI, Edmunds SM, Heilman CJ, Desmond TJ, Frey KA (1994) Localization of muscarinic m3 receptor protein and M3 receptor binding in rat brain. Neuroscience 63:207–221

Levey AI, Edmunds SM, Hersch SM, Wiley RG, Heilman CJ (1995a) Light and electron microscopic study of m2 muscarinic acetylcholine receptor in the basal forebrain of the rat. J Comp Neurol 351:339–356

Levey AI, Edmunds SM, Koliatsos V, Wiley RG, Heilman CJ (1995b) Expression of ml-m4 muscarinic acetylcholine receptor proteins in rat hippocampus and regulation by cholinergic innervation. J Neurosci 15:4077–4092

Li M, Yasuda RP, Wall SJ, Wellstein A, Wolfe BB (1991) Distribution of m2 muscarinic receptors in rat brain using antisera selective for m2 receptors. Mol Pharmacol 40:28–35

Loudon JM, Bromidge SM, Brown F, Clark MS, Hatcher JP, Hawkins J, Riley GJ, Noy G, Orlek BS (1997) SB 202026: a novel muscarinic partial agonist with functional selectivity for M1 receptors. J Pharmacol Exp Ther 283:1059–1068

Ma L, Seager MA, Wittmann M, Jacobson M, Bickel D, Burno M, Jones K, Graufelds VK, Xu G, Pearson M, McCampbell A, Gaspar R, Shughrue P, Danziger A, Regan C, Flick R, Pascarella D, Garson S, Doran S, Kreatsoulas C, Veng L, Lindsley CW, Shipe W, Kuduk S, Sur C, Kinney G, Seabrook GR, Ray WJ (2009) Selective activation of the M1 muscarinic acetylcholine receptor achieved by allosteric potentiation. Proc Natl Acad Sci U S A 106:15950–15955

Marino PJ, Conn PJ (2002) Direct and indirect modulation of the N-methyl-D-aspartate receptor: potential for the development of novel antipsychotic therapies. Curr Drug Targets CNS Neurol Disord 1:1–16

Marino MJ, Rouse ST, Levey AI, Potter LT, Conn PJ (1998) Activation of the genetically defined ml muscarinicreceptor potentiates N-methyl-D-aspartate (NMDA) receptor currents in hippocampal pyramidal cells. Proc Natl Acad Sci U S A 95:11465–11470

Matsuoka N, Maeda N, Ohkubo Y et al (1991) Differential effects of physostigmine and pilocarpine on the spatial memory deficits produced by two septo-hippocampal deafferentations in rats. Brain Res 559:233–240

McDonald AJ, Mascagni F (2010) Neuronal localization of m1 muscarinic receptor immunoreactivity in the rat basolateral amygdala. Brain Struct Funct 215:37–48
McDonough JH Jr (1982) Effects of anticholinergic drugs on DRL performance of rhesus monkeys. Pharmacol Biochem Behav 17:85–90
McGeer PL, McGeer EG, Kamo H, Wong K (1986) Positron emission tomography and the possible origins of cytopathology in Alzheimer's disease. Prog Neuropsychopharmacol Biol Psychiatry 10:501–818
Means LW, Holsten RD, Long M, High KM (1996) Scopolamine- and morphine-induced deficits in water maze alternation: failure to attenuate with glucose. Neurobiol Learn Mem 66:167–175
Mechawar N, Cozzari C, Descarries L (2000) Cholinergic innervation in adult rat cerebral cortex: a quantitative immunocytochemical description. J Comp Neurol 428:305–318
Mesulam MM, Mufson EJ, Wainer BH, Levey AI (1983) Central cholinergic pathways in the rat: an overview based on an alternative nomenclature (Ch1-Ch6). Neuroscience 10:1185–1201
M'Harzi M, Palou AM, Oberlander C, Barzaghi F (1995) Antagonism of scopolamine-induced memory impairments in rats by the muscarinic agonist RU 35,926 (CI-979). Pharmacol Biochem Behav 51:119–124
Mirza NR, Stolerman IP (2000) The role of nicotinic and muscarinic acetylcholine receptors in attention. Psychopharmacology (Berl) 148:243–250
Mishima K, Iwasaki K, Tsukikawa H, Matsumoto Y, Egashira N, Abe K, Egawa T, Fujiwara M (2000) The scopolamine-induced impairment of spatial cognition parallels the acetylcholine release in the ventral hippocampus in rats. Jpn J Pharmacol 84:163–173
Miyachi H, Kiyota H, Uchiki H, Segawa M (1999) Synthesis and antimuscarinic activity of a series of 4-(1-Imidazolyl)-2,2-diphenylbutyramides: discovery of potent and subtype-selective antimuscarinic agents. Bioorg Med Chem 7:1151–1161
Miyakawa T, Yamada M, Duttaroy A et al (2001) Hyperactivity and intact hippocampus-dependent learning in mice lacking the Ml muscarinic acetylcholine receptor. J Neurosci 21:5239–5250
Motter R, Vigo-Pelfrey C, Kholodenko D, Barbour R, Johnson-Wood K, Galasko D, Chang L, Miller B, Clark C, Green R et al (1995) Reduction of beta-amyloid peptide42 in the cerebrospinal fluid of patients with Alzheimer's disease. Ann Neurol 38:643–648
Myhrer T, Enger S, Aas P (2004) Cognitive side effects in rats caused by pharmacological agents used to prevent soman-induced lethality. Eur J Pharmacol 483:271–279
Nakahara N, Iga Y, Saito Y, Mizobe F, Kawanishi G (1989) Beneficial effects of FKS-508 (AF102B), a selective M1 agonist, on the impaired working memory in AF64A-treated rats. Jpn J Pharmacol 51:539–547
Nawaratne V, Leach K, Felder CC, Sexton PM, Christopoulos A (2010) Structural determinants of allosteric agonism and modulation at the M4 muscarinic acetylcholine receptor: identification of ligand-specific and global activation mechanisms. J Biol Chem 285:19012–19021
Newhouse PA, Sunderland T, Tariot PN, Weingartner H, Thomason K, Mellow AM et al (1988) The effects of acute scopolamine in geriatric depression. Arch Gen Psychiatry 45:906–912
Oakman SA, Faris PL, Kerr PE, Cozzari C, Hartman BK (1995) Distribution of pontomesencephalic cholinergic neurons projecting to substantia nigra differs significantly from those projecting to ventral tegmental area. J Neurosci 15:5859–5869
Oda S, Kuroda M, Kakuta S, Kishi K (2001) Differential immunolacalization of m2 and m3 muscarinic receptors in the anteroventral and anderodorsal thalamic nuclei of the rat. Brain Res 894:109–120
Oda S, Sato F, Okada A, Akahane S, Igarashi H, Yokofujita J, Yang J, Kuroda M (2007) Immunolocalization of muscarinic receptor subtypes in the reticular thalamic nucleus of rats. Brain Res Bull 74:376–384
Omelchenko N, Sesack SR (2006) Cholinergic axons in the rat ventral tegmental area synapse preferentially onto mesoaccumbens dopamine neurons. J Comp Neurol 494:863–875
O'Neill J, Fitten LJ, Siembieda D, Halgren E, Kim E, Fisher A, Perryman K (1998) Effects of AF102B and tacrine on delayed match-to-sample in monkeys. Prog Neuropsychopharmacol Biol Psychiatry 22:665–678

O'Neill J, Fitten LJ, Siembieda DW, Crawford KC, Halgren E, Fisher A, Refai D (1999) Divided attention-enhancing effects of AF102B and THA in aging monkeys. Psychopharmacology (Berl) 43:123–130

O'Neill J, Siembieda DW, Crawford KC, Halgren E, Fisher A, Fitten LJ (2003) Reduction in distractibility with AF102B and THA in the macaque. Pharmacol Biochem Behav 76:301–306

Osterholm RK, Camoriano JK (1982) Transdermal scopolamine psychosis. JAMA 247:3081

Pakarinen ED, Moerschbaecher JM (1993) Comparison of the effects of scopolamine and methylscopolamine on the performance of a fixed-ratio discrimination in squirrel monkeys. Pharmacol Biochem Behav 44:815–819

Palacios JM, Bolliger G, Closse A, Enz A, Gmelin G, Malanowski J (1986) The pharmacological assessment of RS 86 (2-ethyl-8-methyl-2,8-diazaspiro-[4,5]-decan-1,3-dion hydrobromide). A potent, specific muscarinic acetylcholine receptor agonist. Eur J Pharmacol 125:45–62

Pazzagli A, Pepeu G (1965) Amnesic properties of scopolamine and brain acetylcholine in the rat. Int J Neuropharmacol 4:291–299

Pepeu G, Giovannini MG (2010) Cholinesterase inhibitors and memory. Chem Biol Interact 187:403–408

Persson CM, Wallin AK, Levander S, Minthon L (2009) Changes in cognitive domains during three years in patients with Alzheimer's disease treated with donepezil. BMC Neurol 9:7

Phelps PE, Houser CR, Vaughn JE (1985) Immunocytochemical localization of choline acetyltransferase within the rat neostriatum: a correlated light and electron microscopic study of cholinergic neurons and synapses. J Comp Neurol 238:286–307

Pinault D, Deschênes M (1998) Anatomical evidence for a mechanism of lateral inhibition in the rat thalamus. Eur J Neurosci 10:3462–3469

Pitsikas N, Rigamonti AE, Cella SG, Locatelli V, Sala M, Muller EE (2001) Effects of molsidomine on scopolamine-induced amnesia and hypermotility in the rat. Eur J Pharmacol 426:193–200

Plummer KL, Manning KA, Levey AI, Rees HD, Uhlrich DJ (1999) Muscarinic receptor subtypes in the lateral geniculate nucleus: a light and electron microscopic analysis. J Comp Neurol 404:408–425

Poulin B, Butcher A, McWilliams P et al (2010) The M3-muscarinic receptor regulates learning and memory in a receptor phosphorylation/arrestin-dependent manner. Proc Natl Acad Sci U S A 107:9440–9445

Price JL, Stern R (1983) Individual cells in the nucleus basalis – diagonal band complex have restricted axonal projections to the cerebral cortex in the rat. Brain Res 269:352–356

Quirion R, Wilson A, Rowe W, Aubert I, Richard J, Doods H, Parent A, White N, Meaney MJ (1995) Facilitation of acetylcholine release and cognitive performance by an M(2)-muscarinic receptor antagonist in aged memory-impaired. J Neurosci 15:1455–1462

Riekkinen P Jr, Serviö J, Aaltonen M, Riekkinen P (1990) Effects of concurrent manipulations of nicotinic and muscarinic receptors on spatial avoidance learning. Pharmacol Biochem Behav 37:405–410

Robbins TW, Everitt BJ, Marston HM, Wilkinson J, Jones GH, Page KJ (1989a) Comparative effects of ibotenic acid- and quisqualic acid-induced lesions of the substantia innominata on attentional function in the rat: further implications for the role of the cholinergic neurons of the nucleus basalis in cognitive processes. Behav Brain Res 35:221–240

Robbins TW, Everitt BJ, Ryan CN, Marston HM, Jones GH, Page KJ (1989b) Comparative effects of quisqualic and ibotenic acid-induced lesions of the substantia innominata and globus pallidus on the acquisition of a conditional visual discrimination: differential effects on cholinergic mechanisms. Neuroscience 28:337–352

Rouse ST, Levey AI (1996) Expression of m1-m4 muscarinic acetylcholine receptor immunoreactivity in septohippocampal neurons and other identified hippocampal afferents. J Comp Neurol 375:406–416

Rouse ST, Levey AI (1997) Muscarinic acetylcholine receptor immunoreactivity after hippocampal commissural/associational pathway lesions: evidence for multiple presynaptic receptor subtypes. J Comp Neurol 380:382–394

Rouse ST, Levey AI (1998) Muscarinic acetylcholine receptor immunoreactivity after hippocampal commissural/associational pathway lesions: evidence for multiple presynaptic receptor subtypes. J Comp Neurol 380:382–394

Rouse ST, Marino MJ, Potter LT, Conn PJ, Levey AI (1999) Muscarinic receptor subtypes involved in hippocampal circuits. Life Sci 64:501–509

Rouse ST, Edmunds SM, Yi H, Gilmor ML, Levey AI (2000) Localization of M(2) muscarinic acetylcholine receptor protein in cholinergic and non-cholinergic terminals in rat hippocampus. Neurosci Lett 284:182–186

Rowe WB, O'Donnell JP, Pearson D, Rose GM, Meaney MJ, Quirion R (2003) Long-term effects of BIBN-99, a selective muscarinic M2 receptor antagonist, on improving spatial memory performance in aged cognitively impaired rats. Behav Brain Res 145:171–178

Rudy JW (1996) Scopolamine administered before and after training impairs both contextual and auditory-cue fear conditioning. Neurobiol Learn Mem 65:73–81

Rupniak NM, Steventon MJ, Field MJ et al (1989) Comparison of the effects of four cholinomimetic agents on cognition in primates following disruption by scopolamine or by lists of objects. Psychopharmacology 99:189–195

Rupniak NM, Samson NA, Tye SJ, Field MJ, Iversen SD (1991) Evidence against a specific effect of cholinergic drugs on spatial memory in primates. Behav Brain Res 43:1–6

Rupniak NM, Tye SJ, Iversen SD (1992) Comparison of the effects of selective and nonselective muscarinic agonists on cognition and thermoregulation in primates. J Neurol Sci 110:222–227

Rusted JM, Warburton DM (1988) The effects of scopolamine on working memory in healthy young volunteers. Psychopharmacology 96:145–152

Saint-Cyr JA, Taylor AE, Lang AE (1988) Procedural learning and neostriatal dysfunction in man. Brain 111:941–959

Sams AG, Hentzer M, Mikkelsen GK, Larsen K, Bundgaard C, Plath N, Christoffersen CT, Bang-Andersen B (2010) Discovery of N-{1-[3-(3-oxo-2,3-dihydrobenzo[1,4]oxazin-4-yl)propyl] piperidin-4-yl}-2-phenylacetamide (Lu AE51090): an allosteric muscarinic M1 receptor agonist with unprecedented selectivity and procognitive potential. J Med Chem 53:6386–6397

Satoh K, Fibiger HC (1986) Cholinergic neurons of the laterodorsal tegmental nucleus: efferent and afferent connections. J Comp Neurol 253:277–302

Savage UC, Faust WB, Lambert P, Moerschbaecher JM (1996) Effects of scopolamine on learning and memory in monkeys. Psychopharmacology (Berl) 123(1):9–14

Schäfer MK, Eiden LE, Weihe E (1998) Cholinergic neurons and terminal fields revealed by immunohistochemistry for the vesicular acetylcholine transporter. I. Central nervous system. Neuroscience 84:331–359

Schwarz RD, Callahan MJ, Coughenour LL, Dickerson MR, Kinsora JJ, Lipinski WJ, Raby CA, Spencer CJ, Tecle H (1999) Milameline (CI-979/RU35926): a muscarinic receptor agonist with cognition-activating properties: biochemical and in vivo characterization. J Pharmacol Exp Ther 291:812–822

Seeger T, Fedorova I, Zheng F et al (2004) M2 muscarinic acetylcholine receptor knock-out mice show deficits in behavioral flexibility, working memory, and hippocampal plasticity. J Neurosci 24:10117–10127

Semba K, Reiner PB, Fibiger HC (1990) Single cholinergic mesopontine tegmental neurons project to both the pontine reticular formation and the thalamus in the rat. Neuroscience 38:643–654

Shannon HE, Love PL (2005) Effects of antiepileptic drugs on attention as assessed by a five-choice serial reaction time task in rats. Epilepsy Behav 7:620–628

Shannon HE, Eberle EL (2006) Effects of biasing the location of stimulus presentation, and the muscarinic cholinergic receptor antagonist scopolamine, on performance of a 5-choice serial reaction time attention task in rats. Behav Pharmacol 17:71–85

Sheffler DJ, Williams R, Bridges TM, Xiang Z, Kane AS, Byun NE, Jadhav S, Mock MM, Zheng F, Lewis LM, Jones CK, Niswender CM, Weaver CD, Lindsley CW, Conn PJ (2009) A novel selective muscarinic acetylcholine receptor subtype 1 antagonist reduces seizures without impairing hippocampus-dependent learning. Mol Pharmacol 76:356–368

Shekhar A, Potter WZ, Lightfoot J et al (2008) Selective muscarinic receptor agonist xanomeline as a novel treatment approach for schizophrenia. Am J Psychiatry 165:1033–1039

Sherman SJ, Atri A, Hasselmo ME, Stern CE, Howard MW (2003) Scopolamine impairs human recognition memory: data and modeling. Behav Neurosci 117:526–539

Shirey JK, Brady AE, Jones PJ et al (2009) A selective allosteric potentiator of the M1 muscarinic acetylcholine receptor increases activity of medial prefrontal cortical neurons and restores impairments in reversal learning. J Neurosci 29:14271–14286

Sipos ML, Burchnell V, Galbicka G (2001) Effects of selected anticholinergics on acoustic startle response in rats. J Appl Toxicol 21(Suppl 1):S95–S101

Skjoldager P, Fowler SC (1991) Scopolamine attenuates the motor disruptions but not the attentional disturbances induced by haloperidol in a sustained attention task in the rat. Psychopharmacology (Berl) 105:93–100

Smith RD, Kistler MK, Cohen-Williams M, Coffin VL (1996) Cholinergic improvement of a naturally-occurring memory deficit in the young rat. Brain Res 707:13–21. doi:10.1016/0006-8993(95)01207-9

Spalding TA, Trotter C, Skjaerbaek N, Messier TL, Currier EA, Burstein ES, Li D, Hacksell U, Brann MR (2002) Discovery of an ectopic activation site on the M(l) muscarinic receptor. Mol Pharmacol 61:1297–1302

Spalding TA, Ma JN, Ott TR, Friberg M, Bajpai A, Bradley SR, Davis RE, Brann MR, Burstein ES (2006) Structural requirements of transmembrane domain 3 for activation by the M1 muscarinic receptor agonists AC-42, AC-260584, clozapine, and N-desmethylclozapine: evidence for three distinct modes of receptor activation. Mol Pharmacol 70:1974–1983

Spencer JP, Middleton LJ, Davies CH (2010) Investigation into the efficacy of the acetylcholinesterase inhibitor, donepezil, and novel procognitive agents to induce gamma oscillations in rat hippocampal slices. Neuropharmacology 59:437–443

Spinelli S, Ballard T, Feldon J, Higgins GA, Pryce CR (2006) Enhancing effects of nicotine and impairing effects of scopolamine on distinct aspects of performance in computerized attention and working memory tasks in marmoset monkeys. Neuropharmacology 51:238–250

Squire LR (1969) Effects of pretrial and posttrial administration of cholinergic and anticholinergic drugs on spontaneous alternation. J Comp Physiol Psychol 69:69–75

Stanhope KJ, Mirza NR, Bickerdike MJ, Bright JL, Harrington NR, Hesselink MB, Kennett GA, Lightowler S, Sheardown MJ, Syed R, Upton RL, Wadsworth G, Weiss SM, Wyatt A (2001) The muscarinic receptor agonist xanomeline has an antipsychotic-like profile in the rat. J Pharmacol Exp Ther 299:782–792

Steidl S, Yeomans JS (2009) M5 muscarinic receptor knockout mice show reduced morphine-induced locomotion but increased locomotion after cholinergic antagonism in the ventral tegmental area. J Pharmacol Exp Ther 328:263–275

Sunderland T, Linker G, Mirza N, Putnam KT, Friedman DL, Kimmel LH, Bergeson J, Manetti GJ, Zimmermann M, Tang B, Bartko JJ, Cohen RM (2003) Decreased beta-amyloid1-42 and increased tau levels in cerebrospinal fluid of patients with Alzheimer disease. JAMA 289:2094–2103

Sugaya K, Clamp C, Bryan D, McKinney M (1997) mRNA for the m4 muscarinic receptor subtype is expressed in adult rat brain cholinergic neurons. Brain Res Mol Brain Res 50:305–313

Sullivan NR, Leventhal L, Harrison J, Smith VA, Cummons TA, Spangler TB, Sun SC, Lu P, Uveges AJ, Strassle BW, Piesla MJ, Ramdass R, Barry A, Schantz J, Adams W, Whiteside GT, Adedoyin A, Jones PG (2007) Pharmacological characterization of the muscarinic agonist (3R,4R)-3-(3-hexylsulfanyl-pyrazin-2-yloxy)-1-aza-bicyclo[2.2.1]heptane (WAY-132983) in in vitro and in vivo models of chronic pain. J Pharmacol Exp Ther 322:1294–1304

Sunderland T, Tariot P, Weingartner H, Murphy D, Newhouse P, Mueller E et al (1986) Pharmacologic modeling of Alzheimer's disease. Prog Neuropsychopharmacol Biol Psychiatry 10:599–610

Suratman S, Leach K, Sexton P et al (2011) Impact of species variability and 'probe-dependence' on the detection and in vivo validation of allosteric modulation at the M4 muscarinic acetylcholine receptor. Br J Pharmacol 162:1659–1670

Strauss ME, Reynolds KS, Jayaram G, Tune LE (1990) Effects of anticholinergic medication on memory in schizophrenia. Schiz Res 3:127–129

Suzuki M, Yamaguchi T, Ozawa Y, Ohyama M, Yamamoto M (1995) Effects of (-)-S-2,8-dimethyl-3-methylene-1-oxa-8-azaspiro[4,5]decane L-tartrate monohydrate (YM796), a novel muscarinic agonist, on disturbance of passive avoidance learning behavior in drug-treated and senescence-accelerated mice. J Pharmacol Exp Ther 275(2):728–36.

Terry AV Jr, Buccafusco JJ, Borsini F, Leusch A (2002) Memory-related task performance by aged rhesus monkeys administered the muscarinic M(1)-preferring agonist, talsaclidine.Psychopharmacology (Berl) 162(3):292–300

Terry AV Jr, Parikh V, Gearhart DA, Pillai A, Hohnadel E, Warner S, Nasrallah HA, Mahadik SP (2006b) Time-dependent effects of haloperidol and ziprasidone on nerve growth factor, cholinergic neurons, and spatial learning in rats. J Pharmacol Exp Ther 318:709–724

Thienel R, Kellermann T, Schall U, Voss B, Reske M, Halfter S, Sheldrick AJ, Radenbach K, Habel U, Shah NJ, Kircher T (2009) Muscarinic antagonist effects on executive control of attention. Int J Neuropsychopharmacol 12:1307–1317

Thomsen M, Wess J, Fulton BS, Fink-Jensen A, Caine SB (2010) Modulation of prepulse inhibition through both M(1) and M (4) muscarinic receptors in mice. Psychopharmacology 208:401–416

Thomsen M, Woldbye DP, Wörtwein G, Fink-Jensen A, Wess J, Caine SB (2005) Reduced cocaine self-administration in muscarinic M5 acetylcholine receptor-deficient mice. J Neurosci 25(36):8141–9

Thomsen M, Wortwein G, Fink-Jensen A, Woldbye DP, Wess J, Caine SB (2007) Decreased prepulse inhibition and increased sensitivity to muscarinic, but not dopaminergic drugs in M5 muscarinic acetylcholine receptor knockout mice. Psychopharmacology (Berl) 192:97–110

Tsai G, Coyle JT (2002) Glutamatergic mechanisms in schizophrenia. Annu Rev Pharmacol Toxicol 42:165–79

Tzavara ET, Bymaster FP, Felder CC et al (2003) Dysregulated hippocampal acetylcholine neurotransmission and impaired cognition in M2, M4 and M2/M4 muscarinic receptor knockout mice. Mol Psychiatry 8:673–679

Tzavara ET, Bymaster FP, Davis RJ et al (2004) M4 muscarinic receptors regulate the dynamics of cholinergic and dopaminergic neurotransmission: relevance to the pathophysiology and treatment of related CNS pathologies. FASEB J 18:1410–1412

Ukai M, Okuda A, Mamiya T (2004) Effects of anticholinergic drugs selective for muscarinic receptor subtypes on prepulse inhibition in mice. Eur J Pharmacol 492:183–187

van der Zee EA, Keijser JN (2011) Localization of pre- and postsynaptic cholinergic markers in rodent forebrain: a brief history and comparison of rat and mouse. Behav Brain Res 221:356–366

van der Zee EA, Matsuyama T, Strosberg AD, Traber J, Luiten PG (1989) Demonstration of muscarinic acetylcholine receptor-like immunoreactivity in the rat forebrain and upper brainstem. Histochemistry 92:475–485

Vanover KE, Veinbergs I, Davis RE (2008) Antipsychotic-like behavioral effects and cognitive enhancement by a potent and selective muscarinic M-sub-1 receptor agonist, AC-260584. Behav Neurosci 122:570–575

Veroff AE, Bodick NC, Offen WW, Sramek JJ, Cutler NR (1998) Efficacy of xanomeline in Alzheimer disease: cognitive im-provement measured using the Computerized Neuro-psychological Test Battery (CNTB). Alzheimer Dis Assoc Disord 12:304–312

Vilaró MT, Palacios JM, Mengod G (1990) Localization of m5 muscarinic receptor mRNA in rat brain examined by in situ hybridization histochemistry. Neurosci Lett 114:154–159

Vilaró MT, Wiederhold KH, Palacios JM, Mengod G (1991) Muscarinic cholinergic receptors in the rat caudate-putamen and olfactory tubercle belong predominantly to the m4 class: in situ hybridization and receptor autoradiography evidence. Neuroscience 40:159–167

Vilaró MT, Wiederhold KH, Palacios JM, Mengod G (1992) Muscarinic M2 receptor mRNA expression and receptor binding in cholinergic and non-cholinergic cells in the rat brain: a correlative study using in situ hybridization histochemistry and receptor autoradiography. Neuroscience 47:367–393

Waelbroeck M (2003) Allosteric drugs acting act muscarinic acetylcholine receptors. Neurochem Res 28:419–422

Wall SJ, Yasuda RP, Hory F, Flagg S, Martin BM, Ginns EI, Wolfe BB (1991) Production of antisera selective for m1 muscarinic receptors using fusion proteins: distribution oof m1 receptors in rat brain. Mol Pharmacol 39:643–649

Warburton EC, Koder T, Cho K, Massey PV, Duguid G, Barker GR, Aggleton JP, Bashir ZI, Brown MW (2003) Cholinergic neurotransmission is essential for perirhinal cortical plasticity and recognition memory. Neuron 38:987–996

Watson J, Brough S, Coldwell MC, Gager T, Ho M, Hunter AJ, Jerman J, Middlemiss DN, Riley GJ, Brown AM (1998) Functional effects of the muscarinic receptor agonist, xanomeline, at 5-HT1 and 5-HT2 receptors. Br J Pharmacol 125:1413–1420

Weiner DM, Levey AI, Brann MR (1990) Expression of muscarinic acetylcholine and dopamine receptor mRNAs in rat basal ganglia. Proc Natl Acad Sci U S A 87:7050–7054

Wienrich M, Ceci A, Ensinger HA, Gaida W, Mendla KD, Osugi T, Raschig A, Weiser T (2002) Talsaclidine (WAL 2014 FU), a muscarinic M1 receptor agonist for the treatment of Alzheimer's disease. Drug Dev Res 56:321–334

Woolf NJ, Hernit MC, Butcher LL (1986) Cholinergic and non-cholinergic projections from the rat basal forebrain revealed by combined choline acetyltransferase and *Phaseolus vulgaris* leucoagglutinin immunohistochemistry. Neurosci Lett 66:281–286

Wu MF, Jenden DJ, Fairchild MD, Siegel JM (1993) Cholinergic mechanisms in startle and prepulse inhibition: effects of the false cholinergic precursor *N*-aminodeanol. Behav Neurosci 107:306–316

Yamada M, Lamping KG, Duttaroy A et al (2001) Cholinergic dilation of cerebral blood vessels is abolished in M5 muscarinic acetylcholine receptor knockout mice. Proc Natl Acad Sci U S A 98:14096–14101

Yamada M, Basile AS, Fedorova I, Zhang W, Duttaroy A, Cui Y, Lamping KG, Faraci FM, Deng CX, Wess J (2003) Novel insights into M5 muscarinic acetylcholine receptor function by the use of gene targeting technology. Life Sci 74:345–353

Yamasaki M, Matsui M, Watanabe M (2010) Preferential localization of muscarinic M1 receptor on dendritic shaft and spine of cortical pyramidal cells and its anatomical evidence for volume transmission. J Neurosci 30:4408–4418

Yasuda RP, Ciesla W, Flores LR, Wall SJ, Li M, Satkus SA, Weisstein JS, Spagnola BV, Wolfe BB (1993) Development of antisera selective for m4 and m5 muscarinic cholinergic receptors: distribution of m4 and m5 receptors in rat brain. Mol Pharmacol 43:149–157

Zang Z, Creese I (1997) Differential regulation of expression of rat hippocampal muscarinic receptor subtypes following fimbria-fornix lesion. Biochem Pharmacol 53:1379–1382

Zhang W, Basile AS, Gomeza J et al (2002) Characterization of central inhibitory muscarinic autoreceptors by the use of muscarinic acetylcholine receptor knock-out mice. J Neurosci 22:1709–1717

Muscarinic Agonists and Antagonists in Schizophrenia

Recent Therapeutic Advances and Future Directions

Amanda R. Bolbecker and Anantha Shekhar

Abstract Existing therapies for schizophrenia have limited efficacy, and significant residual positive, negative, and cognitive symptoms remain in many individuals with the disorder even after treatment with the current arsenal of antipsychotic drugs. Preclinical and clinical data suggest that selective activation of the muscarinic cholinergic system may represent novel therapeutic mechanisms for the treatment of schizophrenia. The therapeutic relevance of earlier muscarinic agonists was limited by their lack of receptor selectivity and adverse event profile arising from activation of nontarget muscarinic receptors. Recent advances in developing compounds that are selective to muscarinic receptor subtypes or activate allosteric receptor sites offer tremendous promise for therapeutic targeting of specific muscarinic receptor subtypes in schizophrenia.

Keywords Acetylcholine • Allosteric • Cholinergic • Cognitive • Muscarinic • Psychosis • Schizophrenia

1 Schizophrenia Symptoms, Treatment, and Challenges

Schizophrenia is a chronic, debilitating neuropsychiatric illness affecting nearly 1% of the population, often requiring lifelong treatment. Symptoms of schizophrenia are broadly categorized into three domains: positive, negative, and cognitive symptoms. The positive symptoms include hallucinations, delusions, and

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disorganized behavior; negative (or deficit) symptoms reflect the absence of normal social and motivational functioning, for example, avolition, alogia, anhedonia, and blunted affect; and cognitive symptoms of schizophrenia include impaired attention, executive functioning, and working memory. Early recognition that antagonism of central dopamine receptors reduces the positive symptoms of the disorder, combined with the evidence that stimulation of the dopamine system by drugs such as amphetamine could induce psychosis, established dopamine as the neurotransmitter central to the disorder and had profound effects on drug development strategies in schizophrenia. Indeed, all clinically available antipsychotic drugs antagonize central dopamine receptors. However, while dopamine's integral role in the positive symptoms of schizophrenia is uncontroversial (see Howes and Kapur 2009), this is not the case with the other symptom domains. Increasing evidence suggests that while antipsychotic drugs can alleviate psychotic symptoms for many individuals with schizophrenia, recovery is often incomplete, leaving patients with residual positive clinical symptoms. For example, some 25% of schizophrenia patients do not respond to dopamine-targeted therapies (Hirsch and Barnes 1995).

In addition, a particularly critical barrier to improving outcomes is the elusiveness of adequate treatments for negative and cognitive symptoms. Even more than the positive clinical symptoms, cognitive deficits contribute to impaired social functioning and poor quality of life (Williams et al. 2008), and predict deficits in occupational functioning (Bellack et al. 1999; Dickinson and Coursey 2002; Gold et al. 2002; Green 1996). Moreover, cognitive deficits are associated with the onset of psychosis in individuals at risk for schizophrenia (Frommann et al. 2010). Finally, in a recent prospective longitudinal analysis, Reichenberg et al. (2010) revealed premorbid neurocognitive deficits in a wide variety of domains in individuals who went on to develop schizophrenia, including executive function, visual and verbal learning and memory, processing speed, attention, visuospatial problem solving, and working memory. These recent studies further underscore the pressing need for novel therapies to improve treatment of schizophrenia.

2 Muscarinic Acetylcholine Receptor System and Schizophrenia

Central cholinergic neurotransmission has long been known to be crucial in CNS functioning. The muscarinic acetylcholine receptor (mAChR) system plays a significant role in memory, learning, arousal, motivation, reward, and attention. Significant evidence links abnormalities in the muscarinic system to the pathophysiology of schizophrenia (for review, see Raedler et al. 2007) and to a number of other debilitating neuropsychiatric illnesses including Alzheimer's disease (Winkler et al. 1998). Moreover, neurochemical, pharmacological, and neuropathological evidence suggests that selective targeting of mAChRs may hold therapeutic potential for schizophrenia (Friedman 2004; Raedler et al. 2007; Sellin et al. 2008; Conn et al. 2009).

2.1 Overview and Distribution of mAChRs

mAChRs are 7-transmembrane G-protein-coupled receptors (GPCRs). Five muscarinic receptor subtypes have been identified (M_1–M_5). The oddly numbered receptors, M_1, M_3, and M_5, are excitatory and couple predominantly through the $G_{q/11}$ pathway to stimulate phosphoinositide hydrolysis and increase intracellular calcium. M_2 and M_4 receptor subtypes have inhibitory effects and couple predominantly with $G_{i/o}$ proteins to inhibit cAMP. The anatomical distribution of these mAChRs places them in key dopamine pathways implicated in psychotic symptoms of schizophrenia, as well as in brain regions relevant to cognitive functioning, especially attention and memory. The M_1 and M_4 mAchR subtypes are concentrated heavily in the forebrain, including cerebral cortex, striatum, and hippocampus. M_4 receptors are also prominently expressed in the midbrain, where their interaction with midbrain dopaminergic mechanisms in VTA and striatum suggests that they influence dopamine release into the nucleus accumbens (Langmead et al. 2008a). The M_2 and M_3 receptor subtypes are located in the periphery as well as in the central nervous system (CNS) and are involved in parasympathetic functions including bronchoconstriction, salivation, smooth muscle relaxation, vasorelaxation, appetite, bradycardia, akinesia, and tremor. Within the brain, M_2 receptors are especially dense in forebrain and hippocampus where they regulate acetylcholine release (Billard et al. 1995; Stoll et al. 2003). Like M_1 receptors, M_4 receptors are also heavily concentrated in the cortex, striatum, and hippocampus. The M_5 receptor subtype is expressed primarily on dopaminergic neurons in the substantia nigra pars compacta, where it modulates dopamine release to the striatum (Weiner et al. 1990). It is also prominently expressed in the ventral tegmental area, which provides dopaminergic input to limbic structures such as the nucleus accumbens (Eglen 2005). However, dissociating the relative contributions of different muscarinic receptor subtypes within the CNS has been challenging, especially in the absence of subtype-specific ligands. Major obstacles include the fact that mAChRs are often co-localized within the same brain regions, often on the same cells (Levey et al. 1991), and sometimes with opposing actions. Of the 5 mAChRs, subtypes M_1 and M_4 have been the target of the most widespread interest for schizophrenia.

2.2 mAChR Manipulations Influence Symptoms of Schizophrenia

One source of evidence for a role of mAChRs in schizophrenia comes from observations that muscarinic antagonists can induce an “antimuscarinic syndrome” that includes psychotic features (Bolden et al. 1991). The hallucinations induced by muscarinic antagonists are remarkably similar in character to those experienced by individuals with schizophrenia (Yeomans 1995). Atropine, scopolamine, quinuclidinyl benzilate, ditran, and other centrally acting antimuscarinic agents

have been known to induce hallucinations in multiple sensory domains, as well as cognitive symptoms that bear marked resemblance to those observed in schizophrenia, including profound disturbances in attention and concentration, impaired memory, and confusion (Abood and Biel 1962; Granacher and Baldessarini 1975; Mego et al. 1988; Gershon and Olariu 1960; Neubauer et al. 1966; Clarke et al. 2004; Fisher 1991; Perry and Perry 1995; Fredrickson et al. 2008). Nonspecific muscarinic antagonists also induce learning and memory deficits in animals (Senda et al. 1997; Rasmussen et al. 2001). Finally, anticholinergic load is also associated with reduced cognitive function in schizophrenia (Minzenberg et al. 2004).

Clinical trials of mAChR agonists provide additional evidence for the putative role of mAChRs. Xanomeline, a relatively selective M_1/M_4 agonist, improved cognition and reduced psychotic symptoms in both schizophrenia (Shekhar et al. 2008) and Alzheimer's disease (Bodick et al. 1997). The nonspecific muscarinic agonist arecoline has also shown cognition-enhancing effects in Alzheimer's disease patients (Christie et al. 1981). Moreover, increasing evidence indicates that some atypical antipsychotics, namely, clozapine and olanzapine (Bymaster et al. 2003a), are partial muscarinic agonists, which has contributed to a new recognition that cholinergic facilitation may contribute to their cognition-enhancing and antipsychotic efficacy. Atypical antipsychotics drugs (i.e., ziprasidone, risperidone, clozapine, and olanzapine), but not conventional antipsychotics, increase acetylcholine release in prefrontal cortex (Ichikawa et al. 2002), which may be one mechanism by which these drugs exert their somewhat modest cognition-enhancing effects. This review will describe evidence suggesting that the muscarinic acetylcholine system is a compelling therapeutic target for schizophrenia, summarize recent progress in understanding the role specific muscarinic receptors may play in schizophrenia, and detail advances in selectively targeting receptors implicated in the pathophysiology of schizophrenia.

2.3 mAChR Abnormalities in Schizophrenia

The anatomy of the CNS cholinergic projections is consistent with a possible role in schizophrenia. The mesopontine cholinergic projection has been most associated with psychotic symptoms. It originates in the pedunculopontine and laterodorsal tegmental nuclei and projects most densely to thalamic nuclei, as well as to the substantia nigra and basal forebrain cholinergic nuclei, lateral hypothalamus, and limbic frontal cortex (Yeomans 1995). The basal forebrain cholinergic system, by virtue of its projection to hippocampal and cortical areas involved in learning and memory, has been strongly associated with cognition.

Notably, postmortem studies have suggested mAChR abnormalities in schizophrenia. Quantitative autoradiography studies measuring the binding of [(3)H] pirenzepine, a muscarinic antagonist that binds selectively to M_1 and M_4 receptors (Doods et al. 1987; Hulme et al. 1990), have consistently demonstrated reductions in the density of these muscarinic receptor subtypes in a number of brain regions

implicated in the pathophysiology of schizophrenia (Dean et al. 1996, 2000, 2002; Crook et al. 2000, 2001; Zavitsanou et al. 2004, 2005; Deng and Huang 2005; Newell et al. 2007; Scarr et al. 2007). For example, [(3)H]pirenzepine binding has revealed low mAChR binding density in prefrontal cortex from subjects with schizophrenia (Brodmann's areas 8, 9, 10, and 46); importantly, this decreased density is also observed in individuals with schizophrenia who had never been treated with anticholinergic drugs (i.e., benzotropine mesylate; Crook et al. 2001). Gene expression studies have also found decreased M_1 (Mancama et al. 2003; Dean et al. 2002) and M_4 (Dean et al. 2002) expression in prefrontal cortex in schizophrenia. In the hippocampus, while M_4 receptor expression levels were significantly decreased in schizophrenia, M_1 receptor levels were comparable to that in controls (Scarr et al. 2007). Reduced [(3)H]pirenzepine binding has also been reported in the anterior cingulate cortex in schizophrenia (Zavitsanou et al. 2004; Newell et al. 2007). A subsequent study by Zavitsanou et al. (2005) using the same cohort of participants tested in their 2004 study showed no differences between schizophrenia patients and other groups on [(3)H]AF-DX384 binding (Zavitsanou et al. 2005), which by inference implicates the M_1 receptor in the previously observed reduction in [^{3}H]pirenzepine binding. Schizophrenia patients also have decreased muscarinic receptor binding in the striatum (Dean et al. 1996, 2000) and throughout the hippocampal formation, including the dentate gyrus, areas CA1–CA4, subiculum, and the parahippocampal gyrus (Crook et al. 2000).

Given the almost ubiquitous exposure to antipsychotic drugs in the schizophrenia population, it is possible that alterations in muscarinic receptor density could be an artifact of medication use. However, in situ hybridization studies suggest that antipsychotic exposure is unlikely to underlie these findings. For example, in rats, M_1 mRNA expression *increased* in the substantia nigra, pars compacta, nucleus accumbens, and hippocampus following exposure to both typical and atypical antipsychotic drugs (Han et al. 2008). This finding supported an earlier study in which long-term exposure to antipsychotic drugs in rats either increased or had no effect on the density of [^{3}H]pirenzepine binding (Crook et al. 2001). Taken together, this evidence suggests that M_1 receptor alterations may be central to the pathophysiology of schizophrenia. It is also consistent with evidence that atypical antipsychotic drug actions at the M_1 receptor may play a critical role in their efficacy in schizophrenia. Similarly, with respect to the M_4 receptor, both typical and atypical antipsychotic drugs have either no effect or increase binding of [3H] pirenzepine and [3H]AF-DX384 (Crook et al.1999), suggesting that decreases in M_4 receptor density in schizophrenia is unlikely to be attributed to antipsychotic drug exposure.

Findings from a study by Raedler et al. (2003) further supported postmortem findings of reductions in muscarinic receptor density in schizophrenia in an in vivo study of 12 unmedicated patients using [I-123]iodoquinuclidinyl benzilate ([(123)I] IQNB) single photon emission computed tomography (SPECT). In comparison to healthy controls matched for gender and age, the schizophrenia group had significant reductions (ranging from ~20 to ~33%) in muscarinic receptor availability in the cortex, basal ganglia, and thalamus. These studies provide compelling evidence

that abnormalities in mAChRs, especially M_1 and M_4 subtypes, exist in schizophrenia independent of treatment effects from antipsychotic drugs.

3 Partially Selective mAChR Agonists

A number of relatively selective muscarinic agonists were developed in the 1990s, each of which preferentially activated either M_1 or M_4 (or both) subtypes. It has been suggested that compounds that selectively enhance M_1 activity are effective in treating cognitive deficits in schizophrenia, while M_4 agonism is effective in treating psychotic symptoms of the disorder (Felder et al. 2001; Bymaster et al. 2003a, b). Evidence in support of this hypothesis comes from studies showing that a number of these partially selective M_1 receptor agonists, including xanomeline, sabcomeline, and milameline, have also demonstrated efficacy in preclinical models of cognition (Bodick et al. 1997; Harries et al. 1998; Dean et al. 2003; Weiner et al. 2004). While the M1 receptor is primarily regarded as a target for enhancing cognition, preclinical studies also implicate this receptor in psychosis. For example, M1 knockout mice show disruptions in pre-pulse inhibition and increased locomotor activity (Gerber et al. 2001; Miyakawa et al. 2001). They also exhibit increased sensitivity to amphetamine and striatal dopamine release is increased twofold compared to wild-type mice, suggesting that M1 activation inhibits dopamine release (Gerber et al. 2001). Importantly, M1 deletion does not appear to result in upregulation of other muscarinic receptor subtypes (Miyakawa et al. 2001; Hamilton et al. 1997). In addition, studies showing that mAChR agonists with partial M_4 selectivity, such as BuTAC, PTAC, xanomeline, and sabcomeline, show efficacy in animal models of psychosis; specifically, they are able to inhibit dopamine agonist-induced behaviors such as conditioned avoidance responding, D_1 and D_2 dopamine agonist-induced rotation, and pre-pulse inhibition (Fink-Jensen et al. 1998; Jones et al. 2005; Rasmussen et al. 2001; Shannon et al. 1999; Bymaster et al. 1998). However, the particular contributions of M_1 versus M_4 to cognition and psychosis are still being elucidated.

Xanomeline has been of particular interest because it is a predominantly M_1/M_4 receptor partial agonist which has shown cognition-enhancing and antipsychotic-like properties. Xanomeline has been demonstrated to exhibit functional dopamine antagonism in vitro (Stanhope et al. 2001; Shannon et al. 2000). Xanomeline's particular affinity for M_1/M_4 receptors has made it of relatively greater interest for schizophrenia due to the suggestions that agonism at the M_1 receptor is relevant to cognitive deficits in the disorder, while M_4 agonism may reduce psychotic symptoms (Felder et al. 2000; Bymaster et al. 2003a, b). Consistent with this hypothesis, xanomeline decreases dopamine cell firing in the ventral tegmental area (Shannon et al. 2000) and increases extracellular levels of dopamine in the prefrontal cortex (Perry et al. 2001). In primates, xanomeline inhibits unrest and stereotypies induced by dopamine agonists (Andersen et al. 2003), in spite of having no affinity for dopamine receptors (Bymaster et al. 1994; 1997).

Of the partially selective muscarinic agonists, xanomeline is the only one to progress to a clinical trial in schizophrenia patients. A small study of xanomeline's efficacy in schizophrenia conducted by our group found statistically significant differences between xanomeline and placebo groups in several measures of learning and memory and PANSS total scores, as well as differences between groups in positive and negative symptom subscales and CGI scores in a randomized placebo-controlled, double-blind, 4-week study (Shekhar et al. 2008). Xanomeline demonstrated similar efficacy in an earlier, relatively large ($n = 343$) multisite clinical trial in patients with Alzheimer's-type dementia (Bodick et al. 1997). In that study, in addition to significant differences between groups on cognitive performance measures, individuals on xanomeline fared significantly better on behavioral measures including vocal outbursts, suspiciousness, delusions, agitation, and hallucinations; moreover, these improvements were dose dependent.

While xanomeline's efficacy in improving cognition and reducing psychotic symptoms in schizophrenia (Shekhar et al. 2008) provided an important proof of concept with respect to mAChRs as therapeutic targets in the disorder, its clinical utility could be limited due to adverse side effects elicited by its agonism at other receptor subtypes (Bodick et al. 1997; Bymaster et al. 1998; Sur and Kinney 2005), as is the case with other multiple muscarinic receptor agonists (Schwarz et al. 1999; Wienrich et al. 2001). These adverse side effects are believed to arise due to M_2 and M_3 receptor activation (Bymaster et al. 2003b, c; Bodick et al. 1997). Indeed, most of the available muscarinic agonists display affinity for most of the five receptor subtypes, with varying levels of selectivity for particular subtypes (Heinrich et al. 2009; Bradley et al. 2010) in spite of early reports suggesting better subtype selectivity. Somewhat conflicting results regarding the selectivity of these agonists are believed to have arisen because they were tested in cell lines where receptor reserve was low; but in native tissue studies, selectivity declined and multiple mAChR subtypes were activated, possibly due to higher receptor reserve and systemic differences in the actions of the various compounds (Conn et al. 2009).

4 M_1 and M_4 Allosteric Activators

The difficulty in designing compounds with true subtype specificity at mAChR orthosteric sites, i.e., the binding site of acetylcholine, derives from their highly conserved sequence homology across the five subtypes (Wess 1996), which has inhibited drug discovery efforts (Felder et al. 2000). Recently, an alternative approach targeting allosteric receptor sites has gained momentum. Allosteric activators enhance the actions of endogenous acetylcholine but bind at a poorly conserved site (removed from the orthosteric site). This approach has proven successful for GPCRs in other neurotransmitter systems including at metabotropic glutamate receptors (Rodriguez et al. 2005; Hemstapat et al. 2007). In the muscarinic system, allosteric activators with antipsychotic-like profiles have been reported for the M_1 receptor (Jones et al. 2008; Ma et al. 2009; Langmead et al.

2008b; Vanover et al. 2008; Bradley et al. 2010; Li et al. 2007, 2008) and the M_4 receptor (Shirey et al. 2008; Brady et al. 2008; Chan et al. 2008; Leach et al. 2010), and are now considered highly promising targets for drug discovery efforts (Christopoulos 2002; Conn et al. 2009).

This new drug development strategy focusing on agonists and potentiators for mAChRs, especially at the M_1 and M_4 receptors, may provide new therapeutic compounds capable of true selectivity with fewer side effects (Conn et al. 2009). Moreover, these allosteric agents could be invaluable in dissociating contributions of different muscarinic receptor subtypes. For example, preclinical models suggest that xanomeline's clinical efficacy is due to actions at either the M_1 or the M_4 receptor, or reciprocal interactions between these two receptor subtypes. However, the differential contributions of M_1 versus M_4 mAChRs to xanomeline's antipsychotic and pro-cognitive effects have been an enduring question, but the lack of subtype selective agents has impeded progress in understanding their specific roles (Brady et al. 2008). Below, the recent progress in developing more selective allosteric mAChR agonists and new knowledge derived from studies using these compounds are summarized.

4.1 Selective M_1 Allosteric Activators

Several M_1-selective allosteric agonists and potentiators have been developed recently that have therapeutic relevance for schizophrenia. M_1 is abundantly expressed in forebrain, especially striatum, hippocampus, and cortical regions (Levey et al. 1991; Wall et al. 1991; Levey 1993; Vilaro et al. 1993), all of which are implicated in the pathogenesis of schizophrenia. M_1 agonism has been specifically suggested as a potential treatment for cognitive impairment in schizophrenia (Friedman 2004), and compounds with varying degrees of selectivity for this receptor have shown efficacy in preclinical animal models of cognition (Bodick et al. 1997; Harries et al. 1998; Cui et al. 2008) and in clinical populations in which cognitive deficits are prominent features of the disorder including Alzheimer's disease (Bodick et al. 1997) and schizophrenia (Shekhar et al. 2008).

4.1.1 AC-42 and Analogs

AC-42 and its structural analogs 77-LH-28-01 and AC-260584 are potent and selective M_1 allosteric agonists as determined by calcium mobilization and inositol phosphate accumulation assays (Spalding et al. 2006; Langmead et al. 2008b; Heinrich et al. 2009; Bradley et al. 2010). These compounds have shown vast improvements in subtype selectivity over orthosteric agonists including xanomeline (Heinrich et al. 2009; Bradley et al. 2010) and have some affinity for D2 and 5HT2b receptors (Vanover et al. 2008; Bradley et al. 2010; Heinrich et al. 2009), a profile

that is consistent with atypical antipsychotic drugs and may confer advantages in this regard.

Results from a study by Langmead et al. (2008) suggest that among the AC-42 family, 77-LH-28-01 may be a better candidate for drug development relative to AC-42 due to its in vitro and in vivo M_1 receptor selectivity. In electrophysiological studies, 77-LH-28-1 showed a full agonist profile, stimulating hippocampal CA1 cell firing in single unit recordings ($pEC_{50} = 6.3$), while AC-42 did not. Carbachol initiated an almost identical response ($pEC_{50} = 5.7$) which was reversed by the M_1 receptor antagonist pirenzepine, suggesting that this effect was mediated by the M_1 receptor. This result also suggests higher potency and efficacy for 77-LH-28-01 relative to AC-42. 77-LH-28-01 also induced gamma oscillatory activity in hippocampus, which studies in knockout mice have demonstrated requires M_1 receptors (Fisahn et al. 2002), and disruptions in gamma oscillations have been linked to schizophrenia (Spencer et al. 2003, 2004) and cognition (Kaiser and Lutzenberger 1999.

Studies of in vitro and in vivo properties of AC-260584 have demonstrated that it has a pharmacological profile similar to that of atypical antipsychotic drugs and orthosteric muscarinic agonists in several important respects. For example, it preferentially increased acetylcholine and dopamine in medial prefrontal cortex compared to that in the nucleus accumbens (Li et al. 2007, 2008). Interestingly, *N*-desmethylclozapine, a metabolite of clozapine, was identified as an M_1 allosteric agonist (Sur et al. 2003), which may account for the pro-cognitive effects of clozapine in schizophrenia (Li et al. 2005; Spalding et al. 2006), and shared the ability of AC-260584 to induce acetylcholine release in the mPFC, an effect that was blocked by the M_1 antagonist telenzepine in the mPFC, but not in the nucleus accumbens (Li et al. 2005). This mPFC finding is consistent with the actions of partial M_1 agonists xanomeline and sabcomeline (Li et al. 2008) and to that of atypical antipsychotics, including clozapine and olanzapine, which increase extracellular dopamine and acetylcholine in the mPFC but not the nucleus accumbens (Kuroki et al. 1999; Ichikawa et al. 2002). Dopamine is believed to modulate critical aspects of prefrontal cortex-mediated working memory function that are compromised in schizophrenia (Braver and Cohen 2000), where dopaminergic hypofunction is believed to contribute to negative and cognitive symptoms of the disorder (Hill et al. 2004; Carter et al. 1998; Perlstein et al. 2001; Riehemann et al 2001; Weinberger et al. 1986; Wolkin et al. 1992; Andreasen et al. 1997). Taken together, these findings add to evidence that atypical antipsychotic drugs and less selective muscarinic agonists could mediate their cognitive effects through M_1 receptor-mediated cholinergic and dopaminergic modulation.

Behaviorally, AC-260584 has demonstrated an antipsychotic-like profile and improved cognitive performance (Vanover et al. 2008; Bradley et al. 2010). An antipsychotic-like profile was demonstrated by AC-260584's ability to reduce amphetamine- and MK-801-induced locomotor hyperactivity as well as reduce apomorphine-induced climbing behavior (Vanover et al. 2008). This finding, along with earlier findings that xanomeline also reduces amphetamine-induced hyperactivity in rodents (Stanhope et al. 2001) and primates (Andersen et al.

2003), suggests that M_1 agonism (versus M_4) may contribute to its antipsychotic effects more than previously believed. However, the activation of D2 and 5HT2b receptors (Vanover et al. 2008; Bradley et al. 2010; Heinrich et al. 2009) by AC-260584 makes it difficult to assess adequately the contribution of M_1 versus M_4 receptors to antipsychotic-like effects observed for compounds like xanomeline (Heinrich et al. 2009).

Preclinical studies have shown cognition-enhancing effects of AC-260584 in two animal models of learning and memory. AC-260584 also improved spatial memory on the Morris water maze (Vanover et al. 2008). Rats treated with AC-260584 demonstrated improved performance on the novel object recognition task, which was reversed by pirenzipene, a muscarinic antagonist (Bradley et al. 2010). $ERK_{1/2}$ phosphorylation, which is associated with important aspects of synaptic plasticity and learning and memory processes (Giovannini 2006), was increased by AC-260584 in hippocampal cells of wild-type but not M_1 knockout mice (Bradley et al. 2010). Moreover, it had low catalepsy rates (Vanover et al. 2008), which is predictive of low EPS in humans (Hoffman and Donovan 1995). Bradley et al. (2010) recently concluded that AC-260584 has high bioavailability, potency, and efficacy, and should serve as a lead compound for drug discovery efforts.

4.1.2 TPBP

Jones and colleagues (2008) recently reported that TPBP is a potent muscarinic allosteric agonist that has shown in vitro M_1 selectivity. TPBP showed robust agonist activity in M_1 transfected cell lines, but not in M_2–M_5 transfected cells. In hippocampal slices, TPBP increased NMDA receptor currents. This is consistent with findings from other studies indicating that this is an M_1-mediated effect. For example, M_1 receptors are co-localized with NMDA receptors in hippocampal neurons, and selective M_1 antagonists block carbachol-induced potentiation of NMDA current (Marino et al. 1998). NMDA-mediated long-term potentiation is enhanced by the muscarinic agonist carbachol in wild-type and M_3 knockout mice, but not in M_1 knockout mice (Shinoe et al. 2005). Potentiation of NMDARs is believed to be critical to synaptic plasticity underlying learning and memory (McBain and Mayer 1994), and is consistent with the finding that the AC-260584 induced $ERK_{1/2}$ phosphorylation in hippocampus (Bradley et al. 2010). Therefore, these effects support a role both for the M_1 receptor in cognition and for the efficacy of TPBP in enhancing cognitive deficits in schizophrenia. Importantly, NMDARs have also been implicated in psychosis, and potentiation of NMDAR current may be a mechanism by which muscarinic agonists mediate their antipsychotic effects (Marino and Conn 2002; Jones et al. 2008). Consistent with this hypothesis, TPBP reversed amphetamine-induced hyperactivity and demonstrated a FOS expression profile similar to both xanomeline and atypical antipsychotics, and these effects were achieved at doses that did not induce catalepsy (Jones et al. 2008).

Indeed, evidence increasingly suggests that in addition to pro-cognitive effects, M_1 receptor activation may also have antipsychotic effects (Vanover et al. 2008;

Mirza et al. 2003; Friedman 2004), consistent with the behavioral effects of TPBP. For example, M_1 knockout mice are hyperactive, most likely due to increased dopamine in the striatum (Wess et al. 2003; Gerber et al. 2001). Striatal hyperdopaminergia has been linked to acute psychotic states in schizophrenia (Schmitt Meisenzahl et al. 2009), striatal neurotransmission in schizophrenia (Gerber et al. 2001), suggesting that M_1 receptor abnormalities may play a role in psychosis.

4.1.3 BQCA

Benzylquinolone carboxylate (BQCA) is a potent and highly selective M_1 positive allosteric modulator that exhibits no agonist properties, but instead greatly enhances the potency of acetylcholine (Ma et al. 2009; Shirey et al. 2009). In wild-type mice but not in $M_1^{-/-}$ mice, oral administration of BQCA induced FOS activation in the cortex, hippocampus, and cerebellum, and significantly increased the ratio of phosophorylated ERK to total ERK (Ma et al. 2008). BQCA ALSO increased contextual fear conditioning in animals that were coadministered scopolamine, but the associative learning was blocked for animals receiving scopolamine only (Ma et al. 2009). The ability of BCQA to counteract the effects of scopolamine in this hippocampus-dependent task suggests that M_1 may enhance learning by reinforcing associative learning; however, M_1 receptors do not appear to be critical for contextual fear conditioning because M_1 knockout mice show no acquisition deficit on this task (Anagnostaras et al. 2003; Miyikawa et al. 2001), and the allosteric selective M_1 antagonist VUO255035 had no effect on contextual fear conditioning (Sheffler et al. 2009). BQCA increased the excitability of mPFC cells in slice preparations from wild-type but not M_1 null mutant mice, and improved impaired PFC-dependent reversal learning in a mouse model of Alzheimer's disease (Shirey et al. 2009).

BQCA, TPBP, and AC-42 all reduced amphetamine-induced hyperactivity; however, TBPB and AC-42 did not counteract scopolamine's effects on fear conditioning, which may be due to a different mechanism of action on the part of BQCA, as suggested by the ability of this drug to induce β-arrestin recruitment (Ma et al. 2009). If M_1 activation has both antipsychotic and pro-cognitive properties, it would be a particularly attractive target for schizophrenia-relevant therapies (Vanover et al. 2008). The finding that BQCA can mimic the antipsychotic-like profile of earlier allosteric M_1 activators and also exhibited pro-cognitive effects in contextual fear conditioning, an animal model of cognition suggests that it may have considerable advantages for treatment of schizophrenia.

4.2 Selective M_4 Allosteric Activators

The M_4 receptor is particularly relevant to schizophrenia for several reasons. This receptor is implicated in the regulation of dopamine levels in brain regions important in the pathophysiology of schizophrenia, including the nucleus accumbens

(Tzavara et al. 2004) and the striatum (Zhang et al. 2002a, b; Gomeza et al. 1999), where it is an inhibitory autoreceptor on cholinergic nerve terminals (Zhang et al. 2002a, b). The M_4 receptor is also believed to play a role in the antipsychotic properties of muscarinic agonists such as xanomeline (Mirza et al. 2003) as well as the atypical antipsychotic drug clozapine (Olianas et al. 1999), whose affinity for the M_4 receptor may be one source of its antipsychotic efficacy. Moreover, although the M_1 receptor has been emphasized as a possible mechanism mediating cognitive improvements observed following xanomeline administration (Felder et al. 2000; Bymaster et al. 2003b) and a wealth of evidence supports a role for this receptor in mediating various aspects of cognition, the presynaptic location of M_4 mAChRs excitatory neurons within the hippocampal formation (Rouse et al. 1999) suggests that they may modulate neurocognitive function as well. The finding that M_4 mRNA expression is decreased in schizophrenia but M_1 density being unchanged supports the argument that reductions in M_4 density may play an important role in learning and memory deficits observed in schizophrenia (Scarr et al. 2007).

4.2.1 VU010010 and Analogs

The first M_4 allosteric potentiator was reported by Shirey et al. (2008). VU010010 selectively enhanced the affinity of acetylcholine for the M_4 receptor and enhanced its efficacy. Recordings from hippocampal cells revealed that VU010010 potentiated carbachol's depression of excitatory postsynaptic potentials at schaffer collateral-CA1 synapses in wild-type but not M_4 knockout mice, suggesting a role for the M_4 receptor in mediating NMDA-mediated excitatory neutrotransmission. Further optimization of VU010010 led to the development of two additional potent and selective allosteric modulators of the M_4 receptor, VU0152099 and VU0152100, which have increased bioavailability and superior pharmacokinetic profiles (Brady et al. 2008; Conn et al. 2009). Both have no agonist effects at M_4, but instead potentiate the effects of acetylcholine. These molecules do not bind with other G-protein-coupled receptors, muscarinic or otherwise, and both potentiated M_4 response to acetylcholine as measured by enhanced calcium mobilization. Importantly, acetylcholine was more potent in the presence of these compounds as demonstrated by a dramatic increase in the ability of ACh to displace [3H] NMS. Behaviorally, both compounds reversed amphetamine-induced hyperactivity, demonstrating antipsychotic-like activity. This is consistent with evidence from M_4 knockout mice that M_4 receptors modulate cholinergic and dopaminergic neurotransmission and that loss of M_4 function results in hyperdopaminergia (Tzavara et al. 2004). In the midbrain, cholinergic excitation activates dopamine release, and data from M_4 knockout mice suggest that these mAChRs serve as inhibitory autoreceptors in the midbrain (Tzavara et al. 2004). Therefore, M_4 agonism could reduce acetylcholine release and subsequent overexcitation of midbrain dopamine neurons, which would decrease dopamine release in subcortical structures. This mechanism may provide an explanation for the antipsychotic-like profile of VU0152099 and VU0152100 (Brady et al. 2008) as well as the

antipsychotic properties of agents with partial M_4 selectivity, including clozapine (Olianas et al. 1999), xanomeline (Stanhope et al. 2001; Andersen et al. 2003; Mirza et al. 2003; Shekhar et al. 2008), and the M_2/M_4 preferring partial agonist PTAC (Fink-Jensen et al. 1998).

4.2.2 LY2033298

The Eli Lilly compound LY2033298 was recently identified as a highly potent (>40-fold increase in potency) and selective allosteric potentiator of M_4 receptors that acts primarily by increasing the affinity of acetylcholine for the M_4 receptor as well as demonstrating agonist activity (Chan et al. 2008; Leach et al. 2010). LY2033298 has shown efficacy in two animal models of psychosis; specifically, it attenuated conditioned avoidance responding and reversed apomorphine-induced disruptions of pre-pulse inhibition (Chan et al. 2008). A reduction in conditioned avoidance responding was also observed in M_4 knockout mice, but the effect was significantly smaller compared to that in wild-type mice (Leach et al. 2010). These findings are consistent with the finding that PTAC and BuTAC, which are M_2/M_4 partial agonists with $M_1/M_3/M_5$ antagonist properties, display antipsychotic-like profiles in animal models, including inhibition of conditioned avoidance responding (PTAC; Bymaster et al. 1998), inhibition of apomorphine-induced climbing, and impaired passive avoidance responding (BuTAC; Rasmussen et al. 2001). Taken together with evidence that M_4 modulates dopaminergic neurotransmission in regions implicated in positive symptoms of schizophrenia (Tzavara et al. 2004; Gomeza et al. 1999), the behavioral effects of LY2033298 provide additional evidence that M_4 agonist activity may be a viable novel therapeutic approach for psychotic symptoms of schizophrenia.

5 M_2 and M_5 mAChRs as Potential Therapeutic Targets

The focus on muscarinic receptor-focused therapies for schizophrenia has overwhelmingly focused on M_1 and M_4 receptors. However, there is intriguing, but limited, evidence that the M_2 and M_5 receptors may also be potential therapeutic targets.

5.1 M_2 Receptor

M_2 receptors are found throughout the brain and CNS, including the basal forebrain, where they act primarily as inhibitory autoreceptors, regulating acetylcholine release from forebrain projections including the hippocampus (Zhang et al. 2002a, b; Kitaichi et al. 1999a, b; Rouse et al. 1999, 2000) and cortex (Zhang et al. 2002a, b). The M_2 receptors have been implicated in cognitive and psychotic symptoms of

schizophrenia (Eglen 2005; Fisher 2008), but are especially believed to play a significant role in learning and memory due to their prominence in the hippocampus, where they are found pre- and postsynaptically.

Numerous studies have reported that M_2 receptor antagonists with various levels of selectivity have increased acetylcholine release in vitro in the hippocampus, cortex, and striatum (Billard et al. 1995; Wang et al. 2002; Carey et al. 2001; Quirion et al. 1995; Vannucchi et al. 1997), presumably through inhibition of this negative feedback mechanism. Corticostriatal recordings in rat slice preparations also showed that an antagonist of M_2-like receptors, methoctramine, facilitates striatal long-term potentiation (Calabresi et al. 2000). The finding that the M_2 selective antagonist SCH 55790 enhanced hippocampal, cortical, and striatal acetylcholine release (Carey et al. 2001) is consistent with reports of increased acetylcholine release in hippocampus and cortex in the presence of less selective M_2 antagonists such as BIBN-99 and AF-DX 384 (Quirion et al. 1995; Vannucchi et al. 1997). Interestingly, anatomical evidence of M_2 receptor localization to non-cholinergic neurons indicates that it also acts a presynaptic heteroreceptor (Rouse et al. 2000).

Behaviorally, a number of M_2 antagonists have shown pro-cognitive effects in animal models. For example, bilateral infusion of methoctramine into the dorsolateral striatum of rats improved performance on a memory task (Lazaris et al. 2003). The compound (+)-14 had high oral efficacy, was highly selective for the M_2 receptor, and significantly decreased passive avoidance response latency in young rats (Wang et al. 2002), a result also reported for the highly selective M_2 antagonist SCH 72788 (Lachowicz et al. 2001). Both SCH 57790 and BIBN-99 induced similar improvements in preclinical models of learning and memory (Carey et al. 2001; Rowe et al. 2003).

It should be noted, however, that in contrast to the findings that M_2 antagonism enhanced performance in pharmacological experiments, Seeger et al. (2004) found that M_2-deficient mice showed impaired learning on a hippocampus-dependent spatial learning task and impaired behavioral flexibility. In marked contrast to M_4 (Gerber et al. 2001) null mutant mice, M_2 knockout mice were not different from wild-type mice on locomotor activity, consistent with the hypothesis that of the two inhibitory mAChRs, M_4 has a greater role in regulating dopaminergic neurotransmission (Tzavara et al. 2004).

Antagonism of presynaptic M_2 receptors increases synaptic acetylcholine levels (Meyer and Otero 1985; Billard et al. 1995; Wang et al. 2002), which could lead to increased M_1 receptor activation (Fisher 2008). Thus, it has been hypothesized that M_2 antagonists may be a possible novel therapeutic direction for the improvement of cognitive impairment and psychotic symptoms (Eglen 2005; Clader and Wang 2005; Fisher 2008). However, to date no clinical studies of M_2 selective agonists have been conducted in schizophrenia. More seriously, although they could be efficacious in treating cognitive and psychotic symptoms in schizophrenia, enthusiasm for M_2-targeted therapies is limited due to their high expression in cardiac tissue (Caulfield 1993; Brodde and Michel 1999), which would likely necessitate

more specific CNS targeting than is currently available (Bymaster et al. 2002; Fisher 2008).

5.2 M_5 Receptor

Although the M_5 receptor is found in the cerebral cortex and hippocampus, it is especially predominant in the substantia nigra and ventral tegmental brain regions, where it is localized to dopaminergic neurons (Vilaró et al. 1990; Weiner et al. 1990). Its localization to the so-called "reward pathways" has prompted speculation that it may be an important target for treatment of schizophrenia (Mirza et al. 2003) as well as drug abuse (Raffa 2009; Basile et al. 2002; Fink-Jensen et al. 2003). Dysregulation of motivational drive is a central feature of schizophrenia, implicating M_5 receptors as potential targets for treatment in the disorder.

In addition to its probable role in modulating reward sensitivity, the M_5 receptor has also been implicated in tonic regulation of mesolimbic and striatal dopamine levels (Blaha et al. 1996; Zhang et al. 2002a, b; Basile et al. 2002; Forster and Blaha 2003). In addition, xanomeline's antipsychotic effects may be attributable in part to its partial agonism of the M_5 receptor in striatum, although M_4 is a more likely mechanism (Mirza et al. 2003). M_5-deficient mice retain phasic but not sustained dopamine release into the nucleus accumbens (Forster et al. 2002). M_5 receptors in VTA activate mesolimbic dopamine input to the nucleus accumbens (Yeomans et al. 2001), and M_5 receptor activation may result in sustained activation of dopaminergic neurons (Forster et al 2002). A study by the same group (Wang et al. 2004) reported that compared to control animals, M_5-deficient mice have improved latent inhibition and decreased amphetamine induced locomotor activity, consistent with reduced dopamine release in the nucleus accumbens. Given that earlier studies have reported that inactivation of dopamine terminals in the nucleus accumbens blocks amphetamine induced locomotion (Joyce and Koob 1981), and reduced nucleus accumbens dopamine activity results in increased latent inhibition (Joseph et al 2000; Moser et al 2000; Russig et al 2002; Gray et al 1997), it is probable that decreased dopamine release in the M_5-deficient mice produced these behavioral results. Taken together, these results suggest that antagonism at the M_5 receptor may reduce psychotic symptoms of schizophrenia by decreasing subcortical dopamine release.

To date, no clinical or preclinical studies of M_5 selective compounds have been undertaken. However, two recent reports have described M_5 allosteric modulators. The first such report characterized VU0238429, which displayed high selectivity ($>$30-fold) for the M_5 receptor in comparison to M_1 and M_3, and no potentiator activity in M_2 and M_4 receptor transfected cells (Bridges et al. 2009). The previous study found that VU0238429 increased the potency of acetylcholine, but had poor brain penetration. The second study described the allosteric properties of the antiarrythmia drug amiodarone, which was found to be an allosteric potentiator at

the M_5 receptor, but not M_1 receptors; interestingly, amiodarone enhanced acetylcholine's efficacy at the M_5 receptor, but not its potency (Stahl and Ellis 2010). Discovery of these molecules provides a significant breakthrough and should lead to additional chemical modifications, electrophysiological studies, and in vivo characterizations of M_5 selective modulators in order to gain additional insight into the role of this receptor in psychosis and addictive behavior.

6 Conclusion

As reviewed above, it has become increasingly evident that the muscarinic system is an attractive novel target for treating cognitive and psychotic symptoms of schizophrenia. The major obstacle to exploiting this receptor system's therapeutic promise has been the lack of selectivity for specific receptor subtypes. Therefore, to date, the few muscarinic agonists that have been tested in humans have shown efficacy, but more selective compounds could make this approach highly fruitful in developing new therapies for schizophrenia. New generations of allosteric activators targeting M_1 and M_4 receptors have now demonstrated improved selectivity and some preclinical evidence of antipsychotic-like and pre-cognitive effects. These compounds may offer substantial therapeutic benefit for the treatment of cognitive and psychotic symptoms of schizophrenia and could be entering clinical trials in the next few years.

References

Abood LG, Biel JH (1962) Anticholinergic psychototmimetic agents. Int Rev Neurobiol 4:217–273

Anagnostaras SG, Murphy GG, Hamilton SE et al (2003) Selective cognitive dysfunction in acetylcholine M_1 muscarinic receptor mutant mice. Nat Neurosci 6:51–58

Andersen MB, Fink-Jensen A, Peacock L et al (2003) The muscarinic M_1/M_4 receptor agonist xanomeline exhibits antipsychotic-like activity in *Cebus apella* monkeys. Neuropsychopharmacology 28:1168–1175

Andreasen NC, O'Leary DS, Flaum M et al (1997) Hypofrontality in schizophrenia: distributed dysfunctional circuits in neuroleptic-naive patients. Lancet 349:1730–1734

Basile AS, Fedorova I, Zapata A et al (2002) Deletion of the M_5 muscarinic acetylcholine receptor attenuates morphine reinforcement and withdrawal but not morphine analgesia. Proc Natl Acad Sci U S A 99:11452–11457

Bellack AS, Gold JM, Buchanan RW (1999) Cognitive rehabilitation for schizophrenia: problems, prospects, and strategies. Schizophr Bull 25:257–274

Billard W, Binch H 3rd, Crosby G et al (1995) Identification of the primary muscarinic autoreceptor subtype in rat striatum as M_2 through a correlation of in vivo microdialysis and in vitro receptor binding data. J Pharmacol Exp Ther 273:273–279

Blaha CD, Allen LF, Das S et al (1996) Modulation of dopamine efflux in the nucleus accumbens after cholinergic stimulation of the ventral tegmental area in intact, pedunculopontine tegmental nucleus-lesioned, and laterodorsal tegmental nucleus-lesioned rats. J Neurosci 16:714–722

Bodick NC, Offen WW, Levey AI et al (1997) (1997) Effects of xanomeline, a selective muscarinic receptor agonist, on cognitive function and behavioral symptoms in Alzheimer disease. Arch Neurol 54:465–473

Bolden C, Cusack B, Richelson E (1991) Clozapine is a potent and selective muscarinic antagonist at the five cloned human muscarinic acetylcholine receptors expressed in CHO-K1 cells. Eur J Pharmacol 192:205–206

Bradley SR, Lameh J, Ohrmund L et al (2010) AC-260584, an orally bioavailable M(1) muscarinic receptor allosteric agonist, improves cognitive performance in an animal model. Neuropharmacology 58:365–373

Brady AE, Jones CK, Bridges TM et al (2008) Centrally active allosteric potentiators of the M_4 muscarinic acetylcholine receptor reverse amphetamine-induced hyperlocomotor activity in rats. J Pharmacol Exp Ther 327:941–953

Braver TS, Cohen JD (2000) On the control of control: the role of dopamine in regulating prefrontal function and working memory. In: Monsell S, Driver J (eds) Control of cognitive processes: attention and performance XVIII. MIT Press, Cambridge

Bridges TM, Marlo JE, Niswender CM (2009) Discovery of the first highly M_5-preferring muscarinic acetylcholine receptor ligand, an M_5 positive allosteric modulator derived from a series of 5-trifluoromethoxy N-benzyl isatins. J Med Chem 52:3445–3448

Brodde OE, Michel MC (1999) Adrenergic and muscarinic receptors in the human heart. Pharmacol Rev 51:651–690

Bymaster FP, Wong DT, Mitch CH et al (1994) Neurochemical effects of the M_1 muscarinic agonist xanomeline (LY246708/NNC11-0232). J Pharmacol Exp Ther 269:282–289

Bymaster FP, Whitesitt CA, Shannon HE et al (1997) Xanomeline: a selective muscarinic agonist for the treatment of Alzheimers disease. Drug Dev Res 40:158–177

Bymaster FP, Shannon HE, Rasmussen K et al (1998) Unexpected antipsychotic-like activity with the muscarinic receptor ligand (5R,6R)6-(3-propylthio-1,2,5-thiadiazol-4-yl)-1-azabicyclo [3.2.1]octane. Eur J Pharmacol 356:109–119

Bymaster FP, Felder C, Ahmed S et al (2002) Muscarinic receptors as a target for drugs treating schizophrenia. Curr Drug Targets CNS Neurol Disord 1:163–181

Bymaster FP, Felder CC, Tzavara E (2003a) Muscarinic mechanisms of antipsychotic atypicality. Prog Neuropsychopharmacol Biol Psychiatry 27:1125–1143

Bymaster FP, McKinzie DL, Felder CC et al (2003b) Use of M_1-M_5 muscarinic receptor knockout mice as novel tools to delineate the physiological roles of the muscarinic cholinergic system. Neurochem Res 28:437–442

Bymaster FP, Carter PA, Yamada M et al (2003c) Role of specific muscarinic receptor subtypes in cholinergic parasympathomimetic responses, in vivo phosphoinositide hydrolysis, and pilocarpine-induced seizure activity. Eur J Neurosci 17:1403–1410

Calabresi P, Centonze D, Gubellini P et al (2000) Acetylcholine-mediated modulation of striatal function. Trends Neurosci 23:120–126

Carey GJ, Billard W, Binch H 3rd et al (2001) SCH 57790, a selective muscarinic M(2) receptor antagonist, releases acetylcholine and produces cognitive enhancement in laboratory animals. Eur J Pharmacol 431:189–200

Carter CS, Perlstein P, Ganguli R et al (1998) Functional hypofrontality and working memory dysfunction in schizophrenia. Am J Psychiatry 155:1285–1287

Caulfield MP (1993) Muscarinic receptors – characterization, coupling and function. Pharmacol Ther 58:319–379

Chan WY, McKinzie DL, Bose S, Mitchell SN, Witkin JM, Thompson RC, Christopoulos A, Lazareno S, Birdsall NJ, Bymaster FP, Felder CC. Allosteric modulation of the muscarinic M4 receptor as an approach to treating schizophrenia. Proc Natl Acad Sci USA. 2008 Aug 5;105 (31):10978–10983. Epub 2008 Aug 4. PubMed PMID: 18678919; PubMed Central PMCID: PMC2495016

Christie JE, Shering A, Ferguson J et al (1981) Physostigmine and arecoline: effects of intravenous infusions in Alzheimer presenile dementia. Br J Psychiatry 138:46–50

Christopoulos A (2002) Allosteric binding sites on cell-surface receptors: novel targets for drug discovery. Nat Rev Drug Discov 1:198–210

Clader JW, Wang Y (2005) Muscarinic receptor agonists and antagonists in the treatment of Alzheimer's disease. Curr Pharm Des 11:3353–3361

Clarke LA, Cassidy CW, Catalano G et al (2004) Psychosis induced by smoking cessation clinic administered anticholinergic overload. Ann Clin Psychiatry 16:171–175

Conn PJ, Jones CK, Lindsley CW (2009) Subtype-selective allosteric modulators of muscarinic receptors for the treatment of CNS disorders. Trends Pharmacol Sci 30:148–155

Crook JM, Tomaskovic-Crook E, Copolov DL et al (2000) Decreased muscarinic receptor binding in subjects with schizophrenia: a study of the human hippocampal formation. Biol Psychiatry 48:381–388

Crook JM, Tomaskovic-Crook E, Copolov DL et al (2001) Low muscarinic receptor binding in prefrontal cortex from subjects with schizophrenia: a study of Brodmann's areas 8, 9, 10, and 46 and the effects of neuroleptic drug treatment. Am J Psychiatry 158:918–925

Cui YH, Si W, Yin L et al (2008) A novel derivative of xanomeline improved memory function in aged mice. Neurosci Bull 24:251–257

Dean B, Crook JM, Opeskin K et al (1996) The density of muscarinic M_1 receptors is decreased in the caudate-putamen of subjects with schizophrenia. Mol Psychiatry 1:54–58

Dean B, Crook JM, Pavey G et al (2000) Muscarinic$_1$ and $_2$ receptor mRNA in the human caudate-putamen: no change in m1 mRNA in schizophrenia. Mol Psychiatry 5:203–207

Dean B, McLeod M, Keriakous D et al (2002) Decreased muscarinic1 receptors in the dorsolateral prefrontal cortex of subjects with schizophrenia. Mol Psychiatry 7:1083–1091

Dean B, Bymaster FP, Scarr E (2003) Muscarinic receptors in schizophrenia. Curr Mol Med 3:419–426

Deng C, Huang XF. Decreased density of muscarinic receptors in the superior temporal gyrusin schizophrenia. J Neurosci Res 2005 Sep 15;81(6):883–890. PubMed PMID: 16041805

Dickinson D, Coursey RD (2002) Independence and overlap among neurocognitive correlates of community functioning in schizophrenia. Schizophr Res 56:161–170

Doods HN, Mathy MJ, Davidesko D et al (1987) Selectivity of muscarinic antagonists in radioligand and in vivo experiments for the putative M_1, M_2 and M_3 receptors. J Pharmacol Exp Ther 242:257–262

Eglen RM (2005) Muscarinic receptor subtype pharmacology and physiology. Prog Med Chem 43:105–136

Felder CC, Bymaster FP, Ward J et al (2000) Therapeutic opportunities for muscarinic receptors in the central nervous system. J Med Chem 43:4333–4353

Fink-Jensen A, Kristensen P, Shannon HE et al (1998) Muscarinic agonists exhibit functional dopamine antagonism in unilaterally 6-OHDA lesioned rats. Neuroreport 9:3481–3486

Fink-Jensen A, Fedorova I, Wortwein G (2003) Role for M_5 muscarinic acetylcholine receptors in cocaine addiction. J Neurosci Res 74:91–96

Fisher CM (1991) Visual hallucinations on eye closure associated with atropine toxicity. A neurological analysis and comparison with other visual hallucinations. Can J Neurol Sci 18:18–27

Fisher A (2008) Cholinergic treatments with emphasis on M_1 muscarinic agonists as potential disease-modifying agents for Alzheimer's disease. Neurotherapeutics 5:433–442

Fisahn A, Yamada M, Duttaroy A, Gan JW, Deng CX, McBain CJ, Wess J. Muscarinic induction of hippocampal gamma oscillations requires coupling of the M1 receptor to two mixed cation currents. Neuron 2002 Feb 14;33(4):615–624. PubMed PMID:11856534

Forster GL, Blaha CD (2003) Pedunculopontine tegmental stimulation evokes striatal dopamine efflux by activation of acetylcholine and glutamate receptors in the midbrain and pons of the rat. Eur J Neurosci 17:751–762

Forster GL, Yeomans JS, Takeuchi J et al (2002) M_5 muscarinic receptors are required for prolonged accumbal dopamine release after electrical stimulation of the pons in mice. J Neurosci 22:RC190

Fredrickson A, Snyder PJ, Cromer J (2008) The use of effect sizes to characterize the nature of cognitive change in psychopharmacological studies: an example with scopolamine. Hum Psychopharmacol 23:425–436

Friedman JI (2004) Cholinergic targets for cognitive enhancement in schizophrenia: focus on cholinesterase inhibitors and muscarinic agonists. Psychopharmacology (Berl) 174:45–53

Frommann I, Pukrop R, Brinkmeyer J et al (2011) Neuropsychological profiles in different at-risk states of psychosis: executive control impairment in the early – and additional memory dysfunction in the late – prodromal state. Schizophr Bull 37(4):861–873

Gerber DJ, Sotnikova TD, Gainetdinov RR et al (2001) Hyperactivity, elevated dopaminergic transmission, and response to amphetamine in M_1 muscarinic acetylcholine receptor-deficient mice. Proc Natl Acad Sci U S A 98:15312–15317

Gershon S, Olariu J (1960) JB 329 degrees—a new spychotomimetic, its antagonism by tetrahydroaminacrin and its comparison with LSD, mescaline and sernyl. J Neuropsychiatr 1:283–292

Giovannini MG (2006) The role of the extracellular signal-regulated kinase pathway in memory encoding. Rev Neurosci 17:619–634

Gold JM, Goldberg RW, McNary SW et al (2002) Cognitive correlates of job tenure among patients with severe mental illness. Am J Psychiatry 159:1395–1402

Gomeza J, Zhang L, Kostenis E et al (1999) Enhancement of D1 dopamine receptor-mediated locomotor stimulation in M_4 muscarinic acetylcholine receptor knockout mice. Proc Natl Acad Sci U S A 96:10483–10488

Granacher RP, Baldessarini RJ (1975) Physostigmine. Its use in acute anticholinergic syndrome with antidepressant and antiparkinson drugs. Arch Gen Psychiatry 32:375–380

Gray JA, Moran PM, Grigoryan G (1997) Latent inhibition: the nucleus accumbens connection revisited. Behav Brain Res 88:27–34

Green MF (1996) What are the functional consequences of neurocognitive deficits in schizophrenia? Am J Psychiatry 153:321–330

Hamilton SE, Loose MD, Qi M et al (1997) Disruption of the M_1 receptor gene ablates muscarinic receptor-dependent M current regulation and seizure activity in mice. Proc Natl Acad Sci U S A 94:13311–13316

Han M, Newell K, Zavitsanou K et al (2008) Effects of antipsychotic medication on muscarinic M_1 receptor mRNA expression in the rat brain. J Neurosci Res 86:457–464

Harries MH, Samson NA, Cilia J et al (1998) The profile of sabcomeline (SB-202026), a functionally selective M_1 receptor partial agonist, in the marmoset. Br J Pharmacol 124:409–415

Heinrich JN, Butera JA, Carrick T et al (2009) Pharmacological comparison of muscarinic ligands: historical versus more recent muscarinic M_1-preferring receptor agonists. Eur J Pharmacol 605:53–56

Hemstapat K, Da Costa H, Nong Y et al (2007) A novel family of potent negative allosteric modulators of group II metabotropic glutamate receptors. J Pharmacol Exp Ther 322:254–264

Hill K, Mann L, Laws KR et al (2004) Hypofrontality in schizophrenia: a meta-analysis of functional imaging studies. Acta Psychiatr Scand 110:243–256

Hirsch S, Barnes TRE (1995) The clinical treatment of schizophrenia with antipsychotic medication. In: Hirsch SR, Weinberger DR (eds) Schizophrenia. Blackwell Science, Oxford

Hoffman DC, Donovan H (1995) Catalepsy as a rodent model for detecting antipsychotic drugs with extrapyramidal side effect liability. Psychopharmacology 120:128–133

Howes OD, Kapur S (2009) The dopamine hypothesis of schizophrenia: version III – the final common pathway. Schizophr Bull 35:549–562

Hulme EC, Birdsall NJ, Buckley NJ (1990) Muscarinic receptor subtypes. Annu Rev Pharmacol Toxicol 30:633–673

Ichikawa J, Dai J, O'Laughlin IA et al (2002) Atypical, but not typical, antipsychotic drugs increase cortical acetylcholine release without an effect in the nucleus accumbens or striatum. Neuropsychopharmacology 26:325–339

Jones CK, Eberle EL, Shaw DB et al (2005) Pharmacologic interactions between the muscarinic cholinergic and dopaminergic systems in the modulation of prepulse inhibition in rats. J Pharmacol Exp Ther 312:1055–1063

Jones CK, Brady AE, Davis AA et al (2008) Novel selective allosteric activator of the M_1 muscarinic acetylcholine receptor regulates amyloid processing and produces antipsychotic-like activity in rats. J Neurosci 28:10422–10433

Joseph MH, Peters SL, Moran PM et al (2000) Modulation of latent inhibition in the rat by altered dopamine transmission in the nucleus accumbens at the time of conditioning. Neuroscience 101:921–930

Joyce EM, Koob GF (1981) Amphetamine-, scopolamine- and caffeine-induced locomotor activity following 6-hydroxydopamine lesions of the mesolimbic dopamine system. Psychopharmacology (Berl) 73:311–313

Kaiser J, Lutzenberger W (2005) Cortical oscillatory activity and the dynamics of auditory memory processing. Rev Neurosci 16:239–254

Kitaichi K, Hori T, Srivastava LK et al (1999a) Antisense oligodeoxynucleotides against the muscarinic M_2, but not M_4, receptor supports its role as autoreceptors in the rat hippocampus. Brain Res Mol Brain Res 67:98–106

Kitaichi K, Day JC et al (1999b) A novel muscarinic M(4) receptor antagonist provides further evidence of an autoreceptor role for the muscarinic M(2) receptor sub-type. Eur J Pharmacol 383:53–56

Kuroki T, Meltzer HY, Icikawa J (1999) Effects of antipsychotic drugs on extracellular dopamine levels in rat medial prefrontal cortex and nucleus accumbens. J Pharmacol Exp Ther 288:774–781

Lachowicz JE, Duffy RA, Ruperto V, Kozlowski J, Zhou G, Clader J, Billard W, Binch H 3rd, Crosby G, Cohen-Williams M, Strader CD, Coffin V. Facilitation of acetylcholine release and improvement in cognition by a selective M2 muscarinic antagonist, SCH 72788. Life Sci 2001 Apr 27;68(22–23):2585–2592. PubMed PMID: 11392630

Lazaris A, Bertrand F, Lazarus C, Galani R, Stemmelin J, Poirier R, Kelche C, Cassel JC. Baseline and 8-OH-DPAT-induced release of acetylcholine in the hippocampus of aged rats with different levels of cognitive dysfunction. Brain Res 2003 Mar 28;967(1–2):181–190. PubMed PMID: 12650979

Langmead CJ, Austin NE, Branch CL (2008a) Characterization of a CNS penetrant, selective M_1 muscarinic receptor agonist, 77-LH-28-1. Br J Pharmacol 154:1104–1115

Langmead CJ, Watson J, Reavill C (2008b) Muscarinic acetylcholine receptors as CNS drug targets. Pharmacol Ther 117:232–243

Leach K, Loiacono RE, Felder CC, McKinzie DL, Mogg A, Shaw DB, Sexton PM, Christopoulos A. Molecular mechanisms of action and in vivo validation of an M_4 muscarinic acetylcholine receptor allosteric modulator with potential antipsychotic properties. Neuropsychopharmacology. 2010 Mar; 35(4):855–869. Epub 2009 Nov 25. PubMed PMID: 19940843; PubMed Central PMCID: PMC3055367

Levey AI (1993) Immunological localization of M1–M5 muscarinic acetylcholine receptors in peripheral tissues and brain. Life Sci 52:441–448

Levey AI, Kitt CA, Simonds WF et al (1991) Identification and localization of muscarinic acetylcholine receptor proteins in brain with subtype-specific antibodies. J Neurosci 11:3218–3226

Li Z, Huang M, Ichikawa J et al (2005) N-desmethylclozapine, a major metabolite of clozapine, increases cortical acetylcholine and dopamine release in vivo via stimulation of M_1 muscarinic receptors. Neuropsychopharmacology 30:1986–1995

Li Z, Bonhaus DW, Huang M et al (2007) AC260584 (4-[3-(4-butylpiperidin-1-yl)-propyl]-7-fluoro-4H-benzo[1,4]oxazin-3-one), a selective muscarinic M_1 receptor agonist, increases acetylcholine and dopamine release in rat medial prefrontal cortex and hippocampus. Eur J Pharmacol 572:129–137

Li Z, Snigdha S, Roseman AS et al (2008) Effect of muscarinic receptor agonists xanomeline and sabcomeline on acetylcholine and dopamine efflux in the rat brain; comparison with effects of 4-[3-(4-butylpiperidin-1-yl)-propyl]-7-fluoro-4H-benzo[1,4]oxazin-3-one (AC260584) and N-desmethylclozapine. Eur J Pharmacol 596:89–97

Ma L, Seager MA, Wittmann M, Jacobson M, Bickel D, Burno M, Jones K, Graufelds VK, Xu G, Pearson M, McCampbell A, Gaspar R, Shughrue P, Danziger A, Regan C, Flick R, Pascarella D, Garson S, Doran S, Kreatsoulas C, Veng L, Lindsley CW, Shipe W, Kuduk S, Sur C, Kinney G, Seabrook GR, Ray WJ. Selective activation of the M_1 muscarinic acetylcholine receptor achieved by allosteric potentiation. Proc Natl Acad Sci USA 2009 Sep 15;106 (37):15950–15955. Epub 2009 Aug 26. Erratum in: Proc Natl Acad Sci USA 2009 Oct 20;106(42):18040. Seager, Matthew [corrected to Seager, Matthew A]. PubMed PMID: 19717450; PubMed Central PMCID:PMC2732705

Mancama D, Arranz MJ, Landau S et al (2003) Reduced expression of the muscarinic 1 receptor cortical subtype in schizophrenia. Am J Med Genet B Neuropsychiatr Genet 119B:2–6

Marino MJ, Conn PJ (2002) Direct and indirect modulation of the N-methyl D-aspartate receptor. Curr Drug Target CNS Neurol Disord 1:1–16

Marino MJ, Rouse ST, Levey AI et al (1998) Activation of the genetically defined M_1 muscarinic receptor potentiates N-methyl-D-aspartate (NMDA) receptor currents in hippocampal pyramidal cells. Proc Natl Acad Sci U S A 95:11465–11470

McBain CJ, Mayer ML (1994) N-methyl-D-aspartic acid receptor structure and function. Physiol Rev 74:723–760

Mego DM, Omori JM, Hanley JF (1988) Transdermal scopolamine as a cause of transient psychosis in two elderly patients. South Med J 81:394–395

Meyer EM, Otero DH (1985) Pharmacological and ionic characterizations of the muscarinic receptors modulating [3H]acetylcholine release from rat cortical synaptosomes. J Neurosci 5:1202–1207

Minzenberg MJ, Poole JH, Benton C et al (2004) Association of anticholinergic load with impairment of complex attention and memory in schizophrenia. Am J Psychiatry 161:116–124

Mirza N, Peters D, Sparks RG (2003) Xanomeline and the antipsychotic potential of muscarinic receptor subtype selective agonists. CNS Drug Rev 9:159–186

Miyakawa T, Yamada M, Duttaroy A, Wess J. Hyperactivity and intact hippocampus-dependent learning in mice lacking the M1 muscarinic acetylcholine receptor. J Neurosci 2001 Jul 15;21 (14):5239–5250. PubMed PMID: 11438599

Moser PC, Hitchcock JM, Lister SD et al (2000) The pharmacology of latent inhibition as an animal model of schizophrenia. Brain Res Rev 33:275–307

Neubauer H, Gershon S, Sundland DM (1966) Differential responses to an anticholinergic psychotomimetic (Ditran) in a mixed psychiatric population. Psychiatr Neurol (Basel) 151:65–80

Newell KA, Zavitsanou K, Jew SK et al (2007) Alterations of muscarinic and GABA receptor binding in the posterior cingulate cortex in schizophrenia. Prog Neuropsychopharmacol Biol Psychiatry 31:225–233

Olianas MC, Maullu C, Onali P (1999) Mixed agonist-antagonist properties of clozapine at different human cloned muscarinic receptor subtypes expressed in Chinese hamster ovary cells. Neuropsychopharmacology 20:263–270

Perlstein WM, Carter CS, Noll DC et al (2001) Relation of prefrontal cortex dysfunction to working memory and symptoms in schizophrenia. Am J Psychiatry 158:1105–1113

Perry EK, Perry RH (1995) Acetylcholine and hallucinations: disease-related compared to drug-induced alterations in human consciousness. Brain Cogn 28:240–258

Perry KW, Nisenbaum LK, George CA et al (2001) The muscarinic agonist xanomeline increases monoamine release and immediate early gene expression in the rat prefrontal cortex. Biol Psychiatry 49:716–725

Quirion R, Wilson A, Rowe W et al (1995) Facilitation of acetylcholine release and cognitive performance by an M(2)-muscarinic receptor antagonist in aged memory-impaired. J Neurosci 15:1455–1462

Raedler TJ, Knable MB, Jones DW et al (2003) In vivo determination of muscarinic acetylcholine receptor availability in schizophrenia. Am J Psychiatry 160:118–127

Raedler TJ, Bymaster FP, Tandon R et al (2007) Towards a muscarinic hypothesis of schizophrenia. Mol Psychiatry 12:232–246

Raffa RB (2009) The M_5 muscarinic receptor as possible target for treatment of drug abuse. J Clin Pharm Ther 34:623–629

Rasmussen T, Fink-Jensen A, Sauerberg P et al (2001) The muscarinic receptor agonist BuTAC, a novel potential antipsychotic, does not impair learning and memory in mouse passive avoidance. Schizophr Res 49:193–201

Reichenberg A, Caspi A, Harrington H et al (2010) Static and dynamic cognitive deficits in childhood preceding adult schizophrenia: a 30-year study. Am J Psychiatry 167:160–169

Riehemann S, Volz HP, Stützer P (2001) Hypofrontality in neuroleptic-naive schizophrenic patients during the Wisconsin card sorting test – a fMRI study. Eur Arch Psychiatry Clin Neurosci 251(2):66–71

Rodriguez AL, Nong Y, Sekaran NK (2005) A close structural analog of 2-methyl-6-(phenylethynyl)-pyridine acts as a neutral allosteric site ligand on metabotropic glutamate receptor subtype 5 and blocks the effects of multiple allosteric modulators. Mol Pharmacol 68:1793–1802

Rouse ST, Marino MJ, Potter LT et al (1999) Muscarinic receptor subtypes involved in hippocampal circuits. Life Sci 64:501–509

Rouse ST, Edmunds SM, Yi H (2000) Localization of M(2) muscarinic acetylcholine receptor protein in cholinergic and non-cholinergic terminals in rat hippocampus. Neurosci Lett 284:182–186

Rowe WB, O'Donnell JP, Pearson D et al (2003) Long-term effects of BIBN-99, a selective muscarinic M_2 receptor antagonist, on improving spatial memory performance in aged cognitively impaired rats. Behav Brain Res 145:171–178

Russig H, Murphy CA, Feldon J (2002) Clozapine and haloperidol reinstate latent inhibition following its disruption during amphetamine withdrawal. Neuropsychopharmacology 26:765–777

Scarr E, Sundram S, Keriakous D (2007) Altered hippocampal muscarinic M_4, but not M_1, receptor expression from subjects with schizophrenia. Biol Psychiatr 61:1161–1170

Schwarz RD, Callahan MJ, Coughenour LL et al (1999) Milameline (CI-979/RU35926): a muscarinic receptor agonist with cognition-activating properties: biochemical and in vivo characterization. J Pharmacol Exp Ther 291:812–822

Seeger T, Fedorova I, Zheng F et al (2004) M_2 muscarinic acetylcholine receptor knock-out mice show deficits in behavioral flexibility, working memory, and hippocampal plasticity. J Neurosci 24:10117–10127

Sellin AK, Shad M, Tamminga C (2008) Muscarinic agonists for the treatment of cognition in schizophrenia. CNS Spectr 13:985–996

Senda T, Matsuno K, Kobayashi T et al (1997) Reduction of scopolamine-induced impairment of passive-avoidance performance by s-receptor agonist in mice. Physiol Behav 61:257–264

Shannon HE, Hart JC, Bymaster FP et al (1999) Muscarinic receptor agonists, like dopamine receptor antagonist antipsychotics, inhibit conditioned avoidance response in rats. J Pharmacol Exp Ther 290:901–907

Shannon HE, Rasmussen K, Bymaster FP et al (2000) Xanomeline, an M(1)/M(4) preferring muscarinic cholinergic receptor agonist, produces antipsychotic-like activity in rats and mice. Schizophr Res 42:249–259

Shekhar A, Potter WZ, Lightfoot J et al (2008) Selective muscarinic receptor agonist xanomeline as a novel treatment approach for schizophrenia. Am J Psychiatry 165:1033–1039

Sheffler DJ, Williams R, Bridges TM, Xiang Z, Kane AS, Byun NE, Jadhav S, Mock MM, Zheng F, Lewis LM, Jones CK, Niswender CM, Weaver CD, Lindsley CW, Conn PJ. A novel selective muscarinic acetylcholine receptor subtype 1 antagonist reduces seizures without impairing hippocampus-dependent learning. Mol Pharmacol 2009 Aug;76(2):356–368. Epub 2009 Apr 30. PubMed PMID: 19407080; PubMed Central PMCID: PMC2713127

Shirey JK, Xiang Z, Orton D et al (2008) An allosteric potentiator of M_4 mAChR modulates hippocampal synaptic transmission. Nat Chem Biol 4:42–50

Shirey JK, Brady AE, Jones PJ et al (2009) A selective allosteric potentiator of the M_1 muscarinic acetylcholine receptor increases activity of medial prefrontal cortical neurons and restores impairments in reversal learning. J Neurosci 29:14271–14286

Shinoe T, Matsui M, Taketo MM, Manabe T. Modulation of synaptic plasticity by physiological activation of M1 muscarinic acetylcholine receptors in the mouse hippocampus. J Neurosci 2005 Nov 30;25(48):11194–11200. PubMed PMID: 16319319

Schmitt GJ, Meisenzahl EM, Dresel S, Tatsch K, Rossmuller B, Frodl T, Preuss UW, Hahn K, Moller HJ. Striatal dopamine D2 receptor binding of risperidone in schizophrenic patients as assessed by 123I-iodobenzamide SPECT: a comparative study with olanzapine. J Psychopharmacol 2002 Sep;16(3):200–206. PubMed PMID: 12236625

Spalding TA, Ma J-N, Ott TR et al (2006) Structural requirements of transmembrane domain 3 for activation by the M_1 muscarinic receptor agonists AC-42, AC-260584, clozapine, and N-desmethylclozapine: evidence for three distinct modes of receptor activation. Mol Pharmacol 70:1974–1983

Spencer KM, Nestor PG, Niznikiewicz MA et al (2003) Abnormal neural synchrony in schizophrenia. J Neurosci 23:7407–7411

Spencer KM, Nestor PG, Perlmutter R et al (2004) Neural synchrony indexes disordered perception and cognition in schizophrenia. Proc Natl Acad Sci U S A 101:17288–17293

Stanhope KJ, Mirza NR, Bickerdike MJ et al (2001) The muscarinic receptor agonist xanomeline has an antipsychotic-like profile in the rat. J Pharmacol Exp Ther 299:782–792

Stoll C, Schwarzwälder U, Johann S et al (2003) Characterization of muscarinic autoreceptors in the rabbit hippocampus and caudate nucleus. Neurochem Res 28:413–417

Stahl E, Ellis J. Novel allosteric effects of amiodarone at the muscarinic M5 receptor. J Pharmacol Exp Ther 2010 Jul;334(1):214–222. Epub 2010 Mar 26. PubMed PMID: 20348203; PubMed Central PMCID: PMC2912050

Sur C, Kinney GG (2005) Selective Targeting of Muscarinic Receptors: Novel Therapeutic Approaches for Psychotic Disorders. Current Neuropharmacology 3:63–71

Sur C, Mallorga PJ, Wittmann M et al (2003) N-desmethylclozapine, an allosteric agonist at muscarinic 1 receptor, potentiates N-methyl-D-aspartate receptor activity. Proc Natl Acad Sci USA 100:13674–13679

Tzavara ET, Bymaster FP, Davis RJ et al (2004) M_4 muscarinic receptors regulate the dynamics of cholinergic and dopaminergic neurotransmission: relevance to the pathophysiology and treatment of related CNS pathologies. FASEB J 18:1410–1412

Vannucchi MG, Pepeu G. Muscarinic receptor modulation of acetylcholine release from rat cerebral cortex and hippocampus. Neurosci Lett 1995 Apr 28;190(1):53–56. PubMed PMID: 7624055

Vannucchi MG, Scali C, Kopf SR et al (1997) Selective muscarinic antagonists differentially affect in vivo acetylcholine release and memory performances of young and aged rats. Neuroscience 79:837–846

Vanover KE, Veinbergs I, Davis RE (2008) Antipsychotic-like behavioral effects and cognitive enhancement by a potent and selective muscarinic M-sub-1 receptor agonist, AC-260584. Behav Neurosci 122:570–575

Vilaro MT, Palacios JM, Mengod G (1994) Multiplicity of muscarinic autoreceptor subtypes? Comparison of the distribution of cholinergic cells and cells containing mRNA for five subtypes of muscarinic receptors in the rat brain. Brain Res Mol Brain Res 21:30–46

Vilaró MT, Palacios JM, Mengod G (1990) Localization of M_5 muscarinic receptor mRNA in rat brain examined by in situ hybridization histochemistry. Neurosci Lett 114:154–159

Wall SJ, Yasuda RP, Hory F (1991) Production of antisera selective for M_1 muscarinic receptors using fusion proteins: distribution of M_1 receptors in rat brain. Mol Pharmacol 39:643–649

Wang Y, Chackalamannil S, Hu Z et al (2002) Improving the oral efficacy of CNS drug candidates: discovery of highly orally efficacious piperidinyl piperidine M_2 muscarinic receptor antagonists. J Med Chem 45:5415–5418

Wang H, Ng K, Hayes D et al (2004) Decreased amphetamine-induced locomotion and improved latent inhibition in mice mutant for the M_5 muscarinic receptor gene found in the human 15q schizophrenia region. Neuropsychopharmacology 29:2126–2139

Weinberger DR, Berman KF, Zec RF (1986) Physiologic dysfunction of dorsolateral prefrontal cortex in schizophrenia: I. Regional cerebral blood flow evidence. Arch Gen Psychiatry 43:114–124

Weiner DM, Levey AI, Brann MR (1990) Expression of muscarinic acetylcholine and dopamine receptor mRNAs in rat basal ganglia. Proc Natl Acad Sci U S A 87:7050–7054

Weiner DM, Meltzer HY, Veinbergs I et al (2004) The role of M_1 muscarinic receptor agonism of N-desmethylclozapine in the unique clinical effects of clozapine. Psychopharmacol 177:207–216

Wess J (1996) Molecular biology of muscarinic acetylcholine receptors. Crit Rev Neurobiol 10:69–99

Wess J, Duttaroy A, Zhang W et al (2003) M_1-M_5 muscarinic receptor knockout mice as novel tools to study the physiological roles of the muscarinic cholinergic system. Receptors Channels 9:279–290

Wienrich M, Ceci A, Ensinger HA et al (2002) Talsaclidine (WAL 2014 FU): a muscarinic M_1 receptor agonist for the treatment of Alzheimer's disease. Drug Develop Res 56:321–334

Williams LM, Whitford TJ, Flynn G et al (2008) General and social cognition in first episode schizophrenia: identification of separable factors and prediction of functional outcome using the IntegNeuro test battery. Schizophr Res 99:182–191

Winkler J, Thal LJ, Gage FH et al (1998) Cholinergic strategies for Alzheimer's disease. J Mol Med 76:555–567

Wolkin A, Sanfilipo M, Wolf AP et al (1992) Negative symptoms and hypofrontality in chronic schizophrenia. Arch Gen Psychiatry 49:959–965

Yeomans JS (1995) Role of tegmental cholinergic neurons in dopaminergic activation, antimuscarinic psychosis and schizophrenia. Neuropsychopharmacology 12:3–16

Yeomans JS, Forster G, Blaha C (2001) M_5 muscarinic receptors are needed for slow activation of dopamine neurons and for rewarding brain stimulation. Life Sci 68:2449–2456

Zavitsanou K, Katsifis A, Mattner F et al (2004) Investigation of M_1/M_4 muscarinic receptors in the anterior cingulate cortex in schizophrenia, bipolar disorder, and major depression disorder. Neuropsychopharmacology 29:619–625

Zavitsanou K, Katsifis A, Yu Y et al (2005) M_2/M_4 muscarinic receptor binding in the anterior cingulate cortex in schizophrenia and mood disorders. Brain Res Bull 65:397–403

Zhang W, Yamada M, Gomeza J et al (2002a) Multiple muscarinic acetylcholine receptor subtypes modulate striatal dopamine release, as studied with M_1-M_5 muscarinic receptor knockout mice. J Neurosci 22:6347–6352

Zhang W, Basile AS, Gomeza J et al (2002b) Characterization of central inhibitory muscarinic autoreceptors by the use of muscarinic acetylcholine receptor knock-out mice. J Neurosci 22:1709–1717

Muscarinic Pain Pharmacology: Realizing the Promise of Novel Analgesics by Overcoming Old Challenges

Dennis F. Fiorino and Miguel Garcia-Guzman

Abstract The antinociceptive and analgesic effects of muscarinic receptor ligands in human and nonhuman species have been evident for more than half a century. In this review, we describe the current understanding of the roles of different muscarinic subtypes in pain modulation and their mechanism of action along the pain signaling pathway, including peripheral nociception, spinal cord pain processing, and supraspinal analgesia. Extensive preclinical and clinical validation of these mechanisms points to the development of selective muscarinic agonists as one of the most exciting and promising avenues toward novel pain medications.

Keywords Adrenergic • Agonist • Antagonist • Anticholinesterase • Antinociception • GPCR • G protein-coupled receptor • Knockout • Mouse • Narcotic • Opioid • Peripheral nerve fiber • Rat • Spinal cord • Supraspinal

Abbreviations

4-DAMP	4-Diphenyl-acetoxy-*N*-methyl-piperidine methiodide
ACh	Acetylcholine
ALCAR	Acetyl-L-carnitine
aODN	Antisense oligodeoxyribonucleotide
ATP	Adenosine triphosphate
BHK	Baby hamster kidney

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BuTAC	[5*R*-(exo)]-6-[4-butylthio-1,2,5-thiadiazol-3-yl]-1-azabicyclo-[3.2.1]-octane
CFA	Complete Freund's adjuvant
CGRP	Calcitonin gene related peptide
CRPS	Complex regional pain syndrome
DRG	Dorsal root ganglion
EPSC	Excitatory postsynaptic current
GABA	Gamma-aminobutyric acid
GTPγS	Guanosine 5′-*O*-[gamma-thio]triphosphate
HC-3	Hemicolinium-3
i.c.v.	Intracerebroventricular
i.t.	Intrathecal
IB4	Isolectin B4
IPSC	Inhibitory postsynaptic current
mAChR	Muscarinic receptor
MT-3	Muscarinic toxin 3
MT-7	Muscarinic toxin 7
NMS	*N*-methylscopolamine
NRM	Nucleus raphe magnus
NSAIDs	Nonsteroidal anti-inflammatory agents
OXO	Oxotremorine
OXO-M	Oxotremorine methiodide
PAG	Periaqueductal gray
PAM	Positive allosteric modulator
PTX	Pertussis toxin
QRT-PCR	Quantitative real-time polymerase chain reaction
RT-PCR	Reverse transcription polymerase chain reaction
RVM	Rostral ventromedial medulla
SCS	Spinal cord stimulation
siRNA	Small interfering ribonucleic acid
STZ	Streptozotocin
substituted TZTP	3-3(Substituted-1,2,5-thiadiazol-4-yl)-12,5,6-tetrahydro-1-methyl pyridine
TNF	Tumor necrosis factor
TRPV1	Transient vanilloid receptor 1; the capsaicin receptor
TTX	Tetrodotoxin
WT	Wild-type

1 Muscarinic Receptors and Pain

Pain is an unpleasant sensory and emotional experience that emerges as a normal response to injury and is a symptom of many diseases. What makes pain different from other sensory modalities is that the stimuli that evoke pain can change or become dissociated from pain in a chronic setting and pain itself becomes a disease. Insufficient management of pain in the hospital setting may have a profound impact

on the subsequent treatment of chronic pain. For example, it is estimated that chronic postoperative pain develops in 10% of surgical patients and becomes an intolerable, intractable chronic pain condition after 1 of every 100 operations, regardless of type of surgery (Kehlet et al. 2006; Katz and Seltzer 2009; Burke and Shorten 2009). Strong, unrelieved pain immediately after surgery and lack of movement after surgery are well-documented risk factors for chronic pain after surgery. Only epidural analgesia and continuous peripheral nerve block analgesia can effectively reduce pain provoked by mobilization thus reducing the risk to develop chronic postoperative pain. Better pain management in the acute hospital setting is critical for the effective treatment or prevention of chronic pain and would be greatly facilitated by the use of effective analgesics that are alternatives to current standard treatment, such as narcotics which carry a high risk of addiction (Breivik and Stubhaug 2008; Popping et al. 2008).

Pain imposes a tremendous burden on society, costing approximately US$1 trillion per year in medical treatment, loss of productivity and disability payments in developed countries (Schappert 1994; Stewart et al. 2003). Not surprisingly, pain is by far the main reason leading to visits to primary care physicians: in 2008, over 100 million adults in the United States – 51% of the estimated 215 million population aged 20 years and older – reported pain at one or more body sites including the joints, low back, neck, face/jaw, or experienced dental pain or headaches/migraines (National Health and Nutrition Examination Survey, 2004).

Despite marked advances in understanding the pathophysiology of pain, and in the context of an urgent medical need to develop safe and efficacious analgesics to treat acute and chronic pain, progress by the pharmaceutical industry in exploiting new mechanisms for clinical efficacy has been limited. Apart from new migraine treatments, the repositioning of existing antidepressants and antiepileptics medication as analgesics, and the development of extended-release opioids preparations, or topical use of old analgesics, pain treatments have changed little since the introduction of nonsteroidal anti-inflammatory agents (NSAIDs) more than three decades ago. There are many reasons for this lack of progress, including our limited understanding of the pathophysiology of human pain and the significant influence of the nonsensory processes of emotion, cognition, and culture on pain (Celestin et al. 2009; Papaioannou et al. 2009; Craig 2003). Although many fundamental mechanisms of pain transmission and pathophysiology appear to be conserved and translate across species, recognizing the limitations of nonhuman animal research is important. Clear risks are taken on when advancing a new mechanism to the clinic based mainly on efficacy observed in nonhuman animals. Until new advances in our understanding of human pathological pain and approaches for better translation to the clinic are achieved, the most reliable path for developing analgesics remains the exploitation of mechanisms with demonstrated efficacy in humans. Among these mechanisms, the activation of muscarinic receptors constitutes one of the most promising strategies to develop novel analgesic agents (Tata 2008; Eisenach 1999; Jones and Dunlop 2007). It is well known that cholinergic stimulation and the subsequent activation of spinal muscarinic receptors (Eisenach 2009) can lead to robust analgesia in humans, demonstrated by administration of cholinesterase inhibitors to treat postoperative pain, labor analgesia (Habib and Gan 2006), and

cancer pain (Lauretti et al. 1999). Indeed, the first clinical proof of concept for the analgesic effects of cholinergic agonists was reported more than 75 years ago (*see* Hartvig et al. 1989). The use of genetic knockouts (Wess 2003; Wess et al. 2003a, b, 2007) has led to a better understanding of the receptor subtypes that are important for pain modulation, and new assay tools and chemistry can now enable the design and optimization of ligands with high subtype selectivity (Avlani et al. 2010) that have the potential to specifically activate muscarinic receptors involved in pain modulation while avoiding those responsible for dose-limiting parasympathetic effects (Stengel et al. 2000; Wess et al. 2007).

This review will describe the current understanding of the roles of different muscarinic subtypes in pain modulation and mechanisms of action along the pain pathway, including peripheral nociception, spinal cord pain processing, and supraspinal analgesia. Among these mechanisms, spinal activation of muscarinic receptors and its role in pain modulation will be described in detail. Nociceptive processing at the level of the spinal cord has been observed in both preclinical models and humans and forms the basis for effective translation of analgesic efficacy to humans.

Acetylcholine (ACh) exerts many of its physiological effects via activation of the five known muscarinic receptor (mAChR) subtypes: M1, M2, M3, M4, and M5 (Caulfield and Birdsall 1998; for a review on mAChR subtypes and physiology as elucidated in knockout mice, *see* Wess et al. 2003a, 2007). It is well known that the odd-numbered mAChRs (i.e., M1, M3, and M5) typically couple via the α subunits of the $G_{q/11}$ family, whereas the even-numbered mAChRs (i.e., M2 and M4) couple via the G_i and G_o α subunits (Caulfield and Birdsall 1998). There is widespread expression of mAChRs in peripheral tissues and in the nervous system (Levey 1993). Two main approaches have been described in the literature to understand the role of distinct subtypes in modulation of pain signaling (1) the use of ligands, most with limited selectivity for specific mAChR subtypes, and (2) the use of mutant mouse strains deficient in the five mAChR subtypes. It is important to realize that these approaches are complementary, providing different types of information. Genetic knockouts reveal subtypes that are required for analgesia, but do not describe which subtypes are sufficient for analgesia. In contrast, ligands with subtype selectivity can reveal subtypes that are sufficient for analgesia but may not be required when nonselective muscarinic agonists are employed (e.g., agonists). Despite their intrinsic differences, these two approaches, genetic and pharmacological, point to the same critical mAChR subtypes capable of modulating pain signaling and illustrate a path forward for the creation of novel analgesics.

2 Systemic Administration of Muscarinic Receptor Ligands Induces Potent Analgesia

The use of mAChR subtype-preferring small molecule agonists and antagonists, selective peptide antagonists, as well as genetic tools, including subtype-specific genetic deletion, antisense, and silencing approaches have established mAChRs as

having key roles in nociceptive processing (Wess 2003; Wess et al. 2003a, 2007; Ghelardini et al. 2000; Cai et al. 2009). Most data have been collected using rodent models of acute nociceptive pain (e.g., tail-flick, hot-plate, grid-shock, acetic acid writhing, formalin) where withdrawal from an acute painful stimulus is the primary measure, but activity in more complex models, such as postincisional, inflammatory, and neuropathic pain models, suggests that mAChR modulators have broader applicability across pain indications (Jones and Dunlop 2007). Systemic administration of the centrally penetrant, nonselective muscarinic agonist oxotremorine (OXO) has repeatedly been shown to yield dramatic antinociceptive effects in rodent models that can be reversed by nonselective muscarinic antagonists, such as atropine and scopolamine (e.g., George et al. 1962; Ireson 1970; Paalzow and Paalzow 1975; Ben-Sreti and Sewell 1982; Sheardown et al. 1997; Capone et al. 1999; Gomeza et al. 1999; Ghelardini et al. 2000; Barocelli et al. 2001). A number of other nonselective mAChRs agonists or mixed agonist/antagonists have shown antinociceptive effects in rodents, as well, including pilocarpine, arecoline, aceclidine, RS86, xanomeline, and xanomeline analogs (Sheardown et al. 1997), OXO analogs, OXO-methiodide (OXO-M) and its derivatives (Barocelli et al. 2001), vedaclidine (LY-297802 or NNC 11-1053; Swedberg et al. 1997; Shannon et al. 1997a), bethanechol (Prado and Segalla 2004), arecaidine (Dussor et al. 2004), and WAY-132983 (Sullivan et al. 2007).

Although muscarinic ligands have been useful in elucidating the physiological function of mAChRs despite limitations of subtype selectivity, Jurgen Wess and colleagues have used mutant mouse strains deficient in the five mAChR subtypes to provide a much clearer understanding of their respective physiological roles, including antinociception (Wess 2003; Wess et al. 2003a, 2007). The behavioral, pharmacological, neurochemical, and electrophysiological study of mice lacking specific mAChRs have highlighted M2, M3, and M4 receptors as important players in nociceptive pathways. A series of excellent studies have been conducted using mouse strains lacking M2 or M4 or both, in conjunction with OXO, to reveal the role of these subtypes in agonist-mediated antinociception. In these studies, the tail-flick and hot-plate tests were used to monitor acute nociceptive pain: the latency for a mouse to either move their tail (tail-flick) or lick/shake their hindpaw (hot-plate) in response to a heat stimulus is the primary endpoint; increased latencies are read as an analgesic response. Using this approach, the role of a mAChR subtype in mediating the antinociceptive effects of a nonselective mAChR agonist is inferred if an attenuation of the agonist effect is observed in the knockout mouse compared to wild-type (WT) control.

There were no differences in baseline responses to thermal pain between the WT, M2+/− and M2−/− mice (Gomeza et al. 1999). Systemically administered OXO yielded a dose-dependent analgesic response in WT mice in both tests. The agonist effect was attenuated dramatically in M2−/− mice over the OXO dose range in both tests, although not completely, suggesting that OXO-induced thermal analgesia is mediated predominantly by M2 receptor activation. A subsequent publication by Duttaroy et al. (2002) highlighted M4 as the other key mAChR mediating the acute antinociceptive effects of OXO. Whereas there was no effect of

Table 1 Reversal of muscarinic agonist-induced analgesia in M2, M4, and M2/M4 knockout mice

	Reversal of nonselective agonist-induced analgesia (oxotremorine)		Reversal of M4-preferring agonist-induced analgesia (CMI-936, CMI-1145)	
Knockout	Tail-flick test	Hot-plate test	Tail-flick test	Hot-plate test
M2	Partial (13.0×)	Partial (3.1×)	Partial (2.1–2.9×)	Partial (1.4–6.1×)
M4	None (1.0×)	None (1.2×)	Partial (1.6–4.9×)	Partial (2.1×)
M2/M4	Complete	Complete	Complete	Complete

Adapted from Duttaroy et al. (2002). Degree of reversal of analgesia induced by the non-selective agonist, oxotremorine, or the M4-preferring agonists, CMI-936 and CMI-1145, in M2, M4, M2/M4 knockout mice: none, partial, or complete. Analgesic responses were assessed in the tail-flick and hot-plate tests. Fold shift in $ED50_{Knockout}/ED50_{Wildtype}$ in parentheses.

M4−/− genotype on the OXO response, lack of both the M2 and M4 receptors completely abolished the OXO analgesic effect. The lack of any effect of M4 knockout on the OXO response is puzzling given that the M4 mAChR presumably accounts for the remaining 20% of antagonist activity in completely reversing the OXO effect in M2/M4−/− mice; the result hints at some functional interplay between subtypes or compensation by M2 in the M4 knockout mice.

Muscarinic agonists that possess some selectivity across mAChRs have also helped shed light on which mAChR subtypes are sufficient to mediate antinociception. Two M4-preferring agonists, CMI-936 and CMI-1145, both analogs of epibatidine, a nonselective cholinergic agonist, delivered potent antinociceptive activity in the mouse tail-flick assay, an effect that was markedly attenuated by systemic administration of the M2/M4-preferring antagonist, himbacine, as well as intrathecal injection of the G-protein signaling antagonist, pertussis toxin (PTX), and the M4-preferring peptide antagonist, MT-3. These data suggest that their antinociceptive effects are primarily mediated via M4, probably at the level of the spinal cord (Ellis et al. 1999). Taking advantage of the M4-preferring properties of CMI-936 and CMI-1145, Duttaroy et al. (2002) were able to reveal a more prominent role for M4 in pain signaling by examining the effect of the analogues in knockout mice. In this study, the analgesic effects of CMI-936 or CMI-1145 were attenuated in *both* M2−/− and M4−/− mice (*see* Table 1 for summary). Taken together, these data suggest that, at least in these mice, OXO exerts it analgesic efficacy primarily via M2, but M4 receptor activation is sufficient for analgesia, given the partial reversal of CMI-936- or CMI-1145 in M4−/− mice. In addition, a portion of the CMI-936 or CMI-1145 effects appears to be mediated by M2. Once again, there was complete reversal of the CMI effect in M2/M4 mice, suggesting that no other mAChR mediates the analgesic efficacy of these muscarinic agonists.

By comparing the in vivo effects of close analogs that differ in their mAChR agonist activity, Sheardown et al. (1997) provided evidence that M1 agonist activity is not required for antinociception as assessed by acute mouse pain models (e.g., tail-flick, hot-plate, grid-shock, writhing). The M1/M4-preferring agonist, xanomeline, was active in the pain models, but so was the 3-3(substituted-1,2,5-thiadiazol-4-yl)-12,5,6-tetrahydro-1-methyl pyridine (substituted TZTP) analogs

of xanomeline, 3-Cl-propylthio-TZTP and propxy-TZTP, despite exhibiting no or little M1 agonist activity in vitro (i.e., M1-tranfected BHK cells) or ex vivo (i.e., rabbit vas deferens). In addition, they found neither OXO nor RS86 to be very potent in these assays, despite delivering strong antinociceptive efficacy. Furthermore, another analog, hexylthio-TZTP which was also effective in the pain models, showed very weak functional activity in the guinea pig atria or ileum model (assessing M2 or M3 activity, respectively), leading the authors to speculate that neither M2 nor M3 agonism contributes to efficacy. In retrospect, it is perhaps more accurate to say that these data support the idea that selective agonism of more than one mAChR subtype may, by itself, be sufficient for antinociception.

Vedaclidine, a selective mAChR ligand that is an agonist at M2 and M4, but an antagonist at M1, M3, and M5 (Shannon et al. 1997a, b), is efficacious across acute nociceptive, inflammatory, and neuropathic rodent pain models (Shannon et al. 2001; Swedberg et al. 1997). Vedaclidine partially reversed intrathecal (*i.t.*) PTX-induced persistent pain in the mouse tail-flick model reflecting allodynia (i.e., painful response to a nonnoxious stimulus), suggesting that agonism at M2 and M4 at the level of the spinal cord mediates at least part of its antiallodynic action (Womer and Shannon 2000). More recently, Sullivan et al. (2007) described WAY-132983, a centrally penetrant M1/M4-preferring agonist, that was effective in a broad range of rodent pain models, including chemical irritant-induced visceral, Complete Freund's adjuvant (CFA)-induced inflammatory, postincisional, and spinal nerve ligation-induced neuropathic pain. The effect of WAY-132983 on CFA-induced mechanical hyperalgesia (i.e., an augmented response to a noxious stimulus) was completely blocked by *i.t.* administration of MT-3, suggesting its behavioral effects are mediated via spinal M4 receptors. Interestingly, WAY-132983 was not effective in models of acute pain (i.e., tail-flick and hot-plate), which may be the result of the compounds lower affinity and potency against M2 (Sullivan et al. 2007). Although speculative, changes in underlying muscarinic signaling pathways due to injury/insult (Mulugeta et al. 2003; Chen and Pan 2003b), which may not be observed in an acute pain setting, could lead to differential sensitivity to the effects of selective mAChR agonists.

3 Muscarinic Receptor Ligands Can Induce Antinociception at the Level of the Peripheral Nerve Fiber

There is evidence for expression of all mAChRs in the rat and chick dorsal root ganglion (DRG), the peripheral sensory nerve fibers (Bernardini et al. 1999; Tata et al. 2000), although M2, M3, and M4 are clearly the predominant subtypes. In the rat, immunochemical localization of M2 and M4 was found to be restricted to small- and medium-sized neurons which were presumed to be the nociceptive C-fibers. In contrast, M1 and M3 receptors were found to be expressed in all DRGs (Bernardini et al. 1999). Tata et al. (2000) used in situ hybridization to

show that M2, M4, and M3 were preferentially localized to small- and medium-sized neurons in the rat and chick, supporting a potential role for these subtypes to modulate nociception. The immunoreaction product for all subtypes was present in the axoplasm of many peripheral and central axons and clustered at the axolemma, suggesting transport of mAChRs to the spinal cord and periphery (Bernardini et al. 1999). mAChRs appeared to accumulate on the proximal side of a sciatic nerve ligation, as assessed by radioligand binding, again suggesting transport to the peripheral nerve terminals (Wamsley et al. 1981). There is also clear evidence for M2 expression in peripheral nerve terminals in the dermal layer of rat glabrous and hairy skin (Haberberger and Bodenbenner 2000), but it is not known if M3 or M4 is present in these nerve endings.

The functional role of peripheral mAChRs in modulating pain signaling was demonstrated in a series of electrophysiological and neurochemical studies (Steen and Reeh 1993; Bernardini et al. 2001a, b, 2002). Bernardini et al. (2001b) examined the effect of local application of various cholinergic agonists and antagonists on nociceptive afferents using an in vitro isolated skin-saphenous nerve preparation that allows for electrophysiological recording of nerves in response to heat or mechanical stimulation of rat hairy skin. Nicotine caused excitation and mild sensitization of C-nociceptor fibers to heat stimulation (but not mechanical stimulation). In contrast, muscarine, while having no effect on spontaneous activity, induced a dramatic desensitization to both heat and mechanical stimulation in all fiber types (i.e., mechanical/heat-sensitive, mechanical/cold-sensitive, and high-threshold mechano-sensitive). Superfusion of the nerve with the M2-preferring agonist arecaidine yielded the same effect as muscarine. Finally, the desensitizing effect of muscarine could be blocked by co-administration of the pan-muscarinic antagonist scopolamine or the M2-preferring antagonist gallamine. These data support the role of M2 in nociceptor desensitization and provides a mechanism by which selective M2 agonists could yield antinociceptive effects at the level of the skin.

In a complementary series of studies, the muscarinic pharmacology underlying in vitro basal and heat-induced calcitonin gene related peptide (CGRP) release was investigated in isolated rat skin (Bernardini et al. 2001a). The results mirrored those from the electrophysiology studies: whereas nicotine enhanced baseline release of CGRP (actually, a bell-shaped concentration response effect) and had no effect on heat-stimulated CGRP release, muscarine and the M2-preferring agonist, arecaidine, both attenuated basal and heat-stimulated CGRP release. It is important to note that keratinocytes in the epidermal layer of the skin express mAChRs, including M2 in the rat (Haberberger and Bodenbenner 2000), raising the possibility that M2 agonists could modulate keratinocyte release of neuromediators of pain, such as CGRP, ATP, or ACh (Grando et al. 1993; Zhao et al. 2008). Blockade of potassium-evoked ATP release from keratinocyte cultures has been demonstrated using the nonselective sodium channel antagonist, TTX (Zhao et al. 2008). At this point, however, the most parsimonious explanation based on the electrophysiology and biochemical data is that M2 agonism attenuates CGRP release from epidermal nerve endings.

The prominent role of cutaneous M2 receptors in modulating nociception was confirmed by examining the effect of muscarine on electrophysiological (i.e., skin-saphenous nerve preparation, C-mechanical/heat-sensitive fibers) and biochemical response (i.e., skin CGRP release preparation) in mAChR knockout mice (Bernardini et al. 2002). While the desensitizing effect of muscarine was observed in WT and M4−/− mice, muscarine was no longer able to attenuate nociceptive signaling in M2−/− mice. In fact, muscarine tended to increase activity in many C-fibers, suggesting a removal of basal inhibitory tone via M2, and induce a mild sensitization to heat (but not mechanical stimulation). There were no differences in basal CGRP release across genotypes. Muscarine attenuated heat-induced release of CGRP in both WT and M4−/− mice (by approx. 56–59%), but had no effect on the heat-induced CGRP release in M2−/− mice.

More recently, the role M2 plays in nociception has been extended to trigeminal sensory neuron pathways of the head and face (Dussor et al. 2004). Perioral injection of formalin induced orofacial grooming behavior associated with irritation/pain that was reversed by co-injection of arecaidine, but not by a subcutaneous arecaidine administered to a site distal from the formalin injection, indicating a local site of action in the buccal mucosa. The agonist effect was blocked by co-injection of atropine. These data suggest that agonism of local M2 receptors is sufficient for antinociception. Superfusion of the muscarnic agonists arecaidine and muscarine was also shown to block in vitro capsaicin-induced CGRP release from buccal mucosa tissue, which is innervated by the trigeminal ganglia. Both effects were antagonized by co-application of gallamine or atropine. It was found that 20% of medium- to small-sized trigeminal ganglion neurons expressed M2 mRNA and 5–9% of those neurons were immunoreactive to CGRP or the transient vanilloid receptor 1 (TRPV1). It may be speculated that, like DRGs, a majority of trigeminal neurons may be IB4 positive (and, thus, likely CGRP negative). Nevertheless, the few CGRP/TRPV1/M2-positive trigeminal neurons may be adequate to mediate the M2 agonist reversal of both capsaicin-induced CGRP release and capsaicin-induced nociceptive behavior (Dussor et al. 2004).

4 A Prominent Spinal Mechanism of Action Underlies Muscarinic Receptor-Mediated Analgesia

M2, M3, and M4 subtypes are the prominent mAChRs expressed in the mouse and rat spinal cord. Radioligand binding studies suggest that M2, M3, and M4 subtypes are present in the superficial lamina of the dorsal horn, where nociceptive C and Aδ fibers terminate (Hoglund and Baghdoyan 1997; Mulugeta et al. 2003). The presence of M2, M3, and M5 mRNA, but not M1 or M4, in the rat spinal cord was revealed by RT-PCR, although the authors note that a suboptimal M4 primer may have led to the negative finding for that subtype (Wei et al. 1994). More recently, Cai et al. (2009) reported that M2, M3, and M4 mRNA was found in the rat DRG

and spinal cord. There is strong evidence for the presence of M2 in dorsal horn laminas I-III of the mouse and rat spinal cord (Duttaroy et al. 2002; Li et al. 2002). M2 labeling was observed throughout the gray matter of the spinal cord with more intense staining in lamina II of WT and M4 KO mice, but little or no label was observed in M2 or M2/M4 KO mice (Duttaroy et al. 2002). Furthermore, little M4 immunoreactivity was observed in the spinal cord of WT mice, although clear labeling was observed in the brain regions known to express M4, such as the striatum. These data are consistent with the lack of change of [^{3}H]-NMS (*N*-methylscopolamine) binding in the spinal cord tissue of M4 KO versus WT mice and suggest very low levels of M4 expression in the mouse spinal cord (Duttaroy et al. 2002), but as revealed by functional studies described later, these low levels of M4 receptors appear to be functionally relevant in pain processing. Li et al. (2002) found that both dorsal rhizotomy and pretreatment with resiniferatoxin, a neurotoxin for capsaicin-sensitive C-fibers, both led to reduced immunoreactivity for spinal M2 in the rat, suggesting that a substantial amount of M2 is located presynaptically on peripheral sensory nerves terminating in the spinal cord. Evidence that M2 is the predominant mAChR in mouse spinal cord comes from [^{35}S]GTPγS binding studies in M2 and M4 KO mice (Chen et al. 2005a). Both muscarine and oxotremorine-M (OXO-M) led to profound increases in [^{35}S]GTPγS binding in spinal cord homogenates from WT mice, indicating the presence of functional G-protein coupled mAChRs (i.e., M2 and/or M4). Muscarinic agonist-induced spinal cord [^{35}S]GTPγS binding was completely abolished in both M2/M4 and M2 KO mice. Interestingly, a small but significant decrease in muscarinic agonist-induced [^{35}S]GTPγS was observed in M4 KO mouse spinal cord, as well, suggesting (1) the presence of functional M4 receptors in mouse spinal cord, and (2) although speculative, that the activity of spinal M4 receptors may require the presence of M2 mAChRs, perhaps as functional M2/M4 mAChR oligomers (Chen et al. 2005a).

The fact that *i.t.* administration of nonselective muscarinic agonists and acetylcholinesterase inhibitors can lead to robust analgesia in rodents and humans (e.g., Yaksh et al. 1985; Iwamoto and Marion 1993; Naguib and Yaksh 1994; Hood et al. 1997; Duttaroy et al. 2002; Li et al. 2002, Naguib and Yaksh 1997), and is blockable by mAChR antagonists (Yaksh et al. 1985; Naguib and Yaksh 1994) strongly suggests that agonism of spinal mAChRs mediates their in vivo efficacy. Blockade of the antinociceptive effects of venaclidine and WAY-132983 by the centrally penetrant nonselective muscarinic antagonist scopolamine, but not by its peripherally restricted quaternary salt, NMS, points to a central site of action (e.g., spinal cord) for these mAChR agonists, as well (Sheardown et al. 1997; Swedberg et al. 1997; Sullivan et al. 2007). Spinal mAChRs also appear to play an important role in a rat cystitis model (Masuda et al. 2009). These authors reported that atropine reversed the analgesic effects of intrathecally administered cholinergic agonists OXO-M and neostigmine on C-fiber-mediated bladder contractions induced by acetic acid.

The relative contributions of spinal and supraspinal mechanisms to muscarinic agonist-induced analgesia were examined by Wess and colleagues by administering compounds directly to the spinal cord region (i.e., via *i.t.* injection) or brain (i.e., via

intracerebroventricular or *i.c.v.* injection) in knockout mice (Duttaroy et al. 2002). The results of *i.t.* and *i.c.v.* muscarinic agonist administration mirrored what was observed following systemic administration: (1) partial attenuation of OXO, CMI-936, and CMI-1145 analgesia in M2−/− mice, (2) no reversal of OXO but partial reversal of CMI-936 and CMI-1145 effects in M4−/− mice, and (3) complete reversal of agonist-induced analgesia in the M2/M4−/− mice. These data suggest a role for both M2 and M4 receptors at spinal and supraspinal levels in modulating pain signaling. Given the presence of M2 and M4 receptors in dorsal root ganglia neurons (DRGs), and the likelihood that compounds administered *i.t.*, likely reach even the cell bodies of these neurons, it is conceivable that the analgesic action of OXO, CMI-936, and CMI-1145 may occur at the level of these nociceptive afferents, as well. As mentioned previously, the ability of *i.t.* MT-3 or PTX to block the effects of CMI-936 and CMI-1145 also supports the idea that spinal M4 or M2/M4 mediates the antinociceptive effects of these compounds (Ellis et al. 1999). Recently, Cai et al. (2009) investigated the role of DRG and spinal M2, M3, and M4 receptors underlying in vivo nociception by small-interference RNA (siRNA) targeting of these subtypes in the rat. Chitosan nanoparticle delivery of siRNA led to successful knockdown of both mRNA and protein in DRGs and dorsal spinal cord, as assessed by QRT-PCR, immunoprecipitation, and receptor binding. Whereas M2 or M4 knockdown led to a large reduction in *i.t.* muscarine-induced nociception, M3 knockdown had no effect, providing functional evidence that M2 and M4, but not M3, contribute to nociceptive modulation at the level of the spinal cord.

It is interesting to note that changes in mAChR expression have been associated with nociception in various animal pain models (Chen and Pan 2003b; Mulugeta et al. 2003). Although causality cannot be inferred from these correlations, it hints that these mAChR are involved in altered pain signaling. Chen and Pan (2003b) reported an increase in both muscarine-stimulated GTPgS binding in spinal cord membranes, as well as an increase in [^{3}H]-AF-DX385 saturation binding in spinal cord homogenates, from the streptozotocin (STZ) rat model of diabetic neuropathy, suggesting augmented spinal M2 expression. The increased sensitivity to noxious heat or pressure stimuli observed in STZ-treated rats was reversed by *i.t.* administration of muscarine, an effect consistent with a spinal site of action. The antinociceptive/antiallodynic effects of *i.t.* muscarine or the anticholinesterase neostigmine in STZ-treated rats were also shown to be antagonized by *i.t.* administration of the $GABA_B$ receptor antagonist, CGP55845, providing functional evidence that a $GABA_B$ mechanism underlies the analgesic properties of *i.t.*-administered cholinergic agonists, as well (Chen and Pan 2003a). Arthritis induced by intradermal injections of heat-killed *Mycobacterium butyricum* in rats was found to decrease the expression of spinal M4 expression assessed by [^{125}I]-MT-3 radioligand binding at 12d and 30d postinoculation (Mulugeta et al. 2003). The consequences of a decrease in spinal M4 expression are not clear. Electrophysiological studies point to functional M4 expression on different populations of dorsal horn interneurons, both excitatory and inhibitory (Pan et al. 2008) and differential changes in M4 expression in these populations, or indeed at another level of the pain signaling pathway, could still allow for a substantial role of M4 agonism in

alleviating inflammatory pain in this model, but this remains to be investigated. Kang and Eisenach (2003) found no evidence of changes in spinal M1 or M4 receptor expression by Western analysis in response to nerve ligation injury in rats.

There is little evidence for a role of spinal M1 in nociceptive processing. However, as mentioned previously, there are some data suggesting that M1 is expressed in the sensory neurons projecting to the spinal cord in the rat (Bernardini et al. 1999). In addition, *i.t.* administration of the M1-preferring agonist, McN-A-343, induced nociception as assessed by the tail-flick and electrical current threshold (ECT) tests (Lograsso et al. 2002). However, ECT changes were observed at the level of the neck indicating there was rostral spread of the compound following *i.t.* injection to higher spinal regions, at least, and raising the possibility that the compound could have engaged a supraspinal mechanism (Lograsso et al. 2002).

Activation of spinal dorsal horn mAChRs inhibits the activity of projection neurons in response to nociceptive stimuli in rats (reviewed in Pan et al. 2008). Activation of M2, M3, and M4 mAChRs leads to modulation of both inhibitory GABAergic and glycinergic and excitatory glutamatergic neurotransmission in a complex dynamic interaction, resulting in a net attenuation of projection neuron activity (*see* Fig. 1). The majority of these mechanistic studies employed whole-cell voltage clamp recording of lamina II dorsal horn neurons in spinal cord slices to elucidate the effect of mAChR modulation on projection neuron activity (Pan et al. 2008).

Zhang et al. (2007a) found that OXO-M application led to concentration-dependent inhibition of both monosynaptic (mono-) and polysynaptic (poly-) excitatory post-synaptic currents (EPSCs, mediated by glutamate) by dorsal root stimulation in rat spinal cord slices. Poly-EPSCs were inhibited to a greater degree than mono-EPSCs. Intrathecal administration of PTX, M2/M4-preferring antagonist himbacine, or the M2-preferring antagonist AFDX-116, blocked the OXO-M-induced attenuation of mono-EPSCs, while the relatively selective M4 toxin, MT-3, had no effect. These data indicate the presence of inhibitory M2 receptors on the terminals of glutamatergic peripheral sensory neurons projecting to the dorsal horn. In some neurons, himbacine completely blocked the OXO-M-induced inhibition of poly-EPSCs, indicating the presence of M2/M4. In other cells, where himbacine had a partial effect, the remaining current was blocked by 4-DAMP, suggesting that the glutamatergic interneurons of the dorsal horn possess M2/M4 and M3. Because 4-DAMP was able to block the frequency of spontaneous EPSCs in a number of cells, Zhang et al. (2007a) suggested that M3 may modulate glutamate release in a subpopulation of these interneurons. Finally, because OXO-M had no effect on miniature EPSCs in all polysynaptic neurons recorded, mAChR expression is probably somatodendritic.

The $GABA_B$ receptor antagonists CGP55845 dramatically attenuated muscarine- or neostigmine-induced suppression of single unit activity of ascending dorsal horn projection neurons induced by mechanical stimulation in the anesthetized rat (Chen and Pan 2004). Given that both the muscarine and neostigmine effects were completely blocked by local atropine or PTX, it appears that the $GABA_B$ receptor appears to play an important modulating role on the in vivo antinociceptive effects

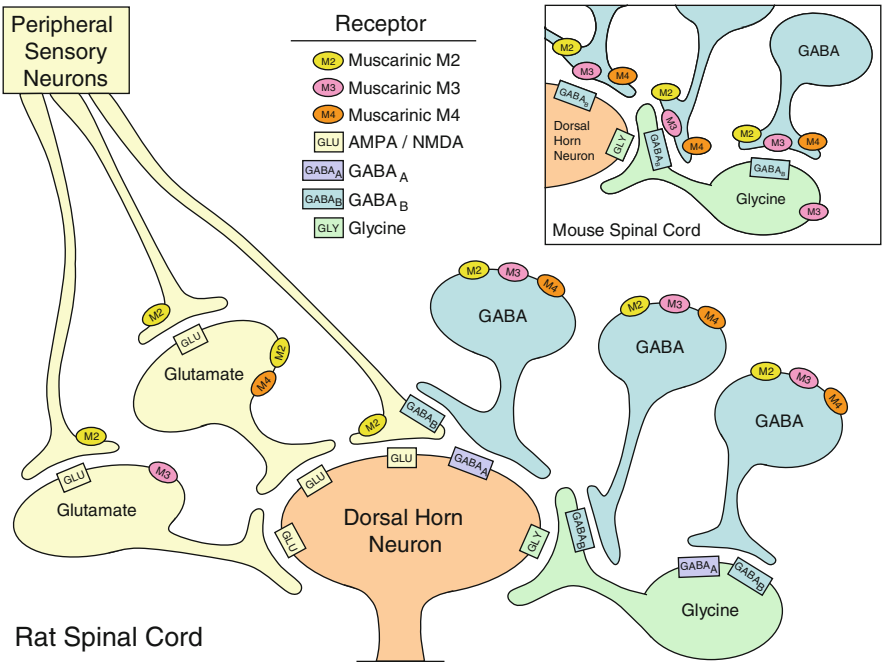

Fig. 1 Representation of the distribution and function of M2, M3, and M4 mAChR subtypes in the modulation of glutamatergic, GABAergic, glycinergic inputs on dorsal horn neurons in rats and mice. In rats, excitatory glutamatergic input to dorsal horn neurons is inhibited by the activation of M2 on primary peripheral sensory neurons and M3 and M2/M4 subtypes on a subset of interneurons. Inhibition of postsynaptic dorsal horn neurons also occurs by facilitated GABAergic transmission via activation of somatodendritic M2, M3, and M4 on GABA interneurons. Activation of somatodendritic M2 and M3 receptors on glycine interneurons also inhibits postsynaptic dorsal horn neurons by increasing glycinergic transmission. GABA released from GABA interneurons can also inhibit both glutamate and glycine release via presynaptic $GABA_B$ receptors. In contrast to rats, dorsal horn M2, M3, and M4 receptors modulate GABAergic transmission via presynaptic receptors in mice (*upper right box*). Activation of M2 and M4 receptors predominantly attenuate inhibitory GABAergic input to dorsal horn neurons, while M3 stimulation facilitates GABA release. Stimulation of M2 and M4 receptors also attenuates inhibitory glycinergic inputs to dorsal horn neurons by a similar presynaptic GABA release mechanism, whereas M3 receptor stimulation leads to glycine release. Adapted from Zhang et al. (2006, 2007a, b), Pan et al. (2008)

of cholinergic agonists in the spinal cord. Whole-cell voltage clamp experiments in rat spinal cord slices provided more detailed information about the underlying GABA mechanism. The ability of CGP55845 and atropine to block ACh-mediated attenuation of miniature EPSCs in projection neurons of the rat dorsal horn suggests that presynaptic $GABA_B$ receptors modulate, at least in part, the mAChR-mediated blunting of antinociceptive transmission at the level of the spinal cord (Li et al. 2002). Indeed, this mechanism was supported by in vivo behavioral data demonstrating that co-administration of CGP55845 concentration dependently reversed the antinociception of *i.t.* muscarine as assessed by withdrawal from

noxious heat. The fact that both CGP55845 and atropine antagonized ACh-induced attenuation of dorsal root evoked EPSCs argues that the effect is mediated via primary sensory glutamate afferents. In a separate study, OXO-M was found to increase GABA interneuron-mediated spontaneous, but not miniature, IPSCs in rat lamina II dorsal horn neurons (Zhang et al. 2005). The OXO-M effect was completely blocked by atropine, indicating a mAChR-mediated effect. In approximately half of the neurons, the OXO-M effect was blocked by *i.t.* PTX, suggesting mediation by M2/M4; in the other half, partial antagonism by PTX was observed and the remaining OXO-M effect was blocked by 4-DAMP, implicating M3. These data suggest that somatodendritic M2, M4, and M3 mAChRs serve to increase GABAergic tone on projection neurons of the dorsal horn in the rat (Zhang et al. 2005).

While M3 agonism appears to increase the GABAergic tone to dorsal horn projection neurons in both rat and mouse, there appear to be differences with respect to localization and function of M2 and M4 receptors in the dorsal horn across species (Zhang et al. 2006). OXO-M decreased GABAergic spontaneous and miniature IPSCs in WT mice. Himbacine not only reversed the OXO-M-induced attenuation of IPSCs, but led to an OXO-M-mediated *increase* in the frequency of spontaneous IPSCs over baseline. The pharmacological effect of the M2/M4 antagonist was recapitulated in M2/M4 KO mice where OXO-M increased spontaneous and miniature IPSCs in all neurons tested, suggesting presynaptic modulation by M2 and M4. The OXO-M effect was completely blocked by 4-DAMP, implicating M3 in the OXO-M-promotion of inhibitory transmission. In M3 or M1/M3 KO mice, himbacine blocked OXO-M-mediated decreases in spontaneous IPSCs, but did not lead to increased inhibitory transmission, which is consistent with the 4-DAMP effects in M2/M4 KO mice. These data suggest that M3 activation serves to increase synaptic GABA transmission in the dorsal horn of mice (Zhang et al. 2006). The effects of OXO-M in M2 and M4 KO mice were varied: OXO-M induced decreased spontaneous IPSCs in some neurons, and increased IPSCs in others, suggesting a heterogenous population of M2/M4 neurons. In general, however, and in contrast to the rat, agonism of M2 and M4 receptors in the spinal cord of mice serves mainly to disinhibit projection neuron activity by presynaptically blocking GABAergic signaling.

In the rat spinal cord, OXO-M was found to increase the frequency of glycinergic spontaneous IPSCs, but not miniature IPSCs, indicating a presynaptic site of action on these interneurons (Wang et al. 2006). The OXO-M effect was not blocked by PTX, himbacine, or AF-DX116, but was completely reversed by 4-DAMP, suggesting that somatodendritic M3 receptors mediate the presynaptic modulation of glycinergic transmission by muscarinic agonists. CGP55845 potentiated the OXO-M effect on glycine release and, under these conditions, the effect was blocked by both himbacine and AF-DX116; by eliminating the influence of GABAergic interneurons, a role for M2 in promotion of inhibitory glycinergic transmission was revealed.

In mice, OXO-M decreased glycinergic spontaneous IPSCs in most neurons, although the response was varied in other cells (Zhang et al. 2007b). While blockade was evident in spinal cord slices from M3 KO mice, OXO-M

decreased spontaneous IPSCs more consistently. This effect was completely blocked by himbacine and partially antagonized by AFDX-116, suggesting that activation of M2 and M4 receptors serves to *decrease* inhibitory glycinergic tone to postsynaptic neurons. In M2/M4 KO mice, OXO-M increased the frequency of spontaneous [glycinergic] IPSCs. In the presence of CGP55845, OXO-M also increased the frequency of spontaneous IPSCs, and this effect was blocked by D-AMP, suggesting that agonism of M3 receptors enhances glycinergic transmission. The effect of M3 receptors on glycinergic neurons appears to be influenced, then, by concurrent M3 modulation of GABAergic interneurons which serves to attenuate glycine release presynaptically via $GABA_B$ receptors. The effect on both spontaneous and miniature IPSCs supports the presence of M3 on both presynaptic and somatodendritic sites of glycinergic interneurons (Zhang et al. 2007b). In line with observations from M2/M4 mice is the finding that, in WT mice, *i.t.* PTX also led to OXO-M-induced increases in the frequency of spontaneous IPSCs. The effects of OXO-M in M2 and M4 KO mice are illustrative of a complex interaction among M2, M3, and M4 receptors such that in the absence of M2 and M4, M3 takes on a prominent role in control of spinal glycine release. In summary, while activation of M3 appears to potentiate spinal glycine transmission in mice and rats, stimulation of M2 and M4 inhibits glycinergic inputs to the spinal horn neurons of mice, but not rats.

Observed species differences in physiology and presumed subcellular distribution of spinal mAChR subtypes (i.e., inferred by electrophysiological studies but not confirmed by immunochemical localization) on GABAergic and glycinergic transmission could lead to potentially disparate antinociceptive effects across species. Yet despite these differences, activation of M2 and M4 subtypes in the spinal cord yields efficacy in both mouse and rat. This suggests other mechanisms, such as attenuation of excitatory glutamatergic inputs to the dorsal horn, may play a more prominent role in mediating the antinoceptive effects of mAChR agonists, or that these agonists are not subtype selective.

5 Muscarinic Receptors: Supraspinal Modulation and Descending Inhibition of Pain

The fact that the intensity of perceived pain is not necessarily proportional to the amount of noxious stimulation reflects a complex regulation of pain perception and proposes the existence of supraspinal modulatory pathways that can influence the efficiency of transmission of peripheral nociception via the spino-thalamic-cortical pathways. There is evidence that mAChRs can modulate pain perception in animals via supraspinal mechanisms that affect both ascending and descending pain pathways between the spinal cord and cortical areas. Supraspinal administration of muscarinic ligands reveals a role for their analgesic effects at the level of the hypothalamus (Franco and Prado 1996), the periaqueductal gray (PAG; Guimaraes et al. 2000), the

rostral ventromedial medulla (RVM; Spinella et al. 1999), and the amygdala (Oliveira and Prado 1994).

Experimental data support a role for M1, M2, and M4 subtypes in supraspinal modulation of pain processing. Intracerebroventricular injection of the M1-preferring agonists, McN-A-343 and AF-102B, induced antinociception in the mouse hot-plate, acetic acid writhing, and paw-pressure tests (Bartolini et al. 1992). While the M1 agonist-induced effects were blocked by *i.c.v.* co-administration with the nonselective mAChR antagonists, atropine, or the M1-preferring antagonists, pirenzepine and dicyclomine, the M2-preferring antagonist, AFDX-116, had no effect, suggesting that activation of supraspinal M1 receptors is sufficient for antinociception. Because the M1 agonist effects were not altered by co-administration of the choline uptake blocker/ACh depletor hemicolinium-3 (HC-3), it also suggests that the M1 effect is postsynaptic. Knockdown of central M1 via *i.c.v.* injection of an antisense oligodeoxyribonucleotide (aODN) prevented the antinociceptive effects of systemically administered OXO, physostigmine, or local *i.c.v.* injection of the M1-preferring agonist, McN-A-343 (Ghelardini et al. 2000). The mediation of central mAChR analgesia via M1 is also supported by the finding that central knockdown of the alpha subunit of $G_{q/11}$ proteins by *i.c.v.* aODN administration blocked the antinociception effects of systemic OXO and physostigmine (Galeotti et al. 2003). Repeated systemic administration of acetyl-L-carnitine (ALCAR), a naturally occurring molecule in the central nervous system, yielded antinociceptive effects in the mouse hot-plate and acetic acid-induced abdominal constrictions tests, as well as the rat paw-pressure test (Ghelardini et al. 2002). These effects were blocked by the nonselective mAChR antagonists atropine, the choline uptake blocker/ACh depletor hemicolinium-3, the M1-preferring antagonists pirenzepine and S-(−)-ET-126, and by *i.c.v.* injection of aODN against M1, which together point to a central presynaptic action of ALCAR, and whose antinociceptive action ultimately is mediated via M1 receptors, also in the CNS (Ghelardini et al. 2002). Atropine blocked the antihyperalgesic effect of repeated ALCAR administration in the rat sciatic nerve ligation model of neuropathic pain (Cesare et al. 2009). Arecoline was also shown to induce antinociception via a central M1 mechanism, based on its antagonism via *i.c.v.* administration of the M1-selective antagonists, pirenzepine and S-(−)-ET-126, as well as aODN-mediated knockdown of M1 (Ghelardini et al. 2001). Ghelardini and colleagues have also described a number of indirect cholinergic agonists, namely, 3-α-tropyl 2-(*p*-bromophenyl) propionate (i.e., (±)PG-9), 3-α-tropanyl-(2-Cl)-acid phenoxybutyrate (i.e., SM-21), and *R*-(+)-hyoscyamine, that exhibit slight binding preferences for M4 and M2 and appear to exert their antinociceptive effects in rodents via a central cholinergic mechanism, perhaps via ACh release (Ghelardini et al. 1997a, b, c, 1998). As mentioned previously, there is evidence for supraspinal M2- and M4-mediated antinociception (Duttaroy et al. 2002). In addition, *i.c.v.* injection of the M2-preferring agonist arecaidine induced antinociception in the mouse hot-plate and paw-pressure tests and these effects were reversed by co-administration of the M2-preferring antagonist, AFDX-116, and the ACh depletor, HC-3, signifying

that agonism of central M2 receptors may mediate antinociceptive effects via modulation of ACh release (Bartolini et al. 1992).

Endogenous descending pain modulation systems are among the most important pain regulatory pathways (Benarroch 2008; Gebhart 2004) and serve to integrate sensory, cognitive, emotional, and motivational information to control the activity of ascending spino-thalamic-cortical sensory pathways. Anatomically, these descending pathways provide a neuronal link between the cortex, hypothalamus, and amygdala to control ascending pain at the level of the reticular formation, midbrain areas, and the spinal cord. This circuitry includes key areas such as the PAG, the nucleus raphe magnus (NRM), and the RVM that project to the dorsal horn of the spinal cord. Activation of these supraspinal areas leads to profound analgesic responses in animals and humans via descending inhibition. Neurons in the PAG activate serotonergic nuclei of NRM in the medulla, which in turn send inhibitory projections to the dorsal horn of the spinal cord to attenuate peripheral pain signals. Activation of muscarinic receptors at the PAG has been shown to induce antinociception in rats (Guimaraes et al. 2000). Local administration of carbachol in the dorsal PAG of rats increased tail-flick latencies and the vocalization thresholds, effects that were blocked by the muscarinic antagonist atropine. These data highlight the role of muscarinic pathways in modulating the affective component of pain responses (e.g., vocalizations) by activating descending inhibition pathways (Guimaraes et al. 2000).

The RVM is another key relay center for descending inhibition, including PAG-mediated analgesia. Activation of mAChRs in the RVM induced strong analgesic effects in rodents (Iwamoto and Marion 1994). It was also reported that the antinociceptive effects of morphine, as measured in the rat tail-flick and hot-plate tests, were blocked by systemically administered atropine, as well as by local injections of the M1-preferring antagonists, MT-7 and pirenzepine, into the RVM (Abe et al. 2003). These data indicate that M1 agonism in the RVM may act to facilitate descending inhibition of spinal nociceptive transmission.

The mechanism of descending inhibition and supraspinal/spinal signal integration involves the activity and release of a number of spinal neurotransmitters that include endogenous opioids, noradrenaline, serotonin, and ACh. Importantly, the spinal release of ACh appears to be a key mechanism by which descending inhibitory pathways induce analgesia in rodents and humans analgesia, and one that is common to many clinical painkillers.

Painful stimuli are known to increase ACh levels in the spinal cord as a consequence of the activation of descending inhibitory pathways (Eisenach et al. 1996) and thought to be mediated by cholinergic neurons projecting from the dorsolateral pontine tegmentum, the RVM, and cholinergic spinal interneurons. This spinal release of ACh and the consequent activation of spinal mAChRs appears to be a key step in the analgesic responses mediated by a number of clinically active drugs, including the α-2-adrenergic agonist clonidine (Duflo et al. 2003; Obata et al. 2005; Hood et al. 1996), morphine (Xu et al. 1997), lidocaine (Abelson and Hoglund 2002b), gabapentin (Hayashida et al. 2007; Takasu et al. 2006),

serotonin agonists (Kommalage and Hoglund 2005), and NSAIDs (Pinardi et al. 2003). Interestingly, mAChR ligands can also induce ACh release at the level of the spinal cord (Hoglund et al. 2000). This raises the possibility that site-specific (i.e., spinal) activation of all spinal mAChR could be achieved if a systemically administered mAChR subtype-selective agonist is able to increase spinal ACh.

In the case of morphine-induced analgesia, it has been reported that its spinal analgesic effect is a result of stimulating cholinergic transmission at the level of the dorsal horn, independent of activation of descending inhibitory pathways (Chen et al. 2005b). The authors reported that the effect of morphine, as assessed by single-unit recording of dorsal horn projection neuron activity in response to mechanical stimulation of the receptive field, can be inhibited by atropine in both intact and spinally transected rats.

The role of spinal ACh release and mAChR activation in analgesia is supported by clinical studies using acetylcholinesterase inhibitors. In humans, intravenous administration of the synthetic opioid alfentanil increased cerebrospinal fluid concentrations of ACh and induced dose-dependent analgesia; both effects were augmented by co-administration of acetylcholinesterase inhibitors (Hood et al. 1997). The *i.t.* or epidural administration of the cholinesterase inhibitor neostigmine or donepezil, alone or in combination with other analgesics, yielded analgesia not only in animals, but in humans, as well, including effects in acute postoperative pain (Habib and Gan 2006; Khan et al. 2008), chronic cancer pain in terminal patients (Lauretti et al. 1999), labor analgesia (Ross et al. 2009; Ho et al. 2005; Van de Velde et al. 2009), and pediatric analgesia (Karaaslan et al. 2009). Unfortunately, *i.t.* neostigmine induces significant nausea in patients, a side effect that limits its use in the hospital setting. Curiously, epidural administration of neostigmine is not associated with as high an incidence of nausea and, given its analgesic properties, has been proposed recently as a potential alternative to soluble opioids for postoperative and labor analgesia (Eisenach 2009). These data clearly suggest that cholinergic activation of mAChRs in the spinal cord, as part of the descending inhibitory pain pathway, is a fundamental mechanism controlling clinical pain.

Morphine-induced analgesia leads to ACh release in the spinal cord (Chen and Pan 2001; Gage et al. 2001) and its analgesic effects appear to be dependent upon activation of spinal mAChRs, probably the M1 and/or M4 subtypes (Honda et al. 2004). Thermal analgesia induced by subcutaneous administration of morphine was inhibited by *i.t.* administration of atropine and the M1-preferring antagonist pirenzepine, in a dose-dependent manner. The M2- and M3-preferring antagonists, methoctramine and 4-DAMP, did not alter morphine-induced analgesia. Interestingly, in this report, *i.t.* administration of a M1-preferring agonist (that has lower partial agonist activity on M4 subtypes) induced analgesia in a dose-dependent manner (Honda et al. 2004). These muscarinic effects appear to mediate the supraspinal, but not spinal, analgesic actions of morphine as intracerebroventricular (*i.c.v*), but not *i.t.*, administration of morphine was sensitive to muscarinic antagonists.

Likewise, gabapentin induces analgesia via a number of spinal mechanisms wherein mAChR activation figures prominently. Gabapentin activates spinal

cholinergic circuits to mediate analgesia and reduced hypersensitivity to noxious stimuli in a synergistic manner with donepezil, a cholinesterase inhibitor (Hayashida et al. 2007). Gabapentin has also been shown to induce analgesia in neuropathic pain rodent models in a mAChP-dependent manner (Clayton et al. 2007). Gabapentin administration into the brain of mice reduced nerve injury-induced allodynia, an effect blocked by *i.t.* atropine and enhanced by the acetylcholinesterase inhibitors neostigmine and donepezil (Hayashida et al. 2007). Pretreatment with atropine (*i.t.*) completely suppressed the effect of *i.c.v.*-injected gabapentin on mechanical hypersensitivity, whereas its effect on thermal hypersensitivity remained unchanged. Similar effects were obtained with *i.t.* pirenzepine, but not with *i.t.* methoctramine, a M2-preferring receptor antagonist, suggesting that the M1/M4 subtypes play a role in gabapentin-induced analgesia (Takasu et al. 2006).

The clinical analgesic and α-2-adrenergic agonist, clonidine, elicits ACh spinal release (Klimscha et al. 1997) and mediates analgesia in a mAChR-dependent manner (Pan et al. 1999; Kang and Eisenach 2003; Obata et al. 2005). The analgesic effect of *i.t.* clonidine in a rat nerve ligation model was reversed by co-administration of the M4 toxin, MT-3 (Kang and Eisenach 2003). Analgesic synergy between activation of spinal cholinergic signaling and α-2-adrenergic receptors is also supported by combination studies co-administering clonidine and the acetylcholinesterase inhibitor neostigmine in humans (Hood et al. 1996).

Spinal cord stimulation (SCS) has proven to be an effective method to manage intractable chronic pain in humans, in that long-lasting pain relief can be achieved in up to 50–70% of patients that are otherwise refractory to analgesic pharmacotherapy (Carter 2004; de Leon-Casasola 2009). Here, the implantation of battery-driven electrodes in the spinal cord is used as a last-resort therapy for patients suffering difficult chronic pain conditions, such as complex regional pain syndrome (CRPS), who do not respond adequately to pharmacotherapy. The mechanisms underlying the pain relieving effect of SCS on neuropathic pain remain unclear, but recently it has was shown that SCS increased spinal concentrations of ACh in the rat and, presumably, activated spinal mAChRs. Indeed, the analgesic effects of SCS were completely blocked by atropine, but were not sensitive to the nicotinic antagonist mecamylamine (Schechtmann et al. 2008). Interestingly, the use of the M4 selective antagonist, MT-3, selectively blocked SCS-induced analgesia, suggesting a key role of this subtype (Schechtmann et al. 2008). Moreover, SCS shows synergy with the nonselective mAChR agonist, OXO (Song et al. 2008). When combining SCS with a subeffective dose of *i.t.* OXO, the effect of SCS on the pain-related symptoms was dramatically enhanced in rats. Enhancing the efficacy of SCS by co-administration of selective mAChR agonists could be an option in patients where SCS alone does not provide sufficient relief.

In summary, it likely mAChR ligands induce analgesia by modulation of the supraspinal cholinergic mechanisms of descending inhibitory pathways. The convergent events of ACh release and mAChR activation in the spinal cord are fundamental mechanisms of pain modulation that extend beyond mAChR ligands to clinically effective analgesics of different classes, including morphine,

gabapentin, clonidine, and SCS. In addition, observations that augmented spinal ACh is associated with analgesia across many different pain indications support the notion that mAChR ligands have broad potential to treat clinical pain and that spinal processing is probably a key site of action for mAChR-mediated analgesia in animals and humans.

6 Other Mechanisms May Contribute to Muscarinic Receptor Ligand-Mediated Analgesia

There is evidence that M1, M2, M3, and M4 subtypes are expressed (Dorje et al. 1991a, b) and can modify neurotransmitter release from or activity of sympathetic neurons (Wanke et al. 1987; Hamilton et al. 1997; Shapiro et al. 2001; Hardouin et al. 2002; Trendelenburg et al. 2003, 2005; Wess et al. 2007; Kubista et al. 2009). Activation of presynaptic M1 can facilitate neurotransmitter release from sympathetic neurons via suppression of the M-type K^+ current or attenuate release by closing the voltage-activated N- and L-type Ca^{++} channels in mice and rats (Shapiro et al. 2001; Hamilton et al. 1997; Trendelenburg et al. 2003; Kubista et al. 2009). Activation presynaptic M2 and M4 inhibit neurotransmitter release by fast inhibition of N- and P/Q-type Ca channels (Shapiro et al. 2001). Carbachol attenuates electrically induced [3H]-noradrenaline release in sympathetically innervated mouse tissues, such as atria and vas deferens; this effect was attenuated to varying degrees in tissue M2, M3, M4, M2/M3, and M2/M4 knockout mice, depending on the tissue (Trendelenburg et al. 2003, 2005). Although these studies have clear implications with respect to therapeutic potential or safety/tolerability issues of a mAChR modulator (e.g., on cardiac function), little is known about the impact of mAChR-mediated modulation of sympathetic activity on pain signaling. Sympathetic activity can sensitize peripheral nociceptors, may mediate nociceptor sensitization initiated by cytokines, and can promote ectopic activity (Janig 2009) to promote and maintain pain. It is important to note that many rodent models of pain, including those used to reveal the analgesic activity of mAChR agonists such as tail-flick, formalin (Coderre et al. 1984), and spinal nerve ligation (Kim and Chung 1991), are sensitive to pharmacological or surgical sympathetic block. Thus, it is possible that the analgesic effects of mAChR agonists may be mediated, at least in part, by an antisympathetic mechanism, especially when the selectivity profile favors activation of M2 and M4. Furthermore, a mAChR agonist that is able to attenuate sympathetic activity may have clinical utility in treating pain indications such as CRPS with sympathetically maintained pain (Burton et al. 2005).

It has been shown that mAChR agonists can stimulate hypothalamic-pituitary-adrenalcortical (HPA) axis activity leading to increases in plasma/serum corticosterone in the mouse and rat (Hedge and Wied 1971; Calogero et al. 1989; Hemrick-Luecke et al. 2002). Systemic administration of the M2/M4-preferring agonist, [5*R*-(exo)]-6-[4-butylthio-1,2,5-thiadiazol-3-yl]-1-azabicyclo-[3.2.1]-octane

(BuTAC), resulted in a dose-dependent increase in serum corticosterone concentrations, which was absent in M2 and M2/M4 knockout mice, suggesting that M2 mediates the mAChR agonist-induced activation of the HPA axis in mice (Hemrick-Luecke et al. 2002). Corticosterone is known to modulate nociceptive signaling and mediates "long-term" stress-induced analgesia (MacLennan et al. 1982), raising the possibility that mAChR agonists exhibit some of their analgesic properties via corticosterone release in the rodent. However, the antinociceptive response of OXO on formalin-induced behaviors has been observed in the absence of significant increases in plasma corticosterone concentrations, suggesting a HPA-independent analgesic mechanism (Capone et al. 1999).

There is some evidence for mAChR ligand-mediated modulation of inflammatory mechanisms (Wessler et al. 1998; Jones and Dunlop 2007). mAChRs are found in cells of the immune system, including mononuclear cells, macrophages, and lymphocytes, and may play an important role in the nonneuronal modulation of immune function (Wessler et al. 1998; Tayebati et al. 2002; Kawashima et al. 2007). Centrally administered mAChR agonists can reduce circulating concentrations of proinflammatory cytokines, such as tumor necrosis factor (TNF; Langley et al. 2004), that can directly modulate neuronal activity and elicit spontaneous neuronal activity (Scholz and Woolf 2007). This suggests that mAChR agonists may act directly on immune cells or indirectly via sympathetic nerve modulation (Janig 2009) to blunt inflammatory mechanisms mediating pain. Although mAChR agonists, such as vedaclindine and WAY-132983, are effective against inflammatory pain (Swedberg et al. 1997; Sullivan et al. 1997), there is no evidence of direct immunomodulation in these studies.

7 Muscarinic Analgesics: The Challenge to Realize Their Potential

It is clear that mAChR agonists have great potential to treat pain. In considering the selectivity profile of mAChR agonists as novel analgesics, it is critical to find the optimal balance of subtype activities that can elicit analgesia while avoiding or minimizing the cholinergic side effects (Wess et al. 2007) observed with nonselective agonists. Within this context, selective agonists targeting M4 or dual agonists with selectivity for M4/M1 may be among the best approaches to elicit analgesia with an acceptable safety profile. Despite the importance of M2 receptors in the modulation of pain signaling, its prominent role in heart and smooth muscle physiology turns its activation into a burden that should be avoided as a systemically administered analgesic. Selective M2 agonists may still be viable as topical analgesic agents for peripherally driven pain (Wess et al. 2003a; Dussor et al. 2004). The role of M5 in the modulation of pain signaling is not known, but the presence of M5 mRNA in DRGs (Tata et al. 2000) and the development of novel M5 agonists (Bridges et al. 2009) may reveal M5 as a target for novel analgesics. Activation of excitatory M5 receptors on midbrain dopamine neurons (Wess et al. 2007)

may contribute to analgesic efficacy (Pellicer et al. 2010), but could also prove to be an addictive liability (Robinson and Berridge 1993; Wanat et al. 2009).

Nonselective muscarinic agonists can induce spinal ACh release by tapping into descending inhibitory pathways in supraspinal areas or at the level of the spinal cord, but it is unclear which subtypes mediate these effects. Based on preclinical pharmacology data, direct activation of M4 receptors, and to some extent, M1 receptors, may be sufficient for the spinal release of ACh. Release of spinal ACh would almost certainly lead to the activation of M2 receptors and subsequent analgesia, as M2 appears to be the primary mAChR mediating spinal analgesia. This raises the intriguing possibility, then, that direct agonism of M4/M1may indirectly lead to spinal M2 mAChR activation via spinal ACh release, and provide a mechanism whereby spinal M2-mediated analgesia may be obtained while avoiding the presumed cardiac liabilities of systemic M2 agonists.

Defining the mAChR selectivity profile for optimal analgesia and safety is only the first step. An equal challenge is to develop molecules that demonstrate functional subtype selectivity, a task that has proven to be extremely difficult. To date, there are few ligands that possess subtype selectivity against mAChRs, and even fewer that can be considered drug candidates based on their physicochemical and pharmacodynamic properties. The recent identification of allosteric agonists and enhancers (Conn et al. 2009b) with subtype selectivity and ligands that interact with mAChRs in sites that both overlap and are distinct from the ACh binding site (e.g., bitopic ligands) has led to new concepts for the development of subtype-selective mAChR activators. Still, their mechanisms present novel challenges (Avlani et al. 2010; Valant et al. 2009).

Positive allosteric enhancers (PAMs) are molecules that bind to allosteric pockets, increase the potency of ACh to activate the receptor and often display subtype selectivity (Conn et al. 2009a). This mechanism of action is presented as a new way to achieve mAChR subtype selectivity, to maintain the spatial and temporal receptor activation that follows the release of ACh, and elicit pharmacological activity while limiting potential side effects. The cooperativity factor of the PAMs, referred as the quantitative factor describing the increase of ACh potency or affinity, limits the maximal activity that can be elicited at saturating concentrations of the PAM in vivo, and defines a ceiling effect for pharmacology that limits potential adverse effects from overdosing. But the cooperativity factor that limits the in vivo pharmacology of the PAM molecule is both a friend and a foe.

For a PAM to elicit in vivo pharmacological responses, sufficient activation of the target receptor by its endogenous neurotransmitter is required; in this case, an adequate basal cholinergic tone in the pain pathway is necessary for an analgesic effect. Systemically administered nonselective mAChR antagonists can decrease pain thresholds in rodent models (Abelson and Hoglund 2002a), suggesting the existence of a certain cholinergic tone under basal conditions. In addition, although increases in spinal ACh have been associated with acute pain in humans (Eisenach et al. 1996), it is unclear if it is common to all pain states. In fact, neuropathic pain as a result of partial nerve ligation in rats was associated with decreased efflux of basal spinal ACh as assessed by microdialysis (Schechtmann et al. 2008).

To date, however, there are no preclinical data to demonstrate the analgesic activity of mAChR PAMs, so it remains to be seen if these molecules have analgesic efficacy and, if so, whether that efficacy can compare favorably to full mAChR agonists.

Besides the challenge to develop mAChR subtype-selective activators, there is an additional complexity of mAChR physiology that contributes to the risk of development of mAChR agonists for analgesia. First, different combinations of mAChRs contribute to parasympathetic effects in different tissues (Trendelenburg et al. 2005) and acute alterations in the balance between sympathetic and parasympathetic modulation with pharmacological agents may display different degrees of sensitivity across species. Evidence of cross-species differences in mAChR distribution and function (e.g., *see* Table 1) and variations in levels of expression in tissues for different subtypes can lead to differences in receptor reserve and agonist sensitivity that could vary across species. So, the activity of selective agonists in rodents with respect to analgesic efficacy and safety characteristics may, or may not, translate across species, always an inherent risk in drug discovery and development.

Despite these challenges, the development of novel analgesics based on selective agonism of mAChRs represents a unique and compelling opportunity. Existing clinical proof of concept for cholinergic-mediated analgesia, improved understanding of the modulation of pain pathways by mAChRs, and the application of novel approaches to develop subtype-selective ligands afford a great position from which to overcome old challenges and realize the analgesic potential of muscarinic ligands.

References

Abe K, Taguchi K, Kato M, Utsunomiya I, Chikuma T, Hojyo H, Miyatake T (2003) Characterization of muscarinic receptor subtypes in the rostral ventrolateral medulla and effects on morphine-induced antinociception in rats. Eur J Pharmacol 465:237–249

Abelson KS, Hoglund AU (2002a) Intravenously administered oxotremorine and atropine, in doses known to affect pain threshold, affect the intraspinal release of acetylcholine in rats. Pharmacol Toxicol 90:187–192

Abelson KS, Hoglund AU (2002b) Intravenously administered lidocaine in therapeutic doses increases the intraspinal release of acetylcholine in rats. Neurosci Lett 317:93–96

Avlani VA, Langmead CJ, Guida E, Wood MD, Tehan BG, Herdon HJ, Watson JM, Sexton PM, Christopoulos A (2010) Orthosteric and allosteric modes of interaction of novel selective agonists of the M1 muscarinic acetylcholine receptor. Mol Pharmacol 78(1):94–104

Barocelli E, Ballabeni V, Bertoni S, De Amici M, Impicciatore M (2001) Evidence for specific analgesic activity of a muscarinic agonist selected among a new series of acetylenic derivatives. Life Sci 68:1775–1785

Bartolini A, Ghelardini C, Fantetti L, Malcangio M, Malmberg-Aiello P, Giotti A (1992) Role of muscarinic receptor subtypes in central antinociception. Br J Pharmacol 105:77–82

Benarroch EE (2008) Descending monoaminergic pain modulation: bidirectional control and clinical relevance. Neurology 71:217–221

Ben-Sreti MM, Sewell RD (1982) Stereospecific inhibition of oxotremorine-induced antinociception by (+)-isomers of opioid antagonists: comparison with opioid receptor agonists. J Pharm Pharmacol 34:501–505

Bernardini N, Levey AI, Augusti-Tocco G (1999) Rat dorsal root ganglia express m1-m4 muscarinic receptor proteins. J Peripher Nerv Syst 4:222–232

Bernardini N, Reeh PW, Sauer SK (2001a) Muscarinic M2 receptors inhibit heat-induced CGRP release from isolated rat skin. Neuroreport 12:2457–2460

Bernardini N, Sauer SK, Haberberger R, Fischer MJ, Reeh PW (2001b) Excitatory nicotinic and desensitizing muscarinic (M2) effects on C-nociceptors in isolated rat skin. J Neurosci 21:3295–3302

Bernardini N, Roza C, Sauer SK, Gomeza J, Wess J, Reeh PW (2002) Muscarinic M2 receptors on peripheral nerve endings: a molecular target of antinociception. J Neurosci 22:RC229

Breivik H, Stubhaug A (2008) Management of acute postoperative pain: still a long way to go! Pain 137:233–234

Bridges TM, Marlo JE, Niswender CM, Jones CK, Jadhav SB, Gentry PR, Plumley HC, Weaver CD, Conn PJ, Lindsley CW (2009) Discovery of the first highly M5-preferring muscarinic acetylcholine receptor ligand, an M5 positive allosteric modulator derived from a series of 5-trifluoromethoxy *N*-benzyl isatins. J Med Chem 52:3445–3448

Burke S, Shorten GD (2009) When pain after surgery doesn't go away. Biochem Soc Trans 37:318–322

Burton AW, Lubenow TR, Prithvi Raj P (2005) Traditional interventional therapies. In: Wilson PR, Stanton-Hicks M, Harden RN (eds) CRPS: current diagnosis and therapies, 1st edn. IASP, Seattle, WA

Cai YQ, Chen SR, Han HD, Sood AK, Lopez-Berestein G, Pan HL (2009) Role of M2, M3, and M4 muscarinic receptor subtypes in the spinal cholinergic control of nociception revealed using siRNA in rats. J Neurochem 111:1000–1010

Calogero AE, Kamilaris TC, Gomez MT, Johnson EO, Tartaglia ME, Gold PW, Chrousos GP (1989) The muscarinic cholinergic agonist arecoline stimulates the rat hypothalamic-pituitary-adrenal axis through a centrally-mediated corticotropin-releasing hormone-dependent mechanism. Endocrinology 125:2445–2453

Capone F, Aloisi AM, Carli G, Sacerdote P, Pavone F (1999) Oxotremorine-induced modifications of the behavioral and neuroendocrine responses to formalin pain in male rats. Brain Res 830:292–300

Carter ML (2004) Spinal cord stimulation in chronic pain: a review of the evidence. Anaesth Intensive Care 32:11–21

Caulfield MP, Birdsall NJ (1998) International union of pharmacology. XVII. Classification of muscarinic acetylcholine receptors. Pharmacol Rev 50:279–290

Celestin J, Edwards RR, Jamison RN (2009) Pretreatment psychosocial variables as predictors of outcomes following lumbar surgery and spinal cord stimulation: a systematic review and literature synthesis. Pain Med 10:639–653

Chen SR, Pan HL (2001) Spinal endogenous acetylcholine contributes to the analgesic effect of systemic morphine in rats. Anesthesiology 95:525–530

Chen SR, Pan HL (2003a) Spinal GABAB receptors mediate antinociceptive actions of cholinergic agents in normal and diabetic rats. Brain Res 965:67–74

Chen SR, Pan HL (2003b) Up-regulation of spinal muscarinic receptors and increased antinociceptive effect of *i.t.* muscarine in diabetic rats. J Pharmacol Exp Ther 307:676–681

Chen SR, Pan HL (2004) Activation of muscarinic receptors inhibits spinal dorsal horn projection neurons: role of GABAB receptors. Neuroscience 125:141–148

Chen SR, Wess J, Pan HL (2005a) Functional activity of the M2 and M4 receptor subtypes in the spinal cord studied with muscarinic acetylcholine receptor knockout mice. J Pharmacol Exp Ther 313:765–770

Chen YP, Chen SR, Pan HL (2005b) Systemic morphine inhibits dorsal horn projection neurons through spinal cholinergic system independent of descending patwhays. J Pharmacol Exp Ther 314:611–617

Clayton BA, Hayashida K, Childers SR, Xiao R, Eisenach JC (2007) Oral donepezil reduces hypersensitivity after nerve injury by a spinal muscarinic receptor mechanism. Anesthesiology 106:1019–1025

Coderre TJ, Abbott FV, Melzack R (1984) Effects of peripheral antisympathetic treatments in the tail-flick, formalin and autotomy tests. Pain 18:13–23

Conn PJ, Christopoulos A, Lindsley CW (2009a) Allosteric modulators of GPCRs: a novel approach for the treatment of CNS disorders. Nat Rev Drug Discov 8:41–54

Conn PJ, Jones CK, Lindsley CW (2009b) Subtype-selective allosteric modulators of muscarinic receptors for the treatment of CNS disorders. Trends Pharmacol Sci 30:148–155

Craig AD (2003) A new view of pain as a homeostatic emotion. Trends Neurosci 26:303–307

de Leon-Casasola OA (2009) Spinal cord and peripheral nerve stimulation techniques for neuropathic pain. J Pain Symptom Manage 38:S28–S38

Di Cesare ML, Ghelardini C, Calvani M, Nicolai R, Mosconi L, Toscano A, Pacini A, Bartolini A (2009) Neuroprotective effects of acetyl-L-carnitine on neuropathic pain and apoptosis: a role for the nicotinic receptor. J Neurosci Res 87:200–207

Dorje F, Levey AI, Brann MR (1991a) Immunological detection of muscarinic receptor subtype proteins (m1-m5) in rabbit peripheral tissues. Mol Pharmacol 40:459–462

Dorje F, Wess J, Lambrecht G, Tacke R, Mutschler E, Brann MR (1991b) Antagonist binding profiles of five cloned human muscarinic receptor subtypes. J Pharmacol Exp Ther 256:727–733

Duflo F, Conklin D, Li X, Eisenach JC (2003) Spinal adrenergic and cholinergic receptor interactions activated by clonidine in postincisional pain. Anesthesiology 98:1237–1242

Dussor GO, Helesic G, Hargreaves KM, Flores CM (2004) Cholinergic modulation of nociceptive responses in vivo and neuropeptide release in vitro at the level of the primary sensory neuron. Pain 107:22–32

Duttaroy A, Gomeza J, Gan JW, Siddiqui N, Basile AS, Harman WD, Smith PL, Felder CC, Levey AI, Wess J (2002) Evaluation of muscarinic agonist-induced analgesia in muscarinic acetylcholine receptor knockout mice. Mol Pharmacol 62:1084–1093

Eisenach JC (1999) Muscarinic-mediated analgesia. Life Sci 64:549–554

Eisenach JC (2009) Epidural neostigmine: will it replace lipid soluble opioids for postoperative and labor analgesia? Anesth Analg 109:293–295

Eisenach JC, Detweiler DJ, Tong C, D'Angelo R, Hood DD (1996) Cerebrospinal fluid norepinephrine and acetylcholine concentrations during acute pain. Anesth Analg 82:621–626

Ellis JL, Harman D, Gonzalez J, Spera ML, Liu R, Shen TY, Wypij DM, Zuo F (1999) Development of muscarinic analgesics derived from epibatidine: role of the M4 receptor subtype. J Pharmacol Exp Ther 288:1143–1150

Franco AC, Prado WA (1996) Antinociceptive effects of stimulation of discrete sites in the rat hypothalamus: evidence for the participation of the lateral hypothalamus area in descending pain suppression mechanisms. Braz J Med Biol Res 29:1531–1541

Gage HD, Gage JC, Tobin JR, Chiari A, Tong C, Xu Z, Mach RH, Efange SM, Ehrenkaufer RL, Eisenach JC (2001) Morphine-induced spinal cholinergic activation: in vivo imaging with positron emission tomography. Pain 91:139–145

Galeotti N, Bartolini A, Ghelardini C (2003) The phospholipase C-IP3 pathway is involved in muscarinic antinociception. Neuropsychopharmacology 28:888–897

Gebhart GF (2004) Descending modulation of pain. Neurosci Biobehav Rev 27:729–737

George R, Haslett WL, Jenden DJ (1962) The central action of a metabolite of tremorine. Life Sci 1:361–363

Ghelardini C, Galeotti N, Gualtieri F, Bellucci C, Manetti D, Giotti A, Malmberg-Aiello P, Galli A, Bartolini A (1997a) Antinociceptive profile of 3-alpha-tropanyl 2-(4-Cl-phenoxy)butyrate (SM-21) [corrected]: a novel analgesic with a presynaptic cholinergic mechanism of action. J Pharmacol Exp Ther 282:430–439

Ghelardini C, Galeotti N, Gualtieri F, Novella Romanelli M, Bartolini A (1997b) Antinociception induced by SM 32 depends on a central cholinergic mechanism. Pharmacol Res 35:141–147

Ghelardini C, Gualtieri F, Novella Romanelli M, Angeli P, Pepeu G, Grazia Giovannini M, Casamenti F, Malmberg-Aiello P, Giotti A, Bartolini A (1997c) Stereoselective increase in cholinergic transmission by R-(+)-hyoscyamine. Neuropharmacology 36:281–294

Ghelardini C, Galeotti N, Gualtieri F, Marchese V, Bellucci C, Bartolini A (1998) Antinociceptive and antiamnesic properties of the presynaptic cholinergic amplifier PG-9. J Pharmacol Exp Ther 284:806–816

Ghelardini C, Galeotti N, Bartolini A (2000) Loss of muscarinic antinociception by antisense inhibition of M(1) receptors. Br J Pharmacol 129:1633–1640

Ghelardini C, Galeotti N, Lelli C, Bartolini A (2001) M1 receptor activation is a requirement for arecoline analgesia. Farmaco 56:383–385

Ghelardini C, Galeotti N, Calvani M, Mosconi L, Nicolai R, Bartolini A (2002) Acetyl-l-carnitine induces muscarinic antinocieption in mice and rats. Neuropharmacology 43:1180–1187

Gomeza J, Shannon H, Kostenis E, Felder C, Zhang L, Brodkin J, Grinberg A, Sheng H, Wess J (1999) Pronounced pharmacologic deficits in M2 muscarinic acetylcholine receptor knockout mice. Proc Natl Acad Sci USA 96:1692–1697

Grando SA, Kist DA, Qi M, Dahl MV (1993) Human keratinocytes synthesize, secrete, and degrade acetylcholine. J Invest Dermatol 101:32–36

Guimaraes AP, Guimaraes FS, Prado WA (2000) Modulation of carbachol-induced antinociception from the rat periaqueductal gray. Brain Res Bull 51:471–478

Haberberger RV, Bodenbenner M (2000) Immunohistochemical localization of muscarinic receptors (M2) in the rat skin. Cell Tissue Res 300:389–396

Habib AS, Gan TJ (2006) Use of neostigmine in the management of acute postoperative pain and labour pain: a review. CNS Drugs 20:821–839

Hamilton SE, Loose MD, Qi M, Levey AI, Hille B, McKnight GS, Idzerda RL, Nathanson NM (1997) Disruption of the m1 receptor gene ablates muscarinic receptor-dependent M current regulation and seizure activity in mice. Proc Natl Acad Sci USA 94:13311–13316

Hardouin SN, Richmond KN, Zimmerman A, Hamilton SE, Feigl EO, Nathanson NM (2002) Altered cardiovascular responses in mice lacking the M(1) muscarinic acetylcholine receptor. J Pharmacol Exp Ther 301:129–137

Hartvig P, Gillberg PG, Gordh T Jr, Post C (1989) Cholinergic mechanisms in pain and analgesia. Trends Pharmacol Sci 10(Suppl):75–79

Hayashida K, Parker R, Eisenach JC (2007) Oral gabapentin activates spinal cholinergic circuits to reduce hypersensitivity after peripheral nerve injury and interacts synergistically with oral donepezil. Anesthesiology 106:1213–1219

Hedge GA, de Wied D (1971) Corticotropin and vasopressin secretion after hypothalamic implantation of atropine. Endocrinology 88:1257–1259

Hemrick-Luecke SK, Bymaster FP, Evans DC, Wess J, Felder CC (2002) Muscarinic agonist-mediated increases in serum corticosterone levels are abolished in m(2) muscarinic acetylcholine receptor knockout mice. J Pharmacol Exp Ther 303:99–103

Ho KM, Ismail H, Lee KC, Branch R (2005) Use of intrathecal neostigmine as an adjunct to other spinal medications in perioperative and peripartum analgesia: a meta-analysis. Anaesth Intensive Care 33:41–53

Hoglund AU, Baghdoyan HA (1997) M2, M3 and M4, but not M1, muscarinic receptor subtypes are present in rat spinal cord. J Pharmacol Exp Ther 281:470–477

Hoglund AU, Hamilton C, Lindblom L (2000) Effects of microdialyzed oxotremorine, carbachol, epibatidine, and scopolamine on intraspinal release of acetylcholine in the rat. J Pharmacol Exp Ther 295:100–104

Honda K, Ando S, Koga K, Takano Y (2004) The spinal muscarinic receptor subtypes contribute to the morphine-induced antinociceptive effects in thermal stimulation in mice. Neurosci Lett 371:235–238

Hood DD, Mallak KA, Eisenach JC, Tong C (1996) Interaction between intrathecal neostigmine and epidural clonidine in human volunteers. Anesthesiology 85:315–325

Hood DD, Mallak KA, James RL, Tuttle R, Eisenach JC (1997) Enhancement of analgesia from systemic opioid in humans by spinal cholinesterase inhibition. J Pharmacol Exp Ther 282:86–92

Ireson JD (1970) A comparison of the antinociceptive actions of cholinomimetic and morphine-like drugs. Br J Pharmacol 40:92–101

Iwamoto ET, Marion L (1993) Characterization of the antinociception produced by intrathecally administered muscarinic agonists in rats. J Pharmacol Exp Ther 266:329–338

Iwamoto ET, Marion L (1994) Pharmacological evidence that nitric oxide mediates the antinociception produced by muscarinic agonists in the rostral ventral medulla of rats. J Pharmacol Exp Ther 269:699–708

Janig W (2009) Autonomic nervous system and pain. In: Basbaum AI, Bushnell MC (eds) Science of pain, 1st edn. Elsevier, San Diego, CA

Jones PG, Dunlop J (2007) Targeting the cholinergic system as a therapeutic strategy for the treatment of pain. Neuropharmacology 53:197–206

Kang YJ, Eisenach JC (2003) Intrathecal clonidine reduces hypersensitivity after nerve injury by a mechanism involving spinal m4 muscarinic receptors. Anesth Analg 96:1403–1408, Table of contents

Karaaslan K, Gulcu N, Ozturk H, Sarpkaya A, Colak C, Kocoglu H (2009) Two different doses of caudal neostigmine co-administered with levobupivacaine produces analgesia in children. Paediatr Anaesth 19:487–493

Katz J, Seltzer Z (2009) Transition from acute to chronic postsurgical pain: risk factors and protective factors. Expert Rev Neurother 9:723–744

Kawashima K, Yoshikawa K, Fujii YX, Moriwaki Y, Misawa H (2007) Expression and function of genes encoding cholinergic components in murine immune cells. Life Sci 80:2314–2319

Kehlet H, Jensen TS, Woolf CJ (2006) Persistent postsurgical pain: risk factors and prevention. Lancet 367:1618–1625

Khan ZH, Hamidi S, Miri M, Majedi H, Nourijelyani K (2008) Post-operative pain relief following intrathecal injection of acetylcholine esterase inhibitor during lumbar disc surgery: a prospective double blind randomized study. J Clin Pharm Ther 33:669–675

Kim SH, Chung JM (1991) Sympathectomy alleviates mechanical allodynia in an experimental animal model for neuropathy in the rat. Neurosci Lett 134:131–134

Klimscha W, Tong C, Eisenach JC (1997) Intrathecal alpha 2-adrenergic agonists stimulate acetylcholine and norepinephrine release from the spinal cord dorsal horn in sheep. An in vivo microdialysis study. Anesthesiology 87:110–116

Kommalage M, Hoglund AU (2005) Involvement of spinal serotonin receptors in the regulation of intraspinal acetylcholine release. Eur J Pharmacol 509:127–134

Kubista H, Kosenburger K, Mahlknecht P, Drobny H, Boehm S (2009) Inhibition of transmitter release from rat sympathetic neurons via presynaptic M(1) muscarinic acetylcholine receptors. Br J Pharmacol 156:1342–1352

Langley RJ, Kalra R, Mishra NC, Sopori ML (2004) Central but not the peripheral action of cholinergic compounds suppresses the immune system. J Neuroimmunol 148:140–145

Lauretti GR, Gomes JM, Reis MP, Pereira NL (1999) Low doses of epidural ketamine or neostigmine, but not midazolam, improve morphine analgesia in epidural terminal cancer pain therapy. J Clin Anesth 11:663–668

Levey AI (1993) Immunological localization of m1-m5 muscarinic acetylcholine receptors in peripheral tissues and brain. Life Sci 52:441–448

Li DP, Chen SR, Pan YZ, Levey AI, Pan HL (2002) Role of presynaptic muscarinic and GABA(B) receptors in spinal glutamate release and cholinergic analgesia in rats. J Physiol 543:807–818

Lograsso M, Nadeson R, Goodchild CS (2002) The spinal antinociceptive effects of cholinergic drugs in rats: receptor subtype specificity in different nociceptive tests. BMC Pharmacol 2:20

MacLennan AJ, Drugan RC, Hyson RL, Maier SF, Jt M, Barchas JD (1982) Corticosterone: a critical factor in an opioid form of stress-induced analgesia. Science 215:1530–1532

Masuda H, Ichiyanagi N, Yokoyama M, Sakai Y, Kihara K, Chancellor MB, de Groat WC, Yoshimura N (2009) Muscarinic receptor activation in the lumbosacral spinal cord ameliorates bladder irritation in rat cystitis models. BJU Int 104:1531–1537

Mulugeta E, El-Bakri N, Karlsson E, Elhassan A, Adem A (2003) Loss of muscarinic M4 receptors in spinal cord of arthritic rats: implications for a role of M4 receptors in pain response. Brain Res 982:284–287

Naguib M, Yaksh TL (1994) Antinociceptive effects of spinal cholinesterase inhibition and isobolographic analysis of the interaction with mu and alpha 2 receptor systems. Anesthesiology 80:1338–1348

Naguib M, Yaksh TL (1997) Characterization of muscarinic receptor subtypes that mediate antinociception in the rat spinal cord. Anesth Analg 85:847–853

National Health and Nutrition Examination Survey. (NHANES), 1999-2004. Centers for Disease Control and Prevention (CDC). National Center for Health Statistics (NCHS). National Health and Nutrition Examination Survey Questionnaire (or Examination Protocol, or Laboratory Protocol). Hyattsville, MD: U.S. Department of Health and Human Services, Centers for Disease Control and Prevention, 2004 http://www.cdc.gov/nchs/nhanes.htm

Obata H, Li X, Eisenach JC (2005) alpha2-Adrenoceptor activation by clonidine enhances stimulation-evoked acetylcholine release from spinal cord tissue after nerve ligation in rats. Anesthesiology 102:657–662

Oliveira MA, Prado WA (1994) Antinociception and behavioral manifestations induced by intracerebroventricular or intra-amygdaloid administration of cholinergic agonists in the rat. Pain 57:383–391

Paalzow G, Paalzow L (1975) Antinociceptive action of oxotremorine and regional turnover of rat brain noradrenaline, dopamine and 5-HT. Eur J Pharmacol 31(2):261–272

Pan HL, Chen SR, Eisenach JC (1999) Intrathecal clonidine alleviates allodynia in neuropathic rats: interaction with spinal muscarinic and nicotinic receptors. Anesthesiology 90:509–514

Pan HL, Wu ZZ, Zhou HY, Chen SR, Zhang HM, Li DP (2008) Modulation of pain transmission by G-protein-coupled receptors. Pharmacol Ther 117:141–161

Papaioannou M, Skapinakis P, Damigos D, Mavreas V, Broumas G, Palgimesi A (2009) The role of catastrophizing in the prediction of postoperative pain. Pain Med 10:1452–1459

Pellicer F, Ortega-Legaspi JM, Lopez-Avila A, Coffeen U, Jaimes O (2010) Dopamine pathways and receptors in nociception and pain. In: Beaulieu P, Lussier D, Porecca F, Dickenson AH (eds) Pharmacology of pain, 1st edn. IASP, Seattle, WA

Pinardi G, Sierralta F, Miranda HF (2003) Atropine reverses the antinociception of nonsteroidal anti-inflammatory drugs in the tail-flick test of mice. Pharmacol Biochem Behav 74:603–608

Popping DM, Zahn PK, Van Aken HK, Dasch B, Boche R, Pogatzki-Zahn EM (2008) Effectiveness and safety of postoperative pain management: a survey of 18 925 consecutive patients between 1998 and 2006 (2nd revision): a database analysis of prospectively raised data. Br J Anaesth 101:832–840

Prado WA, Segalla DK (2004) Antinociceptive effects of bethanechol or dimethylphenylpiperazinium in models of phasic or incisional pain in rats. Brain Res 1018:272–282

Robinson TE, Berridge KC (1993) The neural basis of drug craving: an incentive-sensitization theory of addiction. Brain Res Brain Res Rev 18:247–291

Ross VH, Pan PH, Owen MD, Seid MH, Harris L, Clyne B, Voltaire M, Eisenach JC (2009) Neostigmine decreases bupivacaine use by patient-controlled epidural analgesia during labor: a randomized controlled study. Anesth Analg 109:524–531

Schappert SM (1994) National ambulatory medical care survey: 1992 summary. Adv Data 253:1–20

Schechtmann G, Song Z, Ultenius C, Meyerson BA, Linderoth B (2008) Cholinergic mechanisms involved in the pain relieving effect of spinal cord stimulation in a model of neuropathy. Pain 139:136–145

Scholz J, Woolf CJ (2007) The neuropathic pain triad: neurons, immune cells and glia. Nat Neurosci 10:1361–1368

Shannon HE, Sheardown MJ, Bymaster FP, Calligaro DO, Delapp NW, Gidda J, Mitch CH, Sawyer BD, Stengel PW, Ward JS, Wong DT, Olesen PH, Suzdak PD, Sauerberg P, Swedberg MD (1997a) Pharmacology of butylthio[2.2.2] (LY297802/NNC11-1053): a novel analgesic with mixed muscarinic receptor agonist and antagonist activity. J Pharmacol Exp Ther 281:884–894

Shannon HE, Womer DE, Bymaster FP, Calligaro DO, DeLapp NC, Mitch CH, Ward JS, Whitesitt CA, Swedberg MD, Sheardown MJ, Fink-Jensen A, Olesen PH, Rimvall K, Sauerberg P (1997b) In vivo pharmacology of butylthio[2.2.2] (LY297802/NNC11-1053), an orally acting antinociceptive muscarinic agonist. Life Sci 60:969–976

Shannon HE, Jones CK, Li DL, Peters SC, Simmons RM, Iyengar S (2001) Antihyperalgesic effects of the muscarinic receptor ligand vedaclidine in models involving central sensitization in rats. Pain 93:221–227

Shapiro MS, Gomeza J, Hamilton SE, Hille B, Loose MD, Nathanson NM, Roche JP, Wess J (2001) Identification of subtypes of muscarinic receptors that regulate Ca^{2+} and K^{+} channel activity in sympathetic neurons. Life Sci 68:2481–2487

Sheardown MJ, Shannon HE, Swedberg MD, Suzdak PD, Bymaster FP, Olesen PH, Mitch CH, Ward JS, Sauerberg P (1997) M1 receptor agonist activity is not a requirement for muscarinic antinociception. J Pharmacol Exp Ther 281:868–875

Song Z, Meyerson BA, Linderoth B (2008) Muscarinic receptor activation potentiates the effect of spinal cord stimulation on pain-related behavior in rats with mononeuropathy. Neurosci Lett 436:7–12

Spinella M, Znamensky V, Moroz M, Ragnauth A, Bodnar RJ (1999) Actions of NMDA and cholinergic receptor antagonists in the rostral ventromedial medulla upon beta-endorphin analgesia elicited from the ventrolateral periaqueductal gray. Brain Res 829:151–159

Steen KH, Reeh PW (1993) Actions of cholinergic agonists and antagonists on sensory nerve endings in rat skin, in vitro. J Neurophysiol 70:397–405

Stengel PW, Gomeza J, Wess J, Cohen ML (2000) M(2) and M(4) receptor knockout mice: muscarinic receptor function in cardiac and smooth muscle in vitro. J Pharmacol Exp Ther 292:877–885

Stewart WF, Ricci JA, Chee E, Morganstein D, Lipton R (2003) Lost productive time and cost due to common pain conditions in the US workforce. JAMA 290:2443–2454

Sullivan NR, Leventhal L, Harrison J, Smith VA, Cummons TA, Spangler TB, Sun SC, Lu P, Uveges AJ, Strassle BW, Piesla MJ, Ramdass R, Barry A, Schantz J, Adams W, Whiteside GT, Adedoyin A, Jones PG (2007) Pharmacological characterization of the muscarinic agonist (3R,4R)-3-(3-hexylsulfanyl-pyrazin-2-yloxy)-1-aza-bicyclo[2.2.1]heptane (WAY-132983) in in vitro and in vivo models of chronic pain. J Pharmacol Exp Ther 322:1294–1304

Swedberg MD, Sheardown MJ, Sauerberg P, Olesen PH, Suzdak PD, Hansen KT, Bymaster FP, Ward JS, Mitch CH, Calligaro DO, Delapp NW, Shannon HE (1997) Butylthio[2.2.2] (NNC 11-1053/LY297802): an orally active muscarinic agonist analgesic. J Pharmacol Exp Ther 281:876–883

Takasu K, Honda M, Ono H, Tanabe M (2006) Spinal alpha(2)-adrenergic and muscarinic receptors and the NO release cascade mediate supraspinally produced effectiveness of gabapentin at decreasing mechanical hypersensitivity in mice after partial nerve injury. Br J Pharmacol 148:233–244

Tata AM (2008) Muscarinic acetylcholine receptors: new potential therapeutic targets in antinociception and in cancer therapy. Recent Pat CNS Drug Discov 3:94–103

Tata AM, Vilaro MT, Mengod G (2000) Muscarinic receptor subtypes expression in rat and chick dorsal root ganglia. Brain Res Mol Brain Res 82:1–10

Tayebati SK, El-Assouad D, Ricci A, Amenta F (2002) Immunochemical and immunocytochemical characterization of cholinergic markers in human peripheral blood lymphocytes. J Neuroimmunol 132:147–155

Trendelenburg AU, Gomeza J, Klebroff W, Zhou H, Wess J (2003) Heterogeneity of presynaptic muscarinic receptors mediating inhibition of sympathetic transmitter release: a study with M2- and M4-receptor-deficient mice. Br J Pharmacol 138:469–480

Trendelenburg AU, Meyer A, Wess J, Starke K (2005) Distinct mixtures of muscarinic receptor subtypes mediate inhibition of noradrenaline release in different mouse peripheral tissues, as studied with receptor knockout mice. Br J Pharmacol 145:1153–1159

Valant C, Sexton PM, Christopoulos A (2009) Orthosteric/allosteric bitopic ligands: going hybrid at GPCRs. Mol Interv 9:125–135

Van de Velde M, Berends N, Kumar A, Devroe S, Devlieger R, Vandermeersch E, De Buck F (2009) Effects of epidural clonidine and neostigmine following intrathecal labour analgesia: a randomised, double-blind, placebo-controlled trial. Int J Obstet Anesth 18:207–214

Wamsley JK, Zarbin MA, Kuhar MJ (1981) Muscarinic cholinergic receptors flow in the sciatic nerve. Brain Res 217:155–161

Wanat MJ, Willuhn I, Clark JJ, Phillips PE (2009) Phasic dopamine release in appetitive behaviors and drug addiction. Curr Drug Abuse Rev 2:195–213

Wang XL, Zhang HM, Li DP, Chen SR, Pan HL (2006) Dynamic regulation of glycinergic input to spinal dorsal horn neurones by muscarinic receptor subtypes in rats. J Physiol 571:403–413

Wanke E, Ferroni A, Malgaroli A, Ambrosini A, Pozzan T, Meldolesi J (1987) Activation of a muscarinic receptor selectively inhibits a rapidly inactivated Ca^{2+} current in rat sympathetic neurons. Proc Natl Acad Sci USA 84:4313–4317

Wei J, Walton EA, Milici A, Buccafusco JJ (1994) m1-m5 muscarinic receptor distribution in rat CNS by RT-PCR and HPLC. J Neurochem 63:815–821

Wess J (2003) Novel insights into muscarinic acetylcholine receptor function using gene targeting technology. Trends Pharmacol Sci 24:414–420

Wess J, Duttaroy A, Gomeza J, Zhang W, Yamada M, Felder CC, Bernardini N, Reeh PW (2003a) Muscarinic receptor subtypes mediating central and peripheral antinociception studied with muscarinic receptor knockout mice: a review. Life Sci 72:2047–2054

Wess J, Duttaroy A, Zhang W, Gomeza J, Cui Y, Miyakawa T, Bymaster FP, McKinzie L, Felder CC, Lamping KG, Faraci FM, Deng C, Yamada M (2003b) M1-M5 muscarinic receptor knockout mice as novel tools to study the physiological roles of the muscarinic cholinergic system. Receptors Channels 9:279–290

Wess J, Eglen RM, Gautam D (2007) Muscarinic acetylcholine receptors: mutant mice provide new insights for drug development. Nat Rev Drug Discov 6:721–733

Wessler I, Kirkpatrick CJ, Racke K (1998) Non-neuronal acetylcholine, a locally acting molecule, widely distributed in biological systems: expression and function in humans. Pharmacol Ther 77:59–79

Womer DE, Shannon HE (2000) Reversal of pertussis toxin-induced thermal allodynia by muscarinic cholinergic agonists in mice. Neuropharmacology 39:2499–2504

Xu Z, Tong C, Pan HL, Cerda SE, Eisenach JC (1997) Intravenous morphine increases release of nitric oxide from spinal cord by an alpha-adrenergic and cholinergic mechanism. J Neurophysiol 78:2072–2078

Yaksh TL, Dirksen R, Harty GJ (1985) Antinociceptive effects of intrathecally injected cholinomimetic drugs in the rat and cat. Eur J Pharmacol 117:81–88

Zhang HM, Li DP, Chen SR, Pan HL (2005) M2, M3, and M4 receptor subtypes contribute to muscarinic potentiation of GABAergic inputs to spinal dorsal horn neurons. J Pharmacol Exp Ther 313:697–704

Zhang HM, Chen SR, Matsui M, Gautam D, Wess J, Pan HL (2006) Opposing functions of spinal M2, M3, and M4 receptor subtypes in regulation of GABAergic inputs to dorsal horn neurons revealed by muscarinic receptor knockout mice. Mol Pharmacol 69:1048–1055

Zhang HM, Chen SR, Pan HL (2007a) Regulation of glutamate release from primary afferents and interneurons in the spinal cord by muscarinic receptor subtypes. J Neurophysiol 97:102–109

Zhang HM, Zhou HY, Chen SR, Gautam D, Wess J, Pan HL (2007b) Control of glycinergic input to spinal dorsal horn neurons by distinct muscarinic receptor subtypes revealed using knockout mice. J Pharmacol Exp Ther 323:963–971

Zhao P, Barr TP, Hou Q, Dib-Hajj SD, Black JA, Albrecht PJ, Petersen K, Eisenberg E, Wymer JP, Rice FL, Waxman SG (2008) Voltage-gated sodium channel expression in rat and human epidermal keratinocytes: evidence for a role in pain. Pain 139:90–105

Muscarinic Modulation of Striatal Function and Circuitry

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Abstract Striatal cholinergic interneurons are pivotal modulators of the striatal circuitry involved in action selection and decision making. Although nicotinic receptors are important transducers of acetylcholine release in the striatum, muscarinic receptors are more pervasive and have been more thoroughly studied. In this review, the effects of muscarinic receptor signaling on the principal cell types in the striatum and its canonical circuits will be discussed, highlighting new insights into their role in synaptic integration and plasticity. These studies, and those that have identified new circuit elements driven by activation of nicotinic receptors, make it clear that temporally patterned activity in cholinergic interneurons must play an important role in determining the effects on striatal circuitry. These effects could be critical to the response to salient environmental stimuli that serve to direct behavior.

Keywords Striatum • Medium spiny projection neuron • Acetylcholine • Cholinergic interneuron • Muscarinic receptor • Synaptic integration • Thalamus • Synaptic plasticity • Neuromodulation • Autoreceptor • Parkinson's disease

1 Introduction

The basal ganglia are a richly interconnected set of subcortical nuclei intimately involved in the regulation of action selection and decision making (Albin et al. 1989; DeLong and Wichmann 2009; Frank and Claus 2006; Gerfen 1992; Houk et al. 2007; Kimura et al. 2003; Mink 1996; Morris et al. 2004; Wichmann and DeLong 1996). The striatum is the largest nucleus of this group and serves as the initial integrator of cortical and thalamic information relevant to this process.

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Essentially all cortical areas – sensory, motor, and associational – project to the striatum (Bolam et al. 2000; Gerfen 1992; Wilson 2004). Wide regions of the thalamus also project to the striatum, with a particularly prominent contribution from the intralaminar thalamic nuclei that are responsive to salient or novel sensory events (Doig et al. 2010; Matsumoto et al. 2001; McHaffie et al. 2005; Smith et al. 2004). Both cortical and thalamic projections are glutamatergic, forming excitatory synaptic connections with principal GABAergic, spiny projection neurons (SPNs) and interneurons. SPNs constitute the vast majority of striatal neurons (~90–95%) with each of the four interneuron populations constituting a few percent of the total.

Of the interneuron populations, all but one is GABAergic. The only non-GABAergic interneuron in the striatum is the cholinergic interneuron (Bolam et al. 1984; Kemp and Powell 1971; Phelps et al. 1985). Despite constituting only a few percent of all striatal neurons, these giant, aspiny interneurons are responsible for striatal levels of acetylcholine (ACh), choline acetyltransferase, and choline esterase that are among the highest in the brain (Contant et al. 1996; Mesulam et al. 1992). Because cholinergic interneurons are autonomous pacemakers, whose basal spiking at 3–10 Hz is only transiently modulated up or down by synaptic input, ACh release from the dense interneuronal terminal plexus is virtually continuous, covering all regions of the striatum (Bennett and Wilson 1999; Goldberg and Wilson 2010; Kawaguchi 1993; Wilson et al. 1990).

Both nicotinic and muscarinic receptors transduce ACh signals in the striatum. However, the cellular distribution of nicotinic receptors (nAChRs) is more restricted than that of muscarinic receptors (mAChRs), being limited to interneurons and afferent terminals (Wilson 2004). In contrast, muscarinic receptors are robustly expressed by the axon terminals of major projections systems to the striatum and by all striatal neurons that have been examined, including principal SPNs. The focus of this chapter will be on the part played by muscarinic receptors in the regulation of striatal circuitry in health and disease.

2 Striatal Muscarinic Receptors

Five mAChRs have been cloned (Caulfield and Birdsall 1998; Eglen 2005; Wess 1996). These receptors can be divided into two classes on the basis of their coupling to G-proteins: M1-class (M1, M3, M5) and M2-class (M2, M4). M1-class receptors couple to Gq/11 Gα proteins that activate phospholipase C (PLC) isoforms resulting in phosphatidylinositol 4,5-bisphosphate (PIP2) hydrolysis to inositol 1,4,5-triphosphate (IP3) and diacyl glycerol (DAG). M2-class receptors couple to Gi/o G proteins that inhibit adenylyl cyclase (AC) through Giα subunits and close Cav2 Ca^{2+} channels and open Kir3 channels through associated Gβγ subunits (Wess 1996; Wess et al. 2007). All five of the cloned mAChRs are expressed in the striatum, with the M1 and M4 subtypes being the most abundant at the tissue level (Alcantara et al. 2001; Bernard et al. 1992; Hersch et al. 1994; Yan et al. 2001; Zhang et al. 2002).

3 Muscarinic Modulation of Canonical Striatal Circuits

In an attempt to organize the relevant literature, the effects of mAChRs on three canonical striatal circuits will be discussed. These are (1) the corticostriatal circuit engaging SPNs, (2) the corticostriatal feed-forward circuit through GABAergic interneurons, and (3) the thalamostriatal feed-forward circuit through cholinergic interneurons.

3.1 The Corticostriatal Circuit

The most basic striatal microcircuit is the one formed by glutamatergic cortical pyramidal neurons and SPNs (Bolam et al. 2000; Wilson 2004). The synapses formed by cortical pyramidal neurons are exclusively on dendritic spines of SPNs. These spines are absent from soma and the most proximal dendrites, rising to a peak density (1–2 μm^{-1}) 50–60 μm from the soma and then falling off very gradually in density to the tips of the sparsely branching dendrites (250–400 μm) (Wilson 1994). Individual cortical axons are sparsely connected to any one SPN, typically making one or two en passant synapses (Parent and Parent 2006). There is no obvious organization to the cortical synapses on the dendritic tree of SPNs, but this could simply be that this organization is difficult to see, as the striatum lacks the lamination characteristic of other regions where this is apparent (e.g., cerebral cortex).

Glutamatergic synapses onto SPNs are richly invested with M2-class mAChRs. These presynaptic mAChRs diminish glutamate release, reducing the excitatory effect of a cortical volley on SPNs (Akaike et al. 1988; Briggs et al. 1981; Malenka and Kocsis 1988). Using an elegant paired recording approach, Pakhotin and Bracci (2007) were able to show that a single cholinergic interneuron spike was able to significantly reduce electrically evoked glutamatergic evoked postsynaptic currents (EPSCs) in nearby SPNs. As expected from the signaling linkages of mAChRs, the presynaptic inhibition was mediated by reducing the opening of Cav2 Ca^{2+} channels controlling terminal exocytosis. This modulation appears to be exclusively of Ca^{2+} channels with a Cav2.1 pore-forming subunit (Barral et al. 1999). A recent study using a novel optical quantal analysis has beautifully characterized the mAChR modulation of release probability at this synapse, confirming previous inferences from less direct measurements (Higley et al. 2009). Because the release of ACh is sustained by the autonomous activity of cholinergic interneurons, the presynaptic mAChR signaling results in the tonic inhibition of glutamatergic synapses on SPNs (Pakhotin and Bracci 2007). Thus, either antagonizing M2-class mAChRs or suppressing the activity of interneurons results in an increased frequency of glutamatergic miniature mEPSCs in SPNs.

What has not been fully appreciated by these studies is the heterogeneity of glutamatergic afferent fibers reaching the striatum. As mentioned earlier, both

cortical and thalamic glutamatergic neurons project to the striatum and form synapses on SPNs (Dube et al. 1988; Wilson 2004). Contrary to widely held prejudice, thalamic synapses are nearly as numerous as cortical synapses. Moreover, while some thalamic axons form synapses on dendritic shafts, other thalamic axons synapse on spine heads, just as cortical axons do (Doig et al. 2010; Dube et al. 1988). There seems to be no qualitative difference between SPNs of the direct and indirect pathway (see below) in their innervation by cortex or thalamus (Ding et al. 2008; Doig et al. 2010), in spite of earlier reports that only direct pathway SPNs (dSPNs) were innervated by thalamic axons (Sidibe and Smith 1996; Smith et al. 2004).

Although sharing anatomical features, the physiological properties of these two synapses are quite different. Using a parahorizontal slice that preserves a significant component of the connectivity between cortex, thalamus, and the striatum (Arbuthnott et al. 1985; Kawaguchi et al. 1989; Smeal et al. 2007) Ding et al. (2008) found that corticostriatal synapses exhibited paired-pulse facilitation regardless of which type of SPN they targeted (see below), indicating that glutamate release probability at this synapse was relatively low. In contrast, thalamostriatal synapses exhibited paired-pulse depression, indicating that glutamate release probability was high. Thus, the corticostriatal synapse was "tuned" to repetitive activity, whereas the thalamostriatal synapse was tuned to transient, episodic activity. Thalamostriatal synapses also had a significantly higher complement of NMDA receptors relative to those of the AMPA type. Interestingly, thalamostriatal synapses on cholinergic interneurons were facilitating (not depressing), suggesting a different origin. Activation of M2-class mAChRs decreased release probability at both types of synapse (Ding et al. 2008).

The postsynaptic effects of SPN mAChR activation are more complex and less well characterized. Early studies clearly suggested that M1 mAChR signaling increased the responsiveness of SPNs to both intrasomatic current injection and to synaptic stimulation (Akaike et al. 1988; Dodt and Misgeld 1986; Galarraga et al. 1999; Hsu et al. 1996). Subsequent studies have largely confirmed this view, putting it on a firmer mechanistic footing (Figueroa et al. 2002; Gabel and Nisenbaum 1999; Howe and Surmeier 1995; Lin et al. 2004; Olson et al. 2005; Perez-Burgos et al. 2008; Perez-Rosello et al. 2005; Shen et al. 2005).

One of the complications in sorting out the effects of mAChR signaling that was not fully appreciated until recently is the heterogeneity of SPNs. SPNs can be divided into two broad classes on the basis of their axonal projections (Fujiyama et al. 2011; Gerfen et al. 1990; Robertson et al. 1992). So-called dSPNs have axonal projections to the GABAergic output nuclei of the basal ganglia: the internal segment of the globus pallidus (GPi) and substantia nigra pars reticulata (SNr). These dSPNs also extend an axon collateral to the GABAergic neurons of the external segment of the globus pallidus (GPe). Indirect pathway SPNs (iSPNs) extend axonal projections only to the GPe. GPe neurons project to the glutamatergic neurons of the subthalamic nucleus (STN) and to the output nuclei (GPi/SNr). Thus, the indirect pathway forms a multisynaptic (or indirect) circuit between the striatum and the basal ganglia output nuclei. These differences in axonal trajectory are

paralleled by differences in dendritic anatomy that result in iSPNs being more responsive to intrasomatic current injection than dSPNs (Fujiyama et al. 2011; Gertler et al. 2008). In addition, although both types of SPN co-express M1 and M4 mAChRs, the latter is less abundant in iSPNs than in dSPNs (Bernard et al. 1992; Yan et al. 2001).

Understanding the actions of mAChRs requires some context about SPNs physiology. Both types of SPN have a similar core physiological phenotype. At rest, SPNs are dominated by dendritically positioned, inwardly rectifying, Kir2 K^+ channels that hold the membrane potential near the K^+ equilibrium potential (~−90 mV), far from spike threshold (Mermelstein et al. 1998; Shen et al. 2007; Wilson 1993). This is the so-called down-state (Stern et al. 1998; Wilson and Groves 1981). Synaptic release of glutamate on spine heads can produce a localized depolarization of sufficient magnitude to open voltage-dependent Ca^{2+} channels (Carter and Sabatini 2004). However, if this input lacks spatial or temporal convergence, the constitutively open Kir2 K^+ channels shunt this synaptic current, minimizing the somatodendritic depolarization it produces. Kv4 and Ca^{2+} activated, small conductance K^+ (SK) channels might contribute to this shunting (Day et al. 2008; Higley et al. 2009). On the other hand, if synaptic activity is convergent, the inward currents generated can overwhelm those of the Kir2 K^+ channels, causing them to block as Mg^{2+} and polyamines are swept from the cytoplasm into their pore (Lopatin and Nichols 1996; Wilson and Kawaguchi 1996). Although the battle between synaptically generated currents and Kir2 K^+ channels has been thought to be largely independent of postsynaptic boosting by voltage-dependent channels (Wilson and Kawaguchi 1996), more recent studies have found that in distal dendrites, spatially convergent synaptic input can recruit low threshold Cav3 Ca^{2+} channels and NMDA receptors to produce a regenerative event (Plotkin et al. 2011).

Closure of dendritic Kir2 K^+ channels leads to an elevation of the input impedance of SPN dendrites and a reduction in their electrotonic length (Day et al. 2008; Wilson 1993). With this transition, the SPN *somatic* membrane can reach a potential near spike threshold. Membrane potential transitions to near spike threshold seen in recordings from SPNs in vivo are called an up-state (Stern et al. 1998; Wilson and Groves 1981). Up-states can last hundreds of milliseconds, during which SPNs spike. The voltage trajectory to spike threshold is influenced by slowly inactivating Kv1 and Kv7 (KCNQ) K^+ channels that appear to be localized largely in the somata and axon initial segment (AIS) (Nisenbaum et al. 1994; Shen et al. 2004, 2005). Voltage-dependent Nav1 Na^+ channels and Kv4 K^+ channels also help shape this trajectory (Akins et al. 1990; Carrillo-Reid et al. 2009; Tkatch et al. 2000). During the spike, high-voltage-activated Cav2 Ca^{2+} channels open, leading to activation of SK K^+ channels, which regulate – in concert with Kv1 and Kv7 channels – the relatively slow, modestly adapting discharge of SPNs (Galarraga et al. 2007).

Activation of postsynaptic M1 mAChRs leads to a beautifully coordinated modulation of these channels, resulting in a sustained elevation in the responsiveness to synaptic release of glutamate without modulating the function

of glutamate receptors themselves. In dendrites, M1 receptor activation diminishes the open probability of both Kv4 and Kir2 K^+ channels, increasing input resistance and producing a modest depolarization (Akins et al. 1990; Figueroa et al. 2002; Hsu et al. 1996; Shen et al. 2007). The Kv4 channel modulation is attributable to signaling mechanisms that have been characterized in pyramidal neurons (Hoffman and Johnston 1998). The Kir2 modulation is mediated by depletion of membrane phosphatidylinositol 4,5-bisphosphate (PIP2) by activation of PLC. This modulation is stronger in iSPNs than dSPNs, possibly as a consequence of expression of Kir2 subunits that are more sensitive to fluctuations in membrane PIP2 concentration (Shen et al. 2007). Complementing this dendritic modulation, M1 signaling reduces opening of Kv7 K^+ channels (also likely to be through a PIP2-dependent mechanism) and reduces Cav2 Ca^{2+} channel opening (through a PKC-dependent mechanism), leading to reduced SK K^+ channel opening (Howe and Surmeier 1995; Shen et al. 2005; Vilchis et al. 2000). In addition, M1 receptor signaling enhances the persistent component of the Nav1 Na^+ channel opening (Carrillo-Reid et al. 2009). Thus, by modulating ion channels in both dendritic and somatic compartments, SPNs become transiently more likely to spike repetitively in response to a synaptic barrage from cortical pyramidal neurons.

If one considers then how the release of ACh modulates the corticostriatal microcircuit as a unit, there appears to be a paradox. ACh inhibits presynaptic glutamate release, but potentiates the postsynaptic response to glutamate without changing glutamate receptors themselves (Higley et al. 2009). This paradox is more apparent than real. First, presynaptic "inhibition" preferentially reduces glutamate release to a single action potential; when a burst of action potentials reach the terminal, the effect on glutamate release is much less affected and is enhanced in some circumstances; that is, the reduction in release probability is largely overcome with repetitive spiking. As a consequence, presynaptic inhibition can be viewed as a means of tuning synapses to repetitive stimulation (rather than simply being inhibited). At the same time, the postsynaptic membrane has been modulated to be more responsive to repetitive synaptic input. Thus, cholinergic interneurons serve to bias the corticostriatal circuitry toward a preferential responsiveness to bursts of cortical activity.

It is also important to consider the other mode of cholinergic interneuron spiking. In response to salient stimuli, interneurons will interrupt their tonic, low frequency spiking with a burst of spikes followed by a pause in activity that can last for a second (Aosaki et al. 1994; Apicella et al. 1997; Kimura et al. 1984; Raz et al. 1996). This burst–pause pattern can be evoked by stimulation of thalamic axons in a pattern like that evoked by salient stimuli (Ding et al. 2010). The burst of ACh release produced by this pattern results in a strong, rapid presynaptic modulation that is over in less than a hundred milliseconds, as it relies upon M2-class receptor, membrane delimited G-protein signaling. In contrast, the postsynaptic effects of M1 receptor signaling are slow, because they rely upon membrane enzymes and soluble second messengers; this modulation appears to last about a second – the duration of the thalamically evoked pause. In this situation, the pre- and postsynaptic modulations are largely separated in time. In this way, the thalamically

generated burst–pause pattern of interneuron activity might serve to reset the corticostriatal circuit (allowing a reassessment of ongoing action selection) and then preferentially enhance the responsiveness in iSPNs that are responsible for action suppression.

Within the context of this response, the recently described disynaptic linkage between cholinergic interneurons and SPNs through an undefined GABAergic interneuron makes some sense (Witten et al. 2010). This nicotinic receptor-mediated activation of GABAergic interneurons also links cholinergic interneurons (Sullivan et al. 2008). The identity of the GABAergic interneurons participating in this network remains to be determined, but a likely candidate is the parvalbumin, fast-spiking interneuron (Koos and Tepper 2002). Acting through this network, transient elevation in the spiking of cholinergic interneurons will shut down SPNs at the same time that M2-class receptors are inhibiting their excitatory glutamatergic input.

Although ACh has an important role in modulating the moment-to-moment activity of the corticostriatal network, it also has important part to play in regulating long-term changes in synaptic strength. The best studied form of plasticity in the striatum is long-term depression (LTD) at corticostriatal synapses onto SPNs. Unlike the situation at many other synapses, striatal LTD induction requires pairing of postsynaptic depolarization with moderate- to high-frequency afferent stimulation at physiological temperatures (Kreitzer and Malenka 2005; Lovinger et al. 1993). Typically for the induction to be successful, postsynaptic L-type calcium channels and mGluR5 receptors need to be co-activated. Both L-type calcium channels and mGluR5 receptors are found near glutamatergic synapses on SPN spines, making them capable of responding to local synaptic events (Carter and Sabatini 2004; Carter et al. 2007; Day et al. 2006; Olson et al. 2005; Testa et al. 1994). The induction of LTD requires the postsynaptic generation of endocannabinoids (ECs) (Gerdeman et al. 2002). ECs diffuse retrogradely to activate presynaptic CB1 receptors and decrease glutamate release probability. Ongoing work suggests that both of the abundant striatal ECs, anandamide and 2-arachidonylglycerol (2-AG), are involved in SPN signaling (Gao et al. 2010; Giuffrida et al. 1999; Lerner et al. 2010; Tanimura et al. 2010). A key question about the induction of striatal LTD is whether activation of D_2 receptors is necessary. Activation of D_2 receptors is a potent stimulus for anandamide production (Giuffrida et al. 1999). Studies have consistently found that in iSPNs, D_2 receptor activation is necessary (Kreitzer and Malenka 2007; Shen et al. 2008; Wang et al. 2006). This could be due to the need to suppress A2a adenosine receptor signaling impeding efficient EC synthesis and LTD induction (Fuxe et al. 2007; Shen et al. 2008). Indeed, Lerner et al. (2010) demonstrate quite convincingly that antagonism of A2a receptors promotes EC-dependent LTD induction in iSPNs.

The question then is can EC-dependent LTD be induced in dSPNs that do not express D_2 receptors? When a minimal local stimulation paradigm is used, LTD does not appear to be induced in these SPNs (Kreitzer and Malenka 2007; Shen et al. 2008). However, using macroelectrode stimulation, EC-dependent LTD is readily inducible in identified dSPNs (Wang et al. 2006), consistent with the high

probability of SPN induction seen in previous work (Calabresi et al. 2007). How could induction of LTD in dSPNs be dependent upon D_2 receptors? There are a couple of possibilities. One is that D_2 receptor stimulation reduces DA release through a presynaptic mechanism, preferentially reducing stimulation of D_1 receptors that oppose the induction of LTD in dSPNs (Shen et al. 2008). The other possibility is that for LTD to be induced in dSPNs, ACh release and postsynaptic M1 muscarinic receptor signaling must fall (Calabresi et al. 2007; Wang et al. 2006). D_2 receptor stimulation slows the autonomous spiking of cholinergic interneurons and also inhibits ACh release (Aosaki et al. 1998; Deng et al. 2007; Maurice et al. 2004). Tozzi et al. (2011) have put this latter possibility on firm experimental ground showing that decreasing ACh release and M1 receptor signaling is critical to the reduction of corticostriatal glutamatergic transmission in *both* dSPNs and iSPNs. They also show that the interaction between D_2 and A2a receptors is critical to the regulation of interneuron activity, particularly in parkinsonian states.

Long-term potentiation (LTP) at glutamatergic synapses is less well characterized because it is more difficult to induce in the in vitro preparations typically used to study plasticity. Most of the work describing LTP at glutamatergic synapses has been done with sharp electrodes (either in vivo or in vitro), not with patch clamp electrodes in brain slices that afford greater experimental control and definition of the cellular and molecular determinants of induction. Previous studies have argued that LTP induced in SPNs by pairing HFS of glutamatergic inputs and postsynaptic depolarization depends upon co-activation of M1, D_1, NMDA, and TrkB receptors (Calabresi et al. 2007; Jia et al. 2010; Kerr and Wickens 2001). The involvement of NMDA receptors in LTP induction is clear. The involvement of TrkB receptors and its ligand, brain-derived neurotrophic factor (BDNF), is less well characterized but plausible given the expression of TrkB receptors in both classes of SPN (Lobo et al. 2010). However, the necessity of D_1 receptors is another matter. Although D_1 receptors appear to play an obligatory role in dSPNs, in iSPNs A2a receptor activation, not D_1 receptor activation, is necessary (Shen et al. 2008). The role of M2 and M1 receptors in LTP induction needs more study. Antagonism of M2 receptors appears to promote LTP induction, either by enhancing glutamate or ACh release (Calabresi et al. 1998a, 1999). On the other hand, Calabresi et al. (1999) have suggested that M1 receptors are necessary for LTP induction in SPNs. Although plausible, more mechanistic studies in identified neurons need to be conducted, particularly in light of the apparent lack of M1 receptor effect on postsynaptic glutamate receptors (Higley et al. 2009). If M1 receptors are critical to LTP induction, it would suggest that cholinergic interneurons are full partners with dopaminergic neurons in the regulation of synaptic plasticity with the corticostriatal circuit. In this scenario, bidirectionality of plasticity is dependent not only upon differential expression of dopamine and adenosine receptors in SPNs (Shen et al. 2008), but also by the co-expression of adenosine and dopamine receptors in cholinergic interneurons.

For the sake of completeness, another component of the corticostriatal circuitry needs to be considered. SPNs have a richly branching recurrent axon collateral that

arborizes in the neighborhood of its parent cell body (Fujiyama et al. 2011; Kawaguchi et al. 1989). This feedback could provide the substrate for lateral inhibition (Groves 1983) and has figured prominently in several models of striatal processing (Beiser et al. 1997). However, the functional significance of this feedback circuit has been controversial. In large measure, this is because the synapses formed by recurrent collaterals are onto distal dendrites (Bolam et al. 1983; Wilson and Groves 1980), making their physiological effects difficult to see with a somatic electrode (Jaeger et al. 1994). Using paired patch clamp recordings from neighboring SPNs, it has been possible to more reliably see the effects of collateral activation (Czubayko and Plenz 2002; Guzman et al. 2003; Koos et al. 2004; Taverna et al. 2008; Tunstall et al. 2002), but the percentage of synaptically connected neighbors has been small (~10–15%) in randomly selected SPNs in brain slices. Using D_1 and D_2 BAC transgenic mice to direct sampling, it was found that although iSPNs project to both themselves and dSPNs, dSPNs connect essentially only with other dSPNs (Taverna et al. 2008). The percentage of SPNs showing demonstrable connectivity doubled when sampling was not random. More recent work using optogenetic approaches to activate SPNs has inferred an even higher degree of connectivity (Chuhma et al. 2011). Whether these approaches will yield a pattern of connectivity consistent with that inferred from paired recordings remains to be determined. It is very likely that these connections are modulated by ACh and mAChR signaling, but this has yet to be definitively determined.

3.2 The Feed-Forward Thalamostriatal Circuit

The other major glutamatergic projection to the striatum originates in the thalamus (Smith et al. 2004). This input targets both direct and iSPNs (Ding et al. 2008; Doig et al. 2010). The synapses formed by this projection are found both on dendritic shafts and spine heads, in the same regions as those formed by the corticostriatal projection. In contrast to the corticostriatal synapses, those formed by thalamic axons have a high release probability, making them well suited to signaling transient events (Ding et al. 2008). Another major target of this projection is the cholinergic interneuron. Like the corticostriatal feed-forward circuit involving FS interneurons, the thalamostriatal projection makes a feed-forward connection to SPNs through cholinergic interneurons (Ding et al. 2010). There appear to be two phases to this feed-forward system. The first phase is a rapid and transient inhibition of cortically driven activity in SPNs. This is mediated by a presynaptic, M2/M4 receptor-dependent inhibition of glutamate release (Ding et al. 2010) and a postsynaptic, GABAergic inhibition (Witten et al. 2010). Whether this GABAergic inhibition relies upon nicotinic receptor activation of PV GABAergic interneurons remains to be determined (Koos and Tepper 2002; Sullivan et al. 2008). The second phase is mediated by postsynaptic M1 receptors that enhance the somatic excitability of both SPNs (Perez-Rosello et al. 2005; Pisani et al. 2007), but preferentially enhances the dendritic excitability of iSPNs by decreasing Kir2 K^+

channel opening (Shen et al. 2007). With a burst of thalamic activity like that seen after presentation of a salient stimulus, cholinergic interneurons exhibit a burst–pause pattern of activity that engage both phases of the response, but because the inhibitory effects are fast (milliseconds in duration) and the postsynaptic effects are slow (hundreds of milliseconds), the two modulations do not conflict and lead to a patterned change in SPN activity that could underlie the alerting response.

There also is a feedback component of this microcircuit that is mediated by mAChRs on cholinergic interneurons themselves. Cholinergic interneurons express M1, M2, and M4 receptors (Alcantara et al. 2001; Hersch et al. 1994; Yan and Surmeier 1996). Application of muscarinic agonists can silence cholinergic interneurons, and focal stimulation of the slice can induce what has been described as muscarinic inhibitory postsynaptic potential (IPSP) in these neurons. Both these effects are mediated by postsynaptic M2-class receptors (Bonsi et al. 2008; Calabresi et al. 1998b). Activation of the M2-class receptors downregulates Cav2.1 and Cav2.2 channels in cholinergic interneurons (Yan and Surmeier 1996). Because $Ca_V2.2$ channels activate the SK channels that determine the size of the AHP in these neurons (Goldberg and Wilson 2005), activation of mAChRs reduces AHPs and induces irregular discharge (Ding et al. 2006). This mechanism complements the collateral inhibition mediated by GABAergic interneurons (Sullivan et al. 2008), suggesting that the temporal pattern of activity of cholinergic interneurons is a meaningful network parameter.

3.3 The Feed-Forward Corticostriatal Circuit

Fast-spiking (FS), PV GABAergic interneurons receive a prominent glutamatergic input from cortical pyramidal neurons and, in turn, convey this activity through perisomatic synapses to both dSPNs and iSPNs (Bennett and Bolam 1994; Gittis et al. 2010; Kita 1993; Koos and Tepper 1999; Planert et al. 2010). This feed-forward inhibition is thought to contribute to action selection by suppressing SPN activity in circuits associated with unwanted actions (Gage et al. 2010; Kita et al. 1990; Parthasarathy and Graybiel 1997). Activation of M1 or M4 mAChRs inhibits this synapse – a fact which is puzzling given the potent excitatory impact of nAChRs on the FS interneurons (Barral et al. 1999; Koos and Tepper 2002). Conceptually, this can be resolved by noting that the presynaptic inhibition of GABA release is dependent upon postsynaptic M1 mAChRs that trigger the release of ECs (Narushima et al. 2007). Hence, this mechanism complements the M1-mAChR-mediated modulation of postsynaptic ion channels described earlier that serve to increase SPN excitability. Although both types of SPN are targeted in this circuit, paired recordings in BAC mice have found some preferential connectivity of FS interneurons with dSPNs (Gittis et al. 2010). Whether the M1 mAChR-mediated modulation of FS interneuron synapses is stronger in dSPNs is unclear.

Somatostatin (SOM)/neuropeptide Y (NPY) expressing GABAergic interneurons also form another, less well studied, part of the feed-forward corticostriatal

circuit (Tepper et al. 2010). If these interneurons are like the SOM expressing, Martinotti interneurons of cortex (Wang et al. 2004), their innervation of distal dendrites could make it difficult to accurately judge their importance (Gittis et al. 2010), as with SPN recurrent collaterals. Whether this component of feed-forward circuit differentially controls dSPNs and iSPNs remains to be determined. These interneurons express M3 (M1 class) receptors with their strongest expression being on axon terminals innervating SPNs (Hersch et al. 1994).

4 Muscarinic Signaling in Parkinson's Disease and Dystonia

In Parkinson's disease, striatal DA levels fall as the dopaminergic neurons in the substantia nigra pars compacta (SNc) die. A concomitant of this fall is a rise in striatal cholinergic signaling (Barbeau 1962; DeBoer et al. 1996; Lehmann and Langer 1983; McGeer et al. 1961). This rise is thought to be in large measure responsible for the symptoms of the disease and has motivated the use of mAChR antagonists in the treatment of PD (Lang and Blair 1989; Pisani et al. 2007; Wooten 1990). The elevation in cholinergic signaling is attributable to the loss of negative modulation of interneuron spiking and transmitter release by D_2 dopamine receptors (Aosaki et al. 1998; DeBoer et al. 1996; Maurice et al. 2004; Pisani et al. 2007). In addition, DA depletion triggers an up-regulation in the expression of RGS4 in cholinergic interneurons, resulting in an attenuation of M2-class autoreceptor signaling and enhanced ACh release (Ding et al. 2006; Dolezal and Wecker 1990).

DA depletion and the elevation in cholinergic signaling in PD models have a variety of effects on the striatal circuitry. One dramatic effect is the pruning of iSPN spines and glutamatergic synapses (Day et al. 2006; McNeill et al. 1988). This remodeling requires calcium influx through L-type Ca^{2+} channels that are located near synapses, as it is dramatically reduced in Cav 1.3 KO mice or in wild-type mice treated with L-type channel antagonists (Day et al. 2006; Olson et al. 2005). It is easy to imagine that this structural adaptation is homeostatic (Turrigiano et al. 1998). The loss of inhibitory D_2 receptor signaling and the elevation of excitatory M_1 receptors signaling should drive spiking above the neuronal set point, leading to pruning. Indeed, genetic deletion of M_1 receptors significantly attenuates loss of glutamatergic synapses following DA depletion (Shen et al. 2007). In agreement with a homeostatic model, iSPN pruning can be blunted in vitro by inhibiting calcineurin activity or knocking down the transcriptional regulator MEF2 (Tian et al. 2010). Underscoring the importance of M1 mAChRs in PD, Tozzi et al. (2011) have recently shown that following DA depletion, cholinergic interneurons become more sensitive to inhibition by D_2 receptor signaling and that this shift is likely to be responsible for the enhanced ability of D_2 receptor agonists to inhibit corticostriatal synapses on SPNs.

Another major motor disorder with cholinergic determinants is dystonia. Dystonia, characterized by muscle contraction, involuntary twisting, and abnormal

posture (Fahn 1988), is the third most common movement disorder after PD and essential tremor. DYT1 dystonia, the most common form of early onset generalized dystonia, is a hereditary disorder caused by a deletion in the *dyt1* gene, causing a mutation in the torsinA protein (Ozelius et al. 1997). In a recent series of studies, using a transgenic mouse model of DYT1 expressing mutant torsinA protein, Pisani et al. (2006) found that contrary to the situation in wild-type mice activation of dopamine D2 receptors in mutant mice increased the release of ACh (Pisani et al. 2006; Sciamanna et al. 2009). Additionally, in agreement with the role of M1 receptors in regulating the induction of synaptic plasticity (see above), LTD was lost and LTP enhanced at corticostriatal synapses in mutant mice. Plasticity could be normalized by antagonizing M1 mAChRs (Martella et al. 2009). These studies suggest that mAChR antagonists should be an effective therapy for dystonia and support the notion that dystonia is a muscarinic "disinhibition" disorder (Defazio et al. 2007).

5 Summary

Striatal cholinergic interneurons are pivotal modulators of the striatal circuitry in action selection and decision making. In this chapter, we have described the presynaptic actions of M_2 receptor and postsynaptic actions of M_1 receptor on SPNs and cholinergic interneurons. Recent studies have highlighted the roles of these receptors in synaptic integration and plasticity, and how they differ between the two populations of SPNs. These studies make it clear that temporally patterned activity in cholinergic interneurons is a major determinant of the response to salient environmental stimuli that serve to direct behavior. Moreover, they underscore the need to revisit the clinical potential of anticholinergic therapies for treating movement disorders such as dystonia and Parkinson's disease.

References

Akaike A, Sasa M, Takaori S (1988) Muscarinic inhibition as a dominant role in cholinergic regulation of transmission in the caudate nucleus. J Pharmacol Exp Ther 246:1129–1136

Akins PT, Surmeier DJ, Kitai ST (1990) Muscarinic modulation of a transient K^+ conductance in rat neostriatal neurons. Nature 344:240–242

Albin RL, Young AB, Penney JB (1989) The functional anatomy of basal ganglia disorders. Trends Neurosci 12:366–375

Alcantara AA, Mrzljak L, Jakab RL, Levey AI, Hersch SM, Goldman-Rakic PS (2001) Muscarinic m1 and m2 receptor proteins in local circuit and projection neurons of the primate striatum: anatomical evidence for cholinergic modulation of glutamatergic prefronto-striatal pathways. J Comp Neurol 434:445–460

Aosaki T, Tsubokawa H, Ishida A, Watanabe K, Graybiel AM, Kimura M (1994) Responses of tonically active neurons in the primate's striatum undergo systematic changes during behavioral sensorimotor conditioning. J Neurosci 14:3969–3984

Aosaki T, Kiuchi K, Kawaguchi Y (1998) Dopamine D1-like receptor activation excites rat striatal large aspiny neurons in vitro. J Neurosci 18:5180–5190
Apicella P, Legallet E, Trouche E (1997) Responses of tonically discharging neurons in the monkey striatum to primary rewards delivered during different behavioral states. Exp Brain Res 116:456–466
Arbuthnott GW, MacLeod NK, Rutherfod A (1985) The rate cortico-striatal pathway in vitro. J Physiol 367:102p
Barbeau A (1962) The pathogenesis of Parkinson's disease: a new hypothesis. Can Med Assoc J 87:802–807
Barral J, Galarraga E, Bargas J (1999) Muscarinic presynaptic inhibition of neostriatal glutamatergic afferents is mediated by Q-type Ca^{2+} channels. Brain Res Bull 49:285–289
Beiser DG, Hua SE, Houk JC (1997) Network models of the basal ganglia. Curr Opin Neurobiol 7:185–190
Bennett BD, Bolam JP (1994) Synaptic input and output of parvalbumin-immunoreactive neurons in the neostriatum of the rat. Neuroscience 62:707–719
Bennett BD, Wilson CJ (1999) Spontaneous activity of neostriatal cholinergic interneurons in vitro. J Neurosci 19:5586–5596
Bernard V, Normand E, Bloch B (1992) Phenotypical characterization of the rat striatal neurons expressing muscarinic receptor genes. J Neurosci 12:3591–3600
Bolam JP, Somogyi P, Takagi H, Fodor I, Smith AD (1983) Localization of substance P-like immunoreactivity in neurons and nerve terminals in the neostriatum of the rat: a correlated light and electron microscopic study. J Neurocytol 12:325–344
Bolam JP, Wainer BH, Smith AD (1984) Characterization of cholinergic neurons in the rat neostriatum. A combination of choline acetyltransferase immunocytochemistry, Golgi-impregnation and electron microscopy. Neuroscience 12:711–718
Bolam JP, Hanley JJ, Booth PA, Bevan MD (2000) Synaptic organisation of the basal ganglia. J Anat 196(Pt 4):527–542
Bonsi P, Martella G, Cuomo D, Platania P, Sciamanna G, Bernardi G, Wess J, Pisani A (2008) Loss of muscarinic autoreceptor function impairs long-term depression but not long-term potentiation in the striatum. J Neurosci 28:6258–6263
Briggs RS, Redgrave P, Nahorski SR (1981) Effect of kainic acid lesions on muscarinic agonist receptor subtypes in rat striatum. Brain Res 206:451–456
Calabresi P, Centonze D, Gubellini P, Pisani A, Bernardi G (1998a) Blockade of M2-like muscarinic receptors enhances long-term potentiation at corticostriatal synapses. Eur J Neurosci 10:3020–3023
Calabresi P, Centonze D, Pisani A, Sancesario G, North RA, Bernardi G (1998b) Muscarinic IPSPs in rat striatal cholinergic interneurones. J Physiol 510(Pt 2):421–427
Calabresi P, Centonze D, Gubellini P, Bernardi G (1999) Activation of M1-like muscarinic receptors is required for the induction of corticostriatal LTP. Neuropharmacology 38:323–326
Calabresi P, Picconi B, Tozzi A, Di Filippo M (2007) Dopamine-mediated regulation of corticostriatal synaptic plasticity. Trends Neurosci 30:211–219
Carrillo-Reid L, Tecuapetla F, Vautrelle N, Hernandez A, Vergara R, Galarraga E, Bargas J (2009) Muscarinic enhancement of persistent sodium current synchronizes striatal medium spiny neurons. J Neurophysiol 102:682–690
Carter AG, Sabatini BL (2004) State-dependent calcium signaling in dendritic spines of striatal medium spiny neurons. Neuron 44:483–493
Carter AG, Soler-Llavina GJ, Sabatini BL (2007) Timing and location of synaptic inputs determine modes of subthreshold integration in striatal medium spiny neurons. J Neurosci 27:8967–8977
Caulfield MP, Birdsall NJ (1998) International union of pharmacology. XVII. Classification of muscarinic acetylcholine receptors. Pharmacol Rev 50:279–290
Chuhma N, Tanaka KF, Hen R, Rayport S (2011) Functional connectome of the striatal medium spiny neuron. J Neurosci 31:1183–1192

Contant C, Umbriaco D, Garcia S, Watkins KC, Descarries L (1996) Ultrastructural characterization of the acetylcholine innervation in adult rat neostriatum. Neuroscience 71:937–947

Czubayko U, Plenz D (2002) Fast synaptic transmission between striatal spiny projection neurons. Proc Natl Acad Sci USA 99:15764–15769

Day M, Wang Z, Ding J, An X, Ingham CA, Shering AF, Wokosin D, Ilijic E, Sun Z, Sampson AR, Mugnaini E, Deutch AY, Sesack SR, Arbuthnott GW, Surmeier DJ (2006) Selective elimination of glutamatergic synapses on striatopallidal neurons in Parkinson disease models. Nat Neurosci 9:251–259

Day M, Wokosin D, Plotkin JL, Tian X, Surmeier DJ (2008) Differential excitability and modulation of striatal medium spiny neuron dendrites. J Neurosci 28:11603–11614

DeBoer P, Heeringa MJ, Abercrombie ED (1996) Spontaneous release of acetylcholine in striatum is preferentially regulated by inhibitory dopamine D2 receptors. Eur J Pharmacol 317:257–262

Defazio G, Berardelli A, Hallett M (2007) Do primary adult-onset focal dystonias share aetiological factors? Brain 130:1183–1193

DeLong M, Wichmann T (2009) Update on models of basal ganglia function and dysfunction. Parkinsonism Relat Disord 15(Suppl 3):S237–S240

Deng P, Zhang Y, Xu ZC (2007) Involvement of I(h) in dopamine modulation of tonic firing in striatal cholinergic interneurons. J Neurosci 27:3148–3156

Ding J, Guzman JN, Tkatch T, Chen S, Goldberg JA, Ebert PJ, Levitt P, Wilson CJ, Hamm HE, Surmeier DJ (2006) RGS4-dependent attenuation of M4 autoreceptor function in striatal cholinergic interneurons following dopamine depletion. Nat Neurosci 9:832–842

Ding J, Peterson JD, Surmeier DJ (2008) Corticostriatal and thalamostriatal synapses have distinctive properties. J Neurosci 28:6483–6492

Ding JB, Guzman JN, Peterson JD, Goldberg JA, Surmeier DJ (2010) Thalamic gating of corticostriatal signaling by cholinergic interneurons. Neuron 67:294–307

Dodt HU, Misgeld U (1986) Muscarinic slow excitation and muscarinic inhibition of synaptic transmission in the rat neostriatum. J Physiol 380:593–608

Doig NM, Moss J, Bolam JP (2010) Cortical and thalamic innervation of direct and indirect pathway medium-sized spiny neurons in mouse striatum. J Neurosci 30:14610–14618

Dolezal V, Wecker L (1990) Muscarinic receptor blockade increases basal acetylcholine release from striatal slices. J Pharmacol Exp Ther 252:739–743

Dube L, Smith AD, Bolam JP (1988) Identification of synaptic terminals of thalamic or cortical origin in contact with distinct medium-size spiny neurons in the rat neostriatum. J Comp Neurol 267:455–471

Eglen RM (2005) Muscarinic receptor subtype pharmacology and physiology. Prog Med Chem 43:105–136

Fahn S (1988) Concept and classification of dystonia. Adv Neurol 50:1–8

Figueroa A, Galarraga E, Bargas J (2002) Muscarinic receptors involved in the subthreshold cholinergic actions of neostriatal spiny neurons. Synapse 46:215–223

Frank MJ, Claus ED (2006) Anatomy of a decision: striato-orbitofrontal interactions in reinforcement learning, decision making, and reversal. Psychol Rev 113:300–326

Fujiyama F, Sohn J, Nakano T, Furuta T, Nakamura KC, Matsuda W, Kaneko T (2011) Exclusive and common targets of neostriatofugal projections of rat striosome neurons: a single neuron-tracing study using a viral vector. Eur J Neurosci 33:668–677

Fuxe K, Ferre S, Genedani S, Franco R, Agnati LF (2007) Adenosine receptor-dopamine receptor interactions in the basal ganglia and their relevance for brain function. Physiol Behav 92:210–217

Gabel LA, Nisenbaum ES (1999) Muscarinic receptors differentially modulate the persistent potassium current in striatal spiny projection neurons. J Neurophysiol 81:1418–1423

Gage GJ, Stoetzner CR, Wiltschko AB, Berke JD (2010) Selective activation of striatal fast-spiking interneurons during choice execution. Neuron 67:466–479

Galarraga E, Hernandez-Lopez S, Reyes A, Miranda I, Bermudez-Rattoni F, Vilchis C, Bargas J (1999) Cholinergic modulation of neostriatal output: a functional antagonism between different types of muscarinic receptors. J Neurosci 19:3629–3638

Galarraga E, Vilchis C, Tkatch T, Salgado H, Tecuapetla F, Perez-Rosello T, Perez-Garci E, Hernandez-Echeagaray E, Surmeier DJ, Bargas J (2007) Somatostatinergic modulation of firing pattern and calcium-activated potassium currents in medium spiny neostriatal neurons. Neuroscience 146:537–554

Gao Y, Vasilyev DV, Goncalves MB, Howell FV, Hobbs C, Reisenberg M, Shen R, Zhang MY, Strassle BW, Lu P, Mark L, Piesla MJ, Deng K, Kouranova EV, Ring RH, Whiteside GT, Bates B, Walsh FS, Williams G, Pangalos MN, Samad TA, Doherty P (2010) Loss of retrograde endocannabinoid signaling and reduced adult neurogenesis in diacylglycerol lipase knock-out mice. J Neurosci 30:2017–2024

Gerdeman GL, Ronesi J, Lovinger DM (2002) Postsynaptic endocannabinoid release is critical to long-term depression in the striatum. Nat Neurosci 5:446–451

Gerfen CR (1992) The neostriatal mosaic: multiple levels of compartmental organization. Trends Neurosci 15:133–139

Gerfen CR, Engber TM, Mahan LC, Susel Z, Chase TN, Monsma FJ Jr, Sibley DR (1990) D1 and D2 dopamine receptor-regulated gene expression of striatonigral and striatopallidal neurons. Science 250:1429–1432

Gertler TS, Chan CS, Surmeier DJ (2008) Dichotomous anatomical properties of adult striatal medium spiny neurons. J Neurosci 28:10814–10824

Gittis AH, Nelson AB, Thwin MT, Palop JJ, Kreitzer AC (2010) Distinct roles of GABAergic interneurons in the regulation of striatal output pathways. J Neurosci 30:2223–2234

Giuffrida A, Parsons LH, Kerr TM, Rodriguez de Fonseca F, Navarro M, Piomelli D (1999) Dopamine activation of endogenous cannabinoid signaling in dorsal striatum. Nat Neurosci 2:358–363

Goldberg JA, Wilson CJ (2005) Control of spontaneous firing patterns by the selective coupling of calcium currents to calcium-activated potassium currents in striatal cholinergic interneurons. J Neurosci 25:10230–10238

Goldberg JA, Wilson CJ (2010) The cholinergic interneurons of the striatum: intrinsic properties underlie multiple discharge patterns. In: Steiner H, Tseng K (eds) Handbook of basal gnaglia structure and function. Academic, London, pp 133–149

Groves PM (1983) A theory of the functional organization of the neostriatum and the neostriatal control of voluntary movement. Brain Res 286:109–132

Guzman JN, Hernandez A, Galarraga E, Tapia D, Laville A, Vergara R, Aceves J, Bargas J (2003) Dopaminergic modulation of axon collaterals interconnecting spiny neurons of the rat striatum. J Neurosci 23:8931–8940

Hersch SM, Gutekunst CA, Rees HD, Heilman CJ, Levey AI (1994) Distribution of m1-m4 muscarinic receptor proteins in the rat striatum: light and electron microscopic immunocytochemistry using subtype-specific antibodies. J Neurosci 14:3351–3363

Higley MJ, Soler-Llavina GJ, Sabatini BL (2009) Cholinergic modulation of multivesicular release regulates striatal synaptic potency and integration. Nat Neurosci 12:1121–1128

Hoffman DA, Johnston D (1998) Downregulation of transient K^+ channels in dendrites of hippocampal CA1 pyramidal neurons by activation of PKA and PKC. J Neurosci 18:3521–3528

Houk JC, Bastianen C, Fansler D, Fishbach A, Fraser D, Reber PJ, Roy SA, Simo LS (2007) Action selection and refinement in subcortical loops through basal ganglia and cerebellum. Philos Trans R Soc Lond B Biol Sci 362:1573–1583

Howe AR, Surmeier DJ (1995) Muscarinic receptors modulate N-, P-, and L-type Ca^{2+} currents in rat striatal neurons through parallel pathways. J Neurosci 15:458–469

Hsu KS, Yang CH, Huang CC, Gean PW (1996) Carbachol induces inward current in neostriatal neurons through M1-like muscarinic receptors. Neuroscience 73:751–760

Jaeger D, Kita H, Wilson CJ (1994) Surround inhibition among projection neurons is weak or nonexistent in the rat neostriatum. J Neurophysiol 72:2555–2558

Jia Y, Gall CM, Presynaptic LG (2010) BDNF promotes postsynaptic long-term potentiation in the dorsal striatum. J Neurosci 30:14440–14445
Kawaguchi Y (1993) Physiological, morphological, and histochemical characterization of three classes of interneurons in rat neostriatum. J Neurosci 13:4908–4923
Kawaguchi Y, Wilson CJ, Emson PC (1989) Intracellular recording of identified neostriatal patch and matrix spiny cells in a slice preparation preserving cortical inputs. J Neurophysiol 62:1052–1068
Kemp JM, Powell TP (1971) The synaptic organization of the caudate nucleus. Philos Trans R Soc Lond B Biol Sci 262:403–412
Kerr JN, Wickens JR (2001) Dopamine D-1/D-5 receptor activation is required for long-term potentiation in the rat neostriatum in vitro. J Neurophysiol 85:117–124
Kimura M, Rajkowski J, Evarts E (1984) Tonically discharging putamen neurons exhibit set-dependent responses. Proc Natl Acad Sci USA 81:4998–5001
Kimura M, Yamada H, Matsumoto N (2003) Tonically active neurons in the striatum encode motivational contexts of action. Brain Dev 25(Suppl 1):S20–S23
Kita H (1993) GABAergic circuits of the striatum. Prog Brain Res 99:51–72
Kita H, Kosaka T, Heizmann CW (1990) Parvalbumin-immunoreactive neurons in the rat neostriatum: a light and electron microscopic study. Brain Res 536:1–15
Koos T, Tepper JM (1999) Inhibitory control of neostriatal projection neurons by GABAergic interneurons. Nat Neurosci 2:467–472
Koos T, Tepper JM (2002) Dual cholinergic control of fast-spiking interneurons in the neostriatum. J Neurosci 22:529–535
Koos T, Tepper JM, Wilson CJ (2004) Comparison of IPSCs evoked by spiny and fast-spiking neurons in the neostriatum. J Neurosci 24:7916–7922
Kreitzer AC, Malenka RC (2005) Dopamine modulation of state-dependent endocannabinoid release and long-term depression in the striatum. J Neurosci 25:10537–10545
Kreitzer AC, Malenka RC (2007) Endocannabinoid-mediated rescue of striatal LTD and motor deficits in Parkinson's disease models. Nature 445:643–647
Lang AE, Blair RDG (1989) Anticholinergic drugs and amantadine in the treatment of Parkinson's disease. In: Calne DB (ed) Handbook of experimental pharmacology. Springer, Heidelberg
Lehmann J, Langer SZ (1983) The striatal cholinergic interneuron: synaptic target of dopaminergic terminals? Neuroscience 10:1105–1120
Lerner TN, Horne EA, Stella N, Kreitzer AC (2010) Endocannabinoid signaling mediates psychomotor activation by adenosine A2A antagonists. J Neurosci 30:2160–2164
Lin JY, Chung KK, de Castro D, Funk GD, Lipski J (2004) Effects of muscarinic acetylcholine receptor activation on membrane currents and intracellular messengers in medium spiny neurones of the rat striatum. Eur J Neurosci 20:1219–1230
Lobo MK, Covington HE III, Chaudhury D, Friedman AK, Sun H, Damez-Werno D, Dietz DM, Zaman S, Koo JW, Kennedy PJ, Mouzon E, Mogri M, Neve RL, Deisseroth K, Han MH, Nestler EJ (2010) Cell type-specific loss of BDNF signaling mimics optogenetic control of cocaine reward. Science 330:385–390
Lopatin AN, Nichols CG (1996) [K^+] dependence of polyamine-induced rectification in inward rectifier potassium channels (IRK1, Kir2.1). J Gen Physiol 108:105–113
Lovinger DM, Tyler EC, Merritt A (1993) Short- and long-term synaptic depression in rat neostriatum. J Neurophysiol 70:1937–1949
Malenka RC, Kocsis JD (1988) Presynaptic actions of carbachol and adenosine on corticostriatal synaptic transmission studied in vitro. J Neurosci 8:3750–3756
Martella G, Tassone A, Sciamanna G, Platania P, Cuomo D, Viscomi MT, Bonsi P, Cacci E, Biagioni S, Usiello A, Bernardi G, Sharma N, Standaert DG, Pisani A (2009) Impairment of bidirectional synaptic plasticity in the striatum of a mouse model of DYT1 dystonia: role of endogenous acetylcholine. Brain 132:2336–2349
Matsumoto N, Minamimoto T, Graybiel AM, Kimura M (2001) Neurons in the thalamic CM-Pf complex supply striatal neurons with information about behaviorally significant sensory events. J Neurophysiol 85:960–976

Maurice N, Mercer J, Chan CS, Hernandez-Lopez S, Held J, Tkatch T, Surmeier DJ (2004) D2 dopamine receptor-mediated modulation of voltage-dependent Na^+ channels reduces autonomous activity in striatal cholinergic interneurons. J Neurosci 24:10289–10301

McGeer PL, Boulding JE, Gibson WC, Foulkes RG (1961) Drug-induced extrapyramidal reactions. Treatment with diphenhydramine hydrochloride and dihydroxyphenylalanine. JAMA 177:665–670

McHaffie JG, Stanford TR, Stein BE, Coizet V, Redgrave P (2005) Subcortical loops through the basal ganglia. Trends Neurosci 28:401–407

McNeill TH, Brown SA, Rafols JA, Shoulson I (1988) Atrophy of medium spiny I striatal dendrites in advanced Parkinson's disease. Brain Res 455:148–152

Mermelstein PG, Song WJ, Tkatch T, Yan Z, Surmeier DJ (1998) Inwardly rectifying potassium (IRK) currents are correlated with IRK subunit expression in rat nucleus accumbens medium spiny neurons. J Neurosci 18:6650–6661

Mesulam MM, Mash D, Hersh L, Bothwell M, Geula C (1992) Cholinergic innervation of the human striatum, globus pallidus, subthalamic nucleus, substantia nigra, and red nucleus. J Comp Neurol 323:252–268

Mink JW (1996) The basal ganglia: focused selection and inhibition of competing motor programs. Prog Neurobiol 50:381–425

Morris G, Arkadir D, Nevet A, Vaadia E, Bergman H (2004) Coincident but distinct messages of midbrain dopamine and striatal tonically active neurons. Neuron 43:133–143

Narushima M, Uchigashima M, Fukaya M, Matsui M, Manabe T, Hashimoto K, Watanabe M, Kano M (2007) Tonic enhancement of endocannabinoid-mediated retrograde suppression of inhibition by cholinergic interneuron activity in the striatum. J Neurosci 27:496–506

Nisenbaum ES, Xu ZC, Wilson CJ (1994) Contribution of a slowly inactivating potassium current to the transition to firing of neostriatal spiny projection neurons. J Neurophysiol 71:1174–1189

Olson PA, Tkatch T, Hernandez-Lopez S, Ulrich S, Ilijic E, Mugnaini E, Zhang H, Bezprozvanny I, Surmeier DJ (2005) G-protein-coupled receptor modulation of striatal CaV1.3L-type Ca^{2+} channels is dependent on a Shank-binding domain. J Neurosci 25:1050–1062

Ozelius LJ, Hewett JW, Page CE, Bressman SB, Kramer PL, Shalish C, de Leon D, Brin MF, Raymond D, Corey DP, Fahn S, Risch NJ, Buckler AJ, Gusella JF, Breakefield XO (1997) The early-onset torsion dystonia gene (DYT1) encodes an ATP-binding protein. Nat Genet 17:40–48

Pakhotin P, Bracci E (2007) Cholinergic interneurons control the excitatory input to the striatum. J Neurosci 27:391–400

Parent M, Parent A (2006) Single-axon tracing study of corticostriatal projections arising from primary motor cortex in primates. J Comp Neurol 496:202–213

Parthasarathy HB, Graybiel AM (1997) Cortically driven immediate-early gene expression reflects modular influence of sensorimotor cortex on identified striatal neurons in the squirrel monkey. J Neurosci 17:2477–2491

Perez-Burgos A, Perez-Rosello T, Salgado H, Flores-Barrera E, Prieto GA, Figueroa A, Galarraga E, Bargas J (2008) Muscarinic M(1) modulation of N and L types of calcium channels is mediated by protein kinase C in neostriatal neurons. Neuroscience 155:1079–1097

Perez-Rosello T, Figueroa A, Salgado H, Vilchis C, Tecuapetla F, Guzman JN, Galarraga E, Bargas J (2005) Cholinergic control of firing pattern and neurotransmission in rat neostriatal projection neurons: role of CaV2.1 and CaV2.2 Ca^{2+} channels. J Neurophysiol 93:2507–2519

Phelps PE, Houser CR, Vaughn JE (1985) Immunocytochemical localization of choline acetyltransferase within the rat neostriatum: a correlated light and electron microscopic study of cholinergic neurons and synapses. J Comp Neurol 238:286–307

Pisani A, Martella G, Tscherter A, Bonsi P, Sharma N, Bernardi G, Standaert DG (2006) Altered responses to dopaminergic D2 receptor activation and N-type calcium currents in striatal cholinergic interneurons in a mouse model of DYT1 dystonia. Neurobiol Dis 24:318–325

Pisani A, Bernardi G, Ding J, Surmeier DJ (2007) Re-emergence of striatal cholinergic interneurons in movement disorders. Trends Neurosci 30:545–553

Planert H, Szydlowski SN, Hjorth JJ, Grillner S, Silberberg G (2010) Dynamics of synaptic transmission between fast-spiking interneurons and striatal projection neurons of the direct and indirect pathways. J Neurosci 30:3499–3507

Plotkin JL, Day M, Surmeier DJ (2011) Synaptically driven state transitions in distal dendrites of striatal spiny neurons. Nat Neurosci 14:881–888

Raz A, Feingold A, Zelanskaya V, Vaadia E, Bergman H (1996) Neuronal synchronization of tonically active neurons in the striatum of normal and parkinsonian primates. J Neurophysiol 76:2083–2088

Robertson GS, Vincent SR, Fibiger HC (1992) D1 and D2 dopamine receptors differentially regulate c-fos expression in striatonigral and striatopallidal neurons. Neuroscience 49:285–296

Sciamanna G, Bonsi P, Tassone A, Cuomo D, Tscherter A, Viscomi MT, Martella G, Sharma N, Bernardi G, Standaert DG, Pisani A (2009) Impaired striatal D2 receptor function leads to enhanced GABA transmission in a mouse model of DYT1 dystonia. Neurobiol Dis 34:133–145

Shen W, Hernandez-Lopez S, Tkatch T, Held JE, Surmeier DJ (2004) Kv1.2-containing K^+ channels regulate subthreshold excitability of striatal medium spiny neurons. J Neurophysiol 91:1337–1349

Shen W, Hamilton SE, Nathanson NM, Surmeier DJ (2005) Cholinergic suppression of KCNQ channel currents enhances excitability of striatal medium spiny neurons. J Neurosci 25:7449–7458

Shen W, Tian X, Day M, Ulrich S, Tkatch T, Nathanson NM, Surmeier DJ (2007) Cholinergic modulation of Kir2 channels selectively elevates dendritic excitability in striatopallidal neurons. Nat Neurosci 10:1458–1466

Shen W, Flajolet M, Greengard P, Surmeier DJ (2008) Dichotomous dopaminergic control of striatal synaptic plasticity. Science 321:848–851

Sidibe M, Smith Y (1996) Differential synaptic innervation of striatofugal neurones projecting to the internal or external segments of the globus pallidus by thalamic afferents in the squirrel monkey. J Comp Neurol 365:445–465

Smeal RM, Gaspar RC, Keefe KA, Wilcox KS (2007) A rat brain slice preparation for characterizing both thalamostriatal and corticostriatal afferents. J Neurosci Methods 159:224–235

Smith Y, Raju DV, Pare JF, Sidibe M (2004) The thalamostriatal system: a highly specific network of the basal ganglia circuitry. Trends Neurosci 27:520–527

Stern EA, Jaeger D, Wilson CJ (1998) Membrane potential synchrony of simultaneously recorded striatal spiny neurons in vivo. Nature 394:475–478

Sullivan MA, Chen H, Morikawa H (2008) Recurrent inhibitory network among striatal cholinergic interneurons. J Neurosci 28:8682–8690

Tanimura A, Yamazaki M, Hashimotodani Y, Uchigashima M, Kawata S, Abe M, Kita Y, Hashimoto K, Shimizu T, Watanabe M, Sakimura K, Kano M (2010) The endocannabinoid 2-arachidonoylglycerol produced by diacylglycerol lipase alpha mediates retrograde suppression of synaptic transmission. Neuron 65:320–327

Taverna S, Ilijic E, Surmeier DJ (2008) Recurrent collateral connections of striatal medium spiny neurons are disrupted in models of Parkinson's disease. J Neurosci 28:5504–5512

Tepper JM, Tecuapetla F, Koos T, Ibanez-Sandoval O (2010) Heterogeneity and diversity of striatal GABAergic interneurons. Front Neuroanat 4:150

Testa CM, Standaert DG, Young AB, Penney JB Jr (1994) Metabotropic glutamate receptor mRNA expression in the basal ganglia of the rat. J Neurosci 14:3005–3018

Tian X, Kai L, Hockberger PE, Wokosin DL, Surmeier DJ (2010) MEF-2 regulates activity-dependent spine loss in striatopallidal medium spiny neurons. Mol Cell Neurosci 44:94–108

Tkatch T, Baranauskas G, Surmeier DJ (2000) Kv4.2 mRNA abundance and A-type K(+) current amplitude are linearly related in basal ganglia and basal forebrain neurons. J Neurosci 20:579–588

Tozzi A, de Iure A, Di Filippo M, Tantucci M, Costa C, Borsini F, Ghiglieri V, Giampa C, Fusco FR, Picconi B, Calabresi P (2011) The distinct role of medium spiny neurons and cholinergic interneurons in the D2/A2A receptor interaction in the striatum: implications for Parkinson's disease. J Neurosci 31:1850–1862

Tunstall MJ, Oorschot DE, Kean A, Wickens JR (2002) Inhibitory interactions between spiny projection neurons in the rat striatum. J Neurophysiol 88:1263–1269

Turrigiano GG, Leslie KR, Desai NS, Rutherford LC, Nelson SB (1998) Activity-dependent scaling of quantal amplitude in neocortical neurons. Nature 391:892–896

Vilchis C, Bargas J, Ayala GX, Galvan E, Galarraga E (2000) Ca^{2+} channels that activate Ca^{2+}-dependent K^+ currents in neostriatal neurons. Neuroscience 95:745–752

Wang Y, Toledo-Rodriguez M, Gupta A, Wu C, Silberberg G, Luo J, Markram H (2004) Anatomical, physiological and molecular properties of Martinotti cells in the somatosensory cortex of the juvenile rat. J Physiol 561:65–90

Wang Z, Kai L, Day M, Ronesi J, Yin HH, Ding J, Tkatch T, Lovinger DM, Surmeier DJ (2006) Dopaminergic control of corticostriatal long-term synaptic depression in medium spiny neurons is mediated by cholinergic interneurons. Neuron 50:443–452

Wess J (1996) Molecular biology of muscarinic acetylcholine receptors. Crit Rev Neurobiol 10:69–99

Wess J, Eglen RM, Gautam D (2007) Muscarinic acetylcholine receptors: mutant mice provide new insights for drug development. Nat Rev Drug Discov 6:721–733

Wichmann T, DeLong MR (1996) Functional and pathophysiological models of the basal ganglia. Curr Opin Neurobiol 6:751–758

Wilson CJ (1993) The generation of natural firing patterns in neostriatal neurons. Prog Brain Res 99:277–297

Wilson CJ (1994) Understanding the neostriatal microcircuitry: high-voltage electron microscopy. Microsc Res Tech 29:368–380

Wilson CJ (2004) Basal ganglia. In: Shepherd GM (ed) The synaptic organization of the brain. Oxford University Press, New York, pp 361–413

Wilson CJ, Groves PM (1980) Fine structure and synaptic connections of the common spiny neuron of the rat neostriatum: a study employing intracellular inject of horseradish peroxidase. J Comp Neurol 194:599–615

Wilson CJ, Groves PM (1981) Spontaneous firing patterns of identified spiny neurons in the rat neostriatum. Brain Res 220:67–80

Wilson CJ, Kawaguchi Y (1996) The origins of two-state spontaneous membrane potential fluctuations of neostriatal spiny neurons. J Neurosci 16:2397–2410

Wilson CJ, Chang HT, Kitai ST (1990) Firing patterns and synaptic potentials of identified giant aspiny interneurons in the rat neostriatum. J Neurosci 10:508–519

Witten IB, Lin SC, Brodsky M, Prakash R, Diester I, Anikeeva P, Gradinaru V, Ramakrishnan C, Deisseroth K (2010) Cholinergic interneurons control local circuit activity and cocaine conditioning. Science 330:1677–1681

Wooten GF (1990) Parkinsonism. In: Pearlman AL, Collins RC (eds) Neurobiology of disease. Oxford University Press, New York, pp 454–468

Yan Z, Surmeier DJ (1996) Muscarinic (m2/m4) receptors reduce N- and P-type Ca^{2+} currents in rat neostriatal cholinergic interneurons through a fast, membrane-delimited, G-protein pathway. J Neurosci 16:2592–2604

Yan Z, Flores-Hernandez J, Surmeier DJ (2001) Coordinated expression of muscarinic receptor messenger RNAs in striatal medium spiny neurons. Neuroscience 103:1017–1024

Zhang W, Yamada M, Gomeza J, Basile AS, Wess J (2002) Multiple muscarinic acetylcholine receptor subtypes modulate striatal dopamine release, as studied with M1-M5 muscarinic receptor knock-out mice. J Neurosci 22:6347–6352

Muscarinic Receptors in Brain Stem and Mesopontine Cholinergic Arousal Functions

John S. Yeomans

Abstract All five muscarinic receptor subtypes and mRNAs are found widely in the brain stem, with M_2 muscarinic receptors most concentrated in the hindbrain. Three cholinergic cell groups, Ch5: pedunculopontine (PPT); Ch6: laterodorsal tegmental (LDT); Ch8: parabigeminal (PBG), are found in the tegmentum. Ch5,6 neurons are activated by arousing and reward-activating stimuli, and inhibited via M_2-like autoreceptors. Ch5,6 ascending projections activate many forebrain regions, including thalamus, basal forebrain, and orexin/hypocretin neurons (via M_3 receptors) for waking arousal and attention. Ch5,6 activation of dopamine neurons of the ventral tegmental area and substantia nigra (via M_5 receptors) increases reward-seeking and energizes motor functions. M_5 receptors on dopamine neurons facilitate brain-stimulation reward, opiate rewards and locomotion, and male ultrasonic vocalizations during mating in rodents. Ch5 cholinergic activation of superior colliculus intermediate layers facilitates fast saccades and approach turns, accompanied by nicotinic and muscarinic inhibition of the startle reflex in pons. Ch8 PBG neurons project to the outer layers of the superior colliculus only, where M_2 receptors are associated with retinotectal terminals. Ch5,6 descending projections to dorsal pontine reticular formation contribute to M_2-dependent REM sleep.

Keywords Colliculus • Dopamine • Nicotinic • Opiate • Orexin/hypocretin • Parabigeminal • Pedunculopontine • Sleep • Startle • Thalamus

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1 Brain Stem Cholinergic Cell Groups: Ch5–8 Neurons and Their Projections

Eight cholinergic cell groups were defined by Mesulam et al. (1983) using choline acetyltransferase (ChAT) labeling in rat brain. Three of these (Ch5, 6, 8) are found in the brain stem. The largest is the pedunculopontine tegmental nucleus (PPT, Ch5) which extends from the caudal end of the substantia nigra dorsocaudally into the pons (Fig. 1). The name PPT (sometimes called parabrachial n. in cats and monkeys) is due to the partial overlap of these neurons with the parallel ascending fibers of the superior cerebellar peduncle. The medial border of the elongated PPT in pons is co-extensive with the lateral border of ovoid laterodorsal tegmental nucleus (LDT, Ch6). Ch5,6 neurons together are often called "mesopontine," or sometimes "pontomesencephalic," cholinergic neurons.

The total number of mesopontine Ch5,6 neurons in the human brain was estimated at 19,400 using immunostaining for ChAT (German et al. 1999) and 18,600 neurons using NADPH diaphorase (Garcia-Rill et al. 1995) with the large majority of cells in

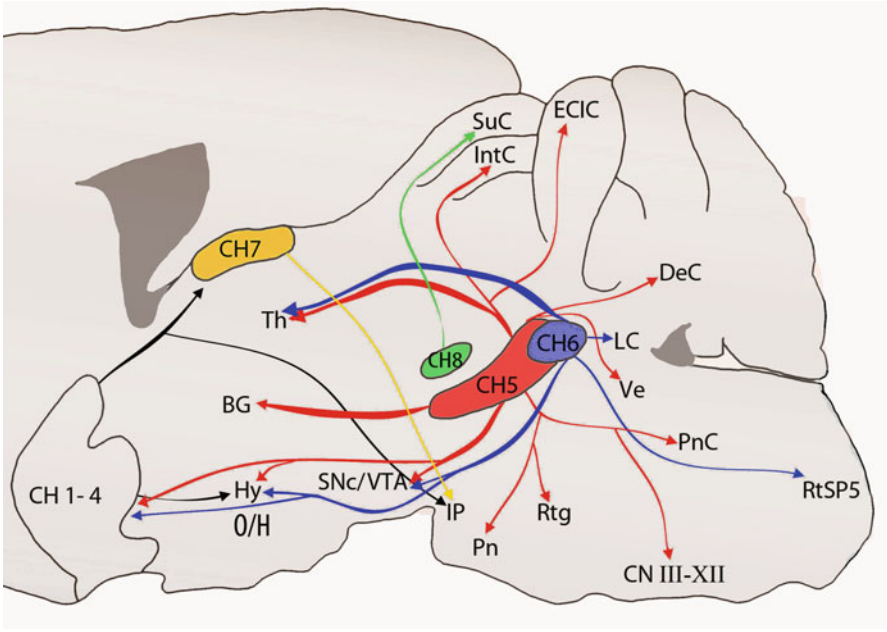

Fig. 1 Cholinergic cell groups (Ch1–8) and their brain stem projections collapsed onto a schematic parasagittal mouse brain section. *Th* thalamus, *BG* basal ganglia, *Hy* hypothalamus, *O/H* orexin/hypocretin neurons, *SuC* superficial layers of superior colliculus, *IntC* intermediate layers of superior colliculus, *ECIC* external cortex of inferior colliculus, *IP* interpeduncular nucleus, *Pn* pontine nuclei, *Rtg* rostral tegmental nucleus, *DeC* deep cerebellar nuclei, *LC* locus coeruleus, *Ve* vestibular nuclei, *PnC* nucleus reticularis pontis caudalis, *CN* cranial nerves, *RtSP5* spinal nucleus of the 5th nerve

Ch5. A smaller midbrain cell group that projects to the outer layers of the superior colliculus is the parabigeminal nucleus (Ch8), on the lateral edge of the rostral tegmentum, lateral to the rostral PPT. All cholinergic cell groups are found in nuclei that include non-cholinergic glutamate and GABA neurons as well.

Ch7 neurons of the medial habenula (dorsal to thalamus) project to the interpeduncular nucleus of the midbrain, just ventromedial to ventral tegmental area (VTA) dopamine neurons. Basal forebrain cholinergic neurons (Ch1–4) project only to the forebrain (especially olfactory bulb, amygdala, cerebral cortex, and hippocampus, with a small projection to the hypothalamus) and to the habenula and midbrain interpeduncular n., and so are discussed in other chapters. Cholinergic interneurons in the striatum and possibly other brain areas will not be discussed here.

Within the brain stem, many groups of cholinergic motoneurons are found (for cranial nerves III, IV, V, VI, VII, IX, X, XI, and XII). Their projections are to muscles and ganglia outside the central nervous system, and so are discussed elsewhere in connection with peripheral muscarinic receptors.

1.1 Functions and Forebrain Projections of Ch5 and Ch6 Neurons

Mesopontine Ch5 and Ch6 neurons (unlike Ch7 and Ch8 neurons) project to dozens of brain stem and subcortical nuclei with many functions (Woolf 1991; Semba and Fibiger 1992; Steininger et al. 1992). Ch5 and Ch6 neurons in many species are active in waking and/or REM sleep states associated with cortical arousal (Steriade and McCarley 2005; Kayama and Koyama 2003) and with reward-associated events (Pan and Hyland 2005; Kobayashi and Okada 2007). Ch5,6 projections activate many neurons in the thalamus, basal forebrain, hypothalamus (e.g., orexin-hypocretin neurons), and tegmentum (e.g., dopamine neurons) (Semba 1993; Yeomans et al. 2001; Sakurai et al. 2005; Yamanaka et al. 2003; Bayer et al. 2005). This "Mesopontine Cholinergic Arousal System" thereby facilitates neocortical electrical activity in waking or REM sleep, and behavioral arousal in waking states (Steriade and McCarley 2005; Yeomans et al. 1993).

Virtually all Ch5 and Ch6 neurons project to the thalamus, but each of these neurons also has axons projecting to other brain stem nuclei (Cornwall et al. 1990; Oakman et al. 1995, 1999; Woolf and Butcher 1986). A few neurons send axons that project to basal forebrain cholinergic neurons, but many more project to basal ganglia (e.g., globus pallidus, subthalamic nucleus), hypothalamus (e.g., suprachiasmatic, ventromedial hypothalamus, orexin/hypocretin neurons of the lateral hypothalamus), midbrain tegmentum (raphe n., rostromedial tegmental n., VTA and substantia nigra), tectum (pretectal nucleus, superior and inferior colliculi), cerebellum deep nuclei and cortex, or to pontine and medullary tegmentum (e.g., pontine nuclei, locus coeruleus, pontine reticular formation, vestibular nuclei, and several cranial nerve nuclei) (Semba and Fibiger 1992; Woolf and

Butcher 1986). Ch5 and Ch6 neurons project to many of the same nuclei with little topographic separation, but rostroventral PPT neurons have stronger projections to basal ganglia, including substantia nigra, subthalamic nucleus, and globus pallidus, while LDT neurons project more to medial hypothalamus and thalamus (Woolf and Butcher 1986; Mena-Segovia et al. 2008).

The functions of the M_1–M_5 muscarinic subtypes in brain stem will be reviewed here, especially in relation to the hypothesis that mesopontine cholinergic neurons act in a coordinated way to facilitate arousal, attention, motor activity, and reward seeking.

2 Localization of Muscarinic Receptors and mRNA in Brain Stem

Immunoprecipitation showed that M_1 receptor proteins are highest in whole telencephalon samples ($M_1 > M_4 > M_2 > M_3 >> M_5$), M_2 receptors are slightly higher in midbrain samples ($M_2 > M_1 > M_4 > M_3 > M_5$) while M_2 receptors are by far the highest in hindbrain and cerebellum samples (>70%) ($M_2 >> M_3 = M_1 = M_4 > M_5$) (Yasuda et al. 1993). Immunocytochemistry localized these receptors, with M_2 receptors widely distributed in brain stem, but most concentrated in the outer layers of the superior colliculus, in pontine and pretectal nuclei, and in Ch5, 6, and 8 cell groups (Levey et al. 1994). Several motoneuron groups (e.g., V and VII) show high levels of M_2 receptors along with lower levels of M_1, M_3, and M_4 receptors. These muscarinic receptor densities are associated with acetylcholinesterase (AChE) staining of these brain stem nuclei, in human (Paxinos and Huang 1995), rat (Paxinos and Watson 2007), or mouse (Franklin and Paxinos 1997).

Low levels of brainstem M_4 receptors are concentrated in the hypothalamus and in brainstem motoneurons. M_5 receptors account for only about 1% of all brain receptors so localization of M_5 receptors using immunocytochemistry was not reported (Levey 1993).

Physiological studies showed that M_1, M_3, and M_5 receptors activate Gαq/11 proteins and phospholipase C in vitro, which can depolarize neurons and stimulate peripheral secretions (Bymaster et al. 2003). M_2 and M_4 receptors, however, activate Gαi/o proteins that inhibit adenylyl cyclase, and hyperpolarize heart muscles and cholinergic neurons. It has been proposed that M_1, M_3, and M_5 are postsynaptic excitatory receptors, while M_2 and M_4 receptors are inhibitory, both pre- and postsynaptic, and as inhibitory autoreceptors on cholinergic neurons (Levey 1993; Wess et al. 2003; Bymaster et al. 2003). In vitro studies suggested that M_2 receptors bind more quickly than M_1 and M_4 receptors, with M_3 and M_5 receptors binding more slowly (Flynn et al. 1997; Ferrari-diLeo et al. 1994).

In situ hybridization locates mRNA for each of the five receptors (Weiner et al. 1990; Lein et al. 2007). This method helps show the cholinergic and non-cholinergic neurons expressing the receptors. For example, detection of M_5 mRNA in VTA

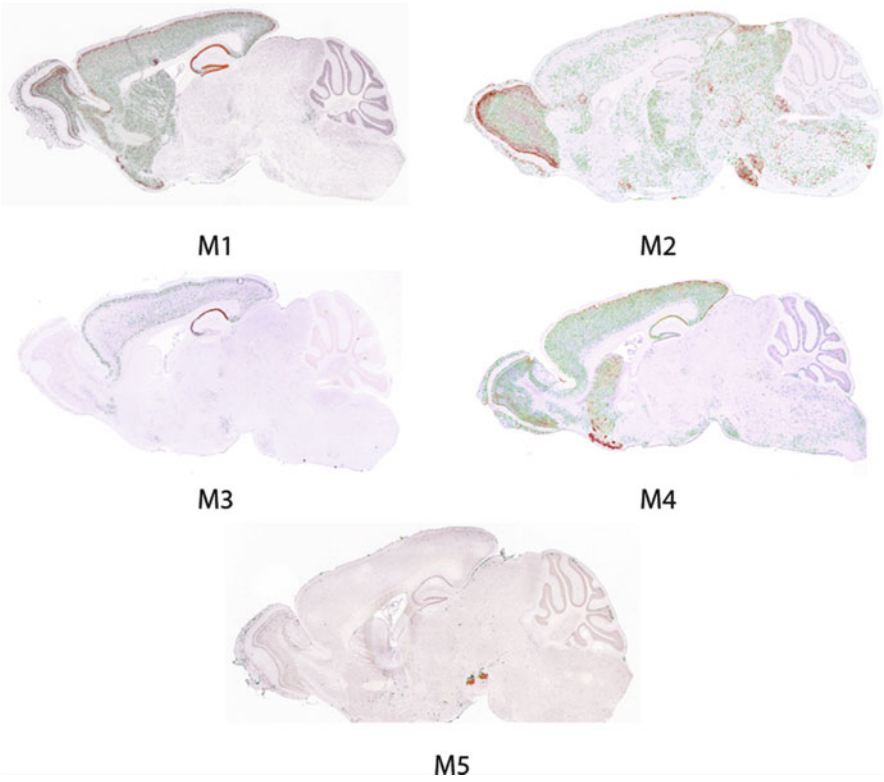

Fig. 2 Muscarinic receptor mRNA density for each of the five muscarinic receptors in mouse. Each section shows density placed on cresyl violet-stained parasagittal sections about 1 mm off the midline of sections from the Allen Mouse Brain Atlas (Lein et al. 2007). The pseudocolor density scales proceed from *green* (low) to *red* (highest) on each section of the e-book, and are set differently on each section to highlight the areas of highest density for each receptor mRNA. For *black* and *white* figures, densities are scaled from *darkest (black)* to *lightest (white)* on a *gray* scale

and substantia nigra, pars compacta, removable by 6-hydroxydopamine, led to evidence that DA neurons are excited by M_5 postsynaptic receptors (Weiner et al. 1990; Vilaro et al. 1990; Yeomans et al. 2001). Figure 2 shows parasagittal sections taken approximately 0.6 mm off the midline for each of the M_1–M_5 mRNAs and AChE mRNA. Again, M_2 mRNA expression is highest in the brain stem, followed by M_3, with M_1, M_4, and M_5 much lower. M_1 mRNA is found at low levels in hypothalamus and motoneuron cell groups (III, V, VI, VII, X, XII).

M_2 mRNA is found near the cell bodies of all Ch5–8 cell groups, with highest levels in pontine nuclei. Moderate levels are found in thalamus (especially the reticular nucleus that inhibits other thalamic nuclei) and widely in the brain stem from the outer layers of the superior colliculus to the caudal medulla. M_2, M_3, and M_5 mRNAs are found near Ch8 parabigeminal neurons, and in the lateral habenula (Vilaro et al. 1994).

M_3 mRNA is found near orexin/hypocretin neurons of the lateral hypothalamus, in several nuclei of the thalamus, and on interpeduncular and pontine n. neurons in the midbrain, as well as superior and inferior colliculus. M_4 mRNA is found near ventral brainstem motoneurons, like M_1, but rarely elsewhere. M_5 mRNA is localized to DA neurons in VTA and SNC and in the ventromedial hypothalamic nucleus.

3 Muscarinic Receptor Functions in Diencephalon and Basal Forebrain: Ascending Mesopontine Cholinergic Arousal

Muscarinic receptors on Ch5,6 neurons are strongly inhibitory, acting via M_2-like receptors and K^+ channels (Leonard and Llinas 1994; Luebke et al. 1993). Both autoreceptors and cholinergic inhibitory synapses are found, suggesting that cholinergic arousal is held in check by somatodendritic autoreceptors and by postsynaptic release of ACh (Leonard, personal communication).

In thalamus, muscarinic inputs are largely excitatory, except for inhibition of reticular n. GABA neurons that tonically inhibit other thalamic nuclei (Steriade 1993; McCormick 1989). These muscarinic inputs, therefore, facilitate thalamic systems, resulting in widespread thalamocortical activation during waking and REM sleep (Steriade and McCarley 2005). Cholinergic facilitation of cortical functions is thereby initiated via mesopontine cholinergic neurons and then is relayed via excitation of thalamus and of basal forebrain cholinergic neurons (Dringenberg and Olmstead 2003).

Muscarinic receptors in the intergeniculate leaflet (IGL) of the thalamus can shift circadian rhythm in hamsters (Cain et al. 2007). In particular, PPT cholinergic neurons that respond to arousing signals are believed to activate IGL neurons that mediate arousal-induced phase shifts by way of direct IGL projections to the suprachiasmatic nucleus (SCN).

Cholinergic inputs to the SCN can alter circadian rhythms especially at night (e.g., Liu and Gillette 1996). Basal forebrain and PPT cholinergic neurons project to SCN (Bina et al. 1993), so either pathway could provide the cholinergic influence on SCN. All five muscarinic mRNAs are expressed in SCN neurons, but M_1- and M_4-like receptors seem to be most effective in mediating carbachol-induced hyperpolarization in SCN neurons, in vitro (Yang et al. 2009). M_1-like receptors are most important in mediating the nighttime effects of carbachol in SCN on circadian rhythms (Gillette et al. 2001).

Orexin/hypocretin neurons in the lateral hypothalamic perifornical area help maintain waking arousal, and are lost in narcolepsy/cataplexy (Thannickal et al. 2001). Many of these neurons are strongly depolarized and excited by 100 μM carbachol, an effect that is blocked by atropine, or the M_3 antagonist 4-DAMP (Yamanaka et al. 2003; Bayer et al. 2005). These cholinergic inputs to orexin/hypocretin neurons could come from either Ch1–4 basal forebrain (Henny and

Jones 2006) or Ch5,6 neurons (Ford et al. 1995). Orexin/hypocretin neurons, in turn, directly and indirectly excite LDT Ch6 cholinergic neurons (Burlet et al. 2002; Takahashi et al. 2002).

4 Functions of Muscarinic Inputs to Substantia Nigra and VTA

Stimulation of PPT or LDT results in monosynaptic excitation of dopamine (DA) neurons of the substantia nigra and VTA (Lacey et al. 1990; Futami et al. 1995; Scarnati et al. 1986). These cholinergic inputs are important for the maintenance of burst firing in DA neurons (Lodge and Grace 2006; Floresco et al. 2003). Anatomical studies show many ChAT-labeled terminals in the vicinity of dopamine and non-dopamine neurons (Beninato and Spencer 1988; Bolam et al. 1991; Sesack and Grace 2010) along with eight nicotinic (alpha 3–7, beta 2–4) and four muscarinic (M_{2-5}) receptor subtypes (Klink et al. 2001). MRNA studies, however, indicate that M_5 receptors are made by only DA neurons, since all M_5 mRNA and M_5-like receptors are lost after 6-hydroxydopamine lesions of DA neurons (Weiner et al. 1990; Vilaro et al. 1990; Reever et al. 1997).

The muscarinic receptors affecting net dopamine output can be studied by recording DA efflux in the nucleus accumbens or striatum. When the PPT or LDT is electrically stimulated, DA efflux is increased in accumbens or striatum for 1–3 min, due to ionotropic nicotinic and glutamate receptors in VTA or SN (Forster and Blaha 2000). DA efflux is reduced from 2 to 8 min after stimulation due to M_2-like receptors in LDT or PPT. Then, a second wave of DA efflux occurs from 8 to 60 min in rats, or 5 to 40 min in mice. This prolonged DA output is completely blocked by muscarinic receptor blockers in the VTA, or by knockout of the M_5 muscarinic receptor in mice (Forster et al. 2002). This very slow M_5 effect is consistent with the very slow binding of M_5 receptors in cell cultures (Ferrari-diLeo et al. 1994) or in salivation (Takeuchi et al. 2002). Therefore, sustained activation of dopamine neurons results from postsynaptic M_5 excitation of DA neurons from PPT and LDT cholinergic neurons (Yeomans et al. 2001).

Muscarinic receptors on non-dopamine neurons and terminals in VTA and substantia nigra have a strong net inhibitory effect on dopamine outputs, and on locomotor activity (Steidl and Yeomans 2009). The muscarinic blocker atropine in VTA facilitated locomotor activity strongly in M_5 knockout mice, for example, but had much less effect in wild type mice. Although M_2 muscarinic receptors are found on terminals and dendrites of many VTA neuron types (Garzón and Pickel 2006), the M_4 muscarinic receptor especially inhibits ACh release from PPT/LDT terminals in the VTA/SN (Tzavara et al. 2004). That is, M_4 knockout mice show increased ACh release in VTA, but M_2 knockout mice do not. M_3 muscarinic receptors are found postsynaptically in VTA, and may provide an excitatory influence on GABA neurons (Michel et al. 2005; Miller et al. 2005). A model of VTA/SN muscarinic effects on DA neurons is shown in Fig. 3.

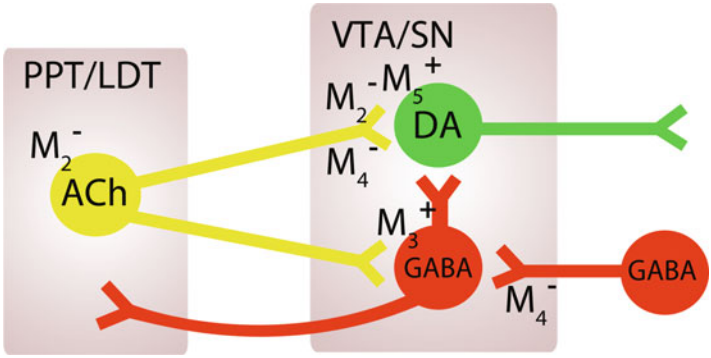

Fig. 3 Muscarinic subtypes affecting PPT/LDT cholinergic, and VTA/SN DA and GABA neurons (model based on Miller et al. 2005; Tzavara et al. 2004; Steidl and Yeomans 2009)

4.1 Reward-Related Behaviors and M_5 Muscarinic Receptors

M_5 receptors are also important for the rewarding effects of hypothalamic stimulation, of carbachol in VTA, of opiates, and of ethanol. Muscarinic blockers in VTA strongly reduce the rewarding effects of hypothalamic stimulation in rats (Kofman et al. 1990; Yeomans et al. 1985). A similar reduction in sensitivity occurs after knockdown of the M_5 muscarinic receptor in VTA by infusions of an antisense oligonucleotide (Yeomans et al. 2001). Muscarinic receptor blockers in VTA also reduced the rewarding effects of food in rats (Sharf et al. 2005; Sharf and Ranaldi 2006). In this regard, hypothalamic brain-stimulation reward, feeding and drinking are known to activate PPT cholinergic neurons and to induce release of acetylcholine into the VTA from cholinergic terminals (Pan and Hyland 2005; Rada et al. 2000).

Fifty kHz ultrasonic vocalizations (USVs) induced in male mice by female urine, or during mating, are reduced by 70–80% in M_5 knockout mice, even though mating appears normal (Wang et al. 2008). This deficit is likely due to reduced activation of dopamine neurons that facilitate USVs. M_5 receptors found in the ventromedial hypothalamic nucleus may be related to USVs, too, because microlesions of ventromedial hypothalamus also reduce male USVs, without disrupting mating (Harding and McGinnis 2005).

Carbachol in VTA is strongly rewarding in rats, either in conditioned place preference tasks (Yeomans et al. 1985) or when self-administered in VTA (Ikemoto and Wise 2002). Although carbachol acts on both nicotinic and muscarinic receptors, the rewarding effects of carbachol are blocked by muscarinic, but not nicotinic, blockers in VTA (Ikemoto and Wise 2002). The weaker rewarding effects of systemic nicotine, however, are blocked by nicotinic blockers in VTA (Corrigall et al. 1994).

4.2 Opiates and Reward

Small bilateral lesions of the caudal PPT block the rewarding effects of opiates, either in conditioned place preference or intravenous self-administration acquisition tasks (Bechara and van der Kooy 1989; Laviolette and van der Kooy 2004; Olmstead and Franklin 1993). Also, carbachol in PPT inhibits sensitivity to brain-stimulation reward or to ethanol intake, presumably by inhibiting cholinergic neurons in PPT (Yeomans et al. 1993; Mathur et al. 1997). By contrast, scopolamine in PPT increases brain-stimulation reward sensitivity and increases striatal dopamine release (Yeomans et al. 1993; Chapman et al. 1997).

Morphine (20–25 mg/kg i.p.) increases accumbal dopamine release in rats or mice (Basile et al. 2002). Ch5,6 lesions, or VTA/nigral infusions of the muscarinic blocker scopolamine, blocked the ability of morphine, but not amphetamine, to increase DA release in the accumbens or striatum (Miller et al. 2005). In M_5 knockout mice, morphine fails to increase accumbal DA release, except in the first 20 min after infusion when ionotropic receptors are used (Basile et al. 2002). Also M_5 knockout mice show less conditioned place preference (1–30 mg/kg) and less locomotion in response to morphine (3–30 mg/kg) (Basile et al. 2002; Steidl and Yeomans 2009). VTA infusions of the muscarinic blocker atropine reduced the locomotor stimulant effect of morphine similarly. Finally, naltrexone had less inhibitory effect on spontaneous locomotion or on USVs in M_5 knockout mice, suggesting that endogenous opiates also work via M_5 receptors in VTA to stimulate dopamine neurons. Therefore, both systemically applied or endogenous opiates depend on muscarinic M_5 activation in VTA/nigra to stimulate dopamine neurons or to stimulate dopamine-dependent forms of locomotion and reward-seeking (Steidl and Yeomans 2009).

A role for muscarinic receptors in PPT and VTA in ethanol intake has been proposed (Katner et al. 1997). The reduction of ethanol intake and ethanol-induced dopamine release by naltrexone (Middaugh et al. 2003) suggests that muscarinic receptors contribute especially to the opiate receptor-dependent part of ethanol drinking.

5 PPT, Basal Ganglia and Parkinson's Disease

Brains of Parkinson's patients often have severe loss of PPT neurons as well as ventrolateral nigral neurons (Pahapill and Lozano 2000; Zweig et al. 1989). Unilateral deep brain stimulation of the PPT has recently been found to relieve gait freezing and postural instability in advanced Parkinson's Disease when dopaminergic drugs are no longer effective (Plaha and Gill 2005; Pereira et al. 2008). Unlike deep brain stimulation of subthalamic nucleus, low frequency stimulation (20–25 Hz) is most effective. The mechanisms of these beneficial effects are not

yet clear, but the ascending pathways from the lateral PPT to basal ganglia are most often considered.

In addition, substantia nigra pars reticulata neurons strongly inhibit PPT cholinergic neurons via monosynaptic GABA receptors (Takakusaki et al. 1996). These connections are relevant to the control of REM sleep and atonia (Takakusaki et al. 2004) as well as ascending influences on basal ganglia. In this regard, the locomotor facilitating effects of PPT stimulation in humans, primates and rats, and the locomotor inhibiting effects of PPT inhibition, are relevant (Nandi et al. 2008; Brudzynski et al. 1988; Mathur et al. 1997). More work is needed on the muscarinic receptors needed for PPT-induced locomotion in M_1–M_5 knockout mice.

6 Descending Cholinergic Pathways in REM Sleep

A subset of Ch5 and Ch6 cholinergic neurons are active just before the onset of REM sleep, suggesting a special role of these neurons in initiating cortical arousal during dreams (Steriade and McCarley 2005; Kayama et al. 1992; Semba 1993). Because these REM-on neurons are interspersed within a larger population of Ch5 neurons that respond only during waking arousal, the critical REM-on neurons have not been identified (Datta 2002). Takakusaki et al. (2004) proposed that REM-on neurons are located in a more dorsal layer of Ch5 cells that, in turn, project to the pontine tegmental area critical for triggering REM onset. In particular, M_2-like muscarinic receptors in the dorsal pontine tegmentum behind PPT and LDT contribute to REM sleep generation in mice (Coleman et al. 2004).

7 Eye Movements, Approach Turns, and Startle Inhibition Due to Muscarinic Receptors in Colliculi and Brain Stem

PPT cholinergic neurons project to intermediate gray layers of the superior colliculus (SCi), where auditory, tactile, and visual inputs converge on premotor neurons that initiate fast saccadic eye movements (Isa and Hall 2009). SCi neurons activate saccadic eye movements and head turns toward visual targets (i.e., "approach turns") by way of crossed tectoreticulospinal axons to premotor nuclei controlling vertical and horizontal eye movements. Both nicotinic and muscarinic inputs to these SCi neurons are excitatory, thereby facilitating fast saccades.

The muscarinic facilitation of SCi neurons in vitro has been shown to involve an inward postsynaptic current working mainly via M_3-like receptors (i.e., blocked by 4-DAMP), with a small M_1 effect blocked by pirenzepine (Sooksawate and Isa 2006). In addition a small inhibitory outward current via M_2 receptors, and a presynaptic reduction in GABA inhibition, occurs.

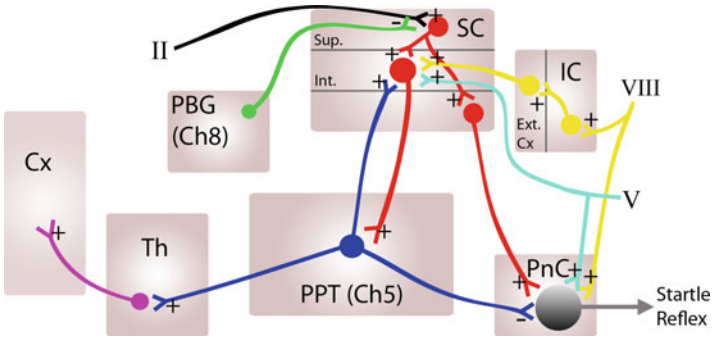

Fig. 4 Model of cholinergic influences on approach turns in superior and inferior colliculi and brain stem, including simultaneous activation of thalamus, and inhibition of startle reflex circuits in pons. Abbreviations are the same as Fig. 1

Auditory inputs to the SCi come from the external cortex of the inferior colliculus, which also receives direct projections from PPT cholinergic neurons. AChE is concentrated in layer 2 of the external cortex, but the muscarinic inputs have not been studied.

By contrast, parabigeminal Ch8 neurons project only to the outer layers of SC, where high levels of M_2 receptors and AChE are found in the outer, visual layers of the SC (see Figs. 1 and 4). ACh results in less depolarization of these superficial neurons by visual glutamate inputs. These inhibitory effects are mediated by parabigeminal inputs to superficial layer neurons (Isa and Hall 2009).

7.1 Cholinergic Inhibition of the Startle Reflex Following Prepulses

Although high-intensity stimulation of the inferior or superior colliculus activates the startle reflex, moderate-intensity stimulation inhibits startle (Fendt et al. 2001; Yeomans et al. 2006). In rats, inferior colliculus lesions block prepulse inhibition of startle by moderate-intensity acoustic prepulses (Leitner and Cohen 1985) while superior colliculus mediates a slower inhibition of startle by visual, tactile, or auditory prepulses (Fendt et al. 2001; Yeomans et al. 2006). The lowest threshold sites for startle inhibition by electrical stimulation are found in SCi associated with activation of tectoreticulospinal neurons initiating approach saccades and turns (Yeomans et al. 2006; Sahibzada et al. 1986; Tehovnik and Yeomans 1986).

Prepulse inhibition results mainly from descending cholinergic projections of the PPT to the pons. PPT lesions block prepulse inhibition of startle in rats (Koch et al. 1993; Swerdlow and Geyer 1993). PPT stimulation inhibits giant neurons in the ventrocaudal pontine reticular formation (PnC) that elicit startle (Bosch and Schmid 2008; Yeomans et al. 2001). Carbachol inhibits PnC giant neurons via both

nicotinic and muscarinic receptors (Bosch and Schmid 2006, 2008). Muscarinic receptors mediate startle inhibition 100–500 ms after prepulse delivery (Yeomans et al. 2010).

These mesopontine cholinergic inhibitory effects on startle parallel the timing of PPT-mediated facilitation of fast saccades in SCi. Accordingly, PPT cholinergic neurons simultaneously facilitate approach saccades via activation of SCi tectoreticulospinal neurons, and inhibit startle-mediated eye closure via inhibition of PnC startle neurons, allowing rapid foveation of targets. Mesopontine cholinergic neurons, therefore, appear to coordinate approach turns, whereby turning to look is accompanied by startle inhibition that prevents eye closure.

Simultaneously, mesopontine cholinergic arousal facilitates thalamocortical systems needed for attention and analysis of incoming signals (Fig. 4). This cortical activation prepares the forebrain for analysis of incoming sensory information.

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References

Basile AS, Federava I, Zapata A, Liu X, Shippenberg T, Duttaroy A, Yamada M, Wess J (2002) Deletion of the M_5 muscarinic acetylcholine receptor attenuates morphine reinforcement and withdrawal but not morphine analgesia. Proc Natl Acad Sci USA 99:11452–11457

Bayer L, Eggermann E, Serafin M, Grivel J, Machard D, Muhlethaler M, Jones BE (2005) Opposite effects of noradrenaline and acetylcholine upon hypocretin/orexin versus melanin concentrating hormone neurons in rat hypothalamic slices. Neuroscience 130:807–811

Bechara A, van der Kooy D (1989) The tegmental pedunculopontine nucleus: a brain-stem output of the limbic system critical for the conditioned place preferences produced by morphine and amphetamine. J Neurosci 9:3400–3409

Beninato M, Spencer RF (1988) The cholinergic innervation of the substantia nigra: a light and electron microscopic immunohistochemical study. Exp Brain Res 72:178–184

Bina K, Rusak B, Semba K (1993) Localization of cholinergic neurons in the forebrain and brainstem that project to the suprachiasmatic nucleus of the hypothalamus in rat. J Comp Neurol 335:295–307

Bolam JP, Francis CM, Henderson Z (1991) Cholinergic input to dopaminergic neurons in the substantia nigra: a double immunocytochemical study. Neuroscience 41:483–494

Bosch D, Schmid S (2006) Activation of muscarinic cholinergic receptors inhibits giant neurons in the caudal pontine reticular nucleus. Eur J Neurosci 24:1967–1975

Bosch D, Schmid S (2008) Cholinergic mechanism underlying prepulse inhibition of the startle response in rats. Neuroscience 155:326–335

Brudzynski SM, Wu M, Mogenson GJ (1988) Modulation of locomotor activity induced by injections of carbachol into the tegmental pedunculopontine nucleus and adjacent areas in the rat. Brain Res 451:119–125

Burlet S, Tyler CJ, Leonard CS (2002) Direct and indirect excitation of laterodorsal tegmental neurons by hypocretin/orexin peptides: implications for wakefulness and narcolepsy. J Neurosci 22:2862–2872

Bymaster FP, McKinzie DL, Felder CC, Wess J (2003) Use of M_1-M_5 muscarinic receptor knockout mice as novel tools to delineate the physiological roles of the muscarinic cholinergic system. Neurochem Res 28:437–442

Cain S, Verwey M, Szybowska M, Ralph MR, Yeomans JS (2007) Carbachol injections into the intergeniculate leaflet induce nonphotic phase shifts. Brain Res 1177:59–65

Chapman CA, Yeomans JS, Blaha CD, Blackburn JR (1997) Increased striatal dopamine efflux follows scopolamine administered systemically or to the tegmental pedunculopontine nucleus. Neuroscience 76:177–186

Coleman CG, Lydic R, Baghdoyan HA (2004) M_2 muscarinic receptors in pontine reticular formation of C57BL/6J mouse contribute to rapid eye movement sleep generation. Neuroscience 126:821–830

Cornwall J, Cooper JD, Phillipson OT (1990) Afferent and efferent connections of the laterodorsal tegmental nucleus in the rat. Brain Res Bull 25:271–284

Corrigall WA, Coen KL, Adamson KM (1994) Self-administered nicotine activates the mesolimbic dopamine system through the ventral tegmental area. Brain Res 653:278–284

Datta S (2002) Evidence that REM sleep is controlled by the activation of brain stem pedunculopontine tegmental kainate receptors. J Neurophysiol 87:1790–1798

Dringenberg HC, Olmstead MC (2003) Integrated contributions of basal forebrain and thalamus to neocortical activation elicited by pedunculopontine tegmental stimulation in urethane-anesthetized rats. Neuroscience 119:839–853

Fendt M, Li L, Yeomans JS (2001) Brain stem circuits mediating prepulse inhibition of the startle reflex. Psychopharmacology 156:216–224

Ferrari-DiLeo G, Waelbroeck M, Mash DC, Flynn DD (1994) Selective labelling and localization of the M_4 (m_4) muscarinic receptor subtype. Mol Pharmacol 46:1028–1035

Floresco SB, West AR, Ash B, Moore H, Grace AA (2003) Afferent modulation of dopamine neuron firing differentially regulates tonic and phasic dopamine transmission. Nat Neurosci 6:968–973

Flynn DD, Reever CM, Ferrari-DiLeo G (1997) Pharmacological strategies to selectively label and localize muscarinic receptor subtypes. Drug Dev Res 40:104–116

Ford B, Holmes CJ, Mainville L, Jones BE (1995) GABAergic neurons in the rat pontomesencephalic tegmentum: codistribution with cholinergic and other tegmental neuronsprojecting to the posterior lateral hypothalamus. J Comp Neurol 363:177–196

Forster G, Blaha CD (2000) Laterodorsal tegmental stimulation elicits dopamine efflux in the rat nucleus accumbens by activation of acetylcholine and glutamate receptors in the ventral tegmental area. Eur J Pharmacol 12:3596–3604

Forster GL, Yeomans JS, Takeuchi J, Blaha CD (2002) M_5 muscarinic receptors are required for prolonged accumbal dopamine release after electrical stimulation of the pons in mice. J Neurosci 22:1–6

Franklin KBJ, Paxinos G (1997) The mouse brain in stereotaxic coordinates. Academic, New York, NY

Futami K, Takakusaki K, Kitai ST (1995) Glutamatergic and cholinergic inputs from the pedunculopontine tegmental nucleus to dopamine neurons in the substantia nigra pars compacta. Neurosci Res 21:331–342

Garcia-Rill E, Biedermann JA, Chambers T, Skinner RD, Mrak RE, Husain M, Karson CN (1995) Mesopontine neurons in schizophrenia. Neuroscience 66:321–335

Garzón M, Pickel VM (2006) Subcellular distribution of M_2 muscarinic receptors in relation to dopaminergic neurons of the rat ventral tegmental area. J Comp Neurol 498:821–839

German DC, Manaye KF, Wu D, Hersh LB, Zweig RM (1999) Mesopontine cholinergic and non-cholinergic neurons in schizophrenia. Neuroscience 94:33–38

Gillette MU, Buchanan GF, Artinian L, Hamilton SE, Nathanson NM, Liu C (2001) Role of the M_1 receptor in regulating circadian rhythms. Life Sci 68:2467–2472

Harding SM, McGinnis MY (2005) Microlesions of the ventromedial nucleus of the hypothalamus: effects on sociosexual behaviors in male rats. Behav Neurosci 119:1227–1234

Henny P, Jones BE (2006) Innervation of orexin/hypocretin neurons by GABAergic, glutamatergic or cholinergic basal forebrain terminals evidenced by immunostaining for

presynaptic vesicular transporter and postsynaptic scaffolding proteins. J Comp Neurol 499:645–661
Ikemoto S, Wise RA (2002) Rewarding effects of the cholinergic agents carbachol and neostigmine in the posterior ventral tegmental area. J Neurosci 22:9895–9904
Isa T, Hall WC (2009) Exploring the superior colliculus in vitro. J Neurophysiol 102:2581–2593
Katner SN, McBride WJ, Lumeng L, Li T, Murphy JM (1997) Alcohol intake of P rats is regulated by muscarinic receptors in the pedunculopontine nucleus and VTA. Pharmacol Biochem Behav 58:497–504
Kayama Y, Koyama Y (2003) Control of sleep and wakefulness by brainstem monoaminergic and cholinergic neurons. Acta Neurochir Suppl 87:3–6
Kayama Y, Ohta M, Jodo E (1992) Firing of "possibly" cholinergic neurons in the rat laterodorsal tegmental nucleus during sleep and wakefulness. Brain Res 569:210–220
Klink R, d'Exaerde AD, Zoli M, Changeux J (2001) Molecular and physiological diversity of nicotinic acetylcholine receptors in the midbrain dopaminergic nuclei. J Neurosci 21:1452–1463
Kobayashi Y, Okada K (2007) Reward prediction error computation in the pedunculopontine tegmental nucleus neurons. Ann N Y Acad Sci 1104:310–323
Koch M, Kungel M, Herbert H (1993) Cholinergic neurons in the pedunculopontine tegmental nucleus are involved in the mediation of prepulse inhibition of the acoustic startle response in the rat. Exp Brain Res 97:71–82
Kofman O, McGlynn S, Olmstead MC, Yeomans JS (1990) Differential effects of atropine, procaine and dopamine in the rat ventral tegmentum on lateral hypothalamic rewarding brain stimulation. Behav Brain Res 38:55–68
Lacey MG, Calabresi P, North RA (1990) Muscarine depolarizes rat substantia nigra zona compacta and ventral tegmental neurons in vitro through M_1-like receptors. J Pharmacol Exp Ther 253:395–400
Laviolette SR, van der Kooy D (2004) The neurobiology of nicotine addiction: bridging the gap from molecules to behaviour. Nat Rev Neurosci 5:55–65
Lein ES, Hawrylycz MJ, Ao N, Ayres M, Bensinger A, Bernard A, Boe AF, Boguski MS, Brockway KS, Byrnes EJ, Chen L, Chen L, Chen TM, Chin MC, Chong J, Crook BE, Czaplinska A, Dang CN, Datta S, Dee NR et al (2007) Genome-wide atlas of gene expression in the adult mouse brain. Nature 445:168–176
Leitner DS, Cohen ME (1985) Role of the inferior colliculus in the inhibition of acoustic startle in the rat. Physiol Behav 34:65–70
Leonard CS, Llinas R (1994) Serotonergic and cholinergic inhibition of mesopontine cholinergic neurons controlling REM sleep: an in vitro electrophysiological study. Neuroscience 59:309–330
Levey AI (1993) Immunological localization of m_1-m_5 muscarinic acetylcholine receptors in peripheral tissues and brain. Life Sci 52:441–448
Levey AI, Edmunds SM, Heilman CJ, Desmond TJ, Frey KA (1994) Localization of muscarinic M_3 receptor protein and M_3 receptor binding in rat brain. Neuroscience 63:207–221
Liu C, Gillette MU (1996) Cholinergic regulation of the suprachiasmatic nucleus circadian rhythm via a muscarinic mechanism at night. J Neurosci 16:744–751
Lodge DJ, Grace AA (2006) The laterodorsal tegmentum is essential for burst firing of ventral tegmental area dopamine neurons. Proc Natl Acad Sci USA 103:5167–5172
Luebke JI, McCarley RW, Greene RW (1993) Inhibitory action of muscarinic agonists on neurons in the rat laterodorsal tegmental nucleus in vitro. J Neurophysiol 70:2128–2135
Mathur A, Shandarin A, LaViolette SR, Parker J, Yeomans JS (1997) Locomotion and stereotypy induced by scopolamine: contributions of muscarinic receptors near the pedunculopontine tegmental nucleus. Brain Res 775:144–155
McCormick DA (1989) Cholinergic and noradrenergic modulation of thalamocortical processing. Trends Neurosci 12:215–221

Mena-Segovia J, Winn P, Bolam JP (2008) Cholinergic modulation of midbrain dopaminergic systems. Brain Res 58:265–271

Mesulam M, Mufson EJ, Wainer BH, Levey AI (1983) Central cholinergic pathways in the rat. An overview based on an alternative nomenclature (Ch1-Ch6). Neuroscience 10:1185–1201

Michel FJ, Fortin G, Martel P, Yeomans J, Trudeau L-E (2005) Muscarinic receptors mediate Ca^{++} influx in rat mesencephalic GABAergic neurons through PKC-dependent activation of receptor-operated channels. Neuropharmacology 48:796–809

Middaugh LD, Szumlinski KK, Van Patten Y, Marlowe AL, Kalivas PW (2003) Chronic ethanol consumption by C57BL/6 mice promotes tolerance to interoceptive cues and increases extracellular dopamine, an effect blocked by naltrexone. Alcohol Clin Exp Res 27:1892–1900

Miller AD, Forster GL, Yeomans JS, Blaha CD (2005) Midbrain muscarinic receptors modulate morphine-induced accumbal and striatal dopamine efflux in the rat. Neuroscience 136:531–538

Nandi D, Jenkinson N, Stein J, Aziz T (2008) The pedunculopontine nucleus in Parkinson's disease: primate studies. Br J Neurosurg 22:S4–S8

Oakman SA, Faris PL, Kerr PE, Cozzari C, Hartman BK (1995) Distribution of pontomesencephalic cholinergic neurons projecting to the substantia nigra differ significantly from those projecting to the ventral tegmental area. J Neurosci 15:5859–5869

Oakman SA, Faris PL, Cozzari C, Hartman BK (1999) Characterization of the extent of pontomesencephalic cholinergic neurons' projections to the thalamus: comparison with projections to midbrain dopaminergic groups. Neuroscience 94:529–547

Olmstead MC, Franklin KBJ (1993) Effects of pedunculopontine tegmental nucleus lesions on morphine-induced conditioned place preference and analgesia in the formalin test. Neuroscience 57:411–418

Pahapill PA, Lozano AM (2000) The pedunculopontine nucleus and Parkinson's disease. Brain 123:1767–1783

Pan WX, Hyland BI (2005) Pedunculopontine tegmental nucleus controls conditioned responses of midbrain dopamine neurons in behaving rats. J Neurosci 25:4725–4732

Paxinos G, Huang XF (1995) Atlas of the human brainstem. Academic, San Diego, CA

Paxinos G, Watson C (2007) The rat brain in stereotaxic coordinates, 6th edn. Elsevier, Amsterdam

Pereira EA, Muthusamy KA, De PN, Joint CA, Aziz TZ (2008) Deep brain stimulation of the pedunculopontine nucleus in Parkinson's disease. Preliminary experience at Oxford. Br J Neurosurg 22:S41–S44

Plaha P, Gill SS (2005) Bilateral deep brain stimulation of the pedunculopontine nucleus for Parkinson's disease. Neuroreport 16:1883–1887

Rada PV, Mark GP, Yeomans JS, Hoebel BG (2000) Acetylcholine release in ventral tegmental area by hypothalamic self-stimulation, eating and drinking. Pharmacol Biochem Behav 55:131–141

Reever CM, Ferrari-DiLeo G, Flynn DD (1997) The M_5 (m_5) receptor subtype: fact or fiction? Life Sci 60:1105–1115

Sahibzada N, Dean P, Redgrave P (1986) Movements resembling orientation or avoidance elicited by electrical stimulation of the superior colliculus in rats. J Neurosci 6:723–733

Sakurai T, Nagata R, Yamanaka A, Kawamura H, Tsujino N, Muraki Y, Kageyama H, Kunita S, Takahashi S, Goto K, Koyama Y, Shioda S, Yanagisawa M (2005) Input of orexin/hypocretin neurons revealed by a genetically encoded tracer in mice. Neuron 46:297–308

Scarnati E, Proia E, Campana E, Pacitti C (1986) A microiontophoretic study on the nature of the putative neurotransmitter involved in pedunculopontine-substantia nigra pars compacta excitatory pathway in the rat. Exp Brain Res 62:470–478

Semba K (1993) Aminergic and cholinergic afferents to REM sleep induction regions of the pontine reticular formation in the rat. J Comp Neurol 330:543–556

Semba K, Fibiger HC (1992) Afferent connections of the laterodorsal and the pedunculopontine tegmental nuclei in the rat: a retro-and antero-grade transport and immunohistochemical study. J Comp Neurol 323:387–410

Sesack SR, Grace AA (2010) Cortico-basal ganglia reward network: microcircuitry. Neuropsychopharmacology 35:27–47

Sharf R, Ranaldi R (2006) Blockade of muscarinic acetylcholine receptors in the ventral tegmental area disrupts food-related learning in rats. Psychopharmacology 184:87–94

Sharf R, Lee DY, Ranaldi R (2005) Microinjections of SCH 23390 in the ventral tegmental area reduce operant responding under a progressive ratio schedule of food reinforcement in rats. Brain Res 1033:179–185

Sooksawate T, Isa T (2006) Properties of cholinergic responses in neurons in the inter-mediate gray layer of the rat superior colliculus. Eur J Neurosci 24:3096–3108

Steidl S, Yeomans JS (2009) M_5 muscarinic receptor knockout mice show reduced morphine-induced locomotion but increased locomotion after cholinergic antagonism in the ventral tegmental area. J Pharmacol Exp Ther 328:263–275

Steininger T, Rye D, Wainer BH (1992) Afferent projections to the cholinergic pedunculopontine tegmental nucleus and adjacent midbrain extrapyramidal area in the albino rat: retrograde tracing studies. J Comp Neurol 22:515–543

Steriade M (1993) Cholinergic blockage of network- and intrinsically generated slow oscillations promotes waking and REM sleep activity patterns in thalamic and cortical neurons. Prog Brain Res 98:345–355

Steriade M, Contreras D (1995) Relations between cortical and thalamic cellular events during transition from sleep patterns to paroxysmal activity. J Neurosci 15:623–642

Steriade M, McCarley RW (2005) Brain control of waking and sleep, 2nd edn. Klüver-Plenum, New York, NY

Swerdlow NR, Geyer MA (1993) Prepulse inhibition of acoustic startle in rats after lesions of the pedunculopontine tegmental nucleus. Behav Neurosci 107:104–117

Takahashi K, Koyama Y, Kayama Y, Yamamoto M (2002) Effects of orexin on the laterodorsal tegmental neurons. Psychiatry Clin Neurosci 56:335–336

Takakusaki K, Shiroyama T, Yamamoto T, Kitai ST (1996) Cholinergic and non-cholinergic tegmental pedunculopontine projection neurons in rats revealed by intracellular labeling. J Comp Neurol 371:345–361

Takakusaki K, Saitoh K, Harada H, Okumura T, Sakamoto T (2004) Evidence for a role of basal ganglia in the regulation of rapid eye movement sleep by electrical and chemical stimulation for the pedunculopontine tegmental nucleus and the substantia nigra pars reticulata in decerebrate cats. Neuroscience 124:207–220

Takeuchi J, Fulton J, Jia ZP, Abramov-Newerly W, Jamot L, Sud M, Coward D, Ralph M, Roder J, Yeomans J (2002) Increased drinking in mutant mice with truncated M_5 muscarinic receptor genes. Pharmacol Biochem Behav 72:117–123

Tehovnik EJ, Yeomans JS (1986) Two converging brainstem pathways mediating circling behavior. Brain Res 385:329–342

Thannickal TC, Nienhuis R, Siegel JM (2001) Localized loss of hypocretin (orexin) cells in narcolepsy without cataplexy. Sleep 32:993–998

Tzavara ET, Bymaster FP, Davis RJ, Wade MR, Perry KW, Wess J, McKinzie DL, Felder C, Nomikos GG (2004) M_4 muscarinic receptors regulate the dynamics of cholinergic and dopaminergic neurotransmission: relevance to the pathophysiology and treatment of related CNS pathologies. FASEB J 12:1410–1412

Vilaro MT, Palacios JM, Mengod G (1990) Localization of m_5 muscarinic receptor mRNA in rat brain examined by in situ hybridization histochemistry. Neurosci Lett 43:31–36

Vilaro MT, Palacios JM, Mengod G (1994) Multiplicity of muscarinic autoreceptor subtypes? Comparison of the distribution of cholinergic cells and cells containing mRNA for five subtypes of muscarinic receptors in the rat brain. Brain Res Mol Brain Res 21:30–46

Wang H, Liang S, Burgdorf J, Wess J, Yeomans J (2008) Ultrasonic vocalizations induced by sex and amphetamine in M_2, M_4, M_5 muscarinic and D2 dopamine receptor knockout mice. PLoS One 3:1–12

Weiner DM, Levey AI, Brann MR (1990) Expression of muscarinic acetylcholine and dopamine receptor mRNAs in rat basal ganglia. Proc Natl Acad Sci USA 87:7050–7054

Wess J, Duttaroy A, Zhang W, Gomeza J, Cui Y, Miyakawa T, Bymaster FP, McKinzie DL, Felder CC, Lamping KG, Faraci FM, Deng C, Yamada M (2003) M_1-M_5 muscarinic receptor knockout mice as novel tools to study the physiological roles of the muscarinic cholinergic system. Receptors Channels 9:279–290

Woolf NJ (1991) Cholinergic systems in the mammalian brain and spinal cord. Prog Neurobiol 37:475–524

Woolf NJ, Butcher LL (1986) Cholinergic systems in the rat brain III. Projections from the pontomesencephalic tegmentum to the thalamus, tectum, basal ganglia, and basal forebrain. Brain Res Bull 16:603–637

Yamanaka A, Beuckmann CT, Willie JT, Hara J, Tsujino N, Mieda M, Tominaga M, Yagami K, Sugiyama F, Goto K, Yanagisawa M, Sakurai T (2003) Hypothalamic orexin neurons regulate arousal according to energy balance in mice. Neuron 38:701–713

Yang S, Liu A, Weidenhammer A, Cooksey RC, McClain D, Kim MK et al (2009) The role of mPer2 clock gene in glucocorticoid and feeding rhythms. Endocrinology 150:2153–2160

Yasuda RP, Ciesla W, Flores LR, Wall SJ, Li M, Satkus SA, Weisstein JS, Spangnola BV, Wolfe BB (1993) Development of antisera for m_4 and m_5 muscarinic cholinergic receptors: distribution of m_4 and m_5 receptors in rat brain. Mol Pharmacol 43:149–157

Yeomans JS, Kofman O, McFarlane V (1985) Cholinergic involvement in lateral hypothalamic rewarding brain stimulation. Brain Res 329:19–26

Yeomans JS, Mathur A, Tampakeras M (1993) Rewarding brain stimulation: role of tegmental cholinergic neurons that activate dopamine neurons. Behav Neurosci 107:1077–1087

Yeomans JS, Forster G, Blaha C (2001) M_5 muscarinic receptors are needed for slow activation of dopamine neurons and for rewarding brain stimulation. Life Sci 68:2449–2456

Yeomans JS, Lee J, Yeomans MH, Steidl S, Li L (2006) Midbrain pathways for prepulse inhibition and startle activation in rat. Neuroscience 142:921–929

Yeomans JS, Bosch D, Alves N, Daros A, Ure RJ, Schmid S (2010) GABA receptors and prepulse inhibition of acoustic startle in mice and rats. Eur J Neurosci 31:2053–2061

Zweig RM, Jankel WR, Hedreen JC, Mayeux R, Price DL (1989) The pedunculopontine nucleus in Parkinson's disease. Ann Neurol 26:41–46

Part III
Muscarinic Receptors in Autonomic Effector Organs

Muscarinic Receptor Agonists and Antagonists: Effects on Ocular Function

Frederick Mitchelson

Abstract Muscarinic agonists act mainly via muscarinic M_3 cholinoceptors to cause contraction of the iris sphincter, ciliary muscle and trabecular meshwork as well as increase outflow facility of aqueous humour. In the iris dilator, the effect of muscarinic agonists is species dependent but is predominantly relaxation via muscarinic M_3 receptors. In the conjunctiva, muscarinic agonists stimulate goblet cell secretion which contributes to the protective tear film. Muscarinic M_2 and M_3 receptors appear mainly involved. In the lens muscarinic agonists act via muscarinic M_1 receptors to produce depolarization and increase $[Ca^{2+}]_i$. All five subtypes of muscarinic receptor are present in the retina. In the developing retina, acetylcholine appears to limit purinergic stimulation of retinal development and decrease cell proliferation. In the adult retina acetylcholine and other muscarinic agonists may have complex effects, for example, enhancing light-evoked neuronal firing in transient ON retinal ganglion cells and inhibiting firing in OFF retinal ganglion cells. In the lacrimal gland, muscarinic agonists activate M_3 receptors on secretory globular acinar cells to stimulate tear secretion and also cause contraction of myoepithelial cells. In Sjögren's syndrome, antibodies to the muscarinic M_3 receptor disrupt normal gland function leading to xerophthalmia although the mechanism of action of the antibody is still not clear. Atropine and pirenzepine are useful in limiting the development of myopia in children probably by an action on muscarinic receptors in the sclera, although many other muscarinic receptor antagonists are not effective.

Keywords Muscarinic agonist • Muscarinic antagonist • Muscarinic cholinoceptor • Ocular function • Sjögren's syndrome • Myopia

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1 Introduction

Drugs affecting muscarinic receptors, both agonists and antagonists, have long been used in the eye to aid in the diagnosis of neurological abnormalities affecting pupil diameter such as the Holmes–Adie syndrome. Agonists, such as pilocarpine and aceclidine, lower intraocular pressure in the treatment of glaucoma and antagonists, such as homatropine and tropicamide, are used as mydriatics and cycloplegics to facilitate ophthalmic examination. All of these uses involve the activation or inhibition of muscarinic receptors associated with cholinergic parasympathetic innervation of structures within the eye.

Additionally, there is growing evidence that acetylcholine (ACh) may have a non-neuronal location in some tissues such as epithelia and immune cells and be involved in regulation of cellular function (Kawashima and Fujii 2008; Wessler et al. 2003) and this may also apply to structures within the eye (Duncan and Collison 2003).

Epithelial cells of the cornea synthesize and store ACh (Mindel and Mittag 1976; Williams and Cooper 1965) and there are muscarinic receptors on the isolated nuclei of rabbit corneal endothelial and epithelial cells (Lind and Cavanagh 1993, 1995) despite the cornea having only a sensory innervation.

The lens is a non-innervated structure without a blood supply, receiving nutrients from the surrounding aqueous and vitreous humours. Muscarinic receptors are present on the epithelial cells of the human lens throughout life (Thomas et al. 1997) and the lens surface contains acetylcholinesterase (Michon and Kinoshita 1968).

In this review, the effects of muscarinic agonists and antagonists and their receptors in various regions of the eye and the associated lacrimal gland are discussed along with their role in some ophthalmic disorders, including myopia and the ophthalmic aspects of Sjögren's syndrome (SS).

2 Iris Sphincter

Muscarinic M_3 receptors appear primarily responsible for the contraction of the iris sphincter muscle induced by muscarinic agonists although other subtypes may be present in the tissue. Mice lacking the M_3 receptor gene showed a more dilated pupil than either wild-type mice or heterozygotes bred from crossing mutant mice lacking the M_3 receptor gene with wild-type mice and pilocarpine had little effect on pupil size in the homozygotes or heterozygotes (Matsui et al. 2000). However, full mydriasis in all the mice was only observed after installation of atropine 1% into the eye, suggesting that other muscarinic receptor subtypes were involved in pupillary constriction as well as the M_3 subtype. Subsequent studies showed $M_2-/-$ mice had pupils of normal size, but paradoxically, $M_2-/-$ $M_3-/-$ mice had smaller pupils than the $M_3-/-$ mice, suggesting that M_2 receptors activated

mydriasis rather than miosis (Matsui et al. 2002). Recently, in the iris/ciliary body of the tree shrew, *Tupaia belangeri*, mRNAs for the five muscarinic receptor subtypes were detected (McBrien et al. 2009).

In human iris, using quantitative reverse transcription-polymerase chain reaction (RT-PCR), Collison et al. (2000) found that mRNA for the M_3 receptor subtype comprised 77.5% of the total. Values for the other subtypes were M_1, 11.8%; M_4, 10.4%; M_5, 0.4%; M_2, undetected. Similarly, antibody immunoprecipitation (Gil et al. 1997) or immunofluorescent antibody studies on human iris sphincter (Ishizaka et al. 1998) showed the highest immunoreactivity for the M_3 receptor subtype-specific antibody (74%) with 5–12% levels for the other four subtypes. Studies with several subtype-preferring antagonists in cultured human iris sphincter cells (WoldeMussie et al. 1993) or human iris sphincter muscle (Ishikawa et al. 1998) indicated that the functional receptor was the M_3 subtype.

In rat iris, only mRNAs encoding the $M_{2–4}$ subtypes were detected using RT-PCR for the five subtypes (Furuta et al. 1998). Interestingly, while the amino acid sequence for the M_2 and M_4 receptors was identical with those in rat brain, the iris M_3 subtype showed minor differences from the brain M_3 subtype, differing by four amino acids at position 165 and 184 at the edge of the second intracellular loop and at positions 337 and 406 in the centre of the i3 loop. Mutations in the latter region of the M_1 receptor have been shown to affect receptor down-regulation and sequestration without affecting G protein coupling (Lameh et al. 1992).

In vivo studies in the anaesthetized rat using pirenzepine, telenzepine, AF-DX 116, 4-DAMP and hexahydrosiladiphenidol indicated that the muscarinic M_3 subtype was involved in mediating miosis (Hagan et al. 1988) [NB: the authors termed the receptor "M_2 (ileal)" as the term "M_3 receptor" was not in common usage at that time]. The potency of topical pirenzepine and telenzepine on the iris was increased up to 60-fold at low pH. In the case of pirenzepine, this was attributed to protonation of the second amine group on the piperazine ring, which facilitated corneal penetration (Hagan et al. 1988).

Early investigations into the nature of the muscarinic receptor mediating contraction of the rabbit iris, inositol trisphosphate (IP_3) accumulation and myosin light chain phosphorylation, using subtype-preferring antagonists, suggested that it was similar to the M_3 subtype (termed "M_2" or "$M_{2\beta}$" in early studies) (Akhtar et al. 1987; Honkanen and Abdel-Latif 1988), although the affinity of some antagonists such as pirenzepine (Bognar et al. 1989) and hexahydrodiphenidol enantiomers (Fuder et al. 1989) did not fully agree with that suggestion.

Bognar et al. (1992) found evidence that the muscarinic receptor mediating contraction of the isolated rabbit iris sphincter had an affinity profile for several subtype-preferring muscarinic receptor antagonists that was different from that for $M_{1–4}$ subtypes. Choppin et al. (1998) were unable to confirm these findings and concluded that the pharmacologically defined M_3 subtype was involved, although they pointed out the involvement of M_5 receptors remained a possibility, due to the lack of a suitable selective antagonist to discriminate between M_3 and M_5 subtypes.

In the dog iris sphincter, low concentrations of carbachol (CCh), <5 μM, produced contraction, increased IP_3 production and inhibited cAMP formation but at

higher concentrations (>5 μM) CCh, after an initial transient contraction, produced relaxation, inhibited IP_3 production and increased cAMP production (Abdel-Latif et al. 1992). This concentration-dependent effect is unusual as in other species, such as the cat, ox and rabbit iris, CCh produces contraction and an increase in IP_3 over the whole concentration range (1–100 μM) (Abdel-Latif et al. 1992). Influx of Ca^{2+} through Ca^{2+} channels as well as intracellular Ca^{2+} appeared to be involved, as nifedipine and the intracellular Ca^{2+} chelator, BAPTA, inhibited CCh-induced cAMP production in the dog iris (Abdel-Latif et al. 1992; Tachado et al. 1994).

Pharmacological evaluation of the receptor subtype involved in the CCh-induced contraction in the dog iris suggested that the M_3 muscarinic cholinoceptor was involved as 4-DAMP (pA_2; 9.0) was a more potent antagonist than pirenzepine (pA_2; 7.1) and AF-DX 116 was ineffective (Tachado et al. 1994). 4-DAMP was also more potent than pirenzepine at inhibiting the CCh-induced increase in IP_3 production and increased cAMP formation suggesting that the same muscarinic receptor subtype was involved in production of both second messengers in the tissue.

More recently, zamifenacin, a potent muscarinic M_3 receptor antagonist, was reported to have lower affinity (pA_2; <6.0) for the muscarinic receptor mediating contraction in the canine iris than for canine ileal M_3 receptor (pA_2; 8.6) suggesting that the M_3 receptor subtype in the iris is atypical (see Eglen et al. 1996).

Muscarinic receptors are also present on sympathetic nerve endings in the human iris–ciliary body or whole iris of rabbit, guinea pig and rat. When activated by muscarinic agonists, electrically stimulated release of ^{3}H-noradrenaline was inhibited (Bognar et al. 1988, 1989; Fuder et al. 1989; Jumblatt and Hackmiller 1994). Studies with various "subtype-preferring" antagonists have shown that this prejunctional inhibitory muscarinic receptor is the M_2 subtype (also termed "$M_{2\alpha}$" in early studies).

3 Iris Dilator

While the main parasympathetic innervation of the iris is directed to the circular sphincter muscle surrounding the pupil there is also parasympathetic innervation of the iris radial dilator muscle.

Narita and Watanabe (1982) found the rat isolated iris dilator relaxed to ACh at low concentrations (1 nM–1 μM) but contracted at higher concentrations (>1 μM). Both responses were enhanced by eserine and antagonized by atropine. Electrical stimulation produced a relaxation at frequencies ≤10 Hz and contraction at 30 Hz, both responses being abolished by tetrodotoxin. Also, the relaxation was abolished by atropine and the contraction was abolished by the adrenoceptor antagonist, phentolamine.

Methacholine and CCh also produced relaxation of the rat iris dilator muscle, but contracted the muscle in higher concentrations, ≤1 μM (Shiraishi and Takayanagi 1993; Masuda et al. 1995). However, arecoline and bethanecol produced mainly relaxation with only a small contraction (<35% ACh maximum) and McN-A-343 or

pilocarpine produced only relaxation (Masuda et al. 1995). Atropine inhibited both responses indicating their muscarinic nature. Ciliary ganglionectomy or pertussis toxin treatment selectively abolished the relaxant response to ACh (Hasegawa et al. 1988; Masuda et al. 1995; Yamahara et al. 1995). Pilocarpine and McN-A-343 produced a small contraction (<30% of ACh maximum) after denervation.

Studies with pirenzepine, AF-DX 116, himbacine and 4-DAMP suggested that the M_3 subtype was involved in both relaxant and contractile responses to muscarinic agonists (Shiraishi and Takayanagi 1993; Masuda et al. 1995; Yamahara et al. 1995). While the investigators at that time did not consider the M_5 receptor as a possibility, a comparison of the pK_B values obtained with representative literature values for the M_1, M_3 and M_5 subtypes with the above antagonists by the current author showed that the best correlations were still obtained for the M_3 subtype.

While the four "subtype-preferring" muscarinic receptor antagonists above produced similar pA_2 values against both responses, methoctramine was an exception in that it inhibited the relaxation (pA_2; 6.4) but was relatively ineffective against the contraction in pertussis toxin-treated preparations (threefold shift at 10 μM) (Yamahara et al. 1995) and completely ineffective after ganglionectomy (Masuda et al. 1995).

It was concluded that the same muscarinic receptor subtype could be responsible for both relaxation and contraction, utilizing different G proteins for each response (Masuda et al. 1995; Yamahara et al. 1995). Furthermore, relaxation required a pertussis toxin-sensitive G protein, the integrity of which required an intact parasympathetic innervation.

In the human (Yoshitomi et al. 1985), canine (Yoshitomi and Ito 1986) and bovine dilator muscle (Suzuki et al. 1983), CCh or ACh produces relaxation over the whole concentration range investigated and the relaxation was blocked by atropine. The relaxant response to CCh in the dog iris dilator did not involve an effect on the resting membrane potential of the muscle (Yoshitomi and Ito 1986) indicating pharmacomechanical, rather than electromechanical, coupling. Relative immunoreactivity of the five muscarinic receptor subtypes in the human iris dilator muscle was lower than in the sphincter muscle, with only M_1 and M_5 immunoreactivity observed at low intensity and inconsistently, with little or no staining for $M_{3/4}$ receptors, and none for M_2 receptors (Ishizaka et al. 1998).

Nerve stimulation of the dilator iris in these three species also provided evidence of an inhibitory cholinergic innervation mediated through muscarinic receptors. In human (Yoshitomi et al. 1985) or canine isolated iris dilator muscle (Yoshitomi and Ito 1986), electrical stimulation produced an initial phasic contraction followed by a prolonged relaxation. Tetrodotoxin abolished both phases of the response while atropine selectively abolished the relaxation. Guanethidine or phentolamine inhibited the contraction.

In bovine iris dilator, electrical stimulation gave rise only to a pronounced relaxation that was enhanced by physostigmine and abolished by tetrodotoxin or atropine (Suzuki et al. 1983). The relaxations produced by ACh or CCh were not affected by adrenoceptor antagonists indicating that their action was not dependent on sympathetic tone.

In the rabbit iris dilator muscle, pilocarpine inhibited noradrenaline release induced by nerve stimulation as well as the resulting contractile response. It was suggested that this action of pilocarpine was responsible for the miotic effect of the partial agonist, as in the sphincter muscle it produces only a small contraction per se and inhibited the contraction produced by CCh (Bognar et al. 1988; Takayanagi et al. 1993).

4 Ciliary Muscle

Studies on cultured human ciliary smooth muscle cells by several groups using variously: pirenzepine, 4-DAMP, pFHHSiD, HHSiD, methoctramine and AF-DX 116 indicated that the functional muscarinic receptor was the M_3 subtype for contraction, phospholipase C activity and intracellular Ca^{2+} accumulation (Matsumoto et al. 1994; Pang et al. 1994; WoldeMussie et al. 1993). Relative receptor protein concentrations, using antibody immunoprecipitation, also showed 73.5% of M_3 receptor; other receptor protein concentrations were M_1 0.8%, M_2 5.4%, M_4 4.9% and M_5 2.4% (Gil et al. 1997).

There are both circular and longitudinal muscles present in the ciliary muscle, the circular ciliary muscle being responsible for changes in accommodation and the longitudinal muscle alters outflow facility. While there are differences between the circular and longitudinal muscles in the levels of mRNA expression for the individual muscarinic receptor subtypes, Zhang et al. (1995a) suggested this could not explain the dissociation observed between accommodation and outflow reported with some muscarinic agonists.

Ishikawa et al. (1998) determined pK_B values for pirenzepine, methoctramine, pFHHSiD and tropicamide in both human isolated circular and longitudinal ciliary muscle and found similar values in the two tissues, concluding that M_3 (or M_4) receptors were not excluded in the contractile response of both muscles. Their data appears more compatible with literature values for the M_3 receptor than for any other of the five receptor subtypes. Similarly, the M_3 subtype appears to mediate contraction in both circular and longitudinal ciliary muscles in the rhesus monkey (Poyer et al. 1994) and the contraction of bovine ciliary muscle induced by transmural electrical stimulation of cholinergic nerves (Masuda et al. 1998).

5 Ciliary Process/Body

CCh increased phosphatidylinositol turnover in the rabbit ciliary process with oxotremorine and pilocarpine acting as partial agonists (Mallorga et al. 1989). All five muscarinic receptor subtypes are present in the tissue, but using antibody immunoprecipitation, the major receptor protein was the M_3 receptor (57.6%) with $<10\%$ for each of the other four subtypes (Gil et al. 1997). Similarly, all

five muscarinic receptor subtypes were expressed in the ciliary body of the tree shrew (McBrien et al. 2009). In the bovine ciliary process, mRNA encoding for the muscarinic M_3 receptor predominated with minor amounts for the M_2 and M_4 receptors; mRNA for the M_1 receptor was not detected (Honkanen et al. 1990). Chick muscarinic receptor subtypes, $M_{2–4}$ were detected in the ciliary body by Fischer et al. (1998a) although only nicotinic receptors are involved in accommodation in the chick (McBrien et al. 1993).

6 Trabecular Meshwork

The trabecular meshwork is now considered to be actively involved in the passage of aqueous humour rather than being a passive structure for outflow (Wiederholt et al. 2000). The tissue contains contractile α-isoactin filaments and various compounds have been demonstrated to contract or relax the isolated tissue. Muscarinic agonists, aceclidine, ACh, CCh and pilocarpine, were found to produce atropine-sensitive contractions of the trabecular meshwork, with pilocarpine producing only 60% of the maximal response to CCh (Lepple-Weinhues et al. 1991; Wiederholt et al. 2000).

Studies have also demonstrated outflow regulation by muscarinic agonists in perfused anterior segments of bovine eye containing the trabecular meshwork but devoid of ciliary muscle (Wiederholt et al. 1995). CCh impeded outflow through the mesh in similar concentrations to that causing contraction of the isolated trabecular meshwork and pilocarpine was a partial agonist, producing ~50% of CCh maximum (Wiederholt et al. 1995).

Muscarinic agonists, aceclidine, CCh, oxotremorine-M and pilocarpine induced an increase in intracellular Ca^{2+} and CCh also increased phosphoinositide production in cultured trabecular meshwork cells (Shade et al. 1996). Both effects of CCh were inhibited by atropine, pirenzepine, 4-DAMP and pFHHSiD; the inhibition profile of the antagonists suggested involvement of muscarinic M_3 receptors for both effects, although it should be noted that no M_2 subtype-preferring antagonist was included in the study.

ACh and CCh have also been shown to activate L-type Ca^{2+} channels in the meshwork (Steinhausen et al. 2000) but this appears to be less important for contraction than in ciliary muscle as the trabecular meshwork can still contract in the absence of extracellular Ca^{2+} (Wiederholt et al. 2000). Contraction appears to involve protein kinase C (PKC). The PKC antagonists H7 and chelerythrine partly reduced the contraction produced by CCh in the trabecular meshwork, while not affecting the action of CCh in the ciliary muscle (Wiederholt et al. 2000). It is of interest that the trabecular meshwork contains PKCε which is a Ca^{++}-independent protein kinase (Thieme et al. 1999). H7 has also been shown to increase outflow facility in the trabecular meshwork by a mechanism independent of the ciliary muscle (Tian et al. 1999).

Rho-associated coiled coil-forming protein kinase (ROCK) may be a regulator of actomyosin-based contractility in the trabecular meshwork. The ROCK inhibitor, YM-39983, lowered intraocular pressure and caused a dose-dependent inhibition of CCh-induced contraction of monkey trabecular meshwork (Nakajima et al. 2005). YM-39983 was also found to be much less effective at relaxing ciliary muscle, raising the possibility of selectively inhibiting the contractile action of cholinomimetics in the trabecular meshwork.

While contraction appears to involve M_3 receptors primarily, there is a small population of M_1 receptors in the tissue (Wiederholt et al. 2000) and there may also be a contribution to contraction from M_2 receptors. Immunoblotting in both human and bovine trabecular meshwork showed the presence of M_2 muscarinic receptors and in the bovine trabecular meshwork, methoctramine (0.1–5 μM) induced a relaxation of a CCh-induced maximal contraction, even in the presence of M_1 and M_3 receptor antagonists (Thieme et al. 2001).

Thus in summary, the contraction of the ciliary muscle by muscarinic agonists to cause opening of the trabecular meshwork with a resulting increase in outflow facility is opposed by the action of the agonists on the meshwork itself and any beneficial effect on intraocular pressure in glaucoma will be the resultant of two opposing actions (Wiederholt et al. 2000).

7 Cornea

The cornea is innervated but the nerves appear to be only sensory, at least in the rabbit (ten Tusscher et al. 1988). Nevertheless, the corneal epithelium contains choline acetylase, cholinesterase and a high content of ACh (Gnädinger et al. 1967, 1973; Mindel and Mittag 1976; Williams and Cooper 1965), which has led to the suggestion that it has a role in the tissue other than as a neurotransmitter (Williams and Cooper 1965).

Furthermore, for a number of species, muscarinic receptors have now been detected in the corneal epithelium and endothelium (Colley and Cavanagh 1982; Lind and Cavanagh 1995; Socci et al. 1996; Walkenbach and Ye 1991; Walkenbach et al. 1993) despite earlier investigators (Olsen and Neufeld 1979; Gupta et al. 1994) reporting a lack of evidence for cholinoceptors in human or rabbit cornea.

For example, Walkenbach and Ye (1991) using cultured human corneal epithelial cells found specific ^{3}H-QNB binding displaced by atropine, CCh and high concentrations of nicotine. However, binding was decreased 90% if the cultured cells were homogenized and fractionated prior to assay.

Using ^{3}H-propylbenzilylcholine mustard, which forms a covalent bond with muscarinc receptors, Lind and Cavanagh (1995) found evidence of muscarinic receptors in epithelium and endothelium layers of fresh-frozen rabbit cornea as well as in cultured rabbit epithelial and endothelial corneal cells and their nuclei. The finding of muscarinic binding sites on the cell nuclei supported a more general regulatory role for ACh in the cells (Lind and Cavanagh 1995). It was hypothesized

that muscarinic receptors on cell nuclei can regulate cyclic nucleotide-dependent protein kinases and stimulate mitotic activity of the corneal epithelium (Cavanagh and Colley 1989; Colley and Law 1987a).

In rabbit cultured corneal epithelial cell homogenates and subcellular fractions, CCh has been shown to increase guanylyl cyclase activity, cGMP levels, cGMP binding, cGMP-dependent protein kinase activity and to decrease cGMP-phosphodiesterase activity in the nuclear and some other fractions (Cavanagh and Colley 1982; Colley and Cavanagh 1982; Colley and Law 1987a; Colley et al. 1985, 1987a, b; Walkenbach and Ye 1991). These effects were inhibited by atropine. The proportion of nuclear to total cGMP-dependent protein kinase activity also increased, suggesting that nuclear translocation of the enzyme may be enhanced by CCh (Colley and Law 1987b). In these studies, cAMP binding and cAMP-dependent kinase activity was decreased and cAMP-phosphodiesterase activity was increased in the nuclear fraction also, without affecting adenyl cyclase activity.

CCh also increased RNA and DNA polymerase activity in the purified nuclear fraction of rabbit corneal epithelial cell cultures by an atropine-dependent mechanism (Colley et al. 1985). In corneal resurfacing studies on rabbits in vivo, following acid burn defects, CCh was shown to elevate V_{max} of both RNA and DNA polymerases and to specifically increase the apparent affinity of RNA polymerase II for UTP and the apparent affinity of the DNA polymerases α and β for dTTP (Colley et al. 1987b).

The subtypes of muscarinic cholinoceptor found in the cornea are subject to considerable speculation due to different findings obtained by various groups. Shepard and Rae (1998) found evidence only for the muscarinic M_4 receptor in rabbit cornea. Freshly isolated mRNA from rabbit corneal epithelium and endothelium was used to construct cDNA libraries, determined by PCR with gene-specific primers for the five muscarinic receptor subtypes. In contrast, CCh was found to increase the accumulation of ^{3}H-IP$_3$ in rabbit corneal epithelial cells (SV40-adenovirus transformed), an effect inhibited by atropine and 4-DAMP, but less effectively by pirenzepine, suggesting muscarinic M_3 rather than M_1 receptor involvement (Zhang et al. 1995b).

Muscarinic cholinoceptors in bovine corneal epithelial cells were characterized by their ligand specificity, cell signalling pathways and gene transcripts (Socci et al. 1996). Pirenzepine and AF-DX 116 were more effective in displacing ^{3}H-NMS binding than 4-DAMP, suggesting the presence of muscarinic M_1 and/or M_4 receptor and M_2 receptor subtypes. CCh increased intracellular Ca^{2+} levels and this response was inhibited by pirenzepine or atropine. CCh also inhibited cAMP accumulation induced by isoprenaline, an effect inhibited by AF-DX 116 or pertussis toxin. However, RT-PCR revealed only the presence of the transcript for the M_2 receptor, but not that for M_1, M_3 or M_4 receptors.

Recently, all five subtypes of muscarinic cholinoceptor were found in human corneal cells in the epithelium, endothelium and the cytoplasm using anti-muscarinic receptor subtype specific antibodies (Liu et al. 2007). Immunofluorescence for the M_4 subtype was more intense on the corneal epithelium while the other four subtypes were evenly distributed between the cell membranes and cytoplasm.

8 Limbus

The limbus is a narrow specialized ring of innervated tissue surrounding the cornea, at the inner edge of the conjunctiva. The limbic epithelial cells of the rabbit are similar to those in the cornea and are a different lineage to the epithelial cells in the conjunctiva (Wei et al. 1996). Corneal epithelial stem cells are located preferentially in the limbus and are now used for limbal stem cell transplantation surgery to restore severely damaged corneal epithelium (Sun and Lavker 2004). The limbus contains parasympathetic nerves as well as sympathetic and sensory nerves.

All five subtypes of muscarinic cholinoceptor were found in cultured human limbal epithelium using RT-PCR (Liu et al. 2007). While M_2, M_3 and M_4 muscarinic receptor subtype transcript levels varied by less than sixfold from the M_1 transcript level, there was an 11-fold higher level of the M_5 transcript.

9 Conjunctiva

The conjuctiva plays an important role in contributing to the protective tear film on the surface of the eye. Parasympathetic nerves, innervating goblet cells in the conjunctival epithelium, increase mucin and other protein secretion into the tear film (Dartt 2002). Stratified squamous cells which contribute fluid to the tear film are stimulated by sympathetic nerves, but appear to be unaffected by parasympathetic nerves.

Nevertheless, muscarinic M_1 receptors were detected, using antibody immunofluorescence, in the stratified squamous cells from day 17 and in the adult animal (Ríos et al. 1999, 2000). M_2 receptors were detected in the stratified squamous cells from day 9 onwards and in goblet cells from day 17 onwards. M_3 receptors were present in the stratified squamous cells on day 9 and 13 but then subsequently declined in number as their presence in goblet cells became established (Ríos et al. 1999, 2000).

While only muscarinic M_3 receptor immunofluorescence was detected in rat cultured goblet cells (Shatos et al. 2001), $M_{1–3}$ receptors were found on human, mouse and rat conjunctival goblet cells by Diebold et al. (2001) using immunofluorescence microscopy on conjunctival tissue cryosections. In the mouse, muscarinic receptors on goblet cells were located above the nuclei and sub-adjacent to the secretory granules. In human stratified squamous epithelium, the three receptor subtypes were detected occasionally, along with intense fluorescence for M_2 and M_3 receptors in the basement epithelium (Diebold et al. 2001). In mouse and rat stratified squamous epithelium M_1 and M_2 receptors were detected, but more uniformly than in the human, while the bulk of the M_3 receptors were on goblet cells.

Motterle et al. (2006) detected M_1 and M_2 receptors over the full thickness of the conjunctival epithelium in biopsy samples obtained from patients during cataract

surgery whereas M_3 receptor immunoreactivity was restricted to the epithelial basal layer. In another study, immunoreactivity to muscarinic $M_{1–3}$ receptors was detected in all conjunctival epithelial cells (Enríquez de Salamanca et al. 2005).

Also in the IOBA-NHC cell line, derived from normal human conjunctiva, muscarinic M_2 and M_3 receptors were detected on cell membranes and in the cytosol (Enríquez de Salamanca et al. 2005). The M_1 receptor was detected only in the cytosol.

More recently, all five subtypes of muscarinic cholinoceptor were found in cultured human conjunctival epithelium and fibroblast cells, using RT-PCR (Liu et al. 2007). The abundance of each of the five subtypes in the two types of conjunctival cells varied. While the level of M_1 transcript was similar in the fibroblasts and epithelium, there were higher levels of $M_{2–5}$ transcripts in the fibroblasts. The epithelial cells had a 24-fold higher level of the M_5 transcript, relative to the M_1 transcript. Other transcripts varied from the M_1 transcript level by less than sixfold. In fibroblasts, the level of M_5 transcript was 44-fold greater, and the M_2 14-fold greater, than the M_1 transcript.

CCh (1–100 μM) caused secretion of glycoconjugate from the goblet cells in the adult animal which was abolished by atropine (10 μM). The subtype-preferring antagonists, 4-DAMP, gallamine and pirenzepine, all at 10 μM, inhibited the response to the agonist (100 μM) by 72, 69 and 54%, respectively (Ríos et al. 1999). It was concluded that parasympathetic nerves acting via M_2 and M_3 receptors could be involved in conjunctival secretions from goblet cells when the eyelids open into adult life (Ríos et al. 1999, 2000). The location of M_2 and M_3 receptors on membranes above the cell nucleus close to the secretory granules of the goblet cells could decrease the latency between receptor activation and apocrine secretion (Dartt 2002).

In rat conjunctiva, goblet cell secretion induced by CCh is dependent on an increase in intracellular Ca^{2+}, presumably due to IP_3 production, release of endoplasmic reticulum Ca^{2+} stores, followed by the influx of extracellular Ca^{2+} and exocytosis of mucin (Dartt et al. 2000). Activation of PKC may also be involved in the action of cholinomimetics but Ca^{2+}/calmodulin-dependent protein kinases do not appear to be important in the secretory process (Dartt et al. 2000; Dartt 2002). CCh also transactivated the EGF receptor through activation of Pyk2 and p60Src in the rat conjunctiva, leading to MAPK activation, which in turn also increased goblet cell mucin secretion (Dartt 2002; Kanno et al. 2003). This effect of CCh was blocked by either pirenzepine, gallamine or 4-DAMP (all at 10 μM) leading to the suggestion that $M_{1–3}$ receptors were all involved (Kanno et al. 2003).

However, it should be appreciated that the subtype preferences of pirenzepine and 4-DAMP, while greater than that of gallamine, are limited. Both pirenzepine and 4-DAMP have lowest affinity at the M_2 receptor, but at 10 μM there will be a receptor occupancy of 90–98% and 99.6–99.9%, respectively, based on a representative range of literature $pK_{B/I}$ values for pirenzepine at the M_2 receptor of 6.0–6.8 and for 4-DAMP, 7.6–8.4.

Activation of MAPK by CCh in cultures of human conjunctival goblet cells, obtained during ocular surgery, appeared similar to that in cultures of rat

conjunctival goblet cells (Horikawa et al. 2003). The ~1.6-fold increase in MAPK induced by CCh (100 μM) had a similar time course in both species; it peaked at 5 min and declined to baseline by ~30 min. The increase in MAPK was also abolished by 4-DAMP (10 μM) or by the EGF receptor antagonist, AG1478, in both species.

CCh also stimulated cell proliferation and p42/44 MAPK activation in the conjunctival epithelial cell line, IOBA-NHC, both effects being inhibited by the MAPK inhibitor, U0126 (Liu et al. 2007). The effect of CCh (150 μM) on cell proliferation was also completely inhibited by atropine (150 μM). This finding was used by Liu et al. (2007) in support of the contention that CCh was acting via muscarinic receptors, but at this high concentration atropine will also inhibit some subtypes of nicotinic receptor (Shirvan et al. 1991; Verbitsky et al. 2000). Furthermore, the three subtype-preferring antagonists, pirenzepine, AF-DX 116 and 4-DAMP all produced only ~42% inhibition of CCh-induced cell proliferation when used at a concentration of 10 μM. A tenfold higher concentration of 4-DAMP (100 μM) produced only ~60% inhibition, although this concentration of the antagonist will occupy >99.9% of all five muscarinic receptor subtypes.

Stratified squamous cells secrete chloride ion leading to fluid secretion, but the basal level of ion secretion is unaffected by ACh (Dartt 2002). Goblet cells can also secrete chloride ion leading to fluid secretion but this property of goblet cells has not been studied (Dartt 2002).

10 Vernal Keratoconjunctivitis

Muscarinic receptor populations in the conjunctiva may be altered in disease states. Vernal keratoconjunctivitis is a rare allergic condition characterized by a non-specific hyper-reactivity to environmental antigens, leading to severe inflammation (Leonardi et al. 2008). In patients with the condition, conjunctival epithelial M_1 receptors were significantly decreased while M_2 and M_3 receptors became more irregularly distributed throughout the epithelium (Motterle et al. 2006). In the conjunctival stroma, M_1, M_2 and M_3 receptors were increased significantly and present throughout the tissue instead of being restricted to a few scattered cells. Motterle et al. (2006) suggested that the decline in epithelial M_1 receptors was consistent with a change to the more viscous secretion of mucus seen in the condition, as M_2 and M_3 receptors located on goblet cells are the prime activators of mucous secretion with the M_1 receptors having a more minor role, perhaps increasing the water content of the secretion. The stromal cells with muscarinic receptors found in vernal keratoconjunctivitis were not positively identified but were suggested to include mast cells and other immune cells. Connective tissue mast cells release histamine when activated by muscarinic agonists; they are present in conjunctival stroma, and markedly increased in vernal keratoconjunctivitis (Irani et al. 1990).

11 Lens

The lens is not innervated but possesses muscarinic receptors (Williams et al. 1993). ACh, CCh and pilocarpine produced depolarization and increased intracellular calcium in the anterior cells of the lens of human and rat (Collison and Duncan 2001; Thomas et al. 1997; Williams et al. 1993). Release of Ca^{2+} from endoplasmic reticulum stores and entry of Ca^{2+} through capacitative Ca^{2+} channels may both occur and Ca^{2+} cycling is important for modulating growth of the lens (Duncan et al. 1996). An increase in $[Ca^{2+}]_i$ has been associated with increased light scatter and localized cortical cataract formation (Duncan et al. 1994; Williams et al. 2001) possibly due to activation of a calcium-dependent protease, such as calpain (Duncan et al. 1994). The lens surface contains high levels of acetylcholinesterase (Michon and Kinoshita 1968) which may offer an explanation for the development of cataracts with the chronic use of anticholinesterases in the treatment of glaucoma (see Duncan and Collison 2003; Thomas et al. 1997). The source of endogenous ACh to act as a substrate for the enzyme is not known, but may possibly arise from cholinergic neurons in the ciliary process or iris (see Williams et al. 1993).

The major subtype of muscarinic receptor mRNA in native human lens epithelium is the M_1 subtype comprising 88.1% of the total with M_2 9.2%; M_3 0.6%; M_4 0.1% and M_5 receptor 6.6% (Collison et al. 2000). Pharmacological studies of the inhibition produced by pirenzepine (pK_B, 8.1) and AF-DX 384 (pK_B, 7.2) on ACh-induced Ca^{2+} mobilization in these cells also suggested that the M_1 subtype was involved.

However, cultures of human lens cells show a different pattern with mRNA for the M_3 receptor subtype predominating. In the human lens epithelial cell line, HLE-B3, the mRNA subtype for the M_3 receptor was 98.8% of the total and in lens primary cultures, 90.6% (Collison et al. 2000). The response to Ca^{++} mobilization by ACh (10 μM) in the HLE-B3 cell lines was delayed in onset and more prolonged than in the native cells, suggestive of a second component in the response. The pK_B values for antagonists were pirenzepine, 6.6; AF-DX 384, 7.4; 4-DAMP, 9.0 and methoctramine, 6.2 which correlated best with tabulated values for the M_3 receptor (Collison et al. 2000).

Shepard and Rae (1998) provide further evidence that lens tissue cultures may have a dissimilar muscarinic receptor profile to native lens tissue. They used freshly isolated mRNA from a single human lens epithelial tissue culture to construct a cDNA library. Only the M_5 muscarinic receptor, out of the five receptor subtypes, was detectable in the library. In the mouse epithelial lens cell line, αTN4, no muscarinic receptor subtype was detected in the cDNA library.

12 Choroid

All five subtypes of muscarinic receptor were detected in the choroid of the tree shrew (McBrien et al. 2009) but only three chick muscarinic receptor subtypes, $M_{2–4}$ were detected in the chick choroid by Fischer et al. (1998a).

13 Sclera

Cell lines of fibroblasts from human sclera expressed mRNA for the five subtypes with greater expression for the M_1 and M_3 receptors, while Western blot analysis detected receptor protein for the five subtypes (Qu et al. 2006). Also, all subtypes were detected in frozen scleral sections by immunohistochemistry. Using quantitative RT-PCR, Collison et al. (2000) found that mRNA for the M_3 receptor subtype in the human sclera comprised 76% of the total. Percentages for the other subtypes were M_1, 23; M_2, 0.0007; M_4, 0.06; M_5, 0.7. Similarly, all five subtypes were detected in the sclera of the tree shrew (McBrien et al. 2009). The muscarinic receptors appeared to be localized to fibroblast processes in both species. The effect of muscarinic receptor antagonists on the sclera is discussed below under *Myopia*.

14 Retina

The only cholinergic cells in the adult retina are starburst amacrine cells but muscarinic receptors are found on amacrine, bipolar, horizontal and ganglion cells in the retina (Fischer et al. 1998a; McBrien et al. 2009; Strang et al. 2010; Townes-Anderson and Vogt 1989; Wong 1995; Yamada et al. 2003).

Using quantitative RT-PCR, Collison et al. (2000) found mRNA for all five muscarinic receptor subtypes in the human retina, and that for the M_3 receptor comprised 86% of the total. Values for the other subtypes were M_1, 2.1%; M_2, 0.07%; M_4, 8.5%; M_5, 3.8%.

McBrien et al. (2009) detected all five muscarinic receptor subtypes in the retina of the tree shrew. M_1 receptors were mainly localized to the outer and inner plexiform layers; M_2 receptors were in the outer segments of photoreceptor layer, plexiform and nerve fibre layers. The M_3 receptors were localized, consistent with Müller cell expression. $M_{4–5}$ receptors had a more diffuse distribution.

ACh plays a role in early retinal development (see Martins and Pearson 2008). Stimulation of muscarinic receptors by ACh, CCh or muscarine in embryonic chick retina was found to release Ca^{2+} from intracellular stores to increase free $[Ca^{2+}]_i$ (Yamashita et al. 1994). The response was maximal at E3 and then declined until E8. ACh (10 μM) was ~tenfold more potent than either of the other agonists, despite the detection of cholinesterase as early as E4 (Yamashita et al. 1994). In vitro ACh was shown to induce curving of the neural retina of E3 chick embryo, at the stage of optic cup formation (Yamashita and Fukuda 1993).

In the chick retina, the subtype of muscarinic receptor varies with the developmental stage; the M_4 subtype is predominant early but later M_2 and M_3 receptors increase (McKinnon and Nathanson 1995; Nadler et al. 1999) whereas in the rabbit neonate, muscarinic M_1 receptors appear transiently to increase $[Ca^{2+}]_i$, in cells of the ventricular zone of the retina, disappearing shortly before the eye opens (Wong 1995). Similarly, ACh, muscarine or McN-A-343 increased $[Ca^{2+}]_i$ in foetal or rat pup retinal neurons, activating M_1 receptors (Wakakura et al. 1998).

Pearson et al. (2002) have proposed that muscarinic receptor activation limits purinergic stimulation of retinal development. In chick embryos at E6, retinal progenitor cells from the ventricular zone, adjacent to the retinal pigment epithelium (RPE), were exposed to CCh. The muscarinic agonist released Ca^{2+} from intracellular stores, frequently causing oscillations in $[Ca^{2+}]_i$ and inhibited mitosis. The effect of CCh was antagonized by pirenzepine (25 μM). Pirenzepine per se also caused an increase in mitosis and increased eye growth, supporting the suggestion that endogenous ACh was modulating retinal development.

dos Santos et al. (2003) found that in vitro exposure of 1–2-day-old rat pup retinal cells to CCh or oxotremorine over 24–48 h decreased cell proliferation. This effect was mediated by muscarinic M_1 receptors activating PKC and was dependent on phosphatidyl inositol 3-kinase and tyrosine kinase activity. Polypeptide release was also involved, possibly BDNF, as the effect of CCh was inhibited by a TrkB receptor antagonist.

In the newborn rabbit retina, after day P3, muscarinic M_1 and M_3 receptor activation was found to propagate spontaneous excitatory waves arising from amacrine starburst cells (Zhou and Zhao 2000). In the foetus, nicotinic receptors were responsible.

Recently, Strang et al. (2010) mapped the location of muscarinic receptors in the rabbit retina and explored the muscarinic effects of choline. All five muscarinic receptor subtypes were detected in subsets of amacrine, bipolar and ganglion cells, revealing a complex organization of muscarinic receptors. For example, cholinergic amacrine cells expressed all five receptor subtypes suggesting multiple feedback mechanisms. Co-localization of muscarinic receptors with the glycine transporter was limited and restricted to M_1, M_2 and M_4 receptors, suggesting muscarinic receptors were localized on GABAergic rather than glycinergic amacrine cells. Sustained OFF, transient OFF, transient ON and ON–OFF retinal ganglion cells were found to have atropine-sensitive responses to choline. Choline enhanced responses to light in transient ON and ON–OFF ganglion cells and inhibited light responses in OFF cells. The findings suggested that choline as well as ACh could contribute to muscarinic modulation of retinal neuronal activity.

Müller cells are a form of glial cell that regulates the extracellular mileau of retinal neurons. Their apical processes extend into the photoreceptor layer and their basal processes end at the inner retina. In Müller cell cultures prepared from 7–10-day-old mice, RT-PCR revealed mRNA only for muscarinic M_1 and M_4 receptors (Da Silva et al. 2008). CCh produced an increase in $[Ca^{2+}]_i$ that was inhibited by atropine or pirenzepine. The sources of the cation were IP_3-sensitive intracellular stores of Ca^{2+} and an influx of extracellular Ca^{2+} through TRP channels. High concentrations of muscarine or McN-A-343 have also been shown to increase $[Ca^{2+}]_i$ in ca 50–70% of rabbit or rat Müller cells, an effect inhibited by pirenzepine or atropine (Wakakura et al. 1998). However, the role of muscarinic receptor activation in the physiological operation of Müller cells is not clear. Hyoscine did not block the transient increase in $[Ca^{2+}]_i$ in the cells induced by exposure of the rat retina to a constant or a flickering light, rather ATP appeared to be the mediator (Newman 2005).

Glycinergic amacrine cells are part of a negative feedback loop for cholinergic amacrine cells in the rat and rabbit retina (Neal and Cunningham 1995). Exposure of the retina to low frequency (3 Hz) flickering light evokes the release of ACh from the cholinergic amacrine cells. These amacrine cells are believed to be innervated by "ON" bipolar cells. The release of ACh can be modulated by activation of inhibitory glycinergic cells to release glycine to act back directly on the cholinergic cell, or alternatively, on another excitatory input to the cholinergic cell. Muscarine was shown to activate muscarinic receptors on the glycinergic cells to potentiate K^+-evoked release of glycine, thereby limiting release of light-evoked release of ACh by 54% (Neal and Cunningham 1995). The glycinergic amacrine cell, containing M_2 muscarinic receptors, is now proposed to be the DAPI-3 cell (Zucker et al. 2005).

CCh can activate muscarinic M_1/M_3 receptors to stimulate nitric oxide synthase (NOS) activity, cGMP production and nNOS mRNA expression in rat retina (Borda et al. 2005). Pirenzepine or 4-DAMP, but not AF-DX 116, blocked the CCh-induced increase in NOS and cGMP, with 4-DAMP producing the greater inhibition. The effect of CCh could also be reduced by blocking phospholipase C (PLC) or Ca^{2+}/calmodulin but not PKC.

In salamander retina, oxotremorine activated M_2 receptors on GABAergic amacrine cells of the inner retina to increase cGMP production via nitric oxide which in turn stimulated GABA release (Cimini et al. 2008). Muscarinic M_4 receptors were also detected in the outer nuclear layer and on bipolar cells, but were not involved in cGMP production.

15 Retinal Pigment Epithelium

In rat RPE [^{3}H]-QNB binding could be detected at postnatal day 5, increasing to a maximum from day 12 to 40 and then declining by ca 50% to adult levels by day 60 (Salceda 1994). Muscarinic receptor activation by CCh in rat RPE led to an increase in IP_3 production and an increased rate of phagocytosis of rod outer segments (Heth et al. 1995) but the latter finding could not be confirmed by Hall et al. (1996).

Naruoka et al. (2003) found ACh or muscarine produced an increase in $[Ca^{2+}]_i$ in explants of RPE from embryonic chick retina (E4–E5). The muscarinic receptors appeared to be the $M_{1/3}$ subtypes rather than the M_2 as muscarine was inhibited by pirenzepine or 4-DAMP, but not by gallamine.

The RPE of the bluegill fish (*Lepomis macrochirus*) expresses only M_5 muscarinic receptors although the retina expresses both muscarinic M_2 and M_5 receptors (Phatarpekar et al. 2005). The M_5 receptor was found to share 65.3% amino acid identity with human M_5 receptor. In the RPE, CCh activated a muscarinic receptor that led to pigment granule dispersion. Dispersion of the granules into the long apical processes of RPE cells interdigitated between rod photoreceptors protects the latter from bleaching in bright light. The effect of CCh was inhibited by a phospholipase C inhibitor or an IP_3 receptor antagonist. Only the muscarinic M_1 and M_3

receptor-preferring antagonists, telenzepine (pIC_{50}, 8.5) and pFHHSiD (pIC_{50}, 7.2), respectively, inhibited the action of CCh, methoctramine (10 μM) was ineffective. No selective M_5 antagonist was available but the order of potency of antagonists that have been tested was 4-DAMP > pirenzepine > telenzepine > pFHHSiD (González et al. 2004; Phatarpekar et al. 2005), supporting the suggestion of an M_5 receptor involvement. However, it should be borne in mind that fish muscarinic receptors may have different affinities to the corresponding mammalian subtypes for subtype-preferring antagonists. For example, the muscarinic M_2 receptor subtype in the zebra fish has been shown to have a high affinity for pirenzepine (pK_i, 7.18) (Hsieh and Liao 2002).

Fischer et al. (1998a) detected $M_{2–4}$ receptors in the chick RPE using specific antibodies. In chick RPE cells, ACh activated muscarinic receptors to raise intracellular Ca^{2+} levels by releasing intracellular stores of Ca^{2+}, an effect abolished by atropine or thapsigargin (Sekiguchi-Tonosaki et al. 2009). These cells have the ability to de-differentiate via intermediates into neuronal or lens cells or back into pigmented epithelial cells and offer the possibility for lens or retinal tissue regeneration. When these cells de-differentiated, ACh was also able to raise intracellular Ca^{2+} by opening L-type Ca^{2+} channels via a nicotinic receptor, as well as retaining the muscarinic response.

16 Lacrimal Gland

Cholinergic nerves passing to the lacrimal gland activate muscarinic receptors to cause lacrimal secretion (Mauduit et al. 1993). The vital role of muscarinic receptor activation can be appreciated by the effects observed on denervation or chronic blockade of muscarinic receptors. Parasympathetic denervation of the rat lacrimal gland leads to severe dry eye, corneal ulceration and dissolution of lacrimal gland structure with the development of pro-inflammatory genes in the gland (Nguyen et al. 2006). The effect occurs without any change in expression of the muscarinic M_3 receptor although it was speculated that loss of muscarinic function was responsible. Cholinergic activation of tear secretion also appears to be necessary to maintain the integrity of the conjunctival surface. Chronic administration of transdermal scopolamine to mice led to a decrease in both tear secretion and the corneal epithelial barrier function of the conjunctiva (Dursun et al. 2002). Exposing the animals to a desiccating environment via a continuous airflow blower exacerbated the condition, leading to a decrease in goblet cell density and an increase in proliferating epithelial cells; changes resembling those occurring in keratoconjunctivitis sicca.

In rat lacrimal gland CCh, oxotremorine, methacholine and pilocarpine stimulated ^{3}H-inositol phosphate production, the latter three being partial agonists for the response. The action of CCh was inhibited competitively by atropine, 4-DAMP, pirenzepine and AF-DX 116 with "functional K_B" values of 0.56, 1.8, 56 and 664 nM, respectively, indicative of M_3 subtype involvement (Mauduit et al.

1993). Also, the antagonists displaced ^{3}H-NMS, from a single site with a similar rank order of potency from lacrimal gland membranes and Northern blot analysis of lacrimal gland mRNA indicated a uniform population of muscarinic M_3 receptors.

There are muscarinic receptors located on secretory globular acinar cells and on stellate myoepithelial cells (Lemullois et al. 1996). The latter cells contract in response to CCh and may play a facilitatory role, assisting in the movement of secretion from the acini into the tear ducts (Satoh et al. 1997).

CCh induced an increase in intracellular Ca^{++} in both cell types but only the response of the myoepithelial cells to CCh was inhibited by lowering extracellular Ca^{++} (Satoh et al. 1997). Activation of secretion by CCh in the acinar cells involved PLCβ activation to generate IP_3, with subsequent release of intracellular Ca^{++} from stores by IP_3 with PKCα/ε and PKCδ activation (see Dartt 2004; Zoukhri et al. 2000). There was also influx of extracellular Ca^{++} through capacitative Ca^{++} entry, stimulated by the depletion of the intracellular Ca^{++} stores (Berridge 1995). Thus, an initial peak in intracellular Ca^{++} levels due to release of Ca^{++} from intracellular stores was followed by a plateau as capacitative influx occurred (Zoukhri et al. 1997a, b, 2000).

Phospholipase D (PLD) in rat lacrimal glands was also activated by CCh (1 mM), independent of both PKC and Ca^{2+}, to increase phosphatidic acid twofold and this effect was prevented by atropine (10 μM) (Zoukhri and Dartt 1995). PLD hydrolyses phosphatidylcholine preferentially to yield phosphatidic acid which may release Ca^{2+} and can be converted by a specific phosphohydrolase to diacylglycerol (DAG), a PKC activator.

In the lacrimal gland acinar cell, CCh does not transactivate the EGF receptor but the agonist activated p42/p44 MAPK by a pathway utilizing Ras, Raf, MEK, c-Src, Pyk2, PKC and Ca^{2+} (Dartt 2004; Hodges et al. 2006; Ota et al. 2003). MAPK, in turn, can inhibit CCh-induced protein secretion (Ota et al. 2003).

Aquaporins (AQPs) are apical plasma membrane water channels found in lacrimal glands, salivary glands and other tissues and facilitate the movement of water across cell membranes (Ishikawa et al. 2006). In mouse lacrimal gland, pilocarpine increased tear secretion 2.5-fold over control and increased the immunoreactivity of AQP5 on the intracellular side of the apical membranes of acinar and duct cells, but not on the extracellular side (Ishida et al. 1997). AQP4, located in the basolateral membrane, showed no change in immunoreactivity to antibody after pilocarpine.

17 Ocular Vascular System

All five muscarinic receptor subtypes were detected in mouse ophthalmic arteries with real-time PCR, the mRNA levels being higher for M_1, M_3 and M_5 subtypes than for the other two subtypes. ACh and CCh produced concentration-dependent vasodilatation in normal wild-type mice, following precontraction with phenylephrine. In receptor subtype-knockout mice, the $M_5-/-$ mice reacted to the agonists as for

the wild type. However, ACh and CCh were almost inactive in $M_3-/-$ mice whereas responses to non-cholinergic vasodilators were unaffected indicating that M_3 receptors mediate the vasodilation to muscarinic agonists (Gericke et al. 2009).

In rat uveal vortex veins, electrical stimulation of the superior salvitory nucleus activated ocular parasympathetic nerves to increase blood flow (Steinle and Smith 2000). This was mediated by nitrergic nerves as the flux response was inhibited by a selective neuronal NOS inhibitor and was unaffected by atropine. Following acute (2 days) or chronic (6 weeks) sympathectomy, the flux increase on stimulation of the parasympathetic nerves was unaltered, but atropine selectively abolished the flux after chronic sympathectomy. It was postulated that sympathectomy led to a down-regulation of nitric oxide release and development of a compensatory cholinergic facilitation. Thus, the effect of atropine was due to blockade of a prejunctional facilitatory muscarinic receptor on the nitrergic nerves. In support of this explanation, bethanecol enhanced parasympathetically mediated vasodilation only in the chronically sympathectomized animals and not in the acutely sympathectomized without affecting basal flow.

It was concluded the muscarinic receptor(s) involved after chronic sympathectomy were both M_3 and M_5 receptor subtypes as 4-DAMP reduced the increased flux by 30% and atropine reduced it further, whereas methoctramine and tropicamide were ineffective. Pirenzepine increased the flux 40% beyond control suggesting the possibility of inhibitory prejunctional M_1 receptors also being present.

In rat retinal vessels, CCh stimulated iNOS activity and iNOS-mRNA gene expression to increase vascular diameter, by activation of muscarinic receptors (Berra et al. 2005). The responses were inhibited by atropine, pirenzepine and 4-DAMP, but not by AF-DX 116 suggesting involvement of M_1/M_3 receptors. The effect of CCh was also reduced by inhibition of PLC or PKC but not by inhibition of Ca^{2+}/calmodulin.

18 Glaucoma

Glaucoma is a condition in which there is excessive intraocular pressure, leading to impairment of the blood supply to the retina, with ensuing blindness. It develops when the outflow of aqueous humour from the eye is impaired. The aqueous humour is produced in the ciliary process, being secreted into the anterior chamber in front of the lens and behind the iris. It flows through the pupillary opening and exits the anterior chamber mainly through the trabecular meshwork in the angle between the front of the iris and the back of the cornea. There is an alternative outflow route, the uveoscleral outflow. This occurs by seepage of the aqueous humour, through an incomplete endothelium on the ciliary body, into the interstitial spaces between the ciliary muscle fibres and then ultimately into the episcleral tissues. In the anaesthetized monkey, the uveoscleral outflow constitutes ca

30–50% of the total aqueous outflow, whereas in humans it is only ca. 5–20% (see Sugrue 1989).

The rationale for the use of muscarinic agonists, such as aceclidine and pilocarpine, in the two main types of glaucoma differs. In narrow angle glaucoma, which constitutes ca 10% of cases, the underlying problem is a narrow angle between the front of the iris and the back of the cornea which limits the access of aqueous humour to the base of the angle where the trabecular meshwork and the canal of Schlemm are situated. This anatomical defect may be exacerbated in conditions leading to pupillary dilatation resulting in the iris being crowded back into the narrow angle. While the ultimate treatment of this condition involves surgical or laser treatment to insert an opening in the iris thereby improving access of aqueous humour to the meshwork, muscarinic agonists may be used acutely to constrict the pupil, causing miosis, drawing the iris out of the angle, thereby lowering the intraocular pressure.

In open-angle glaucoma, the most common form of the condition, there is an obstruction to outflow of aqueous humour within the trabecular meshwork. Muscarinc agonists administered topically can be used to lower intraocular pressure by causing contraction of the ciliary muscle. This results in a conformational alteration of the associated trabecular meshwork, reducing the resistance to outflow of the aqueous humour through the meshwork towards the canal of Schlemm. Studies in cynomolgus monkeys have shown that section of the ciliary muscle's attachment to the region containing the trabecular meshwork and reinsertion of the muscle further back on the inner scleral wall still allowed the ciliary muscle to contract to pilocarpine but without the marked increase in outflow facility seen in the contralateral eye (Kaufman and Bárány 1976; Lütjen-Drecoll et al. 1977).

Recently, Erickson and Schroeder (2000) found that low concentrations (1 nM–1 μM) of the muscarinic agonists, aceclidine, CCh and pilocarpine, were able to increase outflow facility in isolated, perfused, anterior segments of human eye with the lens and ciliary muscle removed. Experiments were conducted on eyes, enucleated within 9 h and dissected within 15.5 h *post-mortem*. The greatest increase in outflow facility (~100%) was obtained with aceclidine, followed by CCh (~50%) and pilocarpine (~30%). High concentrations of the agonists (100 μM–10 mM) were ineffective. In contrast, Kiland et al. (2000) found that low concentrations of pilocarpine (0.1 nM−1 μM) perfused into the anterior chamber in vivo failed to increase outflow facility, accommodation or miosis in the cynomolgus monkey. All three parameters required a concentration of 10 μM to obtain a significant response. It was concluded that the outflow facility would only be increased effectively by concentrations of pilocarpine that contracted the ciliary muscle.

It was concluded that the increase in outflow facility, as well as contraction of the sphincter pupillae and the ciliary muscle, by pilocarpine in the rhesus monkey involved the muscarinic M_3 receptor, based on studies with 4-DAMP, pirenzepine and AF-DX 116 (Gabelt and Kaufman 1992).

CCh has also been reported to inhibit the Na, K-ATPase involved in the production of aqueous humour in the bovine ciliary body by a mechanism utilizing

nitric oxide (NO), soluble guanylate cyclase and cGMP (Ellis et al. 2001) but whether this also contributes to the reduction in intraocular pressure with aceclidine or pilocarpine does not appear to have been tested. However, pilocarpine (0.5%) has been reported to have no practical effect on the production of aqueous humour in humans (Nagataki and Brubaker 1982).

It may be possible to separate the activity of the ciliary muscle on accommodation and on outflow facility since contraction of the circular division of the ciliary muscle is responsible for accommodation while contraction of the longitudinal division influences outflow. Erickson-Lamy and Schroeder (1990) showed that the muscarinic agonist, (±)-aceclidine, produced a greater effect on outflow relative to its effect on accommodation, than did pilocarpine. However, the reason for this finding is not readily apparent. (±)-Aceclidine was found to have EC_{50} values of 25 μM in the human longitudinal ciliary muscle, 20 μM in the circular ciliary muscle, being a partial agonist producing ~85% of the response to CCh in both tissues (Ishikawa et al. 1998). An EC_{50} value of 0.9 μM (E_{max}; 96%) was obtained in the human iris sphincter muscle. Subtype-preferring antagonists had similar affinities in the iris and the two ciliary muscles also. The S-(+)- enantiomer is ca two- to fourfold more potent at all five types of muscarinic receptor than the R-(−)-enantiomer, the latter also being a partial agonist at all subtypes (Ehlert et al. 1996, Griffin et al. 2007). Relative to carbachol, each enantiomer shows ca threefold preference for M_1 and M_2 receptors over M_3 receptors (Ehlert et al. 1996).

It is known that muscarinic agonists increase outflow facility in normal as well as glaucomatous eyes, but because of homeostatic influences there is little change in intraocular pressure in normal eyes (see Potts 1965). Recent findings suggest that there may be a greater cholinergic influence on outflow regulation in glaucomatous eyes. In a rat model of congenital glaucoma, treatment with botulinum toxin A to block cholinergic nerve function, led to an increase in intraocular pressure, not observed in the normotensive control (Gatzioufas et al. 2008).

Clinically, the use of pilocarpine in humans to lower intraocular pressure is not affected by the age-related decline in ciliary muscle mobility (Croft et al. 1996) or the use of argon laser trabeculoplasty (Teus et al. 1997). The use of pilocarpine to treat glaucoma has declined with the increase in the number of alternative drugs to lower intraocular pressure with a lower incidence of side effects, such as the β-adrenoceptor antagonists and prostaglandin $F_{2\alpha}$ analogues. The effect of pilocarpine or latanoprost in combination with timolol has been compared with latanoprost monotherapy in three multicentre, randomized, clinical trials in Europe (Diestelhorst et al. 2002). Latanoprost alone, and the combination of timolol and latanoprost, were both more effective and better tolerated than the combination of timolol and pilocarpine.

Latanoprost lowers intraocular pressure primarily by increasing uveoscleral outflow, whereas pilocarpine increases outflow through the trabecular meshwork and reduces uveoscleral outflow, at least in the cynomolgus monkey (Bill and Wålinder 1966; Bill 1967), probably because it contracts the ciliary muscle. Nevertheless, a review of four published clinical trials showed the effect of latanoprost was not reduced by pilocarpine and the combination of the two drugs

produced a greater decrease in intraocular pressure than either alone (Toris et al. 2002).

19 Myopia

Myopia (near-sightedness) is the state of refraction where parallel rays of light are brought to a focus in front of the retina of a resting eye. The condition is caused by elongation of the eyeball so that the eye has difficulty in focusing on distant objects. In Europe and the USA, the incidence is ~25% whereas in Singapore and other East Asian countries, the incidence may be 70% or more (Saw et al. 1996; Seet et al. 2001). The incidence can vary between ethnic groups within the same community and be influenced by the amount of close work undertaken (Saw et al. 1996, 2006).

Several clinical trials have shown that atropine, 0.05 or 1%, (Chua et al. 2006; Fan et al. 2007; Lee et al. 2006) or pirenzepine (Siatkowski et al. 2008; Tan et al. 2005) is effective in limiting the development of myopia in children. A 2-year study in a group of Asian children found atropine (1% topical ointment daily) almost completely suppressed axial elongation and decreased the rate of progression of low or moderate myopia (Chua et al. 2006). Atropine appeared to be non-toxic to retinal function, producing no effect on multifocal electroretinograms in a 2-year study in children (Luu et al. 2005) and the benefit of atropine was still apparent, 1 year after cessation of the drug (Tong et al. 2009).

Similarly, myopia progressed less in children treated over 1 or 2 years with pirenzepine (2% gel, topical twice daily) than in the placebo groups (Siatkowski et al. 2008; Tan et al. 2005). The drug was well tolerated, only 11% of patients on pirenzepine discontinued treatment over the first year in both trials. Pirenzepine ($\leq$2% gel) produces little mydriasis or effects on accommodation in myopic children (Bartlett et al. 2003; Siatkowski et al. 2008; Tan et al. 2005).

Recently, it was reported that the S2 and S4 polymorphisms of the muscarinic M_1 receptor gene *CHRM1* are associated with susceptibility for developing high myopia and that S1, S2 and S4 *CHRM1* had a cooperative association with high myopia (Lin et al. 2009). No association was found for S3 *CHRM1*.

The mechanism of action of atropine or pirenzepine in myopic eyes has been the subject of several animal studies since Wiesel and Raviola (1977) demonstrated that myopia could be induced experimentally by occlusion of one eye ("form-induced myopia"). Other techniques have included use of a black contact lens in monkeys (Tigges et al. 1999) and use of translucent diffusers in the chick (Diether et al. 2007; Vessey et al. 2002), guinea pig (Qiong et al. 2007) or tree shrew (Cottriall and McBrien 1996; McBrien et al. 2009). Wearing negative lens is another technique ("lens-induced myopia") (Diether et al. 2007; Cottriall and McBrien 1996).

Atropine was shown to inhibit experimental myopia in the rhesus monkey (Tigges et al. 1999) and chick (Stone et al. 1991) and pirenzepine in the tree shrew (Cottriall and McBrien 1996) and chick (Stone et al. 1991). The fact that

pirenzepine as well as atropine is effective in preventing myopia may indicate that the muscarinic M_1 receptor is the important subtype involved. However, while pirenzepine was effective in preventing myopia in the chick, M_1 receptors could not be detected in chick ocular or brain tissue (Yin et al. 2004). Furthermore, the dose of pirenzepine (17.7 μmol daily) was calculated to have produced concentrations of 100 μM in the choroid and sclera, 10 μM in the retina and 1 μM in the vitreous humour. These concentrations would block a substantial percentage of all subtypes of muscarinic receptor in the choroid, sclera and retina, given that pirenzepine has lowest affinity for the M_2 subtype in the reported range of pK_i/pK_b values, 6.0–6.8.

Himbacine (ED_{50} 480 μg, injected intravitreally) inhibited vitreous chamber elongation in the chick leading to a suggestion that muscarinic M_4 receptors also may be important in the development of myopia (Cottriall et al. 2001b) but the subtype preference of himbacine is not as great as pirenzepine and other subtypes could be involved.

Early studies found little or no direct evidence for the involvement of cholinergic nerves or muscarinic receptors. Steady state choline and ACh levels in the retina were not altered during development of experimental myopia in the chick or tree shrew (McBrien et al. 2001). Muscarinic receptors in the chick retina and choroid were not altered in myopia (Vessey et al. 2002) and cholinergic amacrine cells in the retina were not required for the development of form-deprived myopia or its suppression by atropine in the chick (Fischer et al. 1998b). In the rhesus monkey, topical application of atropine or pirenzepine led to a small increase in muscarinic receptor density in the ciliary body and iris, but not in the retina (Tigges et al. 1999). No changes in gene expression or protein expression for the five muscarinic receptor subtypes were detected in the retina, choroid or sclera in form-deprived myopia in the tree shrew (McBrien et al. 2009).

Luft et al. (2003) studied a number of muscarinic receptor antagonists injected intravitreally in Leghorn cockerels and found that only atropine, pirenzepine and oxyphenonium were fully effective at inhibiting form-deprived myopia; AF-DX 116, 4-DAMP, dexetimide, HHSiD, pFHHSiD, propantheline, QNB, scopolamine and tropicamide were partially effective, while dicyclomine, gallamine, methoctramine, mepenzolate and procyclidine were all ineffective or were toxic in the concentrations used. Propantheline and 4-DAMP also produced retinal damage. The antagonists were all used in high concentrations ranging from 0.1 to 10 mM, except for methoctramine (0.5 μM).

Dopamine is another neurotransmitter with a possible involvement in myopia. Both dopamine and its metabolite, DOPAC, were found to be decreased in myopic eyes of the tree shrews and chicks (McBrien et al. 2001). Atropine, injected intravitreally, increased the release of dopamine from the retina in form-deprived myopia in the chick and also produced a spreading depression of light-induced potentials in an in vitro retina-pigmented epithelium-choroid preparation (Schwahn et al. 2000). It was suggested that the spreading depression enhanced neurotransmitter release from tissue stores, inhibiting some presumed retinal signal controlling eye growth and thus, myopia.

It is of interest that the organophosphorus anticholinesterase, dyflos (DFP), injected intravitreally in the form-deprived chick eye over an 8-day period also reduced myopia 58% compared with the contralateral control eye (Cottriall et al. 2001a). Open eyes injected with DFP showed no refractive errors or increase in vitreous chamber depth compared to the contralateral control eye. A single intravitreal injection of DFP also raised dopamine levels 36% and ACh 54% in retinal tissue of the form-deprived eye compared to the control eye. The effect on dopamine was observed 1.5 h after injection of DFP but had returned to control levels by 3 h. Injection of a dopamine D_2 receptor antagonist, spiperone, reduced the inhibitory effect of DFP on myopia development suggesting that retinal dopamine release was an important factor in controlling myopia development. Since DFP produces irreversible inhibition of cholinesterases, it could be acting via a desensitization or down-regulation of muscarinic receptors to influence dopamine release.

Atropine, in high concentrations, was found to inhibit cellular proliferation and extracellular matrix production in chick sclera and along with pirenzepine and 4-DAMP was found to inhibit sulphate incorporation into glycosaminoglycans in isolated scleral chondrocytes (Lind et al. 1998), offering a possible explanation for the effectiveness of atropine and pirenzepine in myopia. Pirenzepine was also ~sevenfold more potent in myopic eyes than in normal eyes. Diether et al. (2007) found that atropine countered both lens-induced myopia and form-induced myopia in the chick, and also suggested that atropine had a direct inhibitory action on scleral chondrocytes. However, Truong et al. (2002) found pirenzepine caused only a transient reduction in glycosaminoglycan synthesis in chick cartilaginous sclera, 2 h after administration, but this was not evident at 6 h. The effect was present in both non-occluded and occluded eyes and was not observed in fibrous sclera. Also, there was no change in scleral DNA at any time point, excluding a toxic effect. The change in glycosaminoglycan synthesis could also be produced by exposing an occluded eye to brief periods of unoccluded vision, a technique that also prevents experimental myopia.

Recently, Qiong et al. (2007) found mRNA for the five subtypes of muscarinic receptor in the retina, choroid, sclera and iris–ciliary body of the guinea pig eye. After induction of form-deprived myopia, over 21 days, there was a significant increase in the posterior sclera for mRNA expression of the M_1 (+18.7%) and M_4 receptor subtype (+26.5%) as well as corresponding protein expression (+24.7%; +49.1%, respectively) with no significant changes in the other regions. These findings contrast with those obtained in the tree shrew by McBrien et al. (2009) where no change was observed in muscarinic receptor gene expression in the sclera, retina or choroid during myopia induction over 5 days.

Thus overall, there is no uniform agreement as to why atropine and pirenzepine are clinically usefully in myopia and produce similar effects at equivalent doses in experimental myopia in the rhesus monkey. The concentrations of muscarinic receptor antagonists required to inhibit RNA and glycosaminoglycan synthesis in chick scleral chondrocytes are high (Lind et al. 1998) and do not equate with the effective clinical doses and furthermore, chondrocytes are not present in the sclera

of monkeys (Tigges et al. 1999). The finding of selective increases in muscarinic M_1 and M_4 receptor protein only in the sclera of the guinea pig with experimental myopia (Qiong et al. 2007) and the transient changes in cartilaginous scleral glycosaminoglycan synthesis induced by pirenzepine in the chick, similar to that induced by brief periods of non-occlusion (Truong et al. 2002) suggest that further investigation of scleral tissue may yield an explanation.

20 Sjögren's Syndrome

SS is an autoimmune disorder characterized by xerophthalmia and xerostomia (Vitali et al. 2002) but may be accompanied by other autonomic symptoms such as mydriasis and bladder hyper-reactivity. These first two symptoms are considered to arise because disruption of the lacrimal and salivary exocrine glands follows lymphocyte infiltration, release of cytokines, inflammation and glandular destruction.

Patients with SS have numerous antibodies in their serum but there is no clear correlation between sicca symptoms of dry eye and dry mouth and the antibody titre and many of these antibodies occur in other autoimmune disorders without sicca. However, Bacman et al. (1996, 1998, 2001) reported that autoantibodies to the M_3 muscarinic receptor were present in the serum IgG of primary and secondary SS patients. These antibodies were detected using the M_3 muscarinic receptor in rat lacrimal and parotid gland.

In both glands the antibodies in IgG could displace [^{3}H]-QNB, non-competitively, from the muscarinic M_3 receptor. In the lacrimal gland the antibodies acted like CCh to increase NOS activity and cGMP production (Bacman et al. 1998). Atropine and 4-DAMP inhibited the action of the antibodies on NOS activity (Bacman et al. 1998) and on phosphoinositide turnover (Bacman et al. 1996). There was one difference between CCh and the antibodies; the PKC inhibitor, staurosporine, partially reduced the effect of CCh on NOS activation but did not alter that of the antibodies. The PLC inhibitor, neomycin, and the Ca^{2+}/calmodulin inhibitor, trifluoperazine, inhibited NOS activation by CCh or the antibodies. These findings suggest that the antibodies used only the calcium/calmodulin-dependent pathway for NOS activation, whereas CCh also used a pathway involving PKC.

It was suggested that continuous NO production by the antibodies may be cytotoxic to the lacrimal gland in line with a previous conclusion of Konttinen et al.(1997) or there may be receptor desensitization, internalization and/or degradation leading to a progressive receptor blockade and dry eye (Bacman et al. 1998).

In contrast, Waterman et al. (2000) suggested that the serum of some patients with SS contains autoantibodies that act as antagonists of muscarinic M_3 receptors. They demonstrated that serum and the purified IgG fraction from patients with primary or secondary SS contained antibodies that maximally inhibited responses

to CCh in mouse isolated bladder by ca 50%, whereas the muscarinic receptor antagonist 4-DAMP abolished the response. Endogenous ACh released by parasympathetic nerve stimulation appeared to be completely inhibited by the antibodies in that the residual response to nerve stimulation could be almost abolished by desensitization of the tissue to the purine receptor ligand α,β-methylene ATP with the subsequent addition of 4-DAMP causing no further reduction. Such a difference in the ability of a compound to inhibit various muscarinic agonists is typical of an allosteric modulator rather than a competitive antagonist. It was also reported, but not commented on in their discussion, that 60% of the sera from primary SS patients (3/5) and 33% of that from secondary SS patients (2/6) produced a contraction of the bladder which could be prevented by prior treatment of the tissue with 4-DAMP. The response when it occurred waned over the course of several minutes after which responses to CCh were inhibited. Thus, in these cases the serum contained antibodies that exhibited initial muscarinic agonist activity, similar to that observed by Bacman et al. (1996, 1998). Perhaps the autoantibodies function as a bitopic agonist (like McN-A-343) having both agonist and antagonist binding sites on the receptor (Valant et al. 2008) or that the tissue is readily desensitized by persistent binding of the antibody which occurs at variable rates such that an underlying agonist action is not always observable. One further complication was that the mouse bladder experiments were conducted in the presence of hexamethonium (C_6) (100 μM) to inhibit nicotinic ganglionic receptors. However, C_6 can interact at muscarinic receptors in the high concentrations employed (Leung and Mitchelson 1982) and therefore may be modifying the action of the antibody with its binding sites on the muscarinic receptor.

As other antibodies to GPCRs couple to epitopes on the extracellular loops of the receptor it has been considered that those in SS may bind similarly. Bacman et al. (2001) reported that serum from SS patients reacted with a 25-mer peptide corresponding to what was thought to be the second extracellular loop of the human muscarinic M_3 receptor, but was in fact the M_4 receptor due to an incorrect entry in GenPept (see Cavill et al. 2002). These findings could not be replicated by Cavill et al. (2002). However, they were able to show that antibodies raised in rabbits against the second extracellular loop of the human muscarinic M_3 receptor mimicked the action of autoantibodies from the serum of SS patients in antagonizing the contractile response to CCh on mouse isolated colon (Cavill et al. 2004).

In a mouse model of SS, the MRL/MpJ-*Fas*^{lpr} mouse, conjunctival changes occur between the 9th and 16th week which are less pronounced than the changes in the lacrimal gland but also involve T cell invasion without tissue destruction. The conjunctival goblet cells remain filled which was attributed to a decreased expression of muscarinic M_3 receptors (Diebold et al. 2007).

Clinically muscarinic agonists, pilocarpine (5 mg qid) and cevimeline (20–30 mg tid) have been shown to alleviate xerophthalmia in trials on SS patients (Vivino et al. 1999; Petrone et al. 2002; Ono et al. 2004) but cevimeline has not been successful in all trials (Leung et al. 2008).

References

Abdel-Latif AA, Yousufzai SYK, De S, Tachado SD (1992) Carbachol stimulates adenylate cyclase and phospholipase C and muscle contraction-relaxation in a reciprocal manner in dog iris sphincter smooth muscle. Eur J Pharmacol 226:351–361

Akhtar RA, Honkanen RE, Howe PH, Abdel-Latif AA (1987) M_2 Muscarinic receptor subtype is associated with inositol trisphosphate accumulation, myosin light chain phosphorylation and contraction in sphincter smooth muscle of rabbit iris. J Pharmacol Exp Ther 243:624–632

Bacman S, Sterin-Borda L, Camusso JJ, Arana R, Hubscher O, Borda E (1996) Circulating antibodies against rat parotid gland M_3 muscarinic receptors in primary Sjögren's syndrome. Clin Exp Immunol 104:454–459

Bacman SR, Berra A, Sterin-Borda L, Borda ES (1998) Human primary Sjögren's syndrome autoantibodies as mediators of nitric oxide release coupled to lacrimal gland muscarinic acetylcholine receptors. Curr Eye Res 17:1135–1142

Bacman S, Berra A, Sterin-Borda L, Borda E (2001) Muscarinic acetylcholine receptor antibodies as a new marker of dry eye Sjögren's syndrome. Invest Ophthalmol Vis Sci 42:321–327

Bartlett JD, Niemann K, Houde B, Allred T, Edmondson MJ, Crockett RS (2003) A tolerability study of pirenzepine ophthalmic gel in myopic children. J Ocul Pharmacol Ther 19:271–279

Berra A, Ganzinelli S, Saravia M, Borda E, Sterin-Borda L (2005) Inducible nitric oxide synthase subserves cholinergic vasodilation in retina. Vis Neurosci 22:371–377

Berridge MJ (1995) Capacitative calcium entry. Biochem J 312:1–11

Bill A (1967) Effects of atropine and pilocarpine on aqueous humour dynamics in cynomolgus monkeys (*Macaca irus*). Exp Eye Res 6:120–125

Bill A, Wålinder P-E (1966) The effects of pilocarpine on the dynamics of aqueous humor in a primate (*Macaca irus*). Invest Ophthalmol 5:170–175

Bognar IT, Pallas S, Fuder H, Muscholl E (1988) Muscarinic inhibition of [^{3}H]-noradrenaline release on rabbit iris in vitro: effects of stimulation conditions on intrinsic activity of methacholine and pilocarpine. Br J Pharmacol 94:890–900

Bognar IT, Baumann B, Dammann F, Knöll B, Meincke M, Pallas S, Fuder H (1989) M_2 muscarinic receptors on the iris sphincter muscle differ from those on iris noradrenergic nerves. Eur J Pharmacol 163:263–274

Bognar IT, Altes U, Beinhauer C, Kessler I, Fuder H (1992) A muscarinic receptor different from the M_1, M_2, M_3 and M_4 subtypes mediates the contraction of the rabbit iris sphincter. Naunyn Schmiedebergs Arch Pharmacol 345:611–618

Borda E, Berra A, Saravia M, Ganzinelli S, Sterin-Borda L (2005) Correlations between neuronal nitric oxide synthase and muscarinic M_3/M_1 receptors in the rat retina. Exp Eye Res 80:391–399

Cavanagh HD, Colley AM (1982) Cholinergic, adrenergic and PGE1 effects on cyclic nucleotides and growth in cultured corneal epithelium. Metab Pediatr Syst Opththalmol 6:63–74

Cavanagh HD, Colley AM (1989) The molecular basis of neurotrophic keratitis. Acta Ophthalmol Suppl 192:115–134

Cavill D, Waterman SA, Gordon TP (2002) Failure to detect antibodies to extracellular loop peptides of the muscarinic M3 receptor in primary Sjögren's syndrome. J Rheumatol 29:1342–1343

Cavill D, Waterman SA, Gordon TP (2004) Antibodies raised against the second extracellular loop of the human muscarinic M3 receptor mimic functional autoantibodies in Sjögren's syndrome. Scand J Immunol 59:261–266

Choppin A, Eglen RM, Hegde SS (1998) Pharmacological characterization of muscarinic receptors in rabbit isolated iris sphincter muscle and urinary bladder smooth muscle. Br J Pharmacol 124:883–888

Chua W-H, Balakrishnan V, Chan Y-H, Tong L, Ling Y, Quah B-L, Tan D (2006) Atropine for the treatment of childhood myopia. Ophthalmology 113:2285–2291

Cimini BA, Strang CE, Wotring VE, Keyser KT, Eldred WD (2008) Role of acetylcholine in nitric oxide production in the salamander retina. J Comp Neurol 507:1952–1963

Colley AM, Cavanagh HD (1982) Binding of [^{3}H]dihydroalprenolol and [^{3}H]quinuclidinyl benzilate in intact cells of cultured corneal epithelium. Metab Pediatr Syst Opththalmol 6:75–86

Colley AM, Law ML (1987a) Effects of carbamylcholine on nuclear cyclic nucleotide-dependent protein kinase activity in cultured corneal epithelial cells of the rabbit. Metab Pediatr Syst Opththalmol 10:24–31

Colley AM, Law ML (1987b) Effects of carbamylcholine on cyclic nucleotide-dependent protein kinase activity in corneal epithelium during resurfacing. Metab Pediatr Syst Opththalmol 10:73–75

Colley AM, Cavanagh HD, Drake LA, Law ML (1985) Cyclic nucleotides in muscarinic regulation of DNA and RNA polymerase activity in cultured corneal epithelial cells of the rabbit. Curr Eye Res 4:941–950

Colley AM, Cavanagh HD, Law ML (1987a) Subcellular localization of muscarinic effects on enzymes of cyclic nucleotide metabolism in cultured corneal epithelial cells of the rabbit. Metab Pediatr Syst Opththalmol 10:36–38

Colley AM, Law ML, Drake LA, Cavanagh HD (1987b) Activity of DNA and RNA polymerases in resurfacing rabbit corneal epithelium. Curr Eye Res 6:477–487

Collison DJ, Duncan G (2001) Regional differences in functional receptor distribution and calcium mobilization in the intact human lens. Invest Ophthalmol Vis Sci 42:2355–2363

Collison DJ, Coleman RA, James RS, Carey J, Duncan G (2000) Characterization of muscarinic receptors in human lens cells by pharmacologic and molecular techniques. Invest Ophthalmol Vis Sci 41:2633–2641

Cottriall CL, McBrien NA (1996) The M_1 muscarinic antagonist pirenzepine reduces myopia and eye enlargement in the tree shrew. Invest Ophthalmol Vis Sci 37:1368–1379

Cottriall CL, Brew J, Vessey KA, McBrien NA (2001a) Diisopropylfluorophosphate alters retinal neurotransmitter levels and reduces experimentally-induced myopia. Naunyn Schmiedebergs Arch Pharmacol 364:372–382

Cottriall CL, Truong H-T, McBrien NA (2001b) Inhibition of myopia development in chicks using himbacine: a role for M_4 receptors? Neuroreport 12:2453–2456

Croft MA, Oyen MJ, Gange SJ, Fisher MR, Kaufman PL (1996) Aging effects on accommodation and outflow facility responses to pilocarpine in humans. Arch Ophthalmol 114:586–592

da Silva N, Herron CE, Stevens K, Jollimore CAB, Barnes S, Kelly MEM (2008) Metabotropic receptor-activated calcium increases and store-operated calcium influx in mouse Müller cells. Invest Ophthalmol Vis Sci 49:3065–3073

Dartt D (2002) Regulation of mucin and fluid secretion by conjunctival epithelial cells. Prog Retin Eye Res 21:555–576

Dartt D (2004) Interaction of EGF family growth factors and neurotransmitters in regulating lacrimal gland secretion. Exp Eye Res 78:337–345

Dartt DA, Rios JR, Kanno H, Rawe IM, Zieske JD, Ralda N, Hodges RR, Zoukhri D (2000) Regulation of conjunctival goblet cell secretion by Ca^{2+} and protein kinase C. Exp Eye Res 71:619–628

Diebold Y, Ríos JD, Hodges RR, Rawe I, Dartt DA (2001) Presence of nerves and their receptors in mouse and human conjunctival goblet cells. Invest Ophthalmal Vis Sci 42:2270–2282

Diebold Y, Chen L-L, Tepavcevic V, Ferdman D, Hodges RR, Dartt DA (2007) Lymphocytic infiltration and goblet cell marker alteration in the conjunctiva of the MRL/MpJ-*Fas^{lpr}* mouse model of Sjögren's syndrome. Exp Eye Res 84:500–512

Diestelhorst M, Nordmann J-P, Toris CB (2002) Combined therapy of pilocarpine or latanoprost with timolol versus latanoprost monotherapy. Surv Ophthalmol 47(Suppl 1):S155–S161

Diether S, Schaeffel F, Lambrou GN, Fritsch C, Trendelenburg A-U (2007) Effects of intravitreally and intraperitoneally injected atropine on two types of experimental myopia in chicken. Exp Eye Res 84:266–274

dos Santos AA, Medina SV, Sholl-Franco A, de Araujo EG (2003) PMA decreases the proliferation of retinal cells in vitro: the involvement of acetylcholine and BDNF. Neurochem Int 42:73–80

Duncan G, Collison DJ (2003) Role of the non-neuronal cholinergic system in the eye: a review. Life Sci 72:2013–2019

Duncan G, Williams MR, Riach RA (1994) Calcium, cell signaling and cataract. Prog Ret Eye Res 13:623–652

Duncan G, Riach RA, Williams MR, Webb SF, Dawson AP, Reddan J (1996) Calcium mobilisation modulates growth of lens cells. Cell Calcium 19:83–89

Dursun D, Wang M, Monroy D, Li D-Q, Lokeshwar BL, Stern ME, Pflugfelder SC (2002) A mouse model of keratoconjunctivitis sicca. Invest Ophthalmol Vis Sci 43:632–638

Eglen RM, Hegde SS, Watson N (1996) Muscarinic receptor subtypes and smooth muscle function. Pharmacol Rev 48:531–565

Ehlert FJ, Griffin MT, Glidden PF (1996) The interaction of the enantiomers of aceclidine with subtypes of the muscarinic receptor. J Pharmacol Exp Ther 279:1335–1344

Ellis DZ, Nathanson JA, Rabe J, Sweadner KJ (2001) Carbachol and nitric oxide inhibition of Na, K-ATPase activity in bovine ciliary processes. Invest Ophthalmol Vis Sci 42:2625–2631

Enríquez de Salamanca A, Siemasko KF, Diebold Y, Calonge M, Gao J, Juárez-Campo M, Stern ME (2005) Expression of muscarinic and adrenergic receptors in normal human conjunctival epithelium. Invest Ophthalmol Vis Sci 46:504–513

Erickson KA, Schroeder A (2000) Direct effects of muscarinic agents on the outflow pathways in human eyes. Invest Ophthalmol Vis Sci 41:1743–1748

Erickson-Lamy K, Schroeder A (1990) Dissociation between the effect of aceclidine on outflow facility and accommodation. Exp Eye Res 50:143–147

Fan DSP, Lam DSC, Chan CKM, Fan AH, Cheung EYY, Rao SK (2007) Topical atropine in retarding myopic progression and axial length growth in children with moderate to severe myopia: a pilot study. Jpn J Ophthalmol 51:27–33

Fischer AJ, McKinnon LA, Nathanson NM, Stell WK (1998a) Identification and localization of muscarinic acetylcholine receptors in the ocular tissues of the chick. J Comp Neurol 392:273–284

Fischer AJ, Miethke P, Morgan IG, Stell WK (1998b) Cholinergic amacrine cells are not required for the progression and atropine-mediated suppression of form-deprivation myopia. Brain Res 794:48–60

Fuder H, Schöpf J, Unckell J, Wesner MTh, Melchiorre C, Tacke R, Mutschler E, Lambrecht G (1989) Different muscarinic receptors mediate the prejunctional inhibition of [^{3}H]-noradrenaline release in rat or guinea-pig iris and the contraction of the rabbit iris sphincter muscle. Naunyn Schmiedebergs Arch Pharmacol 340:597–604

Furuta M, Ohya S, Imaizumi Y, Watanabe M (1998) Molecular cloning of m3 muscarinic acetylcholine receptor in rat iris. J Smooth Muscle Res 34:111–122

Gabelt BT, Kaufman PL (1992) Inhibition of outflow facility and accommodative and miotic responses to pilocarpine in rhesus monkeys by muscarinic receptor subtype antagonists. J Pharmacol Exp Ther 263:1133–1139

Gatzioufas Z, Charalambous P, Seitz B, Evers S, Jonescu-Cuypers C, Krause M, Thanos S (2008) Cholinergic inhibition by botulinum toxin in a rat model of congenital glaucoma raises intraocular pressure. Br J Ophthalmol 92:826–831

Gericke A, Mayer VGA, Steege A, Patzak A, Neumann U, Grus FH, Joachim SC, Choritz L, Wess J, Pfeiffer N (2009) Cholinergic responses of ophthalmic arteries in M3 and M5 muscarinic acetylcholine receptor knockout mice. Invest Ophthalmol Vis Sci 50:4822–4827

Gil DW, Krauss HA, Bogardus AM, WoldeMussie E (1997) Muscarinic receptor subtypes in human iris-ciliary body measured by immunoprecipitation. Invest Ophthalmol Vis Sci 38: 1434–1442

Gnädinger M, Walz D, von Hahn HP, Grün F (1967) Acetylcholine-splitting activity of abraded and cultivated corneal epithelial cells. Exp Eye Res 6:239–242

Gnädinger MC, Heimann R, Markstein R (1973) Choline acetyltransferase in corneal epithelium. Exp Eye Res 15:395–399

González A III, Crittenden EL, García DM (2004) Activation of muscarinic acetylcholine receptors elicits pigment granule dispersion in retinal pigment epithelium isolated from bluegill. BMC Neurosci 5:23, 12 p

Griffin MT, Figueroa KW, Liller S, Ehlert FJ (2007) Estimation of agonist activity at G protein-coupled receptors: analysis of M_2 muscarinic receptor signaling through $G_{i/o}$, G_s and G_{15}. J Pharmacol Exp Ther 321:1193–1207

Gupta N, McAllister R, Drance SM, Rootman J, Cynader MS (1994) Muscarinic receptor M1 and M2 subtypes in the human eye: QNB, pirenzipine, oxotremorine, and AFDX-116 in vitro autoradiography. Br J Ophth 78:555–559

Hagan JJ, van der Heijden B, Broekkamp CLE (1988) The relative potencies of cholinomimetics and muscarinic antagonists on the rat iris in vivo: effects of pH on potency of pirenzepine and telenzepine. Naunyn Schmiedebergs Arch Pharmacol 338:476–483

Hall MO, Burgess BL, Abrams TA, Martinez MO (1996) Carbachol does not correct the defect in the phagocytosis of outer segments by Royal College of Surgeons rat retinal pigment epithelial cells. Invest Ophthalmol Vis Sci 37:1473–1477

Hasegawa N, Imaizuma Y, Watanabe M (1988) Parasympathetic denervation abolishes acetylcholine-induced relaxation in the rat iris dilator. Eur J Pharmacol 156:291–294

Heth CA, Marescalchi PA, Ye L (1995) IP_3 generation increases rod outer segment phagocytosis by cultured Royal College of Surgeons retinal pigment epithelium. Invest Ophthalmol Vis Sci 36:984–989

Hodges RR, Rios JD, Vrouvlianis J, Ota I, Zoukhri D, Dartt DA (2006) Role of protein kinase C, Ca^{2+}, Pyk2, and c-Src in agonist activation of rat lacrimal gland p42/p44 MAPK. Invest Ophthalmol Vis Sci 47:3352–3359

Honkanen RE, Abdel-Latif AA (1988) Characterization of cholinergic muscarinic receptors in the rabbit iris. Biochem Pharmacol 37:2575–2583

Honkanen RE, Howard EF, Abdel-Latif AA (1990) M3-muscarinic receptor subtype predominates in the bovine iris sphincter smooth muscle and ciliary processes. Invest Ophthalmol Vis Sci 31:590–593

Horikawa Y, Shatos MA, Hodges RR, Zoukhri D, Rios JD, Chang EL, Bernardino CR, Rubin PAD, Dartt DA (2003) Activation of mitogen-activated protein kinase by cholinergic agonists and EGF in human compared with rat cultured conjunctival goblet cells. Invest Ophthalmol Vis Sci 44:2535–2544

Hsieh DJ-Y, Liao C-F (2002) Zebrafish M2 muscarinic acetylcholine receptor: cloning, pharmacological characterization, expression patterns and role in embryonic bradycardia. Br J Pharmacol 137:782–792

Irani A-MA, Butrus SI, Tabbara KF, Schwartz LB (1990) Human conjunctival mast cells: distribution of MC_T and MC_{TC} in vernal conjunctivitis and giant papillary conjunctivitis. J Allergy Clin Immunol 86:34–40

Ishida N, Hirai S-I, Mita S (1997) Immunolocalization of aquaporin homologs in mouse lacrimal glands. Biochem Biophys Res Commun 238:891–895

Ishikawa H, DeSantis L, Patil PN (1998) Selectivity of muscarinic agonists including (±)-aceclidine and antimuscarinics on the human intraocular muscles. J Ocul Pharmacol Ther 14:363–373

Ishikawa Y, Cho G, Yuan Z, Inoue N, Nakae Y (2006) Aquaporin-5 water channel in lipid rafts of rat parotid gland. Bichim Biophys Acta 1758:1053–1060

Ishizaka N, Noda M, Yokoyama S, Kawasaki K, Yamamoto M, Higashida H (1998) Muscarinic acetylcholine receptor subtypes in the human iris. Brain Res 787:344–347

Jumblatt JE, Hackmiller RC (1994) M2-type muscarinic receptors mediate prejunctional inhibition of norepinephrine release in the human iris-ciliary body. Exp Eye Res 58:175–180

Kanno H, Horikawa Y, Hodges RR, Zoukhri D, Shatos MA, Ríos JD, Dartt DA (2003) Cholinergic agonists transactivate EGFR and stimulate MAPK to induce goblet cell secretion. Am J Physiol Cell Physiol 284:C988–C998

Kaufman PL, Bárány EH (1976) Loss of acute pilocarpine effect on outflow facility following surgical disinsertion and retrodisplacement of the ciliary muscle from the scleral spur in the cynomolgus monkey. Invest Ophthalmol Vis Sci 15:793–807

Kawashima K, Fujii T (2008) Basic and clinical aspects of non-neuronal acetylcholine: overview of non-neuronal cholinergic systems and their biological significance. J Pharmacol Sci 106: 167–173

Kiland JA, Hubbard WC, Kaufman PL (2000) Low doses of pilocarpine do not significantly increase outflow facility in the cynomolgus monkey. Exp Eye Res 70:603–609

Konttinen YT, Platts LAM, Tuominen S, Eklund KK, Santavirta N, Törnwall J, Sorsa T, Hukkanen M, Polak JM (1997) Role of nitric oxide in Sjögren's syndrome. Arthritis Rheum 40:875–883

Lameh J, Philip M, Sharma YK, Moro O, Ramachandran J, Sadee W (1992) Hm1 muscarinic cholinergic receptor internalization requires a domain in the third cytoplasmic loop. J Biol Chem 267:13406–13412

Lee J-J, Fang P-C, Yang I-H, Chen C-H, Lin P-W, Lin S-A, Kuo H-K, Wu P-C (2006) Prevention of myopia progression with 0.05% atropine solution. J Ocul Pharmacol Ther 22:41–46

Lemullois M, Rossignol B, Mauduit P (1996) Immunolocalization of myoepithelial cells in isolated acini of rat exorbital lacrimal gland: cellular distribution of muscarinic receptors. Biol Cell 86:175–181

Leonardi A, Motterle L, Bortolotti M (2008) Allergy and the eye. Clin Exp Immunol 153 (Suppl 1):17–21

Lepple-Weinhues A, Stahl F, Wiederholt M (1991) Differential smooth muscle-like contractile properties of trabecular meshwork and ciliary muscle. Exp Eye Res 53:33–38

Leung E, Mitchelson F (1982) Modification by hexamethonium of the muscarinic receptor blocking activity of pancuronium and homatropine in isolated tissues of the guinea-pig. Eur J Pharmacol 80:11–17

Leung KCM, McMillan AS, Wong MCM, Leung WK, Mok MY, Lau, CS (2008) The efficacy of cevimeline hydrochloride in the treatment of xerostomia in Sjögren's syndrome in southern Chinese patients: a randomised double-blind, placebo-controlled crossover study. Clin Rheumatol 27: 429–436.

Lin H-J, Wan L, Tsai Y, Chen W-C, Tsai S-W, Tsai F-J (2009) Muscarinic acetylcholine receptor 1 gene polymorphisms associated with high myopia. Mol Vis 15:1774–1780

Lind GJ, Cavanagh HD (1993) Nuclear muscarinic acetylcholine receptors in corneal cells from rabbit. Invest Ophthalmol Vis Sci 34:2943–2952

Lind GJ, Cavanagh HD (1995) Identification and subcellular distribution of muscarinic acetylcholine receptor-related proteins in rabbit corneal and Chinese hamster ovary cells. Invest Ophthalmol Vis Sci 36:1492–1507

Lind GJ, Chew SJ, Marzani D, Wallman J (1998) Muscarinic acetylcholine receptor antagonists inhibit chick scleral chondrocytes. Invest Ophthalmol Vis Sci 39:2217–2231

Liu S, Li J, Tan DTH, Beuerman RW (2007) Expression and function of muscarinic receptor subtypes on human cornea and conjunctiva. Invest Ophthalmol Vis Sci 48:2987–2996

Luft WA, Ming Y, Stell WK (2003) Variable effects of previously untested muscarinic receptor antagonists on experimental myopia. Invest Ophthalmol Vis Sci 44:1330–1338

Lütjen-Drecoll E, Kaufman PL, Bárány EH (1977) Light and electron microscopy of the anterior chamber angle structures following surgical disinsertion of the ciliary muscle in the cynomolgus monkey. Invest Ophthalmol Vis Sci 16:218–225

Luu CD, Lau AMI, Koh AHC, Tan D (2005) Multifocal electroretinogram in children on atropine treatment for myopia. Br J Ophthalmol 89:151–153

Mallorga P, Babilon RW, Buisson S, Sugrue MF (1989) Muscarinic receptors of the albino rabbit ciliary process. Exp Eye Res 48:509–522

Martins RAP, Pearson RA (2008) Control of cell proliferation by neurotransmitters in the developing vertebrate retina. Brain Res 1192:37–60

Masuda Y, Yamahara NS, Tanaka M, Ryang S, Kawai T, Imaizumi Y, Watanabe M (1995) Characterization of muscarinic reptors mediating relaxation and contraction in the rat iris dilator muscle. Br J Pharmacol 114:769–776

Masuda H, Goto M, Tamaoki S, Kamikawatoko S, Tokoro T, Azuma H (1998) M_3-type muscarinic receptors predominantly mediate neurogenic quick contraction of bovine ciliary muscle. Gen Pharmacol 30:579–584

Matsui M, Motomura D, Karasawa H, Fujikawa T, Jiang J, Komiya Y, Takahashi S-I, Taketo MM (2000) Multiple functional defects in peripheral autonomic organs in mice lacking muscarinic acetylcholine receptor gene for the M_3 subtype. Proc Natl Acad Sci USA 97:9579–9584

Matsui M, Motomura D, Fujikawa T, Jiang J, Takahashi S-I, Manabe T, Taketo MM (2002) Mice lacking M_2 and M_3 muscarinic acetylcholine receptors are devoid of cholinergic smooth muscle contractions but still viable. J Neurosci 22:10627–10632

Matsumoto S, Yorio T, DeSantis L, Pang I-H (1994) Muscarinic effects on cellular functions in cultured human ciliary muscle cells. Invest Ophthalmol Vis Sci 35:3732–3738

Mauduit P, Jammes H, Rossignol B (1993) M_3 muscarinic acetylcholine receptor coupling to PLC in rat exorbital lacrimal acinar cells. Am J Physiol 264:C1550–C1560, Cell Physiol 33

McBrien NA, Moghaddam HO, Reeder AP (1993) Atropine reduces experimental myopia and eye enlargement via a nonaccommodative mechanism. Invest Ophthalmol Vis Sci 34: 205–215

McBrien NA, Cottriall CL, Annies R (2001) Retinal acetylcholine content in normal and myopic eyes: a role in ocular growth control? Vis Neurosci 18:571–580

McBrien NA, Jobling AI, Truong HT, Cottriall CL, Gentle A (2009) Expression of muscarinic receptor subtypes in tree shrew ocular tissues and their regulation during development of myopia. Mol Vis 15:464–475

McKinnon LA, Nathanson NM (1995) Tissue-specific regulation of muscarinic acetylcholine receptor expression during embryonic development. J Biol Chem 270:20636–20642

Michon J, Kinoshita JH (1968) Experimental miotic cataract. Arch Ophthalmol 79:79–86

Mindel JS, Mittag TW (1976) Choline acetyltransferase in ocular tissues of rabbits, cats, cattle, and man. Invest Ophthalmol Vis Sci 15:808–814

Motterle L, Diebold Y, Enriquez de Salamanca A, Saez V, Garcia-Vazquez C, Stern ME, Calonge M, Leonardi A (2006) Altered expression of neurotransmitter receptors and neuromediators in vernal keratoconjunctivitis. Arch Ophthalmol 124:462–468

Nadler LS, Rosoff ML, Hamilton SE, Kalaydjian AE, McKinnon LA, Nathanson NM (1999) Molecular analysis of the regulation of muscarinic receptor expression and function. Life Sci 64:375–379

Nagataki S, Brubaker RF (1982) Effect of pilocarpine on aqueous humor formation in human beings. Arch Ophthalmol 100:818–821

Nakajima E, Nakajima T, Minagawa Y, Shearer TR, Azuma M (2005) Contribution of ROCK in contraction of trabecular meshwork: proposed mechanism for regulating aqueous outflow in monkey and human eyes. J Pharm Sci 94:701–708

Narita S, Watanabe M (1982) Response of isolated rat iris dilator to adrenergic and cholinergic agents and electrical stimulation. Life Sci 30:1211–1218

Naruoka H, Kojima R, Ohmasa M, Layer PG, Saito T (2003) Transient muscarinic calcium mobilisation in transdifferentiating as in reaggregating embryonic chick retinae. Brain Res Dev Brain Res 143:233–244

Neal MJ, Cunningham JR (1995) Baclofen enhancement of acetylcholine release from amacrine cells in the rabbit retina by reduction of glycinergic inhibition. J Physiol 482:363–372

Newman EA (2005) Calcium increases in retinal glial cells evoked by light-induced neuronal activity. J Neurosci 25:5502–5510

Nguyen DH, Vadlamudi V, Toshida H, Beuerman RW (2006) Loss of parasympathetic innervation leads to sustained expression of pro-inflammatory genes in the rat lacrimal gland. Auton Neurosci 124:81–89

Olsen JS, Neufeld AH (1979) The rabbit cornea lacks cholinergic receptors. Invest Ophthalmol Vis Sci 18:1216–1225

Ono M, Takamura E, Shinozaki K, Tsumura T, Hamano T, Yagi Y, Tsubota K (2004) Therapeutic effect of cevimeline on dry eye in patients with Sjögren's syndrome: A randomized, double-blind clinical study. Am J Ophthalmol 138: 6–17.

Ota I, Zoukhri D, Hodges RR, Rios JD, Tepavcevic V, Raddassi I, Chen LL, Dartt DA (2003) α_1-Adrenergic and cholinergic agonists activate MAPK by separate mechanisms to inhibit secretion in lacrimal gland. Am J Physiol Cell Physiol 284:C168–C178

Pang I-H, Matsumoto S, Tamm E, DeSantis L (1994) Characterization of muscarinic receptor involvement in human ciliary muscle cell function. J Ocul Pharmacol 10:125–136

Pearson R, Catsicas M, Becker D, Mobbs P (2002) Purinergic and muscarinic modulation of the cell cycle and calcium signaling in the chick retinal ventricular zone. J Neurosci 22:7569–7579

Petrone D, Condemi JJ, Fife R, Gluck O, Cohen S, Dalgin P (2002) A double-blind, randomized, placebo-controlled study of cevimeline in Sjögren's syndrome patients with xerostomia and keratoconjunctivitis sicca. Arthritis Rheum 46:748–754

Phatarpekar PV, Durdan SF, Copeland CM, Crittenden EL, Neece JD, Garcia DM (2005) Molecular and pharmacological characterization of muscarinic receptors in retinal pigment epithelium: role in light-adaptive pigment movements. J Neurochem 95:1504–1520

Potts AM (1965) The effects of drugs upon the eye. In: Root WS, Hofmann FG (eds) Physiological pharmacology, vol II, The nervous system part B, central nervous system drugs. Academic, New York, pp 329–397

Poyer JF, Gabelt BT, Kaufman PL (1994) The effect of muscarinic agonists and selective receptor subtype antagonists on the contractile response of the isolated rhesus monkey ciliary muscle. Exp Eye Res 59:729–736

Qiong L, Jie W, Xinmei W, Junwen Z (2007) Changes in muscarinic acetylcholine receptor expression in form deprivation myopia in guinea pigs. Mol Vis 13:1234–1244

Qu J, Zhou X, Zhang L, Hu D, Li H, Lu F (2006) The presence of m1 to m5 receptors in human sclerea: evidence of the sclera as a potential site of action for muscarinic receptor antagonists. Curr Eye Res 31:587–597

Ríos JD, Zoukhri D, Rawe IM, Hodges RR, Zieske JD, Dartt DA (1999) Immunolocalization of muscarinic and VIP receptor subtypes and their role in stimulating goblet cell secretion. Invest Ophthalmol Vis Sci 40:1102–1111

Ríos JD, Forde K, Diebold Y, Lightman J, Zieske JD, Dartt DA (2000) Development of conjunctival goblet cells and their neuroreceptor subtype expression. Invest Ophthalmol Vis Sci 41:2127–2137

Salceda R (1994) Muscarinic receptors binding in retinal pigment epithelium during rat development. Neurochem Res 19:1207–1210

Satoh Y, Sano K, Habara Y, Kanno T (1997) Effects of carbachol and catecholamines on ultrastructure and intracellular calcium-ion dynamics of acinar and myoepithelial cells of lacrimal glands. Cell Tissue Res 289:473–485

Saw S-M, Katz J, Schein OD, Chew S-J, Chan T-K (1996) Epidemiology of myopia. Epidemiol Rev 18:175–187

Saw S-M, Goh P-P, Cheng A, Shanka A, Tan DTH, Ellwein LB (2006) Ethnicity-specific prevalences of refractive errors vary in Asian children in neighbouring Malaysia and Singapore. Br J Ophthalmol 90:1230–1235

Schwahn HN, Kaymak H, Schaeffel F (2000) Effects of atropine on refractive development, dopamine release, and slow retinal potentials in the chick. Vis Neurosci 17:165–176

Seet B, Wong TY, Tan DTH, Saw S-M, Balakrishnan V, Lee LKH, Lim ASM (2001) Myopia in Singapore: taking a public health approach. Br J Ophthalmol 85:521–526

Sekiguchi-Tonosaki M, Obata M, Haruki A, Himi T, Kosaka J (2009) Acetylcholine induces Ca^{2+} signaling in chicken retinal pigmented epithelial cells during deafferentiation. Am J Physiol Cell Physiol 296:C1195–C1206

Shade DL, Clark AF, Pang I-H (1996) Effects of muscarinic agents on cultured human trabecular meshwork cells. Exp Eye Res 62:201–210

Shatos MA, Rios JD, Tepavcevic V, Kano H, Hodges R, Dartt DA (2001) Isolation, characterization and propagation of rat conjunctival goblet cells in vitro. Invest Ophthalmol Vis Sci 42:1455–1464

Shepard AR, Rae JL (1998) Ion transporters and receptors in cDNA libraries from lens and cornea epithelia. Curr Eye Res 17:708–719

Shiraishi K, Takayanagi I (1993) Subtype of muscarinic receptors mediating relaxation and contraction in the rat iris dilator smooth muscle. Gen Pharmacol 24:139–142

Shirvan MH, Pollard HB, Heldman E (1991) Mixed nicotinic and muscarinic features of cholinergic receptor coupled to secretion in bovine chromaffin cells. Proc Natl Acad Sci USA 88:4860–4864

Siatkowski RM, Cotter SA, Crockett RS, Miller JM, Novack GD, Zadnick K, For the U.S. Pirenzepine Study Group (2008) Two-year multicenter, randomized, double-masked, placebo-controlled, parallel safety and efficacy study of 2% pirenzepine ophthalmic gel in children with myopia. J AAPOS 12:332–339

Socci RR, Tachado SD, Aronstam RS, Reinach PS (1996) Characterization of muscarinic receptor subtypes in the bovine corneal epithelial cells. J Ocul Pharmacol Ther 12:259–269

Steinhausen K, Stumpff F, Strauss O, Thieme H, Wiederholt M (2000) Influence of muscarinic agonists and tyrosine kinase inhibitors on L-type Ca^{2+} channels in human and bovine trabecular meshwork cells. Exp Eye Res 70:285–293

Steinle JJ, Smith PG (2000) Presynaptic muscarinic facilitation of parasympathetic neurotransmission after sympathectomy in the rat choroid. J Pharmacol Exp Ther 294:627–632

Stone RA, Lin T, Laties AM (1991) Muscarinic antagonist effects on experimental chick myopia. Exp Eye Res 52:755–758

Strang CE, Renna JM, Amthor FR, Keyser KT (2010) Muscarinic acetylcholine receptor localization and activation effects on ganglion response properties. Invest Ophthalmol Vis Sci 51:2778–2789

Sugrue MF (1989) The pharmacology of antiglaucoma drugs. Pharmacol Ther 43:91–138

Sun T-T, Lavker RM (2004) Corneal epithelial stem cells: past, present and future. J Investig Dermatol Symp Proc 9:202–207

Suzuki R, Oso T, Koboyashi S (1983) Cholinergic inhibitory response in the bovine iris dilator muscle. Invest Ophthalmol Vis Sci 24:760–765

Tachado SD, Virdee K, Akhtar RA, Abdel-Latif AA (1994) M3 Muscarinic receptors mediate an increase in both inositol trisphosphate production and cyclic AMP formation in dog iris sphincter smooth muscle. J Ocul Pharmacol 10:137–147

Takayanagi I, Kokubu N, Shiraishi K (1993) Inhibitory effect of pilocarpine on norepinephrine release from electrically stimulated iris dilator muscles of rabbits as a most possible mechanism of pilocarpine-induced miosis. J Smooth Muscle Res 29:55–61

Tan DTH, Chua WH, Lam DS, Shu-Ping DF, Crockett RS, For the Asian Pirenzepine Study Group (2005) One-year multicenter, double-masked, placebo-controlled, parallel safety and efficacy study of 2% pirenzepine ophthalmic gel in children with myopia. Ophthalmology 112:84–91

ten Tusscher MPM, Klooster J, Vrensen GFJM (1988) The innervation of the rabbit's anterior eye segment: a retrograde tracing study. Exp Eye Res 46:717–730

Teus MA, Castejón MA, Calvo MA, Fagúndez MA (1997) Ocular hypotensive effect of pilocarpine before and after argon laser trabeculoplasty. Acta Ophthalmol Scand 75:503–506

Thieme H, Nass JU, Nuskovski M, Bechrakis NE, Stumpff F, Strauss O, Wiederholt M (1999) The effects of protein kinase C (PKC) on trabecular meshwork and ciliary muscle contractility. Invest Ophthalmol Vis Sci 40:3254–3261

Thieme H, Hildebrandt J, Choritz L, Strauss O, Wiederholt M (2001) Muscarinic receptors of the M2 subtype in human and bovine trabecular meshwork. Gräefes Arch Clin exp Ophthalmol 239:310–315

Thomas GR, Williams MR, Sanderson S, Duncan G (1997) The human lens possesses acetylcholine receptors that are functional throughout life. Exp Eye Res 64:849–852

Tian B, Gabelt BT, Peterson JA, Kiland JA, Kaufman PL (1999) H-7 increases trabecular facility and facility after ciliary muscle disinsertion in monkeys. Invest Ophthalmol Vis Sci 40:239–242

Tigges M, Iuvone PM, Fernandes A, Sugrue MF, Mallorga PJ, Laties AM, Stone RA (1999) Effects of muscarinic cholinergic receptor antagonists on postnatal eye growth of rhesus monkeys. Optom Vis Sci 76(6):397–407

Tong L, Huang XL, Koh ALT, Zhang X, Tan DTH, Chua W-H (2009) Atropine for the treatment of childhood myopia: effect on myopia progression after cessation of atropine. Ophthalmology 116:572–579

Toris CB, Alm A, Camras CB (2002) Latanoprost and cholinergic agonists in combination. Surv Ophthalmol 47(Suppl 1):S141–S147

Townes-Anderson E, Vogt BA (1989) Distribution of muscarinic acetylcholine receptors on processes of isolated retinal cells. J Comp Neurol 290:369–383

Truong H-T, Cottriall CL, Gentle A, McBrien NA (2002) Pirenzepine affects scleral metabolic changes in myopia through a non-toxic mechanism. Exp Eye Res 74:103–111

Valant C, Gregory KJ, Hall NE, Scammells PJ, Lew MJ, Sexton PM, Christopoulos A (2008) A novel mechanism of G-protein coupled receptor functional selectivity. J Biol Chem 283:29312–29321

Verbitsky M, Rothlin CV, Katz E, Elgoyhen AB (2000) Mixed nicotinic-muscarinic properties of the α9 nicotinic cholinergic receptor. Neuropharmacology 39:2515–2524

Vessey KA, Cottriall CL, McBrien NA (2002) Muscarinic receptor protein expression in the ocular tissues of the chick during normal and myopic eye development. Brain Res Dev Brain Res 135:79–86

Vitali C, Bombardieri S, Jonsson R, Moutsopoulos HM, Alexander EL, Carsons SE, Daniels TE, Fox PC, Fox RI, Kassan SS, Pillemer SR, Talal N, Weisman MH, The European Study Group on Classification Criteria for Sjögren's Syndrome (2002) Classification criteria for Sjögren's syndrome: a revised version of the European criteria proposed by the American-European consensus group. Ann Rheum Dis 61:554–558

Vivino FB, Al-Hashimi I, Khan Z, LeVeque FG, Salisbury PL, Tran-Johnson TK, Muscoplat CC, Trivedi M, Goldlust B, Gallagher SC (1999) Pilocarpine tablets for the treatment of dry mouth and dry eye symptoms in patients with Sjögren's syndrome. A randomized, placebo-controlled, fixed-dose, multicenter trial. Arch Intern Med 159:174–181

Wakakura M, Utsunomiya-Kawasaki I, Ishikawa S (1998) Rapid increase in cytosolic calcium ion concentration mediated by acetylcholine receptors in cultured retinal neurons and Müller cells. Graefes Arch Clin Exp Ophthalmol 236:934–939

Walkenbach RJ, Ye G-S (1991) Muscarinic cholinoceptor regulation of cyclic guanosine monophosphate in human corneal epithelium. Invest Ophthalmol Vis Sci 32:610–615

Walkenbach RJ, Ye GS, Boney F, Duecker DK (1993) Muscarinic cholinoceptors in native and cultured human corneal endothelium. Curr Eye Res 12:155–162

Waterman SA, Gordon TP, Rischmueller M (2000) Inhibitory effects of muscarinic receptor autoantibodies on parasympathetic neurotransmission in Sjögren's syndrome. Arthritis Rheum 43:1647–1654

Wei ZG, Sun T-T, Lavker RM (1996) Rabbit conjunctival and corneal epithelial cells belong to two separate lineages. Invest Ophthalmol Vis Sci 37:523–533

Wessler I, Kilbinger H, Bittinger F, Unger R, Kirkpatrick CJ (2003) The non-neuronal cholinergic system in humans: expression, function and pathophysiology. Life Sci 72:2055–2061

Wiederholt M, Bielka S, Schweig F, Lütjen-Drecoll E, Lepple-Weinhues A (1995) Regulation of outflow rate and resistance in the perfused anterior segment of the bovine eye. Exp Eye Res 61:223–234

Wiederholt M, Thieme H, Stumpff F (2000) The regulation of trabecular meshwork and ciliary muscle contractility. Prog Retin Eye Res 19:271–295

Wiesel TN, Raviola E (1977) Myopia and eye enlargement after neonatal lid fusion in monkeys. Nature 266:66–68

Williams JD, Cooper JR (1965) Acetylcholine in bovine corneal epithelium. Biochem Pharmacol 14:1286–1289

Williams MR, Duncan G, Riach RA, Webb SF (1993) Acetylcholine receptors are coupled to mobilization of intracellular calcium in cultured human lens cells. Exp Eye Res 57: 381–384

Williams MR, Riach RA, Collison DJ, Duncan G (2001) Role of the endoplasmic reticulum in shaping calcium dynamics in human lens cells. Invest Ophthalmol Vis Sci 42:1009–1017

WoldeMussie E, Feldmann BJ, Chen J (1993) Characterization of muscarinic receptors in cultured human iris sphincter and ciliary smooth muscle cells. Exp Eye Res 56:385–392

Wong ROL (1995) Cholinergic regulation of $[Ca^{2+}]_i$ during cell division and differentiation in the mammalian retina. J Neurosci 15:2696–2706

Yamada ES, Dmitrieva N, Keyser KT, Lindstrom JM, Hersch LB, Marshak DW (2003) Synaptic connections of starburst amacrine cells and localization of acetylcholine receptors in primate retinas. J Comp Neurol 461:76–90

Yamahara NS, Tanaka M, Imaizumi Y, Watanabe M (1995) Pertussis toxin-sensitive muscarinic relaxation in the rat iris dilator muscle. Br J Pharmacol 114:777–784

Yamashita M, Fukuda Y (1993) Incurvation of early embryonic neural retina by acetylcholine through muscarinic receptors. Neurosci Lett 163:215–218

Yamashita M, Yoshimoto Y, Fukuda Y (1994) Muscarinic acetylcholine responses in the early embryonic chick retina. J Neurobiol 25:1144–1153

Yin GC, Gentle A, McBrien NA (2004) Muscarinic antagonist control of myopia: a molecular search for the M1 receptor in chick. Mol Vis 10:787–793

Yoshitomi T, Ito Y (1986) Double reciprocal innervations in dog iris sphincter and dilator muscles. Invest Ophthalmol Vis Sci 27:83–91

Yoshitomi T, Ito Y, Inomata H (1985) Adrenergic excitatory and cholinergic inhibitory innervations in the human iris dilator. Exp Eye Res 40:453–459

Zhang X, Hernandez MR, Yang H, Erickson K (1995a) Expression of muscarinic receptor subtype mRNA in the human ciliary muscle. Invest Ophthalmol Vis Sci 36:1645–1657

Zhang Y, Araki-Sasaki K, Handa H, Akhtar RA (1995b) Effect of carbachol on phospholipase C-mediated phosphatidylinositol 4, 5-bisphosphate hydrolysis, and its modulation by isoproterenol in rabbit corneal epithelial cells. Curr Eye Res 14:563–571

Zhou ZJ, Zhao D (2000) Coordinated transitions in neurotransmitter systems for the initiation and propagation of spontaneous retinal waves. J Neurosci 20:6570–6577

Zoukhri D, Dartt DA (1995) Cholinergic activation of phospholipase D in lacrimal gland acini is independent of protein kinase C and calcium. Am J Physiol 268:C713–C720, Cell Physiol 37

Zoukhri D, Hodges RR, Sergheraert C, Toker A, Dartt DA (1997a) Lacrimal gland PKC isoforms are differentially involved in agonist-induced protein secretion. Am J Physiol 272:C263–C269, Cell Physiol 41

Zoukhri D, Hodges RR, Willert S, Dartt DA (1997b) Immunolocalization of lacrimal gland PKC isoforms. Effect of phorbol esters and cholinergic agonists on their cellular distribution. J Membr Biol 157:169–175

Zoukhri D, Hodges RR, Sergheraert C, Dartt DA (2000) Cholinergic-induced Ca^{2+} elevation in rat lacrimal gland acini is negatively modulated by PKCδ and PKCε. Invest Ophthalmol Vis Sci 41:386–392

Zucker CL, Nilson JE, Ehinger B, Grzywacz NM (2005) Compartmental localization of γ-aminobutyric acid type B receptors in the cholinergic circuitry of the rabbit retina. J Comp Neurol 493:448–459

Muscarinic Receptor Agonists and Antagonists: Effects on Cardiovascular Function

Robert D. Harvey

Abstract Muscarinic receptor activation plays an essential role in parasympathetic regulation of cardiovascular function. The primary effect of parasympathetic stimulation is to decrease cardiac output by inhibiting heart rate. However, pharmacologically, muscarinic agonists are actually capable of producing both inhibitory and stimulatory effects on the heart as well as vasculature. This reflects the fact that muscarinic receptors are expressed throughout the cardiovascular system, even though they are not always involved in mediating parasympathetic responses. In the heart, in addition to regulating heart rate by altering the electrical activity of the sinoatrial node, activation of M_2 receptors can affect conduction of electrical impulses through the atrioventricular node. These same receptors can also regulate the electrical and mechanical activity of the atria and ventricles. In the vasculature, activation of M_3 and M_5 receptors in epithelial cells can cause vasorelaxation, while activation of M_1 or M_3 receptors in vascular smooth muscle cells can cause vasoconstriction in the absence of endothelium. This review focuses on our current understanding of the signaling mechanisms involved in mediating these responses.

Keywords Blood vessels • Cardiac muscle • Heart • Vascular endothelium • Vascular smooth muscle

1 Introduction

The parasympathetic branch of the autonomic nervous system plays an integral role in regulating the cardiovascular system. In general, parasympathetic stimulation tends to produce responses that counterbalance those that are associated with

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activation of the sympathetic nervous system (Levy 1971; Löffelholz and Pappano 1985). The tightly orchestrated interactions between sympathetic and parasympathetic responses are essential to maintaining homeostasis of the cardiovascular system under a variety of conditions.

Sympathetic stimulation increases cardiac output by increasing heart rate and contractility through the effects of the neurotransmitter norepinephrine acting on cardiac beta-adrenergic receptors (Bers 2001). Sympathetic stimulation also increases vascular resistance by stimulating vasoconstriction through the effects of norepinephrine acting on alpha-adrenergic receptors in vascular smooth muscle cells (Hirst and Edwards 1989). The primary effect associated with parasympathetic stimulation, on the other hand, is to decrease cardiac output by decreasing heart rate through the effects of the neurotransmitter acetylcholine (ACh) acting on muscarinic receptors (Hartzell 1988; Levy 1971; Löffelholz and Pappano 1985). Under most conditions, parasympathetic stimulation has little effect on cardiac (ventricular) contractility (Levy and Martin 1989). Furthermore, parasympathetic stimulation exerts limited influence on most blood vessels and is not a major factor in regulating total peripheral resistance (Eglen and Whiting 1990; Furchgott and Vanhoutte 1989).

Based on the simplified generalizations just described, the perception is often that muscarinic signaling pathways play an important, yet perhaps more limited physiologic role in regulating cardiovascular function. Yet this notion belies the fact that muscarinic receptors are abundant throughout the cardiovascular system (Eglen and Whiting 1990; Löffelholz and Pappano 1985). As a result, muscarinic agonists as well as antagonists can have profound pharmacologic effects on many aspects of cardiovascular function not normally thought to be under significant parasympathetic influence. For example, muscarinic receptor agonists can actually produce a significant decrease in ventricular contractility in the presence of elevated sympathetic tone (Levy 1977, 1995). Likewise, muscarinic receptor agonists can cause vasodilation of most blood vessels, resulting in a decrease in total peripheral resistance (Furchgott and Zawadzki 1980). These observations, and others, illustrate the more complex nature of the role that muscarinic responses may play in regulating cardiovascular function in health and disease.

2 Cardiovascular Muscarinic Receptors

Five muscarinic receptor subtypes have been identified: M_1, M_2, M_3, M_4, and M_5 (Hulme et al. 1990). In the heart, pharmacologic evidence indicates that most functional responses are associated with activation of M_2 receptors (Harvey and Belevych 2003). This is supported by the inability of ACh to produce bradycardia in mice where expression of the M_2 receptor has been knocked out (Stengel et al. 2000). On the other hand, M_3 receptors appear to play a dominant role in ACh-induced vasodilation of most blood vessels (Beny et al. 2008; Khurana et al. 2004). It should be noted that these are broad generalizations, and that other muscarinic

receptor subtypes have been reported to produce effects in different cell types throughout the cardiovascular system that may or may not be involved in the responses described above. There are also some species-dependent differences in the subtype of receptor associated with different responses (Dhein et al. 2001; Eglen and Whiting 1990).

In general, the signaling pathways most often associated with even-numbered muscarinic receptors involve the heterotrimeric G protein G_i coupled to the inhibition of adenylyl cyclase or the regulation of G protein activated inward rectifying K^+ (GIRK) channels (Lanzafame et al. 2003). Whereas the signaling pathway commonly associated with odd-numbered muscarinic receptors involves G_q activation of phospholipase C (PLC) and subsequent production of diacylglycerol (DAG) and inositoltriphosphate (IP_3) (Lanzafame et al. 2003). While these generalizations explain many of the responses that are mediated by muscarinic receptors in the heart and vasculature, there is evidence that additional signaling mechanisms are important as well.

3 Cardiac Muscarinic Responses

Activation of M_2 muscarinic receptors decreases heart rate by slowing the rate of spontaneous action potential firing in the sinoatrial (SA) node (Irisawa et al. 1993). However, muscarinic agonists can produce significant changes in electrical as well as mechanical function of myocytes throughout all regions of the heart. In the atrioventricular (AV) node, muscarinic stimulation slows the conduction of electrical impulses (Martin 1977). This effect plays a critical role in regulating the propagation of action potentials between the atria and ventricles. The dominant effect that parasympathetic stimulation has on the SA and AV nodes parallels the fact that myocytes that make up the SA and AV node have a greater density of muscarinic receptors and are more heavily innervated by the parasympathetic nervous system than myocytes in other regions of the heart (Löffelholz and Pappano 1985).

Under normal resting conditions, the heart receives significant input from the parasympathetic nervous system. The consequence is that tonic muscarinic receptor activation actually inhibits the intrinsic rate of firing of pacemaker cells and slows heart rate (Levy 1977). The tonic influence of the parasympathetic nervous system also slows AV conduction (Martin 1977). Pharmacologically this is important because muscarinic receptor antagonists such as atropine can increase intrinsic heart rate and facilitate AV conduction. On the other hand, resting sympathetic tone has a less pronounced effect on the heart. This contributes to the misconception that muscarinic receptor stimulation plays little role in regulating ventricular function (see below) (Levy 1995).

The principal effects of parasympathetic stimulation often reflect changes in SA and AV node function. Nevertheless, there is also significant parasympathetic innervation of the atria as well as the ventricles (Standish et al. 1994, 1995), and

muscarinic receptors are expressed throughout all areas of the heart, including the ventricular myocardium (Löffelholz and Pappano 1985). In atrial cells, the primary effect of muscarinic stimulation is a decrease in action potential duration. In ventricular tissue, muscarinic receptor activation has little effect unless it occurs in the presence of concurrent β-adrenergic receptor activation. The primary effect of β-adrenergic stimulation on ventricular function is to increase contractility and stroke volume. Therefore, in the presence of β-adrenergic stimulation, M_2 muscarinic receptor activation can have a significant inhibitory effect on ventricular contractility.

Autonomic responses involved in producing changes in cardiac output, such as those associated with baroreceptor reflexes, are often thought of doing so by altering sympathetic and parasympathetic tone in a reciprocal fashion. For example, the normal autonomic response to an increase in blood pressure detected by arterial baroreceptors would be to decrease sympathetic tone, while at the same time increasing parasympathetic tone. Under those circumstances, parasympathetic activation of muscarinic receptors would be expected to decrease heart rate, while having little or no effect on ventricular contractility. However, there are situations where both sympathetic and parasympathetic activity to the heart change in parallel (Paton et al. 2005). For example, hypoxic chemoreceptor responses (Koizumi et al. 1982) and conditions such as sleep apnea (Leung 2009) are associated with increases in both sympathetic and parasympathetic tone. Under such circumstances, parasympathetic stimulation and muscarinic receptor activation would be expected to have a significant effect on ventricular function.

Another common misconception is that muscarinic receptors in the cardiovascular system are always associated with inhibitory responses. The fact is they are linked to stimulatory effects as well (Dhein et al. 2001; Harvey and Belevych 2003). Perhaps most prominent example in the heart is the rebound stimulatory response observed upon termination of muscarinic receptor activation. This type of stimulatory effect reflects the fact that M_2 receptors simultaneously activate inhibitory and stimulatory signaling pathways. The inhibitory effect tends to dominate the stimulatory response in the presence of muscarinic receptor activation. However, the kinetics of the two responses are distinctly different. The inhibitory effect turns on and off rapidly while the stimulatory response turns on and off much more slowly. This type of rebound stimulatory response has been described in both atrial and ventricular myocytes, and it is believed to be responsible for rebound increases in heart rate and contractility observed during transient changes in vagal stimulation (Harvey and Belevych 2003).

3.1 Muscarinic Receptor Activation of GIRK Channels

One of the primary effects that muscarinic stimulation has on cardiac function is a slowing of the heart rate. This response is due to activation of M_2 receptors in the SA node, and a subsequent decrease in the firing rate of the spontaneous, slow

response action potentials that are characteristic of the cells that make up this region of the heart (Irisawa et al. 1993). Activation of M_2 receptors in the SA node results in a hyperpolarization of the maximum diastolic potential as well as a slowing of the rate of spontaneous depolarization. Both of these effects may contribute to a decrease in the overall rate of firing by increasing the time it takes the membrane potential to reach threshold and fire an action potential.

Hyperpolarization of the maximum diastolic potential produced by muscarinic receptor activation is due to an increase in the open probability of GIRK channels (Sakmann et al. 1983). These are the ion channels that generate the ACh-activated K^+ current ($I_{K(ACh)}$) found in SA nodal cells, atrial cells, AV nodal cells, as well as ventricular myocytes of some species. The GIRK channel family consists of four members: GIRK1, GIRK2, GIRK3, and GIRK4. In the heart, $I_{K(ACh)}$ is generated by a heterotetrameric channel consisting of GIRK1 and GIRK4 (Krapivinsky et al. 1995). The actual functional role of these channels varies, depending on the cell type in which they are found. However, much of what we know about the molecular basis for regulation of these channels actually comes from work conducted using atrial myocytes.

Evidence as to actual mechanism linking M_2 receptor activation to changes in channel activity was demonstrated by a series of elegant experiments by Soejima and Noma (1984). They found that the open probability of these channels was only affected when ACh was able to activate receptors in close proximity to the channel. This suggested that the signaling mechanism does not involve a diffusible second messenger. The idea that receptor activation of $I_{K(ACh)}$ involves a G protein-dependent mechanism came from studies demonstrating the requirement for intracellular GTP in order to activate the current (Kurachi et al. 1986a, b). The fact that receptor activation of $I_{K(ACh)}$ could also be blocked by pertussis-toxin (PTX) indicated that the G protein involved was either G_i or G_o (Kurachi et al. 1986a; Pfaffinger et al. 1985). These observations ultimately led to the idea that the receptor and channel are coupled by a membrane-delimited mechanism, whereby the channel was activated by direct interaction with the G protein. The question then became whether or not the channel was being regulated by the α or $\beta\gamma$ subunits of the activated G protein. Although studies were published supporting both possibilities, it is now generally accepted that activation of $I_{K(ACh)}$ in cardiac myocytes involves the direct interaction of the channel with the $\beta\gamma$ subunits of G_i (see Fig. 1) (Kurachi 1995).

In atrial myocytes, muscarinic activation of $I_{K(ACh)}$ plays a much different role in regulation of cellular function. Atria are actually made up of an inhomogeneous population of cells with varying properties. Some cells exhibit spontaneous electrical activity, while others are quiescent, but they all have a diastolic membrane potential that is typically much more negative than that found in SA nodal cells. As such, the effect that muscarinic activation of $I_{K(ACh)}$ has on the diastolic membrane potential is not as pronounced. Instead, the most significant effect that activation of these channels has on atrial cells is a reduction in action potential duration (Ten Eick et al. 1976). Because $I_{K(ACh)}$ channels are weak inward rectifiers, they can contribute significantly to the conductance of the membrane during the plateau of

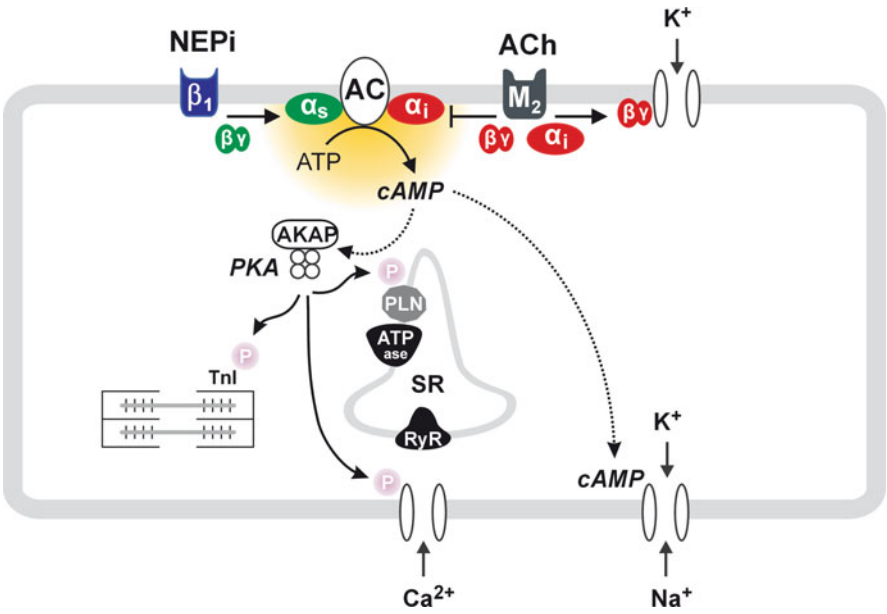

Fig. 1 Muscarinic signaling pathways in supraventricular (sinoatrial, atrial, and atrioventricular) myocytes. Acetylcholine (ACh) acts through M_2 receptors to regulate ACh-activated K^+ channels via a membrane-delimited mechanism involving direct activation by the βγ subunits of the inhibitory G protein G_i. ACh also acts through M_2 receptors to inhibit adenylyl cyclase (AC) activity via the α subunit (α_i) of G_i, resulting in a decrease in cAMP production. This may occur in the absence or presence of agonists that stimulate cAMP production. Norepinephrine (NEPi) acts through β_1-adrenergic receptors to stimulate cAMP synthesis by directly activating all isoforms of adenylyl cyclase (AC) via the α subunit (α_s) of the stimulatory G protein G_s. Changes in cAMP affect targets of protein kinase A (PKA)-dependent phosphorylation such as tropinin I (TnI), phospholamban (PLN), and the L-type Ca^{2+} channel. Changes in cAMP also directly regulate pacemaker channels, which are permeable to both Na^+ and K^+

the action potential, facilitating repolarization. The decrease in action potential duration may also be explained in part by a reduction in cAMP-dependent regulation of the L-type Ca^{2+} current (see below). As a result of the decrease in action potential duration, there is also a decrease in the effective refractory period. This renders these cells more susceptible to excitation by a premature stimulus. This may increase the susceptibility of the atria to arrhythmias (Kovoor et al. 2001). In fact, inhibiting the activation of these channels has been suggested as a treatment for atrial fibrillation (Hashimoto et al. 2006).

Muscarinic activation of $I_{K(ACh)}$ in the AV node plays an important role in regulating action potential propagation. Under normal conditions, the AV node is the only pathway for impulses that originate in the SA node and pass through the atria to reach the ventricles. As such, the AV node plays an essential role in regulating the propagation of impulses from the atria to the ventricles. Activation of muscarinic receptors in the AV node produces a negative dromotropic effect,

or a slowing of impulse propagation. Activation of $I_{K(ACh)}$ may contribute a slowing of conduction by reducing the excitability of AV nodal cells. Reduction in the cAMP-dependent regulation of the L-type Ca^{2+} current may contribute to this effect as well (Nishimura et al. 1988).

Acetylcholine-activated K^+ channels have also been identified in ventricular myocytes of certain species, including frog, ferret, rat, and human (Endoh 1999). However, in those species in which these channels are present in ventricular myocytes, $I_{K(ACh)}$ density is significantly less than that of atrial myocytes. Furthermore, at least in human ventricular myocytes, the channels appear to be much less sensitive to activation by ACh than they are in atrial cells (Koumi and Wasserstrom 1994).

3.2 Muscarinic Regulation of cAMP-Dependent Responses

The other important signaling pathway associated with muscarinic receptor activation in the heart involves modulation of cAMP-dependent responses. As indicated above, the effects of parasympathetic stimulation oppose many of the actions associated with sympathetic stimulation, and sympathetic stimulation exerts many of its acute effects in the heart through β-adrenergic receptor-dependent activation of adenylyl cyclase and subsequent production of cAMP. This pathway modulates a number of key proteins involved in regulating the electrical and mechanical activity of cardiac myocytes (Bers 2002). M_2 muscarinic receptor stimulation can modulate these cAMP-dependent responses through one or more indirect signaling pathways (Harvey and Belevych 2003).

3.2.1 Muscarinic Inhibition of cAMP-Dependent Responses

The dominant effect that M_2 receptor activation has on cAMP-dependent responses is inhibitory and is referred to as "accentuated antagonism" (Levy 1971). This term reflects the fact that the inhibitory response is more prominent in the presence of elevated sympathetic tone. It has been suggested that this type of inhibitory response involves both indirect and direct actions of ACh. The indirect mechanism involves activation of muscarinic receptors on postganglionic sympathetic nerve terminals, which inhibits the release of norepinephrine, preventing subsequent activation of cardiac β-adrenergic receptors. However, muscarinic receptor activation can inhibit responses mediated by β-adrenergic receptor stimulation in isolated myocytes. This demonstrates cAMP-dependent responses can be inhibited by direct activation of cardiac M_2 receptors.

Multiple mechanisms have been suggested to explain how M_2 receptor activation antagonizes cAMP-dependent responses. Perhaps the most widely accepted explanation is based on studies demonstrating that exposure to ACh can reduce cAMP levels in cardiac tissue (Hartzell 1988; Löffelholz and Pappano 1985). This

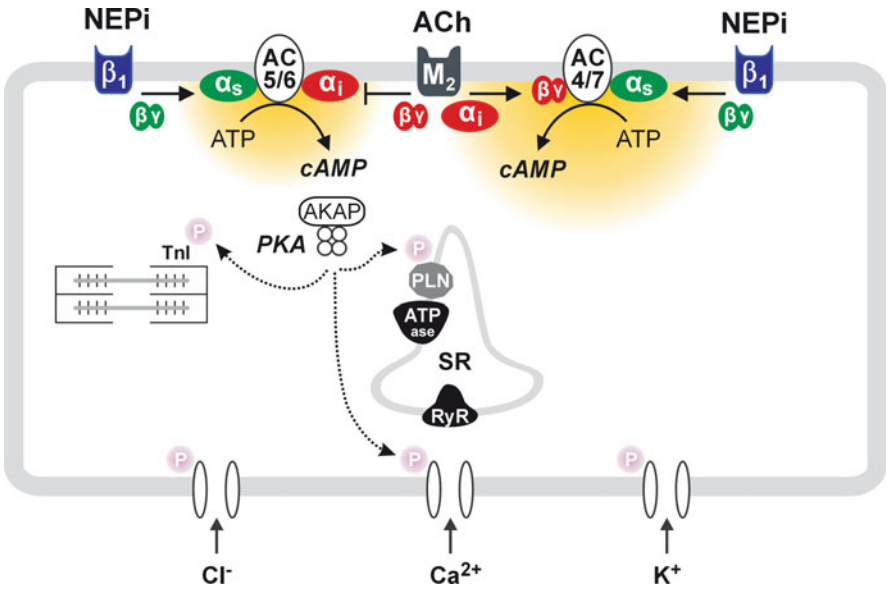

Fig. 2 Muscarinic signaling pathways in ventricular myocytes. Responses to M_2 receptor activation are only observed in the presence of agonists that stimulate cAMP production. Norepinephrine (NEPi) acts through β_1-adrenergic receptors to stimulate cAMP synthesis by directly activating all isoforms of adenylyl cyclase (AC) via the α subunit (α_s) of the stimulatory G protein G_s. Acetylcholine (ACh) acts through M_2 receptors to inhibit AC5/6 activity via the α subunit (α_i) of the inhibitory G protein G_i. ACh acting through M_2 receptors can also stimulate AC4/7 activity via the βγ subunits of G_i. Changes in cAMP affect targets of protein kinase A (PKA)-dependent phosphorylation such as tropinin I (TnI), phospholamban (PLN), as well as L-type Ca^{2+}, delayed rectifier K^+, and CFTR Cl^- channels

effect is due to inhibition of AC activity by a mechanism involving a PTX-sensitive G protein (Endoh et al. 1985). Subsequent biochemical studies have demonstrated that two isoforms of AC expressed in cardiac muscle (AC5 and AC6) can be inhibited by direct interaction with the activated α subunit of the PTX-sensitive G proteins, G_i and G_o (Sunahara et al. 1996). This supports the idea that ACh can antagonize β-adrenergic responses by inhibiting cAMP synthesis (see Fig. 2).

Early studies also demonstrated that exposure to ACh is associated with the production of cGMP in cardiac tissue (George et al. 1970, 1972; Watanabe and Besch 1975). It has been proposed that M_2 receptors stimulate cGMP synthesis through the regulation of endothelial nitric oxide synthase (eNOS) and subsequent production of nitric oxide (NO), which then activates soluble guanylyl cyclase. Furthermore, exogenous cGMP has been reported to inhibit cAMP-dependent responses by activating protein kinase G (PKG) or stimulating type 2 phosphodiesterase activity (Harvey and Belevych 2003; Méry et al. 1997). However, correlations between the effects of ACh and cGMP production have been inconsistent (Hartzell 1988; Löffelholz and Pappano 1985). Furthermore, most studies have found that M_2 receptor antagonism of cAMP response are intact in cardiac

myocytes isolated from the hearts of adult mice in which there has been targeted disruption of eNOS (Belevych and Harvey 2000; Gödecke et al. 2001; Vandecasteele et al. 1999).

Other studies have suggested that the inhibitory effects of ACh do not always correlate with changes in cAMP levels (Hartzell 1988; Lindemann and Watanabe 1989). This has led some to conclude that ACh might antagonize cAMP-dependent responses by stimulating phosphatase activity and enhancing dephosphorylation of proteins phosphorylated by PKA (Ahmad et al. 1989; Gupta et al. 1994). Although such a mechanism could contribute at least partially to the ability of ACh to antagonize cAMP-dependent responses, it has not been possible to demonstrate that ACh directly stimulates the rate of protein dephosphorylation in cardiac myocytes (Stemmer et al. 2000). Furthermore, this mechanism cannot explain the ability of M_2 receptor activation to antagonize responses that do not depend on PKA-dependent phosphorylation, such as direct cAMP-dependent regulation of pacemaker channels (DiFrancesco and Tortora 1991). Dissociation of responses to ACh and changes in cAMP levels may reflect the fact that muscarinic receptor activation appears to affect cAMP production in localized subcellular domains that may be difficult to detect depending on the methods used (Hartzell 1988; Iancu et al. 2007). More recent studies have clearly demonstrated that muscarinic receptor activation causes changes in cAMP activity that can be directly observed in intact, isolated cardiac myocytes using newly developed biosensors (Iancu et al. 2008; Warrier et al. 2005).

The functional consequence of M_2 receptor inhibition of cAMP production varies depending on the cell type involved. In the SA node, muscarinic inhibition of cAMP production contributes to the decrease in heart rate by reversing the effect that cAMP has on the pacemaker channels (DiFrancesco 2010). These channels are regulated by a PKA-independent mechanism that involves direct interaction with cAMP (see Fig. 1). Binding of cAMP shifts the voltage dependence of these channels in a depolarizing direction. This increases their contribution to spontaneous depolarization of the membrane potential during diastole. Muscarinic receptor activation reverses this effect by decreasing cAMP production. This results in a hyperpolarizing shift in the voltage dependence of the channels, reducing their contribution to the rate of spontaneous depolarization. The result is an increase in the amount of time it takes the membrane potential to reach threshold and fire an action potential. The relative importance that activation of $I_{K(ACh)}$ (see above) and inhibition of the pacemaker current play in muscarinic regulation of changes in SA node firing rate and heart rate appear to be concentration dependent. It has been reported that the concentrations of ACh that inhibit the pacemaker current are lower than those required to activate $I_{K(ACh)}$. Muscarinic inhibition of cAMP can also affect the beating rate of SA nodal cells by altering PKA-dependent responses. These include reducing the stimulatory effect that PKA has on L-type Ca^{2+} channel activity (Irisawa et al. 1993). It has also been suggested that inhibition of PKA-dependent regulation of the ryanodine receptor plays an important role in muscarinic inhibition of SA node firing rate, by reducing Ca^{2+} cycling events that

contribute to spontaneous depolarization of the diastolic membrane potential in these cells (Lyashkov et al. 2009).

In atrial myocytes, muscarinic receptor activation can produce a negative inotropic effect (Ten Eick et al. 1976). Part of the inhibitory effect on contractility may be explained by a decrease in cAMP production. The cAMP signaling pathway enhances cardiac myocyte contractility by regulating PKA-dependent phosphorylation of several key proteins. These include, but are not limited to, the L-type Ca^{2+} channel, phospholamban, and troponin I (see Fig. 1). In atrial cells, muscarinic agonists can inhibit contractility by decreasing cAMP production and reversing the actions of PKA-dependent phosphorylation. Some of the inhibitory effect that muscarinic stimulation has on atrial contractility may also be explained by a change in action potential duration that is caused by activation of $I_{K(ACh)}$. Activation of this current contributes to a decrease in action potential duration, which can limit the amount of time available for influx of Ca^{2+} through L-type Ca^{2+} channels, reducing the amplitude of the Ca^{2+} transient.

Muscarinic stimulation can also decrease contractility in ventricular myocytes by inhibiting cAMP production and reversing the effects of PKA-dependent phosphorylation (see Fig. 2). However, ventricular myocyte contractility is not normally influenced of cAMP/PKA-dependent regulation under basal conditions. Therefore, muscarinic inhibition of such responses typically requires prior elevation of cAMP levels through some mechanism that involves increasing adenylyl cyclase activity, such as β-adrenergic receptor stimulation. This type of indirect inhibitory effect is referred to as accentuated antagonism (Levy 1971).

Activation of $I_{K(ACh)}$ does not affect contractility of ventricular myocytes because this current does not contribute significantly to the regulation of membrane potential in most species. On the other hand, muscarinic inhibition of cAMP production does have a significant effect on several channels that do play an important role in regulating the electrical activity of ventricular myocytes. Altering L-type Ca^{2+} channel activity plays an important role in the regulation of cardiac myocyte contractility. However, in addition to affecting contractility, if left unchecked, it would significantly alter action potential duration. Such an effect is potentially arrhythmogenic. To minimize changes in action potential duration, the cAMP/PKA signaling pathway also regulates the activity of ion channels that contribute to repolarization. Depending on the species, these may include delayed rectifier K^+ channels and/or CFTR Cl^- channels (see Fig. 2). Muscarinic receptor stimulation antagonizes the effects that cAMP and PKA have on all of these channels (Hartzell 1988; Harvey and Belevych 2003).

Accentuated antagonism is particularly evident when it comes to explaining the effects of parasympathetic stimulation on ventricular function. In most mammals, muscarinic responses are only observed in adult ventricular myocytes under conditions where cAMP production has been enhanced above basal levels (Hartzell 1988; Harvey and Belevych 2003). This is in contrast to atrial and sinoatrial node cells, where M_2 receptor activation can produce changes in ion channel function typically associated with antagonism of cAMP-dependent responses even in the absence of an agonist that stimulates cAMP production (Dhein et al. 2001; Harvey

and Belevych 2003). This is consistent with the idea that even under basal conditions these cells exhibit a higher basal level of cAMP, which can then be inhibited by muscarinic receptor activation (Méry et al. 1997).

3.2.2 Muscarinic Facilitation of cAMP-Dependent Responses

Despite the fact that M_2 receptor activation can inhibit cAMP-dependent responses, the same receptor acting through the same inhibitory G protein can also produce significant stimulatory effects that are due to facilitation of cAMP production. While both are activated simultaneously, the inhibitory effect dominates. However, upon termination of M_2 receptor activation, the inhibitory effect turns off rapidly, revealing the stimulatory effect, which turns off more slowly. One clear manifestation of such effects is the rebound increase in heart rate and ventricular contractility that can be observed immediately following termination of vagal stimulation or exposure to ACh (Harvey and Belevych 2003).

In atrial myocytes, ACh-induced rebound responses are blocked by inhibition of calmodulin, constitutive NOS activity, soluble guanylyl cyclase, and type 3 phosphodiesterase (PDE3) activity (Wang et al. 1998). This supports the conclusion that ACh-induced rebound stimulation of atrial responses is mediated by Ca^{2+}-calmodulin-dependent activation of NOS, NO-dependent stimulation of soluble guanylyl cyclase, and cGMP-dependent inhibition of PDE3. The result is a decrease in cAMP degradation and facilitation of cAMP-dependent responses. The rebound stimulatory response associated with termination of muscarinic receptor activation has been shown to affect cAMP-dependent regulation of the L-type Ca^{2+} current in atrial myocytes as well as the pacemaker current in SA nodal cells. It has been proposed that this type of response explains the rebound increase in heart rate observed upon termination of vagal stimulation (Wang and Lipsius 1996).

Despite evidence that the NO/cGMP signaling pathway is involved in mediating muscarinic stimulatory responses in atrial myocytes, this is not the case in ventricular myocytes. Rebound stimulatory responses are not blocked by inhibiting this signaling pathway in ventricular cells (Belevych et al. 2001; Zakharov and Harvey 1997). Furthermore, muscarinic stimulatory responses are intact in myocytes isolated from NOS3-KO mice (Belevych and Harvey 2000). In ventricular myocytes, it has been demonstrated that the stimulatory effect of M_2 receptor activation is due to opposing effects that G_i signaling has on the different isoforms of AC expressed in cardiac myocytes (Belevych et al. 2001). In addition to AC5 and AC6, there is also evidence for expression of AC4 and AC7 (Defer et al. 2000). While the activated α subunit of G_i inhibits AC5 and AC6, it has no effect on AC4 and AC7. On the other hand, AC4 and AC7 are stimulated by direct binding of $G\beta\gamma$ subunits (Sunahara and Taussig 2002). Therefore, it has been proposed that muscarinic stimulation can inhibit AC5 and AC6 while at the same time stimulating AC4 and AC7 (see Fig. 2). Furthermore, it has been proposed that muscarinic regulation cAMP inhibitory and stimulatory responses occur in distinct subcellular locations, and that the time-dependent flux of cAMP between these locations can explain the

complex temporal nature of the response (Iancu et al. 2007). In ventricular myocytes, the rebound stimulatory response has been shown to affect L-type Ca^{2+} channels as well as CFTR Cl^- channels. It has also been shown to stimulate spontaneous electrical activity and trigger delayed after depolarizations (Ehara and Mitsuiye 1984; Song et al. 1998). This suggests that the muscarinic receptor activation may contribute to arrhythmogenic activity associated with the complex interaction between parasympathetic and sympathetic stimulation of ventricular myocardium.

3.3 Other Muscarinic Responses in the Heart

In addition to the responses described above, high concentrations of muscarinic receptor agonists have also been reported to produce a positive inotropic effect associated with changes in intracellular Ca^{2+} secondary to an increase in intracellular Na^+ concentration (Korth and Kuhlkamp 1985; Korth et al. 1988). The increase in intracellular Na^+ has been attributed to activation of a tetrodotoxin (TTX)-insensitive Na^+ channel (Matsumoto and Pappano 1989). The resulting change in Na^+ gradient is believed to reduce the driving force for extrusion of intracellular Ca^{2+} by the Na/Ca exchanger (Saeki et al. 1997). This can then explain the increase in intracellular Ca^{2+} concentration and resulting change in force of contraction.

Most muscarinic responses in the heart have been attributed to activation of M_2 receptors. That includes activation of the TTX-insensitive Na^+ current by high agonist concentrations (Matsumoto and Pappano 1991). However, there is evidence for functional responses that are mediated by other types of muscarinic receptors. For example, even though the Na^+ current activated by high muscarinic agonist concentrations has been attributed to activation of M_2 receptors, the corresponding increase in intracellular Ca^{2+} and contractility are supposedly due to activation of M_1 receptors (Sharma et al. 1996). While the explanation for this apparent discrepancy is not clear, M_1 receptor activation has also been reported in to enhance L-type Ca^{2+} channel activity through a PLC-dependent mechanism (Gallo et al. 1993). On the other hand, M_3 receptor activation has been reported to activate a novel delayed rectifier-type K^+ current through a PLC-independent mechanism (Wang et al. 2004).

4 Vascular Muscarinic Responses

Muscarinic agonists can cause both contraction and relaxation of vascular tissue. The actual response can vary depending on the species and the anatomical location of the blood vessel involved, as well as whether or not the endothelial lining of the blood vessel is intact (Eglen et al. 1996). Relaxation is the primary response of most

blood vessels with an intact endothelium. Furchgott and Zawadzki were the first to demonstrate the role of the vascular endothelium in producing vasodilation of blood vessels in response to muscarinic agonist stimulation (Furchgott and Zawadzki 1980). This effect is typically mediated by an indirect mechanism that involves the release of an endothelium-derived relaxing factor (EDRF) following activation of M_3 receptors (Eglen et al. 1996; Eglen and Whiting 1990). Vascular endothelial cells can produce vasodilation by releasing multiple relaxing factors that act on vascular smooth muscle cells in a paracrine fashion. These include prostacyclin and endothelium-dependent hyperpolarization factor (EDHF). However, the most important factor involved in mediating the response to muscarinic agonists is NO (Furchgott and Vanhoutte 1989). This potent vasodilator is generated by eNOS, the isoform of nitric oxide synthase expressed constitutively in endothelial cells. The essential role of eNOS in muscarinic induced vasodilation is consistent with the significant reduction in the relaxation response to ACh observed in blood vessels obtained from eNOS knockout mice (Faraci and Sigmund 1999; Huang et al. 1995).

The signaling mechanism responsible for muscarinic receptor-dependent NO production in endothelial cells involves Ca^{2+} and calmodulin-dependent activation of eNOS (Dinerman et al. 1993). Consistent with this, the muscarinic receptors involved in vasorelaxation can trigger the release of Ca^{2+} from intracellular stores by stimulating PLC-dependent production of IP_3 (Adams et al. 1989). Once produced, NO can readily diffuse from the endothelial cells into adjacent smooth muscle cells. NO may then cause relaxation by one or more different actions. Perhaps the most important mechanism involves stimulation of soluble guanylyl cyclase activity (Pfeifer et al. 1998). This results in the production of cGMP, which can then activate PKG (see Fig. 3). Several mechanisms have been proposed to explain the ability of PKG to cause vascular smooth muscle relaxation (Faraci and Sigmund 1999; Hofmann et al. 2006).

Another indirect mechanism that may contribute to muscarinic relaxation of some blood vessels involves the inhibition of sympathetic neurotransmitter release (Vanhoutte and Shepherd 1983). Sympathetic stimulation of blood vessels causes potent vasoconstriction via the release of the neurotransmitter norepinephrine and subsequent activation of smooth muscle α-adrenergic receptors. Presynaptic inhibition of sympathetic neurotransmitter release by muscarinic agonists involves M_2 receptors (Eglen and Whiting 1990).

In addition to demonstrating the essential role that the vascular endothelium plays in agonist-induced relaxation of blood vessels, Furchgott and Zawadzki also demonstrated that in the absence of endothelium, muscarinic receptor activation can actually cause vascular smooth muscle contraction (Furchgott and Zawadzki 1980). This reflects the fact that vascular smooth muscle cells express G_q-coupled M_1 and M_3 muscarinic receptors capable of stimulating pharmacomechanical coupling. This involves the same PLC- and IP_3-dependent signaling mechanism that muscarinic receptors activate in endothelial cells. However, rather than stimulating NO production, the resulting rise in intracellular Ca^{2+} triggers myocyte contraction by regulating calmodulin-dependent activation of the myosin light chain kinase (see Fig. 3) (Horowitz et al. 1996).

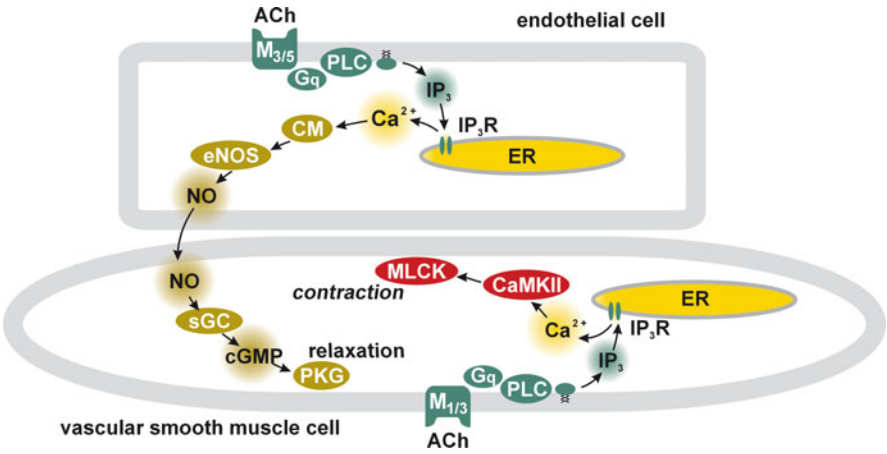

Fig. 3 Muscarinic signaling pathways in the vasculature. In endothelial cells, acetylcholine (ACh) acting through M_3 or M_5 receptors stimulates phospholipase C (PLC) activity through the G protein G_q. Subsequent production of inositoltriphosphate (IP_3) acts on the IP_3 receptor (IP_3R) in the endoplasmic reticulum to release Ca^{2+}. The resulting rise in cytosolic Ca^{2+} activates endothelial nitric oxide synthase (eNOS) via a calmodulin (CM)-dependent mechanism. Activation of eNOS leads to the production of nitric oxide (NO), which can diffuse into adjacent vascular smooth muscle cells, where it stimulates soluble guanylyl cyclase (sGC) to produce cGMP. Protein kinase G (PKG) activated by cGMP promotes relaxation. In vascular smooth muscle cells, ACh acting through M_1 or M_3 receptors stimulates PLC-dependent production of IP_3 and the subsequent release of Ca^{2+} from the ER. This results in Ca^{2+} and CM-dependent kinase (CamKII) activation of myosin light chain kinase (MLCK), which promotes contraction

Despite the significant effect that activation of muscarinic receptors can have on vascular function, parasympathetic stimulation does not play a significant role in autonomic regulation of blood flow in most vascular beds (Furchgott and Vanhoutte 1989). One notable exception to this generalization is in the cerebral circulation, where neurally released ACh is important in regulating vascular tone by causing endothelium-dependent vasodilation. In this case, however, the response to ACh involves the activation of M_5 receptors (Yamada et al. 2001).

5 Summary

Muscarinic receptor activation can regulate many different aspects of cardiovascular function. This review has focused primarily on normal physiological responses. However, there is a growing body of literature demonstrating that there are changes in muscarinic signaling that occur with age and various disease states (Dhein et al. 2001). There is also evidence that parasympathetic stimulation and muscarinic agonists can protect the heart from ischemic damage and prevent some of the deleterious effects associated with heart failure (Kakinuma et al. 2005; Katare

et al. 2009; Li et al. 2004). Because of this, a better understanding of muscarinic signaling pathways and the potential roles they play in regulating the heart and vasculature may provide new therapeutic strategies for treating cardiovascular disease.

References

Adams DJ, Barakeh J, Laskey R, Van Breemen C (1989) Ion channels and regulation of intracellular calcium in vascular endothelial cells. FASEB J 3:2389–2400

Ahmad Z, Green FJ, Subuhi HS, Watanabe AM (1989) Autonomic regulation of type I protein phosphatase in cardiac muscle. J Biol Chem 264:3859–3863

Belevych AE, Harvey RD (2000) Muscarinic inhibitory and stimulatory regulation of the L-type Ca^{2+} current is not altered in cardiac ventricular myocytes from mice lacking endothelial nitric oxide synthase. J Physiol (Lond) 528:279–289

Belevych AE, Sims C, Harvey RD (2001) ACh-induced rebound stimulation of L-type Ca(2+) current in guinea-pig ventricular myocytes, mediated by Gβγ-dependent activation of adenylyl cyclase. J Physiol (Lond) 536:677–692

Beny JL, Nguyen MN, Marino M, Matsui M (2008) Muscarinic receptor knockout mice confirm involvement of M_3 receptor in endothelium-dependent vasodilatation in mouse arteries. J Cardiovasc Pharmacol 51:505–512

Bers DM (2001) Excitation-contraction coupling and cardiac contractile force, 2nd edn. Kluwer, Dordrecht, Netherlands

Bers DM (2002) Cardiac excitation-contraction coupling. Nature 415:198–205

Defer N, Best-Belpomme M, Hanoune J (2000) Tissue specificity and physiological relevance of various isoforms of adenylyl cyclase. Am J Physiol 279:F400–F416

Dhein S, Van Koppen CJ, Brodde OE (2001) Muscarinic receptors in the mammalian heart. Pharmacol Res 44:161–182

DiFrancesco D (2010) The role of the funny current in pacemaker activity. Circ Res 106:434–446

DiFrancesco D, Tortora P (1991) Direct activation of cardiac pacemaker channels by intracellular cyclic AMP. Nature 351:145–147

Dinerman JL, Lowenstein CJ, Snyder SH (1993) Molecular mechanisms of nitric oxide regulation: potential relevance to cardiovascular disease. Circ Res 73:217–222

Eglen RM, Whiting RL (1990) Heterogeneity of vascular muscarinic receptors. J Auton Pharmacol 10:233–245

Eglen RM, Hegde SS, Watson N (1996) Muscarinic receptor subtypes and smooth muscle function. Pharmacol Rev 48:531–565

Ehara T, Mitsuiye T (1984) Adrenergic-cholinergic interactions on membrane potential of K^+-depolarized ventricular muscle. Am J Physiol 247:H244–H250

Endoh M (1999) Muscarinic regulation of Ca^{2+} signaling in mammalian atrial and ventricular myocardium. Eur J Pharmacol 375:177–196

Endoh M, Maruyama M, Iijima T (1985) Attenuation of muscarinic cholinergic inhibition by islet-activating protein in the heart. Am J Physiol 249:H309–H320

Faraci FM, Sigmund CD (1999) Vascular biology in genetically altered mice: smaller vessels, bigger insight. Circ Res 85:1214–1225

Furchgott RF, Vanhoutte PM (1989) Endothelium-derived relaxing and contracting factors. FASEB J 3:2007–2018

Furchgott RF, Zawadzki JV (1980) The obligatory role of endothelial cells in the relaxation of arterial smooth muscle by acetylcholine. Nature 288:373–376

Gallo MP, Alloatti G, Eva C, Oberto A, Levi RC (1993) M_1 muscarinic receptors increase calcium current and phosphoinositide turnover in guinea-pig ventricular cardiocytes. J Physiol (Lond) 471:41–60

George WJ, Polson JB, O'Toole AG, Goldberg ND (1970) Elevation of guanosine 3′-5′-cyclic phosphate in rat heart after perfusion with acetylcholine. Proc Natl Acad Sci USA 66:398–403
George WJ, Wilkerson RD, Kadowitz PJ (1972) Influence of acetylcholine on contractile force and cyclic nucleotide levels in the isolated perfused rat heart. J Pharmacol Exp Ther 184:228–235
Gödecke A, Heinicke T, Kamkin A, Kiseleva I, Strasser RH, Decking UK, Stumpe T, Isenberg G, Schrader J (2001) Inotropic response to beta-adrenergic receptor stimulation and anti- adrenergic effect of ACh in endothelial NO synthase-deficient mouse hearts. J Physiol (Lond) 532:195–204
Gupta RC, Neumann J, Boknik P, Watanabe AM (1994) M_2-specific muscarinic cholinergic receptor-mediated inhibition of cardiac regulatory protein phosphorylation. Am J Physiol 266:H1138–H1144
Hartzell HC (1988) Regulation of cardiac ion channels by catecholamines, acetylcholine and second messenger systems. Prog Biophys Mol Biol 52:165–247
Harvey RD, Belevych AE (2003) Muscarinic regulation of cardiac ion channels. Br J Pharmacol 139:1074–1084
Hashimoto N, Yamashita T, Tsuruzoe N (2006) Tertiapin, a selective IKACh blocker, terminates atrial fibrillation with selective atrial effective refractory period prolongation. Pharmacol Res 54:136–141
Hirst GD, Edwards FR (1989) Sympathetic neuroeffector transmission in arteries and arterioles. Physiol Rev 69:546–604
Hofmann F, Feil R, Kleppisch T, Schlossmann J (2006) Function of cGMP-dependent protein kinases as revealed by gene deletion. Physiol Rev 86:1–23
Horowitz A, Menice CB, Laporte R, Morgan KG (1996) Mechanisms of smooth muscle contraction. Physiol Rev 76:967–1003
Huang PL, Huang Z, Mashimo H, Bloch KD, Moskowitz MA, Bevan JA, Fishman MC (1995) Hypertension in mice lacking the gene for endothelial nitric oxide synthase. Nature 377: 239–242
Hulme EC, Birdsall NJ, Buckley NJ (1990) Muscarinic receptor subtypes. Annu Rev Pharmacol Toxicol 30:633–673
Iancu RV, Jones SW, Harvey RD (2007) Compartmentation of cAMP signaling in cardiac myocytes: a computational study. Biophys J 92:3317–3331
Iancu RV, Ramamurthy G, Warrier S, Nikolaev VO, Lohse MJ, Jones SW, Harvey RD (2008) Cytoplasmic cAMP concentrations in intact cardiac myocytes. Am J Physiol Cell Physiol 295: C414–C422
Irisawa H, Brown HF, Giles W (1993) Cardiac pacemaking in the sinoatrial node. Physiol Rev 73:197–227
Kakinuma Y, Ando M, Kuwabara M, Katare RG, Okudela K, Kobayashi M, Sato T (2005) Acetylcholine from vagal stimulation protects cardiomyocytes against ischemia and hypoxia involving additive non-hypoxic induction of HIF-1alpha. FEBS Lett 579:2111–2118
Katare RG, Ando M, Kakinuma Y, Arikawa M, Handa T, Yamasaki F, Sato T (2009) Vagal nerve stimulation prevents reperfusion injury through inhibition of opening of mitochondrial permeability transition pore independent of the bradycardiac effect. J Thorac Cardiovasc Surg 137:223–231
Khurana S, Chacon I, Xie G, Yamada M, Wess J, Raufman JP, Kennedy RH (2004) Vasodilatory effects of cholinergic agonists are greatly diminished in aorta from M3R−/− mice. Eur J Pharmacol 493:127–132
Koizumi K, Terui N, Kollai M, Brooks CM (1982) Functional significance of coactivation of vagal and sympathetic cardiac nerves. Proc Natl Acad Sci USA 79:2116–2120
Korth M, Kuhlkamp V (1985) Muscarinic receptor-mediated increase of intracellular Na^+-ion activity and force of contraction. Pflugers Arch 403:266–272
Korth M, Sharma VK, Sheu SS (1988) Stimulation of muscarinic receptors raises free intracellular Ca^{2+} concentration in rat ventricular myocytes. Circ Res 62:1080–1087
Koumi S-I, Wasserstrom JA (1994) Acetylcholine-sensitive muscarinic K^+ channels in mammalian ventricular myocytes. Am J Physiol 266:H1812–H1821

Kovoor P, Wickman K, Maguire CT, Pu W, Gehrmann J, Berul CI, Clapham DE (2001) Evaluation of the role of I(KACh) in atrial fibrillation using a mouse knockout model. J Am Coll Cardiol 37:2136–2143

Krapivinsky G, Gordon EA, Wickman K, Velimirovic B, Krapivinsky L, Clapham DE (1995) The G-protein-gated atrial K^+ channel I_{KACh} is a heteromultimer of two inwardly rectifying K^+-channel proteins. Nature 374:135–141

Kurachi Y (1995) G protein regulation of cardiac muscarinic potassium channel. Am J Physiol 269:C821–C830

Kurachi Y, Nakajima T, Sugimoto T (1986a) Acetylcholine activation of K^+ channels in cell-free membrane of atrial cells. Am J Physiol 251:H681–H684

Kurachi Y, Nakajima T, Sugimoto T (1986b) On the mechanism of activation of muscarinic K^+ channels by adenosine in isolated atrial cells: involvement of GTP-binding proteins. Pflugers Arch 407:264–274

Lanzafame AA, Christopoulos A, Mitchelson F (2003) Cellular signaling mechanisms for muscarinic acetylcholine receptors. Receptors Channels 9:241–260

Leung RS (2009) Sleep-disordered breathing: autonomic mechanisms and arrhythmias. Prog Cardiovasc Dis 51:324–338

Levy MN (1971) Sympathetic-parasympathetic interactions in the heart. Circ Res 29:437–445

Levy MN (1977) Parasympathetic control of the heart. In: Randall WC (ed) Neural regulation of the heart. Oxford University Press, New York, NY, pp 95–129

Levy MN (1995) Neural control of the heart: the importance of being ignorant. J Cardiovasc Electrophysiol 6:283–293

Levy MN, Martin PJ (1989) Autonomic neural control of cardiac function. In: Sperelakis N (ed) Physiology and pathophysiology of the heart, 2nd edn. Kluwer Academic, Boston, MA, pp 361–380

Li M, Zheng C, Sato T, Kawada T, Sugimachi M, Sunagawa K (2004) Vagal nerve stimulation markedly improves long-term survival after chronic heart failure in rats. Circulation 109: 120–124

Lindemann JP, Watanabe AM (1989) Mechanisms of adrenergic and cholinergic regulation of myocardial contractility. In: Sperelakis N (ed) Physiology and pathophysiology of the heart, 2nd edn. Kluwer Academic, Boston, MA, pp 423–452

Löffelholz K, Pappano AJ (1985) The parasympathetic neuroeffector junction of the heart. Pharmacol Rev 37:1–24

Lyashkov AE, Vinogradova TM, Zahanich I, Li Y, Younes A, Nuss HB, Spurgeon HA, Maltsev VA, Lakatta EG (2009) Cholinergic receptor signaling modulates spontaneous firing of sinoatrial nodal cells via integrated effects on PKA-dependent Ca(2+) cycling and I(KACh). Am J Physiol Heart Circ Physiol 297:H949–H959

Martin P (1977) The influence of the parasympathetic nervous system on atrioventricular conduction. Circ Res 41:593–599

Matsumoto K, Pappano AJ (1989) Sodium-dependent membrane current induced by carbachol in single guinea-pig ventricular myocytes. J Physiol (Lond) 415:487–502

Matsumoto K, Pappano AJ (1991) Carbachol activates a novel sodium current in isolated guinea pig ventricular myocytes via M_2 muscarinic receptors. Mol Pharmacol 39:359–363

Méry P-F, Abi-Gerges N, Vandecasteele G, Jurevicius J, Eschenhagen T, Fischmeister R (1997) Muscarinic regulation of the L-type calcium current in isolated cardiac myocytes. Life Sci 60:1113–1120

Nishimura M, Habuchi Y, Hiromasa S, Watanabe Y (1988) Ionic basis of depressed automaticity and conduction by acetylcholine in rabbit AV node. Am J Physiol 255:H7–H14

Paton JF, Boscan P, Pickering AE, Nalivaiko E (2005) The yin and yang of cardiac autonomic control: vago-sympathetic interactions revisited. Brain Res Brain Res Rev 49:555–565

Pfaffinger PJ, Martin JM, Hunter DD, Nathanson NM, Hille B (1985) GTP-binding proteins couple cardiac muscarinic receptors to a K channel. Nature 317:536–538

Pfeifer A, Klatt P, Massberg S, Ny L, Sausbier M, Hirneiss C, Wang GX, Korth M, Aszodi A, Andersson KE, Krombach F, Mayerhofer A, Ruth P, Fassler R, Hofmann F (1998) Defective smooth muscle regulation in cGMP kinase I-deficient mice. EMBO J 17:3045–3051

Saeki T, Shen JB, Pappano AJ (1997) Carbachol promotes Na^+ entry and augments Na/Ca exchange current in guinea pig ventricular myocytes. Am J Physiol 273:H1984–H1993

Sakmann B, Noma A, Trautwein W (1983) Acetylcholine activation of single muscarinic K^+ channels in isolated pacemaker cells of the mammalian heart. Nature 303:250–253

Sharma VK, Colecraft HM, Wang DX, Levey AI, Grigorenko EV, Yeh HH, Sheu SS (1996) Molecular and functional identification of m_1 muscarinic acetylcholine receptors in rat ventricular myocytes. Circ Res 79:86–93

Soejima M, Noma A (1984) Mode of regulation of the Ach-sensitive K-channel by the muscarinic receptor in rabbit atrial cells. Pflugers Arch 400:424–431

Song Y, Shryock JC, Belardinelli L (1998) Potentiating effect of acetylcholine on stimulation by isoproterenol of L-type Ca^{2+} current and arrhythmogenic triggered activity in guinea pig ventricular myocytes. J Cardiovasc Electrophysiol 9:718–726

Standish A, Enquist LW, Schwaber JS (1994) Innervation of the heart and its central meduallary origin defined by viral tracing. Science 263:232–234

Standish A, Enquist LW, Escardo JA, Schwaber JS (1995) Central neuronal circuit innervating the rat heart defined by transneuronal transport of pseudorabies virus. J Neurosci 15:1998–2012

Stemmer PM, Ledyard TH, Watanabe AM (2000) Protein dephosphorylation rates in myocytes after isoproterenol withdrawal. Biochem Pharmacol 59:1513–1519

Stengel PW, Gomeza J, Wess J, Cohen ML (2000) M(2) and M(4) receptor knockout mice: muscarinic receptor function in cardiac and smooth muscle in vitro. J Pharmacol Exp Ther 292:877–885

Sunahara RK, Taussig R (2002) Isoforms of mammalian adenylyl cyclase: multiplicities of signaling. Mol Interv 2:168–184

Sunahara RK, Dessauer CW, Gilman AG (1996) Complexity and diversity of mammalian adenylyl cyclases. Annu Rev Pharmacol Toxicol 36:461–480

Ten Eick RE, Nawrath J, McDonald RF, Trautwein W (1976) On the mechanism of the negative inotropic effect of acetylcholine. Pflugers Arch 361:207–213

Vandecasteele G, Eschenhagen T, Scholz H, Stein B, Verde I, Fischmeister R (1999) Muscarinic and beta-adrenergic regulation of heart rate, force of contraction and calcium current is preserved in mice lacking endothelial nitric oxide synthase. Nat Med 5:331–334

Vanhoutte PM, Shepherd JT (1983) Muscarinic and beta-adrenergic prejunctional modulation of adrenergic neurotransmission in the blood vessel wall. Gen Pharmacol 14:35–37

Wang YG, Lipsius SL (1996) A cellular mechanism contributing to post-vagal tachycardia studied in isolated pacemaker cells from cat right atrium. Circ Res 79:109–114

Wang YG, Rechenmacher CE, Lipsius SL (1998) Nitric oxide signaling mediates stimulation of L-type Ca^{2+} current elicited by withdrawal of acetylcholine in cat atrial myocytes. J Gen Physiol 111:113–125

Wang Z, Shi H, Wang H (2004) Functional M_3 muscarinic acetylcholine receptors in mammalian hearts. Br J Pharmacol 142:395–408

Warrier S, Belevych AE, Ruse M, Eckert RL, Zaccolo M, Pozzan T, Harvey RD (2005) Beta-adrenergic and muscarinic receptor induced changes in cAMP activity in adult cardiac myocytes detected using a FRET based biosensor. Am J Physiol 289:C455–C461

Watanabe AM, Besch HR (1975) Interaction between cyclic adenosine monophosphate and cyclic guanosine monophosphate in guinea pig ventricular myocardium. Circ Res 37:309–317

Yamada M, Lamping KG, Duttaroy A, Zhang W, Cui Y, Bymaster FP, McKinzie DL, Felder CC, Deng CX, Faraci FM, Wess J (2001) Cholinergic dilation of cerebral blood vessels is abolished in M(5) muscarinic acetylcholine receptor knockout mice. Proc Natl Acad Sci USA 98:14096–14101

Zakharov SI, Harvey RD (1997) Rebound stimulation of the cAMP-regulated Cl- current by acetylcholine in guinea-pig ventricular myocytes. J Physiol (Lond) 499:105–120

Muscarinic Receptor Antagonists: Effects on Pulmonary Function

Kalmia S. Buels and Allison D. Fryer

Abstract In healthy lungs, muscarinic receptors control smooth muscle tone, mucus secretion, vasodilation, and inflammation. In chronic obstructive pulmonary disease (COPD) and asthma, cholinergic mechanisms contribute to increased bronchoconstriction and mucus secretion that limit airflow. This chapter reviews neuronal and nonneuronal sources of acetylcholine in the lung and the expression and role of M_1, M_2, and M_3 muscarinic receptor subtypes in lung physiology. It also discusses the evidence for and against the role of parasympathetic nerves in asthma, and the current use and therapeutic potential of muscarinic receptor antagonists in COPD and asthma.

Keywords Lung • Parasympathetic nerves • Asthma • COPD • Atropine • Ipratropium • Tiotropium • Bronchoconstriction • Bronchodilation • Hyperresponsiveness

Abbreviations

ChAT	Choline acetyltransferase
COPD	Chronic obstructive pulmonary disease
FEV_1	Forced expiratory volume in 1 s

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1 Acetylcholine in the Lung

The lung's primary role is gas exchange, but it also serves as both a barrier and defense against pathogens and environmental contaminants. Acetylcholine produced and released by both neuronal and nonneuronal sources acts through muscarinic receptors to regulate these important physiological functions. Acetylcholine contracts airway smooth muscle to control tone and regulate patency of the conducting airways. In blood vessels, acetylcholine causes smooth muscle relaxation and vasodilation. At mucosal glands and epithelial cells, acetylcholine regulates mucus secretion and, via ciliary beat frequency, mucus clearance. Acetylcholine also modulates inflammation.

1.1 Neuronal Acetylcholine in the Lungs

Parasympathetic nerves synthesize and release acetylcholine and are the primary source of acetylcholine in the lung. They innervate all conducting airways, from the trachea to the bronchioles (Canning and Fischer 1997), and pulmonary blood vessels (Cavallotti et al. 2005; Haberberger et al. 1997).

Preganglionic parasympathetic nerves originate in the brain in the medulla oblongata, and their axons travel in the right and left vagus nerves to synapse with postganglionic nerves in the trachea and bronchi (Kalia 1981; McAllen and Spyer 1978). Postganglionic nerve cell bodies are clustered in ganglia of 2–38 cells (Baker et al. 1986; Canning and Fischer 1997), with axons projecting to airway smooth muscle (Canning and Fischer 1997; Daniel et al. 1986), mucous glands (Basbaum 1984), and blood vessels (both arteries and large veins) (Cavallotti et al. 2005; Haberberger et al. 1997; Knight et al. 1981). Acetylcholine released from cholinergic parasympathetic nerves induces smooth muscle contraction (Cabezas et al. 1971; Olsen et al. 1965) and mucus secretion (Baker et al. 1985; Gallagher et al. 1975; Ramnarine et al. 1996) in the conducting airways, and vasodilation in pulmonary arteries and large veins (Laitinen et al. 1987).

Cholinergic parasympathetic nerves fire tonically (Widdicombe 1966; Widdicombe et al. 1962) to contract airway smooth muscle during normal breathing in humans and animals (Jammes and Mei 1979; Kesler and Canning 1999; Roberts et al. 1988; Sheppard et al. 1982; Widdicombe 1966). Thus, vagotomy (severing the vagus nerve) relaxes smooth muscle and decreases airway tone while electrically stimulating the distal ends of cut vagus nerves contracts airway smooth muscle. Airway smooth muscle contraction narrows conducting airways resulting in bronchoconstriction, which decreases the volume of conducting airways and increases resistance to airflow (Cabezas et al. 1971; Kesler and Canning 1999; Olsen et al. 1965; Severinghaus and Stupfel 1955).

Acetylcholine, released by parasympathetic nerves upon stimulation, acts directly at muscarinic receptors on airway smooth muscle to cause bronchoconstriction.

Therefore, as with vagotomy, muscarinic receptor antagonists decrease smooth muscle tone (Kesler and Canning 1999; Severinghaus and Stupfel 1955; Sheppard et al. 1982) and prevent bronchoconstriction induced by electrical stimulation of the vagus nerves. Furthermore, bronchoconstriction is increased by acetylcholinesterase inhibitors, which prevent metabolism of acetylcholine (Colebatch and Halmagyi 1963).

Cholinergic parasympathetic nerves are the efferent arm of vagal reflexes initiated by both physical (ex. dust and cold air) and chemical (ex. allergens, histamine, methacholine, and irritant gasses such as SO_2) stimuli (Gold et al. 1972; Holtzman et al. 1980; Sheppard et al. 1982; Wagner and Jacoby 1999; Widdicombe et al. 1962). These stimuli directly or indirectly activate afferent sensory nerves in the respiratory tract, which conduct signals to the brain via the vagus nerve (Widdicombe et al. 1962). Parasympathetic neurons carry the reflex response back to the lungs via acetylcholine release that leads to bronchoconstriction and mucus secretion in the airways.

Convincing evidence for reflex bronchoconstriction comes from experiments where a stimulus is applied to one location in the respiratory tract and a cholinergic response is measured in a separate location innervated by the efferent arm of the reflex arc (Nadel et al. 1965; Wagner and Jacoby 1999). For example, SO_2 gas applied to the larynx of tracheostomized animals causes bronchoconstriction in the trachea and bronchi. Severing either the afferent sensory nerves that leave the larynx or the efferent vagus nerves completely prevents this reflex bronchoconstriction (Boushey et al. 1972; Nadel et al. 1965). Mucus secretion is also initiated by reflexes but is challenging to measure in the lower airways. However, in the human upper respiratory tract, histamine applied to one nostril induces secretions in both nostrils. Secretions in the contralateral untreated nostril are completely prevented by muscarinic receptor antagonists, supporting a cholinergic reflex (Baroody et al. 1993). From a therapeutic perspective, vagal reflexes are important because muscarinic receptor antagonists are capable of blocking bronchoconstriction and mucus secretion initiated by a variety of chemical and physical stimuli in humans (Baroody et al. 1993; Holtzman et al. 1980; Sheppard et al. 1982).

It is also important to note that some chemicals act both directly at smooth muscle and indirectly through vagal reflexes to cause bronchoconstriction. This is true for both inhaled methacholine (muscarinic agonist) and inhaled histamine (Belmonte et al. 1998; Holtzman et al. 1980; Wagner and Jacoby 1999), two drugs commonly used to test airway responsiveness. For instance, most bronchoconstriction induced by inhaled histamine in humans is prevented by pharmacological blockade of vagal ganglionic neurotransmission with a neuronal nicotinic receptor antagonist (Holtzman et al. 1980). While no evidence exists for a similar effect of methacholine in people, there is substantial evidence for methacholine-induced reflex bronchoconstriction in diverse species such as sheep and rats, strongly suggesting that a similar reflex may also occur in humans (Belmonte et al. 1998; Wagner and Jacoby 1999).

1.2 Nonneuronal Acetylcholine in the Lungs

Acetylcholine synthesis and release is not limited to cholinergic neurons in the lung. Both epithelial cells (Proskocil et al. 2004; Reinheimer et al. 1998) and endothelial cells (Haberberger et al. 1997, 2000) in the lung contain the cellular machinery required to synthesize and release acetylcholine including choline acetyltransferase (ChAT), which catalyzes the synthesis of acetylcholine; hemicholinium-3 sensitive choline transporters that transport choline into cells (Ferguson et al. 2003); and vesicular acetylcholine transporters, which package acetylcholine into vesicles. In addition, ciliated epithelial cells express organic cation transporters OCT1 and OCT2 in their luminal membranes. These polyspecific organic cation transporters can transport acetylcholine and are believed to directly release acetylcholine into the airway lumen (Kummer et al. 2006; Lips et al. 2005).

Airway epithelial cells contain and release acetylcholine as measured by high-pressure liquid chromatography (Proskocil et al. 2004; Reinheimer et al. 1996, 1998). Epithelial cells from freshly isolated human bronchi contain 23±6 pmol acetylcholine per gram bronchus. This is just 1% of the 2,600±500 pmol acetylcholine per gram bronchus contained in the whole bronchial wall, but it is likely physiologically important for increasing ciliary beat frequency via muscarinic receptors on ciliated airway epithelial cells (Corssen and Allen 1959; Klein et al. 2009; Reinheimer et al. 1998; Wong et al. 1988).

Acetylcholine acts at muscarinic receptors in pulmonary arteries to induce vasodilation (Greenberg et al. 1987; McMahon and Kadowitz 1992). One source of acetylcholine is presumably the endothelial cells since they contain the machinery for synthesizing and releasing acetylcholine. In pulmonary arteries, endothelial ChAT expression is mosaic suggesting acetylcholine production may be related to differences in local mechanical forces due to blood flow (Haberberger et al. 2000).

2 Muscarinic Receptors in the Lungs

Sir Henry Dale first divided the actions of acetylcholine, and other choline derivatives, into nicotinic and muscarinic, based on their similarity to responses elicited by either nicotine or muscarine (Dale 1914). Nicotinic acetylcholine receptors are ligand-gated ion channels and muscarinic receptors are G protein-coupled. Although nicotinic receptors are also present throughout the lungs and are crucial for neurotransmission between pre- and postganglionic parasympathetic nerves, muscarinic receptors are a major physiological target for acetylcholine in the lungs and are the primary focus of this chapter.

Five muscarinic receptor subtypes, M_1, M_2, M_3, M_4, and M_5, are recognized by the International Union of Pharmacology (Caulfield and Birdsall 1998). M_1, M_3, and M_5 receptors typically couple to $G\alpha_{q/11}$ while M_2 and M_4 receptors typically

couple to $G\alpha_{i/o}$. All five muscarinic receptor subtypes are expressed in the lungs. Currently, strong evidence for a functional role only exists for M_1, M_2, and M_3 receptors, and muscarinic receptor antagonists that target these receptors are used to treat several lung diseases, including asthma and chronic obstructive pulmonary disease (COPD).

The earliest studies of muscarinic receptor distribution in lung tissues employed autoradiographic labeling of muscarinic receptor density in lung sections (van Koppen et al. 1987, 1988). These studies demonstrated that muscarinic receptor density is actually highest in parasympathetic ganglia, followed by mucous glands, smooth muscle, and nerve fibers (van Koppen et al. 1988). Subtype-specific distribution and function has subsequently been determined using pharmacological analysis, in situ hybridization, RT-PCR, and knockout mice. The distribution of receptor subtypes in tissues based on these assays is in good agreement. However, it is important to note that most "selective" muscarinic antagonists have at most a 10-fold selectivity for one muscarinic receptor subtype over other subtypes, where selectivity is usually defined as 100-fold higher affinity (Caulfield and Birdsall 1998). In addition, many commercially available receptor antibodies may not be specific, based on the presence of similar antibody staining patterns in wild-type and muscarinic receptor gene-deficient mice (Jositsch et al. 2009; Pradidarcheep et al. 2008).

2.1 Muscarinic Receptors on Airway Nerves and Ganglia

Both pre- and postganglionic parasympathetic nerves innervating the lungs express muscarinic receptors that are densest at the ganglia (van Koppen et al. 1987, 1988). Muscarinic receptors on parasympathetic nerves modulate synaptic neurotransmission between the pre- and postganglionic nerves, and also limit release of acetylcholine by postganglionic nerves at target tissues (smooth muscle, glands).

Preganglionic autonomic nerves release acetylcholine onto nicotinic receptors at their synapses with postganglionic nerves. In the lungs, muscarinic receptors modulate neurotransmission across this synapse (Myers 2001). Preganglionic neurons contain inhibitory M_2 receptors at the synapse, which limit acetylcholine release in guinea pig bronchi. Thus, activating M_2 receptors during electrical stimulation of the preganglionic nerves reduces acetylcholine release into the synapse, which in turn decreases the amplitude of nicotinic fast excitatory postsynaptic potentials recorded in bronchial ganglia (Myers and Undem 1996).

M_1 muscarinic receptors are found in cell bodies of postganglionic nerves, although there are species differences regarding the importance of these receptors in modulating synaptic neurotransmission. In guinea pigs, M_1 receptors depolarize the resting membrane potential in approximately 50% of ganglion cells, which would be expected to facilitate neurotransmission at the synapse and increase bronchoconstriction (Myers and Undem 1996). However, blocking ganglion M_1 receptors does not reduce smooth muscle contraction induced by electrically stimulating vagal preganglionic nerves in guinea pigs (Undem et al. 1990).

Similarly, no functional role for M_1 receptors has been identified in rat tracheal ganglia (Murai et al. 1998). This contrasts with rabbit, where blocking M_1 receptors in the bronchi reduces smooth muscle contraction following vagal stimulation (Bloom et al. 1988). Finally, in atopic humans, blocking M_1 receptors in the lung with pirenzepine decreases inhaled SO_2-induced reflex bronchoconstriction by approximately 50%. These data indirectly support a role for M_1 receptors facilitating parasympathetic neurotransmission in allergic humans. However, the importance of M_1 receptors in healthy humans is unclear, since pirenzepine inhibits only 17% of vagal tone in healthy women (Fujimura et al. 1992).

Of greater physiological importance are inhibitory M_2 receptors on postganglionic parasympathetic nerves that were first described in guinea pig (Fryer and Maclagan 1984). These neuronal M_2 receptors are activated by acetylcholine to inhibit further acetylcholine release in a feedback mechanism that limits vagally induced bronchoconstriction and mucus secretion in healthy animals and humans (Ayala and Ahmed 1989; Blaber et al. 1985; Fryer et al. 1996; Fryer and Maclagan 1984; Minette et al. 1989; Ramnarine et al. 1996). When neuronal M_2 receptors in trachea are blocked, the amount of acetylcholine released by nerve stimulation significantly increases (Baker et al. 1992). Pharmacologically blocking inhibitory M_2 receptors with gallamine in guinea pigs or deleting them genetically in mice significantly potentiates vagally induced bronchoconstriction in vivo, while selectively activating M_2 receptors with low doses of pilocarpine inhibits vagally induced bronchoconstriction 80% (Fisher et al. 2004; Fryer and Maclagan 1984). Similarly, in healthy humans, low doses of muscarinic agonists reduce bronchoconstriction induced by a vagal reflex, demonstrating a role for inhibitory neuronal M_2 receptors in limiting acetylcholine release (Ayala and Ahmed 1989; Minette et al. 1989).

2.2 Muscarinic Receptors on Airway Smooth Muscle

In the lungs, acetylcholine causes bronchoconstriction via smooth muscle contraction (Haddad et al. 1991; Roffel et al. 1988, 1990; Stengel et al. 2000; Struckmann et al. 2003). The presence of M_2 and M_3 receptors on airway smooth muscle is supported by radioligand binding data, autoradiography, in situ hybridization, and genetic deletion in humans, cows, guinea pigs, dogs, and mice (Fernandes et al. 1992; Haddad et al. 1991; Mak and Barnes 1990; Mak et al. 1992; Roffel et al. 1987; Struckmann et al. 2003). Physiological data support M_3 receptors as having the dominant role in smooth muscle contraction.

Functional experiments demonstrate that contraction induced by muscarinic ligands in isolated trachea and bronchi is mediated by M_3 receptors in all species including humans (Haddad et al. 1991; Roffel et al. 1988, 1990; Struckmann et al. 2003). In addition, in vivo experiments in muscarinic receptor gene-deficient mice demonstrate that only M_3 receptors contribute to bronchoconstriction induced by electrical stimulation of the vagus nerves or intravenous methacholine (Fisher et al.

2004). Recently, it has also been shown that smooth muscle M_3 receptors are activated in the absence of acetylcholine by membrane depolarization induced chemically with KCl (Liu et al. 2009). This ligand-independent activation of M_3 receptors has only thus far been demonstrated in mice. It is not known whether membrane depolarization activates M_3 receptors in vivo.

M_2 receptors often outnumber M_3 receptors but have an indirect role in airway smooth muscle contraction. M_2 receptors on airway smooth muscle inhibit relaxation induced both by β-adrenoreceptor agonists and adenylyl cyclase activation with forskolin (Fernandes et al. 1992). Thus, M_2 receptors contribute to smooth muscle contraction by functionally antagonizing $G\alpha_s$-induced relaxation. In isolated trachea from mice deficient for M_2 receptors, muscarinic agonist potency is reduced, however maximum contraction is still achieved (Stengel et al. 2000). This suggests M_2 receptors contribute to acetylcholine-induced smooth muscle contraction, but that M_3 receptors alone are sufficient for smooth muscle contraction. In vitro, airway narrowing induced by muscarine can only be completely prevented in airways from mice deficient in both M_2 and M_3 receptor genes. In vivo, however, only M_3 receptors contribute to bronchoconstriction induced by vagal stimulation and intravenous methacholine, since bronchoconstriction is absent in mice deficient for M_3 receptors.

2.3 Muscarinic Receptors on Airway Submucosal Glands

Parasympathetic nerves also stimulate mucus secretion from submucosal glands in the lungs. Mucus is an aqueous solution that includes electrolytes, mucins (large glycoproteins), enzymes, and antibacterial agents (Rogers 2001), and is beneficial in airway defense and for trapping particles. Particles are then removed along with the mucus by ciliary clearance into the mouth and esophagus.

Both constitutive and induced release of mucus occur in vivo (Gallagher et al. 1975) and in isolated glands in vitro (Baker et al. 1985; Dwyer et al. 1992; Gallagher et al. 1975). Constitutive mucus release does not depend on cholinergic nerves, since neither tetrodotoxin, which prevents action potentials in nerves by blocking sodium channels, nor vagotomy change baseline mucus secretion (Baker et al. 1985; Borson et al. 1984; Gallagher et al. 1975). However, vagal stimulation and exogenous acetylcholine both increase mucus secretion from submucosal glands (Borson et al. 1984; Gallagher et al. 1975). Acetylcholine-induced mucus release is rapid and transient, lasting only 2–6 min, followed by a relative refractory period where further mucus cannot be released by additional acetylcholine exposure. This could be due to receptor desensitization or acetylcholine may initiate a slower inhibitory response along with the secretory response (Dwyer et al. 1992).

Mucus secretion caused by vagal stimulation or exogenous acetylcholine is blocked by the nonselective muscarinic antagonist atropine (Borson et al. 1984; Gallagher et al. 1975). In submucosal glands, muscarinic receptors are found on both serous cells that secrete fluid and mucous cells that secrete mucins (Mak and

Barnes 1990; Ramnarine et al. 1996; van Koppen et al. 1988). Both M_1 and M_3 receptors are present in human and animal submucosal glands (Mak and Barnes 1990; Mak et al. 1992). M_3 receptors are responsible for both vagal and exogenous acetylcholine-induced mucin secretion. This is supported by experiments that use selective antagonists (4-DAMP, methoctramine, and telenzepine) at concentrations that only block high-affinity binding sites (Ramnarine et al. 1996). Despite their presence, a direct role for M_1 receptors has not been demonstrated in airway submucosal glands, but it has been hypothesized that these receptors may be responsible for fluid or electrolyte release by serous cells (Yang et al. 1988).

2.4 Muscarinic Receptors on Pulmonary Arteries

While neuronal acetylcholine does not contribute to resting tone in pulmonary blood vessels, stimulation of the vagus nerves causes vasodilation (Laitinen et al. 1987). However, exogenous acetylcholine will only relax precontracted human pulmonary arteries if the endothelium is intact (Greenberg et al. 1987). Acetylcholine likely acts at muscarinic receptors on endothelial cells to stimulate production of nitric oxide, which relaxes smooth muscle (Furchgott and Zawadzki 1980; Greenberg et al. 1987; McMahon and Kadowitz 1992).

M_3 muscarinic receptors are important for vasodilation in vivo. This is supported by the inability of electrical stimulation of the vagus nerves in M_3 receptor-deficient mice to maximally decrease blood pressure (Fisher et al. 2004). Endothelial cells isolated from pulmonary trunk arteries in pigs express mRNA for both M_2 and M_3 receptors. Moreover, acetylcholine increases intracellular calcium concentrations in these cells in a manner consistent with M_3 receptor activation (Kummer and Haberberger 1999). Arterial smooth muscle cells may also contain M_2 and M_3 muscarinic receptors, however this needs to be confirmed because the evidence is based only on immunohistochemistry (Kummer and Haberberger 1999).

2.5 Muscarinic Receptors on Airway Epithelium

Activating muscarinic receptors in epithelial cells transiently increases intracellular calcium (Salathe et al. 1997) and increases ciliary beat frequency (Klein et al. 2009; Salathe et al. 1997; Seybold et al. 1990), which would increase transport of mucus and particulates out of the lung. Muscarinic signaling also increases the velocity of liquid (Seybold et al. 1990) and particle transport (Klein et al. 2009) upward in isolated tracheas.

M_3 receptor mRNA is found in human airway epithelium by in situ hybridization (Mak et al. 1992), while mRNA for both M_3 and M_1 receptors has been identified in mouse epithelia (Klein et al. 2009). Experiments using muscarinic receptor gene-deficient mice demonstrate that M_3 receptors are both required and sufficient for the

full increase in ciliary beat frequency and particle transport speed induced by muscarine in wild-type mice. A role for M_3 receptors is further supported by pharmacological experiments in sheep showing that an antagonist with selectivity for M_3 receptors (4-DAMP) blocks acetylcholine-induced calcium signaling and ciliary beat frequency, while an antagonist with selectivity for M_1 receptors (pirenzepine) does not have this effect (Salathe et al. 1997).

While M_3 receptors provide the dominant control of ciliary beat frequency, M_1 and M_2 receptors can also contribute. M_1 receptors increase ciliary transport speed, but this function is only uncovered in mice that are deficient for both M_2 and M_3 receptors. M_2 receptor activation prevents increases in ciliary beat frequency initiated by M_1 receptors and also by nonmuscarinc stimuli such as ATP. Inhibition of ciliary beat frequency mediated by M_2 receptors is likely indirect, since M_2 mRNA and protein are not detectable in epithelial cells but are found in neighboring cells (Klein et al. 2009).

2.6 Muscarinic Receptors and Immune Responses

A functional role for muscarinic receptors in immune responses has been demonstrated in lung mast cells, alveolar macrophages, and airway epithelial cells.

Experimentally, anti-IgE antibodies and calcium ionophore both evoke histamine release from mast cells, an effect that is blocked by acetylcholine and other muscarinic agonists in isolated human bronchi (Reinheimer et al. 1997, 2000; Wessler et al. 2007). The role of inhibitory muscarinic receptors in human mast cells is confirmed since atropine (nonselective muscarinic antagonist) blocks the ability of acetylcholine to inhibit evoked histamine release (Reinheimer et al. 1997). Pharmacological data suggest this is mediated by M_1 receptors. In rats, however, acetylcholine enhances rather than inhibits evoked histamine release (Reinheimer et al. 2000).

Alveolar macrophages phagocytose foreign substances and initiate immune responses against invading pathogens. Acetylcholine induces release of leukotriene B_4 and other factors from alveolar macrophages that induce human peripheral blood monocyte, neutrophil, and eosinophil chemotaxis, and M_3 receptor antagonists prevent acetylcholine-induced release of chemotactic activity from macrophages (Reinheimer et al. 1998). Additionally, acetylcholine may also contribute to inflammation by inducing release of chemotactic factors from airway epithelial cells (Koyama et al. 1992, 1998).

2.7 Muscarinic Receptors and Airway Remodeling

Airway remodeling describes measureable changes in airway structure that occur as a pathological feature of lung diseases such as asthma and COPD. Acetylcholine

may contribute to airway remodeling by acting at muscarinic receptors to increase proliferation of both fibroblasts and smooth muscle cells. In primary cultures of human fibroblasts and fibroblast cell lines, acetylcholine stimulates collagen production and proliferation through MAPK activation (Haag et al. 2008; Matthiesen et al. 2006, 2007; Pieper et al. 2007). While acetylcholine does not directly increase smooth muscle cell proliferation, it enhances proliferation induced by growth factors, including platelet-derived growth factor and epidermal growth factor (Gosens et al. 2003; Krymskaya et al. 2000). Muscarinic receptor antagonists block the proliferative effects of acetylcholine in both fibroblast and smooth muscle cells.

Human lung fibroblasts contain mRNA for M_1, M_2, and M_3 muscarinic receptors with trace levels of M_4 receptors (Haag et al. 2008; Matthiesen et al. 2006). It is likely that M_2 receptors are dominant since the proliferative response in fibroblasts is pertusis toxin sensitive and can be blocked with selective muscarinic antagonists that suggest M_2 receptors are responsible (Matthiesen et al. 2006). Acetylcholine-enhanced proliferation of human airway smooth muscle cells is M_3 receptor-dependent and is lost when M_3 receptor expression is decreased with cell passage in vitro (Gosens et al. 2003).

2.8 Muscarinic Receptors in Normal Lung Function

The contribution of M_1, M_2, and M_3 receptors to pulmonary physiology is summarized in Table 1. Most lung tissues express more than one muscarinic receptor subtype, but the function of one muscarinic subtype is often dominant. Where the functions of additional muscarinic receptor subtypes are known, they either inhibit or supplement the dominant receptor's function. For example, M_2 receptors on postganglionic parasympathetic nerves inhibit acetylcholine release, and this function is inhibited by M_1 receptors in ganglia, which increase acetylcholine release by facilitating neurotransmission. In airway smooth muscle, M_2 receptors supplement contraction mediated via M_3 receptors. In healthy individuals, this muscarinic physiology is balanced and results in airway smooth muscle tone, vasodilation, mucus secretion, and mucociliary clearance.

However, in obstructive lung diseases some muscarinic receptor functions contribute to disease symptoms. For example, excessive bronchoconstriction and increased mucus secretion limit airflow in asthma. Acetylcholine released by the vagus nerves onto M_3 receptors mediates both of these physiological functions. Blocking M_3 receptors is therefore therapeutically very beneficial for reducing symptoms and improving lung function. Conversely, blocking inhibitory M_2 receptors on parasympathetic nerves is counterproductive since this increases acetylcholine release, resulting in increased bronchoconstriction and mucus secretion.

Table 1 Function of muscarinic receptor subtypes in lung

	M_1	M_2	M_3
Parasympathetic nerves	Increase neurotransmission at ganglia	Limit acetylcholine release	
Smooth muscle		Inhibit relaxation[a]	Contraction
Submucosal glands	Unknown		Mucus secretion
Endothelial cells		Unknown	Vasodilation[a]
Airway epithelium	Increase ciliary beat frequency[a] (if M_2 and M_3 blocked)	Reduce ciliary beat frequency[a]	Increase ciliary beat frequency
Immune function	Limit evoked histamine release from mast cells		Induce release of chemotactic factors from alveolar macrophages[a]
Airway remodeling		Increase proliferation in fibroblasts	Enhance proliferation induced by growth factors in smooth muscle

Receptor subtype involvement is based on human data unless noted with [a]. Shading indicates physiological functions that are especially important in COPD and asthma, lung diseases characterized by airflow limitation

3 Effects of Therapeutic Muscarinic Antagonists in Lung Disease

Muscarinic antagonists are used therapeutically as bronchodilators to treat both COPD and asthma, lung diseases characterized by airflow obstruction and underlying airway inflammation.

In COPD, airflow limitation is not fully reversible, and is caused by structural changes and narrowing of peripheral airways along with parenchymal destruction (GOLD 2009). Muscarinic antagonists increase airflow in COPD by blocking cholinergic tone at airway smooth muscle. However, asthma is different in that airflow limitation is generally fully reversible and caused by bronchoconstriction. In more severe asthma, edema due to mucus hypersecretion also contributes to airflow limitation. The airways are hyperresponsive and bronchoconstrictor responses are exaggerated (NHLBI 2007). Muscarinic antagonists increase airflow in asthma by blocking cholinergic tone and also by blocking reflex bronchoconstriction mediated by the vagus nerves. They may also inhibit secretion and clearance of mucus.

3.1 Therapeutic Muscarinic Receptor Antagonists

Atropine and other naturally occurring muscarinic receptor antagonists found in plants of the Datura genus have been effectively used as bronchodilators for

Table 2 Comparison of binding affinities and duration of binding for atropine, ipratropium, and tiotropium at human muscarinic receptors

		Atropine	Ipratropium	Tiotropium
K_i (nM)[a]	M_1	0.170	0.398	0.016
	M_2	0.339	0.295	0.020
	M_3	0.209	0.263	0.010
	M_4	0.107	0.224	0.010
	M_5	0.316	0.851	0.110
Dissociation half-life (h)[a, b]	M_1		0.10	10.5
	M_2		0.03	2.6
	M_3	0.04	0.22	27.0

[a]Antagonist affinities determined in heterologous competition binding experiments against [^{3}H] NMS. Dissociation kinetics using Motulski and Mahan method (Casarosa et al. 2009)
[b]Dowling and Charlton (2006)

centuries. In western medicine, the leaves and roots of *D. stramonium* were administered in cigarettes to treat respiratory diseases starting in the 1800s (Gross and Skorodin 1984). However, while atropine is an effective bronchodilator, its use is associated with side effects. Therefore, when beta adrenoreceptor agonists, which directly relax airway smooth muscle by stimulating β_2 receptors became available they largely replaced atropine. Since then, however, synthetic derivatives of atropine have been developed that contain a quaternary ammonium. This next generation of drugs, which include ipratropium and tiotropium, have limited bioavailability and are unable to cross the blood–brain barrier, and thus have fewer side effects. They are currently administered by inhalation to treat both COPD and asthma. Atropine, ipratropium, and tiotropium are all competitive antagonists (Casarosa et al. 2009), and thus contribute to bronchodilation primarily by blocking acetylcholine binding to M_3 receptors on airway smooth muscle. The pharmacological properties of atropine, ipratropium, and tiotropium are discussed below and summarized in Table 2.

3.1.1 Atropine

Atropine is a nonselective muscarinic antagonist with similar affinities for all five muscarinic receptor subtypes (Casarosa et al. 2009). Relative to the quaternary ammonium derivatives, atropine is also well absorbed across the gastrointestinal tract into systemic circulation. Total absorption of atropine across the intestine is approximately 25% in rat (Levine 1959), while bioavailability following intramuscular injection in humans is reported to be 50% (Goodman et al. 2006). As a result, atropine has many undesirable side effects including at low doses dry mouth, urinary retention, and accelerated heart rate. In addition, atropine is also able to cross the blood–brain barrier (Virtanen et al. 1982). Thus, at high doses side effects include coma, fever, and hallucinations.

3.1.2 Ipratropium Bromide

Ipratropium bromide is a quaternary ammonium derivative of atropine used clinically as a second-line bronchodilator behind β_2-agonists. It was also the first muscarinic antagonist widely used to treat COPD. Like atropine, ipratropium is nonselective and has similar affinities for all five muscarinic receptor subtypes (Casarosa et al. 2009). The major differences between ipratropium and atropine are the inability of ipratropium to cross the blood–brain barrier and its poor absorption in the gastrointestinal tract. Ipratropium is better absorbed when administered by inhalation (Ensing et al. 1989), which may be due to uptake by organic cation/carnitine transporters (OCTN) in airway epithelium. OCTN2, and to a lesser extent OCTN1, transport both ipratropium and tiotropium in a human bronchial epithelial cell line (Nakamura et al. 2010). Ipratropium produces peak bronchodilation within 60–90 min of inhalation and its duration of action is 4–6 h, requiring four times daily administration.

3.1.3 Tiotropium Bromide

Like ipratropium, tiotropium bromide also contains a quaternary ammonium. However, tiotropium has a much higher affinity for muscarinic receptors and a much longer duration of binding to muscarinic receptors than either atropine or ipratropium (see Table 2). However, tiotropium's most interesting property is its significantly greater duration of binding to M_1 and M_3 receptors than M_2 receptors, which provides tiotropium with kinetic selectivity for these receptors (Casarosa et al. 2009; Disse et al. 1993). Functionally, tiotropium blocks M_2 receptors on parasympathetic nerves early after administration to increase acetylcholine release. However, following washout, neuronal acetylcholine release returns to baseline within 2 h, a time point when smooth muscle contraction via M_3 receptors is still completely blocked. M_3 receptor function only begins to return after 7 h (Takahashi et al. 1994). Tiotropium's onset of bronchodilation in humans is very slow, reaching peak bronchodilation in 3–4 h, but tiotropium then has a very long duration of action (1–2 days) and can be administered daily (Maesen et al. 1995). The slow onset of action makes tiotropium inappropriate for a rescue medication, but the duration of action makes it useful as a once-daily bronchodilator.

3.2 Therapeutic Use of Muscarinic Receptor Antagonists in COPD

In COPD patients, airflow is limited by destructive and fibrotic changes in the lungs that narrow the airways. These changes are not reversible, but some bronchodilation can be achieved by blocking cholinergic tone. Because of the limited treatment

options for COPD, bronchodilators are central to the management of symptoms. Cholinergic tone may be higher in patients with COPD than in healthy patients and is effectively reversed with muscarinic receptor antagonists (Gross et al. 1989). Ipratropium is currently recommended for use as a four times daily short-acting bronchodilator by the Global Initiative for Chronic Obstructive Lung Disease 2009 global strategy for diagnosis, management, and prevention of COPD (GOLD 2009). Tiotropium is recommended for use as a once-daily long-acting bronchodilator. In COPD patients, a single inhaled dose (10–80 μg) of tiotropium results in a dose-dependent 19–26% improvement in the volume of air that is exhaled during the first second of forced exhalation (FEV_1) (Maesen et al. 1995). However, once steady-state plasma concentrations are reached following multiple once-daily dosings (4.5–36 μg), higher doses add little additional improvement. Thus, low doses with limited adverse side effects can be effectively used. Based on these data repeated daily dosing with the recommended 18 μg leads to continued bronchodilation (Littner et al. 2000; van Noord et al. 2002).

In the 4-year UPLIFT randomized, double-blind, placebo-controlled trial, 5,993 COPD patients were treated with either tiotropium or a placebo control. Tiotropium improved airflow (as measured by FEV_1), improved health-related quality of life scores, significantly delayed onset of exacerbations and associated hospitalizations, and reduced respiratory failure (Tashkin et al. 2008). These results are consistent with results from previous smaller and shorter studies (Casaburi et al. 2000; Dusser et al. 2006; Niewoehner et al. 2005). However, COPD is a progressive disease, and while tiotropium remained efficacious over the study period it was not able to significantly slow the rate of decline in mean FEV_1 (Tashkin et al. 2008). This is not unexpected, since only cessation of smoking has been shown to reduce this decline in patients with COPD (Anthonisen et al. 1994).

3.3 Therapeutic Use of Muscarinic Antagonists in Asthma

Asthma is characterized by inflammation and airway hyperresponsiveness, which is defined as excessive bronchoconstriction to contractile stimuli. There is no correlation between contractile responses of bronchial smooth muscle isolated from asthma and nonasthma patients and methacholine responsiveness in these same patients in vivo (Roberts et al. 1984; Whicker et al. 1988). Thus airway hyperresponsiveness in asthma is not simply due to increased smooth muscle sensitivity to contractile agents. However, maximum contractile responses in tracheal smooth muscle are greater in tissues from humans who died of fatal asthma than controls (Bai 1990; Haddad et al. 1996). It is unclear whether these results reflect the use of tracheal tissue instead of bronchi or are unique to fatal asthma patients. Since airway hyperresponsiveness occurs in vivo where vagal reflexes are present but not in vitro where reflexes are absent, this supports the role of parasympathetic nerves in airway hyperresponsiveness in asthma patients.

The contribution of parasympathetic nerves to airway hyperresponsiveness is further supported by published reports of surgical treatment in humans with severe asthma, where autonomic and sensory nerves supplying the lung were severed. These uncontrolled studies show improvements in 50% or more of the patients (Balogh et al. 1957; Overholt 1959; Phillips and Scott 1929). Dimitrov-Szokodi et al. denervated the lungs in 19 patients. Prior to surgery, asthma attacks were actually induced in eight patients with histamine. In these patients, histamine-induced asthma attacks ceased when neurotransmission in autonomic and sensory nerves was blocked with novocaine administered in the neck. Surgeries to denervate the airways were then carried out in all patients, and the cut vagus nerves were carefully sutured to prevent reinnervation. Following surgical denervation of the airways, exogenous histamine no longer induced asthma attacks. Of the 19 patients treated, 10 no longer needed any pharmacological treatment for their asthma, 7 had improved symptoms that could easily be controlled with drugs, and only 2 patients were not improved. In addition, denervation of the airways altered airway inflammation by reducing or abolishing eosinophils in sputum and blood (Balogh et al. 1957).

However, pharmacological evidence supporting a role for parasympathetic nerves and vagal reflexes in asthma was controversial for many years. Numerous studies in humans showed limited or no benefit of muscarinic receptor antagonists in their ability to block bronchoconstriction induced by nonspecific stimuli such as histamine, sulfur dioxide, exercise, cold air, and antigens (Casterline et al. 1976; Chan-Yeung et al. 1971; Cockcroft et al. 1978; Fish et al. 1977; Fisher et al. 1970; Nadel et al. 1965; Rosenthal et al. 1977; Ruffin et al. 1978; Woenne et al. 1978). The majority of these studies used a single dose of inhaled muscarinic antagonist, which was chosen because it effectively blocked either cholinergic tone or bronchoconstriction induced by inhaled methacholine. Conversely, other studies showed that muscarinic antagonists are effective, and they were able to inhibit bronchoconstriction induced by these same stimuli (Chan-Yeung 1977; Chen et al. 1981; Holtzman et al. 1980; Nadel et al. 1965; Sheppard et al. 1982; Widdicombe et al. 1962; Yu et al. 1972). These discrepancies are due to the dose of muscarinic antagonist administered, the degree of bronchoconstriction induced, and the method by which the antagonist is administered, all of which contribute to the degree of blockade of bronchoconstriction induced by vagal reflexes.

In acute asthma, the dose of muscarinic antagonist administered is very important because of the competitive nature of therapeutic antagonists. Doses of muscarinic receptor antagonists that only block or reduce airway tone may be ineffective for inhibiting asthma attacks when acetylcholine concentrations are increased. For example, in asthma patients low doses of inhaled atropine inhibit baseline cholinergic tone and prevent bronchoconstriction induced by inhaled methacholine. However, much higher doses of atropine are required to inhibit cold air-induced bronchoconstriction that is mediated by the vagus nerves. In one study, cold air-induced bronchoconstriction could be abolished in five and reduced in two of seven patients with higher doses of atropine. In these two patients, atropine could abolish cold air-induced bronchoconstriction when the level of cold

air-induced bronchoconstriction was decreased by reducing the exposure time (Sheppard et al. 1982). Thus, effective blockade can be achieved by increasing the muscarinic antagonist concentration or decreasing the agonist challenge (in this case cold air-induced acetylcholine release). Similarly, when increasing doses of ipratropium were given by nebulization to patients admitted to the hospital with acute asthma, 500 μg were required to achieve maximum bronchodilation, presumably via blockade of endogenous acetylcholine from vagus nerves. This is a ten times greater dose than the 40–80 μg dose (from a metered dose inhaler) that blocks vagal cholinergic tone and bronchoconstriction induced by inhaled exogenous methacholine (Baigelman and Chodosh 1977; Cockcroft et al. 1978). Thus, it is not surprising that the lower dose (80 μg) used in early studies had very little benefit in acute asthma (Cockcroft et al. 1978; Ruffin et al. 1978).

The method by which muscarinic receptor antagonists are administered is also important for achieving complete vagal blockade in animals and humans (Holtzman et al. 1983; Sheppard et al. 1983). In dogs, intravenous atropine blocks bronchoconstriction induced by inhaled acetylcholine or electrical stimulation of the vagal nerves equally well. This is in contrast to atropine administered by inhalation, which blocks bronchoconstriction induced by inhaled acetylcholine at significantly lower doses than are required to block bronchoconstriction induced by vagal stimulation (Holtzman et al. 1983). This study shows that intravenous administration of muscarinic receptor antagonists results in effective blockade of bronchoconstriction regardless of whether the agonist is inhaled or released by nerves, whereas inhaled antagonists, which are deposited in similar sites in the airway as inhaled agonists, are not able to block neuronal acetylcholine as effectively. Similar results are also found in humans (Sheppard et al. 1983).

One of the best-understood mechanisms for airway hyperresponsiveness in asthma is loss of inhibitory M_2 receptor function on the parasympathetic nerves (Ayala and Ahmed 1989; Minette et al. 1989). Loss of negative feedback through M_2 receptors in the efferent half of vagal reflexes leads to increased acetylcholine release and excessive bronchoconstriction to diverse stimuli.

In animals, airway hyperreactivity is due to M_2 receptor dysfunction following antigen challenge (Fryer and Wills-Karp 1991), ozone exposure (Schultheis et al. 1994), and viral infection (Fryer and Jacoby 1991), and it is closely associated with airway inflammation. Eosinophils, which are inflammatory cells associated with asthma, are clustered around the nerves in airways of sensitized guinea pigs and humans who have died of fatal asthma (Costello et al. 1997). Following antigen challenge, eosinophils are recruited to airway nerves (Fryer et al. 2006) and activated to release an endogenous M_2 receptor-selective antagonist, major basic protein (Jacoby et al. 1993). M_2 receptor dysfunction and hyperreactivity mediated by the vagus nerves are prevented by eosinophil depletion (Elbon et al. 1995), and by neutralizing eosinophil major basic protein or removing it from M_2 receptors (Evans et al. 1997; Fryer and Jacoby 1992). Eosinophils also mediate M_2 receptor dysfunction following ozone exposure (Yost et al. 1999) and virus infection in sensitized guinea pigs (Adamko et al. 1999). In addition, M_2 receptor dysfunction on parasympathetic nerves also occurs through eosinophil-independent

mechanisms. In the absence of antigen sensitization, viral neuraminidases reduce agonist affinity for M_2 receptors by removing sialic acid. The muscarinic agonist, carbachol, has tenfold lower affinity for M_2 receptors following desialation with neuraminidase (Fryer et al. 1990). Additionally, interferon-γ and tumor necrosis factor-α, cytokines produced during the inflammatory response to virus or inhaled antigen, reduce M_2 receptor gene expression resulting in decreased function (Jacoby et al. 1998; Nie et al. 2009). The mechanisms by which neuronal M_2 receptor function is lost in humans with asthma are not known, but the increased association of eosinophils with nerves in the lungs of humans who died of fatal asthma (Costello et al. 1997) suggests a role for eosinophils.

In humans, muscarinic receptor antagonists have been shown to provide significant bronchodilation in virus-induced asthma (Aquilina et al. 1980; Empey et al. 1976), allergic asthma (Yu et al. 1972), exercise-induced bronchospasm (Borut et al. 1977; Godfrey and Konig 1975), nocturnal asthma (Catterall et al. 1988; Morrison et al. 1988), and psychogenic asthma (McFadden et al. 1969; Rebuck and Marcus 1979). Muscarinic receptor antagonists are effective at blocking vagally induced bronchoconstriction and decreasing tone but are less effective for blocking direct effects of noncholinergic agents on airway smooth muscle. Current guidelines for asthma management recommend β_2-agonists be used as first-line bronchodilators, and ipratropium be used in combination with short-acting β_2-agonists in moderate-to-severe asthma exacerbations. Ipratropium is also recommended as the treatment of choice for bronchospasm due to beta-blocker medications (NHLBI 2007).

A double-blind, randomized, prospective trial compared the use of short-acting β_2-agonists and β_2-agonists combined with high doses of ipratropium (504 μg per hour for 3 h) in 180 patients admitted to the emergency department with acute asthma. Patients who received both ipratropium and β_2-agonist had a 48.1% greater improvement in FEV_1 than those who received β_2-agonist alone. Additionally, they had a 49% reduction in the risk of hospital admission. Patients with severe airway obstruction and patients with a longer duration of symptoms were more likely to benefit from the addition of ipratropium (Rodrigo and Rodrigo 2000). Similarly, a meta-analysis of 32 randomized controlled trials in children and adults with acute asthma showed that use of muscarinic receptor antagonists along with β_2-agonists significantly improved airflow measurements and decreased the risk of hospital admissions by 30% when compared to treatment with β_2-agonists alone. Muscarinic receptor antagonists were particularly beneficial in patients with moderate to severe obstruction, and there was a greater bronchodilation benefit when patients were treated with more than one dose of muscarinic receptor antagonist (Rodrigo and Castro-Rodriguez 2005).

Muscarinic receptor antagonists are not recommended for long-term management of stable asthma because β_2-agonists inhibit bronchoconstriction and corticosteroids effectively inhibit inflammation (NHLBI 2007). However, studies in animals suggest that tiotropium is as effective as budesonide (corticosteroid) in inhibiting several aspects of allergen-induced airway remodeling and inflammation including smooth muscle thickening, mucous gland hypertrophy, and eosinophilia

in airway tissue (Bos et al. 2007; Gosens et al. 2005). In addition, a small study in humans with severe asthma found that tiotropium bromide administered daily for 4 weeks improved FEV_1, and this was especially true for patients who had a large proportion of neutrophils in their sputum (Iwamoto et al. 2008). This may be due to muscarinic receptor antagonist inhibition of neutrophil recruitment into lungs, since tiotropium inhibits acetylcholine-induced release of chemotactic factors for neutrophils from human alveolar macrophages in vitro (Buhling et al. 2007). Together these data suggest that muscarinic receptor antagonists such as tiotropium may have additional anti-inflammatory benefits that need to be researched further and could be exploited in the future.

4 Conclusions

In the lungs, acetylcholine released from parasympathetic nerves provides the dominant control over airway smooth muscle tone. Muscarinic receptors found on glands, airway smooth muscle, and nerves control airway tone and mucus secretion. Additionally, nonneuoronal acetylcholine stimulates muscarinic receptors on epithelial cells and endothelial cells to increase ciliary beat frequency and cause vasodilation. Parasympathetic nerves act as the efferent arm in vagal reflexes to various chemical and physical stimuli, and in asthma changes in neuronal M_2 receptor function contribute to airway hyperresponsiveness. Muscarinic receptor antagonists are currently used as bronchodilators to treat airflow limitation in COPD and asthma and recent data suggest they may also be useful for treating airway remodeling.

References

Adamko DJ, Yost BL, Gleich GJ, Fryer AD, Jacoby DB (1999) Ovalbumin sensitization changes the inflammatory response to subsequent parainfluenza infection. Eosinophils mediate airway hyperresponsiveness, m(2) muscarinic receptor dysfunction, and antiviral effects. J Exp Med 190:1465–1478

Anthonisen NR, Connett JE, Kiley JP, Altose MD, Bailey WC, Buist AS, Conway WA Jr, Enright PL, Kanner RE, O'Hara P et al (1994) Effects of smoking intervention and the use of an inhaled anticholinergic bronchodilator on the rate of decline of FEV_1. The Lung Health Study. JAMA 272:1497–1505

Aquilina AT, Hall WJ, Douglas RG Jr, Utell MJ (1980) Airway reactivity in subjects with viral upper respiratory tract infections: the effects of exercise and cold air. Am Rev Respir Dis 122:3–10

Ayala LE, Ahmed T (1989) Is there loss of protective muscarinic receptor mechanism in asthma? Chest 96:1285–1291

Bai TR (1990) Abnormalities in airway smooth muscle in fatal asthma. Am Rev Respir Dis 141: 552–557

Baigelman W, Chodosh S (1977) Bronchodilator action of the anticholinergic drug, ipratropium bromide (Sch 1000), as an aerosol in chronic bronchitis and asthma. Chest 71:324–328

Baker B, Peatfield AC, Richardson PS (1985) Nervous control of mucin secretion into human bronchi. J Physiol 365:297–305
Baker DG, McDonald DM, Basbaum CB, Mitchell RA (1986) The architecture of nerves and ganglia of the ferret trachea as revealed by acetylcholinesterase histochemistry. J Comp Neurol 246:513–526
Baker DG, Don HF, Brown JK (1992) Direct measurement of acetylcholine release in guinea pig trachea. Am J Physiol 263:L142–L147
Balogh G, Dimitrov-Szokodi D, Husveti A (1957) Lung denervation in the therapy of intractable bronchial asthma. J Thorac Surg 33:166–184
Baroody F, Wagenmann M, Naclerio R (1993) Comparison of the secretory response of the nasal mucosa to methacholine and histamine. J Appl Physiol 74:2661–2671
Basbaum C (1984) Innervation of the airway mucosa and submucosa. Semin Respir Med 5:308–313
Belmonte KE, Fryer AD, Costello RW (1998) Role of insulin in antigen-induced airway eosinophilia and neuronal M2 muscarinic receptor dysfunction. J Appl Physiol 85:1708–1718
Blaber LC, Fryer AD, Maclagan J (1985) Neuronal muscarinic receptors attenuate vagally-induced contraction of feline bronchial smooth muscle. Br J Pharmacol 86:723–728
Bloom JW, Baumgartener-Folkerts C, Palmer JD, Yamamura HI, Halonen M (1988) A muscarinic receptor subtype modulates vagally stimulated bronchial contraction. J Appl Physiol 65: 2144–2150
Borson DB, Charlin M, Gold BD, Nadel JA (1984) Neural regulation of 35SO4-macromolecule secretion from tracheal glands of ferrets. J Appl Physiol 57:457–466
Borut TC, Tashkin DP, Fischer TJ, Katz R, Rachelefsky G, Siegel SC, Lee E, Harper C (1977) Comparison of aerosolized atropine sulfate and SCH 1000 on exercise-induced bronchospasm in children. J Allergy Clin Immunol 60:127–133
Bos IS, Gosens R, Zuidhof AB, Schaafsma D, Halayko AJ, Meurs H, Zaagsma J (2007) Inhibition of allergen-induced airway remodelling by tiotropium and budesonide: a comparison. Eur Respir J 30:653–661
Boushey H, Richardson P, Widdicombe J (1972) Reflex effects of laryngeal irritation on the pattern of breathing and total lung resistance. J Physiol 224:501–513
Buhling F, Lieder N, Kuhlmann UC, Waldburg N, Welte T (2007) Tiotropium suppresses acetylcholine-induced release of chemotactic mediators in vitro. Respir Med 101:2386–2394
Cabezas GA, Graf PD, Nadel JA (1971) Sympathetic versus parasympathetic nervous regulation of airways in dogs. J Appl Physiol 31:651–655
Canning BJ, Fischer A (1997) Localization of cholinergic nerves in lower airways of guinea pigs using antisera to choline acetyltransferase. Am J Physiol 272:L731–L738
Casaburi R, Briggs DD Jr, Donohue JF, Serby CW, Menjoge SS, Witek TJ Jr (2000) The spirometric efficacy of once-daily dosing with tiotropium in stable COPD: a 13-week multicenter trial. The US Tiotropium Study Group. Chest 118:1294–1302
Casarosa P, Bouyssou T, Germeyer S, Schnapp A, Gantner F, Pieper M (2009) Preclinical evaluation of long-acting muscarinic antagonists: comparison of tiotropium and investigational drugs. J Pharmacol Exp Ther 330:660–668
Casterline CL, Evans R III, Ward GW Jr (1976) The effect of atropine and albuterol aerosols on the human bronchial response to histamine. J Allergy Clin Immunol 58:607–613
Catterall JR, Rhind GB, Whyte KF, Shapiro CM, Douglas NJ (1988) Is nocturnal asthma caused by changes in airway cholinergic activity? Thorax 43:720–724
Caulfield MP, Birdsall NJ (1998) International Union of Pharmacology. XVII. Classification of muscarinic acetylcholine receptors. Pharmacol Rev 50:279–290
Cavallotti C, D'Andrea V, Cavallotti C, Cameroni M (2005) Distribution of acetylcholinesterase and cholineacetyl-transferase activities in the human pulmonary vessels of younger and older adults. Geriatr Gerontol Int 5:286–292
Chan-Yeung M (1977) The effect of Sch 1000 and disodium cromoglycate on exercise-induced asthma. Chest 71:320–323

Chan-Yeung MM, Vyas MN, Grzybowski S (1971) Exercise-induced asthma. Am Rev Respir Dis 104:915–923
Chen WY, Brenner AM, Weiser PC, Chai H (1981) Atropine and exercise-induced bronchoconstriction. Chest 79:651–656
Cockcroft DW, Ruffin RE, Hargreave FE (1978) Effect of Sch1000 in allergen-induced asthma. Clin Allergy 8:361–372
Colebatch HJ, Halmagyi DF (1963) Effect of vagotomy and vagal stimulation on lung mechanics and circulation. J Appl Physiol 18:881–887
Corssen G, Allen CR (1959) Acetylcholine: its significance in controlling ciliary activity of human respiratory epithelium in vitro. J Appl Physiol 14:901–904
Costello RW, Schofield BH, Kephart GM, Gleich GJ, Jacoby DB, Fryer AD (1997) Localization of eosinophils to airway nerves and effect on neuronal M2 muscarinic receptor function. Am J Physiol 273:L93–L103
Dale HH (1914) The action of certain esters and ethers of choline, and their relation to muscarine. J Pharmacol Exp Ther 6:147–190
Daniel EE, Kannan M, Davis C, Posey-Daniel V (1986) Ultrastructural studies on the neuromuscular control of human tracheal and bronchial muscle. Respir Physiol 63:109–128
Disse B, Reichl R, Speck G, Traunecker W, Ludwig Rominger KL, Hammer R (1993) Ba 679 BR, a novel long-acting anticholinergic bronchodilator. Life Sci 52:537–544
Dowling MR, Charlton SJ (2006) Quantifying the association and dissociation rates of unlabelled antagonists at the muscarinic M3 receptor. Br J Pharmacol 148:927–937
Dusser D, Bravo ML, Iacono P (2006) The effect of tiotropium on exacerbations and airflow in patients with COPD. Eur Respir J 27:547–555
Dwyer TM, Szebeni A, Diveki K, Farley JM (1992) Transient cholinergic glycoconjugate secretion from swine tracheal submucosal gland cells. Am J Physiol 262:L418–L426
Elbon CL, Jacoby DB, Fryer AD (1995) Pretreatment with an antibody to interleukin-5 prevents loss of pulmonary M2 muscarinic receptor function in antigen-challenged guinea pigs. Am J Respir Cell Mol Biol 12:320–328
Empey DW, Laitinen LA, Jacobs L, Gold WM, Nadel JA (1976) Mechanisms of bronchial hyperreactivity in normal subjects after upper respiratory tract infection. Am Rev Respir Dis 113:131–139
Ensing K, de Zeeuw RA, Nossent GD, Koeter GH, Cornelissen PJ (1989) Pharmacokinetics of ipratropium bromide after single dose inhalation and oral and intravenous administration. Eur J Clin Pharmacol 36:189–194
Evans CM, Fryer AD, Jacoby DB, Gleich GJ, Costello RW (1997) Pretreatment with antibody to eosinophil major basic protein prevents hyperresponsiveness by protecting neuronal M2 muscarinic receptors in antigen-challenged guinea pigs. J Clin Invest 100:2254–2262
Ferguson SM, Savchenko V, Apparsundaram S, Zwick M, Wright J, Heilman CJ, Yi H, Levey AI, Blakely RD (2003) Vesicular localization and activity-dependent trafficking of presynaptic choline transporters. J Neurosci 23:9697–9709
Fernandes LB, Fryer AD, Hirshman CA (1992) M2 muscarinic receptors inhibit isoproterenol-induced relaxation of canine airway smooth muscle. J Pharmacol Exp Ther 262:119–126
Fish JE, Rosenthal RR, Summer WR, Menkes H, Norman PS, Permutt S (1977) The effect of atropine on acute antigen-mediated airway constriction in subjects with allergic asthma. Am Rev Respir Dis 115:371–379
Fisher HK, Holton P, Buxton RS, Nadel JA (1970) Resistance to breathing during exercise-induced asthma attacks. Am Rev Respir Dis 101:885–896
Fisher JT, Vincent SG, Gomeza J, Yamada M, Wess J (2004) Loss of vagally mediated bradycardia and bronchoconstriction in mice lacking M2 or M3 muscarinic acetylcholine receptors. FASEB J 18:711–713
Fryer AD, Jacoby DB (1991) Parainfluenza virus infection damages inhibitory M2 muscarinic receptors on pulmonary parasympathetic nerves in the guinea-pig. Br J Pharmacol 102: 267–271

Fryer AD, Jacoby DB (1992) Function of pulmonary M2 muscarinic receptors in antigen-challenged guinea pigs is restored by heparin and poly-L-glutamate. J Clin Invest 90:2292–2298
Fryer AD, Maclagan J (1984) Muscarinic inhibitory receptors in pulmonary parasympathetic nerves in the guinea-pig. Br J Pharmacol 83:973–978
Fryer AD, Wills-Karp M (1991) Dysfunction of M2-muscarinic receptors in pulmonary parasympathetic nerves after antigen challenge. J Appl Physiol 71:2255–2261
Fryer AD, el-Fakahany EE, Jacoby DB (1990) Parainfluenza virus type 1 reduces the affinity of agonists for muscarinic receptors in guinea-pig lung and heart. Eur J Pharmacol 181:51–58
Fryer AD, Elbon CL, Kim AL, Xiao HQ, Levey AI, Jacoby DB (1996) Cultures of airway parasympathetic nerves express functional M2 muscarinic receptors. Am J Respir Cell Mol Biol 15:716–725
Fryer AD, Stein LH, Nie Z, Curtis DE, Evans CM, Hodgson ST, Jose PJ, Belmonte KE, Fitch E, Jacoby DB (2006) Neuronal eotaxin and the effects of CCR3 antagonist on airway hyperreactivity and M2 receptor dysfunction. J Clin Invest 116:228–236
Fujimura M, Kamio Y, Matsuda T (1992) Effect of a M1-selective muscarinic receptor antagonist (pirenzepine) on basal bronchomotor tone in young women. Respiration 59:102–106
Furchgott RF, Zawadzki JV (1980) The obligatory role of endothelial cells in the relaxation of arterial smooth muscle by acetylcholine. Nature 288:373–376
Gallagher JT, Kent PW, Passatore M, Phipps RJ, Richardson PS (1975) The composition of tracheal mucus and the nervous control of its secretion in the cat. Proc R Soc Lond B Biol Sci 192:49–76
Godfrey S, Konig P (1975) Suppression of exercise-induced asthma by salbutamol, theophylline, atropine, cromolyn, and placebo in a group of asthmatic children. Pediatrics 56:930–934
GOLD (2009) Global initiative for chronic obstructive lung disease. Global strategy for the diagnosis, management, and prevention of chronic obstructive pulmonary disease. National Heart Lung and Blood Institute, World Health Organization. http://www.goldcopd.com. Accessed 8 July 2010
Gold WM, Kessler GF, Yu DY (1972) Role of vagus nerves in experimental asthma in allergic dogs. J Appl Physiol 33:719–725
Goodman LS, Gilman A, Brunton LL, Lazo JS, Parker KL (2006) Goodman & Gilman's the pharmacological basis of therapeutics, 11th edn. McGraw-Hill, New York, NY
Gosens R, Nelemans SA, Grootte Bromhaar MM, McKay S, Zaagsma J, Meurs H (2003) Muscarinic M3-receptors mediate cholinergic synergism of mitogenesis in airway smooth muscle. Am J Respir Cell Mol Biol 28:257–262
Gosens R, Bos IS, Zaagsma J, Meurs H (2005) Protective effects of tiotropium bromide in the progression of airway smooth muscle remodeling. Am J Respir Crit Care Med 171:1096–1102
Greenberg B, Rhoden K, Barnes PJ (1987) Endothelium-dependent relaxation of human pulmonary arteries. Am J Physiol 252:H434–H438
Gross NJ, Skorodin MS (1984) Anticholinergic, antimuscarinic bronchodilators. Am Rev Respir Dis 129:856–870
Gross NJ, Co E, Skorodin MS (1989) Cholinergic bronchomotor tone in COPD. Estimates of its amount in comparison with that in normal subjects. Chest 96:984–987
Haag S, Matthiesen S, Juergens UR, Racke K (2008) Muscarinic receptors mediate stimulation of collagen synthesis in human lung fibroblasts. Eur Respir J 32:555–562
Haberberger R, Schemann M, Sann H, Kummer W (1997) Innervation pattern of guinea pig pulmonary vasculature depends on vascular diameter. J Appl Physiol 82:426–434
Haberberger RV, Bodenbenner M, Kummer W (2000) Expression of the cholinergic gene locus in pulmonary arterial endothelial cells. Histochem Cell Biol 113:379–387
Haddad EB, Landry Y, Gies JP (1991) Muscarinic receptor subtypes in guinea pig airways. Am J Physiol 261:L327–L333
Haddad EB, Mak JC, Belvisi MG, Nishikawa M, Rousell J, Barnes PJ (1996) Muscarinic and beta-adrenergic receptor expression in peripheral lung from normal and asthmatic patients. Am J Physiol 270:L947–L953

Holtzman M, Sheller J, Dimeo M, Nadel J, Boushey H (1980) Effect of ganglionic blockade on bronchial reactivity in atopic subjects. Am Rev Respir Dis 122:17–25

Holtzman MJ, McNamara MP, Sheppard D, Fabbri LM, Hahn HL, Graf PD, Nadel JA (1983) Intravenous versus inhaled atropine for inhibiting bronchoconstrictor responses in dogs. J Appl Physiol 54:134–139

Iwamoto H, Yokoyama A, Shiota N, Shoda H, Haruta Y, Hattori N, Kohno N (2008) Tiotropium bromide is effective for severe asthma with noneosinophilic phenotype. Eur Respir J 31: 1379–1380

Jacoby DB, Gleich GJ, Fryer AD (1993) Human eosinophil major basic protein is an endogenous allosteric antagonist at the inhibitory muscarinic M2 receptor. J Clin Invest 91:1314–1318

Jacoby DB, Xiao HQ, Lee NH, Chan-Li Y, Fryer AD (1998) Virus- and interferon-induced loss of inhibitory M2 muscarinic receptor function and gene expression in cultured airway parasympathetic neurons. J Clin Invest 102:242–248

Jammes Y, Mei N (1979) Assessment of the pulmonary origin of bronchoconstrictor vagal tone. J Physiol 291:305–316

Jositsch G, Papadakis T, Haberberger RV, Wolff M, Wess J, Kummer W (2009) Suitability of muscarinic acetylcholine receptor antibodies for immunohistochemistry evaluated on tissue sections of receptor gene-deficient mice. Naunyn Schmiedebergs Arch Pharmacol 379:389–395

Kalia M (1981) Brain stem localization of vagal preganglionic neurons. J Auton Nerv Syst 3:451–481

Kesler BS, Canning BJ (1999) Regulation of baseline cholinergic tone in guinea-pig airway smooth muscle. J Physiol 518(Pt 3):843–855

Klein MK, Haberberger RV, Hartmann P, Faulhammer P, Lips KS, Krain B, Wess J, Kummer W, Konig P (2009) Muscarinic receptor subtypes in cilia-driven transport and airway epithelial development. Eur Respir J 33:1113–1121

Knight DS, Ellison JP, Hibbs RG, Hyman AL, Kadowitz PJ (1981) A light and electron microscopic study of the innervation of pulmonary arteries in the cat. Anat Rec 201:513–521

Koyama S, Rennard SI, Robbins RA (1992) Acetylcholine stimulates bronchial epithelial cells to release neutrophil and monocyte chemotactic activity. Am J Physiol 262:L466–L471

Koyama S, Sato E, Nomura H, Kubo K, Nagai S, Izumi T (1998) Acetylcholine and substance P stimulate bronchial epithelial cells to release eosinophil chemotactic activity. J Appl Physiol 84:1528–1534

Krymskaya VP, Orsini MJ, Eszterhas AJ, Brodbeck KC, Benovic JL, Panettieri RA Jr, Penn RB (2000) Mechanisms of proliferation synergy by receptor tyrosine kinase and G protein-coupled receptor activation in human airway smooth muscle. Am J Respir Cell Mol Biol 23:546–554

Kummer W, Haberberger R (1999) Extrinsic and intrinsic cholinergic systems of the vascular wall. Eur J Morphol 37:223–226

Kummer W, Wiegand S, Akinci S, Wessler I, Schinkel AH, Wess J, Koepsell H, Haberberger RV, Lips KS (2006) Role of acetylcholine and polyspecific cation transporters in serotonin-induced bronchoconstriction in the mouse. Respir Res 7:65

Laitinen LA, Laitinen MV, Widdicombe JG (1987) Parasympathetic nervous control of tracheal vascular resistance in the dog. J Physiol 385:135–146

Levine RM (1959) The intestinal absorption of the quaternary derivatives of atropine and scopolamine. Arch Int Pharmacodyn Ther 121:146–149

Lips KS, Volk C, Schmitt BM, Pfeil U, Arndt P, Miska D, Ermert L, Kummer W, Koepsell H (2005) Polyspecific cation transporters mediate luminal release of acetylcholine from bronchial epithelium. Am J Respir Cell Mol Biol 33:79–88

Littner MR, Ilowite JS, Tashkin DP, Friedman M, Serby CW, Menjoge SS, Witek TJ Jr (2000) Long-acting bronchodilation with once-daily dosing of tiotropium (Spiriva) in stable chronic obstructive pulmonary disease. Am J Respir Crit Care Med 161:1136–1142

Liu QH, Zheng YM, Korde AS, Yadav VR, Rathore R, Wess J, Wang YX (2009) Membrane depolarization causes a direct activation of G protein-coupled receptors leading to local Ca^{2+} release in smooth muscle. Proc Natl Acad Sci USA 106:11418–11423

Maesen FP, Smeets JJ, Sledsens TJ, Wald FD, Cornelissen PJ (1995) Tiotropium bromide, a new long-acting antimuscarinic bronchodilator: a pharmacodynamic study in patients with chronic obstructive pulmonary disease (COPD). Dutch Study Group. Eur Respir J 8:1506–1513
Mak J, Barnes P (1990) Autoradiographic visualization of muscarinic receptor subtypes in human and guinea pig lung. Am Rev Respir Dis 141:1559–1568
Mak JC, Baraniuk JN, Barnes PJ (1992) Localization of muscarinic receptor subtype mRNAs in human lung. Am J Respir Cell Mol Biol 7:344–348
Matthiesen S, Bahulayan A, Kempkens S, Haag S, Fuhrmann M, Stichnote C, Juergens UR, Racke K (2006) Muscarinic receptors mediate stimulation of human lung fibroblast proliferation. Am J Respir Cell Mol Biol 35:621–627
Matthiesen S, Bahulayan A, Holz O, Racke K (2007) MAPK pathway mediates muscarinic receptor-induced human lung fibroblast proliferation. Life Sci 80:2259–2262
McAllen R, Spyer K (1978) Two types of vagal preganglionic motoneurones projecting to the heart and lungs. J Physiol 282:353–364
McFadden ER Jr, Luparello T, Lyons HA, Bleecker E (1969) The mechanism of action of suggestion in the induction of acute asthma attacks. Psychosom Med 31:134–143
McMahon TJ, Kadowitz PJ (1992) Methylene blue inhibits neurogenic cholinergic vasodilator responses in the pulmonary vascular bed of the cat. Am J Physiol 263:L575–L584
Minette PA, Lammers JW, Dixon CM, McCusker MT, Barnes PJ (1989) A muscarinic agonist inhibits reflex bronchoconstriction in normal but not in asthmatic subjects. J Appl Physiol 67:2461–2465
Morrison JF, Pearson SB, Dean HG (1988) Parasympathetic nervous system in nocturnal asthma. Br Med J (Clin Res Ed) 296:1427–1429
Murai Y, Ishibashi H, Akaike N, Ito Y (1998) Acetylcholine modulation of high-voltage-activated calcium channels in the neurones acutely dissociated from rat paratracheal ganglia. Br J Pharmacol 123:1441–1449
Myers AC (2001) Transmission in autonomic ganglia. Respir Physiol 125:99–111
Myers AC, Undem BJ (1996) Muscarinic receptor regulation of synaptic transmission in airway parasympathetic ganglia. Am J Physiol 270:L630–L636
Nadel JA, Salem H, Tamplin B, Tokiwa Y (1965) Mechanism of Bronchoconstriction during Inhalation of Sulfur Dioxide. J Appl Physiol 20:164–167
Nakamura T, Nakanishi T, Haruta T, Shirasaka Y, Keogh JP, Tamai I (2010) Transport of ipratropium, an anti-chronic obstructive pulmonary disease drug, is mediated by organic cation/carnitine transporters in human bronchial epithelial cells: implications for carrier-mediated pulmonary absorption. Mol Pharm 7:187–195
NHLBI (2007) National Heart, Lung, and Blood Institute. Expert panel report 3 (EPR3): guidelines for the diagnosis and management of asthma. U.S. Department of Health and Human Services, National Institutes of Health, National Heart, Lung, and Blood Institute. http://purl.access.gpo.gov/GPO/LPS93946. Accessed 8 July 2010
Nie Z, Jacoby DB, Fryer AD (2009) Etanercept prevents airway hyperresponsiveness by protecting neuronal M2 muscarinic receptors in antigen-challenged guinea pigs. Br J Pharmacol 156:201–210
Niewoehner DE, Rice K, Cote C, Paulson D, Cooper JA Jr, Korducki L, Cassino C, Kesten S (2005) Prevention of exacerbations of chronic obstructive pulmonary disease with tiotropium, a once-daily inhaled anticholinergic bronchodilator: a randomized trial. Ann Intern Med 143:317–326
Olsen C, Colebatch H, Mebel P, Nadel J, Staub N (1965) Motor control of pulmonary airways studied by nerve stimulation. J Appl Physiol 20:202–208
Overholt RH (1959) Pulmonary denervation and resection in asthmatic patients. Ann Allergy 17:534–545
Phillips EW, Scott WJM (1929) The surgical treatment of bronchial asthma. Arch Surg 19: 1425–1456
Pieper MP, Chaudhary NI, Park JE (2007) Acetylcholine-induced proliferation of fibroblasts and myofibroblasts in vitro is inhibited by tiotropium bromide. Life Sci 80:2270–2273

Pradidarcheep W, Labruyere WT, Dabhoiwala NF, Lamers WH (2008) Lack of specificity of commercially available antisera: better specifications needed. J Histochem Cytochem 56: 1099–1111

Proskocil BJ, Sekhon HS, Jia Y, Savchenko V, Blakely RD, Lindstrom J, Spindel ER (2004) Acetylcholine is an autocrine or paracrine hormone synthesized and secreted by airway bronchial epithelial cells. Endocrinology 145:2498–2506

Ramnarine SI, Haddad EB, Khawaja AM, Mak JC, Rogers DF (1996) On muscarinic control of neurogenic mucus secretion in ferret trachea. J Physiol 494(Pt 2):577–586

Rebuck AS, Marcus HI (1979) SCH 1000 in psychogenic asthma. Scand J Respir Dis Suppl 103:186–191

Reinheimer T, Bernedo P, Klapproth H, Oelert H, Zeiske B, Racke K, Wessler I (1996) Acetylcholine in isolated airways of rat, guinea pig, and human: species differences in role of airway mucosa. Am J Physiol 270:L722–L728

Reinheimer T, Baumgartner D, Hohle KD, Racke K, Wessler I (1997) Acetylcholine via muscarinic receptors inhibits histamine release from human isolated bronchi. Am J Respir Crit Care Med 156:389–395

Reinheimer T, Munch M, Bittinger F, Racke K, Kirkpatrick CJ, Wessler I (1998) Glucocorticoids mediate reduction of epithelial acetylcholine content in the airways of rats and humans. Eur J Pharmacol 349:277–284

Reinheimer T, Mohlig T, Zimmermann S, Hohle KD, Wessler I (2000) Muscarinic control of histamine release from airways. Inhibitory M1-receptors in human bronchi but absence in rat trachea. Am J Respir Crit Care Med 162:534–538

Roberts JA, Raeburn D, Rodger IW, Thomson NC (1984) Comparison of in vivo airway responsiveness and in vitro smooth muscle sensitivity to methacholine in man. Thorax 39:837–843

Roberts AM, Coleridge HM, Coleridge JC (1988) Reciprocal action of pulmonary vagal afferents on tracheal smooth muscle tension in dogs. Respir Physiol 72:35–46

Rodrigo GJ, Castro-Rodriguez JA (2005) Anticholinergics in the treatment of children and adults with acute asthma: a systematic review with meta-analysis. Thorax 60:740–746

Rodrigo GJ, Rodrigo C (2000) First-line therapy for adult patients with acute asthma receiving a multiple-dose protocol of ipratropium bromide plus albuterol in the emergency department. Am J Respir Crit Care Med 161:1862–1868

Roffel AF, in't Hout WG, de Zeeuw RA, Zaagsma J (1987) The M2 selective antagonist AF-DX 116 shows high affinity for muscarine receptors in bovine tracheal membranes. Naunyn Schmiedebergs Arch Pharmacol 335:593–595

Roffel AF, Elzinga CR, Van Amsterdam RG, De Zeeuw RA, Zaagsma J (1988) Muscarinic M2 receptors in bovine tracheal smooth muscle: discrepancies between binding and function. Eur J Pharmacol 153:73–82

Roffel AF, Elzinga CR, Zaagsma J (1990) Muscarinic M3 receptors mediate contraction of human central and peripheral airway smooth muscle. Pulm Pharmacol 3:47–51

Rogers DF (2001) Motor control of airway goblet cells and glands. Respir Physiol 125:129–144

Rosenthal RR, Norman PS, Summer WR, Permutt S (1977) Role of the parasympathetic system in antigen-induced bronchospasm. J Appl Physiol 42:600–606

Ruffin RE, Cockcroft DW, Hargreave FE (1978) A comparison of the protective effect of fenoterol and Sch 1000 on allergen-induced asthma. J Allergy Clin Immunol 61:42–47

Salathe M, Lipson EJ, Ivonnet PI, Bookman RJ (1997) Muscarinic signaling in ciliated tracheal epithelial cells: dual effects on Ca^{2+} and ciliary beating. Am J Physiol 272:L301–L310

Schultheis AH, Bassett DJ, Fryer AD (1994) Ozone-induced airway hyperresponsiveness and loss of neuronal M2 muscarinic receptor function. J Appl Physiol 76:1088–1097

Severinghaus JW, Stupfel M (1955) Respiratory dead space increase following atropine in man, and atropine, vagal or ganglionic blockade and hypothermia in dogs. J Appl Physiol 8:81–87

Seybold ZV, Mariassy AT, Stroh D, Kim CS, Gazeroglu H, Wanner A (1990) Mucociliary interaction in vitro: effects of physiological and inflammatory stimuli. J Appl Physiol 68: 1421–1426

Sheppard D, Epstein J, Holtzman M, Nadel J, Boushey H (1982) Dose-dependent inhibition of cold air-induced bronchoconstriction by atropine. J Appl Physiol: Respir Environ Exercise Physiol 53:169–174

Sheppard D, Epstein J, Holtzman MJ, Nadel JA, Boushey HA (1983) Effect of route of atropine delivery on bronchospasm from cold air and methacholine. J Appl Physiol 54:130–133

Stengel PW, Gomeza J, Wess J, Cohen ML (2000) M(2) and M(4) receptor knockout mice: muscarinic receptor function in cardiac and smooth muscle in vitro. J Pharmacol Exp Ther 292:877–885

Struckmann N, Schwering S, Wiegand S, Gschnell A, Yamada M, Kummer W, Wess J, Haberberger RV (2003) Role of muscarinic receptor subtypes in the constriction of peripheral airways: studies on receptor-deficient mice. Mol Pharmacol 64:1444–1451

Takahashi T, Belvisi MG, Patel H, Ward JK, Tadjkarimi S, Yacoub MH, Barnes PJ (1994) Effect of Ba 679 BR, a novel long-acting anticholinergic agent, on cholinergic neurotransmission in guinea pig and human airways. Am J Respir Crit Care Med 150:1640–1645

Tashkin DP, Celli B, Senn S, Burkhart D, Kesten S, Menjoge S, Decramer M (2008) A 4-year trial of tiotropium in chronic obstructive pulmonary disease. N Engl J Med 359:1543–1554

Undem BJ, Myers AC, Barthlow H, Weinreich D (1990) Vagal innervation of guinea pig bronchial smooth muscle. J Appl Physiol 69:1336–1346

van Koppen CJ, Blankesteijn WM, Klaassen AB, Rodrigues de Miranda JF, Beld AJ, van Ginneken CA (1987) Autoradiographic visualization of muscarinic receptors in pulmonary nerves and ganglia. Neurosci Lett 83:237–240

van Koppen CJ, Blankesteijn WM, Klaassen AB, Rodrigues de Miranda JF, Beld AJ, van Ginneken CA (1988) Autoradiographic visualization of muscarinic receptors in human bronchi. J Pharmacol Exp Ther 244:760–764

van Noord JA, Smeets JJ, Custers FL, Korducki L, Cornelissen PJ (2002) Pharmacodynamic steady state of tiotropium in patients with chronic obstructive pulmonary disease. Eur Respir J 19:639–644

Virtanen R, Kanto J, Iisalo E, Iisalo EU, Salo M, Sjovall S (1982) Pharmacokinetic studies on atropine with special reference to age. Acta Anaesthesiol Scand 26:297–300

Wagner EM, Jacoby DB (1999) Methacholine causes reflex bronchoconstriction. J Appl Physiol 86:294–297

Wessler I, Holper B, Kortsik C, Buhl R, Kilbinger H, Kirkpatrick CJ (2007) Dysfunctional inhibitory muscarinic receptors mediate enhanced histamine release in isolated human bronchi. Life Sci 80:2294–2297

Whicker SD, Armour CL, Black JL (1988) Responsiveness of bronchial smooth muscle from asthmatic patients to relaxant and contractile agonists. Pulm Pharmacol 1:25–31

Widdicombe JG (1966) Action potentials in parasympathetic and sympathetic efferent fibres to the trachea and lungs of dogs and cats. J Physiol 186:56–88

Widdicombe JG, Kent DC, Nadel JA (1962) Mechanism of bronchoconstriction during inhalation of dust. J Appl Physiol 17:613–616

Woenne R, Kattan M, Orange RP, Levison H (1978) Bronchial hyperreactivity to histamine and methacholine in asthmatic children after inhalation of SCH 1000 and chlorpheniramine maleate. J Allergy Clin Immunol 62:119–124

Wong LB, Miller IF, Yeates DB (1988) Stimulation of ciliary beat frequency by autonomic agonists: in vivo. J Appl Physiol 65:971–981

Yang CM, Farley JM, Dwyer TM (1988) Muscarinic stimulation of submucosal glands in swine trachea. J Appl Physiol 64:200–209

Yost BL, Gleich GJ, Fryer AD (1999) Ozone-induced hyperresponsiveness and blockade of M2 muscarinic receptors by eosinophil major basic protein. J Appl Physiol 87:1272–1278

Yu DY, Galant SP, Gold WM (1972) Inhibition of antigen-induced bronchoconstriction by atropine in asthmatic patients. J Appl Physiol 32:823–828

Muscarinic Agonists and Antagonists: Effects on Gastrointestinal Function

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Abstract Muscarinic agonists and antagonists are used to treat a handful of gastrointestinal (GI) conditions associated with impaired salivary secretion or altered motility of GI smooth muscle. With regard to exocrine secretion, the major muscarinic receptor expressed in salivary, gastric, and pancreatic glands is the M_3 with a small contribution of the M_1 receptor. In GI smooth muscle, the major muscarinic receptors expressed are the M_2 and M_3 with the M_2 outnumbering the M_3 by a ratio of at least four to one. The antagonism of both smooth muscle contraction and exocrine secretion is usually consistent with an M_3 receptor mechanism despite the major presence of the M_2 receptor in smooth muscle. These results are consistent with the conditional role of the M_2 receptor in smooth muscle. That is, the contractile role of the M_2 receptor depends on that of the M_3 so that antagonism of the M_3 receptor eliminates the response of the M_2. The physiological roles of muscarinic receptors in the GI tract are consistent with their known signaling mechanisms. Some so-called tissue-selective M_3 antagonists may owe their selectivity to a highly potent interaction with a nonmuscarinic receptor target.

Keywords Adenylate cyclase • Ca^{2+}-activated potassium channels • Colon • Gastroparesis • Ileum • Irritable bowel syndrome • M_2 muscarinic receptor • M_3 muscarinic receptor • Nonselective cation conductance • Pancreas • Parotid gland • Pertussis toxin • Sjogren's Syndrome • Stomach

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Abbreviations

A23187	5-(Methylamino)-2-({(2*R*,3*R*,6*S*,8*S*,9*R*,11*R*)-3,9,11-trimethyl-8-[(1*S*)-1-methyl-2-oxo-2-(1*H*-pyrrol-2-yl)ethyl]-1,7-dioxaspiro[5.5]undec-2-yl}methyl)-1,3-benzoxazole-4-carboxylic acid
AF-DX 116	11-[[2-[(Diethylamino)methyl]-1-piperidinyl]acetyl]-5,11-dihydro-6*H*-pyrido[2,3-*b*][1,4]benzodiazepin-6-one
AQ-RA 741	11-[[4-[4-(Diethylamino)butyl]-1-piperidinyl]acetyl]-5,11-dihydro-6*H*-pyrido[2,3-*b*][1,4]benzodiazepin-6-one
4-DAMP	*N*,*N*-dimethyl-4-piperidinyl diphenlyacetate
4-DAMP mustard	*N*-(2-chloroethyl)-4-piperidinyl diphenylacetate
GI	Gastrointestinal
HHSiD	Hexahydro-sila-difenidol
[^{3}H]NMS	[^{3}H]N-methylscopolamine
I_{cat}	Nonselective cation conductance
K_B	Dissociation constant of the antagonist
KO	Knockout
pK_B	Negative log dissociation constant
pK_D	Log binding affinity constant
p-F-HHSiD	*para*-Fluoro-hexahydro-sila-difenidol
TRP	Transient receptor potential
K_{Ca}	Ca^{2+}-activated potassium channel

1 Introduction

Muscarinic receptors are present on myenteric neurons and various organs of the gastrointestinal (GI) tract. When activated by acetylcholine or muscarinic agonists, these receptors cause contraction of smooth muscle; inhibition of neurotransmitter release; stimulation or inhibition of neuronal firing; and secretion of protein, electrolyte, and mucous from exocrine glands. Not surprisingly, muscarinic agonists and antagonists have marked effects on the function of the GI tract.

These actions can be explained on the basis of the proximate signaling mechanisms of subtypes of muscarinic receptors, which often interact with one another and complicate the interpretation of drug effects and the design of novel muscarinic and antimuscarinic drugs for the treatment of GI disorders.

In this chapter, therefore, we review the distribution and proximate signaling mechanisms of muscarinic receptors in GI tissues, describe the pharmacological antagonism of responses mediated by two receptors (i.e., the M_2 and M_3 muscarinic receptors), review the effects of muscarinic agonists and antagonists on various organs of the GI tract, and briefly summarize the therapeutic uses of muscarinic agonists and antagonists in the treatment of some GI disorders.

2 Distribution of Muscarinic Receptors in the Gastrointestinal Tract

The function of the GI tract is to digest food, absorb nutrients, and eliminate waste. These are mediated by the propulsive effect of GI smooth muscle, the absorptive capacity of the mucosa, and the secretory action of the mucosa and salivary glands. Thus, much of the pharmacology of muscarinic agonists and antagonists can be attributed to their effects on the mucosa, exocrine glands, and smooth muscle of the GI tract.

In the smooth muscle of the esophagus (Preiksaitis et al. 2000), fundus (Herawi et al. 1988), duodenum (Liebmann et al. 1992), intestine (Giraldo et al. 1987; Michel and Whiting 1987; Candell et al. 1990), and colon (Zhang et al. 1991), the binding properties of muscarinic receptors are consistent with a major population of M_2 muscarinic receptors and very often a minor population of M_3 receptors. The interpretation of radioligand binding data is potentially complicated because of the expression of multiple receptor subtypes, but the former studies are consistent, nonetheless, with the distribution of mRNA in the GI tract. A relatively large amount of M_2 and small amount of M_3 mRNA has been measured in the small intestine (Maeda et al. 1988) and colon (Zhang et al. 1991). More recent studies using RT-PCR have also shown an abundance of M_2 and M_3 mRNA in the muscle of the esophagus, fundus, pyloris, ileum, and colon (Lin et al. 1997; Preiksaitis et al. 2000; Wang et al. 2000; Aihara et al. 2005; Ontsouka et al. 2007). The levels of the M_2 and M_3 transcripts are similar, however, and other muscarinic transcripts are present in amounts equal to or lower than that of the M_2 receptor. Immunohistochemical studies have demonstrated a widespread distribution of the M_2 receptor throughout the smooth muscle of the GI tract and in the interstitial cells of Cajal (Iino and Nojyo 2006).

Ito and coworkers (2009) have measured the binding capacity of the muscarinic antagonist, [^{3}H]N-methylscopolamine ([^{3}H]NMS) in peripheral tissues from wild type and whole body muscarinic receptor knockout (KO) mice and have observed large decreases (94, 89, and 72%) in muscarinic receptor density in stomach, ileum, and colon from M_2 KO mice, respectively, illustrating the abundance of M_2 receptors in these tissues. Moderate decreases (25, 37, and 19%) in muscarinic receptors density were noted in the same respective tissues from M_3 KO mice, whereas little or no decreases were noted in the same tissues from M_1, M_4, and M_5 KO mice.

Muscarinic receptors are also expressed abundantly in the mucosa of the stomach, ileum, jejunum (Rimele et al. 1981; Rossowski et al. 1988), and colon (Tien et al. 1985). The binding properties are consistent with that of an M_3 muscarinic receptor in the stomach (Hammer 1980; Herawi et al. 1988) and colon (Tien et al. 1985). The oxyntic mucosa expresses an abundance of M_3 mRNA, but also moderate amounts of M_2 and M_1 mRNA (Aihara et al. 2005).

Muscarinic receptors are also expressed in exocrine glands of the GI tract including the pancreas and salivary glands. The binding properties of muscarinic

receptors in the parotid and submandibular glands are consistent with a major population of M_3 receptors and a minor population of M_1 receptors (Hammer et al. 1980; Watson et al. 1996). Pancreatic acini also exhibit a similar profile (Louie and Owyang 1986; Korc et al. 1987; Kato et al. 1992). Both the pancreas and salivary glands express an abundance of M_3 receptor mRNA and a smaller amount of M_1 receptor mRNA (Gautam et al. 2005).

Thus, the most abundant muscarinic receptors expressed in the mucosa and smooth muscle of the GI tract are the M_3 and M_2 subtypes. When expressed in heterologous cells, these receptors are known to signal primarily through the G_q and $G_{i/o}$ family of G proteins, respectively (Peralta et al. 1988), and they maintain this preference in the GI tract as described next.

3 Proximate Signaling Mechanisms of Muscarinic Receptors in Gastrointestinal Tissue

3.1 Adenylate Cyclase

Muscarinic agonists cause a pertussis toxin-sensitive inhibition of adenylate cyclase activity in homogenates of smooth muscle from the ileum (Candell et al. 1990; Thomas and Ehlert 1994) and colon (Zhang and Buxton 1991). The demonstration of this effect in broken cell preparations suggests that the muscarinic inhibition of adenylate cyclase is mediated through direct coupling to G_i and not the result of the accumulation of another second messenger inside the cell. The pharmacological antagonism of this response is consistent with that expected for an M_2 receptor (Candell et al. 1990; Zhang and Buxton 1991). M_2 receptor-mediated inhibition of adenylate cyclase activity is readily demonstrable in homogenates of the myocardium (Ehlert 1985), which expresses the type V and VI adenylate cyclases (Ishikawa et al. 1992; Manolopoulos et al. 1995). Adenylate cyclase V is also expressed in the smooth muscle of the esophagus (Shin et al. 2007), stomach, and intestines (Hu et al. 2009).

In smooth muscle, cyclic AMP elicits relaxation (Andersson and Nilsson 1972). The M_2 receptor-mediated inhibition of adenylate cyclase is expected, therefore, to oppose the relaxant effects of receptors that stimulate adenylate cyclase. Evidence for this mechanism is described in Sect. 4.4.

3.2 Phosphoinositide Hydrolysis

Muscarinic agonists were shown to stimulate the turnover of phosphatidylinositol in intestinal smooth muscle (Jafferji and Michell 1976), and the effect is now known to be mediated by phospholipase Cβ-mediated hydrolysis of

phosphatidylinositol-4,5-bisphosphate (see review by Berridge 1984). This muscarinic response is inhibited by subtype-selective muscarinic antagonists in a manner consistent with an M_3 mechanism in the longitudinal muscle of the ileum (Candell et al. 1990) and the circular muscle of the colon (Zhang and Buxton 1991). The response is insensitive to pertussis-toxin treatment (Zhang and Buxton 1991; Thomas and Ehlert 1994), which is consistent with a G_q mechanism.

In the longitudinal muscle of the ileum, muscarinic receptor-stimulated phosphoinositide hydrolysis undergoes a large 80% loss in the M_3 KO mouse (Tran et al. 2006). The residual response can be attributed to M_1 (15%) and M_2 (5%) receptors.

Many receptors elicit contraction of smooth muscle signal through G_q to mobilize Ca^{2+}. The coupling of the M_3 receptor to phosphoinositide hydrolysis in intestinal smooth muscle is consistent with its role in eliciting contraction as described later. The signaling mechanism for contraction is unclear, however, because the source of Ca^{2+} for the tonic phase of contraction is usually extracellular (Chang and Triggle 1973; Bolger et al. 1983) and not through the IP_3-mediated release of Ca^{2+} from the endoplasmic reticulum.

In the gastric mucosa, muscarinic agonists elicit phosphoinositide hydrolysis and the muscarinic antagonists inhibit the response with potencies that agree with inhibition of an M_3 response. Specifically the pK_B values (negative log dissociation constant) of the muscarinic antagonists 11-[[2-[(diethylamino)methyl]-1-piperidinyl]acetyl]-5,11-dihydro-6*H*-pyrido[2,3-*b*][1,4]benzodiazepin-6-one (AF-DX 116) (5.5), hexahydro-sila-difenidol (HHSiD) (8.3), and pirenzepine (6.9) for antagonizing carbachol-stimulated phosphoinositide hydrolysis in the gastric parietal cells (Pfeiffer et al. 1988, 1990) are in best agreement with their binding affinities (pK_D) for the M_3 subtype [6.1, 7.7, and 6.6, respectively (Ehlert et al. 1997b)]. The structures of the muscarinic agonists and antagonists described in this study are shown in Figs. 1–3.

The parotid gland exhibits a robust stimulation of phosphoinositide hydrolysis when activated by muscarinic agonists, and this response also exhibits an M_3 profile when inhibited by subtype-selective muscarinic antagonists (Gil and Wolfe 1985; Barras et al. 1999).

3.3 Nonselective Cation Conductance

Muscarinic agonists elicit a nonselective cation conductance (I_{cat}) in the longitudinal muscle of the guinea-pig ileum (Inoue and Isenberg 1990). This muscarinic receptor-induced inward current is thought to provide the requisite depolarization for an influx of Ca^{2+} through voltage-dependent Ca^{2+} channels to sustain the tonic phase of contraction of smooth muscle. It has a reversal potential of approximately 10 mV, which is consistent with an inward current of mainly sodium as well as some Ca^{2+}. It has been measured in isolated smooth muscle cells from a variety of

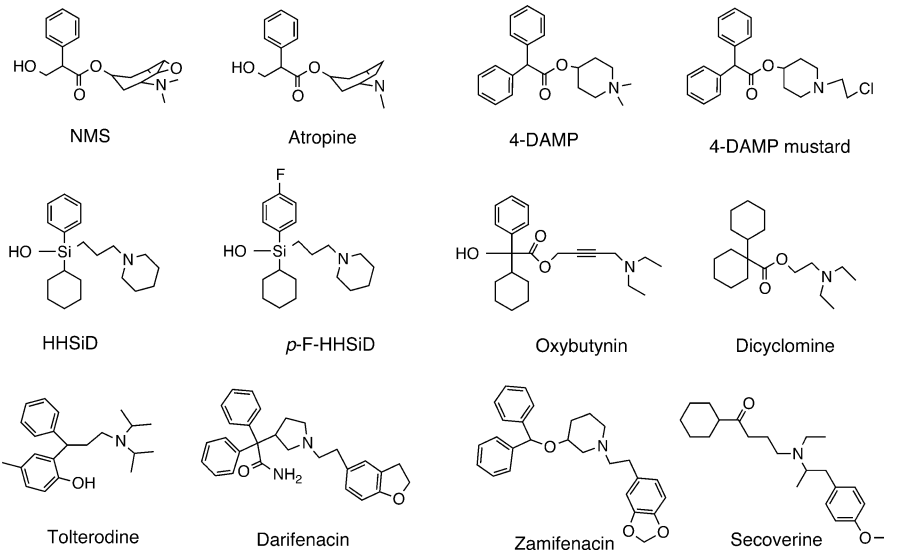

Fig. 1 Structures of some muscarinic antagonists mentioned in the text. The figure shows compounds related to the prototypical muscarinic antagonist atropine, including derivatives with a large substituent to the cationic amine group

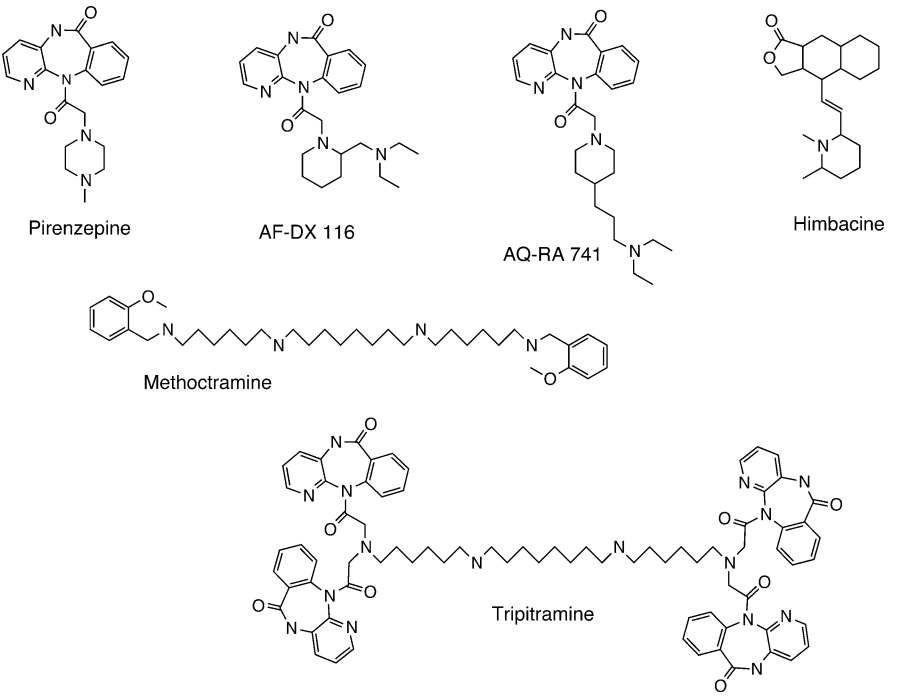

Fig. 2 Structures of some muscarinic antagonists mentioned in the text related to pirenzepine and methoctramine

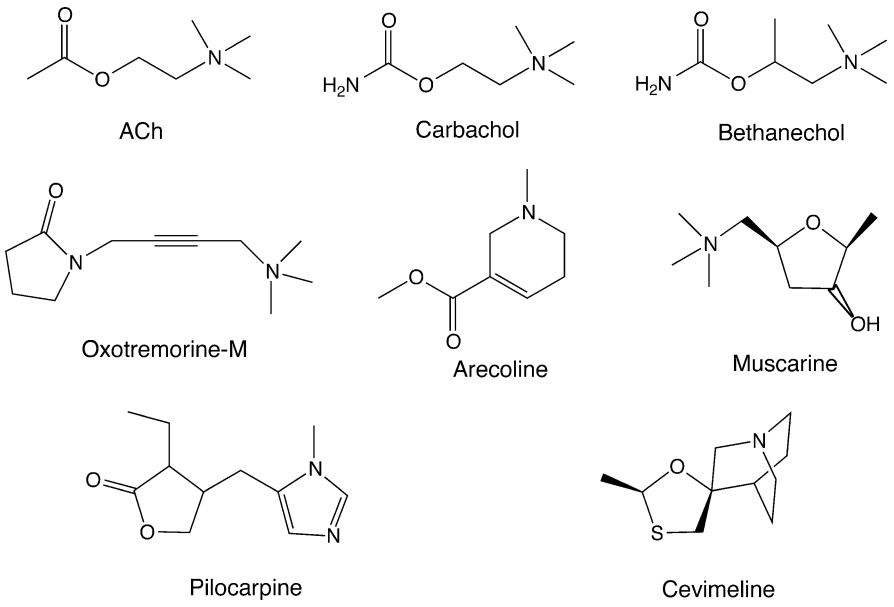

Fig. 3 Structures of the muscarinic agonists mentioned in the text

tissues including stomach (Kim et al. 1998), colon (Lee et al. 1993), and ileum (Bolton 1979; Inoue and Isenberg 1990).

Muscarinic receptor stimulation of I_{cat} is enhanced by Ca^{2+} (Inoue and Isenberg 1990; Pacaud and Bolton 1991), suggesting that the coupling of the M_3 receptor to phosphoinositide hydrolysis through G_q increases I_{cat}. To study G protein activation of I_{cat}, therefore, several investigators have measured muscarinic activation of the current when intracellular Ca^{2+} is buffered at a low value (e.g., 0.1 μM). Under this condition, muscarinic stimulation of I_{cat} exhibits a U-shaped dependence on membrane voltage, with a peak conductance at about −40 mV (Zholos and Bolton 1994). The conductance steadily declines to a low value at −120 mV, yet is still at least half maximal at −80 mV. At depolarizing potentials above −40 mV, the conductance rapidly declines and reverses around +10 mV and sometimes exhibits modest rectification depending on the tissue. The U-shaped dependence on voltage shows that muscarinic activation is greater after some depolarization has already occurred, suggesting that the conductance operates physiologically to reinforce depolarization and contraction of smooth muscle once triggered through another mechanism.

The muscarinic stimulation of I_{cat} is pertussis toxin sensitive (Inoue and Isenberg 1990; Unno et al. 1995), which suggests that the M_2 receptor mediates the current through activation of a member of the $G_{i/o}$ family of G proteins. Because the stimulation of I_{cat} is inhibited by pertussis-toxin treatment and enhanced by Ca^{2+}, it was suggested that both M_2 and M_3 receptors mediate the current (Ehlert et al. 1997a, b; Ehlert 2003a). That is, the M_2 receptor gates the current by directly

coupling with G_i or G_o, and the M_3 receptor enhances the current by G_q-mediated Ca^{2+} mobilization. This conclusion is consistent with the observation that antibodies to G_q and G_o, but not G_i, inhibit I_{cat} in mouse small intestine (Sakamoto et al. 2006). Bolton and Zholos (1997) showed that the competitive antagonists, methoctramine, himbacine, and tripitramine (M_2 selective relative to M_3), potently shifted the concentration–response curve of carbachol for stimulating I_{cat} to the right without affecting the E_{max}, whereas zamifenacin, *p*-fluorohexahyrosiladifenidol (*p*-F-HHSiD), and 4-DAMP (M_3-selective relative to M_2), reduced the E_{max} of the response. Based on this behavior, Bolton and Zholos (1997) and Zholos and Bolton (1997) interpreted this data as evidence that the M_2 receptor gates the current, whereas the M_3 receptor modulates the current. It would be expected that all competitive antagonists would shift the concentration–response curve to the right, regardless of their selectivity for M_2 and M_3 receptors. The decline in E_{max} by atropine, 4-DAMP, *p*-F-HHSiD, and zamifenacin can be explained by their slow dissociation from muscarinic receptors relative to the short period over which the conductance was measured (about 1–2 min) (Ehlert et al. 1997a, b; Ehlert 2003a). This issue is particularly relevant because the EC_{50} value of carbachol for the response (10 μM) is high relative to its predicted dissociation constant for M_2 and M_3 muscarinic receptors (about 4–10 μM in the presence of guanine nucleotides). It follows that the maximal I_{cat} response to carbachol occurs at approximately full receptor occupancy and that there is insufficient time for carbachol to displace the slowly dissociating antagonists from the receptor during the short time that the response was measured.

The result of studies on intestinal smooth muscle cells from muscarinic receptor KO mice clearly shows that M_2 and M_3 muscarinic receptors interact to elicit I_{cat}. When measured with Ca^{2+} buffered to about 0.1 μM, the muscarinic agonist, carbachol, elicited a robust I_{cat} that exhibited the typical U-shape dependence on membrane voltage (Dresviannikov et al. 2006; Sakamoto et al. 2006). The response in smooth muscle cells from either M_2 or M_3 KO mice was greatly attenuated (Sakamoto et al. 2007). When measured in physiological buffer containing Ca^{2+}, the muscarinic agonist-stimulated I_{cat} was substantial in smooth muscle from wild-type mouse, but not from either the M_3 or M_2 KO mouse (Sakamoto et al. 2007). These results suggest that M_2 receptor-mediated stimulation of I_{cat} in mouse ileum is not potentiated by Ca^{2+} and that the Ca^{2+} enhancement of I_{cat} noted in guinea-pig smooth muscle may require both M_2 and M_3 receptors.

It is often assumed that the muscarinic stimulation of I_{cat} is the initial trigger for contraction of smooth muscle by muscarinic agonists. But the conductance depends on activation of the M_2 receptor, whereas the most potent contractile mechanism of muscarinic agonists in guinea-pig ileum is pertussis toxin insensitive and is selectively inhibited by M_3 antagonists, but not M_2 antagonists. It is unclear, therefore, how the M_3 receptor initiates contraction through I_{cat} unless either Ca^{2+} can activate the channel under physiological conditions or else the hydrolysis of PIP_2 prevents the latter's tonic inhibition of the channel.

Members of the transient receptor potential (TRP) channel family are likely candidates for the muscarinic agonist-induced I_{cat} because these channels exhibit

the appropriate conductance properties (high Na conductance), are expressed in GI smooth muscle, and some members are activated by Ca^{2+} (Lee et al. 2003) or inhibited by phosphatidylinositol-4,5-bisphosphate (Otsuguro et al. 2008). Hydrolysis of the latter could lead to TRP channel activation by a G_q-linked G protein-coupled receptor. Muscarinic agonist-induced activation of I_{cat} is greatly inhibited in ileal myocytes from KO mice lacking the TRPC4 channel (Tsvilovskyy et al. 2009). The very small residual conductance in intestinal smooth muscle from the TRPC4 KO mouse is lost in the TRPC4/TRPC6 double KO mouse, suggesting that the conductance is mediated mainly by TRPC4 but also to a small extent by TRPC6. The properties of muscarinic agonist activation of I_{cat} in gastric smooth muscle suggest that the TRPC5 channel mediates the conductance (Lee et al. 2003).

3.4 Calcium-Activated Potassium Channels

It has been known for quite some time that muscarinic agonists elicit a large increase in K^+ efflux from guinea pig intestinal smooth muscle (Burgen and Spero 1968; Bolton and Clark 1981). In the absence of muscarinic stimulation, spontaneous outward currents occur in intestinal smooth muscle cells clamped to depolarizing potentials (e.g., −40 mV) (Bolton and Lim 1989). The size of the current depends on the extracellular concentration of K^+, and the reversal potential is consistent with a K^+ equilibrium potential. Caffeine-induced release of Ca^{2+} from intracellular stores increases the K^+ current, whereas buffering of intracellular Ca^{2+} with EGTA reduces it suggesting that intracellular Ca^{2+} triggers K^+ efflux (Bolton and Lim 1989; Wade and Sims 1993). Direct evidence for this hypothesis has been obtained in colonic smooth muscle, where the Ca^{2+} ionophore A23187 was shown to elicit a K^+ current, even though inhibitors of voltage-sensitive Ca^{2+} channels did not (Wade and Sims 1993). Acetylcholine causes an increase in the potassium current discharge followed by a prolonged decay (Bolton and Lim 1989). The mechanism for the increase in conductance by ACh is thought to involve muscarinic receptor stimulation of phospholipase Cβ, which causes the formation of IP_3 and the subsequent release of Ca^{2+} from intracellular stores. The elevated Ca^{2+} near the plasma membrane then opens large-conductance, Ca^{2+}-activated potassium channels (K_{Ca}). Thus, the G_q-linked M_3 receptor is expected to elicit the increase in K_{Ca} current in intact smooth muscle. K_{Ca} channels are present in a variety of smooth muscles including intestine (Vogalis and Goyal 1997), trachea (Wade and Sims 1993), and urinary bladder (Nakamura et al. 2002), and muscarinic receptor-stimulated K_{Ca} currents have been measured in intact smooth muscle cells from a variety of sources. This K_{Ca} current behaves as a negative feedback circuit that limits the degree of cellular excitation following activation of G_q-linked receptors in smooth muscle.

In addition to activating K_{Ca} indirectly through Ca^{2+} release, muscarinic receptor stimulation also directly inhibits the channel (Wade and Sims 1993). This has

been demonstrated in excised membrane patches of smooth muscle in which the role of Ca^{2+} release has been effectively eliminated. In outside-out patches of tracheal smooth muscle, muscarinic agonists cause a pertussis toxin-sensitive inhibition of K_{Ca} (Wade and Sims 1993). The pertussis-toxin sensitivity of the response suggests that it is mediated by the $G_{i/o}$-linked M_2 muscarinic receptor. A similar muscarinic inhibition of K_{Ca} has been demonstrated in colon (Cole et al. 1989) and esophagus (Muinuddin et al. 2005). Thus, M_2 receptor activation directly inhibits the inhibitory K_{Ca} current that occurs following M_3 receptor activation. The net effect is that the simultaneous activation of both M_2 and M_3 receptors causes a greater stimulation of the smooth muscle cell than if only the M_3 receptor were activated.

4 Effects of Muscarinic Agonists and Antagonists on Contraction of Smooth Muscle

Inhibitors of receptors, enzymes, and other signaling proteins are often used at single blocking concentrations in vivo or in vitro to probe the role of a protein of interest in a physiological response. If, for example, an inhibitor of enzyme A blocks a particular physiological response, then it is often assumed that enzyme A is required for the response. This might be a safe bet if the inhibitor is highly potent, but it is possible that the inhibitor might also have an as yet undiscovered highly potent inhibitory effect on a different protein that mediates the response. With regard to subtype-selective muscarinic antagonists, many lack high selectivity for receptor subtypes. There is also a large variation in the sensitivity of responses mediated by the same receptor in different tissues. For example, it only requires occupancy of one-half of 1% of the M_3 receptor population in the guinea-pig ileum by the muscarinic agonist, oxotremorine-M, for elicitation of half-maximal contraction (Ringdahl 1987). In the urinary bladder, however, occupancy of a 20-fold greater fraction of the M_3 receptor population (about 10%) is required for the same level of response. Thus, we would expect that an M_3 antagonist would be much more effective at blocking the contraction elicited by a high concentration of a muscarinic agonist in the bladder than in the ileum, even though the same receptor is involved in mediating the contraction in the two tissues. Thus, many muscarinic antagonists are virtually useless in identifying the specific receptor subtype mediating a response in experimental paradigms in which a single concentration of antagonist is used to block a physiological response in vivo or the response to a single concentration of agonist in an in vitro experiment.

Nonetheless, it is possible to design an in vitro experiment so that an estimate of the dissociation constant of the antagonist for the receptor mediating the response is obtained. The method involves determining equiactive concentrations of agonist in the presence and absence of a muscarinic antagonist. The dissociation constant (K_B) of the antagonist can be estimated from the ratio of equiactive agonist concentrations using a competitive inhibition relationship (Arunlakshana and

Schild 1959). The estimate of K_B can then be compared with the dissociation constant of the antagonist measured for the recombinant receptor in a binding experiment using the same physiological buffer. Notwithstanding potential differences in receptor oligomerization and species differences in receptor sequence, the estimate of binding affinity should agree with the K_B measured in the functional assay if the recombinant receptor is the same as that mediating the response. If a handful of subtype-selective antagonists are employed, it should be possible to determine which muscarinic receptor subtype mediates a given response unequivocally.

This approach is complicated when more than one receptor mediates the response, which we might expect in smooth muscle because both M_2 and M_3 muscarinic receptors mediate ionic conductances (see Sect. 3). In the following section, therefore, we review how to interpret the pharmacological antagonism of a response elicited by two receptors.

While antagonism studies on isolated smooth muscle preparations are useful for determining which receptors can be activated to elicit contraction, these experiments do not provide information about which receptors are activated under physiological conditions. For example, it is often assumed that contraction of urinary bladder is mediated by the M_3 receptor, with little input from the M_2 (Abrams et al. 2006). The rationale is that the K_B values of muscarinic antagonists for blocking contraction agree best with those of the M_3 receptor and not the M_2. While the M_3 receptor undoubtedly has an important role in contraction, the method for estimating K_B does not address the conditional nature of M_2 receptor signaling mechanisms (see Sect. 4.1). Activation of the M_2 receptor enhances contractions elicited by the M_3 receptor. This enhancement mechanism is less potent than the M_3 mechanism, and maximal contractions through M_3 receptor activation occur at agonist concentrations that are too low to activate the M_2 enhancement mechanism (Sawyer and Ehlert 1999b; Ehlert et al. 2005a). Hence, elimination of the M_2 mechanism has no effect on the concentration–response curve of a muscarinic agonist in many isolated smooth muscles, which has led to the conclusion that the M_2 receptor has little effect on contraction. At a dynamic synapse in the bladder, however, it is entirely possible that local concentrations of ACh activate both M_2 and M_3 mechanisms and that the former is critically important for normal transfer of information across the synapse in vivo. The mechanism could predominate in synapses and have unique kinetic properties that are not apparent in equilibrium assays on isolated tissues.

4.1 Analysis of the Competitive Inhibition of a Response Mediated by Both M_2 and M_3 Receptors

The ability of an antagonist to block the response elicited by an agonist acting on two types of receptors depends on (1) the selectivity of the antagonist for the two receptors, (2) the sensitivity of the signaling pathway of each receptor, and

(3) whether the response elicited by each receptor is direct or conditional. A direct response is one that occurs following activation of the receptor by itself. If activation of a receptor elicits no response, but causes an increase in the response of a second directly or conditionally acting receptor, then the response of the first receptor is conditional. In the following theoretical examples, we consider responses mediated by a combination of directly and conditionally acting receptors. The dissociation constants of the R1-selective antagonist (A1) for R1 and R2 receptors are 10^{-9} and 10^{-8} M, respectively, whereas the corresponding values for the R2-selective antagonist (A2) are the converse (10^{-8} and 10^{-9} M, respectively). In all of the theoretical examples, the concentration–response curve of the agonist is measured in the absence and presence of either A1 or A2 at a concentration of 10^{-8} M. Further details are given in the legend to Fig. 4.

Panels a–c in Fig. 4 show concentration–response curves elicited by an agonist acting with equivalent affinity and efficacy on two directly acting receptors – R1 and R2. The sensitivity of the signaling pathway is high enough so that each receptor is capable of eliciting a maximal response by itself when activated by the agonist. Figure 4a shows the condition where the sensitivity of each receptor-signaling pathway is about the same. Pontari et al. (2004) and colleagues have described the consequences of this situation. The R1-selective antagonist (A1) inhibits the response with moderate potency (pK_B, 8.6), but less than that characteristic of its ability to block an R1 response (pK_B, 9.0) because the R2 receptor is able to rescue the response whenever the R1 receptor is blocked. Analogously, the R2-selective antagonist (A2) inhibits the response with moderate potency (pK_B, 8.6), but less than that characteristic of its ability to block an R2 response (pK_B, 9.0). Thus, even though the response is mediated by both R1 and R2 receptors, neither R1- nor R2-selective antagonists block the response with high potency. As the sensitivity of the R1 (Fig. 4b) or R2 (Fig. 4) signaling pathway increases relative to that of the other receptor, then the profile for pharmacological antagonism tends to shift toward that of the receptor having the more potent signaling pathway (i.e., R1-like for panel b and R2-like for panel c).

Panels d–f in Fig. 4 show the response elicited by an agonist interacting with directly (R1) and conditionally (R2) acting receptors. Activation of the R2 pathway by itself has no effect, but it amplifies the response to R1 by a certain amount (i.e., fivefold). The pharmacological antagonism of this system tends to follow the expected profile of the directly acting receptor (Sawyer and Ehlert 1999b; Ehlert 2003b). That is, the R1-selective antagonist blocks the response with high potency (pK_B, 9.0), whereas the R2-selective antagonist does not (pK_B, 8.5). One possible exception is when the sensitivities of the two pathways are similar (panel d). In this case, the potency of antagonists for blocking the response may be in between that expected of an R1 and R2 response depending upon the concentration of the antagonist used. The lower the concentration of antagonist, the more likely the antagonism will shift toward that of the R2 receptor under the condition shown in panel d.

Panels g–i in Fig. 4 show a response contingent upon activation of two conditionally acting receptors. This situation corresponds to an AND gate or a

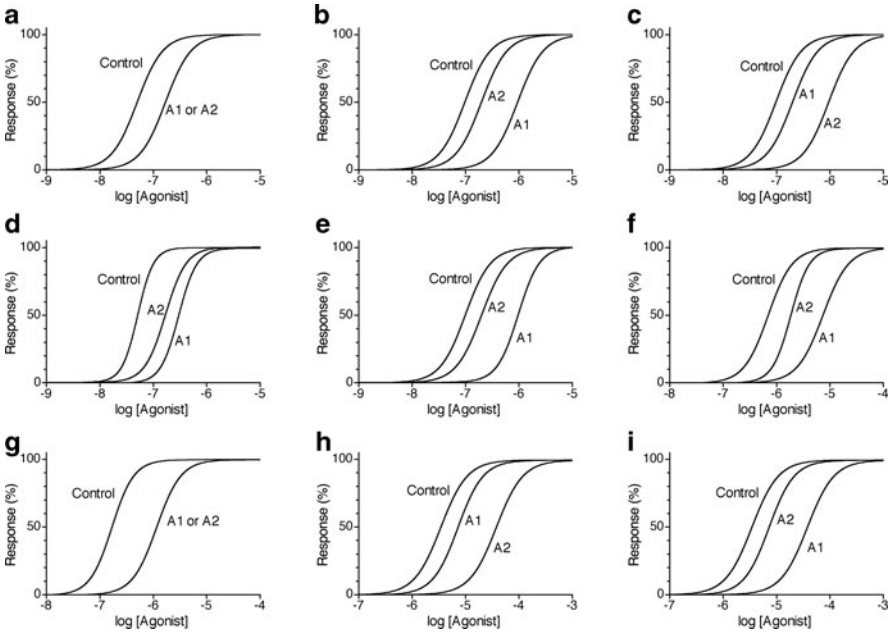

Fig. 4 Theoretical simulation of the competitive antagonism of a response mediated by an interaction between two receptors, R1 and R2. The plots show the concentration–response curves of an agonist having equivalent affinity and intrinsic efficacy for R1 and R2 in the absence (control) and presence of an R1-preferring antagonist (A1) or an R2-preferring antagonist (A2). The dissociation constants of A1 and A2 for the R1 receptor are 9.0 and 8.0, respectively, and the corresponding values for the R2 receptor are the converse (8.0 and 9.0, respectively). The panels show the effects of A1 and A2 when both R1 and R2 elicit a direct response (**a**–**c**), when the response to R1 is direct and that to R2 is contingent upon activation of R1 (**d**–**f**), and when the responses to both R1 and R2 are contingent upon simultaneous activation of both receptors (**g**–**i**). The sensitivities of the signaling pathways of the R1 and R2 receptors were varied in the simulations. For the left most panel in each row (**a**, **d**, **g**), the sensitivities are the same; for the middle panel in each row (**b**, **e**, **h**), the sensitivity of the R2 pathway is only 3% that of R1, and for the right most panel in each row (**c**, **f**, **i**), the sensitivity of R1 is only 3% that of R2. When considered in the simulations, the concentrations of A1 and A2 were 10 nM. The simulations have been carried out using a strategy similar to that described in Sawyer and Ehlert (1999b)

coincidence detector. That is, no response occurs unless both receptors are activated. The pharmacological antagonism of this pathway resembles that of the receptor with the less sensitive signaling pathway (Sawyer and Ehlert 1999b; Ehlert 2003b). Thus, the response in panel g is blocked with high potency by both R_1- and R_2-selective antagonists; that in panel h is blocked preferentially by the R_2-selective antagonist; and that in panel i is preferentially blocked by the R_1-selective antagonist.

As described next, the examples shown in Fig. 4 provide the basis for interpreting the antagonism of muscarinic responses in GI smooth muscle.

4.2 Antagonism of Agonist-Induced Contraction of Gastrointestinal Smooth Muscle

Studies on isolated GI smooth muscle from muscarinic receptor KO mice provide clear evidence for the role of M_2 and M_3 muscarinic receptors in eliciting contraction. The contractions elicited by carbachol or oxotremorine-M in the ileum (Matsui et al. 2002; Unno et al. 2005; Griffin et al. 2009) and gastric fundus and antrum (Stengel et al. 2000, 2002; Kitazawa et al. 2007) undergo a small loss of function in the M_2 KO mouse (two-fold increase in agonist EC_{50}, with no decrease in E_{max}), a large loss of function in the M_3 KO mouse (50–70% reduction in E_{max}), and a complete loss in the M_2/M_3 double KO mouse. The contractions measured in antrum, fundus, and ileum from the M_2 KO mouse are competitively inhibited by the antagonists, *N*,*N*-dimethyl-4-piperidinyl diphenylacetate (4-DAMP) (M_3- over M_2-selective) and AF-DX 116 (M_2- over M_3-selective) with pK_B values (approximately 9.0 and 6.0, respectively) that agree with M_3 binding affinity [8.8 and 6.1, respectively (Esqueda et al. 1996; Griffin et al. 2004)]. Conversely, the pK_B values of 4-DAMP and AF-DX 116 in the same tissues from the M_3 KO mouse (approximately 8.3 and 7.3, respectively) agree with M_2-binding affinity [7.9 and 7.3, respectively (Esqueda et al. 1996; Griffin et al. 2004)]. The muscarinic contractile response in intestinal smooth muscle from the M_3 KO mouse is pertussis toxin sensitive, whereas that measured in tissue from the M_2 KO mouse are insensitive to pertussis toxin (Unno et al. 2005). The data are consistent with the postulate that both M_2 and M_3 receptors mediate a direct contraction of GI smooth muscle in the mouse, although the magnitude of the M_3 receptor component is larger. In smooth muscle from the wild-type mouse, the pK_B values of 4-DAMP and AF-DX 116 are intermediate between the binding affinities of M_2 and M_3 receptors, although much closer to that of the M_3. This result is consistent with the model described earlier in connection with panels a–c.

A useful tool for characterizing the contractile role of muscarinic receptors is *N*-(2-chloroethyl)-4-piperidinyl diphenyl acetate (4-DAMP mustard) (Thomas et al. 1992). It can be used to inactivate the M_3 receptor without affecting the M_2 in smooth muscle, which is advantageous in studies on species for which receptor KO strains are unavailable. The effects of 4-DAMP mustard treatment on [^{3}H]NMS binding to human M_1–M_5 receptors expressed in CHO cells is shown in Fig. 5. Treatment of mouse ileum with 4-DAMP mustard converts the agonist concentration–response curve for contraction into that observed in the M_3 KO mouse (Griffin et al. 2009). The E_{max} value is 43% that of wild type, and the contractions are antagonized by AF-DX 116 and 4-DAMP with pK_B values consistent with an M_2 response. The pEC_{50} value of oxotremorine-M exhibits high potency in the wild-type mouse after 4-DAMP mustard treatment (6.9) and is similar to that measured in ileum from the M_3 KO mouse (7.15). In contrast, 4-DAMP mustard treatment of the ileum from the M_2 KO mouse causes a large reduction in the E_{max} (33% that of wild type) and pEC_{50} value of oxotremorine-M (5.8).

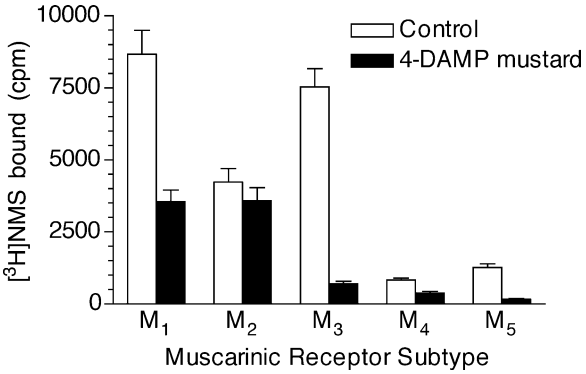

Fig. 5 The effects of 4-DAMP mustard treatment (10 nM, 1 h) in combination with AF-DX 116 (1 μM) on the specific binding of [^{3}H]NMS to CHO cells expressing muscarinic receptor subtypes. Intact CHO cells were incubated for 1 h with AF-DX 116 (1 μM) and a solution of 4-DAMP mustard (10 nM) that had been previously incubated for 30 min at pH 7.4 to allow for the formation of the aziridinium ion. The cells were washed, and the specific binding of [^{3}H]NMS was measured at a concentration of 1 nM using an intact cell assay (unpublished observations)

GI smooth muscle from the guinea pig and rat behaves similarly to that of the mouse with respect to the major role for the M_3 receptor in eliciting a direct contractile response. Muscarinic agonist-induced contraction of the longitudinal muscle of the ileum (Lambrecht et al. 1989; Eglen and Harris 1993; Thomas et al. 1993), colon (Sawyer and Ehlert 1998, 1999b), esophagus (Kamikawa et al. 1985; Eglen and Whiting 1988), and stomach (Del Tacca et al. 1990) is competitively inhibited by antagonists with pK_B values that are indicative of an M_3 response. The contractile response of the ileum and colon and field-stimulated contraction of the ileum is slightly increased or unaffected by pertussis toxin treatment (Eglen et al. 1988; Thomas and Ehlert 1994; Sawyer and Ehlert 1999a, b). These results are consistent with a major role for the M_3 receptor in mediating direct contraction in smooth muscle from the guinea pig. Unlike the mouse ileum, however, selective inactivation of M_3 receptors in the ileum and colon of the guinea pig does not unmask a highly potent, M_2 receptor-mediated contraction. Following 4-DAMP mustard treatment, the pEC_{50} value of oxotremorine-M is reduced to 1/80th of control in the ileum, and the residual contractile response exhibits an M_3 profile in competitive antagonism studies (Griffin et al. 2009). Similar results have been observed in the colon, although pertussis toxin treatment greatly inhibits the residual muscarinic agonist-induced contractions after 4-DAMP mustard treatment of the colon, even though the contraction is weakly antagonized by M_2-selective antagonists (Sawyer and Ehlert 1999b). Why is this residual response M_3-like in its antagonistic profile yet M_2-like in its sensitivity to pertussis toxin?

A possible explanation is that the residual contraction in the colon after 4-DAMP mustard treatment represents an interaction between M_2 and M_3 receptor mechanisms. That is, a direct M_3 receptor-mediated contraction may be enhanced by a conditional M_2 receptor mechanism. There is a precedent for this mechanism

because both the muscarinic stimulation of I_{cat} and the M_2 receptor-mediated inhibition of K_{Ca} channels require a simultaneous activation of M_2 and M_3 receptors (Sakamoto et al. 2007), and the M_3 receptor can elicit contraction on its own when activated. The pharmacological antagonism of this type of interaction resembles that of the directly acting receptor (M_3) and not that of the conditionally acting receptor (M_2) (see Fig. 4). Thus, the pertussis toxin sensitivity and M_3 profile of this response can be explained by such an M_2/M_3 interaction.

This interaction is expected to trigger a contractile stimulus much greater than that required to elicit maximal contraction because occupancy of only a small fraction of the M_3 receptor population is all that is required for highly efficacious agonist to elicit a maximal response. This M_2/M_3 interaction would probably increase the rate of contraction and reduce its susceptibility to relaxant eicosanoids and β-adrenoceptor activation. Thus, even though this M_2/M_3 interaction is masked by a more potent agonist-mediated M_3 contractile mechanism in isolated tissue bath assays, it could have an important physiological role at a dynamic synapse where the concentration of ACh is likely to approach receptor-saturating concentrations.

One consequence of a powerful muscarinic contractile stimulus in the guinea-pig ileum is a subsequent period of heterologous desensitization (Cantoni and Eastman 1946; Dale 1958; Paton 1961). Treatment of mouse ileum with ACh for 20 min causes a subsequent reduction in the potency of $PGF_{2\alpha}$ for eliciting contraction (Griffin et al. 2004). This desensitizing effect of acetylcholine is absent in ileum from either the M_2 KO or M_3 KO mouse. Similarly, acetylcholine-mediated desensitization of histamine-induced contraction of the guinea-pig ileum is prevented by pertussis toxin treatment (i.e., an uncoupling of M_2 receptor signaling) or inactivation of M_3 muscarinic receptors with 4-DAMP mustard (Shehnaz et al. 2001). Both of these treatments have no influence on histamine-induced contractions in ileum not treated with ACh. The data in mouse and guinea pig are consistent with the postulate that acetylcholine-induced heterologous desensitization is contingent upon activation of both M_2 and M_3 muscarinic receptors and that activation of either receptor by itself is insufficient to cause desensitization. Thus, the response of each receptor is conditional upon activation of the other receptor. This type of interaction exhibits a pharmacological profile for antagonism consistent with that of the less-sensitive signaling pathway (see discussion of Fig. 4h–j). In the guinea-pig ileum, acetylcholine-mediated heterologous desensitization is blocked by M_2-selective antagonists with high potency relative to M_3-selective antagonists, indicating that the conditional M_2-receptor mechanism is less sensitive than that of the M_3 (Griffin et al. 2004).

If heterologous desensitization is ultimately caused by a prior contractile stimulus, then the conditional M_2 receptor-mediated enhancement (pertussis toxin sensitive) of direct M_3 receptor-mediated contraction noted in the guinea-pig colon after 4-DAMP mustard treatment may represent the initial contractile mechanism that ultimately causes heterologous desensitization.

One enigma regarding muscarinic agonist induced-contraction of the guinea-pig ileum and other smooth muscles is that, when measured in isolated smooth muscle, the tonic phase of contraction is nearly completely inhibited by voltage-sensitive

Ca^{2+} channel antagonists (Chang and Triggle 1973; Rosenberger et al. 1979; Bolger et al. 1983) and is unaffected by pertussis toxin treatment (Eglen et al. 1988; Thomas and Ehlert 1994). It seems unlikely, therefore, that muscarinic stimulation of I_{cat} or inhibition of K_{Ca} is involved in mediating the high potency M_3 receptor-mediated contraction, even though the latter ionic mechanisms are often assumed to be involved. Rather, these mechanisms may have a role in the less potent M_2 receptor-enhancement of M_3 receptor-mediated contraction as described earlier.

4.3 Tissue-Selective Muscarinic Antagonists?

A few antagonists have been reported to cause a differential antagonism of muscarinic agonist-induced contraction of smooth muscle from different tissues even though contraction is thought to be elicited mainly by the M_3 receptor. For example, the compound zamifenacin inhibits muscarinic agonist-induced contraction of the guinea-pig ileum, trachea, and urinary bladder with high ($pK_B = 9.3$), intermediate ($pK_B = 8.2$), and low potency ($pK_B = 7.6$), respectively (Wallis 1995; Watson et al. 1995), and the compound *p*-F-HHSiD exhibits a similar pattern of selectivity with regard to ileum and trachea (Eglen et al. 1990; Ehlert et al. 2005b). These compounds exhibit high affinity for M_3, low affinity for M_2, and variable affinity for the other muscarinic subtypes when measured in radioligand binding assays on Chinese hamster ovary (CHO) cells stably expressing human muscarinic receptor subtypes. The log affinity constants of zamifenacin for M_1, M_2, M_3, and M_4 muscarinic receptors are 7.5, 7.1, 7.9, and 6.7, respectively (Eglen et al. 1996a), and the corresponding values for *p*-F-HHSiD at M_1–M_5 are 7.1, 6.1, 7.3, 7.1, and 6.3, respectively (Ehlert et al. 1997b). Thus, the highest affinity that zamifenacin exhibits for a muscarinic receptor is approximately 10^8 M^{-1} (M_3) and that for *p*-F-HHSiD is approximately 10^7 M^{-1} (M_3). Somehow, both compounds antagonize muscarinic agonist-induced contraction of intestinal smooth muscle with tenfold higher affinity. These results suggest that these antagonists inhibit contraction of intestinal smooth muscle by interacting with a target other than a muscarinic receptor.

In contrast to the variable pK_B value of *p*-F-HHSiD noted in contractile studies, similar pK_B values were estimated when the antagonism of muscarinic agonist-induced phosphoinositide hydrolysis in guinea-pig ileum and bovine trachea was measured (Ehlert et al. 2005b). The corresponding pK_B values were approximately the same as the binding affinity (pK_D value) of the human M_3 muscarinic receptor. These results suggest that *p*-F-HHSiD antagonizes M_3 responses with similar potency in different tissues, and that the high potency inhibitory effect of *p*-F-HHSiD on contraction of intestinal smooth muscle is through inhibition of a process downstream from phosphoinositide hydrolysis.

Resultant analysis is an ingenious method for estimating the competitive component of the total effect of an antagonist when the antagonist also possesses another action (inhibitory or stimulatory) on the signaling pathway (Black et al. 1986). This technique enables one to estimate the pK_B of the competitive

component based on the ability of the antagonist to interfere with the competitive effect of a purely competitive antagonist, like atropine, for example. When this approach was used on guinea-pig ileum and trachea to measure the competitive component of the inhibitory effect of *p*-F-HHSiD on muscarinic agonist-induced contraction, it was found that *p*-F-HHSiD exhibited similar pK_B values for inhibition of agonist-induced contraction of ileum and trachea (Ehlert et al. 2005b). Thus, there is no reason to suggest that *p*-F-HHSiD competitively antagonizes muscarinic responses with different affinities in different tissues.

p-F-HHSiD has been shown to inhibit histamine-induced contractions of the guinea-pig ileum, although its potency for doing so is less than that measured for inhibition of muscarinic agonist-induced contraction (Ehlert et al. 2005b). The compound has also been shown to inhibit GTPγS-stimulated I_{cat} in guinea pig ileal smooth muscle suggesting that it can directly block the cationic channel. But again, the potency is less than that for inhibiting muscarinic agonist-induced contraction (Zholos and Bolton 1997). Thus, *p*-F-HHSiD appears to exhibit a highly potent inhibitory effect on intestinal smooth muscle that is unrelated to blockade of muscarinic receptors. Its high affinity target is unknown, and it is possible that other tissue selective antagonists, like zamifenacin, also share the same target. This nonmuscarinic, inhibitory action raises ambiguities when *p*-F-HHSiD and zamifenacin are used to assess the role of M_2 and M_3 muscarinic receptors in eliciting contraction in different smooth muscles.

4.4 M_2 Receptor-Mediated Inhibition of Relaxation

Cyclic AMP and agents that activate adenylate cyclase directly (e.g., forskolin) or indirectly through receptors that couple to G_s (e.g., ß-adrenoceptors) elicit relaxation of smooth muscle (Andersson and Nilsson 1972). Not surprisingly, activation of the M_2 receptor inhibits this relaxation through G_i-mediated inhibition of adenylate cyclase (Ehlert 2003a). The simplest demonstration of this mechanism is that forskolin causes a greater relaxation of muscarinic agonist-induced contraction of ileum, trachea, and urinary bladder from the M_2 KO as compared to wild-type mice (Matsui et al. 2003). When the ß-adrenoceptor agonist isoproterenol is used in the same type of experiment, the relaxant effect is only slightly greater in intestinal smooth muscle from the M_2 KO compared to wild-type mouse. These results suggest that only part of the relaxant effect of isoproterenol is mediated through inhibition of adenylate cyclase. A greater M_2 receptor-mediated inhibition of isoproterenol-induced relaxation was observed in mouse urinary bladder, whereas no effect was observed in mouse trachea (Matsui et al. 2003).

The role of the M_2 receptor in opposing cAMP-mediated relaxation in smooth muscle has also been investigated by first inactivating M_3 muscarinic receptors with 4-DAMP mustard and then measuring the concentration–response curve of a muscarinic agonist in the presence of a combination of both contractile (e.g., histamine or $PGF_{2\alpha}$) and cAMP-stimulating (forskolin or isoproterenol) agents.

When present together, histamine and forskolin have little or no net contractile effect because the contraction elicited by histamine is prevented by forskolin. When measured as just described, the highly efficacious agonist, oxotremorine-M, elicits potent contractions of the guinea-pig ileum ($pEC_{50} = 8.0$), and the pK_B value of the M_2-selective antagonist AF-DX 116 for antagonizing these contractions is approximately 7.0, which is in good agreement with its binding affinity for the M_2 receptor (Thomas et al. 1993). These oxotremorine-M-induced contractions are pertussis toxin sensitive, unlike the contraction elicited to oxotremorine-M in the absence of other contractile and relaxant agents, which is pertussis toxin insensitive (Thomas and Ehlert 1994). Thus, oxotremorine-M appears to activate the M_2 receptor to inhibit the relaxant effect of forskolin on histamine-induced contractions, and this mechanism can be verified with subtype-selective antagonists, provided that the M_3 receptor is first inactivated. Several variations of this experimental paradigm have been reported and used to demonstrate M_2 receptor-mediated inhibition of relaxation in ileum (Reddy et al. 1995; Ostrom and Ehlert 1997), colon (Sawyer and Ehlert 1998; Shen and Mitchelson 1998), and esophagus (Eglen et al. 1996b).

One might expect that if the concentration–response curve of a muscarinic agonist is measured in the presence of isoproterenol or forskolin, then the potency of M_2-selective antagonists should increase from the low value typical of M_3 receptor-mediated contraction of smooth muscle to a higher value midway between M_2- and M_3-like to reflect the role of the M_2 receptor in opposing the relaxation of M_3-mediated contractions. This is usually not observed, however, and the explanation is consistent with a role for the M_2 receptor in contraction, nonetheless. When the M_2 receptor inhibits relaxation, the mechanism is conditional because M_2 receptor activation elicits little or no contraction by itself in guinea pig intestinal smooth muscle. The M_2 response ultimately depends on the contraction elicited by the M_3 receptor, which is inhibited by forskolin, and this relaxation is inhibited by M_2 receptor activation. As described earlier in connection with Fig. 4, the competitive antagonism of an M_2 receptor-mediated conditional enhancement of a directly mediated M_3 receptor response exhibits an M_3 profile when subtype-selective antagonists are used to characterize the response.

It is often observed that the relaxant effect of isoproterenol is much greater when measured against histamine-, as compared to, muscarinic agonist-induced contraction. Part of the explanation in GI and urinary bladder smooth muscle (but not tracheal) is that the M_2 receptor inhibits relaxation through inhibition of adenylate cyclase. Another explanation is related to the observation that the differential relaxant effect of isoproterenol is particularly great when high, maximally effective concentrations of the contractile agents are used. At high concentrations of muscarinic agonist, the low potency, M_2 receptor-mediated enhancement of M_3 receptor-mediated contraction occurs. As described earlier, this M_2 mechanism may involve an inhibition of K_{Ca} or a stimulation of I_{cat}. Histamine, acting mainly through the H_1 receptor in smooth muscle, is unable to elicit these additional mechanisms. Thus, the low potency M_2 receptor mechanism also contributes to the refractoriness of muscarinic agonist-induced contractions to the relaxant effect of isoproterenol. Finally, it also seems likely that maximal occupancy of M_3 receptors elicits

a greater Ca^{2+} mobilization as compared to that elicited by maximal occupation of the H_1 receptor by histamine (Hoiting et al. 1996).

5 Effects of Muscarinic Agonists and Antagonists on Gastric Secretions

When administered in vivo, muscarinic antagonists block gastric acid and pepsinogen secretion (Soll and Walsh 1979). The M_1 selective antagonist, pirenzepine, is moderately selective in this action, and blocks gastric secretions at doses that have little effect on salivary secretion, heart rate, and intestinal motility (Bianchi Porro and Petrillo 1982; Feldman 1984). The mechanism of its action in vivo is thought to be an antagonism of M_1 receptors in parasympathetic ganglia in the stomach. This site of action is consistent with its high potency for inhibiting vagally mediated acid secretion, but not that elicited by muscarinic agonists (Daly et al. 1982; Bertaccini and Coruzzi 1989).

In isolated rabbit gastric glands, carbachol-stimulated acid secretion is weakly antagonized by M_1- (pirenzepine) and M_2- (AF-DX 116) selective muscarinic antagonists, but rather potently by 4-DAMP (Wilkes et al. 1991), which exhibits high affinity for all subtypes of the muscarinic receptor except the M_2. Given the high expression of M_3 muscarinic receptors on gastric parietal cells (Kajimura et al. 1992), the pharmacological antagonism studies are not inconsistent with a major role for the parietal cell M_3 muscarinic receptor in mediating the stimulatory effect of muscarinic agonists on acid secretion. Carbachol-stimulated gastric acid secretion in vivo is greatly inhibited in M_3 KO, but not in M_1 KO mice (Aihara et al. 2003, 2005). These results are consistent with the idea that carbachol acts mainly postjunctionally on the gastric parietal cell to elicit HCl secretion via the M_3 muscarinic receptor.

In isolated chief cells from the guinea-pig stomach, the muscarinic agonists acetylcholine, carbachol, muscarine, arecoline, and bethanechol elicit pepsinogen secretion with EC_{50} values of approximately 0.19, 1.8, 1.3, 3.4, and 32 μM, respectively (Sutliff et al. 1989). The maximal response of each agonist was approximately the same and represented a tenfold increase over basal secretion. Sutliff and coworkers (1989) measured the IC_{50} values of various muscarinic antagonists for inhibiting the pepsinogen secretion elicited by a maximally effective concentration of carbachol (10 μM). It follows that equiactive-carbachol concentrations in the absence and presence of the IC_{50} concentrations of the antagonists were approximately 1.8 and 10 μM, respectively, which yield an estimate of 5.6-fold for the shift in the carbachol concentration–response curve caused by each antagonist at its IC_{50}. Using a competitive inhibition relationship, it is possible to calculate the pK_B values of the antagonists from the published IC_{50} values of Sutliff et al. (1989). The corresponding pK_B values of NMS, scopolamine, 4-DAMP, atropine, pirenzepine, and AF-DX 116 are 9.2, 8.8, 8.9, 9.0, 6.5, and 5.8, respectively. These values agree best with the binding affinity

of the M_3 muscarinic receptor, but the contribution of other muscarinic receptors is also possible.

Using chief cells isolated from gastric glands of muscarinic receptor KO mice, Xie and coworkers (2005) showed that the there was a small loss of carbachol-stimulated pepsinogen in glands prepared from M_1 and M_3 KO mice, and a complete loss of function in the M_1/M_3 double KO mouse. Thus, it appears that the response is mediated by both M_1 and M_3 receptors and that the response to either receptor can be rescued by the other in isolated gastric glands prepared from the mouse stomach.

6 Effects of Muscarinic Agonists and Antagonists on the Exocrine Pancreas

Isolated rat pancreatic acinar cells release amylase in response to muscarinic agonists. The pEC_{50} values of ACh and carbachol for eliciting release are approximately 7.5 and 6.5, respectively, and the average pA_2 values of the muscarinic antagonists atropine (9.3), 4-DAMP (8.8), pirenzepine (6.7), and AF-DX 116 (5.9) generally agree with their respective binding affinities for the human M_3 muscarinic receptor (Louie and Owyang 1986; Korc et al. 1987; Kato et al. 1992). Using isolated pancreatic acini from muscarinic receptor KO mice, Gautam and coworkers (2005) found that carbachol-stimulated amylase secretion exhibited a partial loss in both M_1 and M_3 receptor KO mice and a complete loss in M_1/M_3 double KO mice. The data indicate that both receptors mediate the response.

7 Effects of Muscarinic Agonists and Antagonists on Salivary Secretion

When administered in vivo muscarinic agonists elicit salivation, whereas muscarinic antagonists cause dry mouth. Lesion of the medial-septal area or centrally administered *N*-methylatropine partially inhibits the salivary response to peripherally administered pilocarpine, suggesting that at least part of the salivary response to pilocarpine is mediated through central muscarinic receptors (Takakura et al. 2003; Paulin et al. 2009).

In isolated dispersed cells of the rat submandibular gland, carbachol elicits a modest increase in acidification, which can be easily quantified using microphysiometry. The pEC_{50} value of carbachol for this response is approximately 6.0. The pA_2 values of 4-DAMP (8.88), 11-[[4-[4-(Diethylamino)butyl]-1-piperidinyl]acetyl]-5,11-dihydro-6*H*-pyrido[2,3-*b*][1, 4]benzodiazepin-6-one (AQ-RA 741) (6.63), atropine (8.85), himacine (6.82), methoctramine (5.9), oxybutynin (7.94),

pirenzepine (6.85), S-secoverine (7.18), and tolterodine (8.51) are generally consistent with the binding affinities of either the M_3 or M_5 subtype of the muscarinic receptor (Meloy et al. 2001).

Studies on muscarinic receptor KO mice show a major role for the M_3 receptor as well as a minor role for the M_1 in mediating salivation in the mouse. When a low dose of pilocarpine (1 mg/kg) was administered to mice in vivo, the salivary response characteristic of wild-type mice was completely eliminated in the M_3 KO mouse (Matsui et al. 2000). When higher doses of pilocarpine (5 and 15 mg/kg) were administered, however, the salivary response was hardly impaired in either M_3 KO or M_1 KO mice, although it was completely eliminated in the M_1/M_3 double KO mouse (Gautam et al. 2004). These results show that the salivary response to high doses of pilocarpine involves activation of both M_1 and M_3 receptors and that either receptor by itself is able to trigger a maximal response. These results are also consistent with the expression of mainly M_3 but also M_1 muscarinic receptor in the submandibular (Hammer et al. 1980) and parotid (Watson et al. 1996) glands as well as a possible central M_1 receptor contribution to pilocarpine-induced salivation.

Studies on dispersed acinar cells from the submandibular glands of muscarinic receptor KO mice show a major role for the M_3 receptor in eliciting Ca^{2+} mobilization as measured by fura-2 fluorescence (Nakamura et al. 2004). The concentration–response curve of carbachol was greatly suppressed in cells from the M_3 KO mouse, but hardly affected in the M_1 KO mouse. Imaging studies on clusters of acinar cells revealed that some cells within clusters elicited small Ca^{2+} responses in tissue from the M_3 KO mouse, while those from the M_1/M_3 double KO mouse were nearly completely inactive. Thus, both M_1 and M_3 receptor appear to contribute to Ca^{2+} mobilization postjunctionally, although the M_3 receptor has a much greater role (Nakamura et al. 2004).

The contribution of both the M_1 and M_3 receptor to the salivary response has implications with regard to the effects of muscarinic antagonists. Several M_3-selective antagonists have been shown in vivo and in vitro to antagonize contraction of the urinary bladder more potently than salivation (Wallis et al. 1993; Wallis 1995; Wallis and Napier 1999; Maruyama et al. 2006), which has led to the suggestion that these agents somehow exhibit functional selectivity for M_3 receptors in the urinary bladder over the salivary glands. This conclusion is based on the assumption that both urination and salivation are mediated mainly by the M_3 receptor. The contribution of the M_1 receptor to the response in the salivary glands indicates that the potency of M_3-selective antagonists, like zamifenacin and darifenacin, should be less than that expected for a pure M_3 response, whereas the potency of an M_1/M_3-selective antagonist, like 4-DAMP, for example, should be equivalent to that expected for an M_3 response. This behavior follows from concepts explained in Fig. 4a–c regarding the antagonism of a response mediated by two directly acting receptors. In urinary bladder, contraction is elicited by a directly acting M_3 receptor and an indirectly acting M_2 receptor. Hence, M_3-selective antagonist should inhibit urination with potencies consistent with an M_3

mechanism. Thus, the bladder selectivity (relative to salivary glands) of some "M_3-selective antagonists" might be explained by the direct role of the M_3 receptor in the bladder and the direct roles of both M_1 and M_3 receptors in the salivary glands.

8 Clinical Uses of Muscarinic Agonists and Antagonists in Gastrointestinal Disorders

8.1 Xerostomia and Sjogren's Syndrome

Sjogren's syndrome is a chronic inflammatory autoimmune disease of the exocrine glands (Nikolov and Illei 2009). Patients suffering from this condition have dry eyes, reduced tear production, dry mouth, swollen salivary glands, and usually require liquids to swallow food. Upon histological examination, the salivary glands for Sjogren's patients show infiltration of activated T cells and B cells around the salivary ducts. The prevalence of this syndrome in women relative to men is about 9:1.

Muscarinic agonists are used symptomatically to improve salivary and lacrimal flow in Sjogren's syndrome. The two agents used most commonly are cevimeline and pilocarpine (Ramos-Casals et al. 2010; Braga et al. 2009). Both of these agents are tertiary amines that can cross the blood–brain barrier. Also both compounds exhibit partial-agonist activity in a variety of assays for muscarinic activity. Their activity at human muscarinic receptor subtypes has been measured in cells expressing muscarinic receptor subtypes. One way to quantify the activity of agonists in a manner that is independent of the nature of the particular signaling pathway measured is to obtain a relative estimate of its affinity for the active state of a given receptor (Tran et al. 2009). This estimate, which is known as intrinsic relative activity (RA_i), has been estimated for pilocarpine at M_1–M_4 muscarinic receptors (Figueroa et al. 2008) and can also be estimated from the published EC_{50} and E_{max} values of cevimeline for eliciting responses in CHO cells expressing M_1–M_5 muscarinic receptors (Heinrich et al. 2009). When expressed relative to those of carbachol, the RA_i values of pilocarpine at M_1, M_2, M_3, and M_4 muscarinic receptors are 0.49, 0.02, 0.10, and 0.01, respectively. The corresponding values for cevimeline at M_1–M_5 receptors are 0.13, 0.0097, 0.035, 0.011, and 0.061. Both compounds exhibit selectivity for the M_1 muscarinic receptor. The activities of pilocarpine at the M_2, M_3, and M_4 subtypes relative to M_1 are 0.031, 0.20, and 0.027, respectively. For cevimeline, the activities at M_2, M_3, M_4, and M_5 relative to M_1 are 0.075, 0.27, 0.085, and 0.47, respectively. Thus, both compounds also exhibit a modest preference for M_3 receptors, but little activity at M_2 and M_4 receptors. Cevimeline also exhibits a moderate preference for M_5 receptors. This agonist selectivity (M_1 and M_3) is consistent with the role of both M_1 and M_3 receptors in salivation as described earlier.

8.2 Irritable Bowel Syndrome

Irritable bowel syndrome is a condition characterized by abdominal pain or discomfort associated with an alteration in bowel habit, including constipation or diarrhea and a change in the appearance of stool (Maxwell et al. 1997; Drossman et al. 2002; Spinelli 2007). The underlying cause is thought to involve some of the following including psychological factors, hypersensitivity and inflammation of the bowel, dysfunction in GI motility, and aberrant autonomic function. The ileum, colon, and rectum exhibit a hyper-responsiveness to a variety of stimuli including food, bowel distention, and various drugs (e.g., neostigmine and cholecystokinin).

Muscarinic antagonists have been used to treat the condition, and the two compounds most commonly used are atropine and dicyclomine (Callahan 2002; Chang et al. 2006). Atropine lacks selectivity for muscarinic receptor subtypes when binding assays are run in physiological buffers, and dicyclomine exhibits a similar profile (Wallis and Napier 1999). Both compounds have been reported to improve conditions in irritable bowel syndrome although reports of antimuscarinic side effects are common including dry mouth, dizziness, and blurred vision (Chang et al. 2006). Given the important role of the M_3 muscarinic receptor in intestinal motility, it would seem that M_3-selective antagonists, like zamifenacin, would exhibit fewer side effects (Callahan 2002). Such an agent would avoid a blockade of the M_2 receptor in the heart and perhaps cause less inhibition of salivation because of the role of the M_1 receptor in this response as described earlier.

8.3 Gastroparesis

Gastroparesis is a condition characterized by delayed gastric emptying (Soykan et al. 1998; Parkman et al. 2004). It can occur postsurgically with partial gastric resection. It also occurs in diabetes mellitus, in GI disorders, including peptic ulcer, gastroesophageal reflux disease, and in a variety of other non-GI disorders. The symptoms associated with this condition include nausea, vomiting, bloating, and early satiety.

A variety of prokinetic agents are used to treat gastroparesis, and the directly acting muscarinic agonist, bethanechol, is also used (Parkman et al. 2004). This compound has a quaternary ammonium structure and is resistant to hydrolysis by cholinesterase. It lacks selectivity for subtypes of the muscarinic receptor. When administered orally, its stimulatory actions are usually confined to the GI and urinary tracts. It increases gastric emptying and intestinal transit. It can also elicit typical muscarinic side effects including blurred vision, bladder spasms, salivation, and abdominal pain.

9 Conclusions

The M_2 and M_3 muscarinic receptors are the major receptors for acetylcholine located postjunctionally that elicit contraction of GI smooth muscle. In mouse, but not other rodents, the M_2 receptor elicits a small (40% of E_{max}), direct contraction of intestinal smooth muscle. Whether the M_2 receptor elicits direct contraction of human GI smooth muscle is unknown. The M_2 receptor does elicit a conditional enhancement of M_3 receptor-mediated contraction of GI smooth muscle, and this mechanism may have an important role physiologically. This M_2 mechanism is potently antagonized by M_3-selective antagonists, indicating that M_3 receptor antagonism by itself inhibits this M_2–M_3 interaction. In other words, if the action of the M_2 receptor depends on the M_3, then blockade of the M_3 receptor will block the action of the M_2. It follows that antagonists with selectivity for the M_3 receptor would be more selective therapeutic agents in the treatment of conditions associated with hypermotility than antagonists that block both M_2 and M_3 receptors. The mechanism for this interaction is probably an M_2 receptor-mediated stimulation of I_{cat} or inhibition of K_{Ca}.

The observation that some antagonists (e.g., *p*-F-HHSiD and zamifenacin) appear to discriminate among muscarinic responses with potencies that exceed by about tenfold their affinity constant for any known muscarinic receptor subtype suggests that these compounds may antagonize contraction by inhibiting an important signaling protein downstream from receptor activation. This putative site of action may represent a useful target for the development of tissue-selective inhibitors of smooth muscle contraction.

References

Abrams P, Andersson KE, Buccafusco JJ, Chapple C, de Groat WC, Fryer AD, Kay G, Laties A, Nathanson NM, Pasricha PJ, Wein AJ (2006) Muscarinic receptors: their distribution and function in body systems, and the implications for treating overactive bladder. Br J Pharmacol 148:565–578

Aihara T, Fujishita T, Kanatani K, Furutani K, Nakamura E, Taketo MM, Matsui M, Chen D, Okabe S (2003) Impaired gastric secretion and lack of trophic responses to hypergastrinemia in M_3 muscarinic receptor knockout mice. Gastroenterology 125:1774–1784

Aihara T, Nakamura Y, Taketo MM, Matsui M, Okabe S (2005) Cholinergically stimulated gastric acid secretion is mediated by M(3) and M(5) but not M(1) muscarinic acetylcholine receptors in mice. Am J Physiol Gastrointest Liver Physiol 288:G1199–G1207

Andersson R, Nilsson K (1972) Cyclic AMP and calcium in relaxation in intestinal smooth muscle. Nat New Biol 238:119–120

Arunlakshana O, Schild HO (1959) Some quantitative uses of drug antagonists. Br J Pharmacol 14:48–58

Barras M, Coste A, Eon MT, Guillot E (1999) Pharmacological characterization of muscarinic receptors implicated in rabbit detrusor muscle contraction and activation of inositol phospholipid hydrolysis in rabbit detrusor and parotid gland. Fundam Clin Pharmacol 13:562–570

Berridge MJ (1984) Inositol trisphosphate and diacylglycerol as second messengers. Biochem J 220:345–360

Bertaccini G, Coruzzi G (1989) Control of gastric acid secretion by histamine H2 receptor antagonists and anticholinergics. Pharmacol Res 21:339–352

Bianchi Porro G, Petrillo M (1982) Pirenzepine in the treatment of peptic ulcer disease. Review and commentary. Scand J Gastroenterol Suppl 72:229–236

Black JW, Gerskowitch VP, Leff P, Shankley NP (1986) Analysis of competitive antagonism when this property occurs as part of a pharmacological resultant. Br J Pharmacol 89:547–555

Bolger GT, Gengo P, Klockowski R, Luchowski E, Siegel H, Janis RA, Triggle AM, Triggle DJ (1983) Characterization of binding of the Ca++ channel antagonist, [3H]nitrendipine, to guinea-pig ileal smooth muscle. J Pharmacol Exp Ther 225:291–309

Bolton TB (1979) Mechanisms of action of transmitters and other substances on smooth muscle. Physiol Rev 59:606–718

Bolton TB, Clark JP (1981) Actions of various muscarinic agonists on membrane potential, potassium efflux, and contraction of longitudinal muscle of guinea-pig intestine. Br J Pharmacol 72:319–334

Bolton TB, Lim SP (1989) Properties of calcium stores and transient outward currents in single smooth muscle cells of rabbit intestine. J Physiol 409:385–401

Bolton TB, Zholos AV (1997) Activation of M_2 muscarinic receptors in guinea-pig ileum opens cationic channels modulated by M_3 muscarinic receptors. Life Sci 60:1121–1128

Braga MA, Tarzia O, Bergamaschi CC, Santos FA, Andrade ED, Groppo FC (2009) Comparison of the effects of pilocarpine and cevimeline on salivary flow. Int J Dent Hyg 7:126–130

Burgen AS, Spero L (1968) The action of acetylcholine and other drugs on the efflux of potassium and rubidium from smooth muscle of the guinea-pig intestine. Br J Pharmacol 34:99–115

Callahan MJ (2002) Irritable bowel syndrome neuropharmacology. A review of approved and investigational compounds. J Clin Gastroenterol 35:S58–S67

Candell LM, Yun SH, Tran LL, Ehlert FJ (1990) Differential coupling of subtypes of the muscarinic receptor to adenylate cyclase and phosphoinositide hydrolysis in the longitudinal muscle of the rat ileum. Mol Pharmacol 38:689–697

Cantoni GL, Eastman G (1946) On the response of the intestine to smooth muscle stimulants. J Pharmacol Exp Ther 87:392–399

Chang KJ, Triggle DJ (1973) Quantitative aspects of drug-receptor interactions. I. Ca^{2+} and cholinergic receptor activation in smooth muscle: a basic model for drug-receptor interactions. J Theor Biol 40:125–154

Chang HY, Kelly EC, Lembo AJ (2006) Current gut-directed therapies for irritable bowel syndrome. Curr Treat Options Gastroenterol 9:314–323

Cole WC, Carl A, Sanders KM (1989) Muscarinic suppression of Ca^{2+}-dependent K current in colonic smooth muscle. Am J Physiol 257:C481–C487

Dale MM (1958) An inhibitory effect of acetylcholine on the response of the guinea-pig ileum to histamine. Br J Pharmacol 13:17–19

Daly MJ, Humphray JM, Stables R (1982) Effects of H2-receptor antagonists and anticholinoceptor drugs on gastric and salivary secretion induced by bethanechol in the anaesthetized dog. Br J Pharmacol 76:361–365

Del Tacca M, Danes R, Blandizzi C, Bernardini MC (1990) Differential affinities of AF-DX 116, atropine, and pirenzepine for muscarinic receptors in guinea pig gastric fundus, atria and urinary bladder: might atropine distinguish among muscarinic receptor subtypes? Pharmacology 40:241–249

Dresviannikov AV, Bolton TB, Zholos AV (2006) Muscarinic receptor-activated cationic channels in murine ileal myocytes. Br J Pharmacol 149:179–187

Drossman DA, Camilleri M, Mayer EA, Whitehead WE (2002) AGA technical review on irritable bowel syndrome. Gastroenterology 123:2108–2131

Eglen RM, Harris GC (1993) Selective inactivation of muscarinic M_2 and M_3 receptors in guinea-pig ileum and atria in vitro. Br J Pharmacol 109:946–952

Eglen RM, Whiting RL (1988) Comparison of the muscarinic receptors of the guinea-pig oesophageal muscularis mucosae and trachea in vitro. J Auton Pharmacol 8:181–189

Eglen RM, Huff MM, Montgomery WW, Whiting RL (1988) Differential effects of pertussis toxin on muscarinic responses in isolated atria and smooth muscle. J Auton Pharmacol 8:29–37

Eglen RM, Cornett CM, Whiting RL (1990) Interaction of p-F-HHSiD (p-Fluoro-hexahydrosiladifenidol) at muscarinic receptors in guinea-pig trachea. Naunyn Schmiedebergs Arch Pharmacol 342:394–399

Eglen RM, Hegde SS, Watson N (1996a) Muscarinic receptor subtypes and smooth muscle function. Pharmacol Rev 48:531–565

Eglen RM, Peelle B, Pulido-Rios MT, Leung E (1996b) Functional interactions between muscarinic M_2 receptors and 5-hydroxytryptamine (5-HT)4 and β3-adrenoceptors in isolated oesophageal muscularis mucosae of the rat. Br J Pharmacol 119:595–601

Ehlert FJ (1985) The relationship between muscarinic receptor occupancy and adenylate cyclase inhibition in the rabbit myocardium. Mol Pharmacol 28:410–421

Ehlert FJ (2003a) Contractile role of M_2 and M_3 muscarinic receptors in gastrointestinal, airway and urinary bladder smooth muscle. Life Sci 74:355–366

Ehlert FJ (2003b) Pharmacological analysis of the contractile role of M_2 and M_3 muscarinic receptor in smooth muscle. Receptors Channels 9:261–277

Ehlert FJ, Ostrom RS, Sawyer GW (1997a) Subtypes of the muscarinic receptor in smooth muscle. Life Sci 61:1729–1740

Ehlert FJ, Thomas EA, Gerstin EH, Griffin MT (1997b) Muscarinic receptors and gastrointestinal smooth muscle. In: Eglen RM (ed) Muscarinic receptor subtypes in smooth muscle. CRC, Boca Raton, FL, pp 92–147

Ehlert FJ, Griffin MT, Abe DM, Vo TH, Taketo MM, Manabe T, Matsui M (2005a) The M_2 muscarinic receptor mediates contraction through indirect mechanisms in mouse urinary bladder. J Pharmacol Exp Ther 313:368–378

Ehlert FJ, Hsu JC, Leung K, Lee AG, Shehnaz D, Griffin MT (2005b) Comparison of the antimuscarinic action of p-fluorohexahydrosiladifenidol in ileal and tracheal smooth muscle. J Pharmacol Exp Ther 312:592–600

Esqueda EE, Gerstin EH Jr, Griffin MT, Ehlert FJ (1996) Stimulation of cyclic AMP accumulation and phosphoinositide hydrolysis by M3 muscarinic receptors in the rat peripheral lung. Biochem Pharmacol 52:643–658

Feldman M (1984) Inhibition of gastric acid secretion by selective and nonselective anticholinergics. Gastroenterology 86:361–366

Figueroa KW, Griffin MT, Ehlert FJ (2008) Selectivity of agonists for the active state of M_1–M_4 muscarinic receptor subtypes. J Pharmacol Exp Ther 328:331–342

Gautam D, Heard TS, Cui Y, Miller G, Bloodworth L, Wess J (2004) Cholinergic stimulation of salivary secretion studied with M_1 and M_3 muscarinic receptor single- and double-knockout mice. Mol Pharmacol 66:260–267

Gautam D, Han SJ, Heard TS, Cui Y, Miller G, Bloodworth L, Wess J (2005) Cholinergic stimulation of amylase secretion from pancreatic acinar cells studied with muscarinic acetylcholine receptor mutant mice. J Pharmacol Exp Ther 313:995–1002

Gil DW, Wolfe BB (1985) Pirenzepine distinguishes between muscarinic receptor-mediated phosphoinositide breakdown and inhibition of adenylate cyclase. J Pharmacol Exp Ther 232(3):608–616

Giraldo E, Monferini E, Ladinsky H, Hammer R (1987) Muscarinic receptor heterogeneity in guinea pig intestinal smooth muscle: binding studies with AF-DX 116. Eur J Pharmacol 141:475–477

Griffin MT, Matsui M, Shehnaz D, Ansari KZ, Taketo MM, Manabe T, Ehlert FJ (2004) Muscarinic agonist-mediated heterologous desensitization in isolated ileum requires activation of both muscarinic M_2 and M_3 receptors. J Pharmacol Exp Ther 308:339–349

Griffin MT, Matsui M, Ostrom RS, Ehlert FJ (2009) The guinea pig ileum lacks the direct, high-potency, M(2)-muscarinic, contractile mechanism characteristic of the mouse ileum. Naunyn Schmiedebergs Arch Pharmacol 380:327–335

Hammer R (1980) Muscarinic receptors in the stomach. Scand J Gastroenterol Suppl 66:5–11

Hammer R, Berrie CP, Birdsall NJ, Burgen AS, Hulme EC (1980) Pirenzepine distinguishes between different subclasses of muscarinic receptors. Nature 283:90–92

Heinrich JN, Butera JA, Carrick T, Kramer A, Kowal D, Lock T, Marquis KL, Pausch MH, Popiolek M, Sun SC, Tseng E, Uveges AJ, Mayer SC (2009) Pharmacological comparison of muscarinic ligands: historical versus more recent muscarinic M_1-preferring receptor agonists. Eur J Pharmacol 605:53–56

Herawi M, Lambrecht G, Mutschler E, Moser U, Pfeiffer A (1988) Different binding properties of muscarinic M_2-receptor subtypes for agonists and antagonists in porcine gastric smooth muscle and mucosa. Gastroenterology 94:630–637

Hoiting BH, Meurs H, Schuiling M, Kuipers R, Elzinga CR, Zaagsma J (1996) Modulation of agonist-induced phosphoinositide metabolism, Ca^{2+} signalling and contraction of airway smooth muscle by cyclic AMP-dependent mechanisms. Br J Pharmacol 117:419–426

Hu CL, Chandra R, Ge H, Pain J, Yan L, Babu G, Depre C, Iwatsubo K, Ishikawa Y, Sadoshima J, Vatner SF, Vatner DE (2009) Adenylyl cyclase type 5 protein expression during cardiac development and stress. Am J Physiol Heart Circ Physiol 297:H1776–H1782

Iino S, Nojyo Y (2006) Muscarinic M(2) acetylcholine receptor distribution in the guinea-pig gastrointestinal tract. Neuroscience 138:549–559

Inoue R, Isenberg G (1990) Acetylcholine activates nonselective cation channels in guinea pig ileum through a G protein. Am J Physiol 258:C1173–C1178

Ishikawa Y, Katsushika S, Chen L, Halnon NJ, Kawabe J, Homcy CJ (1992) Isolation and characterization of a novel cardiac adenylylcyclase cDNA. J Biol Chem 267:13553–13557

Ito Y, Oyunzul L, Seki M, Fujino Oki T, Matsui M, Yamada S (2009) Quantitative analysis of the loss of muscarinic receptors in various peripheral tissues in M_1-M_5 receptor single knockout mice. Br J Pharmacol 156:1147–1153

Jafferji SS, Michell RH (1976) Muscarinic cholinergic stimulation of phosphatidylinositol turnover in the longitudinal smooth muscle of guinea-pig ileum. Biochem J 154:653–657

Kajimura M, Reuben MA, Sachs G (1992) The muscarinic receptor gene expressed in rabbit parietal cells is the m3 subtype. Gastroenterology 103:870–875

Kamikawa Y, Uchida K, Shimo Y (1985) Heterogeneity of muscarinic receptors in the guinea pig esophageal muscularis mucosae and ileal longitudinal muscle. Gastroenterology 88:706–716

Kato M, Ohkuma S, Kataoka K, Kashima K, Kuriyama K (1992) Characterization of muscarinic receptor subtypes on rat pancreatic acini: pharmacological identification by secretory responses and binding studies. Digestion 52:194–203

Kim SJ, Koh EM, Kang TM, Kim YC, So I, Isenberg G, Kim KW (1998) Ca^{2+} influx through carbachol-activated non-selective cation channels in guinea-pig gastric myocytes. J Physiol 513(Pt 3):749–760

Kitazawa T, Hashiba K, Cao J, Unno T, Komori S, Yamada M, Wess J, Taneike T (2007) Functional roles of muscarinic M_2 and M_3 receptors in mouse stomach motility: studies with muscarinic receptor knockout mice. Eur J Pharmacol 554:212–222

Korc M, Ackerman MS, Roeske WR (1987) A cholinergic antagonist identifies a subclass of muscarinic receptors in isolated rat pancreatic acini. J Pharmacol Exp Ther 240:118–122

Lambrecht G, Feifel R, Moser U, Wagner-Roder M, Choo LK, Camus J, Tastenoy M, Waelbroeck M, Strohmann C, Tacke R, Rodrigues de Miranda JF, Christophe J, Mutschler E (1989) Pharmacology of hexahydro-difenidol, hexahydro-sila-difenidol and related selective muscarinic antagonists. Trends Pharmacol Sci (Suppl): 60–64

Lee HK, Bayguinov O, Sanders KM (1993) Role of nonselective cation current in muscarinic responses of canine colonic muscle. Am J Physiol 265:C1463–C1471

Lee YM, Kim BJ, Kim HJ, Yang DK, Zhu MH, Lee KP, So I, Kim KW (2003) TRPC5 as a candidate for the nonselective cation channel activated by muscarinic stimulation in murine stomach. Am J Physiol Gastrointest Liver Physiol 284:G604–G616

Liebmann C, Nawrath S, Schnittler M, Schubert H, Jakobs KH (1992) Binding characteristics and functional G protein coupling of muscarinic acetylcholine receptors in rat duodenum smooth muscle membranes. Naunyn Schmiedebergs Arch Pharmacol 345:7–15

Lin S, Kajimura M, Takeuchi K, Kodaira M, Hanai H, Kaneko E (1997) Expression of muscarinic receptor subtypes in rat gastric smooth muscle: effect of M_3 selective antagonist on gastric motility and emptying. Dig Dis Sci 42:907–914

Louie DS, Owyang C (1986) Muscarinic receptor subtypes on rat pancreatic acini: secretion and binding studies. Am J Physiol 251:G275–G279

Maeda A, Kubo T, Mishina M, Numa S (1988) Tissue distribution of mRNAs encoding muscarinic acetylcholine receptor subtypes. FEBS Lett 239:339–342

Manolopoulos VG, Liu J, Unsworth BR, Lelkes PI (1995) Adenylyl cyclase isoforms are differentially expressed in primary cultures of endothelial cells and whole tissue homogenates from various rat tissues. Biochem Biophys Res Commun 208:323–331

Maruyama S, Oki T, Otsuka A, Shinbo H, Ozono S, Kageyama S, Mikami Y, Araki I, Takeda M, Masuyama K, Yamada S (2006) Human muscarinic receptor binding characteristics of antimuscarinic agents to treat overactive bladder. J Urol 175:365–369

Matsui M, Motomura D, Karasawa H, Fujikawa T, Jiang J, Komiya Y, Takahashi S, Taketo MM (2000) Multiple functional defects in peripheral autonomic organs in mice lacking muscarinic acetylcholine receptor gene for the M_3 subtype. Proc Natl Acad Sci USA 97:9579–9584

Matsui M, Motomura D, Fujikawa T, Jiang J, Takahashi S, Manabe T, Taketo MM (2002) Mice lacking M_2 and M_3 muscarinic acetylcholine receptors are devoid of cholinergic smooth muscle contractions but still viable. J Neurosci 22:10627–10632

Matsui M, Griffin MT, Shehnaz D, Taketo MM, Ehlert FJ (2003) Increased relaxant action of forskolin and isoproterenol against muscarinic agonist-induced contractions in smooth muscle from M_2 receptor knockout mice. J Pharmacol Exp Ther 305:106–113

Maxwell PR, Mendall MA, Kumar D (1997) Irritable bowel syndrome. Lancet 350:1691–1695

Meloy TD, Daniels DV, Hegde SS, Eglen RM, Ford AP (2001) Functional characterization of rat submaxillary gland muscarinic receptors using microphysiometry. Br J Pharmacol 132: 1606–1614

Michel AD, Whiting RL (1987) Direct binding studies on ileal and cardiac muscarinic receptors. Br J Pharmacol 92:755–767

Muinuddin A, Naqvi K, Sheu L, Gaisano HY, Diamant NE (2005) Regional differences in cholinergic regulation of potassium current in feline esophageal circular smooth muscle. Am J Physiol Gastrointest Liver Physiol 288:G1233–G1240

Nakamura T, Kimura J, Yamaguchi O (2002) Muscarinic M2 receptors inhibit Ca^{2+}-activated K^+ channels in rat bladder smooth muscle. Int J Urol 9:689–696

Nakamura T, Matsui M, Uchida K, Futatsugi A, Kusakawa S, Matsumoto N, Nakamura K, Manabe T, Taketo MM, Mikoshiba K (2004) M(3) muscarinic acetylcholine receptor plays a critical role in parasympathetic control of salivation in mice. J Physiol 558:561–575

Nikolov NP, Illei GG (2009) Pathogenesis of Sjogren's syndrome. Curr Opin Rheumatol 21:465–470

Ontsouka EC, Bruckmaier RM, Steiner A, Blum JW, Meylan M (2007) Messenger RNA levels and binding sites of muscarinic acetylcholine receptors in gastrointestinal muscle layers from healthy dairy cows. J Recept Signal Transduct Res 27:147–166

Ostrom RS, Ehlert FJ (1997) M_2 muscarinic receptor inhibition of agonist-induced cyclic adenosine monophosphate accumulation and relaxation in the guinea pig ileum. J Pharmacol Exp Ther 280:1–11

Otsuguro K, Tang J, Tang Y, Xiao R, Freichel M, Tsvilovskyy V, Ito S, Flockerzi V, Zhu MX, Zholos AV (2008) Isoform-specific inhibition of TRPC4 channel by phosphatidylinositol 4,5-bisphosphate. J Biol Chem 283:10026–10036

Pacaud P, Bolton TB (1991) Relation between muscarinic receptor cationic current and internal calcium in guinea-pig jejunal smooth muscle cells. J Physiol 441:477–499

Parkman HP, Hasler WL, Fisher RS (2004) American Gastroenterological Association technical review on the diagnosis and treatment of gastroparesis. Gastroenterology 127:1592–1622

Paton WDM (1961) A theory of drug action based on the rate of drug-receptor combination. Proc Roy Soc Lond B 154:21–69

Paulin RF, Menani JV, Colombari E, De Paula PM, Colombari DS (2009) Role of the medial septal area on pilocarpine-induced salivary secretion and water intake. Brain Res 1298:145–152

Peralta EG, Ashkenazi A, Winslow JW, Ramachandran J, Capon DJ (1988) Differential regulation of PI hydrolysis and adenylyl cyclase by muscarinic receptor subtypes. Nature 334:434–437

Pfeiffer A, Rochlitz H, Herz A, Paumgartner G (1988) Stimulation of acid secretion and phosphoinositol production by rat parietal cell muscarinic M_2 receptors. Am J Physiol 254: G622–G629

Pfeiffer A, Rochlitz H, Noelke B, Tacke R, Moser U, Mutschler E, Lambrecht G (1990) Muscarinic receptors mediating acid secretion in isolated rat gastric parietal cells are of M_3 type. Gastroenterology 98:218–222

Pontari MA, Braverman AS, Ruggieri MR Sr (2004) The M_2 muscarinic receptor mediates in vitro bladder contractions from patients with neurogenic bladder dysfunction. Am J Physiol Regul Integr Comp Physiol 286:R874–R880

Preiksaitis HG, Krysiak PS, Chrones T, Rajgopal V, Laurier LG (2000) Pharmacological and molecular characterization of muscarinic receptor subtypes in human esophageal smooth muscle. J Pharmacol Exp Ther 295:879–888

Ramos-Casals M, Tzioufas AG, Stone JH, Siso A, Bosch X (2010) Treatment of primary Sjogren syndrome: a systematic review. JAMA 304:452–460

Reddy H, Watson N, Ford AP, Eglen RM (1995) Characterization of the interaction between muscarinic M_2 receptors and beta-adrenoceptor subtypes in guinea-pig isolated ileum. Br J Pharmacol 114:49–56

Rimele TJ, O'Dorisio MS, Gaginella TS (1981) Evidence for muscarinic receptors on rat colonic epithelial cells: binding of [3H]quinuclidinyl benzilate. J Pharmacol Exp Ther 218:426–434

Ringdahl B (1987) Selectivity of partial agonists related to oxotremorine based on differences in muscarinic receptor reserve between the guinea pig ileum and urinary bladder. Mol Pharmacol 31:351–356

Rosenberger LB, Ticku MK, Triggle DJ (1979) The effect of Ca^{2+} antagonists on mechanical responses and Ca^{2+} movements in guinea pig ileal longitudinal smooth muscle. Can J Physiol Pharmacol 57:333–347

Rossowski WJ, Ozden A, Ertan A, Maumus M, Arimura A (1988) Somatostatin, gastrin, and cholinergic muscarinic binding sites in rat gastric, duodenal, and jejunal mucosa. Scand J Gastroenterol 23:717–725

Sakamoto T, Unno T, Matsuyama H, Uchiyama M, Hattori M, Nishimura M, Komori S (2006) Characterization of muscarinic receptor-mediated cationic currents in longitudinal smooth muscle cells of mouse small intestine. J Pharmacol Sci 100:215–226

Sakamoto T, Unno T, Kitazawa T, Taneike T, Yamada M, Wess J, Nishimura M, Komori S (2007) Three distinct muscarinic signalling pathways for cationic channel activation in mouse gut smooth muscle cells. J Physiol 582:41–61

Sawyer GW, Ehlert FJ (1998) Contractile role of the M_2 and M_3 muscarinic receptors in the guinea pig colon. J Pharmacol Exp Ther 284:269–277

Sawyer G, Ehlert F (1999a) Pertussis toxin increases isoproterenol induced relaxation in field-stimulated ileum. Eur J Pharmacol 367:81–84 [published erratum appears in Eur J Pharmacol 1999 May 21;372(3):329]

Sawyer GW, Ehlert FJ (1999b) Muscarinic M_3 receptor inactivation reveals a pertussis toxin-sensitive contractile response in the guinea pig colon. J Pharmacol Exp Ther 289:464–476

Shehnaz D, Ansari KZ, Ehlert FJ (2001) Acetylcholine-induced desensitization of the contractile response to histamine in guinea pig ileum is prevented by either pertussis toxin-treatment or by selective inactivation of muscarinic M_3 receptors. J Pharmacol Exp Ther 297:1152–1159

Shen A, Mitchelson F (1998) Muscarinic M_2 receptor-mediated contraction in the guinea pig Taenia caeci: possible involvement of protein kinase C. Biochem Pharmacol 56:1529–1537

Shin CY, Lee YP, Song HJ, Je HD, Sohn UD (2007) Cyclic AMP dependent down regulation in the relaxation of smooth muscle cells of cat esophagitis. Arch Pharm Res 30:715–722

Soll AH, Walsh JH (1979) Regulation of gastric acid secretion. Annu Rev Physiol 41:35–53

Soykan I, Sivri B, Sarosiek I, Kiernan B, McCallum RW (1998) Demography, clinical characteristics, psychological and abuse profiles, treatment, and long-term follow-up of patients with gastroparesis. Dig Dis Sci 43:2398–2404

Spinelli A (2007) Irritable bowel syndrome. Clin Drug Investig 27:15–33

Stengel PW, Gomeza J, Wess J, Cohen ML (2000) M(2) and M(4) receptor knockout mice: muscarinic receptor function in cardiac and smooth muscle in vitro. J Pharmacol Exp Ther 292:877–885

Stengel PW, Yamada M, Wess J, Cohen ML (2002) M(3)-receptor knockout mice: muscarinic receptor function in atria, stomach fundus, urinary bladder, and trachea. Am J Physiol Regul Integr Comp Physiol 282:R1443–R1449

Sutliff VE, Rattan S, Gardner JD, Jensen RT (1989) Characterization of cholinergic receptors mediating pepsinogen secretion from chief cells. Am J Physiol 257:G226–G234

Takakura AC, Moreira TS, Laitano SC, De Luca Junior LA, Renzi A, Menani JV (2003) Central muscarinic receptors signal pilocarpine-induced salivation. J Dent Res 82:993–997

Thomas EA, Ehlert FJ (1994) Pertussis toxin blocks M_2 muscarinic receptor-mediated effects on contraction and cyclic AMP in the guinea pig ileum, but not M_3-mediated contractions and phosphoinositide hydrolysis. J Pharmacol Exp Ther 271:1042–1050

Thomas EA, Hsu HH, Griffin MT, Hunter AL, Luong T, Ehlert FJ (1992) Conversion of *N*-(2-chloroethyl)-4-piperidinyl diphenylacetate (4-DAMP mustard) to an aziridinium ion and its interaction with muscarinic receptors in various tissues. Mol Pharmacol 41:718–726

Thomas EA, Baker SA, Ehlert FJ (1993) Functional role for the M_2 muscarinic receptor in smooth muscle of guinea pig ileum. Mol Pharmacol 44:102–110

Tien XY, Wahawisan R, Wallace LJ, Gaginella TS (1985) Intestinal epithelial cells and musculature contain different muscarinic binding sites. Life Sci 36:1949–1955

Tran JA, Matsui M, Ehlert FJ (2006) Differential coupling of muscarinic M_1, M_2, and M_3 receptors to phosphoinositide hydrolysis in urinary bladder and longitudinal muscle of the ileum of the mouse. J Pharmacol Exp Ther 318:649–656

Tran JA, Chang A, Matsui M, Ehlert FJ (2009) Estimation of relative microscopic affinity constants of agonists for the active state of the receptor in functional studies on M_2 and M_3 muscarinic receptors. Mol Pharmacol 75:381–396

Tsvilovskyy VV, Zholos AV, Aberle T, Philipp SE, Dietrich A, Zhu MX, Birnbaumer L, Freichel M, Flockerzi V (2009) Deletion of TRPC4 and TRPC6 in mice impairs smooth muscle contraction and intestinal motility in vivo. Gastroenterology 137:1415–1424

Unno T, Komori S, Ohashi H (1995) Inhibitory effect of muscarinic receptor activation on Ca^{2+} channel current in smooth muscle cells of guinea-pig ileum. J Physiol 484:567–581

Unno T, Matsuyama H, Sakamoto T, Uchiyama M, Izumi Y, Okamoto H, Yamada M, Wess J, Komori S (2005) M(2) and M(3) muscarinic receptor-mediated contractions in longitudinal smooth muscle of the ileum studied with receptor knockout mice. Br J Pharmacol 146:98–108

Vogalis F, Goyal RK (1997) Activation of small conductance Ca(2+)-dependent K^+ channels by purinergic agonists in smooth muscle cells of the mouse ileum. J Physiol 502(Pt 3):497–508

Wade GR, Sims SM (1993) Muscarinic stimulation of tracheal smooth muscle cells activates large-conductance Ca(2+)-dependent K^+ channel. Am J Physiol 265:C658–C665

Wallis RM (1995) Pre-clinical and clinical pharmacology of selective muscarinic M(3) receptor antagonists. Life Sci 56:861–868

Wallis RM, Napier CM (1999) Muscarinic antagonists in development for disorders of smooth muscle function. Life Sci 64:395–401

Wallis RM, Alker D, Burges RA, Cross PE, Newgreen DT, Quinn P (1993) Zamifenacin: a novel gut selective muscarinic receptor antagonist. Br J Pharmacol 109:36P

Wang J, Krysiak PS, Laurier LG, Sims SM, Preiksaitis HG (2000) Human esophageal smooth muscle cells express muscarinic receptor subtypes M(1) through M(5). Am J Physiol Gastrointest Liver Physiol 279:G1059–G1069

Watson N, Reddy H, Stefanich E, Eglen RM (1995) Characterization of the interaction of zamifenacin at muscarinic receptors in vitro. Eur J Pharmacol 285:135–142

Watson EL, Abel PW, DiJulio D, Zeng W, Makoid M, Jacobson KL, Potter LT, Dowd FJ (1996) Identification of muscarinic receptor subtypes in mouse parotid gland. Am J Physiol 271: C905–C913

Wilkes JM, Kajimura M, Scott DR, Hersey SJ, Sachs G (1991) Muscarinic responses of gastric parietal cells. J Membr Biol 122:97–110

Xie G, Drachenberg C, Yamada M, Wess J, Raufman JP (2005) Cholinergic agonist-induced pepsinogen secretion from murine gastric chief cells is mediated by M_1 and M_3 muscarinic receptors. Am J Physiol Gastrointest Liver Physiol 289:G521–G529

Zhang LB, Buxton IL (1991) Muscarinic receptors in canine colonic circular smooth muscle. II. Signal transduction pathways. Mol Pharmacol 40:952–959

Zhang LB, Horowitz B, Buxton IL (1991) Muscarinic receptors in canine colonic circular smooth muscle. I. Coexistence of M_2 and M_3 subtypes. Mol Pharmacol 40:943–951

Zholos AV, Bolton TB (1994) G-protein control of voltage dependence as well as gating of muscarinic metabotropic channels in guinea-pig ileum. J Physiol 478:195–202

Zholos AV, Bolton TB (1997) Muscarinic receptor subtypes controlling the cationic current in guinea-pig ileal smooth muscle. Br J Pharmacol 122:885–893

Muscarinic Agonists and Antagonists: Effects on the Urinary Bladder

Donna J. Sellers and Russ Chess-Williams

Abstract Voiding of the bladder is the result of a parasympathetic muscarinic receptor activation of the detrusor smooth muscle. However, the maintenance of continence and a normal bladder micturition cycle involves a complex interaction of cholinergic, adrenergic, nitrergic and peptidergic systems that is currently little understood. The cholinergic component of bladder control involves two systems, acetylcholine (ACh) released from parasympathetic nerves and ACh from non-neuronal cells within the urothelium. The actions of ACh on the bladder depend on the presence of muscarinic receptors that are located on the detrusor smooth muscle, where they cause direct (M_3) and indirect (M_2) contraction; pre-junctional nerve terminals where they increase (M_1) or decrease (M_4) the release of ACh and noradrenaline (NA); sensory nerves where they influence afferent nerve activity; umbrella cells in the urothelium where they stimulate the release of ATP and NO; suburothelial interstitial cells with unknown function; and finally, other unidentified sites in the urothelium from where prostaglandins and inhibitory/relaxatory factors are released. Thus, the actions of muscarinic receptor agonists and antagonists on the bladder may be very complex even when considering only local muscarinic actions. Clinically, muscarinic antagonists remain the mainstay of treatment for the overactive bladder (OAB), while muscarinic agonists have been used to treat hypoactive bladder. The antagonists are effective in treating OAB, but their precise mechanisms and sites of action (detrusor, urothelium, and nerves) have yet to be established. Potentially more selective agents may be developed when the cholinergic systems within the bladder are more fully understood.

Keywords Afferent nerves • Detrusor • Muscarinic agonist • Muscarinic antagonist • Muscarinic receptor subtypes • Urinary bladder • Urothelium

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1 The Urinary Bladder and Control of Micturition

Simplistically, the urinary bladder has two functions: storage of urine, which requires the organ to relax and fill with little or no increase in intravesical pressure, and voiding, which requires contraction of the detrusor smooth muscle with simultaneous relaxation of the bladder outlet and urethra. This micturition cycle, of filling and emptying, is dependent on the coordinated efferent and afferent activity of the parasympathetic and sympathetic nervous systems, which regulate the bladder body, outlet and urethra (see de Groat 2006 for review). The somatic nervous system controls the external urethral sphincter via the pudendal nerve to provide some voluntary control over micturition and continence. During bladder filling the sympathetic system, via the hypogastric nerve, relaxes the bladder wall (via β-adrenoceptors) and contracts the bladder outlet (via α1-adrenoceptors) to maintain continence. During bladder emptying, the parasympathetic nervous system, via the pelvic nerve, initiates and maintains detrusor contraction and relaxes the bladder outlet. In addition to ACh, other nonadrenergic noncholinergic (NANC) transmitters are co-released, including ATP (see Burnstock 2009 for review). It is thought that ATP initiates detrusor contraction during voiding, while ACh acting on muscarinic receptors maintains contraction to allow complete emptying of the bladder (Chancellor et al. 1992). In the bladder outlet, nitric oxide is the main neurotransmitter released to induce relaxation during micturition (Persson et al. 1992).

In recent years, the contributions of the *urothelium* (innermost layer of transitional epithelium) and the *suburothelium* (underlying layer containing nerves, interstitial cells, fibroblasts, blood vessels, etc.) to bladder function have gained significance and it is now known that these layers have their own cholinergic system and that cells within the (sub)urothelium are important in regulating bladder contraction (Hawthorn et al. 2000), as well as being involved in sensory mechanisms (reviewed by Birder 2010). The urothelium can respond to chemical, mechanical and thermal stimuli and expresses a number of key ion channels and receptors including nicotinic, muscarinic, tachykinin, adrenergic, bradykinin and transient potential receptors (see Birder 2010). It has close association with the suburothelial afferent nerves and also releases a number of chemical mediators including acetylcholine (Yoshida et al. 2006), ATP (Ferguson et al. 1997), prostaglandins (Masunaga et al. 2006), nitric oxide (Birder et al. 1998) and unidentified inhibitory factors that can modulate tone of the underlying smooth muscle (Fovaeus et al. 1999; Hawthorn et al. 2000) (see Fig. 1). The suburothelium also possesses a population of cells with similar morphology to the interstitial cells of Cajal (ICC) of the gastrointestinal tract. Subtypes of these cells have been found in the suburothelium and within the detrusor. The function of the ICCs in the bladder is currently unknown but based on their role in the gastrointestinal tract and what is known of their physiological properties in the bladder, it has been suggested that they act to process sensory information, act as pacemakers for contractile activity and modulate detrusor contractility (for review see McCloskey 2010).

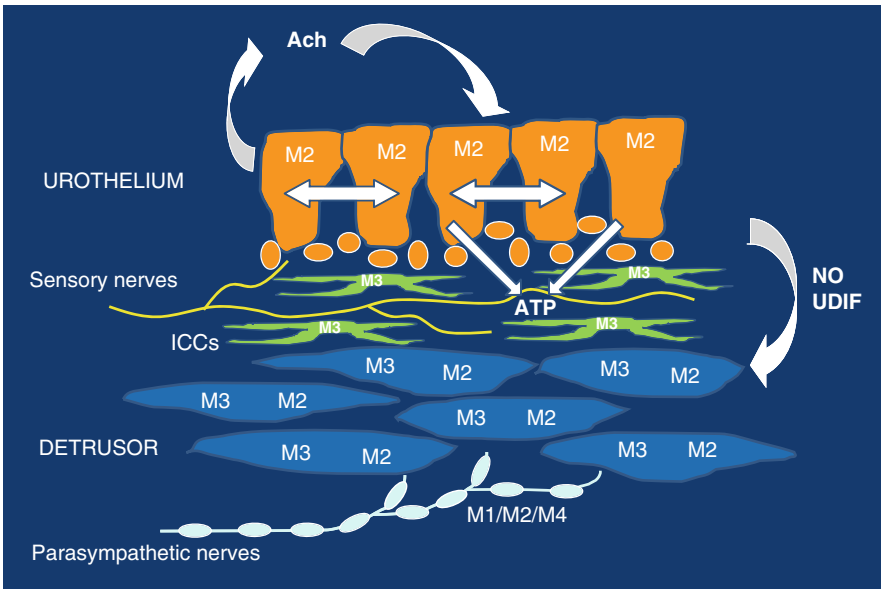

Fig. 1 Diagrammatic representation of the bladder wall. The location of the muscarinic receptor subtypes mediating bladder function is shown, along with factors released from the urothelium/suburothelium. *ACh* acetylcholine, *NO* nitric oxide, *UDIF* unidentified inhibitory/relaxatory factor, *ATP* adenosine triphosphate, *ICCs* interstitial cell of Cajal-like cells

2 Sources of ACh in the Bladder

Both neuronal and non-neuronal sources of ACh exist in the bladder.

2.1 Neuronal Sources of ACh

During micturition powerful bladder contractions are produced when ACh is released from parasympathetic cholinergic nerves and acts on muscarinic receptors in the bladder smooth muscle (de Groat 2006). However, other excitatory transmitters, e.g. ATP, are co-released with ACh and elicit a nonadrenergic, noncholinergic (NANC) mediated contraction of the detrusor (Burnstock 2001). The relative contributions of ACh via muscarinic receptors and NANC to contraction vary greatly between species. In healthy human bladders responses to electrical field stimulation are mediated by ACh and muscarinic receptors only (Sjogren et al. 1982; Sibley 1984; Kinder and Mundy 1985; Mills et al. 2000), but in other species the cholinergic component is usually about 40–60% (Yokota and Yamaguchi 1996; Banks et al. 2005; Fabiyi and Brading 2006). However, the relative importance of ACh and NANC is altered in pathological states. In the human bladder the

contribution of NANC transmission is increased in the overactive bladder (OAB) and in the obstructed bladder (Sjogren et al. 1982; Bayliss et al. 1999). Similar findings in animal models of disease have been reported following spinal cord injury (Somogyi et al. 1998; Yokota and Yamaguchi 1996), bladder cryo-injury (Somogyi et al. 2002) and bladder outlet obstruction (Banks et al. 2005).

ACh release from parasympathetic neurons in the bladder is known to be under complex modulation. Firstly, the released ACh can act back on pre-junctional facilitatory (Somogyi et al. 1994; Inadome et al. 1998) and inhibitory (D'agostino et al. 2000; Inadome et al. 1998; Braverman et al. 1998a) muscarinic receptors on the cholinergic nerve endings to increase or decrease its own release (see below). Furthermore, ACh release can be modulated by a number of other neurotransmitters acting pre-junctionally, including 5-hydroxytryptamine (Sellers et al. 2000; Chapple et al. 2004), nitric oxide (Miyamoto et al. 2001) and noradrenaline (Tobin and Sjogren 1998). It has also been suggested (Zagorodnyuk et al. 2009) that there is a spontaneous, tetrodotoxin-resistant release of ACh from cholinergic nerves during states when there is no parasympathetic outflow from the spinal cord and this may be relevant to detrusor overactivity, where detrusor contractions occur during the storage phase of the micturition cycle.

2.2 Non-neuronal ACh

Non-neuronal sources of ACh have been a focus of intense research in recent years. In the bladder, urothelial cells express the high-affinity choline transporter (CHT1), acetylcholine-synthesising enzymes, choline acetyltransferase (ChAT) and carnitine acetyltransferase (CarAT), but they do not express the vesicular acetylcholine transporter VAChT and the release of ACh is not inhibited by brefeldin-A, which blocks vesicle formation (Hanna-Mitchell et al. 2007). Instead urothelial cells have been shown to express a subtype of polyspecific organic cation transporter (OCT3, Hanna-Mitchell et al. 2007) that may be involved in ACh release from non-neuronal cells (Wessler et al. 2001). This suggests that ACh release from urothelial cells is mediated by a mechanism different to the vesicular storage and exocytosis found in neuronal release of ACh.

Stretch of the urothelium stimulates release of ACh and recent studies have suggested that this non-neuronal ACh is released during bladder filling and exerts paracrine effects on the suburothelial sensory nerves and ICCs and on detrusor muscle. Yoshida et al. (2004, 2006) have also shown basal ACh release from the human urothelium and there appears to be an auto-feedback mechanism via muscarinic receptors to suppress its own release. Thus, atropine enhances ACh-evoked release of ACh from cultured urothelial cells (Hanna-Mitchell et al. 2007). The release of ACh in the bladder wall may play a role in bladder pathology, since Yoshida et al. (2004) reported increased ACh content and release in the bladder mucosa (urothelium/suburothelium) from aged patients, who have an increased incidence of bladder overactivity. In addition, there is evidence to suggest that

muscarinic antagonists act during the storage phase of micturition, when the afferent nerves would normally be silent, suggesting a role for non-neuronal Ach release in bladder dysfunction (Andersson and Yoshida 2003). Thus, during the storage phase, ACh may be released from both neuronal and non-neuronal sources and directly and indirectly influence bladder function by actions at a number of sites where muscarinic receptors are found.

3 Distribution and Function of Bladder Muscarinic Receptors

Muscarinic receptors are found at a number of sites within the detrusor muscle and urothelium and also on the efferent and afferent innervation.

3.1 Muscarinic Receptors on Detrusor Smooth Muscle

Molecular studies of the human bladder have identified equal quantities of mRNA encoding the M_2 and M_3 subtypes, but have been unable to detect any expression of the M_1, M_4 and M_5 subtypes (Yamaguchi et al. 1996). At the protein level, immunoprecipitation studies and radioligand binding studies have confirmed the presence of both M_2- and M_3-receptor subtypes in several species including humans (Wang et al. 1995; Baselli et al. 1999; Yamanishi et al. 2000). However, in all species examined to date, it is the M_2-receptor subtype that predominates at the protein level. In the human bladder immunoprecipitation studies have shown a majority population of M_2 receptors (about 70%) with a smaller M_3-receptor population (about 30%, Wang et al. 1995), while Mansfield et al. (2005), using radioligand binding, found the muscarinic receptor subtypes present in the detrusor to be composed of 70% M_2, 20% M_3 and 10% M_1 receptors. In the pig bladder there is a similar ratio of M_2 and M_3 receptors (Yamanishi et al. 2000), while in the rat, M_3 receptors make up only 10% of the population (Wang et al. 1995).

Surprisingly, although the M_2 receptors predominate in number, it is the minority population of M_3 receptors that mediates direct contractile responses in vitro in all species studied to date including the mouse (Choppin 2002; Canda et al. 2009), rat (Wang et al. 1995; Longhurst et al. 1995), rabbit (Choppin et al. 1998), guinea pig (Noronha-Blob et al. 1989), pig (Sellers et al. 2000) and humans (Chess-Williams 2001; Fetscher et al. 2002). In the detrusor, stimulation of the M_3 receptors activates phosphoinositide hydrolysis leading to inositol triphosphate (IP3) and diacylglycerol (DAG) formation, which causes calcium release from intracellular stores and the influx of extracellular calcium, respectively (Hegde and Eglen 1999). At low acetylcholine concentrations, calcium influx is more important in raising calcium, while at high agonist concentration the release of Ca^{2+} from intracellular stores becomes increasingly important (Masters et al. 1999). Also, further M_3-receptor signalling pathways have been identified in the detrusor

muscle including a Ca^{2+} sensitisation pathway involving the small GTPase Rho and one of its downstream effectors, Rho-associated kinase (ROCK) (Wu et al. 1999; Fry et al. 2002; Schneider et al. 2004; Peters et al. 2006). Activated ROCK phosphorylates the regulatory subunit of myosin light chain phosphatase and inhibits the phosphatase activity, resulting in Ca^{2+} sensitisation of the detrusor smooth muscle to calcium and contraction (Wibberley et al. 2003).

The role of detrusor M_2 receptors has long been questioned, and under normal in vitro conditions, these play no role in detrusor contraction. However, M_2 receptors couple to $G_{i/o}$-proteins and inhibit adenylate cyclase and thus in vitro they may aid bladder contraction by reversing cAMP-induced relaxation. However, other pathways leading to smooth muscle contraction may also occur such as activation of ion channels (non-selective cation channels, K_{ATP} and BK potassium channels and transient receptor channels) and activation of rho kinase (see Chap. X). Furthermore these transduction pathways may interact, which may explain why smooth muscles usually have a mixed M_2 and M_3 population (Ehlert et al. 2005). In vitro, manipulation of the experimental conditions unmasks M_2-receptor-mediated effects in the detrusor (Yamanishi et al. 2000; Hegde et al. 1997) (see later). This suggests that activation of M_2 receptors may 'switch off' sympathetic β-adrenoceptor-mediated relaxation of the detrusor, which would aid urine storage. Such responses involving M_2 receptors have been observed in vivo (Hegde et al. 1997). Gene knock-out studies have also been used to investigate the roles of the muscarinic receptors in bladder contraction. In M_3 knock-out mice bladder contraction is severely defective, being reduced by 95% (Matsui et al. 2002a, b), with the remaining response being completely lost in mice lacking both M_2 and M_3 receptors (Matsui et al. 2004), implicating the M_2 receptor in mediating a small direct contractile response. This is supported by studies on M_2 knock-out mice, in which responses to a muscarinic agonist in vitro were only slightly depressed and the affinity for a M_2-receptor-selective antagonist was reduced (Stengel et al. 2000). Furthermore, in an in vivo study, the M_3 receptor was found to be the dominant receptor, but the lack of the M_2 receptor had a small effect on cystometric parameters (Igawa et al. 2004).

3.2 Pre-junctional Muscarinic Receptors on Nerves

Muscarinic receptors are located on pre-junctional sympathetic and parasympathetic nerve varicosities in the bladder and regulate release of neurotransmitters. Generally M_1 receptors have been found to enhance, while M_2 receptors inhibit, release of ACh (Inadome et al. 1998; see Chess-Williams 2002 for review). However, it is difficult to distinguish between M_2 and M_4 receptors pharmacologically and it has been suggested that the M_4 receptor may be the subtype inhibiting ACh release in humans (D'Agostino et al. 2000). Recently, gene knock-out animals have been used to study the pre-junctional regulation of ACh release. ACh release was found to be similar to wild-type mice in all muscarinic receptor knock-out mice except the mice lacking the M_4 receptor (Takeuchi et al. 2008). Similarly in the

mouse bladder, carbachol has been shown to reduce the release of *noradrenaline* from sympathetic nerves and this response is reduced in M_2 knock-out animals and abolished in M_2/M_4 double knock-out animals (Trendelenburg et al. 2003, 2005). In contrast, carbachol inhibition of transmitter release in mice lacking the M_3 receptor or the M_4 receptor gene was found to be normal, suggesting that M_2 receptors are the relevant subtype regulating noradrenaline release, at least in the mouse bladder (Trendelenburg et al. 2003, 2005). However, responses may be altered in pathological conditions and in rats with spinal cord injuries the enhancement of ACh release is upregulated and the receptor involved appears to change from the M_1- to the M_3-receptor subtype (Somogyi and de Groat 1999).

3.3 Muscarinic Receptors on Urothelium

Muscarinic receptors have been shown to be expressed in the urothelium/suburothelium of rat (Tong et al. 2006) and human bladder (Mansfield et al. 2005; Tyagi et al. 2006), and in the pig bladder are found at a density twice that of the detrusor (Hawthorn et al. 2000). All five muscarinic receptor subtypes are expressed in the urothelium of the mouse (Zarghooni et al. 2007) and human bladder (Bschleipfer et al. 2007), although the M_2 receptor appears to be the most highly expressed in the urothelium/suburothelium of the human bladder body at a mRNA and protein level (Mansfield et al. 2005; Bschleipfer et al. 2007). The distribution of muscarinic receptor subtypes varies within the different layers of the urothelium and in the mouse and human bladder M_2 receptors are found exclusively in the umbrella cells, while M_3, M_4 and M_5 receptors are distributed throughout the urothelium (Bschleipfer et al. 2007; Zarghooni et al. 2007). M_1 receptors are also found in the human urothelium, but they are located exclusively in the basal cells (Bschleipfer et al. 2007).

Activation of these urothelial muscarinic receptors induces the release of a range of factors. In cultured urothelial cells, stimulation of M_1, M_2 and M_3 receptors releases ATP (Kullmann et al. 2008) which is involved in sensory mechanisms of the bladder (Burnstock 2009). Muscarinic agonists also stimulate the production of nitric oxide by the urothelium (Birder 1998) as well as an unidentified factor that inhibits detrusor contraction (Fovaeus 1999; Hawthorn et al. 2000). These receptors and the responses they mediate may be altered in disease, since both M_2 and M_3 receptors were found to be increased in the urothelium of rats with partial bladder outlet obstruction (Kim et al. 2008).

3.4 Muscarinic Receptors on ICC in the Suburothelium

Various terms have been used to describe the cells of the bladder that resemble the ICC in the gastrointestinal tract, including myofibroblasts, interstitial cells,

interstitial cells of Cajal-like (ICC-like) cells and pacemaking cells, but at the Vth International Symposium on ICC in 2007, a recent consensus was reached to term them 'ICCs'. These cells appear to express muscarinic receptors and Mukerji et al. (2006) have shown cellular localisation of M_2 and M_3 receptors in the suburothelium of the human bladder using immunostaining. These authors also showed increased receptor expression in these suburothelial ICCs in painful bladder syndrome and idiopathic detrusor overactivity. Recently, in the guinea pig bladder Grol et al. (2009) showed M_3-receptor immunoreactivity in a dense network of vimentin-positive cells just below the urothelium, i.e. the suburothelial interstitial cells. These cells were more prominent in the lateral wall of the bladder, diminished towards the bladder base and were absent in the bladder–urethral junction. They also showed interstitial cells (vimentin-positive cells) in the bladder–urethral junction that were not immunoreactive for the M_3 receptor. However, a number of recent reviews on the use of muscarinic antibodies have questioned the specificity of these agents (Michel et al. 2009; Pradidarcheep et al. 2008, 2009; Jositsch et al. 2009) and current debate on ICC muscarinic receptor distribution centres on the quality of these experimental tools (Lamers 2009). The subject is further confused by the existence of different types of ICCs that have yet to be fully characterised. At least in the guinea pig ICCs associated with the detrusor muscle respond to cholinergic stimulation by firing calcium transients (Johnston et al. 2008), while those in the suburothelium are unresponsive to muscarinic agonist (Sui et al. 2004). Reliable methods of ICC characterisation therefore need to be developed at the same time as specific antibodies for this field to develop.

3.5 Muscarinic Receptors on Afferent Nerves

It is well known that bladder afferent nerves are responsible for the initiation of the micturition reflex (Andersson 2002). Sensory information from the lower urinary tract is relayed to the spinal cord via primary afferent neurons in the hypogastric and pelvic nerves and are classified according to their response to bladder distension, namely low (<20 mmHg) and high (>20 mmHg) threshold afferents. Small myelinated Aδ fibres (low threshold) respond to changes in wall tension caused by bladder filling during normal micturition, while unmyelinated C-fibres (high threshold) are associated with painful sensations (de Groat 1997) and play a more prominent role in disease (see de Groat and Yoshimura 2010).

Currently the role of anti-muscarinic agents in afferent nerve function is not clear. In vivo studies have found that muscarinic antagonists can increase micturition interval and bladder capacity (Hedlund et al. 2007). Studies using in vivo models, cystometry and urodynamics have assessed this issue indirectly. In a study by de Wachter and Wyndaele (2003) intravesical instillation of oxybutynin into rat bladders was shown to decrease activity of C-fibres, and a similar effect was seen when this agent was administered systemically (de Laet et al. 2006). Tolterodine, administered intravenously or intravesically at low doses, increased bladder

capacity during the storage phase by an effect mediated via C-fibres. Yokoyama et al. (2005) also showed that intravenous and intravesical administration of tolterodine at low doses inhibited C-fibre afferent nerves. In addition, darifenacin reduced bladder afferent activity in both Aδ and C-fibres of the rat bladder in vivo (Iijima et al. 2007). In contrast, Hedlund et al. (2007) have shown that tolterodine increases micturition interval and bladder capacity in normal and resiniferatoxin (RTX)-treated rats, suggesting that RTX-sensitive C-fibres are not involved in the effects of this agent and the results may be explained by the antagonists affecting both Aδ and C-fibres.

Intravesical administration of M_2-selective antagonists suppresses bladder overactivity induced by intravesical administration of oxotremorine in rats (Matsumoto et al. 2010). This suggests that oxotremorine acts to induce bladder overactivity by activating M_2 receptors on the urothelium/suburothelium. However, intravesical administration of the antagonists alone had no effect on voiding frequency. Furthermore, a recent study where afferent nerve activity was recorded directly during bladder filling reported *depressed* activity during muscarinic receptor stimulation (Daly et al. 2010). Clinically Boy et al. (2007) reported that oral tolterodine significantly increased the perception threshold to intravesical electrical stimulation in healthy women, but there was no effect on subjective bladder sensations during cystometry. Thus, the effect of anti-muscarinics on afferent nerves still requires further investigation, and it is possible that muscarinic pathways may only be involved in pathological conditions.

4 Experimental Studies of Muscarinic Receptors in the Bladder

Studies on muscarinic receptors in the bladder have been hampered by the lack of receptor subtype selective agents, both agonists and antagonists. Functional studies have mostly made use of the relative selectivity of the available antagonists to characterise the muscarinic receptors of the bladder. Table 1 shows the affinities of the most commonly used antagonists in experimental studies.

Table 1 Affinities of experimentally used selective antagonists

Antagonist	M_1	M_2	M_3	M_4	M_5	Pig bladder	Human bladder
Pirenzapine	8.0[a]	6.3[a]	6.8[a]	7.0[a]	6.9[a]	6.8[b]	6.7[c]
Methoctramine	7.2[d]	8.2[d]	6.3[d]	7.1[d]	6.3[a]	6.1[b]	6.0[c]
4-DAMP	9.1[e]	8.0[e]	9.0[e]	8.9[e]	8.7[e]	9.4[b]	9.8[c]
Tropicamide	7.3[d]	7.3[d]	7.3[d]	7.8[d]	?	?	?

Values for M_1–M_5 are for radioligand binding data to human receptors expressed in CHO cells
[a]Hegde et al. (1997)
[b]Lazareno and Birdsall (1993)
[c]Wuest et al. (2006)
[d]Sellers et al. (2000)
[e]Stevens et al. (2007)

Pirenzepine has a relatively high affinity at M_1 receptors and is tenfold selective for this subtype over the other receptors. On pig and human bladder, pirenzepine has a relatively low affinity (Table 1) ruling out any involvement of M_1 receptors in mediating contractile responses. To identify the role of the majority M_2-receptor population, methoctramine has been employed. This antagonist has an affinity tenfold greater at M_2 receptors than at the other receptor subtypes, but on the detrusor it also has a relatively low affinity (Table 1). In contrast, 4-DAMP has an affinity tenfold lower at M_2 than at the M_3 receptor. At detrusor muscarinic receptors 4-DAMP has a high affinity indicating the involvement of M_3, M_4 or M_5 receptors in contraction. Furthermore, the Schild plots for antagonists on detrusor muscle have slopes of unity, indicating that only one receptor is involved in mediating responses (Sellers et al. 2000; Chess-Williams et al. 2001). It is difficult to distinguish pharmacologically between the M_3- and M_5-receptor subtypes, but generally antagonist affinity values correlate best with the M_3 receptor (Sellers et al. 2000; Chess-Williams et al. 2001) and no mRNA for the M_4 or M_5 receptor could be detected in human bladder (Yamaguchi et al. 1996) indicating that the M_3-receptor subtype is the important receptor mediating detrusor contraction. Since the first report that tropicamide was a selective M_4 antagonist, it has been used in many studies as an M_4-selective tool with a pK_i value of 7.8 at this receptor subtype (Lazareno et al. 1990). However, as seen in Table 1, this antagonist is only very slightly selective for the M_4 receptor over the other subtypes and no suitable high-affinity antagonist with high selectivity for the M_4 or M_5 receptor is currently available.

Several muscarinic agonists have been used in functional studies of the muscarinic receptors of the bladder. Carbachol, or carbamylcholine, is a non-specific cholinergic agonist that acts at both muscarinic and nicotinic receptors. It is commonly used experimentally in in vitro investigations, since it is not easily metabolised by the endogenous cholinesterase enzymes. However, it is not selective for any of the five muscarinic subtypes. (+)-cis-dioxolane is a high-affinity muscarinic agonist, which is also not specific for any of the muscarinic receptor subtypes, while oxotremorine methiodide (oxotremorine-M) is a mixed action cholinergic agonist, originally thought to be a pure muscarinic agonist, but it has been shown to act at nicotinic receptors in xenopus myocytes (Reitstetter et al. 1994). Arecaidine is another potent muscarinic receptor agonist which is thought to have higher selectivity for muscarinic receptors over nicotinic receptors, and has been used in functional studies of bladder for this reason (Gillespie et al. 2003; Finney et al. 2007). It also shows some selectivity for cardiac versus ileal M_2 receptors (Barlow and Weston-Smith 1985).

In the pig and human bladder carbachol acts via the M_3 receptor to evoke contraction (Sellers et al. 2000; Chess-Williams et al. 2001; Fetscher et al. 2002). 4-DAMP and darifenacin antagonise carbachol-induced contraction of pig bladder with high affinity (pK_B values of 9.4 and 8.6), while oxybutynin, tolterodine and pirenzepine show affinities of 8.2, 8.1 and 6.8. The M_2-selective agent methoctramine has a relatively low affinity (pK_B 6.1, Sellers et al. 2000).

Similarly in the human bladder pirenzepine and methoctramine antagonise carbachol-induced contractions with low affinity (pK_B 6.8 and 6.9), while the M_3-selective antagonist 4-DAMP shows high affinity (9.5, Chess-Williams et al. 2001). There appears to be little difference in potency of the muscarinic agonists in inducing bladder contraction, since in the mouse bladder, oxotremorine has potency similar to carbachol, acetylcholine and (+)-cis-dioxolane (Choppin and Eglen 2001a). In addition antagonist affinities appear similar regardless of the contracting agonist used (Choppin and Eglen 2001a).

These agents have also been used in pharmacological manipulations of the muscarinic receptor population of the bladder. In particular it has been found that carbachol can contract the bladder under conditions where M_3 receptors have been selectively inactivated by the alkylating agent 4-DAMP mustard and cAMP levels elevated, in both the rat (Hegde et al. 1997) and pig (Yamanishi et al. 2000). In pig bladder tissues under these conditions the affinity of 4-DAMP at reducing carbachol-induced contractions is significantly reduced to 8.5, but that of methoctramine is significantly increased to 6.5, suggesting the involvement of M_2 receptors in contraction (Yamanishi et al. 2000).

Muscarinic agonists and antagonists have also been used experimentally to study the role of muscarinic receptors in bladder dysfunction. The sensitivity and responsiveness of the detrusor muscle to muscarinic agonists have been shown to be increased in several pathological conditions of the bladder including neurogenic OAB (Saito et al. 1993; Stevens et al. 2007), bladder outlet obstruction (Harrison et al. 1987) and denervation, spinal cord injury and diabetic-induced detrusor overactivity in rats (Gunasena et al. 1995; Braverman et al. 1998b; Stevens et al. 2006). There may also be changes in receptor subtype function in bladder disorders, and muscarinic antagonists have been used to determine the pharmacological profile of the key muscarinic receptors mediating detrusor contractions in the diseased bladder. In neurogenic OAB the affinity of darifenacin (M_3 selective) in antagonising carbachol-evoked contractions is reduced to around 7.6 compared to 7.9–8.9 in the normal bladder, while the affinity of methoctramine increases compared to normal bladders (Pontari et al. 2004), suggesting a role for M_2 in bladder contraction. Similar findings have been observed in detrusor strips from rat models of bladder outlet obstruction (Ruggieri and Braverman 2006) and denervation (Braverman et al. 2006). However, an in-depth study, using full Schild analysis of several selective antagonists, could find no difference between samples of bladders from idiopathic and neurogenic OAB patients compared with controls (Stevens et al. 2007) These authors showed that carbachol-induced responses of detrusor muscle were antagonised with high affinitiy by 4-DAMP and darifenacin, and with low affinity by methoctramine and pirenzapine, showing that the M_3 receptor is solely responsible for mediating contraction in bladder pathology. Krichevsky et al. (1999) also demonstrated that in obstructed rat bladder, M_3 receptors continue to play the dominant role in mediating detrusor contraction. The role for M_2 receptors in human bladder pathology is therefore still unclear.

5 Clinical Use of Muscarinic Agonists and Antagonists

5.1 Agonists

Detrusor underactivity is an underdiagnosed condition defined as reduced detrusor contractility that results in prolonged voiding and/or failure to achieve complete bladder emptying within a normal time span, and can lead to urinary retention (Abrams et al. 2003). Bladder contractility is reduced with old age, with 22% of men and 11% of women aged 60 and older reporting difficulty in bladder emptying (Diokno et al. 1986). However, detrusor underactivity can occur in men of all ages (Abrams et al. 1981), and the symptoms are indistinguishable from bladder outlet obstruction, such as that seen in benign prostatic hyperplasia. This condition has received little clinical and research attention, and is a challenge to diagnose because symptoms are non-specific (Abrams et al. 2003).

Bethanechol (Urecholine®) is a cholinergic agonist that has commonly been prescribed for treatment of urinary retention. Although it has been used to treat hypocontractile or underactive detrusor, most studies have shown little clinical benefit (Wein et al. 1980; Awad 1985). If administered subcutaneously at high doses, bethanechol can favourably alter bladder performance, although in randomised, placebo-controlled trials it does not appear to improve clinically relevant outcomes in detrusor underactivity (Finkbeiner 1985). In a recent review (Krishnamoorthy and Kekre 2009) it was concluded that the data currently available suggest that using bethanechol in patients with detrusor underactivity offers no definite clinical benefit. It has been suggested (Taylor and Kuchel 2006) that this may be due to the fact that in detrusor underactivity there is muscle cell degeneration as well as axonal degeneration (Elbadawi et al. 1993). One condition where bethanechol has been shown to be of benefit is in the treatment of patients undergoing radical hysterectomy who experience impaired detrusor function (Madeiro et al. 2006).

Another confounding factor is that detrusor underactivity is also often associated with detrusor overactivity, termed detrusor overactivity with impaired contractility (DO-IC). About 17% of all men with lower urinary tract symptoms such as urgency due to bladder outlet obstruction also have detrusor underactivity (Abrams et al. 1981). This mixed condition is even more challenging to treat, since the use of muscarinic antagonists to treat LUTS may be expected to worsen the urinary retention. However, in spite of this several studies have suggested that muscarinic antagonists may be safe to use in this condition, and solifenacin (Ronchi et al. 2009) and immediate release oxybutynin (Miller et al. 2002) have been shown to result in few voiding difficulties and a low incidence of acute urinary retention.

5.2 Antagonists

Disorders of the lower urinary tract manifest as either storage symptoms or voiding symptoms. Urinary incontinence is a disorder of storage in which there is an

involuntary loss of urine from the bladder during the filling phase (Abrams et al. 2010). It can be further classified depending on symptoms that may include urgency incontinence, stress incontinence, mixed incontinence and nocturnal enuresis (loss of urine during sleep). OAB syndrome is a symptom complex characterised by urgency, with or without urgency incontinence, usually with frequency and nocturia (Abrams et al. 2010). Urodynamically, OAB is characterised by involuntary contractions of the detrusor muscle during the filling phase, and can be idiopathic or neurogenic in origin. OAB is a common and distressing condition that has a significant impact on the quality of life (Tubaro 2004), and has a prevalence of between 12 and 17% in Europe and the United States (Stewart et al. 2003; Irwin et al. 2006). Incontinence is present in around one third of cases and prevalence increases with age (Reilly et al. 2006). The first line in pharmacotherapy for OAB syndrome and urinary incontinence is a muscarinic antagonist.

Six muscarinic antagonists are currently marketed for treatment of OAB syndrome and urinary incontinence: propiverine, oxybutynin, tolterodine, trospium, darifenacin and solifenacin. Of the more established agents, oxybutynin, trospium and tolterodine are used worldwide, while propiverine is available only in the UK. Darifenacin and solfenacin are the more recently introduced agents to the market (Abrams and Andersson 2007). In addition, a newly licensed pro-drug, fesoterodine, has been shown to be effective in reducing symptoms of OAB syndrome (Khullar et al. 2008), probably acting via its metabolite (Michel and Hegde 2006). Imidafenacin is also a newly developed muscarinic antagonist that has been approved for treatment of OAB and is marketed in Japan. These muscarinic antagonists differ in structure, muscarinic receptor subtype selectivity and bladder selectivity and therefore adverse effect profile. Although they remain the first line in pharmacotherapy for OAB syndrome and urinary incontinence, their use is hindered by adverse effects, particularly dry mouth, constipation, headache and blurred vision. These adverse effects are caused by actions at muscarinic receptors in the salivary glands, gastrointestinal smooth muscle and ciliary and iris sphincter muscles, particularly the M_3 subtype (Hegde et al. 2004). Of these adverse effects dry mouth is by far the most common. Recently there have been concerns over cardiovascular side effects, such as increased heart rate, QT interval prolongation and induction of ventricular tachycardia (torsade de pointes), although there appears to be little evidence to support this (Abrams and Andersson 2007). In addition, use of muscarinic antagonists may impair detrusor contractility and cause urinary retention, especially in men with bladder outlet obstruction due to benign prostatic hyperplasia. However, again, there is scant evidence of this, and indeed muscarinic antagonists can be combined with α1-adrenoceptor antagonists to treat men with bladder overactivity and outlet obstruction, which may be more effective than with α1-antagonists alone (Athanasopoulos et al. 2003). Kaplan et al. (2006) reported a low incidence (0.4%) of acute retention in patients taking tolterodine with tamsulosin, although this was greater than that seen in patients taking placebo or tamsulosin alone (both 0%). Furthermore, two recent reviews of the literature have concluded that muscarinic antagonists are safe to use in BPH patients. The antagonists had no effect on maximum urinary flow rates and importantly rates of acute urinary retention were low (Blake-James et al. 2007; Reynard 2004).

Table 2 Affinities of muscarinic receptor antagonists in clinical use

Antagonist	M_1	M_2	M_3	M_4	M_5
Tolterodine	7.8[a]	8.0[a]	8.3[a]	8.6[a]	8.6[a]
Darifenacin	7.2[a]	7.0[a]	8.4[a]	7.5[a]	7.6[a]
Solifenacin	7.6[b]	6.8[b]	7.9[b]	7.0[b]	7.5[b]
Oxybutynin	8.6[c]	8.2[c]	9.2[c]	8.7[c]	8.0[c]
Propiverine	6.6[d]	5.8[d]	6.4[d]	6.5[d]	6.4[d]
Trospium	8.5[a]	8.9[a]	9.0[a]	8.8[a]	8.2[a]
Fesoterodine	8.0[e]	7.7[e]	7.4[e]	7.3[e]	7.5[e]
Imidafenacin	8.1[f]	7.6[f]	8.8[f]	8.0[f]	8.6[f]

All values are from radioligand binding data to human receptors expressed in CHO cells
[a]Mansfield et al. (2009)
[b]Ohtake et al. (2007)
[c]Nilvebrant et al. (1997)
[d]Wuest et al. (2006)
[e]Ney et al. (2008)
[f]Kobayashi et al. (2007)

The newly developed agents such as darifenacin and solifenacin seem to be better tolerated clinically and this may be related to their selectivity for the M_3 receptor, the main receptor involved in bladder function. Table 2 shows the affinities of the clinically used muscarinic antagonists at the five muscarinic receptor subtypes. All the antagonists have a relatively high affinity at the M_3 receptor, with oxybutynin and trospium having the greatest values. Selectivity for the M_3 receptor however varies between the antagonists with tolterodine, trospium, fesoterodine and propiverine being non-selective, oxybutynin and solifenacin displaying moderate selectivity whilst darifenacin displays the greatest selectivity for this receptor over the M_2 receptor. Imidafenacin has high affinity for both the M1 and M3 receptor.

In addition to their action at the muscarinic receptor subtypes some of these agents are known to have additional actions, which may contribute to their effects on the bladder. In particular, propiverine and oxybutynin have anti-spasmolytic effects and are thought to act as calcium antagonists (reviewed by Andersson et al. 1999).

Although the selectivity of muscarinic antagonists for the M_3 receptor is key, it has become recognised that tissue selectivity may be just as important for limiting adverse effects. Some of the least selective agents such as oxybutynin and tolterodine still prove effective in reducing symptoms of OAB syndrome and urinary incontinence. Tolterodine and solifenacin have been shown to be more selective for the bladder compared with the salivary glands (Nilvebrant et al. 1997; Ikeda et al. 2002), and imidafenacin is even more bladder selective over salivary gland (15 fold) than both of these (solifenacin 1.7 fold, tolterodine 2.5 fold) (Yamazaki et al. 2011). There is conflicting data over the tissue selectivity of oxybutynin, darifenacin and propiverine, and this is confounded by the data being available only from animal studies, and no data appear to be available for trospium (see Abrams and Andersson 2007).

Another factor that has had an impact on clinical outcomes for muscarinic antagonists is the development of innovative drug delivery systems. Transdermal

sustained release formulations of oxybutynin avoid first-pass metabolism and so result in lower concentrations of the active metabolite, *N*-desethyloxybutynin. This metabolite has greater affinity for the muscarinic receptors of the parotid gland and trials have shown 94% versus 38% incidence of dry mouth with immediate release versus transdermal oxybutynin. Extended transdermal release (ER) of oxybutynin in a recent large-scale trial (MATRIX) was well tolerated with a few incidences of side effects apart from some skin reactions (Sand et al. 2007). Oxybutynin is available in multiple immediate and extended release oral formulations and two transdermal formulations. Oxybutynin topical gel (OTG) was approved in the USA in 2009 (Gelnique) and designed to provide consistent plasma oxybutynin levels with daily application, with fewer side effects than the transdermal patches, and appears to be a safe, efficacious and convenient alternative to other oxybuytnin formulations (see Staskin and Robinson 2009 for review). Extended release tolterodine has also been shown to improve overactive symptoms in men, with few adverse effects (Kaplan et al. 2006), as did controlled release darifenacin (Hill et al. 2006). Another innovative approach is double muscarinic antagonist therapy for treatment of refractory OAB. Bolduc et al. (2009) have shown combination therapy with oxybutynin, tolterodine and/or solifenacin to improve continence in children with refractory OAB.

There have been few comparative studies of the new muscarinic antagonists against the more established ones. In a meta-analysis of 83 randomised controlled trials of licensed muscarinic antagonists all appeared to be similar in terms of efficacy, although in some cases higher doses of propiverine, fesoterodine and solifenacin seemed to be more efficacious (Chapple et al. 2008). Dry mouth was the most commonly reported adverse effect with all agents (29.6% versus 7.9% on placebo); however, the newer agents and extended delivery systems seemed better tolerated. It was concluded that these agents are efficacious, safe and well tolerated. In another meta-analysis of 50 randomised controlled trials tolterodine immediate release had a more favourable profile for adverse effects than oxybutynin immediate release (Novara et al. 2008). Higher doses of the agents did offer some improvements in efficacy, but at the cost of increased adverse effects, while extended release formulations showed some advantages over immediate release in terms of efficacy and safety. Transdermal administration did not provide any advantages over oral routes in this meta-analysis (Novara et al. 2008). Interestingly in a meta-analysis of the placebo response in trials of muscarinic antagonists for OAB, the placebo response was found to be substantial and highly heterogenous (Lee et al. 2009) highlighting the difficulties in undertaking these clinical urological studies.

5.3 Muscarinic Antagonist Efficacy and Adverse Effects in the Elderly

The prevalence of OAB increases with age and one important question therefore is whether efficacy or side-effect profile is different in the elderly compared to

younger patients. Griebling et al. (2009) compared the effects of tolterodine ER in elderly patients (>75 years) and younger (<65 years) patients with bladder overactivity. Discontinuation rates relative to placebo were similar in the two groups. Generally the occurrence of anti-cholinergic symptoms was low (<5%) and there was no significant difference between the age groups. Dry mouth was the only significant adverse effect and this was consistent between the older (17%) and younger (16%) group. Thus, the effectiveness and side-effect profile of tolterodine ER are independent of age. Similar findings have been reported for solifenacin (Wagg et al. 2006) and darifenacin (Foote et al. 2005), with treatment resulting in significant improvement in urinary symptoms and the incidence of side effects being low in both young and older populations.

5.4 Cognitive Side Effects of Muscarinic Antagonists

A substantial proportion of OAB patients are elderly and therefore at risk of CNS impairment, particularly when administered muscarinic antagonists. Muscarinic receptors within the central nervous system are involved in many processes including learning, memory and cognition. The receptor involved appears to be predominantly the M_1 subtype, but with M_2 receptors also involved. Thus, mice lacking the M_1 receptor (Anagnostara et al. 2003; Matsui et al. 2004) exhibit cognitive deficits and in mutant mice lacking the M_2 receptor (Seeger et al. 2004) memory is impaired, while mice lacking the M_3-receptor protein exhibit normal cognition (Yamada et al. 2001). In addition to the cognitive decrease, urinary incontinence is a substantial symptom in patients with dementia and may be considered a form of neurogenic overactivity. In a study of Alzheimer's patients 58% were found to have OAB and 6% to have low compliance bladders (Mori et al. 1999). In patients with vascular dementia, these figures were 91% and 9%, respectively. Two questions arise. Firstly, are muscarinic antagonists as effective in treating OAB symptoms in these dementia patients as they are in the rest of the population, and secondly, are muscarinic antagonists safe to use in patients with cognitive impairment? Kay and Ebinger (2008) have reviewed the literature on darifenacin and concluded that this drug is indeed effective and also has no effect on memory. Another study reported that propiverine was effective in dementia patients and that this was independent of the cause of dementia (Mori et al. 1999).

5.5 Cholinesterase Inhibitors and the Bladder

Cholinesterase inhibitors are used in patients with Alzheimer's disease to elevate ACh levels in the central nervous system. However, adverse effects on the bladder might be predicted since ACh levels may also be enhanced within the bladder. Sakakibara et al. (2005) have investigated the effects of donepezil on urinary

function using questionnaires and cystometry. Although only eight dementia patients were studied (seven had neurogenic bladder overactivity) treatment with donepezil did not worsen any urinary symptoms. During detrusor overactivity there was an increase in detrusor pressure, but mean bladder capacity was increased while taking the drug. Also, one previously incontinent patient became continent. Larger clinical trials are now required to confirm these unexpected findings.

The dual treatment of dementia patients with OAB has also been studied, where muscarinic antagonists for OAB are administered concomitantly with cholinesterase inhibitors for dementia. Sink et al. (2008) reported a greater decline in cognitive function and daily activity when patients were taking muscarinic antagonists (oxybutynin or tolterodine). Although this was only observed in patients who were starting the study with a high initial cognitive function, it does highlight a possible risk that needs further investigation, particularly when the high number of dementia patients with bladder overactivity is considered.

6 Summary

There are several potential sites of action for muscarinic antagonists administered for the treatment of bladder overactivity. For most of the drugs used clinically, their actions at sites other than the detrusor muscle have not been investigated. Table 3, however, summarises the key factors that may influence the actions of a muscarinic antagonist in the treatment of bladder overactivity.

Table 3 Summary of actions of muscarinic antagonists used in the treatment of bladder overactivity

Antagonist	M_3 affinity	M_3:M_2 selectivity	Direct actions on muscle/nerve	Pre-junctional actions	Effects on cognition/memory
Tolterodine	8.3	2-fold	N[a]	Y[b]	Y[c,d]
Darifenacin	8.4	25-fold	?	Y[b]	N[d,e]
Solifenacin	7.9	13-fold	Y[f]	Y[f]	?
Oxybutynin	9.2	10-fold	Y[f]	Y[b,f]	Y[b,d,e]
Propiverine	6.4	4-fold	Y[f]	N[f]	N[b]
Trospium	9.0	None	?	?	N[d]
Fesoterodine	7.3	0.5-fold	?	Y[h]	?
Imidafenacin	8.8	16-fold	Y[b]	Y[b]	N[g]

M_3-receptor affinities and M_3:M_2 selectivities have been derived from affinity values in Table 2
[a]de Wachter and Wyndaele (2003)
[b]Kobayashi et al. (2007a)
[c]Womack and Heilman (2003)
[d]Staskin (2005)
[e]Kay and Ebinger (2008)
[f]Masunaga et al. (2008)
[g]Kobayashi et al. (2007b)
[h]Ney et al. (2008)

Affinity at the M_3 receptors is important as this receptor is the main functional receptor in the bladder. A high-affinity at M_3 receptors suggests a high potency when antagonising contractile responses of the detrusor smooth muscle. In terms of M_2:M_3 selectivity, the greater the selectivity for M_3 receptors the less effect the drug will have on cardiac M_2 receptors. This is important since the main use of these drugs is in elderly patients. Direct inhibitory actions on detrusor muscle or nerves may also contribute to the inhibitory effects of the antagonist on the bladder. In theory this is a useful property, as it will result in depression of detrusor responses mediated via ACh and also NANC transmitters such as ATP. This is particularly relevant as NANC neurotransmission is thought to enhance and contribute a greater component of the contractile response in the diseased bladder (Sjogren et al. 1982; Bayliss et al. 1999). Pre-junctional effects are also of consideration since most of the antagonists have a high affinity at M_1 receptors, which may reduce ACh release from cholinergic nerves innervating the bladder detrusor and thus depress responses, while drugs with a high affinity at M_2 receptors may cause an increase in transmitter release and enhanced contraction to nerve stimulation. As can be seen in the table, where pre-junctional effects have been noted for these antagonists, the effect has always been a reduction in ACh release, suggesting that M_1 receptors predominate at the pre-junctional level in the bladder. Thus, pre-junctional effects for these antagonists will aid their depressant action on the detrusor. In terms of depressed cognition, OAB is more common in the elderly (Reilly et al. 2006) and in dementia patients (Mori et al. 1999). It is therefore important in these patients that treatment of bladder conditions does not exert CNS actions, particularly effects on memory and cognition. Thus, the main therapeutic use for muscarinic agents on the bladder is the treatment of overactivity. The drugs are effective but the precise mechanisms of action have yet to be determined and may involve a range of actions at multiple sites in the bladder including the muscle, urothelium and autonomic innervation.

References

Abrams P, Andersson KE (2007) Muscarinic receptor antagonists for overactive bladder. BJU Int 100:987–1006

Abrams P, Shah PJR, Feneley RCL (1981) Voiding disorders in the young male adult. Urology 18:107–113

Abrams P, Cardozo L, Fall M et al (2003) The standardisation of terminology of lower urinary tract function: report from the standardisation sub-committee of the International Continence Society. Urology 61:37–49

Abrams P, Andersson KE, Birder L, Brubaker L, Cardozo L, Chapple C et al (2010) Fourth International Consultation on Incontinence Recommendations of the International Scientific Committee: evaluation and treatment of urinary incontinence, pelvic organ prolapse, and fecal incontinence. Neurourol Urodyn 29:213–240

Anagnostara SG, Murphy GG, Hamilton SE, Mitchell SL, Rahnama NP, Nathanson NM, Silva AJ (2003) Selective cognition dysfunction in acetylcholine m_1 muscarinic receptor mutant mice. Nat Neurosci 6:51–58

Andersson KE (2002) Bladder activation: afferent mechanisms. Urology 59(5 Suppl 1):43–50

Andersson KE, Yoshida M (2003) Antimuscarinics and the overactive detrusor – which is the main mechanism of action? Eur Urol 43:1–5

Andersson KE, Appell R, Cardozo LD, Chapple C, Drutz HP, Finkbeiner AE, Haab F, Vela Navarrete R (1999) The pharmacological treatment of urinary incontinence. BJU Int 84(9):923–947

Athanasopoulos A, Gyftopoulos K, Giannitsas K, Fisfis J, Perimenis P, Barbalias G (2003) Combination treatment with an alpha-blocker plus an anticholinergic for bladder outlet obstruction: a prospective, randomized, controlled study. J Urol 169(6):2253–2256

Awad S (1985) Clinical use of bethanechol. J Urol 134:523–524

Banks FCL, Knight GE, Calvert RC, Morgan RJ, Burnstock G (2005) Alterations in purinergic and cholinergic components of contractile responses of isolated detrusor contraction in a rat model of partial bladder outlet obstruction. BJU Int 97:372–378

Barlow RB, Weston-Smith P (1985) The relative potencies of some agonists at M_2 muscarinic receptors in guinea pig ileum, atria and bronchi. Br J Pharmacol 85:437–440

Baselli EC, Brandes SB, Luthin GR, Ruggieri MR (1999) The effect of pregnancy and contractile activity on bladder muscarinic receptor subtypes. Neurourol Urodyn 18(5):511–520

Bayliss M, Wu C, Newgreen AR, Mundy CH, Fry CH (1999) A quantitative study of atropine-resistant contractile responses in human bladder detrusor smooth muscle, from stable, unstable and obstructed bladders. J Urol 162:1833–1839

Birder LA (2010) Urothelial signaling. Auton Neurosci 153(1–2):33–40

Birder LA, Apodaca G, de Groat WC, Kanai AJ (1998) Adrenergic- and capsaicin-evoked nitric oxide release from urothelium and afferent nerves in urinary bladder. Am J Physiol 275(2 Pt 2): F226–F229

Blake-James BT, Rashidian A, Ikeda Y, Emberton M (2007) The role of anticholinergics in men with lower urinary tract symptoms suggestive of benign prostatic hyperplasia: a systematic review and meta-analysis. BJU Int 99(1):85–96

Bolduc S, Moore K, Lebel S, Lamontagne P, Hamel M (2009) Double anticholinergic therapy for refractory overactive bladder. J Urol 182(4 Suppl):2033–2038

Boy S, Schurch B, Mehnert U, Mehring G, Karsenty G, Reitz A (2007) The effects of tolterodine on bladder-filling sensations and perception thresholds to intravesical electrical stimulation: method and initial results. BJU Int 100(3):574–578

Braverman AS, Kohn IJ, Luthin GR, Ruggieri MR (1998a) Prejunctional M_1 facilitory and M_2 inhibitory muscarinic receptors mediate rat bladder contractility. Am J Physiol 274: R517–R523

Braverman AS, Luthin GR, Ruggieri MR (1998b) M_2 muscarinic receptor contributes to contraction of the denervated rat urinary bladder. Am J Physiol Regul Integr Comp Physiol 275: R1654–R1660

Braverman AS, Doumanian LR, Ruggieri MR (2006b) M_2 and M_3 muscarinic receptor activation of urinary bladder contractile signal transduction. II. Denervated rat bladder. J Pharmacol Exp Ther 316:875–880

Bschleipfer T, Schukowski K, Weidner W, Grando SA, Schwantes U, Kummer W, Lips KS (2007) Expression and distribution of cholinergic receptors in the human urothelium. Life Sci 80:2303–2307

Burnstock G (2001) Purinergic signalling in the lower urinary tract. In: Abbracchio MP, Williams M (eds) Handbook of experimental pharmacology. Springer, Berlin, pp 423–515

Burnstock G (2009) Purinergic cotransmission. Exp Physiol 94(1):20–24

Canda AE, Chapple CR, Chess-Williams R (2009) Pharmacologic responses of the mouse urinary bladder. Cent Eur J Med 4(2):192–197

Chancellor MB, Kaplan SA, Blavias JG (1992) The cholinergic and purinergic components of detrusor contractility in a whole rabbit bladder model. J Urol 148:906–909

Chapple CR, Radley SC, Martin SW, Sellers DJ, Chess-Williams R (2004) Serotonin-induced potentiation of cholinergic responses to electrical field stimulation in normal and neurogenic overactive human detrusor muscle. BJU Int 93(4):599–604

Chapple CR, Khullar V, Gabriel Z, Muston D, Bitoun CE, Weinstein D (2008) The effects of antimuscarinic treatments in overactive bladder: an update of a systematic review and meta-analysis. Eur Urol 54:543–562

Chess-Williams R (2002) Muscarinic receptors of the bladder: detrusor, urothelial and prejunctional. Auton Autocoid Pharmacol 22(3):133–145

Chess-Williams R, Chapple CR, Yamanishi T, Sellers DJ (2001) The minor population of M_3 receptors mediate contraction of human detrusor muscle in vitro. J Auton Pharmacol 21(5–6): 243–248

Choppin A (2002) Muscarinic receptors in isolated urinary bladder smooth muscle from different mouse strains. Br J Pharmacol 137(4):522–528

Choppin A, Eglen RM (2001a) Pharmacological characterization of muscarinic receptors in mouse isolated urinary bladder smooth muscle. Br J Pharmacol 133(7):1035–1040

Choppin A, Eglen RM (2001b) Pharmacological characterization of muscarinic receptors in dog ciliary and urinary bladder smooth muscle. Br J Pharmacol 132:835–842

Choppin A, Eglen RM, Hedge SS (1998) Pharmacological characterization of muscarinic receptors in rabbit isolated iris sphincter muscle and urinary bladder smooth muscle. Br J Pharmacol 124:883–888

D'Agostino G, Bolognesi ML, Lucchelli A, Vicini D, Balestra B, Spelta V, Melchiorre C, Tonini M (2000) Prejunctional muscarinic inhibitory control of acetylcholine release in the human isolated detrusor: involvement of the M_4 receptor subtype. Br J Pharmacol 129:493–500

Daly DM, Chess-Williams R, Chapple C, Grundy D (2010) The inhibitory role of acetylcholine and muscarinic receptors in bladder afferent activity. Eur Urol 58(1):22–28

de Groat C (1997) A neurologic basis for the overactive bladder. Urology 50 (6A Suppl):36–52; discussion 53–56

de Groat WC (2006) Integrative control of the lower urinary tract: preclinical perspective. Br J Pharmacol 147:S25–S40

de Groat WC, Yoshimura N (2010) Changes in afferent activity after spinal cord injury. Neurourol Urodyn 29(1):63–76

De Laet K, De Wachter S, Wyndaele JJ (2006) Systemic oxybutynin decreases afferent activity of the pelvic nerve of the rat: new insights into the working mechanism of antimuscarinics. Neurourol Urodyn 25:156–161

De Wachter S, Wyndaele JJ (2003) Intravesical oxybutynin: a local anesthetic effect on bladder C afferents. J Urol 169(5):1892–1895

Diokno AC, Brock BM, Brown MB et al (1986) Prevalence of urinary incontinence and other urological symptoms in the non-institutionalized elderly. J Urol 136:1022–1025

Ehlert FJ, Griffin MT, Abe DM, Vo TH, Taketo MM, Manabe T, Matsui M (2005) The M_2 muscarinic receptor mediates contraction through indirect mechanisms in mouse urinary bladder. J Pharmacol Exp Ther 313(1):368–378

Elbadawi A, Yalla SV, Resnick NM (1993) Structural basis of geriatric voiding dysfunction. II Aging detrusor: normal versus impaired contractility. J Urol 150:1657–1667

Fabiyi AC, Brading AF (2006) The use of the isolated mouse whole bladder for investigating bladder overactivity. J Pharmacol Exp Ther 319(3):1386–1394

Ferguson DR, Kennedy I, Burton TJ (1997) ATP is released from rabbit urinary bladder epithelium cells by hydrostatic pressure changes: a possible sensory mechanism? J Physiol 505:503–511

Fetscher C, Fleichman M, Schmidt M, Krege S, Michel MC (2002) M_3 muscarinic receptors mediate contraction of human urinary bladder. Br J Pharmacol 136:641–643

Finkbeiner AE (1985) Is bethanechol chloride clinically effective in promoting bladder emptying? A literature review. J Urol 134:443–449

Finney SM, Stewart LH, Gillespie JI (2007) Cholinergic activation of phasic activity in the isolated bladder: possible evidence for M_3- and M_2-dependent components of a motor/sensory system. BJU Int 100(3):668–678

Foote J, Glavind K, Kralidis G, Wyndaele JJ (2005) Treatment of overactive bladder in the older patient: pooled analysis of three phase III studies of darifenacin, an M_3 selective receptor antagonist. Eur Urol 48(3):471–477

Fovaeus M, Fujiwara M, Högestätt ED, Persson K, Andersson KE (1999) A non-nitrergic smooth muscle relaxant factor released from rat urinary bladder by muscarinic receptor stimulation. J Urol 161(2):649–653

Fry CH, Skennerton D, Wood D, Wu C (2002) The cellular basis of contraction in human detrusor smooth muscle from patients with stable and unstable bladders. Urology 59:3–12

Gillespie JI, Harvey IJ, Drake MJ (2003) Agonist- and nerve-induced phasic activity in the isolated whole bladder of the guinea pig: evidence for two types of bladder activity. Exp Physiol 88(3): 343–357

Griebling TL, Kraus SR, Richter HE, Glasser DB, Carlsson M (2009) Tolterodine extended release is well tolerated in older subjects. Int J Clin Pract 63(8):1198–1204

Grol S, Essers PBM, van Koeveringe GA, Martinez-Martinez P, de Vente J, Gillespie JI (2009a) M_3 muscarinic receptor expression on suburothelial interstitial cells. BJU Int 104:398–405

Gunasena KT, Nimmo AJ, Morrison JF, Whitaker EM (1995) Effects of denervation on muscarinic receptors in the rat bladder. Br J Urol 76(3):291–296

Hanna-Mitchell AT, Beckel JM, Barbadora S, Kanai AJ, de Groat WC, Birder LA (2007) Non-neuronal acetylcholine and urinary bladder urothelium. Life Sci 80(24–25):2298–2302

Harrison SC, Hunnam GR, Farman P, Ferguson DR, Doyle PT (1987) Bladder instability and denervation in patients with bladder outflow obstruction. Br J Urol 60(6):519–522

Hawthorn MH, Chapple CR, Cock M, Chess-Williams R (2000) Urothelium-derived inhibitory factor(s) influence detrusor muscle contractility in vitro. Br J Pharmacol 129:416–419

Hedlund P, Streng T, Lee T, Andersson K-E (2007) Effects of tolterodine on afferent neurotransmission in normal and resiniferatoxin treated conscious rats. J Urol 178:326–331

Hegde SS, Choppin A, Bonhaus D, Briaud S, Loeb M, Moy TM, Loury D, Eglen RM (1997) Functional role of M_2 and M_3 muscarinic receptors in the urinary bladder of rats in vitro and in vivo. Br J Pharmacol 120:1409–1418

Hegde SS, Eglen R (1999) Muscarinic receptor subtypes modulating smooth muscle contractility on the urinary bladder. Life Sci 64(6–7):419–428

Hegde SS, Mammen M, Jasper JR (2004) Antimuscarinics for the treatment of overactive bladder: current options and emerging therapies. Curr Opin Investig Drugs 5(1):40–49

Hill S, Khullar V, Wyndaele JJ et al (2006) Dose response with darifenacin, a novel once-daily M3 selective receptor antagonist for the treatment of overactive bladder: results of a fixed dose study. Int Urogynecol J Pelvic Floor Dysfunct 17:239–247

Igawa Y, Zhang X, Nishizawa O, Umeda M, Iwata A, Taketo MM, Manabe T, Matsui M, Andersson KE (2004) Cystometric findings in mice lacking muscarinic M_2 or M_3 receptors. J Urol 172(Part 1 of 2):2460–2464

Iijima K, De Wachter S, Wyndaele JJ (2007) Effects of the M_3 receptor selective muscarinic antagonist darifenacin on bladder afferent activity of the rat pelvic nerve. Eur Urol 52:842–849

Ikeda K, Kobayashi S, Suzuki M, Miyata K, Takeuchi M, Yamada T, Honda K (2002) M(3) receptor antagonism by the novel antimuscarinic agent solifenacin in the urinary bladder and salivary gland. Naunyn Schmiedebergs Arch Pharmacol 366(2):97–103

Inadome A, Yoshida M, Takahashi W, Yono M, Seshita H, Miyamoto Y, Kawano T, Ueda S (1998) Prejunctional muscarinic receptors modulating acetylcholine release in rabbit detrusor smooth muscles. Urol Int 61:135–141

Irwin DE, Milsom I, Hunskaar S et al (2006) Population-based survey of urinary incontinence, overactive bladder, and other lower urinary tract symptoms in five countries: results of the EPIC study. Eur Urol 50:1306–1315

Johnston L, Carson C, Lyons AD, Davidson RA, McCloskey KD (2008) Cholinergic-induced Ca^{2+} signaling in interstitial cells of Cajal from the guinea pig bladder. Am J Physiol Renal Physiol 294(3):F645–F655

Jositsch G, Papdakis T, Haberberger RV, Wolff M, Wess J, Kummer W (2009) Suitability of muscarinic acetylcholine receptor antibodies for immunohistochemistry evaluated on tissue sections of receptor gene-deficient mice. Naunyn Schmiedebergs Arch Pharmacol 379:389–395

Kaplan SA, Roehrborn CG, Dmochowski R et al (2006) Tolterodine extended release improves overactive bladder symptoms in men with overactive bladder and nocturia. Urology 68(2): 328–332

Kay GG, Ebinger U (2008) Preserving cognitive function for patients with overactive bladder: evidence for a differential effect with darifenacin. Int J Clin Pract 62(11):1792–1800

Khullar V, Rovner ES, Dmochowski R, Nitti V, Wang J, Guan Z (2008) Fesoterodine DOSE response in subjects with overactive bladder syndrome. Urology 71(5):839–843

Kim JC, Yoo JS, Park EY, Hong SH, Seo SI, Hwang TK (2008) Muscarinic and purinergic receptor expression in the urothelium of rats with detrusor overactivity induced by bladder outlet obstruction. BJU Int 101:371–375

Kinder RB, Mundy AR (1985) Atropine blockade of nerve-mediated stimulation of the human detrusor. Br J Urol 57:418–421

Kobayashi F, Yageta Y, Segawa M, Matsuzawa S (2007a) Effects of imidafenacin (KRP-197/ ONO-8025), a new anti-cholinergic agent, on muscarinic acetylcholine receptors. High affinities for M_3 and M_1 receptor subtypes and selectivity for urinary bladder over salivary gland. Arzneimittelforschung 57(2):92–100

Kobayashi F, Yageta Y, Yamazaki T, Wakabayashi E, Inoue M, Segawa M, Matsuzawa S (2007b) Pharmacological effects of imidafenacin (KRP-197/ONO-8025), a new bladder selective anti-cholinergic agent, in rats. Comparison of effects on urinary bladder capacity and contraction, salivary secretion and performance in the Morris water maze task. Arzneimittelforschung 57(3):147–154

Krichevsky VP, Pagala MK, Vaydovsky I, Damer V, Wise GJ (1999) Function of M_3 muscarinic receptor in the rat urinary bladder following partial outlet obstruction. J Urol 161:1644–1650

Krishnamoorthy S, Kekre NS (2009) Detrusor underactivity: to tone or not to tone the bladder? Indian J Urol 25(3):407–408

Kullmann FA, Artim DE, Beckel JM, Barrick S, de Groat WC, Birder LA (2008) Heterogeneity of muscarinic receptor mediated Ca^{2+} responses in cultured urothelial cells from rat. Am J Physiol Renal Physiol 294:F971–F981

Lamers WH (2009) BJUI Letters. BJU Int 104:1538–1539

Lazareno S, Birdsall NJ (1993) Pharmacological characterization of acetylcholine-stimulated [35S]-GTP gamma S binding mediated by human muscarinic m_1-m_4 receptors: antagonist studies. Br J Pharmacol 109(4):1120–1127

Lazareno S, Buckley NJ, Roberts FF (1990) Characterization of muscarinic M_4 binding sites in rabbit lung, chicken heart, and NG108-15 cells. Mol Pharmacol 38(6):805–815

Lee S, Malhotra B, Creanga D, Carlsson M, Glue P (2009) A meta-analysis of the placebo response in antimuscarinic drug trials for overactive bladder. BMC Med Res Methodol 9:55

Longhurst PA, Leggett RE, Briscoe AK (1995) Characterization of the functional muscarinic receptors in the rat urinary bladder. Br J Pharmacol 116:2279–2285

Madeiro AP, Rufino AC, Sartori MGF, Baracat EC, Lima GR, Girao MJ (2006) The effects of bethanechol and cisapride on urodynamic parameters in patients undergoing radical hysterectomy for cervical cancer. A randomised, double-blind, placebo controlled study. Int Urogynecol J Pelvic Floor Dysfunct 17:248–252

Mansfield KJ, Lui L, Michelson FJ, Moore KH, Millard RJ, Burcher E (2005) Muscarinic receptor subtypes in human bladder detrusor and mucosa, studied by radioligand binding and quantitative competitive RT-PCR: changes in ageing. Br J Pharmacol 144:1089–1099

Mansfield KJ, Chandran JJ, Vaux KJ, Millard RJ, Christopoulos A, Mitchelson FJ, Burcher E (2009) Comparison of receptor binding characteristics of commonly used muscarinic antagonists in human bladder detrusor and mucosa. J Pharmacol Exp Ther 328(3):893–899

Masters JG, Neal DE, Gillespie JI (1999) The contribution of intracellular Ca^{2+} release to contraction in human bladder smooth muscle. Br J Pharmacol 127:996–1002

Masunaga K, Yoshida M, Inadome A, Murakami S, Sugiyama Y, Satoji Y, Maeda Y, Ueda S (2008) Pharmacogical effects of solifenacin on human isolated urinary bladder. Pharmacology 82(1):43–52

Masunaga K, Yoshida M, Inadome A, Iwashita H, Miyamae K, Ueda S (2006) Prostaglandin E2 release from isolated bladder strips in rats with spinal cord injury. Int J Urol 13(3): 271–276

Matsui M, Motomura D, Fujikawa T, Jiang J, Takahashi S, Manabe T, Taketo MM (2002a) Mice lacking M_2 and M_3 muscarinic acetylcholine receptors are devoid of cholinergic smooth muscle contractions but still viable. J Neurosci 22:10627–10632

Matsui M, Yamada S, Oki T, Manabe S, Taketo MM, Ehlert FJ (2004) Functional analysis of muscarinic acetylcholine receptors using knockout mice. Life Sci 75:2971–2981

Matsumoto Y, Miyazato M, Furata A, Torimoto K, Hirao Y, Chancellor MB, Yoshimura N (2010) Differential roles of M_2 and M_3 muscarinic receptor subtypes in modulation of bladder afferent activity in rats. Urology 75(4):862–867

McCloskey KD (2010) Interstitial cells in the urinary bladder–localization and function. Neurourol Urodyn 29(1):82–87

Michel MC, Hegde SS (2006) Treatment of the overactive bladder syndrome with muscarinic receptor antagonists: a matter of metabolites? Naunyn Schmiedebergs Arch Pharmacol 374(2):79–85

Michel MC, Weiland T, Tsujimoto G (2009) How reliable are G-protein coupled receptor antibodies? Naunyn Schmiedebergs Arch Pharmacol 379:385–388

Miller K, DuBeau C, Bergmann M et al (2002) How does drug treatment improve urge incontinence? Neurourol Urodyn 32:186–199

Mills IW, Greenland JE, McMurray G et al (2000) Studies of the pathophysiology of idiopathic detrusor instability: the physiological properties of the detrusor smooth muscle and its pattern of innervation. J Urol 163:646–651

Miyamoto Y, Yoshida M, Takahashi W, Inadome A, Yono M, Seshita H, Murakami S, Ueda S (2001) The effect of nitric oxide on acetylcholine release in the rabbit bladder. Eur J Pharmacol 428:59–67

Mori S, Kojima M, Sakai Y, Nakajima K (1999) Bladder dysfunction in dementia patients showing urinary incontinence: evaluation with cystometry and treatment with propiverine hydrochloride. Nippon Ronen Igakkai Zasshi 36(7):489–494

Mukerji G, Yiangou Y, Grogono J, Underwood J, Agarwal SK, Khullar V, Anand P (2006) Localisation of M_2 and M_3 muscarinic receptors in human bladder disorders and their clinical correlations. J Urol 176(1):367–373

Ney P, Kumar Pandita R, Newgreen DT, Breidenbach A, Stöhr T, Andersson KE (2008) Pharmacological characterisation of a novel investigational antimuscarinic drug, fesoterodine, in vitro and in vivo. BJU Int 101:1036–1042

Nilvebrant L, Andersson KE, Gillberg PG et al (1997) Tolterodine: a new bladder-selective antimuscarinic agent. Eur J Pharmacol 327:195

Noronha-Blob L, Lowe V, Patton A, Canning B, Costello D, Kinnier WJ (1989) Muscarinic receptors: relationships among phosphoinositide breakdown, adenylate cyclase inhibition, in vitro detrusor muscle contractions and in vivo cystometrogram studies in guinea pig bladder. J Pharmacol Exp Ther 249:843–851

Novara G, Galfano A, Secco S, D'Elia C, Cavalleri S, Ficarra V, Artibani W (2008) A systematic review and meta-analysis of randomised controlled trials with antimuscarinic drugs for overactive bladder. Eur Urol 54:740–764

Ohtake A, Saitoh C, Yuyama H, Ukai M, Okutsu H, Noguchi Y, Hatanaka T, Suzuki M, Sato S, Sasamata M, Miyata K (2007) Pharmacological characterization of a new antimuscarinic agent, solifenacin succinate, in comparison with other antimuscarinic agents. Biol Pharm Bull 30(1):54–58

Persson K, Igawa Y, Mattiasson A, Andersson KE (1992) Effects of inhibition of the L-arginine/nitric oxide pathway in the rat lower urinary tract in vivo and in vitro. Br J Pharmacol 107(1): 178–184

Peters SLM, Schmidt M, Michel MC (2006) Rho kinase. A target for treating urinary bladder dysfunction? Trends Pharmacol Sci 27:492–497

Pontari MA, Braverman AS, Ruggieri MR et al (2004) The M_2 muscarinic receptor mediates in vitro bladder contractions from patients with neurogenic bladder dysfunction. Am J Physiol Regul Integr Comp Physiol 286:R874–R880

Pradidarcheep W, Labruyere WT, Dabhoiwala NF, Lamers WH (2008) Lack of specificity of commercially available antisera: better specifications needed. J Histochem Cytochem 56: 1099–1111

Pradidarcheep W, Stallen J, Labruyere WT, Dabhoiwala NF, Michel MC (2009) Lack of specificity of commercially available antisera against muscarinergic and adrenergic receptors. Naunyn Schmiedebergs Arch Pharmacol 379:397–402

Reilly K, Milsom I, Irwin D et al (2006) Prevalence of incontinence and overactive bladder: European results from the EPIC study. Eur Urol 5(Suppl):116

Reitstetter R, He DS, Gruener R (1994) Oxotremorine-M activates single nicotinic acetylcholine receptor channels in cultured Xenopus myocytes. Eur J Pharmacol 264(1):27–32

Reynard JM (2004) Does anticholinergic medication have a role for men with lower urinary tract symptoms/benign prostatic hyperplasia either alone or in combination with other agents? Curr Opin Urol 14(1):13–16

Ronchi P, Gravina GL, Galatioto GP, Costa AM, Martella O, Vicentini C (2009) Urodynamic parameters after solifenacin treatment in men with overactive bladder symptoms and detrusor underactivity. Neurourol Urodyn 28:52–57

Ruggieri MR, Braverman AS (2006) Regulation of bladder muscarinic receptor subtype by experimental pathologies. Auton Autocoid Pharmacol 26:311–325

Saito M, Kondo A, Kato T, Hasegawa S, Miyake K (1993) Response of the human neurogenic bladder to KCl, carbachol, ATP and $CaCl_2$. Br J Urol 72(3):298–302

Sakakibara R, Uchiyama T, Yoshiyama M, Yamanishi T, Hattori T (2005) Preliminary communication: urodynamic assessment of donepezil hydrochloride in patients with Alzheimer's disease. Neurourol Urodyn 24(3):273–275

Sand P, Zinner N, Newman D, Lucente V, Dmochowski R, Kelleher C, Dahl NV (2007) Oxybutynin transdermal system improves the quality of life in adults with overactive bladder: a multi-centre, community-based, randomized study. BJU Int 99(4):836–844

Schneider T, Fetscher C, Krege S, Michel MC (2004) Signal transduction underlying carbachol-induced contraction of human urinary bladder. J Pharmacol Exp Ther 309:1148–1153

Seeger T, Fedorova I, Zheng F, Miyakawa T, Koustova E, Gomeza J, Basile AS, Alzheimer C, Wess J (2004) M_2 muscarinic acetylchline receptor knock-out mice show deficits in behavioural flexibility, working memory and hippocampal plasticity. J Neurosci 24:10117–10127

Sellers DJ, Yamanishi T, Yasuda K, Couldwell C, Chapple CR, Chess-Williams R (2000) M_3 muscarinic receptors but not M_2 mediated contraction of the porcine detrusor muscle in vitro. J Auton Pharmacol 20(3):171–176

Sibley GNA (1984) A comparison of spontaneous and nerve-mediated activity in bladder muscle from man, pig and rabbit. J Physiol 354:431–443

Sink KM, Thomas J III, Xu H, Craig B, Kritchevsky S, Sands LP (2008) Dual use of bladder anticholinergics and cholinesterase inhibitors: long-term functional and cognitive outcomes. J Am Geriatr Soc 56(5):847–853

Sjogren C, Andersson KE, Husted S, Mattiasson A, Moller-Madsen B (1982) Atropine resistance of transmurally stimulated isolated human bladder muscle. J Urol 128:1368–1371

Somogyi GT, de Groat WC (1999) Function, signal transduction mechanisms and plasticity of presynaptic muscarinic receptors in the urinary bladder. Life Sci 64(6–7):411–418

Somogyi GT, Tanowitz M, De Groat WC (1994) M_1 muscarinic receptor-mediated facilitation of acetylcholine release in the rat urinary bladder. J Physiol 480(Pt 1):81–89

Somogyi GT, Zernova GV, Yoshiyama M, Yamamoto T, de Groat WC (1998) Frequency dependence of muscarinic facilitation of transmitter release in urinary bladder strips from neurally intact or chronic spinal cord transected rats. Br J Pharmacol 125(2):241–246

Somogyi GT, Yokoyama EA, Szell EA, Smith CP, de Groat WC, Huard J, Chancellor MB (2002) Effect of cryoinjury on the contractile parameters of bladder strips: a model of impaired detrusor contractility. Brain Res Bull 59:23–28

Staskin DR (2005) Overactive bladder in the elderly: a guide to pharmacological management. Drugs Aging 22(12):1013–1028

Staskin DR, Robinson D (2009) Oxybutynin chloride topical gel: a new formulation of an established antimuscarinic therapy for overactive bladder. Expert Opin Pharmacother 10(18): 3103–3111

Stengel PW, Gomeza J, Wess J, Cohen ML (2000) M_2 and M_4 receptor knockout mice: muscarinic receptor function in cardiac and smooth muscle in vitro. J Pharmacol Exp Ther 292:877–885

Stevens LA, Sellers DJ, McKay NG, Chapple CR, Chess-Williams R (2006) Muscarinic receptor function, density and G-protein coupling in the overactive diabetic rat bladder. Auton Autocoid Pharmacol 26(3):303–309

Stevens LA, Cr C, Chess-Williams R (2007) Human idiopathic and neurogenic overactive bladders and the role of M(2) muscarinic receptors in contraction. Eur Urol 52(2):531–538

Stewart WF, Van Rooyen JB, Cundiff GW et al (2003) Prevalence and burden of overactive bladder in the United States. World J Urol 20:327–336

Sui GP, Wu C, Fry C (2004) Electrical characteristics of suburothelial cells isolated from the human bladder. J Urol 171:938–943

Takeuchi T, Yamashiro N, Kawasaki T, Nakajima H, Azuma YT, Matusui M (2008) The role of muscarinic receptor subtypes in acetylcholine release from the urinary bladder obtained from muscarinic receptor knockout mouse. Neuroscience 156:381–389

Taylor JA, Kuchel GA (2006) Detrusor underactivity: clinical features and pathogenesis of an underdiagnosed geriatric condition. J Am Geriatr Soc 54:1920–1932

Tobin G, Sjogren C (1998) Prejunctional facilitatory and inhibitory modulation of parasympathetic nerve transmission in the rabbit urinary bladder. J Auton Nerv Syst 68:153–156

Tong YC, Cheng JT, Hsu CT (2006) Alterations of M_2 muscarinic receptor protein and mRNA expression in the urothelium and muscle layer of the streptozotocin-induced diabetic rat urinary bladder. Neurosci Lett 406:216–221

Trendelenburg AU, Gomeza J, Klebroff W, Zhou H, Wess J (2003) Heterogeneity of presynaptic muscarinic receptors mediating inhibition of sympathetic transmitter release: a study with M_2- and M_4-receptor-deficient mice. Br J Pharmacol 138(3):469–480

Trendelenburg AU, Meyer A, Wess J, Starke K (2005) Distinct mixtures of muscarinic receptor subtypes mediate inhibition of noradrenaline release in different mouse peripheral tissues, as studied with receptor knockout mice. Br J Pharmacol 145(8):1153–1159

Tubaro A (2004) Defining overactive bladder: epidemiology and burden of disease. Urology 64(6 Suppl 1):2–6

Tyagi S, Tyagi P, Van-le S, Yoshimura N, Chancellor MB, de Miguel F (2006) Qualitative and quantitative expression profile of muscarinic receptors in human urothelium and detrusor. J Urol 176:1673–1678

Wagg A, Wyndaele JJ, Sieber P (2006) Efficacy and tolerability of solifenacin in elderly subjects with overactive bladder syndrome: a pooled analysis. Am J Geriatr Pharmacother 4(1):14–24

Wang P, Luthin GR, Ruggieri MR (1995) Muscarinic acetylcholine receptor subtypes mediating urinary bladder contractility and coupling to GTP binding proteins. J Pharmacol Exp Ther 273:959–966

Wein AJ, Malloy TR, Shofer F, Raezer DM (1980) The effects of bethanechol chloride on urodynamic parameters in normal women and in women with significant residual urine volumes. J Urol 124:397–399

Wessler I, Roth E, Deutsch C, Brockerhoff P, Bittinger F, Kirkpatrick CJ, Kilbinger H (2001) Release of non-neuronal acetylcholine from the isolated human placenta is mediated by organic cation transports. Br J Pharmacol 134:951–956

Wibberley A, Chen Z, Hu E, Hieble JP, Westfall TD (2003) Expression and functional role of Rho-kinase in rat urinary bladder smooth muscle. Br J Pharmacol 138:757–766

Womack KB, Heilman KM (2003) Tolterodine and memory: dry but forgetful. Arch Neurol 60(5):771–773

Wu C, Bayliss M, Newgreen D, Mundy AR, Fry CH (1999) A comparison of the mode of action of ATP and carbachol on isolated human detrusor smooth muscle. J Urol 162:1840–1847

Wuest M, Weiss A, Waelbroeck M, Braeter M, Kelly LU, Hakenberg OW, Ravens U (2006) Propiverine and metabolites: differences in binding to muscarinic receptors and in functional models of detrusor contraction. Naunyn Schmiedebergs Arch Pharmacol 374(2):87–97

Yamada M, Miyakawa T, Duttaroy A, Yamanaka A, Moriguchi T, Makita R, McKinzie DL, Felder CC, Deng CX, Faraci FM, Wess J (2001) Mice lacking the M_3 muscarinic acetylcholine receptor are hypophagic and lean. Nature 410:207–212

Yamaguchi O, Shishido K, Tamura K, Ogawa T, Fujimura T, Ohtsuka M (1996) Evaluation of mRNAs encoding muscarinic receptor subtypes in human detrusor muscle. J Urol 156: 1208–1213

Yamanishi T, Yasuda K, Chapple CR, Chess-Williams R (2000) The role of M_2-muscarinic receptors in mediating contraction of the pig urinary bladder in vitro. Br J Pharmacol 131(7):1482–1488

Yamazaki T, Muraki Y, Anraku T (2011) In vivo bladder selectivity of imidafenacin, a novel antimuscarinic agent, assessed by using an effectiveness index for bladder capacity in rats. Naunyn-Schmiedeberg's Arch Pharmacol 384:319–329

Yokota T, Yamaguchi O (1996) Changes in cholinergic and purinergic neurotransmission in pathologic bladder of chronic spinal rabbit. J Urol 156(5):1862–1866

Yokoyama O, Yusup A, Miwa Y, Oyama N, Aoki Y, Akino H (2005) Effects of tolterodine on an overactive bladder depend on suppression of C-fiber bladder afferent activity in rats. J Urol 174(5):2032–2036

Yoshida M, Miyamae K, Iwashita H, Otani M, Inadome A (2004) Management of detrusor dysfunction in the elderly: changes in acetylcholine and adenosine triphosphate release during aging. Urology 63:17–23

Yoshida M, Inadome A, Maeda Y, Satoji Y, Masunaga K, Sugiyama Y, Murakami S (2006) Non-neuronal cholinergic system in human bladder urothelium. Urology 67:425–430

Zagorodnyuk VP, Gregory S, Costa M, Brookes SJ, Tramontana M, Giuliani S, Maggi CA (2009) Spontaneous release of acetylcholine from autonomic nerves in the bladder. Br J Pharmacol 157(4):607–619

Zarghooni S, Wunsch J, Bodenbenner M, Bruggmann D, Grando SA, Schwantes U, Wess J, Kummer W, Lips KS (2007) Expression of muscarinic and nicotinic acetylcholine receptors in the mouse urothelium. Life Sci 80:2308–2313

Part IV
Muscarinic Receptors and Mediation of Hormonal Effects of Acetylcholine

Muscarinic Receptor Agonists and Antagonists: Effects on Inflammation and Immunity

Norah G. Verbout and David B. Jacoby

Abstract In this chapter, we will review what is known about muscarinic regulation of immune cells and the contribution of immune cell muscarinic receptors to inflammatory disease and immunity. In particular, immune cell expression of cholinergic machinery, muscarinic receptor subtypes and functional consequences of agonist stimulation will be reviewed. Lastly, this chapter will discuss the potential therapeutic effects of selective antagonists on immune cell function and inflammatory disease in recent animal studies and human clinical trials.

Keywords Immune cells • Inflammatory disease • Non-neuronal cholinergic system

1 Introduction

It is increasingly apparent that cells of the immune system express muscarinic receptors that directly regulate their function. Since immune cells play an important role in defense against pathogens and disease pathophysiology, it seems likely that muscarinic regulation of immune cells contributes to pathology. In particular, muscarinic modulation of immune cell function may be a significant target under inflammatory settings. Indeed, several muscarinic receptor agonists and antagonists are approved to treat several clinical conditions, including glaucoma, Sjogren's syndrome, chronic obstructive pulmonary disease (COPD), and asthma. In this chapter, we will review what is known about muscarinic regulation of immune

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cells and the contribution of immune cell muscarinic receptors to inflammatory disease and immunity.

1.1 Non-neuronal Cholinergic System in Immune Cells

The emergence of a non-neuronal cholinergic system has expanded the conventional assumption that acetylcholine production is limited to the nervous system. Acetylcholine is synthesized by nearly all mammalian cells and can play an integral role in regulating the interactions of non-neuronal cells with their external environment. Indeed, many immune cells have been demonstrated to express the molecular machinery required to synthesize, store, and release acetylcholine. Evidence for acetylcholine synthesis, storage, release, and breakdown in immune cells has been demonstrated using multiple methods, including immunoreactivity for choline-acetyltransferase (ChAT), vesicular acetylcholine transporter (VAChT), and choline transferase (CHT1) in isolated immune cells, reviewed in (Kawashima and Fujii 2004).

While the essential components of the cholinergic system are present in many immune cells (Table 1), the method by which immune cells produce and release acetylcholine differs from the nervous system. Neuronal cells store acetylcholine in discrete neurosecretory vesicles and release acetylcholine via exocytosis. In contrast, non-neuronal cells appear to actively transport acetylcholine directly upon synthesis (Wessler et al. 1999). The current understanding of how non-neuronal cells release acetylcholine is based upon human placenta as a model

Table 1 Expression of cholinergic machinery in immune cells

Immune cell	ACh	ChAT	AChE	ChT 1	VAChT	References
Lymphocyte	+	+	+	+ (*Cl*)	+	Kawashima et al. (1993), Neumann et al. (2007), and Tayebati et al. (2002)
Monocyte	+	+ (*A*)	NR	NR	NR	Hecker et al. (2006) and Neumann et al. (2007)
Macrophage	NR	+	NR	NR	NR	Wessler and Kirkpatrick (2001)
Neutrophil	+ (<)	+	NR	NR	NR	Hagforsen et al. (2000) and Neumann et al. (2007)
Dendritic cell	NR	+ (*A*)	+(*A*)	NR	NR	Kawashima et al. (2007)
Mast cell	NR	+	+ (*Cl*)	NR	NR	Nechushtan et al. (1996) and Wessler et al. (2003)
Eosinophil	NR	+/−	NR	NR	−	Durcan et al. (2006) and Hagforsen et al. (2000)

Abbreviations: ACh acetylcholine; *ChAT* choline acetyltransferase; *AChE* acetylcholinesterase; *VAChT* vesicular acetylcholine transporter; *ChT1* high affinity choline transporter; *NR* not reported
Symbols: + present; − absent; +/− conflicting reports; *Cl* detected in cell lines, but not primary cells; *A* detected in experimental animal; < detected in very low amounts

system (Wessler et al. 2001), in which active transport of acetylcholine is mediated by members of the organic cation transporter (OCT) family. Inhibitors of organic cation transporters suppress acetylcholine release in human placenta (Wessler et al. 2001), organic cation transporter-transfected oocytes (Lips et al. 2005) and airway epithelium (Kummer et al. 2006; Lips et al. 2005). Although it is not yet confirmed that organic cation transporters control acetylcholine release from immune cells, these transporters are expressed on nearly every cell type, making them a probable candidate for immune cell acetylcholine release.

1.2 Acetylcholine in the Blood

Leukocytes are derived from a multipotent progenitor cell in the bone marrow. Once produced, leukocytes migrate to various tissues throughout the body via the systemic circulation and the lymphatic system, which consists of the thymus, spleen, and lymphatic vessels. Although there is no evidence of cholinergic innervation of the bone marrow or lymphatic system, it is likely that leukocytes are exposed to acetylcholine in the blood. Indeed, human blood contains physiologically relevant concentrations of acetylcholine (8.66 +/− 1.02 nM in whole blood and 3.12 nM in plasma). This concentration is similar to values measured in blood of chimpanzees, pigs, and rabbits (Fujii et al. 1995; Kawashima et al. 1993).

The source of acetylcholine in the circulation is not definitively known. Kawashima et al. purport that lymphocytes are the main source of blood acetylcholine, since 60% of blood acetylcholine has been located in lymphocytes (Kawashima et al. 1993). Acetylcholine degradation is regulated in part by acetylcholinesterase (AChE), an enzyme that is present at sufficiently high concentrations in the blood. The local concentration of acetylcholine at sites of muscarinic receptors on immune cells in the blood is not known, but it is likely that acetylcholine hydrolysis in the plasma occurs at a much slower rate than what occurs at neuromuscular junctions (Kawashima and Fujii 2000). Thus, physiologic levels of acetylcholine are present in the blood, and may affect immune cells during migration to sites of inflammation.

Inflammation is one of the initial responses of the immune system to infection. Symptoms of inflammation include redness and edema, caused by increased blood flow into tissues. Injured or infected cells produce specific mediators that attract immune cells to the site of inflammation. These pro-inflammatory mediators include eicosanoids, cytokines, and chemokines. Eicosanoids include prostaglandins that cause vasodilation of the local vasculature and leukotrienes that attract leukocytes. Cytokines mediate communication between leukocytes and chemokines promote leukocyte chemotaxis. In addition, growth factors and other cytotoxic factors are present at sites of inflammation. These mediators act in concert

to selectively recruit immune cells from the blood to the site of inflammation to clear pathogens and promote healing of damaged tissue.

1.3 Early Evidence of Muscarinic Regulation of Immune Cells

Muscarinic modulation of the immune system is an evolving story. In 1976, Levy et al. demonstrated that vagotomy and atropine protect against histamine shock and lethal anaphylaxis in rats (Levy et al. 1976). Anaphylactic shock is an acute physiological response to an allergen, characterized by systemic release of inflammatory mediators, leading to circulatory and respiratory collapse. Thus, this study suggested that muscarinic blockade may modulate the immune response to allergen.

A subsequent study conducted in guinea pigs demonstrated that carbacholine increased granulocytes and lymphocytes in venous splenic blood and decreased spleen weight (Sandberg 1994). During an immune response, lymphoid tissues, such as spleen are sites of immune cell proliferation. The splenic vein drains blood from the spleen into the portal vein. Atropine alone had no effect on any other parameters, but blocked the effects of carbacholine, indicating an effect on muscarinic receptors. It is known that the spleen has adrenergic innervation, though direct cholinergic innervation of the spleen is sparse or unreported. However, there is some evidence indicating that lymphoid tissues are innervated by parasympathetic fibers in rodents (Bulloch and Pomerantz 1984; Kendall and al-Shawaf 1991). Indeed, in vitro, acetylcholine at 10^{-9} to 10^{-4} mol/l significantly increased spleen cell proliferation induced by concanavalin A (Con A), a lymphocyte mitogen (Qiu et al. 1995, 1996). Altogether, these studies suggested that muscarinic stimulation has an immunomodulatory effect on cells of the immune system.

2 Expression of Muscarinic Receptors on Immune Cells

Table 2 gives an overview of the expression of muscarinic receptors on immune cells in several different species. Recently, the specificity of antibodies raised against subtype specific muscarinic receptors has been questioned (Jositsch et al. 2009). However, it should be noted that in the majority of the references given in Table 1, more than one method had been utilized to demonstrate expression of muscarinic receptors. The different methods used are indicated in the legend.

Table 2 Expression of muscarinic receptor subtypes in immune cells

Immune cell	M1	M2	M3	M4	M5	Species	Source	Detection	References
Lymphocytes	+ (*V*)	+ (V)	+	+	+	Human	Blood	W, I, B	Tayebati et al. (2002), Ricci et al. (2002), Tayebati et al. (1999) Kawashima et al. (2007), and Costa et al. (1994)
	+	+	+	+	+	Mouse	Blood	I, M	
		+	+	+	+	Rat	Blood	M	
Monocytes	+	+ (<)	+	+		Human	Blood	M	Kawashima et al. (2007), Costa et al. (1994), and Pahl et al. (2006)
	+	+	+	+	+	Mouse	Blood	I, M	
	–		+	–	–	Rat	Blood	M	
Macrophages	+	+	+			Human	Sputum	I	Profita et al. (2005), Gwilt et al. 2007, and Kawashima et al. (2007)
	+	+	+			Human	Alveolar	I	
	+	+	+	+	+	Mouse	Peritoneal	I, M	
Neutrophils	+	+	+ (*S*)			Human	Sputum	I	Profita et al. (2005) and Bany et al. (1999)
			+	+	+	Human	Blood	M	
Dendritic cells			+			Human	Skin	I	Liu et al. (2010), Ma et al. (2007), andKawashima et al. (2007)
	+	+	+	+	+	Mouse	Bone-marrow	I, M	
Mast cells	+					Rat		F	Masini et al. (1983) and Nemmar et al. (1999)
	+					Rabbit	Lung	F	
Eosinophils	+ (*S*)	–	–			Human	Sputum	I	Profita et al. (2005), Durcan et al. (2006) and Profita et al. (2005)
	–	–	+	+	+	Human	Blood	I, M	
		–				Human	Blood	M	
	–	–	+	+	–	Guinea pig	Peritoneal and blood	I, M	

Abbreviations: B binding experiments; *F* functional experiments with agonists and antagonists; *I* immunoreactivity; *M* detection of subtype specific mRNA; *W* Western blot
Symbols: +present; −absent; *V* varied by individual subject; < present in very low amounts; *S* present in smoker or COPD

2.1 Lymphocytes

2.1.1 Role in the Immune System

Lymphocytes are derived from a hematopoietic precursor in the thymus. Lymphocytes can be broadly characterized into three major types: T cells, B cells, and natural killer (NK) cells. NK cells are a part of innate immune system and play a major role in host defense against tumors and viruses. T cells and B cells are the major immune cells of the adaptive response. T cells participate in cell-mediated immunity and B cells are associated with antibody production.

B and T lymphocytes coordinate the immune response to "non-self" antigens, during a process known as antigen presentation. B cells respond to pathogens by producing large quantities of antibodies that can neutralize foreign objects such as bacteria and viruses. In response to pathogens, T helper cells secrete cytokines that

coordinate the immune response, and cytotoxic T cells release toxic proteins that induce the death of pathogen-infected cells. Of the immune cells, lymphocytes are the best characterized with regard to regulation by muscarinic receptors.

2.1.2 Cholinergic Components

Lymphocytes express most cholinergic components found in the nervous system, including muscarinic, nicotinic, acetylcholine, choline acetyltransferase, vesicular acetylcholine transporter, choline transferase 1, and acetylcholinesterase (Fujii et al. 1999; Kawashima and Fujii 2004). Expression of muscarinic receptors has been demonstrated in lymphocytes isolated from blood, lymph nodes, spleen and thymus of mouse, rat, and human (Kawashima and Fujii 2000).

2.1.3 Muscarinic Receptors

The presence of muscarinic receptors has been detected via multiple methods in lymphocytes obtained from experimental animals and humans. Radioligand binding studies demonstrate muscarinic binding in mouse (Atweh et al. 1984; Genaro et al. 1993; Gordon et al. 1978; Kawashima et al. 2007) and rat lymphocytes (Costa et al. 1994; Krzystyniak et al. 1982; Maslinski et al. 1980; Tominaga et al. 1992). In human lymphocytes, muscarinic receptors have been detected by multiple methods, including radioligand binding (Adem et al. 1986; Bidart et al. 1983; Ferrero et al. 1991; Rabey et al. 1986; Zalcman et al. 1981), reverse-transcription-polymerase chain reaction (RT-PCR) and immunocytochemistry with monoclonal antibodies (see Table 2 for subtypes).

All five muscarinic subtypes have been detected in human lymphocytes; however, receptor subtype expression appears to vary by individual. For example, Tayebati et al. detected expression of M3, M4, and M5 muscarinic receptor subtypes in all subjects tested, whereas expression of M1 and M2 varied by individual (Tayebati et al. 2002). In healthy individuals, relative expression of each subtype exhibited a pattern, with M3 being the most abundantly expressed, followed in order by M5, M4, and M2, as determined by immunocytochemistry (Tayebati et al. 2002) and radioligand binding (Ricci et al. 2002; Tayebati et al. 1999) techniques. It may be that receptor subtype differs under pathophysiological conditions, since asthmatic patients have greater expression of M2 and M5 subtypes in mononuclear lymphocytes (Ricci et al. 2002).

2.1.4 Functional Changes in Lymphocytes Induced by Muscarinic Agonists

Most available data of the effects of muscarinic stimulation on lymphocytes have been derived from in vitro studies (see review (Kawashima and Fujii 2004)). Stimulation of muscarinic receptors on lymphocytes by acetylcholine or other

agonists initiates intracellular signaling via increased inositol-1, 4,5-triphosphate (IP_3) content, inhibition of cAMP production, and increased intracellular calcium $[Ca^{2+}]_i$ via muscarinic receptor coupling to either phospholipase C (PLC) (M1, M3, M5) or adenylyl cyclase (M2, M4).

Muscarinic receptor subtype expression has been well characterized in human lymphocyte cell lines (Kawashima and Fujii 2004), and these have been used as a model system to examine subtype specific responses to muscarinic stimulation in lymphocytes. In human B and T cell lines, both acetylcholine and oxotremorine-M (Oxo-M), a non-selective muscarinic agonist, induced intracellular calcium release and increased c-fos gene expression, a transcription factor upregulated in activated lymphocytes (Fujii and Kawashima 2000a, b). These effects of muscarinic stimulation were inhibited by 4-DAMP (M1, M3, M4, M5), YM905 (M1, M3) and atropine (Fujii and Kawashima 2000a, b, c). Conversely, neither pirenzipine (M1) nor AF-DX 16 (M2, M4) had any effect. Taken together, these experiments suggest that muscarinic agonists increase intracellular signaling and increase gene expression in lymphocytes via M3 and/or M5 muscarinic receptors.

2.1.5 Cytokine Production and Proliferation

During an immune response, lymphoid tissues, such as the thymus or spleen are sites of lymphocyte proliferation. Activated cytotoxic T cells undergo proliferation induced by IL-2, a cytokine that induces growth and differentiation. This IL-2 mediated activation increases the number of antigen-specific lymphocytes, thereby enhancing the immune response.

In vitro, acetylcholine enhances mitogen (ConA)-induced T-cell proliferation in rat spleen cell cultures, an effect blocked by atropine (Qiu et al. 1995). Similarly, IL-2 production is enhanced by Oxo-M following stimulation with phytohaemagglutinin (PHA), a T cell mitogen (Fujino et al. 1997). Pretreating T cells with the acetylcholine or Oxo-M in the presence of an acetylcholinesterase inhibitor enhances mitogen-induced IL-2 production (Nomura et al. 2003; Okuma and Nomura 2001), suggesting that acetylcholine produced by lymphocytes acts in a paracrine/autocrine fashion. It is probable that IL-2 mediated signal transduction in lymphocytes is also regulated by muscarinic receptors since treatment with Oxo-M also increased gene expression of the IL-2 receptor. Similarly, Fujino et al. (1997) reported stimulatory effects of Oxo-M on IL-2 production and proliferation in T cells (Fujino et al. 1997). Altogether, these studies suggest that IL-2 acts as an autocrine factor via muscarinic receptors during immunological interactions. It is known that many immune tasks performed by T cells depend on IL-2 production, which is a key cytokine for regulating immunity.

It is interesting to note that different muscarinic agonists affect lymphocyte function in diverse ways. For instance, arecoline, a partial muscarinic agonist used clinically in Alzheimer's disease, has immunosuppressive, rather than stimulatory effects on lymphocytes. Chronic administration of arecoline reduced spleen size compared to untreated control mice (Wen et al. 2006). Other lymphoid organs

including the thymus and mesenteric lymph nodes have also been found to have modest weight reductions after chronic arecoline treatment (Selvan et al. 1989). In vitro, Wen et al. found that chronic arecoline treatment decreased splenocyte proliferation induced by Con A or lipopolysaccharide (LPS), and reduced IL-2 secretion, effects that were reversed by pretreatment with atropine. Yet another study found that pilocarpine, an M2 agonist, had no effect on IL-2 production; however, it did decrease the number of IL-2 receptor expressing cells (Prync et al. 1992).

The underlying reason for opposing effects of different muscarinic agonists on IL-2 expression and signaling is not known, but it may reflect differences in receptor subtypes on discrete lymphocyte subpopulations, since it is known that an array of subtype combinations are expressed among lymphocytes within individual subjects (Tayebati et al. 2002). Alternatively, it may be that lymphocyte populations differ in their phenotype, for example, the muscarinic receptor targeted by the arecoline could be expressed on a suppressor lymphocyte, and the muscarinic receptor targeted by Oxo-M found on an activating type.

2.1.6 Differentiation

Upon stimulation, naïve CD8+ lymphocytes differentiate into cytolytic T cells, which are the main effector mechanism by which the immune system clears pathogen-infected cells. It is known that differentiation of naïve CD8+ lymphocytes into cytolytic T cells requires activation. Since acetylcholine regulates T cell proliferation via muscarinic receptors, it is conceivable that muscarinic signaling may modulate generation of cytolytic T cells. In support of this, Zimring et al. reported that CD8+ T cells from M1 receptor knockout mice were defective in their ability to differentiate into cytolytic T lymphocytes in vitro (Zimring et al. 2005). However, a subsequent study published by the same authors found evidence to the contrary. In vivo, there was no identifiable defect in virus-induced CD8+ T cell expansion in M1 mice knockout mice (Vezys et al. 2007). Some potential explanations for these discrepancies include differences in experimental stimulus conditions (standard proliferation assay versus whole animal viral infection) and differences in antigenicity, mouse strain, or compensation by redundant pathways. Despite these potential reasons for contrasting results, it appears that the data supporting muscarinic regulation of T cell differentiation are somewhat limited.

2.1.7 Immunoglobulin Class Switching

There is evidence that muscarinic receptors on lymphocytes may modulate antibody class switching. Fujii et al. examined this hypothesis in dual M1/M5 knockout mice exposed to ovalbumin (OVA) protein (Fujii et al. 2007). They found that serum levels of total and OVA specific IgG were significantly lower in M1/M5 knockout mice compared to wildtype mice. In addition, IL-6 secretion was reduced

in activated spleen cells from M1/M5 KO mice, suggesting that M1 and or M5 receptors contribute to IL-6 production, leading to modulation of antibody class switching from the IgM type to the IgG1. There were no differences in serum level of total and anti-OVA specific IgM between the KO and WT, thus M1 and M5 do not appear to contribute to the initial generation of antibodies.

2.2 Monocytes

2.2.1 Role in the Immune System

Monocytes are produced by the bone marrow, travel via the bloodstream to populate various tissues where they differentiate into macrophages or dendritic cells. Monocytes and their macrophage and dendritic cell progeny serve three main functions in the immune system. These are phagocytosis, antigen presentation, and cytokine production.

2.2.2 Cholinergic Components

Human mononuclear cells (monocytes and lymphocytes) isolated from peripheral blood produce acetylcholine (Neumann et al. 2007). In rat monocytes, expression of ChAT has been detected (Hecker et al. 2006); however, there are no reports of ChAT expression in human primary monocytes or in a monocytic cell line (Fujii et al. 1999).

2.2.3 Muscarinic Receptors

Expression of muscarinic receptors on monocytes appears to be mixed. Early studies suggested that human peripheral monocytes do not express muscarinic receptors. Using radiolabeling techniques, Eva et al. found that human peripheral monocytes do not bind NMS (Eva et al. 1989) and neither was mRNA for any of the five subtypes detected by RT-PCR (Hellstrom-Lindahl and Nordberg 1996). These reports contrast with a study conducted by Pahl et al., which found that human monocytes express mRNA for M3, M4 and M1 muscarinic receptors and possibly M2 (Pahl et al. 2006). M5 mRNA was not detected. Inflammatory status may also affect receptor subtype expression in monocytes, since treatment with LPS, a component of bacterial cell walls, modulates gene expression of muscarinic receptor subtypes (Pahl et al. 2006). Despite the conflicting reports, functional data suggest that monocytes probably do express muscarinic receptors.

2.2.4 Functional Changes in Monocytes Induced by Muscarinic Agonists

Acetylcholine stimulates ERK1/2 signaling and leukotriene (LTB4) production in blood monocytes, an effect blocked by oxitropium bromide (Profita et al. 2005).

2.3 Macrophages

2.3.1 Role in the Immune System

Macrophages are derived from a monocytic precursor produced in the bone marrow. In the blood, monocytes are recruited to the tissues, where they differentiate into tissue-specific resident macrophages. Macrophages play an important role in the innate response to pathogens by phagocytosing cellular debris and pathogens and releasing factors that stimulate lymphocytes and other immune cells.

2.3.2 Cholinergic Components

In the lung, human alveolar macrophages express ChAT (Wessler and Kirkpatrick 2001) and likely produce acetylcholine (Wessler et al. 1999). This may not be the case in other species, since peritoneal macrophages from C57BL/6J mice do not appear to express mRNA for ChAT (Kawashima et al. 2007).

2.3.3 Muscarinic Receptors

Lung macrophages (Gwilt et al. 2007) and macrophages isolated from human sputum express M2 and M3 receptors (Profita et al. 2005). Similarly, bovine alveolar macrophages express M3 receptors (Sato et al. 1998). In mice, mRNAs encoding all five muscarinic receptor subtypes are expressed in peritoneal macrophages from C57BL/6J mice (Kawashima et al. 2007).

2.3.4 Functional Changes in Macrophages Induced by Muscarinic Agonists

In bovine alveolar and human alveolar and sputum macrophages, acetylcholine stimulates release of lipoxygenase-derived inflammatory mediators, in particular leukotriene B4 (LTB4) acting via M3 muscarinic receptors (Buhling et al. 2007; Profita et al. 2005; Sato et al. 1998). LTB4 is a potent inflammatory mediator that increases leukocyte adhesion, activation, and neutrophil recruitment. Thus acetylcholine acting via muscarinic receptors may increase inflammation by recruiting inflammatory cells via release of macrophage-derived chemotactic mediators.

2.4 Neutrophils

2.4.1 Role in the Immune System

Neutrophils are granulocytic leukocytes that are produced in the bone marrow. They are the most abundant leukocyte found in the blood. Neutrophils are part of the acute inflammatory response and are robustly recruited from the vasculature to sites of injury, inflammation, or infection via chemoattractant mediators.

2.4.2 Cholinergic Components

Choline acetyltransferase has been detected in human skin neutrophils, peripheral blood neutrophils (Hagforsen et al. 2000), and peripheral granulocytes (Wessler et al. 1999). Human peripheral blood granulocytes have also been found to contain low levels of acetylcholine (Neumann et al. 2007).

2.4.3 Muscarinic Receptors

M3, M4, and M5, but not M1 or M2 muscarinic receptors have been detected on neutrophils by immunocytochemistry (Profita et al. 2005) and RT-PCR (Bany et al. 1999).

2.4.4 Functional Changes in Macrophages Induced by Muscarinic Agonists

There is little evidence to indicate that neutrophil function is regulated by muscarinic receptors. According to Profita et al., acetylcholine increased chemotactic activity and LTB4 production in sputum neutrophils, though it is not clear whether this is a muscarinic or nicotinic effect, since specific antagonists were not tested (Profita et al. 2005).

2.5 Dendritic Cells

2.5.1 Role in the Immune System

Dendritic cells are professional antigen-presenting cells produced in the bone marrow. Immature dendritic cells migrate via the bloodstream to the tissues. As a part of their antigen-sensing role, dendritic cells are located in tissues that interface with the external environment, for example the skin, the nose, lungs, stomach, and

intestines. Once activated, dendritic cells migrate to the lymph node where they interact with lymphocytes and initiate the adaptive immune response.

2.5.2 Cholinergic Components

In mice, unstimulated bone-marrow derived dendritic cells do not appear to express ChAT; however, stimulation with the bacterial wall component, LPS induces ChAT expression, suggesting that acetylcholine synthesis is an outcome of dendritic cell activation (Kawashima et al. 2007). AChE mRNA is expressed in mouse bone marrow-derived dendritic cells (Kawashima et al. 2007), and dendritic cells may also contain acetylcholine (Wessler et al. 1999).

2.5.3 Muscarinic Receptors

In mice, mRNAs encoding all five muscarinic receptor subtypes are expressed in bone marrow-derived dendritic cells from C57BL/6J mice (Kawashima et al. 2007), and M2 muscarinic receptors have been detected in dendritic cells in mouse gut (Ma et al. 2007). In humans, dendritic cells from nasal mucosa express M3 muscarinic receptors; however, very little expression was detected by flow cytometry in peripheral blood dendritic cells (Liu et al. 2010), underscoring the observation that the local environment may modulate subtype expression.

2.5.4 Functional Changes in Dendritic Cells Induced by Muscarinic Agonists

Methacholine induces dendritic cells to produce OX40L, a ligand expressed on activated dendritic cells that contributes to immune cell interactions (Liu et al. 2010). It is likely that this effect is mediated by muscarinic receptors, since methacholine has little effect on nicotinic receptors.

2.6 Mast Cells

2.6.1 Mast Cells in the Immune System

Mast cells are resident tissue cells derived from a hematopoetic precursor produced in the bone marrow. Immature mast cells migrate to the tissues via the blood stream and mature in the tissues. Mast cells play a key role in the inflammatory process by producing large quantities of protein mediators. Activated mast cells rapidly release protein granules and various inflammatory mediators into the interstitium. Mast cell degranulation is triggered by tissue injury, cross-linking of Immunoglobulin E (IgE) receptors, or by activated complement proteins. Mast cells are a major source

of histamine, which dilates post capillary venules, activates the endothelium and increases blood vessel permeability. This leads to local edema, warmth, redness, and recruitment of other inflammatory cells to the site of release.

2.6.2 Cholinergic Components

At present, there is no direct evidence that mast cells produce acetylcholine; however, ChAT immunoreactivity has been detected in human mast cells in the skin (Wessler et al. 2003) and mRNA for AChE has been detected in a murine mast cell line (Nechushtan et al. 1996), suggesting that mast cells may participate in acetylcholine regulation.

2.6.3 Muscarinic Receptors

Muscarinic receptors have been identified on rodent mast cells (Masini et al. 1983), with M1 as the best characterized subtype. Data from studies on airway disease suggest that mast cells from humans and rabbits also express M1 muscarinic receptors (see below).

2.6.4 Functional Changes in Mast Cells Induced by Muscarinic Agonists

Muscarinic regulation of mast cell degranulation differs by species. For example, in rabbits and rats, muscarinic agonists stimulate mast cell degranulation (Masini et al. 1985; Nemmar et al. 1999). Similarly, carbachol induces degranulation in a rat basophil leukemic cell line transfected with M1 receptors (Jones et al. 1991). There is also some indication that allergy may affect rodent mast cell responses to acetylcholine, since mast cells in allergen-sensitized rats are more sensitive to acetylcholine-induced histamine release compared to non-sensitized rats (Masini et al. 1985).

While muscarinic agonists promote histamine release in rats and rabbits, in human airways, muscarinic receptors are inhibitory. Acetylcholine inhibits ionophore induced histamine release in human mucosal mast cells through an M1-mediated pathway (Reinheimer et al. 2000). Allergen-induced histamine release is similarly inhibited in human airways (Reinheimer et al. 1997). Altogether, these studies suggest that this inhibitory pathway may be important in pathological conditions, such as asthma or COPD. Wessler et al. have examined histamine release in mucosal mast cells in tracheas. In healthy controls, oxotremorine reduced ionophore induced histamine release, but had little effect in COPD patients, suggesting that muscarinic inhibition of mast cells in COPD patients is dysregulated (Wessler et al. 2007). It is not known whether the interaction between acetylcholine and mast cells, a key effector cell in asthma, contribute to the pathophysiology of asthma. One possibility is that inhibitory muscarinic receptors

on mast cells normally limit allergen-induced histamine release, but in asthma, this pathway is dysfunctional. If this were the case, blockade of M1 muscarinic receptors on mast cells may worsen allergic asthma. It may be important to emphasize that studies using human mast cells have only been performed in whole tissue (Reinheimer et al. 1997, 2000; Wessler et al. 2007), therefore one cannot rule out the contribution of other cell types, for instance structural cells and nerves within the trachea.

2.7 Eosinophils

2.7.1 Role in the Immune System

Eosinophils are granulocytic leukocytes that play a role in immune defense and are implicated in pathogenesis of allergic disorders including asthma, rhinitis, and atopic dermatitis (Hogan 2007). In the bone marrow, eosinophils develop and mature in response to specific cytokines, IL-3, IL-5, and GM-CSF. Following maturation, eosinophils circulate in the blood and migrate to inflammatory sites within tissues in response to chemoattractant mediators, including CCL11 and CCL2, and CCL5. Activated eosinophils release granular proteins and inflammatory mediators at sites of infection or inflammation.

2.7.2 Cholinergic Components

There is conflicting evidence of cholinergic components or muscarinic receptors in eosinophils. Very small amounts of ChAT have been reported to be present in peripheral blood eosinophils (Hagforsen et al. 2000) via Western blot. This contrasts with Durcan et al., who did not detect mRNA for ChAT or VAChT in human peripheral blood eosinophils via RT-PCR (Durcan et al. 2006). There are no reports of eosinophils producing acetylcholine.

2.7.3 Muscarinic Receptors

Profita et al. (2005) report that human sputum eosinophils are negative for M1, M2 and M3 muscarinic receptors (Profita et al. 2005). However, it may be that muscarinic receptors on eosinophils are inducible, since the same study detected positive M1 immunostaining in sputum eosinophils from patients with COPD. In human peripheral blood eosinophils, mRNA and protein expression of M_3, M_4, and M_5, but not M_1, M_2 muscarinic receptors has been detected (Verbout 2008). In addition, guinea pig peritoneal and blood eosinophils express M_3 and M_4, but not M_1, M_2, or M_5 muscarinic receptors (Verbout 2008).

2.7.4 Functional Changes in Eosinophils Induced by Muscarinic Agonists

Muscarinic inhibition of eosinophil function may be important under pathophysiological conditions, for example allergy. In guinea pigs and in humans, allergic airway disease is associated with increased lung eosinophils that release proteins that damage tissue and increase airway reactivity (Costello et al. 1997, 2000). In guinea pigs, pretreatment with atropine at the time of allergen inhalation increases eosinophil activation and airway reactivity (Verbout 2007, 2009), suggesting that muscarinic antagonists affect eosinophil function. If muscarinic receptors on eosinophils inhibit eosinophil activation (Verbout 2008), then muscarinic antagonists that block inhibitory muscarinic receptors on eosinophils may be problematic in patients with allergic airway disease. This potential mechanism is consistent with clinical data indicating that perennial allergic rhinitis patients taking ipratropium have increased eosinophils and nasal reactivity compared to patients taking placebo (Gorski et al. 1993). Thus, it appears that ipratropium may potentiate inflammatory mechanisms when used in subjects with an allergy in the nasal mucosa.

3 Immune Cells as Targets for Muscarinic Drugs

It is well established that cells within the immune system express functional muscarinic receptors that modulate their function. Recent evidence suggests that muscarinic regulation of immune cells may be altered in disease states or inflammatory conditions, making them a significant therapeutic target for selective muscarinic drugs (Table 3).

One difficulty with interpreting data from such studies is overlap between the nicotinic and muscarinic pathways. Furthermore, since some muscarinic receptor subtypes share common signaling pathways, and often more than one receptor subtype is expressed by a given cell, it is difficult to tease out the clinical effects of muscarinic drugs on immune cell function. Further, the clinical usefulness of muscarinic drugs has historically been plagued by their lack of selectivity. Not only are the binding pockets on each of the five subtypes highly conserved, but it is also well established that muscarinic receptors are expressed in most tissues or cell types in a complex, overlapping pattern, thereby adding greater difficulty in targeting a specific muscarinic receptor subtype on a given cell. This has been a major obstacle in the development of clinically prescribed selective muscarinic drugs. The recent generation of mice deficient in each muscarinic receptor subtype, each with their own distinct phenotypes, may have stimulated a renewed interest in developing subtype selective drugs. Indeed, we have witnessed a resurgence of novel, highly selective muscarinic drugs, largely due to rational drug design and small-molecule screening assays. Studies conducted with selective muscarinic agonists and antagonists are therefore extremely useful in determining the contribution of

Table 3 Muscarinic regulation of inflammation in immune cells

Cell type	Muscarinic receptors	Role in inflammation	References
Lymphocytes	M1–M5	Increased cytotoxicity, cytokine production, proliferation	Kawashima and Fujii (2004)
Macrophages	M1, M2, M3	LTB4 production	Gosens et al. (2005) and Profita et al. (2005)
Monocytes	M1–M5	LTB4 production	Profita et al. (2005)
Neutrophils	M1, M2, M3	Chemotaxis, LTB4 production	Profita et al. (2005)
Dendritic cells	M3	OX40 ligand expression	Liu et al. (2010)
Mast cells	M1	Inhibition of histamine release in allergy	Reinheimer et al. (1997, 2000) and Wessler et al. (2007)
Eosinophil	M3, M4, M5	Regulation of major basic protein release in guinea pigs	Verbout et al. 2007, 2009)

Abbreviations: LTB4 leukotriene B4

muscarinic receptors to immune cell function. In particular, recent studies using muscarinic drugs in animal models of inflammatory disease have provided a strong basis for treating human disease. These new drugs are now just beginning to be used clinically to treat inflammatory disorders in humans.

3.1 Effects of Muscarinic Antagonists in Animal Models of Inflammation

3.1.1 Abscess Formation

In a rat model of turpentine-induced abscess formation, Razani-Boroujerdi et al. found that atropine treatment reduced leukocyte migration toward the site of abscess in vivo (Razani-Boroujerdi et al. 2008). Further, they found that peripheral blood mononuclear cells obtained from turpentine-treated rats exhibited decreased chemotaxis and chemokinesis when rats were given atropine, suggesting that muscarinic blockade inhibits leukocyte motility migration.

3.1.2 Particulates

Muscarinic antagonists may also affect neutrophil recruitment to sites of inflammation. In a diesel particle-induced rat model of pulmonary neutrophilic inflammation, bilateral vagotomy and atropine pretreatment reduced neutrophil lung inflammation (McQueen et al. 2007). This finding is supported by another study demonstrating that ipratropium decreases cadmium-induced neutrophil recruitment to the lung (Zhang et al. 2010). In a cigarette-smoke model of COPD, inhaled tiotropium dose-

dependently inhibited cigarette smoke-induced neutrophilic inflammation and production of chemotactic factors in lungs of mice (Wollin and Pieper 2010).

3.1.3 Gastroesophageal Reflux

Gastro-esophageal reflux is a common condition in patients with chronic airway diseases, such as asthma or COPD, and is considered to be a trigger for airway symptoms. It is characterized by inflammation of the lung and airway remodeling. In animal models, intra-esophageal instillation of the acid HCl induces features of gastro-esophageal reflux disease. In a mouse model of gastro-esophageal reflux, inhaled tiotropium or intraperitoneal atropine prevent lung inflammation as effectively as intraperitoneal dexamethasone (Cui et al. 2010). Altogether, these studies demonstrate that muscarinic antagonists have the potential to prevent lung inflammation with effectiveness similar to anti-inflammatory drugs, providing additional evidence that anticholinergics might contribute to the control of inflammatory processes in airway diseases.

3.1.4 Allergic Airway Remodeling

The use of anticholinergics in obstructive airways diseases, like asthma and COPD, is primarily based on their acute bronchodilatory effects on airway smooth muscle. Thus, muscarinic receptor antagonists provide acute relief from vagally induced bronchoconstriction in obstructive airway disease. Several recent studies have begun to examine whether muscarinic antagonists have protective effects on pulmonary inflammation, remodeling and injury.

Tiotropium, a muscarinic antagonist with kinetic selectivity for M3 muscarinic receptors, may have beneficial effects on immune cells under pathological conditions such as allergic asthma. Airway remodeling is a feature of allergic airway disease. In particular, airway remodeling is associated with increased inflammatory mediators and growth factors (Bousquet et al. 2000; Woodruff et al. 2004). Additionally, there are characteristic pathological changes in the airway architecture, for instance, increased airway smooth muscle thickness and goblet cell hyperplasia (Moir et al. 2003). These features are replicated in animal models; repeated exposure to inhaled allergen causes lung inflammation, increased airway responsiveness, and airway smooth muscle remodeling.

In guinea pigs, chronic exposure to allergen increases airway smooth muscle mass, pulmonary contractile protein expression, contractility of tracheal smooth muscle, mucous gland hypertrophy, and airway eosinophilia. Nearly all of these features were partially or fully prevented by inhaled tiotropium bromide (Bos et al. 2007; Gosens et al. 2005), indicating that muscarinic blockade may have beneficial effects on remodeling. Animals that received tiotropium in the absence of allergen challenge were unaffected histologically, thus it appears that the protective effects

of tiotropium only occur under pathological conditions of increased inflammatory mediators.

The mechanism by which tiotropium reduced airway remodeling is not known, but it could involve direct effects on immune cells. For example, tiotropium may have inhibitory effects on lymphocytes, since they are known to express M3 receptors. This is supported by a study conducted in mice, in which tiotropium inhibited allergen-induced airway remodeling, inflammation, and Th2 cytokine production (Ohta et al. 2010). In that study, Th2 cytokine production by spleen cells was inhibited by tiotropium and 4-DAMP, both selective M3 antagonists, suggesting that tiotropium may attenuate airway remodeling by suppressing Th2 cytokine production by T lymphocytes.

3.1.5 Chronic Obstructive Pulmonary Disease

Chronic obstructive lung disease is characterized by chronic inflammation of the main airways, airway obstruction, lung remodeling, and emphysematous lung destruction. Development of COPD is associated with increased inflammatory cells from both the innate and immune response, which cause remodeling and tissue destruction.

Accumulation of inflammatory cells in the lung is thought to be mediated in part by chemotactic factors, which are released by activated immune and structural cells within the lung. Increased cells of the innate immune system are characteristic of COPD and in particular, neutrophils and macrophages are increased in lungs of patients with COPD. Activation of these cells at sites of inflammation is thought to trigger airway remodeling in COPD.

Bronchodilators play a significant role in COPD management and anticholinergics are considered to be an effective and safe therapeutic. Ipratropium, though effective, requires frequent dosing (Casaburi et al. 2002; Disse 2001), thus tiotropium, with the kinetic selectivity and longer duration of action is now more commonly used for COPD therapy. In humans with COPD, airway epithelial cells and macrophages are likely to be the first cells in the lung to come into contact with inhaled cigarette smoke and tiotropium. In vitro studies have demonstrated that muscarinic receptor stimulation triggers release of pro-inflammatory mediators involved in neutrophil recruitment, such as IL-8, LTB4 from airway smooth muscle, epithelial cells and alveolar macrophages (Buhling et al. 2007; Gosens et al. 2005; Profita et al. 2005). Therefore, these cell types are all candidates for increased neutrophil recruitment. Macrophages, which also express M2 and M3 receptors (Buhling et al. 2007; Gwilt et al. 2007; Profita et al. 2005), may also contribute to these effects. Tiotropium antagonizes the effects of acetylcholine on human alveolar macrophage production of LTB4 (Buhling et al. 2007). Thus, tiotropium may have direct effects on macrophages as well.

3.2 *Effects of Tiotropium on Inflammation in Humans*

3.2.1 Clinical Trials in Humans

Two recent clinical trials have examined the anti-inflammatory effectiveness of anti-muscarinic drugs in humans, both of which show little effect on inflammatory outcomes (Perng et al. 2009; Powrie et al. 2007). In a clinical trial examining the effect of tiotropium on sputum inflammatory markers and exacerbation frequency, there was no added benefit of tiotropium (Powrie et al. 2007). The study examined sputum levels of IL-6 and neutrophil myeloperoxidase, a destructive enzyme, both of which were not reduced in patients that received tiotropium. Surprisingly, IL-8, a potent neutrophil chemoattractant was increased in individuals that received tiotropium compared to placebo. Neither was serum IL-6 or C-reactive protein decreased by addition of tiotropium compared to baseline levels at the start of the trial.

Another clinical study examined COPD outcomes in COPD patients using tiotropium alone in combination with fluticasone or combined salmeterol/fluticasone. This study found increased IL-8 and MMP-9 levels in the group treated with tiotropium alone (Perng et al. 2009). IL-8 is a potent neutrophil chemoattractant and MMP-9 is a protease produced by activated neutrophils and alveolar macrophages. Both are increased in COPD patients and MMP-9 is associated with emphysematous remodeling (Atkinson and Senior 2003; Ohnishi et al. 1998; Vernooy et al. 2004). Compared to salmeterol/fluticasone or tiotropium/fluticasone, tiotropium alone had no effect on inflammatory cells, pulmonary function, or quality of life assessment. However, due to ethical considerations, there was no placebo control to compare the effects of these drugs on reducing inflammation.

3.2.2 Interpretation of Clinical Data

One possible explanation for the lack of an effect on sputum inflammatory marker is decreased airway mucus (due to cholinergic inhibition), thus causing an increase in concentration in the sputum samples taken from the patients. This is supported by subjective reporting of decreased sputum production. In mice, tiotropium decreases mucin production, probably by acting directly on epithelial cells (Arai et al. 2010). Thus it is possible that measurement of sputum cytokines and cell number is a poor means of assessing airway inflammation.

At this time, it is not clear yet whether tiotropium exerts direct anti-inflammatory effects on immune cells in COPD. Tiotropium has been tested for antiinflammatory properties in preclinical studies and has shown anti-inflammatory effects on human cells in vitro and in animal studies. However, in many cases, the effects of tiotropium on inflammation were mediated via suppressing production of neutrophil chemotactic factors produced by epithelial cells (Gosens et al. 2005).

Currently, the beneficial effects of muscarinic antagonists in COPD can be explained by inhibition of vagal nerve-induced smooth muscle contraction in the bronchi. The effects on inflammation in humans are confusing and are inconsistent with findings in experimental animals.

4 Conclusion

The collective work presented in this chapter reveals that muscarinic regulation of the immune system is an emerging, but underdeveloped, field. Undoubtedly, there is considerable overlap between the immune and cholinergic systems. The basic machinery required for muscarinic regulation exists in nearly all immune cells. The expansion of more selective muscarinic agonists and antagonists has further fueled research on immune cells as cholinergic drug targets. Indeed, numerous studies have shown direct effects of muscarinic drugs on cells derived from multiple species, and there is evidence that receptor expression is altered in various diseases. Animal studies suggest that targeting muscarinic pathways may have beneficial effects on inflammation and disease, but to date, it is unclear whether these benefits are manifest in human studies.

References

Adem A, Nordberg A, Slanina P (1986) A muscarinic receptor type in human lymphocytes: a comparison of 3H-QNB binding to intact lymphocytes and lysed lymphocyte membranes. Life Sci 38:1359–1368

Arai N, Kondo M, Izumo T, Tamaoki J, Nagai A (2010) Inhibition of neutrophil elastase-induced goblet cell metaplasia by tiotropium in mice. Eur Respir J 35:1164–1171

Atkinson JJ, Senior RM (2003) Matrix metalloproteinase-9 in lung remodeling. Am J Respir Cell Mol Biol 28:12–24

Atweh SF, Grayhack JJ, Richman DP (1984) A cholinergic receptor site on murine lymphocytes with novel binding characteristics. Life Sci 35:2459–2469

Bany U, Gajewski M, Ksiezopolska-Pietrzak K, Jozwicka M, Klimczak E, Ryzewski J, Chwalinska-Sadowska H, Maslinski W (1999) Expression of mRNA encoding muscarinic receptor subtypes in neutrophils of patients with rheumatoid arthritis. Ann N Y Acad Sci 876:301–304

Bidart JM, Moingeon P, Bohuon C (1983) Muscarinic cholinergic receptors on circulating human lymphocytes: evidence for a particulate binding site. Res Commun Chem Pathol Pharmacol 39:169–172

Bos IS, Gosens R, Zuidhof AB, Schaafsma D, Halayko AJ, Meurs H, Zaagsma J (2007) Inhibition of allergen-induced airway remodelling by tiotropium and budesonide: a comparison. Eur Respir J 30:653–661

Bousquet J, Jeffery PK, Busse WW, Johnson M, Vignola AM (2000) Asthma. From bronchoconstriction to airways inflammation and remodeling. Am J Respir Crit Care Med 161:1720–1745

Buhling F, Lieder N, Kuhlmann UC, Waldburg N, Welte T (2007) Tiotropium suppresses acetylcholine-induced release of chemotactic mediators in vitro. Respir Med 101:2386–2394

Bulloch K, Pomerantz W (1984) Autonomic nervous system innervation of thymic-related lymphoid tissue in wildtype and nude mice. J Comp Neurol 228:57–68

Casaburi R, Mahler DA, Jones PW, Wanner A, San PG, ZuWallack RL, Menjoge SS, Serby CW, Witek T Jr (2002) A long-term evaluation of once-daily inhaled tiotropium in chronic obstructive pulmonary disease. Eur Respir J 19:217–224

Costa P, Traver DJ, Auger CB, Costa LG (1994) Expression of cholinergic muscarinic receptor subtypes mRNA in rat blood mononuclear cells. Immunopharmacology 28:113–123

Costello RW, Schofield BH, Kephart GM, Gleich GJ, Jacoby DB, Fryer AD (1997) Localization of eosinophils to airway nerves and effect on neuronal M2 muscarinic receptor function. Am J Physiol 273:L93–L103

Costello RW, Jacoby DB, Gleich GJ, Fryer AD (2000) Eosinophils and airway nerves in asthma. Histol Histopathol 15:861–868

Cui Y, Devillier P, Kuang X, Wang H, Zhu L, Xu Z, Xia Z, Zemoura L, Advenier C, Chen H (2010) Tiotropium reduction of lung inflammation in a model of chronic gastro-oesophageal reflux. Eur Respir J 35:1370–1376

Disse B (2001) Antimuscarinic treatment for lung diseases from research to clinical practice. Life Sci 68:2557–2564

Durcan N, Costello RW, McLean WG, Blusztajn J, Madziar B, Fenech AG, Hall IP, Gleich GJ, McGarvey L, Walsh MT (2006) Eosinophil-mediated cholinergic nerve remodeling. Am J Respir Cell Mol Biol 34:775–786

Eva C, Ferrero P, Rocca P, Funaro A, Bergamasco B, Ravizza L, Genazzani E (1989) [3H]N-methylscopolamine binding to muscarinic receptors in human peripheral blood lymphocytes: characterization, localization on T-lymphocyte subsets and age-dependent changes. Neuropharmacology 28:719–726

Ferrero P, Rocca P, Eva C, Benna P, Rebaudengo N, Ravizza L, Genazzani E, Bergamasco B (1991) An analysis of lymphocyte 3H-N-methyl-scopolamine binding in neurological patients. Evidence of altered binding in Alzheimer's disease. Brain 114(Pt 4):1759–1770

Fujii T, Kawashima K (2000a) Ca^{2+} oscillation and c-fos gene expression induced via muscarinic acetylcholine receptor in human T- and B-cell lines. Naunyn Schmiedebergs Arch Pharmacol 362:14–21

Fujii T, Kawashima K (2000b) Calcium signaling and c-Fos gene expression via M3 muscarinic acetylcholine receptors in human T- and B-cells. Jpn J Pharmacol 84:124–132

Fujii T, Kawashima K (2000c) YM905, a novel M3 antagonist, inhibits Ca^{2+} signaling and c-fos gene expression mediated via muscarinic receptors in human T cells. Gen Pharmacol 35:71–75

Fujii T, Yamada S, Yamaguchi N, Fujimoto K, Suzuki T, Kawashima K (1995) Species differences in the concentration of acetylcholine, a neurotransmitter, in whole blood and plasma. Neurosci Lett 201:207–210

Fujii T, Tajima S, Yamada S, Watanabe Y, Sato KZ, Matsui M, Misawa H, Kasahara T, Kawashima K (1999) Constitutive expression of mRNA for the same choline acetyltransferase as that in the nervous system, an acetylcholine-synthesizing enzyme, in human leukemic T-cell lines. Neurosci Lett 259:71–74

Fujii YX, Tashiro A, Arimoto K, Fujigaya H, Moriwaki Y, Misawa H, Fujii T, Matsui M, Kasahara T, Kawashima K (2007) Diminished antigen-specific IgG1 and interleukin-6 production and acetylcholinesterase expression in combined M1 and M5 muscarinic acetylcholine receptor knockout mice. J Neuroimmunol 188:80–85

Fujino H, Kitamura Y, Yada T, Uehara T, Nomura Y (1997) Stimulatory roles of muscarinic acetylcholine receptors on T cell antigen receptor/CD3 complex-mediated interleukin-2 production in human peripheral blood lymphocytes. Mol Pharmacol 51:1007–1014

Genaro AM, Cremaschi GA, Borda ES (1993) Muscarinic cholinergic receptors on murine lymphocyte subpopulations. Selective interactions with second messenger response system upon pharmacological stimulation. Immunopharmacology 26:21–29

Gordon MA, Cohen JJ, Wilson IB (1978) Muscarinic cholinergic receptors in murine lymphocytes: demonstration by direct binding. Proc Natl Acad Sci U S A 75:2902–2904

Gorski P, Pazdrak K, Ruta U (1993) Effect of ipratropium on nasal reactivity to histamine and eosinophil influx in perennial allergic rhinitis. Eur J Clin Pharmacol 44:545–547
Gosens R, Bos IS, Zaagsma J, Meurs H (2005) Protective effects of tiotropium bromide in the progression of airway smooth muscle remodeling. Am J Respir Crit Care Med 171:1096–1102
Gwilt CR, Donnelly LE, Rogers DF (2007) The non-neuronal cholinergic system in the airways: an unappreciated regulatory role in pulmonary inflammation? Pharmacol Ther 115:208–222
Hagforsen E, Einarsson A, Aronsson F, Nordlind K, Michaelsson G (2000) The distribution of choline acetyltransferase- and acetylcholinesterase-like immunoreactivity in the palmar skin of patients with palmoplantar pustulosis. Br J Dermatol 142:234–242
Hecker A, Lips KS, Pfeil U, Kummer W, Padberg W, Grau V (2006) Peripheral choline acetyltransferase is expressed by monocytes and upregulated during renal allograft rejection in rats. J Mol Neurosci 30:23–24
Hellstrom-Lindahl E, Nordberg A (1996) Muscarinic receptor subtypes in subpopulations of human blood mononuclear cells as analyzed by RT-PCR technique. J Neuroimmunol 68:139–144
Hogan SP (2007) Recent advances in eosinophil biology. Int Arch Allergy Immunol 143(Suppl 1):3–14
Jones SV, Choi OH, Beaven MA (1991) Carbachol induces secretion in a mast cell line (RBL-2H3) transfected with the ml muscarinic receptor gene. FEBS Lett 289:47–50
Jositsch G, Papadakis T, Haberberger RV, Wolff M, Wess J, Kummer W (2009) Suitability of muscarinic acetylcholine receptor antibodies for immunohistochemistry evaluated on tissue sections of receptor gene-deficient mice. Naunyn Schmiedebergs Arch Pharmacol 379:389–395
Kawashima K, Fujii T (2000) Extraneuronal cholinergic system in lymphocytes. Pharmacol Ther 86:29–48
Kawashima K, Fujii T (2004) Expression of non-neuronal acetylcholine in lymphocytes and its contribution to the regulation of immune function. Front Biosci 9:2063–2085
Kawashima K, Kajiyama K, Suzuki T, Fujimoto K (1993) Presence of acetylcholine in blood and its localization in circulating mononuclear leukocytes of humans. Biog Amine 9:251–258
Kawashima K, Yoshikawa K, Fujii YX, Moriwaki Y, Misawa H (2007) Expression and function of genes encoding cholinergic components in murine immune cells. Life Sci 80:2314–2319
Kendall MD, al-Shawaf AA (1991) Innervation of the rat thymus gland. Brain Behav Immun 5:9–28
Krzystyniak K, Ryzewski J, Maslinski W (1982) Cholinergic receptors of rat lymphocytes during adjuvant polyarthritis. Biochim Biophys Acta 691:341–344
Kummer W, Wiegand S, Akinci S, Schinkel AH, Wess J, Koepsell H, Haberberger RV, Lips KS (2006) Role of acetylcholine and muscarinic receptors in serotonin-induced bronchoconstriction in the mouse. J Mol Neurosci 30:67–68
Levy RM, Rose JE, Johnson JS (1976) Effect of vagotomy on anaphylaxis in the rat. Clin Exp Immunol 24:96–101
Lips KS, Volk C, Schmitt BM, Pfeil U, Arndt P, Miska D, Ermert L, Kummer W, Koepsell H (2005) Polyspecific cation transporters mediate luminal release of acetylcholine from bronchial epithelium. Am J Respir Cell Mol Biol 33:79–88
Liu T, Xie C, Chen X, Zhao F, Liu AM, Cho DB, Chong J, Yang PC (2010) Role of muscarinic receptor activation in regulating immune cell activity in nasal mucosa. Allergy 65:969–977
Ma B, von Wasielewski R, Lindenmaier W, Dittmar KE (2007) Immmunohistochemical study of the blood and lymphatic vasculature and the innervation of mouse gut and gut-associated lymphoid tissue. Anat Histol Embryol 36:62–74
Masini E, Fantozzi R, Blandina P, Brunelleschi S, Mannaioni PF (1983) Muscarinic cholinergic receptor binding in rat mast cells. Agents Actions 13:327–332
Masini E, Fantozzi R, Conti A, Blandina P, Brunelleschi S, Mannaioni PF (1985) Immunological modulation of cholinergic histamine release in isolated rat mast cells. Agents Actions 16:152–154

Maslinski W, Grabczewska E, Ryzewski J (1980) Acetylcholine receptors of rat lymphocytes. Biochim Biophys Acta 633:269–273

McQueen DS, Donaldson K, Bond SM, McNeilly JD, Newman S, Barton NJ, Duffin R (2007) Bilateral vagotomy or atropine pre-treatment reduces experimental diesel-soot induced lung inflammation. Toxicol Appl Pharmacol 219:62–71

Moir LM, Leung SY, Eynott PR, McVicker CG, Ward JP, Chung KF, Hirst SJ (2003) Repeated allergen inhalation induces phenotypic modulation of smooth muscle in bronchioles of sensitized rats. Am J Physiol Lung Cell Mol Physiol 284:L148–L159

Nechushtan H, Soreq H, Kuperstein V, Tshori S, Razin E (1996) Murine and human mast cell express acetylcholinesterase. FEBS Lett 379:1–6

Nemmar A, Delaunois A, Beckers JF, Sulon J, Bloden S, Gustin P (1999) Modulatory effect of imetit, a histamine H3 receptor agonist, on C-fibers, cholinergic fibers and mast cells in rabbit lungs in vitro. Eur J Pharmacol 371:23–30

Neumann S, Razen M, Habermehl P, Meyer CU, Zepp F, Kirkpatrick CJ, Wessler I (2007) The non-neuronal cholinergic system in peripheral blood cells: effects of nicotinic and muscarinic receptor antagonists on phagocytosis, respiratory burst and migration. Life Sci 80:2361–2364

Nomura J, Hosoi T, Okuma Y, Nomura Y (2003) The presence and functions of muscarinic receptors in human T cells: the involvement in IL-2 and IL-2 receptor system. Life Sci 72:2121–2126

Ohnishi K, Takagi M, Kurokawa Y, Satomi S, Konttinen YT (1998) Matrix metalloproteinase-mediated extracellular matrix protein degradation in human pulmonary emphysema. Lab Invest 78:1077–1087

Ohta S, Oda N, Yokoe T, Tanaka A, Yamamoto Y, Watanabe Y, Minoguchi K, Ohnishi T, Hirose T, Nagase H, Ohta K, Adachi M (2010) Effect of tiotropium bromide on airway inflammation and remodelling in a mouse model of asthma. Clin Exp Allergy 40:1266–1275

Okuma Y, Nomura Y (2001) Roles of muscarinic acetylcholine receptors in interleukin-2 synthesis in lymphocytes. Jpn J Pharmacol 85:16–19

Pahl A, Bauhofer A, Petzold U, Cnota PJ, Maus J, Brune K, Szelenyi S (2006) Synergistic effects of the anti-cholinergic R, R-glycopyrrolate with anti-inflammatory drugs. Biochem Pharmacol 72:1690–1696

Perng DW, Tao CW, Su KC, Tsai CC, Liu LY, Lee YC (2009) Anti-inflammatory effects of salmeterol/fluticasone, tiotropium/fluticasone or tiotropium in COPD. Eur Respir J 33:778–784

Powrie DJ, Wilkinson TM, Donaldson GC, Jones P, Scrine K, Viel K, Kesten S, Wedzicha JA (2007) Effect of tiotropium on sputum and serum inflammatory markers and exacerbations in COPD. Eur Respir J 30:472–478

Profita M, Giorgi RD, Sala A, Bonanno A, Riccobono L, Mirabella F, Gjomarkaj M, Bonsignore G, Bousquet J, Vignola AM (2005) Muscarinic receptors, leukotriene B4 production and neutrophilic inflammation in COPD patients. Allergy 60:1361–1369

Prync AE, Arzt E, Fernandez Castelo S, Finkielman S, Nahmod V (1992) The inhibitory effect of the muscarinic agonist pilocarpine on lymphocyte activation involves the IL-2 pathway and the increase in suppressor cell function. Int J Neurosci 62:277–285

Qiu YH, Peng YP, Zhang QQ, Wang JH (1995) Effect of acetylcholine on the proliferation of T lymphocyte of rat spleen. Sheng Li Xue Bao 47:275–280

Qiu Y, Peng Y, Wang J (1996) Immunoregulatory role of neurotransmitters. Adv Neuroimmunol 6:223–231

Rabey JM, Shenkman L, Gilad GM (1986) Cholinergic muscarinic binding by human lymphocytes: changes with aging, antagonist treatment, and senile dementia of the Alzheimer type. Ann Neurol 20:628–631

Razani-Boroujerdi S, Behl M, Hahn FF, Pena-Philippides JC, Hutt J, Sopori ML (2008) Role of muscarinic receptors in the regulation of immune and inflammatory responses. J Neuroimmunol 194:83–88

Reinheimer T, Baumgartner D, Hohle KD, Racke K, Wessler I (1997) Acetylcholine via muscarinic receptors inhibits histamine release from human isolated bronchi. Am J Respir Crit Care Med 156:389–395

Reinheimer T, Mohlig T, Zimmermann S, Hohle KD, Wessler I (2000) Muscarinic control of histamine release from airways. Inhibitory M1-receptors in human bronchi but absence in rat trachea. Am J Respir Crit Care Med 162:534–538

Ricci A, Amenta F, Bronzetti E, Mannino F, Mariotta S, Tayebati SK (2002) Expression of peripheral blood lymphocyte muscarinic cholinergic receptor subtypes in airway hyperresponsiveness. J Neuroimmunol 129:178–185

Sandberg G (1994) Leukocyte mobilization from the guinea pig spleen by muscarinic cholinergic stimulation. Experientia 50:40–43

Sato E, Koyama S, Okubo Y, Kubo K, Sekiguchi M (1998) Acetylcholine stimulates alveolar macrophages to release inflammatory cell chemotactic activity. Am J Physiol 274:L970–L979

Selvan RS, Venkateswaran KS, Rao AR (1989) Influence of arecoline on immune system: I. Short term effects on general parameters and on the adrenal and lymphoid organs. Immunopharmacol Immunotoxicol 11:347–377

Tayebati SK, Codini M, Gallai V, Mannino F, Parnetti L, Ricci A, Sarchielli P, Amenta F (1999) Radioligand binding assay of M1-M5 muscarinic cholinergic receptor subtypes in human peripheral blood lymphocytes. J Neuroimmunol 99:224–229

Tayebati SK, El-Assouad D, Ricci A, Amenta F (2002) Immunochemical and immunocytochemical characterization of cholinergic markers in human peripheral blood lymphocytes. J Neuroimmunol 132:147–155

Tominaga K, Hato F, Kinoshita Y, Tominaga M, Yamada M (1992) Enhancement of DNA synthesis in rat thymocytes by stimulating their muscarinic acetylcholine receptors. Cell Mol Biol 38:815–822 (Noisy-le-grand)

Verbout NG, Lorton JK, Jacoby DB, Fryer AD (2007) Atropine pretreatment enhances airway hyperreactivity in antigen-challenged guinea pigs through an eosinophil-dependent mechanism. Am J Physiol Lung Cell Mol Physiol 292:L1126–L1135

Verbout NG (2008) Role of eosinophils and muscarinic receptors in paradoxical airway hyperreactivity associated with antigen challenge, Dissertation, Oregon Health and Science University

Verbout NG, Jacoby DB, Gleich GJ, Fryer AD (2009) Atropine-enhanced, antigen challenge-induced airway hyperreactivity in guinea pigs is mediated by eosinophils and nerve growth factor. Am J Physiol Lung Cell Mol Physiol 297:L228–L237

Vernooy JH, Lindeman JH, Jacobs JA, Hanemaaijer R, Wouters EF (2004) Increased activity of matrix metalloproteinase-8 and matrix metalloproteinase-9 in induced sputum from patients with COPD. Chest 126:1802–1810

Vezys V, Masopust D, Desmarets M, Wess J, Zimring JC (2007) Analysis of CD8+ T cell-mediated anti-viral responses in mice with targeted deletions of the M1 or M5 muscarinic cholinergic receptors. Life Sci 80:2330–2333

Wen XM, Zhang YL, Liu XM, Guo SX, Wang H (2006) Immune responses in mice to arecoline mediated by lymphocyte muscarinic acetylcholine receptor. Cell Biol Int 30:1048–1053

Wessler IK, Kirkpatrick CJ (2001) The non-neuronal cholinergic system: an emerging drug target in the airways. Pulm Pharmacol Ther 14:423–434

Wessler I, Kirkpatrick CJ, Racke K (1999) The cholinergic 'pitfall': acetylcholine, a universal cell molecule in biological systems, including humans. Clin Exp Pharmacol Physiol 26:198–205

Wessler I, Roth E, Deutsch C, Brockerhoff P, Bittinger F, Kirkpatrick CJ, Kilbinger H (2001) Release of non-neuronal acetylcholine from the isolated human placenta is mediated by organic cation transporters. Br J Pharmacol 134:951–956

Wessler I, Reinheimer T, Kilbinger H, Bittinger F, Kirkpatrick CJ, Saloga J, Knop J (2003) Increased acetylcholine levels in skin biopsies of patients with atopic dermatitis. Life Sci 72:2169–2172

Wessler I, Holper B, Kortsik C, Buhl R, Kilbinger H, Kirkpatrick CJ (2007) Dysfunctional inhibitory muscarinic receptors mediate enhanced histamine release in isolated human bronchi. Life Sci 80:2294–2297

Wollin L, Pieper MP (2010) Tiotropium bromide exerts anti-inflammatory activity in a cigarette smoke mouse model of COPD. Pulm Pharmacol Ther 23:345–354

Woodruff PG, Dolganov GM, Ferrando RE, Donnelly S, Hays SR, Solberg OD, Carter R, Wong HH, Cadbury PS, Fahy JV (2004) Hyperplasia of smooth muscle in mild to moderate asthma without changes in cell size or gene expression. Am J Respir Crit Care Med 169:1001–1006

Zalcman SJ, Neckers LM, Kaayalp O, Wyatt RJ (1981) Muscarinic cholinergic binding sites on intact human lymphocytes. Life Sci 29:69–73

Zhang W, Fievez L, Cheu E, Bureau F, Rong W, Zhang F, Zhang Y, Advenier C, Gustin P (2010) Anti-inflammatory effects of formoterol and ipratropium bromide against acute cadmium-induced pulmonary inflammation in rats. Eur J Pharmacol 628:171–178

Zimring JC, Kapp LM, Yamada M, Wess J, Kapp JA (2005) Regulation of CD8+ cytolytic T lymphocyte differentiation by a cholinergic pathway. J Neuroimmunol 164:66–75

Muscarinic Receptor Agonists and Antagonists: Effects on Keratinocyte Functions

Sergei A. Grando

Abstract The stratified epithelium enveloping the skin and lining the surfaces of oral and vaginal mucosa is comprised by keratinocytes that synthesize, secrete, degrade, and respond to acetylcholine via muscarinic and nicotinic receptors. The two pathways may compete or synergize with one another, so that net biologic effect represents the biologic sum of the effects of distinct acetylcholine receptors expressed by a keratinocyte at a particular stage of its development. Keratinocytes express a unique combination of muscarinic receptor subtypes at each stage of their development. Experimental results indicate that muscarinic receptors expressed in human keratinocytes regulate their viability, proliferation, migration, adhesion, and terminal differentiation, hair follicle cycling, and secretion of humectants, cytokines, and growth factors. Learning the muscarinic pharmacology of keratinocyte development and functions has salient clinical implications for patients with nonhealing wounds, mucocutaneous cancers, and various autoimmune and inflammatory diseases. Successful therapy of pemphigus lesions with topical pilocarpine and disappearance of psoriatic lesions due to systemic atropine therapy illustrate that such therapeutic approach is feasible.

Keywords Oral and epidermal keratinocytes • Proliferation • Migration • Adhesion • Apoptosis • Differentiation

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1 Introduction

The stratified epithelium enveloping the skin and lining the surfaces of oral and vaginal mucosa is comprised by keratinocytes (KCs) that synthesize, secrete, degrade, and respond to acetylcholine (ACh) via two classes of classic cholinergic receptors: the muscarinic and the nicotinic receptors (mAChRs and nAChRs). The continuous cycle of keratinocyte birth and death is a self-sustained process controlled, in part, by auto/paracrine acetylcholine (ACh) through the signaling pathways coupling each type of ACh receptors to a particular cell function. The higher concentration of free ACh is found in human skin, 1,000 pmol ACh/g, compared to oral mucosa, 8 pmol (Klapproth et al. 1997). Human KCs synthesize approximately 2×10^{-17} moles and secrete 7×10^{-19} moles of ACh per min (Grando et al. 1993b). The nonneuronal cholinergic system of human KCs represents a previously not appreciated regulatory pathway, or "ACh axis" (Fig. 1). ACh can stimulate KCs simultaneously through two distinct types of ACh signaling pathways: (1) the ionic events, generated by opening of nAChR channels; and (2) the metabolic events, elicited by ACh binding to the G-protein-coupled mAChRs. Simultaneous stimulation of nAChRs and mAChRs by ACh may be required to synchronize and balance ionic and metabolic events in a single cell, and the net biologic response is determined by a unique combination of nAChRs and mAChRs expressed by an individual cell. For instance, the stimulatory effect of ACh on Ca^{2+} influx in KCs, mediated by its nicotinic action, is balanced by an inhibitory effect, mediated by its muscarinic action (Grando and Horton 1997). Simultaneous activation of both pathways may produce a kind of a yin–yang regulatory balance rendering ACh the "pacemaker" function in the mucocutaneous

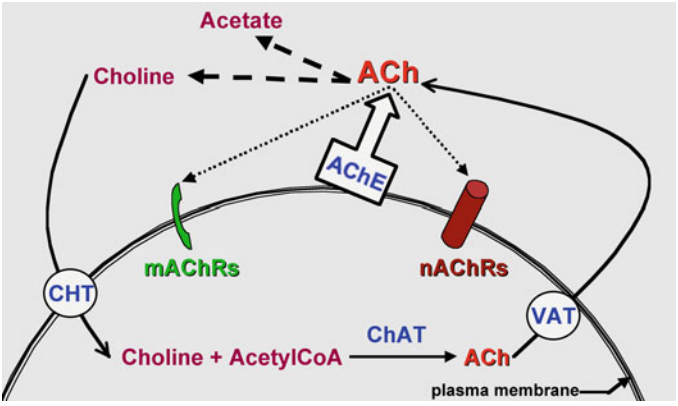

Fig. 1 Scheme of ACh metabolism and signaling in KCs (Grando 2006). *ACh* acetylcholine; *AChE* acetylcholinesterase; *ChAT* choline acetyltransferase; *CHT* high-affinity choline transporter; *CoA* coenzyme A; *mAChRs* muscarinic acetylcholine receptor subtypes identified in human KCs; *nAChRs* nicotinic acetylcholine receptor identified in human KCs; *VAT* vesicular acetylcholine transporter

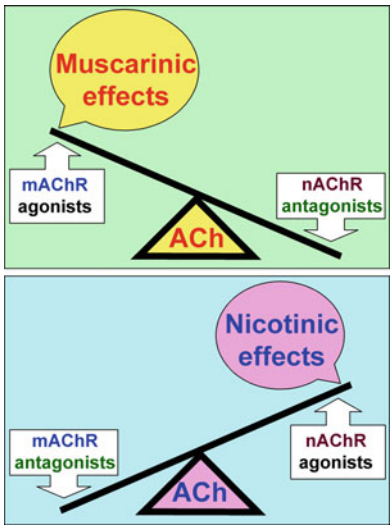

Fig. 2 Pharmacologic approaches to skew an equilibrium of auto/paracrine ACh signaling in a single keratinocyte

epithelium. Hence, the dynamic equilibrium between nicotinic and muscarinic signaling can be skewed by acting upon either class of ACh receptors (Fig. 2).

The external environment, hormones, growth factors, cytokines, and the neural system can modify the ACh axis by altering the expression of genes encoding cholinergic enzymes and receptors. This is how ACh can play an intermediary role in the interactions of host with the environment. In this model, binding of ACh to the cell membrane simultaneously elicits several diverse biochemical events, the "biologic sum" of which, taken together with cumulative effects of other hormonal and environmental stimuli, determines a distinct change in cell cycle and function. The detailed reviews of the structure and function of the keratinocyte ACh axis have been published (Grando 1997; Grando et al. 2006; Kurzen and Schallreuter 2004; Kurzen et al. 2007). This work analyzes the cellular and molecular mechanisms mediating the biologic effects of agonists and antagonists acting at keratinocyte mAChRs.

2 Localization of Keratinocyte mAChR Subtypes in the Mucocutaneous Tissues

The early radioligand binding experiments utilizing [^{3}H]atropine and [^{3}H] quinuclidinyl benzilate demonstrated that human KCs both in vitro and in vivo express heterogeneous population of mAChRs, totaling to approximately 2.5×10^5 receptors per cell (Grando and Dahl 1993; Grando et al. 1995b). Thereafter, M_1, M_2, M_3, M_4, and M_5 mAChR subtypes have been shown to be expressed in KCs at the mRNA and protein levels and characterized in functional assays (Elwary et al. 2004; Kurzen et al. 2004; Kurzen and Schallreuter 2004; Ndoye et al. 1998).

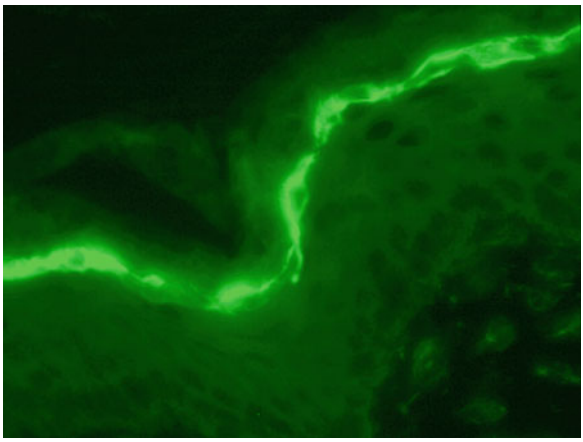

Fig. 3 Immunolocalization of M_2 mAChR subtype in human epidermis**.** The cryostat section of freshly frozen normal human foreskin was fixed with phosphate buffered saline containing 3% formaldehyde and 7% sucrose, and incubated overnight at 4°C with rabbit anti-M_2 antibody (Research & Diagnostic Antibodies, North Las Vegas, NV) diluted 1:1,000. Binding of the primary antibody was visualized using secondary, FITC-labeled swine anti-rabbit IgG antibody (DAKO Corporation, Carpinteria, CA). Both preincubation of the anti-peptide immune sera with the synthetic peptides used for immunization and omitting the primary antibody abolished the fluorescent staining

To visualize keratinocyte mAChRs, we raised rabbit antisera to synthetic peptide analogues of the carboxyl terminal regions of each receptor subtype (Ndoye et al. 1998). The immunofluorescent staining patterns produced by each antibody in epidermis suggested that the profile of keratinocyte mAChRs changes during the epidermal turnover. A semiquantitative analysis of the intensity of fluorescence revealed that basal cells predominantly express M_3, prickle cells have equally high levels of M_4 and M_5, and the uppermost granular cells possess chiefly the M_1 subtype (Ndoye et al. 1998; Nguyen et al. 2001). These results were corroborated, in the most part, by Kurzen et al. (Kurzen et al. 2004), who, however, found M_5 predominantly in the basal layer. The reports on M_2 mAChR localization in the epidermis are controversial. According to Kurzen et al. (2004), M_2 is expressed by basal KCs, whereas Elwary et al. (2004) observed it throughout epidermis. In our studies, M_2 immunofluorescence was localized to the granular epidermal layer (Fig. 3). This discrepancy may be explained by age-, gender-, and anatomic region-dependent variations in the M_2 expression by KCs. In the rat epidermis, M_2 immunoreactivity is predominant in KCs of the granular layer (Haberberger and Bodenbenner 2000).

All anti-mAChR antibodies produced an intercellular, web-like epidermal staining, which is consistent with antibody binding to the cell membrane (Beutner et al. 1985). The confocal and electron microscopic studies employing the pan-mAChR antibody M35 (Carsi-Gabrenas et al. 1997) demonstrated that an intercellular, pemphigus-like staining pattern could be explained by an accumulation of

receptor molecules at the cell membrane areas associated with desmosomes (Grando et al. 1995b). The M35 antibody recognizes an extracellular epitope of mAChRs involved in receptor signaling (Leiber et al. 1984). The presence of mAChRs on the cell membrane areas overlying desmosomes at the sites of cell–cell contacts would be useful if these receptors foster intercellular communications between neighboring KCs.

The mAChRs are also expressed in hair follicles and skin adnexae (Kurzen et al. 2004). In the subinfundibular outer root sheath of the hair follicle, M_1 and M_3 are predominantly present in the basal cell layer, whereas M_4 and M_5 are present in the central cell layer. M_1, M_2, M_3, and M_4 are strongly expressed in the inner root sheath. Undifferentiated sebocytes express M_3–M_5, whereas M_2 and M_4 are found in mature sebocytes. In sweat glands, M_2–M_5 receptors are most prominent in the myoepithelial cells, whereas M_1, M_3, and M_4 are present in the acinar cells.

Human gingival KCs express M_2, M_3, M_4, and M_5 mAChR subtypes (Arredondo et al. 2003). The antibodies mapped these receptors in the epithelium of human attached gingiva and also visualized them on the cell membrane of cultured cells. The M_2 antibody stained the entire mucosa, being most abundant in the middle epithelial compartment. In contrast, the bulk of M_3 and M_4 immunoreactivities were localized to the lowermost rows of the epithelial cells, especially to the rete ridges. The M_5 antibody stained predominantly the lower 2/3 portion of the epithelium. Exposure of cultured gingival KCs to cigarette smoke or pure nicotine downregulated expression of mRNA encoding the M_2 and M_3 (Arredondo et al. 2005) subtypes.

Since KCs appeared to express a unique combination of mAChR subtypes at each stage of their development in the stratified epithelium, each receptor subtype may regulate a specific cell function. Hence, ACh and muscarinic drugs should exert distinct biological effects on KCs being at different stages of their development.

3 Muscarinic Regulation of Keratinocyte Cell Cycle Progression

Blocking the mAChRs expressed in epidermal KCs cultured at low calcium medium with propylbenzilylcholine mustard (PrBCM) for 48 h significantly increased cell number (Grando et al. 1993a). Long-term incubation with another muscarinic antagonist, scopolamine, resulted in the maintenance of an immature phenotype when KCs were exposed to agents usually causing differentiation (Grando and Lynch 1993). In keeping with these findings, treatment of the organotypic culture of human epidermis with atropine upregulating the hyperproliferation-associated markers cytokeratins 6/16 and downregulating the differentiation marker filaggrin (Kurzen et al. 2004). Taken together, these in vitro experiments suggested that the major biologic role of ACh signaling through the muscarinic pathway in immature KCs is to control their growth and facilitate

differentiation. This conclusion has been corroborated by the results of experiments employing arecoline – the pan-muscarinic agonist that can bind/activate M_1 (Ghelardini et al. 2001), M_2 (Yang et al. 2000), M_3 (Xie et al. 2004), and M_4 (McKinney et al. 1991) receptor subtypes. Arecoline suppressed keratinocyte growth in a dose-dependent fashion (Jeng et al. 1999) and induced cell cycle arrest at the G_1/G_0 phase (Chang et al. 2001; Thangjam and Kondaiah 2009).

To characterize the role of keratinocyte mAChRs in the regulation of cell cycle progression, we identified the muscarine-induced changes of the regulatory genes that can be abolished in the presence of atropine (Arredondo et al. 2003). Muscarine produced a severalfold increase of Ki-67, a proliferation marker expressed in the nucleolus during G_1, S, G_2, and M phase (MacCallum and Hall 1999), and PCNA (proliferating cell nuclear antigen), an auxiliary factor for DNA polymerase δ and ε that is expressed primarily at G_1–S phase and plays a role in chromosomal DNA replication and repair (Tsurimoto 1999). Simultaneously with upregulation of these cell cycle progression-associated markers, stimulation of keratinocyte mAChRs also led to a reciprocal increase of p53 and p21, which play a significant role in the induction of cell cycle arrest at G_1/S. p53, known as a tumor suppressor gene, regulates the transcription of p21, thereby inhibiting S phase entry primarily via inhibition of cyclin-dependent protein kinases and p21 binding and inhibition of PCNA. Stimulation of keratinocyte mAChRs with arecoline induced expression of human telomerase reverse transcriptase mRNA and protein (Gao et al. 2007).

Altogether, these findings suggest that downstream signaling from mAChRs expressed in KCs initiates complex changes in cell cycle regulation, including differentiation-inducing effects, DNA repair and replication anomalies, and proapoptotic gene activation. Therefore, a major biological function of keratinocyte mAChRs could be coordination of the process of cell development in epidermis.

4 Muscarinic Regulation of Keratinocyte Crawling Locomotion

It is widely accepted that keratinocyte migration is an essential step of repair of epithelial wounds. A few hours after wounding, KCs migrate laterally over the wound bed both as a cellular sheet and as individually crawling cells (Donaldson and Mahan 1988). Directional migration is coupled to the formation of new actin filaments at the leading lamella (Theriot and Mitchison 1991). Crawling locomotion of all KCs comprising the advancing sheet, regardless of distance from the wound edge, depends on the cell-to-substrate attachment. Because KCs synthesize extracellular matrix (ECM) proteins and express them on their surfaces (O'Keefe et al. 1984; Schafer et al. 1991), integrin receptors of one cell may interact with ECM on the surface of an adjacent cell, thus providing an abutment for the upper cell that crawls over the lower one. The process of keratinocyte migration can be subdivided into four component events (1) forward protrusion of cell cytoplasm, (2) focal adhesion of the protruded part to a substrate, (3) contraction of the rear end to push the cell "body" over this focal adhesion, and (4) detachment of the rear focal

adhesion to free the cell tail (Harris 1990). Focal adhesions provide the primary stabilizing force for leading lamella and serve as an abutment during forward relocation of cell body. Integrins are frequently clustered into focal adhesions, and focal complexes, in which numerous signaling components are concentrated (Schoenwaelder and Burridge 1999). Different integrins mediate different types of interactions of KCs with ECM. To attach to fibronectin, vitronectin, thrombospondin, and other proteins comprising the provisional matrix, crawling KCs express the $\alpha_5\beta_1$, $\alpha_V\beta_5$, and $\alpha_V\beta_6$ integrins (Larjava et al. 1993). In contrast, the $\alpha_2\beta_1$ and $\alpha_3\beta_1$ integrins are present at the sites of cell–cell contacts (Marchisio et al. 1991). Thus, based on their differential expression in wounded versus intact epidermis and their relationship to keratinocyte migration, the integrin proteins expressed by KCs can be tentatively divided into two groups, "sedentary," or nonmotile (α_2 and α_3), versus "migratory," or motile (α_5, α_v, and β_5) integrins.

Galvanotropism in a direct current (DC) electric field represents a natural model for studying the intrinsic mechanisms mediating reorientation of KCs prior to the onset of crawling locomotion toward a chemoattractant. The exposed KCs orient the axis of direction of their migration parallel to the field lines and migrate toward the cathode (Nishimura et al. 1996). Recent results indicate that keratinocyte galvanotaxis toward the cathode is, in effect, chemotaxis toward the concentration gradient of ACh that it creates in a DC field due to its highly positive charge (Chernyavsky et al. 2005). We observed that M_1 mAChR and $\alpha7$ nAChR relocate to and cluster at the cell pole facing cathode within 15–30 min of DC field application. Redistribution of M_1 immunoreactivity to the leading edge of KCs precedes crescent shape formation required for directional migration, indicating that among the M_1–M_5 mAChR subtypes expressed in human KCs, the M_1 is primarily involved in ACh chemotaxis. When the cells were treated with the M_1 inhibitor MT7, their ability to turn to the cathode was completely blocked. MT7 decreased the number of KCs responding to the DC field. Silencing the M_1 gene expression with siRNA also partially blocked the galvanotropism. The galvanotropism was completely blocked, however, when KCs were coexposed to the $\alpha7$ nAChR inhibitor α-bungarotoxin, indicating that the effects of M_1 synergize with those of $\alpha7$ nAChR in mediating directional migration of KCs toward the ACh gradient. Indeed, simultaneous silencing of the $\alpha7$ and M_1 genes produced a stronger inhibiting effect compared to silencing of each receptor gene individually. The downstream signaling from M_1 mAChR and $\alpha7$ nAChR regulating directional migration proceeded through common steps of the Ras/Raf-1/MEK1/ERK pathway with an endpoint effect of upregulation of the sedentary integrins α_2 and α_3. Thus, relocation of M_1 to the pole of the cell facing a chemoattractant may provide for a compartmentalized upregulation of α_2 and α_3 integrins via the MEK1/ERK pathways. Since both α_2 and α_3 integrins accumulate at the lamellipodium, activation of the M_1-coupled signaling pathway at the very beginning of migration may be required for an extension of the leading lamella and its anchoring to the substrate.

To characterize the physiologic control of crawling locomotion of KCs by ACh, we developed an in vitro model of skin reepithelialization, termed agarose gel keratinocyte outgrowth system (AGKOS), that allows accurate evaluation of

pharmacologic effects on lateral migration of KCs (Grando et al. 1993a). AGKOS proved to be a reliable and highly specific in vitro technique for investigating keratinocyte functions mediating reepithelialization (Chernyavsky et al. 2004a; Chernyavsky et al. 2007). To explore the role of mAChRs in regulating the keratinocyte migratory function and elucidate the mechanisms responsible, we used a combination of three overlapping approaches to inhibit mAChR-coupled signaling pathways (a) pharmacologic blockade with receptor antagonist and regulatory enzyme inhibitors; (b) mAChR gene silencing; and (c) mAChR gene knockout (KO) in mice (Chernyavsky et al. 2004b). AGKOS assays showed that the two anti-M_3 and anti-M_4 mAChR subtype siRNAs differentially affected chemokinesis of transfected human KCs. The siRNA-M_4 significantly decreased and the siRNA-M_3 significantly increased random migration distance of KCs. The enhanced migration of siRNA-M_3 transfected KCs could be inhibited when the cells were fed with the M_4-preferring antagonist MT3. Conversely, the inhibition of migration of the siRNA-M_4 transfected KCs could be prevented in the presence of 4-DAMP that blocks M_3 (but also other mAChR subtypes). When given alone, MT3 reduced migration distance by >50%, whereas 4-DAMP did not produce any significant effect, as could be expected when the two receptors that exhibit reciprocal effects on keratinocyte migration are blocked at the same time. In a separate study, activation of M_3 expressed in HaCaT cells significantly reduced the stimulatory effect of epidermal growth factor on the keratinocyte migration measured by the phagokinetic track assay (Metzger et al. 2005). Taken together, these results indicated that M_3 and M_4 have opposing roles in regulating keratinocyte migration.

The roles of M_3 and M_4 mAChRs in regulating keratinocyte crawling locomotion were further investigated in AGKOS plates loaded with KCs from the epidermis of wild-type (WT) and $M_3-/-$ and $M_4-/-$ mice (Chernyavsky et al. 2004b). As expected, the lack of M_3 receptors was associated with an increase of keratinocyte migration distance by approximately 85%. The $M_4-/-$ KCs showed a reduced migration distance (by >50%). MT3 significantly decreased the migration distances of $M_3-/-$ KCs, whereas 4-DAMP increased the migration distance of $M_4-/-$ KCs.

To elucidate possible involvement of the sedentary and migratory integrins in M_3- and M_4-mediated signaling pathways, we tested the effects of mAChR null mutations on the ability of receptor KO cells to move over the ECM proteins that represent known ligands for the integrin receptors (Chernyavsky et al. 2004b). $\alpha_v\beta_6$ and $\alpha_5\beta_1$ preferentially bind to fibronectin, $\alpha_3\beta_1$ and $\alpha_6\beta_4$ to laminin 5, $\alpha_2\beta_1$ to collagen types I and IV, and $\alpha_5\beta_5$ to vitronectin. To avoid confounding effects of the ECM proteins secreted and deposited underneath the ventral membrane of individual KCs in long-term cultures, we employed a short-term, 24-h-long "scratch assay" in ECM-coated dishes, and treated cells with Mitomycin C to inhibit proliferation. Compared to migration of WT KCs over the ligands of the migratory integrins fibronectin and vitronectin, the migration of $M_3-/-$ KCs was upregulated and that of $M_4-/-$ KCs downregulated. Transfection of human KCs with siRNA-M_3 resulted in a decrease of the relative amounts of α_2 and α_3 integrins by ~50 and 40%, respectively, and an increase of α_5, α_v, and β_5 expression by ~30, 100, and

70%, respectively. The KCs transfected with siRNA-M_4 showed changes in the integrin expression pattern which were, in most cases, reciprocal to those observed in KCs transfected with siRNA-M_3. This suggested that silencing of M_3 favors the expression of the migratory integrins, whereas silencing of M_4 upregulates the sedentary integrins. Taken together, these results indicated that the M_3 and M_4 receptor-dependent changes in keratinocyte crawling locomotion rate were mediated, at least in part, by reciprocal shifts in the integrin expression patterns.

To clarify the molecular mechanism of regulatory effects of M_3 and M_4 on keratinocyte migration, we pharmacologically blocked, or activated, key steps of the signaling pathways known to mediate functions of the $G_{q/11}$- and $G_{i/o}$-coupled mAChRs. It was established that inhibition of migration by M_3 was mediated through Ca^{2+}-dependent guanylyl cyclase/cGMP/protein kinase G signaling pathway. The M_4 effects resulted from inhibition of the inhibitory pathway involving the adenylyl cyclase/cAMP/protein kinase A pathway. Both signaling pathways intersected at Rho, indicating that Rho kinase provides a common effector for M_3 and M_4 regulation of cell migration.

Thus, the M_1/M_3 receptor-induced downstream signaling may support the establishment and/or maintenance of stable connections of KCs with the substrate in culture, and of the epidermis to the underlying dermis in the skin. An M_4-dependent shift in the integrin phenotype to a migratory pattern may be sufficient to launch keratinocyte crawling locomotion. The hypothetical scheme of the signaling mechanisms linking M_1, M_3, and M_4 to regulation of keratinocyte migration and integrin expression is shown in Fig. 4.

The regulatory effects of mAChRs on the keratinocyte migratory function may be responsible for the therapeutic action of cholinomimetics in wound healing. A lotion containing the muscarinic agonist methacholine speeded up healing of neuropathic ulcers, whereas a lotion containing the muscarinic antagonist atropine delayed healing compared with placebo (Dillon 1991). Carbachol, a mixed muscarinic-and-nicotinic agonist and reversible acetylcholinesterase inhibitor (DiPalma 1994), enhanced epithelial resurfacing of mucosal and cutaneous wounds (Colley et al. 1987; Grabovoi et al. 1994). Topical treatment with ACh, carbachol, or the M_1 agonist pilocarpine expedited reepithelialization of corneal defects in rabbits, and it was therefore proposed that ACh may have a place in the treatment of corneal epithelial injuries (Er 1997; Ozturk et al. 1999).

5 Muscarinic Regulation of Epidermal Cohesion

The ability of KCs to adhere to each other determines the integrity of the epithelium enveloping the human body and lining the upper digestive tract. Stable cell-to-cell adhesion of KCs is mediated by the adherence (*zonula adherence*) and desmosomal (*macula adherence*) junctions. Recent research convincingly demonstrated that the physiologic control of keratinocyte adhesion involves auto/paracrine ACh signaling through both muscarinic and nicotinic pathways (reviewed in (Grando 2006; Grando

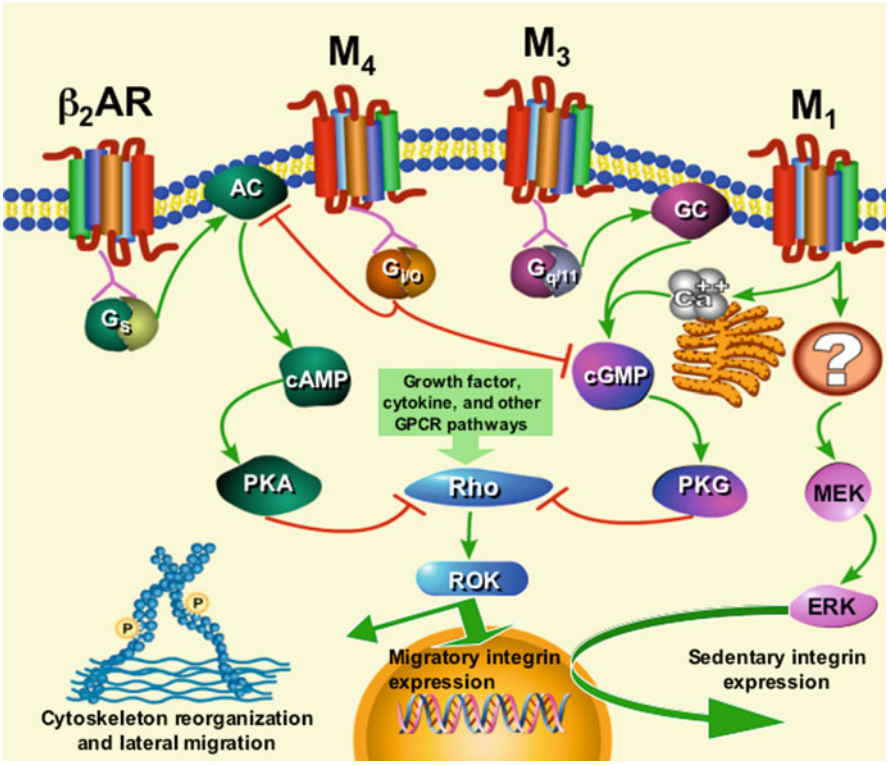

Fig. 4 Hypothetical scheme of stimulatory (*right arrow*) and inhibitory (—|) events within the muscarinic signaling cascades regulating keratinocyte migration (Grando et al. 2006). The downstream signaling from keratinocyte mAChRs involves the second messenger pathways that control expression and activity of the effector molecules mediating crawling locomotion of KCs. *Abbreviations*: *AC* adenylyl cyclase; *AR* adrenergic receptor; Ca^{2+} intracellular free calcium; *cAMP* cyclic AMP; *cGMP* cyclic GMP; *ERK* extracellular signal-regulated kinase; *GC* guanylyl cyclase; *MEK* mitogen activated protein kinase; *PKA* protein kinase A; *PKG* protein kinase G; *ROK* Rho-associated protein kinase

and Kurzen 2009)). Constant stimulation of mAChRs is required to sustain intercellular adhesion in a monolayer, because interrupting the auto/paracrine ACh signaling with mAChR antagonists causes cell–cell detachment (acantholysis) (Grando and Dahl 1993). The rate of acantholysis depends on the dose of drug, but the sequence of morphologic changes is always the same (Fig. 5). Within 3–5 min of addition of an antagonist, the cells of a confluent monolayer abruptly lose their polygonal shape (as if their cytoskeleton has suddenly collapsed), retract their cytoplasmic aprons to separate themselves from neighboring cells, internalize the remaining spindle-shaped cytoplasmic protrusions, and then initiate peripheral cytoplasmic blebbing that gives them a rosette-like appearance. Within 30 min after washing, the cascade of antagonist-induced morphologic changes reverses. The KCs flatten and the blebs on the periphery of their cytoplasm evolve into cytoplasmic aprons that spread

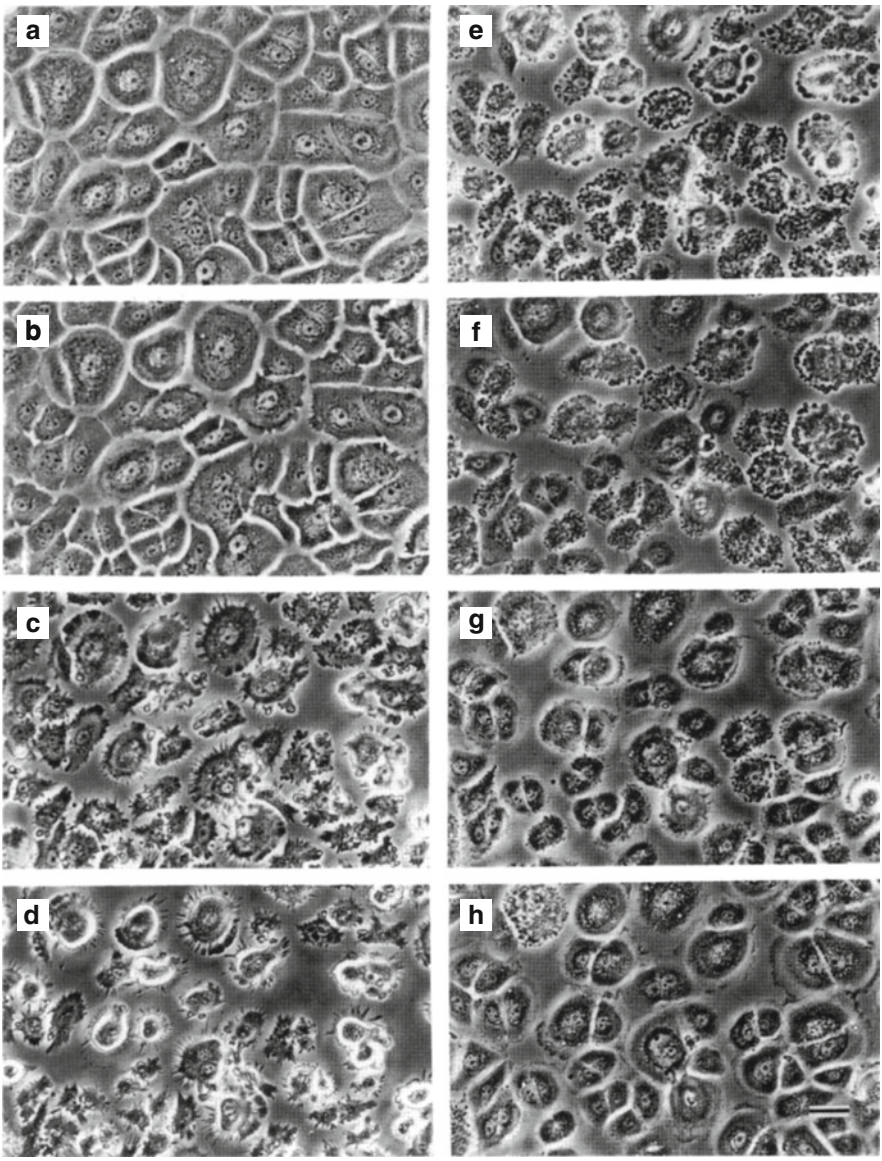

Fig. 5 Reversible acantholysis in keratinocyte monolayer due to blockade of mAChRs (Grando and Horton 1997). A confluent keratinocyte monolayer in 6-well tissue culture plate before treatment (**a**), and after incubation with 10 mM atropine for 1 min (**b**), 2 min (**c**), and 3 min (**d**). At this point, the medium containing atropine was replaced with fresh medium and the cells were observed for 5 min (**e**), 10 min (**f**), 20 min (**g**), and 30 min (**h**). The phase-contrast images were taken from the same microscopic field. Scale bar: 57 μm

outwards giving the cells a new polygonal shape. The spreading of keratinocyte cytoplasm continues until the cell borders reach neighboring cells. Having touched, KCs re-form intercellular contacts and reestablish a confluent monolayer (Fig. 5). Atropine also increases permeability of keratinocyte monolayers (Nguyen et al. 2004b), causes acantholysis in the lower epidermal layers of the organotypic cultures of human epidermis (Kurzen et al. 2006), and enlarges the intercellular space between epidermal KCs in vivo (Wessler et al. 1999). Remarkably, the nicotinic antagonists produce similar effects (Grando et al. 1995a; Kurzen et al. 2006; Wessler 1998), suggesting a synergistic control of keratinocyte cell–cell adhesion through both the muscarinic and nicotinic pathways.

Cholinergic antagonist-induced acantholysis is associated with increased phosphorylation of keratinocyte adhesion molecules (Nguyen et al. 2003, 2004b). Blocking of keratinocyte mAChRs with atropine increased the phosphorylation of E-cadherin by 120%, desmoglein (Dsg) 3 by 33%, and that of β- and γ-catenins by 50%, suggesting that keratinocyte adhesion can be regulated through the mAChR-dependent changes in the phosphorylation status of adhesion molecules. Long-term blockade of the M_3 signaling pathway with antisense oligonucleotides resulted in cell–cell detachment associated with changes in the expression levels of E-cadherin, and β- and γ-catenins (Nguyen et al. 2004b). Moreover, altered cell–cell adhesion was found in the stratified epithelium of M_3 KO mice that exhibit clefting in the epithelium lining of the oral mucosa, and the upper portion of esophagus. KCs from these mice feature abnormal expression of adhesion molecules at both the protein and the mRNA levels. Simultaneous inhibition of several receptor subtypes, i.e., M_3 mAChR together with α3 and α9 nAChR subunits, produced intensified abnormalities of keratinocyte adhesion (Nguyen et al. 2004b). In the organotypic cultures, atropine altered expression of the adhesion molecules E-cadherin, β- and γ-catenins, Dsg 1, Dsg 3, desmoplakin, and desmocollin 1 as well as the tight junction protein ZO-1 (Kurzen et al. 2006).

On the other hand, cholinergic agonists stimulate re-adhesion of dispersed KCs in cell culture, reverse acantholysis induced by cholinergic antagonists, the serine proteinase trypsin and the calcium chelator EDTA, and also suppress atropine-dependent phosphorylation of adhesion molecules (Grando and Dahl 1993; Nguyen et al. 2003, 2004b). The agonists stimulate expression of adhesion molecules in KCs. When the level of expression of intercellular adhesion molecules was studied in monolayers of normal human KCs treated with carbachol or the acetylcholinesterase inhibitor pyridostigmine bromide (Mestinon), the relative amounts of E-cadherin, Dsg 1, and Dsg 3 were increased (Nguyen et al. 2003, 2004a). Treatment of the organotypic culture of human epidermis elevated ZO-1 (Kurzen et al. 2006). Most importantly, ACh, carbachol, and the specific muscarinic agonists bethanechol and methacholine reversed acantholysis and restored the integrity of monolayers in cultures of human KCs affected by autoantibodies from patients with pemphigus (Grando and Dahl 1993).

Pemphigus is an IgG autoantibody-mediated autoimmune disease of skin and mucosa leading to progressive blistering and nonhealing erosions. Therapy of patients relies on the long-term use of systemic glucocorticosteroids in relatively

large doses, which is life saving but causes severe side effects, including death. Pemphigus patients develop autoantibodies to keratinocyte adhesion molecules, such as Dsg 1 and Dsg 3, as well as cell-surface receptors, including cholinergic receptors (reviewed in Grando 2000; Kurzen and Brenner 2006). Preincubation of KCs with pemphigus, but not normal, IgG significantly diminished the amount of [^{3}H]atropine specifically bound to keratinocyte cell membranes (Grando and Dahl 1993). In the radioimmunoprecipitation assay utilizing cell membranes of human KCs covalently labeled with the muscarinic radioligand [^{3}H]PrBCM, the mean radioactivity precipitated by pemphigus vulgaris (PV) and pemphigus foliaceus sera significantly exceeded both normal and disease-control levels (Nguyen et al. 1998). In addition to mAChRs (Curtis et al. 1989), [^{3}H]PrBCM could label pemphaxin, a novel keratinocyte annexin-like molecule identified by PV IgG in human keratinocyte λgt11 cDNA library (Nguyen et al. 2000b), and α9 nAChR subunit that forms receptors with mixed muscarinic-and-nicotinic pharmacology (Elgoyhen et al. 1994), and serves as an autoantigen of PV autoantibodies (Nguyen et al. 2000a).

To develop a steroid hormone-free treatment of pemphigus, we tested anti-acantholytic activities of cholinergic agonists in the in vivo model of pemphigus acantholysis produced in the skin of neonatal mice injected with PV IgG. Simultaneous injections of carbachol or pyridostigmine bromide significantly reduced the extent of PV IgG-induced intraepidermal dyshesion (Nguyen et al. 2004a). Mestinon (ICS Pharmaceuticals; 60 mg tablets) has been used in the clinical trial of pemphigus treatment and showed therapeutic efficacy (Grando 2004; Nguyen et al. 2004a). Noteworthily, nicotinamide (niacinamide) – a well-known steroid-sparing agent in pemphigus (Chaffins et al. 1993) – exhibits cholinomimetic effects (Romanenko 1987) due to both stimulation of ACh release (Koeppen et al. 1997) and inhibition of acetylcholinesterase (Stoytcheva and Zlatev 1996).

One of the hypothetical mechanisms that could explain anti-acantholytic effect of the cholinomimetics was their competition with PV IgG for binding to KCs. However, quantitative analysis of the intensity of epidermal binding of PV IgG in mice treated with cholinomimetics indicated that steric hindrance could not entirely account for the anti-acantholytic effect (Nguyen et al. 2004a). In addition to upregulation of keratinocyte adhesion molecules (Kurzen et al. 2006; Nguyen et al. 2003, 2004a), the therapeutic effect of cholinomimetics in pemphigus apparently stems from their ability to abrogate PV IgG-induced phosphorylation of E-cadherin and γ-catenin (Nguyen et al. 2004a), and to abolish the detrimental effect of PV IgG on keratinocyte monolayer repair (Lanza et al. 2009).

Most recently, partial evaluation of PV autoantibody profile using the protein array technology revealed M_1 mAChR among the keratinocyte antigens significantly differentially reactive with PV sera (Kalantari et al. 2011). This is in keeping with the fact that pilocarpine – preferential M_1 agonist (Kebabian and Neumeyer 1994) – exhibits therapeutic efficacy in PV patients (Iraji and Yoosefi 2006; Namazi 2004). In a double-blind, placebo-controlled study, skin erosions of PV patients were treated with either 4% pilocarpine or placebo gel (Iraji and Yoosefi 2006). After 15 days of treatment, the epithelialization index in two groups of patients was

compared. In skin lesions treated with pilocarpine, the epithelialization index was 40.3 ± 1.7, compared to 24.4 ± 3.3 ($p < 0.001$). The contribution of the signaling pathway coupled by M_1 mAChR to the anti-acantholytic activity of cholinomimetic agents is being elucidated. As in the case with directing migration of KCs toward ACh gradient (Chernyavsky et al. 2005), the downstream signaling from M_1 mAChR that produces anti-acantholytic effect in PV synergizes with α7 nAChR signaling (Chernyavsky et al. 2008). It has been demonstrated that pilocarpine ameliorated pemphigus acantholysis in vitro by inhibiting protein kinase C-dependent serine phosphorylation of β-catenins and tyrosine phosphorylation of p120-catenin via activation of type II serine/threonine protein phosphatase and protein tyrosine phosphatase, respectively (Chernyavsky et al. 2008). This novel paradigm of regulation of the mAChR-receptor-associated signaling kinases and phosphatases can be further exploited for the development of novel therapeutic strategies to control mucocutaneous blistering, accelerate wound epithelialization, and prevent cancer metastases.

6 Muscarinic Regulation of Epidermal Cornification

The programmed cell death of KCs comprising human epidermis culminates in abrupt transition of viable granular KCs into dead corneocytes sloughed by the skin. The granular cell–corneocyte transition is associated with a loss in volume and dry cell weight in a range of 45–86%. Biological significance of cornification stems from the ability of the stratum corneum to protect the body from water loss and external chemical injury. The synthetic products of granular KCs subserve barrier function of the stratum corneum, forming a "mortar" in a "bricks and mortar" model of human stratum corneum (Menon et al. 1986), and calcium-dependent enzymes crosslink intracellular proteins that envelop a corneocyte, a "brick." In the cytosol of terminally differentiated KCs, the humectant precursor protein profilaggrin is associated with optically dense keratohyaline granules, and the barrier lipids are contained within lamellar bodies. The dissolution of keratohyaline granules coincides with enzymatic processing of profilaggrin into mature filaggrin. We have demonstrated that terminally differentiated KCs extrude into the intercellular spaces of living epidermis, the cytoplasmic buds containing filaggrin, and randomly congregated components of the cytosol reminiscent of holocrine secretion (Nguyen et al. 2001). Auto/paracrine ACh is a secretagogue for this humectant secretion, and its secretagogue action is mediated by the synergistic action of the M_1 mAChR and α7 and α9 nAChRs that elevate intracellular levels of free calcium ($[Ca^{2+}]_i$) in terminally differentiated KCs (Nguyen et al. 2001).

To elucidate the physiologic mechanism regulating humectant secretion by KCs, we developed an in vitro pharmacologic model in which the discharge can be achieved using the following combinations of cholinergic agonists and antagonists: bromacetylcholine plus atropine, tropicamide, or PrBCM; and carbachol plus atropine, tropicamide, or PrBCM (Nguyen et al. 2001). A typical sequence of

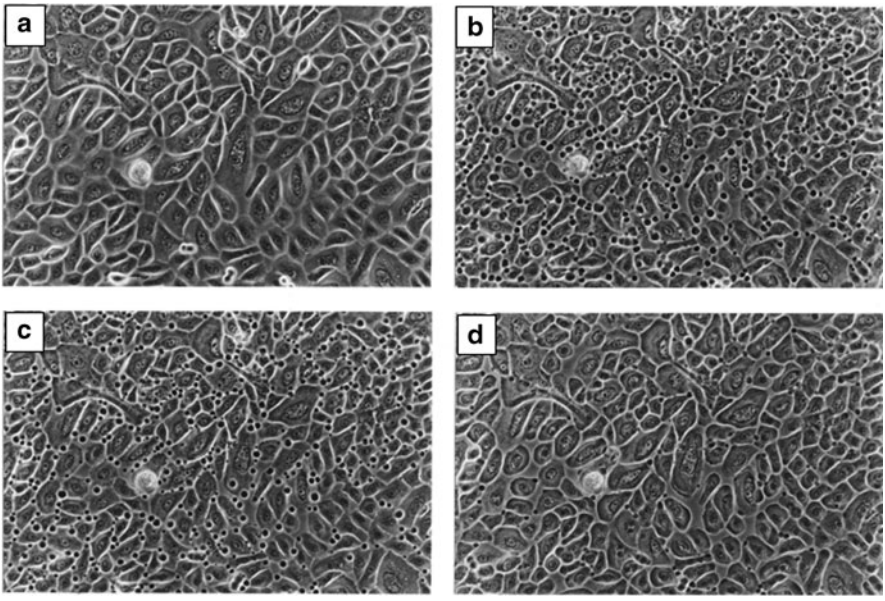

Fig. 6 Time-course observation of extrusion of cytoplasmic buds in response to cholinergic stimulation observed in a monolayer of normal human KCs (Nguyen et al. 2001). (**a**) The intact monolayer of second passage human foreskin KCs. (**b**) Same microscopic field 5 min after the culture received prewarmed (37°C) medium supplemented to contain 0.05 mM bromacetylcholine and 0.1 mM atropine. Note extensive cytoplasmic budding. (**c**) Same field 10 min after exposure. The buds have grown in size and number, and many spheres pinched off and float free. (**d**) Same field after the pinched off spheres were removed by collecting the chasing medium

morphologic events observed in both high Ca^{2+}-induced and naturally differentiated keratinocyte monolayers exposed to nicotinic agonist in the presence of a muscarinic antagonist is shown on Fig. 6**.** Upon exposure, optically dense cytoplasmic knobs start to protrude at the periphery of KCs. These small, approximately 1–3 μm in diameter, knobs grow into large, 5–7 μm in diameter, spheres that pinch off and float away from the cells. The spheres can be easily removed from the cultures by collecting chasing medium overlaying the monolayers. The cells in the monolayers exposed to a muscarinic antagonist alone produce reversible cytoplasmic blebbing without discharge of the cytoplasmic buds. As determined by immunoblotting, the cytoplasmic buds extruded by stimulated KCs contain filaggrin (Nguyen et al. 2001). The filaggrin degradation products are the natural humectants (a.k.a. natural moisturizing factor; NMF) that render epidermis exceptional plasticity and resilience. The filaggrin-containing portion of the secretory product may become a part of intercellular cementing substance (glycocalyx) and serve as an internal NMF that counterbalances the osmotic pressure imposed by the external NMF associated with corneocytes in the stratum corneum.

We investigated the receptor-mediated pathway of ACh-induced humectant secretion by measuring muscarinic effects on Ca^{2+} metabolism in KCs.

Tropicamide, a specific M_4 antagonist, rapidly increased the concentration of $[Ca^{2+}]_i$ above the basal level, suggesting that activation of this "inhibitory" receptor subtype might be opposing a "stimulatory" pathway that increases the $[Ca^{2+}]_i$ through an odd-numbered receptor. Since KCs secreting filaggrin in the epidermis express M_1, we exposed cultured KCs to the M_1/M_4 inhibitor pirenzepine which prevented the tropicamide-induced increase in $[Ca^{2+}]_i$. These results indicated that the rise of $[Ca^{2+}]_i$ is an essential element of the biological mechanism mediating humectant secretion by KCs. Apparently, the discharge does not occur precociously at earlier stages of keratinocyte differentiation in epidermis because ACh signaling through the "prosecretory" M_1 receptor that elevates $[Ca^{2+}]_i$ is counterbalanced by simultaneous activation of the "anti-secretory" M_4 receptor. Disappearance of M_4 from the cell membrane of KCs during the granular cell–corneocyte transition allows an unopposed activation of the "prosecretory" receptors, which disturbs the physiologic equilibrium of oscillations of $[Ca^{2+}]_i$, providing for a sudden rise in $[Ca^{2+}]_i$ that actually launches extrusion of cytoplasmic buds. Thus, it is an intracellular biochemical event, triggered by the activation of a specific combination of ACh receptors expressed by a keratinocyte at the latest stage of its development in epidermis that triggers humectant secretion.

7 Muscarinic Regulation of Hair Follicle Cycling

Hair follicle morphogenesis is a well-defined physiological process reflecting complex neuroectodermal–mesodermal interactions (Paus et al. 1999). We have exploited murine hair follicle cycling to elucidate the physiologic role of mAChRs in the biologic mechanisms mediating dramatic functional and phenotypic metamorphoses of follicular KCs. The quantitative morphometric analysis of hair follicle development was performed in mAChR KO mice (Chernyavsky et al. 2004b). One-day-old $M_3-/-$ pups displayed accelerated hair follicle morphogenesis, as could be judged from a significantly increased number of hair follicles in advanced stages of perinatal follicle morphogenesis and a reciprocal decrease of the percentage of follicles at the early stages of their development, compared to WT controls. In marked contrast, the $M_4-/-$ neonates showed a retardation of hair follicle morphogenesis evidenced by a significant increase in the number of hair follicles at the immature developmental stages and a correspondent reduction of the percentage of hair follicles at more mature stages. On the 17th day postpartum (dpp), when murine hair follicles enter the first catagen phase, thereby initiating hair follicle cycling, we observed a slight delay in catagen development in $M_4-/-$ mice (Hasse et al. 2007). A dramatic difference between hair follicle cycling in KO and WT mice was observed on dpp34. At this time point, WT skin showed only fully pigmented, growing anagen IV–VI hair follicles. In marked contrast, the hair follicles of $M_4-/-$ mice were still in telogen, indicating a substantially retarded progression through the first hair follicle cycle, i.e., from the first catagen at around dpp17 via the first telogen (dpp 21–25) through the first anagen phase. On dpp42,

the majority of skin hair follicles in both KO and WT mice were in telogen (Hasse et al. 2007). These results suggest that the M_4-coupled pathway plays a fundamental role in the physiologic regulation of hair follicle cycling.

8 Regulation of Cutaneous Homeostasis Through Keratinocyte mAChRs

Muscarinic stimulation of KCs has been shown to alter functions of other cell types residing in the mucocutaneous tissues via secretion of various cytokines and growth factors by KCs. Activation of mAChRs expressed in KCs induced secretion of putative humoral facts that upregulated production of collagen, matrix metalloproteinases, and tissue inhibitor of metalloproteinase by fibroblasts in culture (Xia et al. 2009). Stimulation of KCs via their mAChRs can also influence local inflammation due to the elevated production of IL-1α, IL-8, and PGE_2 and suppressed production of IL-6 observed in KCs treated with arecoline (Cheng et al. 2000; Jeng et al. 2003; Thangjam and Kondaiah 2009). The muscarinic signaling induced by arecoline in human KCs proceeds via the pathway involving p38 MAPK activation and leads to overexpression of the following stress-responsive genes: heme oxygenase-1, ferritin light chain, glucose-6-phosphate dehydrogenase, glutamatecysteine ligase catalytic subunit, and glutathione reductase (Thangjam and Kondaiah 2009). These findings indicate that keratinocyte mAChRs contribute to the physiologic regulation of mucocutaneous tissue remodeling and inflammation.

9 Conclusion

The physiologic regulation of KCs by auto/paracrine ACh via the muscarinic signaling pathways is inseparable from its nicotinergic signaling. The two pathways may compete or synergize with one another, so that the net biologic effect represents the biologic sum of the effects of distinct ACh receptors expressed by a keratinocyte at a particular stage of its development in the stratified epithelium. Apparently, an interplay of the ACh receptor signals governs progression of KCs through the major differentiation stages via an intricate level of fine-tuning of the homeostatic control of the gene expression at the transcriptional and translational levels, and posttranslational modifications. Experimental results indicate that a group of keratinocyte mAChRs is jointly responsible for the regulation of keratinocyte viability, proliferation, migration, adhesion, terminal differentiation, and hair follicle cycling as well as secretion of humectants, cytokines, and growth factors. Elucidation of the cholinergic control of KCs via mAChRs merits further consideration because of its strong potential for the development of novel therapies. Learning the muscarinic pharmacology of keratinocyte development and functions

has silent clinical implications for patients with nonhealing wounds, mucocutaneous cancers, and various autoimmune and inflammatory diseases. Successful therapy of pemphigus lesions with topical pilocarpine (Iraji and Yoosefi 2006) and disappearance of psoriatic lesions due to systemic atropine therapy (Gajewski 1970) hint that such therapeutic approach is feasible.

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References

Arredondo J, Hall LL, Ndoye A, Chernyavsky AI, Jolkovsky DL, Grando SA (2003) Muscarinic acetylcholine receptors regulating cell cycle progression are expressed in human gingival keratinocytes. J Periodontal Res 38:79–89

Arredondo J, Chernyavsky AI, Marubio LM, Beaudet AL, Jolkovsky DL, Pinkerton KE, Grando SA (2005) Receptor-mediated tobacco toxicity: regulation of gene expression through α3β2 nicotinic receptor in oral epithelial cells. Am J Pathol 166:597–613

Beutner EH, Chorzelski TP, Jablonska S (1985) Immunofluorescence tests. Clinical significance of sera and skin in bullous diseases. Int J Dermatol 24:405–421

Carsi-Gabrenas JM, Van Der Zee EA, Luiten PGM, Potter LT (1997) Non-selectivity of the monoclonal antibody M35 for subtypes of muscarinic acetylcholine receptors. Brain Res Bull 44:25–31

Chaffins ML, Collison D, Fivenson DP (1993) Treatment of pemphigus and linear IgA dermatosis with nicotinamide and tetracycline: a review of 13 cases. J Am Acad Dermatol 28:998–1000

Chang MC, Ho YS, Lee PH, Chan CP, Lee JJ, Hahn LJ, Wang YJ, Jeng JH (2001) Areca nut extract and arecoline induced the cell cycle arrest but not apoptosis of cultured oral KB epithelial cells: association of glutathione, reactive oxygen species and mitochondrial membrane potential. Carcinogenesis 22:1527–1535

Cheng YA, Shiue LF, Yu HS, Hsieh TY, Tsai CC (2000) Interleukin-8 secretion by cultured oral epidermoid carcinoma cells induced with nicotine and/or arecoline treatments. Kaohsiung J Med Sci 16:126–133

Chernyavsky AI, Arredondo J, Marubio LM, Grando SA (2004a) Differential regulation of keratinocyte chemokinesis and chemotaxis through distinct nicotinic receptor subtypes. J Cell Sci 117:5665–5679

Chernyavsky AI, Arredondo J, Wess J, Karlsson E, Grando SA (2004b) Novel signaling pathways mediating reciprocal control of keratinocyte migration and wound epithelialization by M_3 and M_4 muscarinic receptors. J Cell Biol 166:261–272

Chernyavsky AI, Arredondo J, Karlsson E, Wessler I, Grando SA (2005) The Ras/Raf-1/MEK1/ERK signaling pathway coupled to integrin expression mediates cholinergic regulation of keratinocyte directional migration. J Biol Chem 280:39220–39228

Chernyavsky AI, Arredondo J, Vetter DE, Grando SA (2007) Central role of alpha9 acetylcholine receptor in coordinating keratinocyte adhesion and motility at the initiation of epithelialization. Exp Cell Res 313:3542–3555

Chernyavsky AI, Arredondo J, Piser T, Karlsson E, Grando SA (2008) Differential coupling of M_1 muscarinic and α7 nicotinic receptors to inhibition of pemphigus acantholysis. J Biol Chem 283:3401–3408

Colley AM, Cavanagh HD, Law ML (1987) Effects of topical carbamylcholine on corneal epithelial resurfacing. Metab Pediatr Syst Ophthalmol 10:71–72

Curtis CA, Wheatley M, Bansal S, Birdsall NJ, Eveleigh P, Pedder EK, Poyner D, Hulme EC (1989) Propylbenzilylcholine mustard labels an acidic residue in transmembrane helix 3 of the muscarinic receptor. J Biol Chem 264:489–495

Dillon RS (1991) Role of cholinergic nervous system in healing neuropathic lesions: preliminary studies and prospective, double-blinded, placebo-controlled studies. Angiology 42:767–778

DiPalma JR (1994) Basic pharmacology in medicine, 4th edn. McGraw-Hill, New York

Donaldson DJ, Mahan JT (1988) Keratinocyte migration and the extracellular matrix. J Invest Dermatol 90:623–628

Elgoyhen AB, Johnson DS, Boulter J, Vetter DE, Heinemann S (1994) α9: an acetylcholine receptor with novel pharmacological properties expressed in rat cochlear hair cells. Cell 79:705–715

Elwary SM, Hasse S, Schallreuter KU (2004) m2 muscarinic acetylcholine receptor (mAchR) subtype is present in human epidermal keratinocytes in situ and in vivo. J Invest Dermatol 123:1206–1207

Er H (1997) The effect of topical parasympathomimetics on corneal epithelial healing in rabbits. Doc Ophthalmol 93:327–335

Gajewski W (1970) Disappearance of psoriatic skin rashes in the course of atropine therapy. Pol Tyg Lek 25:1815–1816

Gao YJ, Ling TY, Yin XM, Li X, Huang Y (2007) Effects of arecoline and nicotine on the expression of hTERT in oral keratinocytes. Zhonghua Kou Qiang Yi Xue Za Zhi 42:26–30

Ghelardini C, Galeotti N, Lelli C, Bartolini A (2001) M1 receptor activation is a requirement for arecoline analgesia. Farmaco 56:383–385

Grabovoi AN, Prosha MV, Shcherbak LF (1994) Effects of carbacholine and noradrenaline on tissue reactions in healing of skin wounds (in Russian). Klin Khir 6:54–56

Grando SA (1997) Biological functions of keratinocyte cholinergic receptors. J Investig Dermatol Symp Proc 2:41–48

Grando SA (2000) Autoimmunity to keratinocyte acetylcholine receptors in pemphigus. Dermatology (Basel) 201:290–295

Grando SA (2004) New approaches to the treatment of pemphigus. J Investig Dermatol Symp Proc 9:84–91

Grando SA (2006) Cholinergic control of epidermal cohesion in norm and pathology. Exp Dermatol 15:265–282

Grando SA, Dahl MV (1993) Activation of keratinocyte muscarinic acetylcholine receptors reverses pemphigus acantholysis. J Eur Acad Dermatol Venereol 2:72–86

Grando SA, Horton RM (1997) The keratinocyte cholinergic system with acetylcholine as an epidermal cytotransmitter. Curr Opin Dermatol 4:262–268

Grando SA, Kurzen H (2009) Cholinergic control of keratinocyte cohesion. In: Cirillo N, Lanza A, Gombo F (eds) Pathophysiology of the desmosome. Research Signpost, Kerela, India, pp 87–104

Grando SA, Lynch PJ (1993) Muscarinic antagonist scopolamine protects keratinocyte monolayers from morphological transformations induced by enalapril and thiol drugs. J Invest Dermatol 100:541

Grando SA, Crosby AM, Zelickson BD, Dahl MV (1993a) Agarose gel keratinocyte outgrowth system as a model of skin re-epithelization: requirement of endogenous acetylcholine for outgrowth initiation. J Invest Dermatol 101:804–810

Grando SA, Kist DA, Qi M, Dahl MV (1993b) Human keratinocytes synthesize, secrete and degrade acetylcholine. J Invest Dermatol 101:32–36

Grando SA, Horton RM, Pereira EFR, Diethelm-Okita BM, George PM, Albuquerque EX, Conti-Fine BM (1995a) A nicotinic acetylcholine receptor regulating cell adhesion and motility is expressed in human keratinocytes. J Invest Dermatol 105:774–781

Grando SA, Zelickson BD, Kist DA, Weinshenker D, Bigliardi PL, Wendelschafer-Crabb G, Kennedy WR, Dahl MV (1995b) Keratinocyte muscarinic acetylcholine receptors: immunolocalization and partial characterization. J Invest Dermatol 104:95–100

Grando SA, Pittelkow MR, Schallreuter KU (2006) Adrenergic and cholinergic control in the biology of epidermis: physiological and clinical significance. J Invest Dermatol 126:1948–1965

Haberberger RV, Bodenbenner M (2000) Immunohistochemical localization of muscarinic receptors (M2) in the rat skin. Cell Tissue Res 300:389–396

Harris AK (1990) Protrusive activity of the cell surface and the movements of tissue cells. In: Akkas N (ed) Biomechanics of active movement and deformation of cells, vol 42, NATO ASI Series H. Springer, Heidelberg, pp 249–294

Hasse S, Chernyavsky AI, Grando SA, Paus R (2007) The M4 muscarinic acetylcholine receptor plays a key role in the control of murine hair follicle cycling and pigmentation. Life Sci 80:2248–2252

Iraji F, Yoosefi A (2006) Healing effect of Pilocarpine gel 4% on skin lesions of pemphigus vulgaris. Int J Dermatol 45:743–746

Jeng JH, Hahn LJ, Lin BR, Hsieh CC, Chan CP, Chang MC (1999) Effects of areca nut, inflorescence piper betle extracts and arecoline on cytotoxicity, total and unscheduled DNA synthesis in cultured gingival keratinocytes. J Oral Pathol Med 28:64–71

Jeng JH, Wang YJ, Chiang BL, Lee PH, Chan CP, Ho YS, Wang TM, Lee JJ, Hahn LJ, Chang MC (2003) Roles of keratinocyte inflammation in oral cancer: regulating the prostaglandin E2, interleukin-6 and TNF-alpha production of oral epithelial cells by areca nut extract and arecoline. Carcinogenesis 24:1301–1315

Kalantari M, Molina DM, Farhadieh M, Morrow WJ, Liang X, Felgner PL, Grando SA (2011) New targets of pemphigus vulgaris antibodies identified by protein array technology. Exp Dermatol 20(2):154–156

Kebabian JW, Neumeyer JL (1994) The RBI handbook of receptor classification. Research Biochemicals International, Natick, p 122

Klapproth H, Reinheimer T, Metzen J, Munch M, Bittinger F, Kirkpatrick CJ, Hohle KD, Schemann M, Racke K, Wessler I (1997) Non-neuronal acetylcholine, a signalling molecule synthezised by surface cells of rat and man. Naunyn Schmiedebergs Arch Pharmacol 355:515–523

Koeppen A, Klein J, Erb C, Loeffelholz K (1997) Acetylcholine release and choline availability in rat hippocampus: effects of exogenous choline and nicotinamide. J Pharmacol Exp Ther 282:1139–1145

Kurzen H, Brenner S (2006) Significance of autoimmunity to non-desmoglein targets in pemphigus. Autoimmunity 39:549–556

Kurzen H, Schallreuter KU (2004) Novel aspects in cutaneous biology of acetylcholine synthesis and acetylcholine receptors. Exp Dermatol 13(Suppl 4):27–30

Kurzen H, Berger H, Jager C, Hartschuh W, Naher H, Gratchev A, Goerdt S, Deichmann M (2004) Phenotypical and molecular profiling of the extraneuronal cholinergic system of the skin. J Invest Dermatol 123:937–949

Kurzen H, Henrich C, Booken D, Poenitz N, Gratchev A, Klemke CD, Engstner M, Goerdt S, Maas-Szabowski N (2006) Functional characterization of the epidermal cholinergic system in vitro. J Invest Dermatol 126:2458–2472

Kurzen H, Wessler I, Kirkpatrick CJ, Kawashima K, Grando SA (2007) The non-neuronal cholinergic system of human skin. Horm Metab Res 39:125–135

Lanza A, Stellavato A, Heulfe I, Landi C, Gombos F, Cirillo N (2009) Serum of patients with oral pemphigus vulgaris impairs keratinocyte wound repair in vitro: a time-lapse study on the efficacy of methylprednisolone and pyridostigmine bromide. Oral Dis 15:478–483

Larjava H, Salo T, Haapasalmi K, Kramer RH, Heino J (1993) Expression of integrins and basement membrane components by wound keratinocytes. J Clin Invest 92:1425–1435

Leiber D, Harbon S, Guillet JG, Andre C, Strosberg AD (1984) Monoclonal antibodies to purified muscarinic receptor display agonist-like activity. Proc Natl Acad Sci U S A 81:4331–4334

MacCallum DE, Hall PA (1999) Biochemical characterization of pKi67 with the identification of a mitotic-specific form associated with hyperphosphorylation and altered DNA binding. Exp Cell Res 252:186–198

Marchisio PC, Bondanza S, Cremona O, Cancedda R, De Luca M (1991) Polarized expression of integrin receptors ($\alpha_6\beta_4$, $\alpha_2\beta_1$, $\alpha_3\beta_1$, and $\alpha_V\beta_5$) and their relationship with the cytoskeleton and basement membrane matrix in cultured human keratinocytes. J Cell Biol 112:761–773

McKinney M, Miller JH, Gibson VA, Nickelson L, Aksoy S (1991) Interactions of agonists with M2 and M4 muscarinic receptor subtypes mediating cyclic AMP inhibition. Mol Pharmacol 40:1014–1022

Menon GK, Brown BE, Elias PM (1986) Avian epidermal differentiation: role of lipids in permeability barrier formation. Tissue & Cell 18:71–82

Metzger M, Just L, Boss A, Drews U (2005) Identification and functional characterization of the muscarinic receptor M3 in the human keratinocyte cell line HaCaT. Cells Tissues Organs 180:96–105

Namazi MR (2004) Practice pearl: gargling with cholinergic ophthalmic drops for treating the oral lesions of pemphigus vulgaris. J Drugs Dermatol 3:484–485

Ndoye A, Buchli R, Greenberg B, Nguyen VT, Zia S, Rodriguez JG, Webber RJ, Lawry MA, Grando SA (1998) Identification and mapping of keratinocyte muscarinic acetylcholine receptor subtypes in human epidermis. J Invest Dermatol 111:410–416

Nguyen VT, Lee TX, Ndoye A, Shultz LD, Pittelkow MR, Dahl MV, Lynch PJ, Grando SA (1998) The pathophysiological significance of non-desmoglein targets of pemphigus autoimmunity. Pemphigus vulgaris and foliaceus patients develop antibodies against keratinocyte cholinergic receptors. Arch Dermatol 134:971–980

Nguyen VT, Ndoye A, Grando SA (2000a) Novel human α9 acetylcholine receptor regulating keratinocyte adhesion is targeted by pemphigus vulgaris autoimmunity. Am J Pathol 157:1377–1391

Nguyen VT, Ndoye A, Grando SA (2000b) Pemphigus vulgaris antibody identifies pemphaxin – a novel keratinocyte annexin-like molecule binding acetylcholine. J Biol Chem 275: 29466–29476

Nguyen VT, Ndoye A, Hall LL, Zia S, Arredondo J, Chernyavsky AI, Kist DA, Zelickson BD, Lawry MA, Grando SA (2001) Programmed cell death of keratinocytes culminates in apoptotic secretion of a humectant upon secretagogue action of acetylcholine. J Cell Sci 114:1189–1204

Nguyen VT, Arredondo J, Chernyavsky AI, Kitajima Y, Grando SA (2003) Keratinocyte acetylcholine receptors regulate cell adhesion. Life Sci 72:2081–2085

Nguyen VT, Arredondo J, Chernyavsky AI, Pittelkow MR, Kitajima Y, Grando SA (2004a) Pemphigus vulgaris acantholysis ameliorated by cholinergic agonists. Arch Dermatol 140:327–334

Nguyen VT, Chernyavsky AI, Arredondo J, Bercovich D, Orr-Urtreger A, Vetter DE, Wess J, Beaudet AL, Kitajima Y, Grando SA (2004b) Synergistic control of keratinocyte adhesion through muscarinic and nicotinic acetylcholine receptor subtypes. Exp Cell Res 294:534–549

Nishimura KY, Isseroff RR, Nuccitelli R (1996) Human keratinocytes migrate to the negative pole in direct current electric fields comparable to those measured in mammalian wounds. J Cell Sci 109:199–207

O'Keefe EJ, Woodley D, Castillo G, Russell N, Payne RE Jr (1984) Production of soluble and cell-associated fibronectin by cultured keratinocytes. J Invest Dermatol 82:150–155

Ozturk F, Kurt E, Inan UU, Emiroglu L, Ilker SS (1999) The effects of acetylcholine and propolis extract on corneal epithelial wound healing in rats. Cornea 18:466–471

Paus R, Muller-Rover S, Van Der Veen C, Maurer M, Eichmuller S, Ling G, Hofmann U, Foitzik K, Mecklenburg L, Handjiski B (1999) A comprehensive guide for the recognition and classification of distinct stages of hair follicle morphogenesis. J Invest Dermatol 113:523–532

Romanenko AV (1987) The action of nicotinamide on neuromuscular transmission. Fiziol Zh 33:51–56

Schafer IA, Kovach M, Price RL, Fratianne RB (1991) Human keratinocytes cultured on collagen gels form an epidermis which synthesizes bullous pemphigoid antigens and alpha 2 beta 1 integrins and secretes laminin, type IV collagen, and heparan sulfate proteoglycan at the basal cell surface. Exp Cell Res 195:443–457

Schoenwaelder SM, Burridge K (1999) Bidirectional signaling between the cytoskeleton and integrins. Curr Opin Cell Biol 11:274–286

Stoytcheva M, Zlatev R (1996) Bioelectrocatalytical studies of the effect of some pharmaceuticals on the acetylcholinesterase activity. Electroanalysis 8:676–679

Thangjam GS, Kondaiah P (2009) Regulation of oxidative-stress responsive genes by arecoline in human keratinocytes. J Periodontal Res 44:673–682

Theriot JA, Mitchison TJ (1991) Actin microfilament dynamics in locomoting cells. Nature 352:126–131

Tsurimoto T (1999) PCNA binding proteins. Front Biosci 4:849–858

Wessler I (1998) New use of active ingredients which affect non-neuronal acetylcholine functions PCT Gazette WO 98/00119. Boehringer Ingelheim KG, Germany

Wessler I, Kirkpatrick CJ, Racke K (1999) The cholinergic 'pitfall': acetylcholine, a universal cell molecule in biological systems, including humans. Clin Exp Pharmacol Physiol 26:198–205

Xia L, Tian-You L, Yi-Jun G, Dong-Sheng T, Wen-Hui L (2009) Arecoline and oral keratinocytes may affect the collagen metabolism of fibroblasts. J Oral Pathol Med 38:422–426

Xie DP, Chen LB, Liu CY, Zhang CL, Liu KJ, Wang PS (2004) Arecoline excites the colonic smooth muscle motility via M3 receptor in rabbits. Chin J Physiol 47:89–94

Yang YR, Chang KC, Chen CL, Chiu TH (2000) Arecoline excites rat locus coeruleus neurons by activating the M2-muscarinic receptor. Chin J Physiol 43:23–28

Muscarinic Receptor Agonists and Antagonists: Effects on Cancer

Eliot R. Spindel

Abstract Many epithelial and endothelial cells express a cholinergic autocrine loop in which acetylcholine acts as a growth factor to stimulate cell growth. Cancers derived from these tissues similarly express a cholinergic autocrine loop and ACh secreted by the cancer or neighboring cells interacts with M3 muscarinic receptors expressed on the cancer cells to stimulate tumor growth. Primary proliferative pathways involve MAPK and Akt activation. The ability of muscarinic agonists to stimulate, and M3 antagonists to inhibit tumor growth has clearly been demonstrated for lung and colon cancer. The ability of muscarinic agonists to stimulate growth has been shown for melanoma, pancreatic, breast, ovarian, prostate and brain cancers, suggesting that M3 antagonists will also inhibit growth of these tumors as well. As yet no clinical trials have proven the efficacy of M3 antagonists as cancer therapeutics, though the widespread clinical use and low toxicity of M3 antagonists support the potential role of these drugs as adjuvants to current cancer therapies.

Keywords Muscarinic receptors • Acetylcholine • M3 muscarinic receptor • Lung cancer • Colon cancer • Therapy

1 Introduction

The majority of cancers derived from epithelial and endothelial cells express muscarinic acetylcholine receptors (mAChR) and activation of the Gq-linked muscarinic receptors (M1, M3 and M5) leads to increased cell proliferation. In addition, many of those cancers also secrete acetylcholine (ACh) which stimulates cell growth; thus for

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many cancers, ACh acts as an autocrine growth factor. For cancers that do not synthesize ACh, muscarinic receptor activation can also come from neuronal, endocrine or paracrine sources of ACh or from constitutive activity of muscarinic receptors. To a large extent, expression of muscarinic receptors by cancers follows expression of the receptors by the normal tissue, though patterns of both muscarinic receptors and ACh synthesis can change between normal tissues and tumors. The ability of muscarinic activation to stimulate cancer growth clearly suggests that muscarinic antagonists will have the potential to inhibit lung cancer growth.

2 The Non-neuronal Cholinergic Autocrine Loop

The expression of muscarinic receptors in cancer derives from the continued expression of the non-neuronal cholinergic autocrine and paracrine signaling loop that exists in most endothelial and epithelial tissues. The best characterized non-neuronal cholinergic autocrine loop is in lung, and elements of that loop and how they pertain to cancer are discussed below.

2.1 The Cholinergic Autocrine Loop Expressed in Normal Lung

Bronchial epithelial cells (BEC) synthesize and secrete ACh which interacts with mAChR and nicotinic ACh receptors (nAChR) expressed by the BEC (Klapproth et al. 1997; Proskocil et al. 2004; Reinheimer et al. 1998). ACh secretion and signaling by BEC are similar in some ways to cholinergic signaling by neurons and different in other ways (Fig. 1). In BEC, as in neurons, ACh is synthesized from choline and acetyl-CoA by the enzyme choline acetyltransferase (ChAT). ACh secreted by BEC interacts with the same receptors (nAChR and mAChR) as ACh secreted by neurons; and ACh secreted by BEC is inactivated by acetylcholinesterase and butyryl cholinesterase just like neuronal ACh. The key differences between neuronal cholinergic signaling and BEC cholinergic signaling is the transport of choline into the cell, the secretory process and signal transduction mechanisms. Understanding these differences is important as it has implications for how muscarinic receptors stimulate cancer growth and how that stimulation can be potentially targeted. Because the key focus of this chapter is on muscarinic signaling in cancer, detail is provided on how these mechanisms affect cancer growth. A more general discussion of non-neuronal cholinergic signaling is in Wessler and Kirkpatrick (2011).

2.2 The Cholinergic Autocrine Loop Expressed in Lung Cancer

The cholinergic autocrine loop expressed in normal BEC is similarly expressed in lung cancers that derive from airway epithelial cells (Song et al. 2003) (Fig. 2).

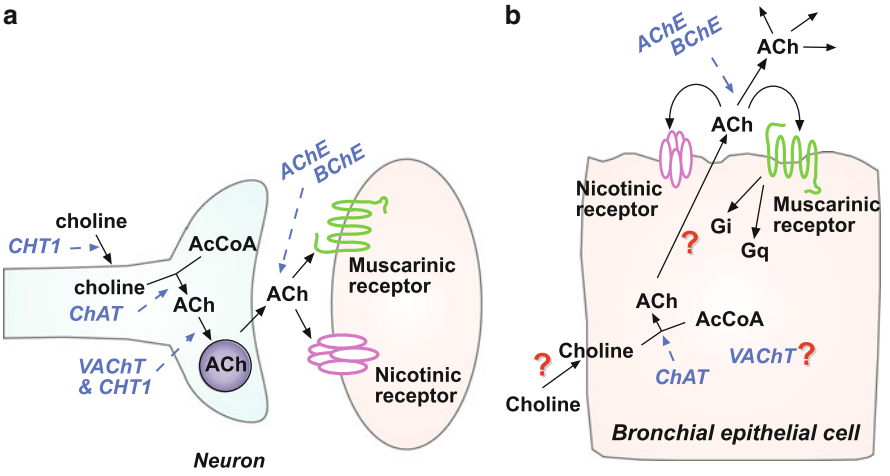

Fig. 1 Cholinergic signaling in neurons and bronchial epithelial cells. (**a**) In neurons, choline for ACh synthesis is transported by the choline high-affinity transporter (CHT1). ACh is then synthesized by the action of choline acetyltransferase (ChAT), and packaged into synaptic vesicles by the action of the vesicular acetylcholine transporter (VAChT) and CHT1. ACh is then secreted by the complex processes that control synaptic release. Released ACh then interacts with postsynaptic nAChR and mAChR as well as presynaptic receptors. Signaling is terminated by acetylcholinesterase (AChE) and butyrylcholinesterase (BChE). Key signal transduction events lead to the generation of action potentials, opening of membrane and internal ion channels, muscle contraction and kinase activation. (**b**) In bronchial epithelial cells (BEC), though CHT1 is present, CHT1 does not appear necessary for choline transport for ACh synthesis. In BEC, as for neurons, ChAT is utilized for ACh synthesis, though since there are multiple isoforms of ChAT, different splicing products may be utilized in different cell types. Since CHT1 is not required, and BEC do not have synaptic vesicles, the role of VAChT and CHT1 in ACh secretion is unknown, though both are expressed in BEC (Proskocil et al. 2004). ACh released by BEC is inactivated by the same cholinesterases as expressed in neurons. A key difference is that released ACh is not limited just to synaptic communication, but can also signal multiple neighboring cells as a paracrine factor or more distal cells as a hormone

The overwhelming majority of lung cancers derive from airway epithelial cells. Lung cancers are classified as small cell lung carcinoma (SCLC), which accounts for approximately 15–20% of the cases and non-small cell lung carcinoma (NSCLC), which accounts for the remaining 80–85% (Gabrielson 2006). SCLC derives from cells related to pulmonary neuroendocrine cells (Kumar et al. 2009). The two most common forms of NSCLC are squamous cell lung carcinoma (SCC) and lung adenocarcinoma, which together represent at least 80% of all NSCLC (Gabrielson 2006). Based on histology, gene expression and location, SCC is considered to arise from BEC of large airways and adenocarcinoma from epithelial cells of smaller airways (Kumar et al. 2009). These cell types of origin of SCLC, lung adenocarcinoma and SCC all express muscarinic receptors and synthesize ACh, thus not surprisingly the majority of these cancers also synthesize ACh and express muscarinic receptors.

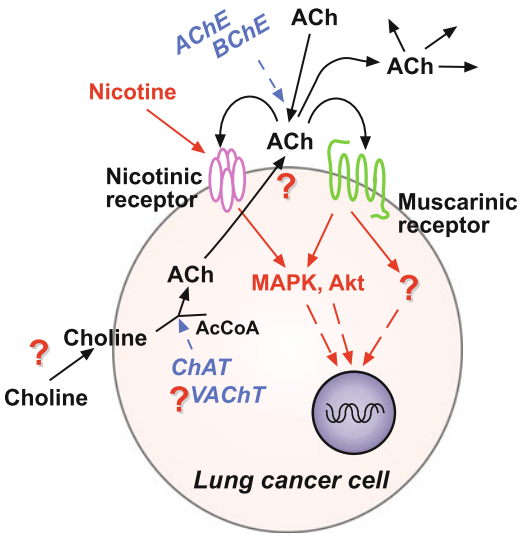

Fig. 2 Cholinergic signaling by lung cancer cells. Cholinergic signaling by lung cancer cells is similar to normal bronchial epithelial cells. Steps for ACh synthesis and signal transduction in lung cancer provide the potential steps to target for development of therapies. In particular, inhibition of choline transport and muscarinic receptor antagonists offer unique advantages as discussed in Sect. 5. Targeting proliferative kinase pathways such as MAPK and Akt is an area of major development for cancer therapy in general since so many growth factors activate those pathways

3 M3 Muscarinic Receptors and Lung Cancer Growth

As described above, muscarinic receptors are expressed by lung cancers as part of a cholinergic autocrine loop expressed in both normal and neoplastic lung. Proliferation is stimulated by several mechanisms. First, activation of M3 receptors leads to increased intracellular calcium which in turn leads to activation of Akt and MAPK (Figs. 3 and 4) (Song et al. 2003, 2007) . As shown in Fig. 3, ACh rapidly increases intracellular calcium in lung cancer cell lines and the increase is blocked by M3 antagonists and by knockdown of M3 RNA by siRNAs (Song et al. 2003, 2007). As shown in Fig. 4, M3 receptor activation leads in turn to Akt and MAPK activation, which is also blocked by M3 antagonists. This activation then leads to cell proliferation as shown in Fig. 5, and as for activation of signaling, cell growth can be inhibited by M3 antagonists (Song et al. 2003, 2007).

As can be seen in Figs. 4 and 5, the addition of M3 antagonists inhibits kinase activation and cell proliferation, in the absence of added ligand. This implies either a role for ACh secreted by the cells into the cell culture medium or constitutive activity of the M3 receptor. Constitutive (unliganded) activity is well established for G-protein coupled receptors in general (Kenakin 2004) and has been specifically demonstrated for the M3 muscarinic receptor (Casarosa et al. 2010). Most likely, both of these mechanisms are involved in muscarinic stimulation of lung cancer

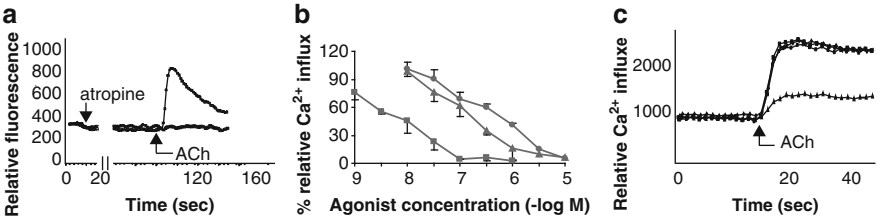

Fig. 3 Calcium responses to muscarinic agonists and antagonists in H82 cells. (**a**) A representative trace of the $[Ca^{2+}]_I$ response of H82 cells to ACh in the absence (−) or presence (+) of atropine. (**b**) Rank order potency of selective muscarinic antagonists to inhibit the $[Ca^{2+}]_I$ increase elicited by ACh in H82 cells. Antagonists tested were 4-DAMP (*filled square*, a selective M3 antagonist), pirenzepine (*filled triangle*, a selective M1 antagonist) and AFDX 116 (*filled circle*, a selective M2/M4 antagonist). The rank order potency of these antagonists is most consistent with mediation by the M3 mAChR. (**c**) siRNA knockdown of M3 mAChR blocked the ACh induced increase in $[Ca^{2+}]_I$ but control, M1 and M5 mAChR knockdowns had no effect. *Filled circle* = control siRNA, *filled square* = M1 siRNA, *filled triangle* = M3 siRNA, *filled diamond* = M5 siRNA. Data are presented as mean ± SE of at least 12 replicates from 3 separate experiments. Modified after Song et al. (2007)

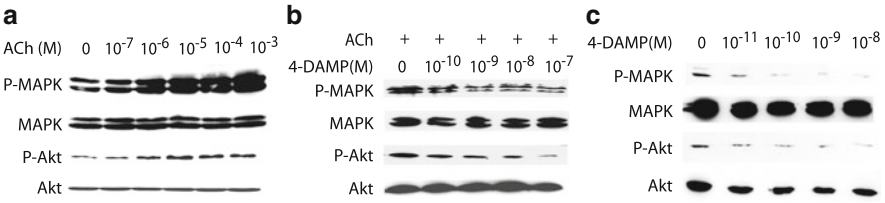

Fig. 4 Effect of ACh on phosphorylation of MAPK and Akt in H82 SCLC cells. (**a**) Western blot showing increased MAPK and Akt phosphorylation induced by concentrations of ACh shown. (**b**) Western blot showing that phosphorylation of Akt and MAPK induced by 3×10^{-5} M ACh was decreased by the M3 antagonist 4-DAMP in a concentration-dependent fashion. (**c**) Western blot showing that 4-DAMP alone decreased basal phosphorylation of Akt and MAPK. Modified after Song et al. (2007)

growth. M3 antagonists inhibit growth of cell lines in vitro which express very little ChAT (Song et al. 2007) which implies a role for constitutive activity. However, the ability of M3 antagonists to inhibit cell proliferation and generation of IP3 metabolites is decreased (though not eliminated) by the addition of cholinesterase to cell culture medium which implies a role for autocrine cholinergic activation as well (Spindel, unpublished observation). Therefore, in patients, as discussed further below, the growth of lung cancers expressing muscarinic M3 receptors can be stimulated by ACh secreted from the tumor by paracrine sources of ACh from neighboring airway epithelium and by ACh from distal sources present in blood (Fujii et al. 1995).

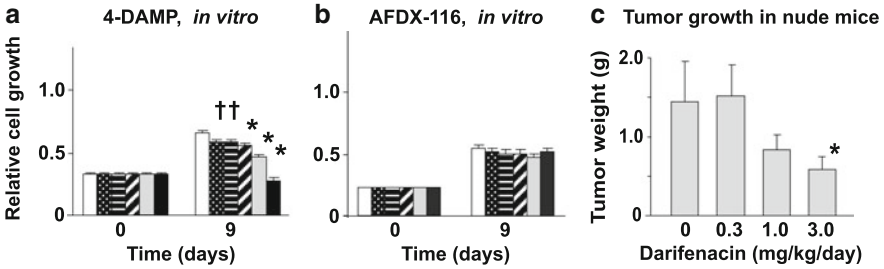

Fig. 5 Regulation of H82 cell proliferation by mAChR subtype antagonists. The MTS assay was used to detect H82 cell growth after treatment with 4-DAMP and AFDX-116. (**a**) The M3 mAChR antagonist 4-DAMP inhibited H82 cell proliferation in a concentration-dependent manner. (**b**) The M2/M4 selective mAChR antagonist, AFDX 116 had no significant effect on cell growth. All data are expressed as the mean ± SE of 24 replicates of two separate experiments. White column, control; dotted-pattern column, 10^{-9} M; horizontal-pattern column, 10^{-8} M; diagonal-pattern column, 10^{-7} M; gray column, 10^{-6} M; black column, 10^{-5} M. $*p < 0.001$ and $\dagger p < 0.05$ compared to control at 9 days by Tukey–Kramer multiple comparison test after 2-way ANOVA. (**c**) Effect of darifenacin on growth of H82 tumor xenografts in nude mice. (**c**) Tumor weight. $*p < 0.05$ compared to control by *t* test. Modified after Song et al. (2007)

Table 1 Frequency of ChAT and M3 coexpression in selected cancers

Cancer type	*N*	% M3	% ChAT	% M3 and chat coexpression
Lung (SCLC)	24	70	92	70
Lung (BAC)	20	85	80	70
Lung (SCC)	31	71	58	45
Pancreatic	32	78.1	65.6	50
Cervical	14	50	71	43

Frequency of M3 mAChR, ChAT and their coexpression in archival samples of SCLC, bronchoalveolar lung carcinoma (BAC), squamous cell lung carcinoma (SCC), pancreatic carcinoma and cervical carcinoma as determined by immunostaining. Sample size of each series as shown (*N*). Modified after Song et al. (2007)

4 Muscarinic Receptors and Specific Cancers

The ability of ACh to stimulate the growth of normal and neoplastic lung suggests that growth of any cancers that express M3 receptors can potentially be inhibited by muscarinic antagonists and that cancers that express both ACh and M3 receptors should be especially sensitive. As outlined in Table 1, this includes most lung cancers, pancreatic cancer and cervical cancer as analyzed by our laboratory, as well as other cancers as discussed below. Broadening the range of potentially sensitive cancers even further, we have observed that lung cancers that express M1 or M5 receptors can also be inhibited, suggesting that these Gq-linked subtypes may also confer sensitivity to lung cancers (Song et al. 2009).

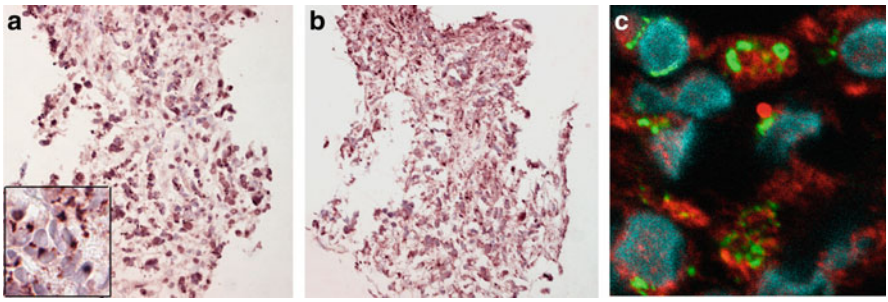

Fig. 6 Immunohistochemistry of ChAT and M3 mAChR expression in an SCLC biopsy. (**a**) ChAT immunostaining (400×, chromogen = VIP), insert box = 1,000×. (**b**) M3R immunostaining (400×, chromogen = VIP). (**c**) Confocal image showing coexpression of M3 mAChR (*red*) and ChAT (*green*) in tumor cells in same sample as (**a**) and (**b**). Modified after Song et al. (2007)

4.1 Lung Cancer

The initial report of muscarinic receptor expression in normal lung was in 1984 by Whitsett and Hollinger (1984) based on QNB binding. Subsequently, Mak et al. (1992) demonstrated that in airway epithelium, expression of the M3 receptor predominated. Studies by Wessler and co-workers (Klapproth et al. 1997; Reinheimer et al. 1996; Wessler and Kirkpatrick 2001) and Proskocil et al. (2004) then established that airway epithelium also synthesized ACh. Expression of muscarinic receptors in lung cancers was initially shown by Cunningham et al. (1985) and Morin et al. (1987) though effects on proliferation were not clearly determined. Subsequently, studies by Spindel and co-workers (Song et al. 2003, 2007, 2008) demonstrated that the majority of SCLC and NSCLC expressed M3 receptors as shown in Table 1. As discussed above, Song et al. also showed that both SCLC and NSCLC synthesized and secreted ACh (Song et al. 2003, 2007, 2008) which acted as an autocrine growth factor for lung cancers. An example of expression of ChAT and M3 receptors in SCLC tumor and a cell line is shown in Fig. 6.

Song et al. (2008) also demonstrated an apparent activation of cholinergic signaling in lung cancer with increased levels of ChAT and ACh, and decreased levels of cholinesterase in lung cancers compared to normal lung. Martinez-Moreno has similarly reported that cholinesterase levels are reduced in lung cancer, thus increasing the available ACh to stimulate tumor growth (de Martinez-Lopez et al. 2008; Martinez-Moreno et al. 2006).

The potential of M3 muscarinic receptor antagonists to inhibit lung cancer growth was demonstrated by Song et al. (2003, 2007, 2008, 2010) who showed that multiple M3 antagonists, including 4-diphenyl-acetoxy-N-methyl-piperidine (4-DAMP), para-fluoro-hexahydrosila-difenidol (P-F-HHSiD), darifenacin and tiotropium all could inhibit lung cancer cell proliferation in vitro and the effectiveness of darifenacin and tiotropium to inhibit lung cancer cell growth in vivo in nude mice was also demonstrated. While most reports suggest that M3 receptors are most

important for lung cancer proliferation, Matthiesen et al. (2006) has suggested for lung fibroblasts that M2 receptors may be more important, though Pieper et al. (2007) also support a key role for M3 receptors in mediating lung fibroblast proliferation. It is important to note that fibroblasts are not, however, the primary cell of origin for most lung cancers. Interestingly, in a preliminary observation, Song et al. (2004) has suggested that M2 receptors might play an inhibitory role in the growth of lung cancers. This observation which needs further study would suggest that greater selectivity of M3 over M2 for muscarinic antagonists used for lung cancer therapy would be desirable.

4.2 Skin Cancer

Skin cancer is by far the most common form of cancer. Basal and squamous cell skin carcinomas are most frequent and arise from keratinocytes though rarely cause significant morbidity or mortality. Melanomas arise from melanocytes and while they represent only about 3% of skin cancer, they cause by far the majority of skin cancer morbidity and mortality.

4.2.1 Squamous and Basal Cell Skin Carcinoma

Squamous and basal cell skin carcinomas arise from keratinocytes. As discussed in "Muscarinic Receptor Agonists and Antagonists: Effects on Keratinocyte Function" by Grando (2011), non-neuronal cholinergic signaling by keratinocytes has been extensively described by Grando and co-workers and the ability of acetylcholine and muscarinic receptors to stimulate keratinocyte proliferation and muscarinic antagonists to inhibit proliferation is well characterized (Arredondo et al. 2003; Chernyavsky et al. 2004; Grando et al. 1993, 2006). As squamous and basal cell carcinomas are treated by local curative surgery, the role of muscarinic antagonists to inhibit their growth is not likely to be clinically significant.

4.2.2 Melanoma and Merkel Cell Carcinoma

Expression of M2–M5 muscarinic receptors in normal melanocytes was reported by Buchli et al. (2001). Subsequently multiple reports have established that melanomas primarily express M3 muscarinic receptors (Boss et al. 2005; Lammerding-Koppel et al. 1997; Noda et al. 1998; Oppitz et al. 2008); and, critically, that M3 muscarinic receptors expression appears elevated in leading edges of tumors and in metastases (Lammerding-Koppel et al. 1997; Oppitz et al. 2008). Consistent with this, Boss et al. (2005) have shown that M3 receptors play a role in chemotaxis of melanoma cells. This would suggest a potential for M3

antagonists to inhibit melanoma growth or metastasis, but this remains to be determined.

Merkel cell carcinomas derive from skin neuroendocrine cells and, though relatively rare, can have an aggressive clinical course. By immunohistochemistry, Bowers et al. (2008) reported that 15 of 15 primary cutaneous cases of Merkel cell carcinoma expressed M3 and M5 receptors. Given that the proliferation of other types of neuroendocrine cells such as pulmonary neuroendocrine cells that express muscarinic receptors can be inhibited by M3 antagonists, this would suggest that muscarinic antagonists might also inhibit growth of Merkel Cell Carcinomas, but this again needs to be determined.

4.3 Colon Cancer

Initial reports of muscarinic receptor expression in colon adenocarcinoma were by Frucht et al. (1992) based on the presence N-methylscopolamine and carbamylcholine binding to most colon cancer cell lines. Follow-up studies by Frucht and co-workers (Frucht et al. 1999; Yang and Frucht 2000) demonstrated that the receptors were primarily M3, were increased approximately eightfold in tumor versus normal, and that carbamylcholine stimulated proliferation of colon carcinoma cell lines expressing M3 receptors. Raufman et al. (2003) and Ukegawa et al. (2003) confirmed those findings, again showing the importance of M3 receptors and also demonstrated that the proliferative action of M3 receptors depended in part on the transactivation of EGF receptors. The actual role of M3 receptors in colon cancer development was further confirmed by Raufman et al. (2008) who showed that M3 receptor knockout mice were resistant to the development of colon tumors in the azoxymethane-induced colon neoplasia model. This suggests that M3 receptor antagonists may be useful for colon cancer treatment or chemoprevention.

Showing that the cholinergic autocrine loop also played an important role in colon cancer, Cheng et al. (2008) showed that most colon cancers, such as lung cancers, synthesize ACh and that ChAT expression is higher in colon adenocarcinoma than in normal colon enterocytes. The role of ACh as an autocrine growth factor for colon cancer was further confirmed by showing that the choline transport inhibitor hemicholinium-3 could inhibit growth of colon adenocarcinoma cell lines and that the addition of cholinesterase inhibitors to the cell culture medium could stimulate colon cancer cell growth (Cheng et al. 2008). This observation may be clinically important since the levels of cholinesterase appear decreased in colon cancer (Montenegro et al. 2005). Thus in colon cancer, as in lung cancer, there is upregulation of the cholinergic autocrine loop with increased levels of M3 receptors, increased ChAT expression and decreased cholinesterase expression.

Raufmann et al. have also demonstrated that some bile acids can bind to the M3 receptor; thus bile acids may represent another endogenous ligand to stimulate colon cancer growth through muscarinic receptors (Raufman et al. 2002, 2003).

4.4 Gastric Cancer

M1, M3 and M5 receptors are expressed in normal gastric epithelium consistent with their role in regulation of acid and enzyme secretion (Aihara et al. 2005; Leonard et al. 1991; Xie et al. 2005). Consistent with this, gastric carcinomas also express muscarinic receptors. In early studies, two out of four gastric carcinoma cell lines established by Park et al. expressed muscarinic receptors (Park et al. 1990) though muscarinic subtype was not determined. Subsequently, Kodaira et al. (1999) demonstrated that five out of eight gastric carcinoma cell lines examined expressed M3 receptors and that carbachol treatment stimulated MAP kinase in those cell lines but did not stimulate cell proliferation. This would argue against a proliferative role for muscarinic receptors in stomach cancer.

4.5 Pancreatic Cancer

While a key role for muscarinic receptors in regulating pancreatic endocrine and exocrine function of the pancreas is well established (Gautam et al. 2005, 2006; Williams 2006), muscarinic effects on pancreatic carcinoma are less well defined. In normal pancreas, M3 receptors play a role in regulating insulin and glucagon secretion (Gautam et al. 2006; Gromada and Hughes 2006), while M1 and M3 receptors are involved in acinar secretion (Gautam et al. 2005). In an examination of pancreatic carcinoma cell lines, two out of five lines expressed muscarinic receptors, though the subtype was not identified (Ackerman et al. 1989). Similarly, nafenopin-induced pancreatic carcinomas in rats expressed muscarinic receptors that were linked to calcium mobilization, though the subtype and muscarinic effects on cell proliferation were not determined (Chien and Warren 1985, 1986). Notably, approximately 50% of pancreatic adenocarcinomas examined by Sekhon et al. (2002) expressed ChAT; so depending on the degree of muscarinic receptor expression by pancreatic carcinomas, there is potential for autocrine stimulation. Effects of muscarinic antagonists on pancreatic carcinoma growth have not been characterized.

4.6 Breast Cancer

The degree of expression of muscarinic receptors in breast cancer has not been well characterized. It has, however, been clearly demonstrated that muscarinic activation stimulates growth of MCF-7 human breast carcinoma cells (Jimenez and Montiel 2005; Schmitt et al. 2010). As shown by siRNA studies, stimulation of proliferation is mediated by M3 receptors leading to Erk 1/2 activation with partial dependence on Src and Cam Kinase pathways. Negroni et al. (2010) have also demonstrated the presence of autoantibodies in blood of breast cancer patients that

directly stimulates MCF-7 cell proliferation in an M3-dependent manner. Similar M3-dependent stimulation of proliferation has also been observed for mouse breast cancer cell lines (Espanol et al. 2007; Fiszman et al. 2007). Interestingly, Cabello et al. (2001) have demonstrated that organophosphorus pesticides lead to rat mammary tumors perhaps by inhibiting cholinesterase through a muscarinic mechanism since the effect could be blocked by atropine. The potential for cholinergic stimulation to lead to development of breast cancer is further supported by the recent paper linking α9 nAChR to breast cancer (Lee et al. 2010).

4.7 Ovarian Cancer

Initial studies by Batra et al. (1993) showed the presence of muscarinic receptors in ovarian adenocarcinoma with binding profile most consistent with M3 receptors. Studies by Oppitz et al. (2002) reported that 23 of 39 ovarian cancers studied expressed muscarinic receptors. Studies by Mayerhofer and co-workers (Fritz et al. 2001; Mayerhofer and Kunz 2005) have clearly demonstrated a clear cholinergic autocrine loop expressed by normal ovary. As for colon and lung, cholinergic agonists stimulate the growth of ovarian cells, which would suggest that muscarinic antagonists might have a beneficial effect in ovarian cancer. Consistent with this, expression of muscarinic receptors by ovarian cancer is associated with decreased patient survival (Oppitz et al. 2002).

4.8 Prostate Cancer

Relatively less is known about the role of muscarinic receptors in prostate cancer and the potential of anti-muscarinics to inhibit cancer growth (Witte et al. 2008). In normal prostate gland epithelium, M1 receptors predominate (Ruggieri et al. 1995) and sparse M2 receptors are found in the stroma (Obara et al. 2000). M1 receptors similarly predominate in benign prostate adenomas and benign prostatic hyperplasia (BPH) (Ruggieri et al. 1995). Luthin et al. (1997) showed that carbachol stimulated proliferation in three out of three prostate carcinoma cell lines (PC3, LnCaP, DU145) tested and based on antagonists, the primary mechanism appeared to be M1 activation of the Erk cascade, though M3 effects could not be excluded. Rayford et al. (1997) similarly showed that carbachol stimulated proliferation of LnCaP cells and that the effect appeared mediated by M3 receptors. Neither the studies by Luthin et al. nor Rayford et al. used siRNA techniques, so determinations of specificity of receptor mediation was based only on relative antagonist potencies. Rayford et al. (1997) also reported that carbachol stimulated the proliferation of primary cultures of normal prostate, BPH and prostate carcinoma. Notably they also reported that the ability of carbachol to stimulated proliferation was significantly increased in prostate carcinoma compared to normal prostate or BPH

(Rayford et al. 1997). This suggests that muscarinic antagonists with M3 or M3 combined with M1 selectivity might be helpful for prostate carcinoma therapy. In addition, whether there will be differences between responses of androgen-dependent and -independent prostate carcinomas remains to be determined.

4.9 Brain Cancer (Astrocytoma and Neuroblastoma)

One of the earliest reports on the ability of acetylcholine to stimulate cell proliferation through Gq-linked muscarinic receptors was by Ashkenazi et al. (1989) who reported that carbachol could stimulate proliferation of primary cultures of astrocytes, and astrocyte and neuroblastoma cell lines. This was confirmed by Guizzetti et al. (1996) who also demonstrated that astrocytes expressed M2, M3 and M5 receptors. Wessler et al. (1997) then demonstrated that astrocytes also synthesized acetylcholine, thus establishing that normal astrocytes, such as lung epithelial cells express the cholinergic autocrine loop. As for lung, carbachol activation of M3 receptors leads to rapid activation of MAPK and Akt (Guizzetti and Costa 2001; Tang et al. 2002; Yagle et al. 2001). These data suggest that muscarinic antagonists may have the potential to inhibit growth of both astrocytomas and neuroblastomas.

5 Targeting Muscarinic Signaling for Lung Cancer Therapy

Muscarinic signaling in lung cancer is summarized in Fig. 2 which shows potential levels to target proliferation. Muscarinic activation of lung cancer growth can potentially be targeted at the following levels: (1) by blocking choline transport into the cancer cell; (2) by blocking ChAT activity in the cancer cell; (3) by blocking ACh secretion from the cancer cell; (4) by muscarinic antagonists; and (5) by blocking muscarinic receptor activated proliferative pathways. Some of these approaches are clearly more promising than others.

Clearly of great promise is the use of M3 muscarinic antagonists to block cancer growth. This has been demonstrated in multiple in vitro studies and in limited mouse studies as discussed above. If additional preclinical studies appear promising, then clinical trials should be considered. Given that multiple muscarinic antagonists are already in routine clinical use for overactive bladder and COPD with minimal side effects, the barriers for clinical studies should be relatively low and even small effects of muscarinic antagonists on survival or quality of life would suggest a place for these drugs as adjuvants to existing therapeutic regimes. In addition, because of the common use of these drugs, there may be epidemiologic data that could be mined to determine if there is indeed therapeutic potential for their use as cancer therapeutics.

Blocking choline transport into cancer cells so as to prevent ACh synthesis is potentially promising since the choline transporters used for ACh synthesis by lung cancer cells are different than the transporters used for neuronal ACh synthesis. Neurons use the choline high-affinity transporter (CHT1) to mediate ACh synthesis (Ferguson et al. 2004; Okuda et al. 2000) while cancer cells do not need CHT1 and may use the choline transporter-like proteins (CTL1-5) (Machova et al. 2009; Song and Spindel 2007; Wang et al. 2007). In addition, choline is needed for membrane phospholipids, so this approach would potentially block cancer growth both by limiting ACh synthesis and phospholipid synthesis (Glunde et al. 2006).

Blocking ChAT activity in cancer cells is not likely to be a viable approach as the same enzyme is also used in neurons (Song et al. 2003), thus resulting in impaired neurotransmission, respiration and muscle contraction. Similarly, stimulating cholinesterase activity in the tumors would likely be highly toxic as that would also affect neuronal and muscular neurotransmission. At present very little is known about the regulation of ACh secretion by cancers to determine if that could be successfully targeted. Discussion of strategies to block kinase pathways activated by muscarinic receptors is clearly promising, but is not unique just for muscarinic activation, since multiple factors activate the same pathways in many cancers. Inhibition of these pathways is a major area of cancer therapy development in general (Bennasroune et al. 2004; Engelman 2009; Friday and Adjei 2008; Natoli et al. 2010).

Thus the near term prospects for targeting muscarinic activation of cancer growth rests with muscarinic antagonists and downstream kinase inhibitors. Future approaches will likely include blocking ACh synthesis in cancers by targeting choline transport.

In summary, there are considerable data suggesting that muscarinic receptors may be therapeutically useful as an adjunct to existing cancer therapies. The case is most compelling for M3 antagonists for lung and colon cancer; and additional studies are clearly warranted for melanoma, pancreatic, breast, ovarian, prostate and brain cancers. As for many potential uses of muscarinic antagonists, more selective antagonists with greater ratios of M1, M3 and M5 selectivity relative to M2 and M4 would be desirable, though siRNA-based approaches may solve this problem.

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References

Ackerman MS, Roeske WR, Heck RJ, Korc M (1989) Identification and characterization of muscarinic receptors in cultured human pancreatic carcinoma cells. Pancreas 4:363–370

Aihara T, Nakamura Y, Taketo MM, Matsui M, Okabe S (2005) Cholinergically stimulated gastric acid secretion is mediated by M(3) and M(5) but not M(1) muscarinic acetylcholine receptors in mice. Am J Physiol Gastrointest Liver Physiol 288:G1199–G1207

Arredondo J, Hall LL, Ndoye A, Chernyavsky AI, Jolkovsky DL, Grando SA (2003) Muscarinic acetylcholine receptors regulating cell cycle progression are expressed in human gingival keratinocytes. J Periodontal Res 38:79–89

Ashkenazi A, Ramachandran J, Capon DJ (1989) Acetylcholine analogue stimulates DNA synthesis in brain-derived cells via specific muscarinic receptor subtypes. Nature 340:146–150

Batra S, Popper LD, Iosif CS (1993) Characterisation of muscarinic cholinergic receptors in human ovaries, ovarian tumours and tumour cell lines. Eur J Cancer 29A:1302–1306

Bennasroune A, Gardin A, Aunis D, Cremel G, Hubert P (2004) Tyrosine kinase receptors as attractive targets of cancer therapy. Crit Rev Oncol Hematol 50:23–38

Boss A, Oppitz M, Drews U (2005) Muscarinic cholinergic receptors in the human melanoma cell line SK-Mel 28: modulation of chemotaxis. Clin Exp Dermatol 30:557–564

Bowers JW, Schlauder SM, Calder KB, Morgan MB (2008) Acetylcholine receptor expression in Merkel cell carcinoma. Am J Dermatopathol 30:340–343

Buchli R, Ndoye A, Arredondo J, Webber RJ, Grando SA (2001) Identification and characterization of muscarinic acetylcholine receptor subtypes expressed in human skin melanocytes. Mol Cell Biochem 228:57–72

Cabello G, Valenzuela M, Vilaxa A, Duran V, Rudolph I, Hrepic N, Calaf G (2001) A rat mammary tumor model induced by the organophosphorous pesticides parathion and malathion, possibly through acetylcholinesterase inhibition. Environ Health Perspect 109:471–479

Casarosa P, Kiechle T, Sieger P, Pieper MP, Gantner F (2010) The constitutive activity of the human muscarinic M3 receptor unmasks differences in the pharmacology of anticholinergics. J Pharmacol Exp Ther 333:201–209

Cheng K, Samimi R, Xie G, Shant J, Drachenberg C, Wade M, Davis RJ, Nomikos G, Raufman JP (2008) Acetylcholine release by human colon cancer cells mediates autocrine stimulation of cell proliferation. Am J Physiol Gastrointest Liver Physiol 295(3):G591–G597

Chernyavsky AI, Arredondo J, Wess J, Karlsson E, Grando SA (2004) Novel signaling pathways mediating reciprocal control of keratinocyte migration and wound epithelialization through M3 and M4 muscarinic receptors. J Cell Biol 166:261–272

Chien JL, Warren JR (1985) Differentiation of muscarinic cholinergic receptors in acinar carcinoma of rat pancreas. Cancer Res 45:4858–4863

Chien JL, Warren JR (1986) Muscarinic receptor coupling to intracellular calcium release in rat pancreatic acinar carcinoma. Cancer Res 46:5706–5714

Cunningham JM, Lennon VA, Lambert EH, Scheithauer B (1985) Acetylcholine receptors in small cell carcinomas. J Neurochem 45:159–167

de Martinez-Lopez CA, Nieto-Ceron S, Pons-Castillo A, Galbis-Martinez L, Latour-Perez J, Torres-Lanzas J, Tovar-Zapata I, Martinez-Hernandez P, Rodriguez-Lopez JN, Cabezas-Herrera J (2008) Cancer-associated differences in the acetylcholinesterase activity in bronchial aspirates of lung cancer patients. Clin Sci (Lond) 115(8):245–253

Engelman JA (2009) Targeting PI3K signalling in cancer: opportunities, challenges and limitations. Nat Rev Cancer 9:550–562

Espanol AJ, de la TE, Fiszman GL, Sales ME (2007) Role of non-neuronal cholinergic system in breast cancer progression. Life Sci 80:2281–2285

Ferguson SM, Bazalakova M, Savchenko V, Tapia JC, Wright J, Blakely RD (2004) Lethal impairment of cholinergic neurotransmission in hemicholinium-3-sensitive choline transporter knockout mice. Proc Natl Acad Sci U S A 101:8762–8767

Fiszman GL, Middonno MC, De la TE, Farina M, Espanol AJ, Sales ME (2007) Activation of muscarinic cholinergic receptors induces MCF-7 cells proliferation and angiogenesis by stimulating nitric oxide synthase activity. Cancer Biol Ther 6:1106–1113

Friday BB, Adjei AA (2008) Advances in targeting the Ras/Raf/MEK/Erk mitogen-activated protein kinase cascade with MEK inhibitors for cancer therapy. Clin Cancer Res 14:342–346

Fritz S, Wessler I, Breitling R, Rossmanith W, Ojeda SR, Dissen GA, Amsterdam A, Mayerhofer A (2001) Expression of muscarinic receptor types in the primate ovary and evidence for nonneuronal acetylcholine synthesis. J Clin Endocrinol Metab 86:349–354

Frucht H, Gazdar AF, Park JA, Oie H, Jensen RT (1992) Characterization of functional receptors for gastrointestinal hormones on human colon cancer cells. Cancer Res 52:1114–1122

Frucht H, Jensen RT, Dexter D, Yang WL, Xiao Y (1999) Human colon cancer cell proliferation mediated by the M3 muscarinic cholinergic receptor. Clin Cancer Res 5:2532–2539

Fujii T, Yamada S, Yamaguchi N, Fujimoto K, Suzuki T, Kawashima K (1995) Species differences in the concentration of acetylcholine, a neurotransmitter, in whole blood and plasma. Neurosci Lett 201:207–210

Gabrielson E (2006) Worldwide trends in lung cancer pathology. Respirology 11:533–538

Gautam D, Han SJ, Heard TS, Cui Y, Miller G, Bloodworth L, Wess J (2005) Cholinergic stimulation of amylase secretion from pancreatic acinar cells studied with muscarinic acetylcholine receptor mutant mice. J Pharmacol Exp Ther 313:995–1002

Gautam D, Han SJ, Hamdan FF, Jeon J, Li B, Li JH, Cui Y, Mears D, Lu H, Deng C, Heard T, Wess J (2006) A critical role for beta cell M3 muscarinic acetylcholine receptors in regulating insulin release and blood glucose homeostasis in vivo. Cell Metab 3:449–461

Glunde K, Ackerstaff E, Mori N, Jacobs MA, Bhujwalla ZM (2006) Choline phospholipid metabolism in cancer: consequences for molecular pharmaceutical interventions. Mol Pharm 3:496–506

Grando SA (2011) Muscarinic receptor agonists and antagonists: effects on keratinocyte function. In: Fryer AD (ed) Muscarinic receptors, Handbook of experimental pharmacology. Springer, Heidelberg

Grando SA, Kist DA, Qi M, Dahl MV (1993) Human keratinocytes synthesize, secrete, and degrade acetylcholine. J Invest Dermatol 101:32–36

Grando SA, Pittelkow MR, Schallreuter KU (2006) Adrenergic and cholinergic control in the biology of epidermis: physiological and clinical significance. J Invest Dermatol 126: 1948–1965

Gromada J, Hughes TE (2006) Ringing the dinner bell for insulin: muscarinic M3 receptor activity in the control of pancreatic beta cell function. Cell Metab 3:390–392

Guizzetti M, Costa LG (2001) Activation of phosphatidylinositol 3 kinase by muscarinic receptors in astrocytoma cells. Neuroreport 12:1639–1642

Guizzetti M, Costa P, Peters J, Costa LG (1996) Acetylcholine as a mitogen: muscarinic receptor-mediated proliferation of rat astrocytes and human astrocytoma cells. Eur J Pharmacol 297: 265–273

Jimenez E, Montiel M (2005) Activation of MAP kinase by muscarinic cholinergic receptors induces cell proliferation and protein synthesis in human breast cancer cells. J Cell Physiol 204:678–686

Kenakin T (2004) Efficacy as a vector: the relative prevalence and paucity of inverse agonism. Mol Pharmacol 65:2–11

Klapproth H, Reinheimer T, Metzen J, Munch M, Bittinger F, Kirkpatrick CJ, Hohle KD, Schemann M, Racke K, Wessler I (1997) Non-neuronal acetylcholine, a signalling molecule synthezised by surface cells of rat and man. Naunyn Schmiedebergs Arch Pharmacol 355: 515–523

Kodaira M, Kajimura M, Takeuchi K, Lin S, Hanai H, Kaneko E (1999) Functional muscarinic m3 receptor expressed in gastric cancer cells stimulates tyrosine phosphorylation and MAP kinase. J Gastroenterol 34:163–171

Kumar V, Abbas AK, Fausto N, Aster JC (2009) Robbins and Cotran pathologic basis of disease, 8th edn. W.B. Saunders Company, Philadelphia, PA

Lammerding-Koppel M, Noda S, Blum A, Schaumburg-Lever G, Rassner G, Drews U (1997) Immunohistochemical localization of muscarinic acetylcholine receptors in primary and metastatic malignant melanomas. J Cutan Pathol 24:137–144

Lee CH, Huang CS, Chen CS, Tu SH, Wang YJ, Chang YJ, Tam KW, Wei PL, Cheng TC, Chu JS, Chen LC, Wu CH, Ho YS (2010) Overexpression and activation of the {alpha}9-nicotinic receptor during tumorigenesis in human breast epithelial cells. J Natl Cancer Inst 102: 1322–1335

Leonard A, Cuq P, Magous R, Bali JP (1991) M3-subtype muscarinic receptor that controls intracellular calcium release and inositol phosphate accumulation in gastric parietal cells. Biochem Pharmacol 42:839–845

Luthin GR, Wang P, Zhou H, Dhanasekaran D, Ruggieri MR (1997) Role of m1 receptor-G protein coupling in cell proliferation in the prostate. Life Sci 60:963–968

Machova E, O'Regan S, Newcombe J, Meunier FM, Prentice J, Dove R, Lisa V, Dolezal V (2009) Detection of choline transporter-like 1 protein CTL1 in neuroblastoma x glioma cells and in the CNS, and its role in choline uptake. J Neurochem 110(4):1297–1309

Mak JC, Baraniuk JN, Barnes PJ (1992) Localization of muscarinic receptor subtype mRNAs in human lung. Am J Respir Cell Mol Biol 7:344–348

Martinez-Moreno P, Nieto-Ceron S, Torres-Lanzas J, Ruiz-Espejo F, Tovar-Zapata I, Martinez-Hernandez P, Rodriguez-Lopez JN, Vidal CJ, Cabezas-Herrera J (2006) Cholinesterase activity of human lung tumours varies according to their histological classification. Carcinogenesis 27:429–436

Matthiesen S, Bahulayan A, Kempkens S, Haag S, Fuhrmann M, Stichnote C, Juergens UR, Racke K (2006) Muscarinic receptors mediate stimulation of human lung fibroblast proliferation. Am J Respir Cell Mol Biol 35:621–627

Mayerhofer A, Kunz L (2005) A non-neuronal cholinergic system of the ovarian follicle. Ann Anat 187:521–528

Montenegro MF, Nieto-Ceron S, Ruiz-Espejo F, Paez dlC, Rodriguez-Berrocal FJ, Vidal CJ (2005) Cholinesterase activity and enzyme components in healthy and cancerous human colorectal sections. Chem Biol Interact 157–158:429–430

Morin D, Zini R, Lange F, Lange J, Tillement JP (1987) Alterations of beta-adrenergic, muscarinic cholinergic receptors and imipramine binding sites in human lung tumors. Int J Clin Pharmacol Ther Toxicol 25:605–608

Natoli C, Perrucci B, Perrotti F, Falchi L, Iacobelli S (2010) Tyrosine kinase inhibitors. Curr Cancer Drug Targets 10:462–483

Negroni MP, Fiszman GL, Azar ME, Morgado CC, Espanol AJ, Pelegrina LT, De la TE, Sales ME (2010) Immunoglobulin G from breast cancer patients in stage I stimulates muscarinic acetylcholine receptors in MCF7 cells and induces proliferation. Participation of nitric oxide synthase-derived nitric oxide. J Clin Immunol 30:474–484

Noda S, Lammerding-Koppel M, Oettling G, Drews U (1998) Characterization of muscarinic receptors in the human melanoma cell line SK-Mel-28 via calcium mobilization. Cancer Lett 133:107–114

Obara K, Arai K, Miyajima N, Hatano A, Tomita Y, Takahashi K (2000) Expression of m2 muscarinic acetylcholine receptor mRNA in primary culture of human prostate stromal cells. Urol Res 28:196–200

Okuda T, Haga T, Kanai Y, Endou H, Ishihara T, Katsura I (2000) Identification and characterization of the high-affinity choline transporter. Nat Neurosci 3:120–125

Oppitz M, Mobus V, Brock S, Drews U (2002) Muscarinic receptors in cell lines from ovarian carcinoma: negative correlation with survival of patients. Gynecol Oncol 85:159–164

Oppitz M, Busch C, Garbe C, Drews U (2008) Distribution of muscarinic receptor subtype M3 in melanomas and their metastases. J Cutan Pathol 35:809–815

Park JG, Frucht H, LaRocca RV, Bliss DP Jr, Kurita Y, Chen TR, Henslee JG, Trepel JB, Jensen RT, Johnson BE (1990) Characteristics of cell lines established from human gastric carcinoma. Cancer Res 50:2773–2780

Pieper MP, Chaudhary NI, Park JE (2007) Acetylcholine-induced proliferation of fibroblasts and myofibroblasts in vitro is inhibited by tiotropium bromide. Life Sci 80:2270–2273

Proskocil BJ, Sekhon HS, Jia Y, Savchenko V, Blakely RD, Lindstrom J, Spindel ER (2004) Acetylcholine is an autocrine or paracrine hormone synthesized and secreted by airway bronchial epithelial cells. Endocrinology 145:2498–2506

Raufman JP, Chen Y, Cheng K, Compadre C, Compadre L, Zimniak P (2002) Selective interaction of bile acids with muscarinic receptors: a case of molecular mimicry. Eur J Pharmacol 457:77–84

Raufman JP, Cheng K, Zimniak P (2003) Activation of muscarinic receptor signaling by bile acids: physiological and medical implications. Dig Dis Sci 48:1431–1444

Raufman JP, Samimi R, Shah N, Khurana S, Shant J, Drachenberg C, Xie G, Wess J, Cheng K (2008) Genetic ablation of M3 muscarinic receptors attenuates murine colon epithelial cell proliferation and neoplasia. Cancer Res 68:3573–3578

Rayford W, Noble MJ, Austenfeld MA, Weigel J, Mebust WK, Shah GV (1997) Muscarinic cholinergic receptors promote growth of human prostate cancer cells. Prostate 30:160–166

Reinheimer T, Bernedo P, Klapproth H, Oelert H, Zeiske B, Racke K, Wessler I (1996) Acetylcholine in isolated airways of rat, guinea pig, and human: species differences in role of airway mucosa. Am J Physiol 270:L722–L728

Reinheimer T, Munch M, Bittinger F, Racke K, Kirkpatrick CJ, Wessler I (1998) Glucocorticoids mediate reduction of epithelial acetylcholine content in the airways of rats and humans. Eur J Pharmacol 349:277–284

Ruggieri MR, Colton MD, Wang P, Wang J, Smyth RJ, Pontari MA, Luthin GR (1995) Human prostate muscarinic receptor subtypes. J Pharmacol Exp Ther 274:976–982

Schmitt JM, Abell E, Wagner A, Davare MA (2010) ERK activation and cell growth require CaM kinases in MCF-7 breast cancer cells. Mol Cell Biochem 335:155–171

Sekhon H, Sauer D, Corless CL, Lupo SL, Lindstrom J, Spindel ER (2002) Expression of nicotinic acetylcholine receptors, choline acetyltransferase and lynx1 in pancreatic carcinoma. Proc AACR 43:A2569; abstract

Song P, Spindel ER (2007) Novel Na-independent choline transporters mediate choline transport and acetylcholine induced-proliferation in small cell lung carcinoma. Am J Respir Crit Care Med 175:A47; abstract

Song P, Sekhon HS, Jia Y, Keller JA, Blusztajn JK, Mark GP, Spindel ER (2003) Acetylcholine is synthesized by and acts as an autocrine growth factor for small cell lung carcinoma. Cancer Res 63:214–221

Song P, Sekhon HS, Duan J, Mark GP, Spindel ER (2004) Inhibitory regulation by M2 muscarinic acetylcholine receptors is decreased in lung cancers. Am J Respir Crit Care Med 169:A290; abstract

Song P, Sekhon HS, Lu A, Arredondo J, Sauer D, Gravett C, Mark GP, Grando SA, Spindel ER (2007) M3 muscarinic receptor antagonists inhibit small cell lung carcinoma growth and mitogen-activated protein kinase phosphorylation induced by acetylcholine secretion. Cancer Res 67:3936–3944

Song P, Sekhon HS, Fu XW, Maier M, Jia Y, Duan J, Proskosil BJ, Gravett C, Lindstrom J, Mark GP, Saha S, Spindel ER (2008) Activated cholinergic signaling provides a target in squamous cell lung carcinoma. Cancer Res 68:4693–4700

Song P, Maier M, Olivas AS, Spindel ER (2009) Inhibition of lung cancer cell growth by tiotropium: mechanism of action. Am J Respir Crit Care Med 179:A2675; abstract

Song P, Olivas AS, Spindel ER (2010) Tiotropium inhibits growth of squamous cell lung carcinoma (SCC) cell lines in vitro and also inhibits SCC growth in vivo in nude mice by inhalation. Eur Respir J 36(946S); abstract

Tang X, Batty IH, Downes CP (2002) Muscarinic receptors mediate phospholipase C-dependent activation of protein kinase B via Ca^{2+}, ErbB3, and phosphoinositide 3-kinase in 1321N1 astrocytoma cells. J Biol Chem 277:338–344

Ukegawa JI, Takeuchi Y, Kusayanagi S, Mitamura K (2003) Growth-promoting effect of muscarinic acetylcholine receptors in colon cancer cells. J Cancer Res Clin Oncol 129:272–278

Wang T, Li J, Chen F, Zhao Y, He X, Wan D, Gu J (2007) Choline transporters in human lung adenocarcinoma: expression and functional implications. Acta Biochim Biophys Sin (Shanghai) 39:668–674

Wessler IK, Kirkpatrick CJ (2001) The non-neuronal cholinergic system: an emerging drug target in the airways. Pulm Pharmacol Ther 14:423–434

Wessler IK, Kirkpatrick CJ (2011) Activation of muscarinic receptors by non-neuronal acetylcholine. In: Fryer AD (ed) Muscarinic receptors, Handbook of experimental pharmacology. Springer, Heidelberg

Wessler I, Reinheimer T, Klapproth H, Schneider FJ, Racke K, Hammer R (1997) Mammalian glial cells in culture synthesize acetylcholine. Naunyn Schmiedebergs Arch Pharmacol 356:694–697

Whitsett JA, Hollinger B (1984) Muscarinic cholinergic receptors in developing rat lung. Pediatr Res 18:1136–1140

Williams JA (2006) Regulation of pancreatic acinar cell function. Curr Opin Gastroenterol 22:498–504

Witte LP, Chapple CR, de la Rosette JJ, Michel MC (2008) Cholinergic innervation and muscarinic receptors in the human prostate. Eur Urol 54:326–334

Xie G, Drachenberg C, Yamada M, Wess J, Raufman JP (2005) Cholinergic agonist-induced pepsinogen secretion from murine gastric chief cells is mediated by M1 and M3 muscarinic receptors. Am J Physiol Gastrointest Liver Physiol 289:G521–G529

Yagle K, Lu H, Guizzetti M, Moller T, Costa LG (2001) Activation of mitogen-activated protein kinase by muscarinic receptors in astroglial cells: role in DNA synthesis and effect of ethanol. Glia 35:111–120

Yang WL, Frucht H (2000) Cholinergic receptor up-regulates COX-2 expression and prostaglandin E(2) production in colon cancer cells. Carcinogenesis 21:1789–1793

Activation of Muscarinic Receptors by Non-neuronal Acetylcholine

Ignaz Karl Wessler and Charles James Kirkpatrick

Abstract The biological role of acetylcholine and the cholinergic system is revisited based particularly on scientific research early and late in the last century. On the one hand, acetylcholine represents the classical neurotransmitter, whereas on the other hand, acetylcholine and the pivotal components of the cholinergic system (high-affinity choline uptake, choline acetyltransferase and its end product acetylcholine, muscarinic and nicotinic receptors and esterase) are expressed by more or less all mammalian cells, i.e. by the majority of cells not innervated by neurons at all. Moreover, it has been demonstrated that acetylcholine and "cholinergic receptors" are expressed in non-neuronal organisms such as plants and protists. Acetylcholine is even synthesized by bacteria and algae representing an extremely old signalling molecule on the evolutionary timescale. The following article summarizes examples, in which non-neuronal acetylcholine is released from primitive organisms as well as from mammalian non-neuronal cells and binds to muscarinic receptors to modulate/regulate phenotypic cell functions via auto-/paracrine pathways. The examples demonstrate that non-neuronal acetylcholine and the non-neuronal cholinergic system are vital for various types of cells such as epithelial, endothelial and immune cells.

Keywords Bacteria • Cell functions • Choline acctyltnnsloves • Epithelial – Endothelial – Mesenchymal – immune cells • Evolution • Plants • Muscarinic receptors • Non-neuronal acetylcholine • Release • Urtica diocie

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1 Change of Paradigm: Acetylcholine Revisited as a Ubiquitous Signalling Molecule in Nature

The impressive work of Otto Loewi in 1921 provided first experimental evidence for chemical neurotransmission (Loewi 1921). The vagus-substance "parasympathin" was identified as acetylcholine 5 years later (Loewi and Navratil 1926). Since that time, acetylcholine and the cholinergic system (high-affinity choline uptake [HACU], choline acetyltransferase [ChAT], vesicular transporter [VAChT], cholinesterase and nicotinic and muscarinic receptors [n- and m-AChR]) have been characterized as the most exemplary system mediating chemical neurotransmission. In addition, acetylcholine has been regarded as the foremost prototype of neurotransmitter. In contrast to this idea, the scientific community had unfortunately neglected the first experiments by Ewins and Dale when they were investigating depressor effects of ergot extracts (Ewins 1914; Dale 1914). The biological effect of lowering blood pressure was attributed to acetylcholine. In conclusion, in one of the first experiments illuminating the biological role of acetylcholine, the "substance" was extracted from fungi, i.e. from non-neuronal organisms. Later on (1963) Whittaker stated that "acetylcholine occurs in non-nervous tissues and is so widely distributed in nature to suggest a non-nervous function of it" and Koelle speculated that acetylcholine represents a phylogenetically very old molecule, which in primitive organisms such as plants and unicellular organisms might be involved in the regulation of transport processes (Whittaker 1963; Koelle 1963).

In fact, important contributions in the last century showed the synthesis of acetylcholine in bacteria, algae, tubellaria, yeast, fungi, protozoa, nematodes, sponges and plants (Dale and Dudley 1929; Beyer and Wense 1936; Comline 1946; Bülbring et al. 1953; Lentz 1966; Erzen and Brzin 1979; Stephenson and Rowatt 1947; Fischer 1971; Sastry and Sadavongvivad 1979; Smallman and Maneckjee 1981; Wessler et al. 1998, 1999; Horiuchi et al. 2003). Therewith, acetylcholine is as far as we know one of the oldest signalling molecules in the evolutionary process. In this context, it has been postulated that when the first neurons were established in marine organisms (molluscs; about 500 million years ago) these cells took advantage of the already well-established cholinergic signalling system (Wessler et al. 1999). The existing system was further specialized by the generation of three important properties: the generation of a vesicular storage system; the generation of a synchronized vesicular release machinery; and finally, the upregulation (hot spots) for n- and m-AChRs and cholinesterase. Thus, rapid and repetitive cellular communication on the ms timescale was established. The electrical organ of some fishes may be regarded as an interesting lateral branch of this evolutionary process (see Fig. 1).

Despite this clear evidence about non-neuronal acetylcholine in the first half of the last century, acetylcholine has attracted scientific interest more or less exclusively as a neurotransmitter, its synaptic function and related neuronal diseases. Only late in the last century new experimental evidence using modern analytic techniques demonstrated the expression of acetylcholine and the main components

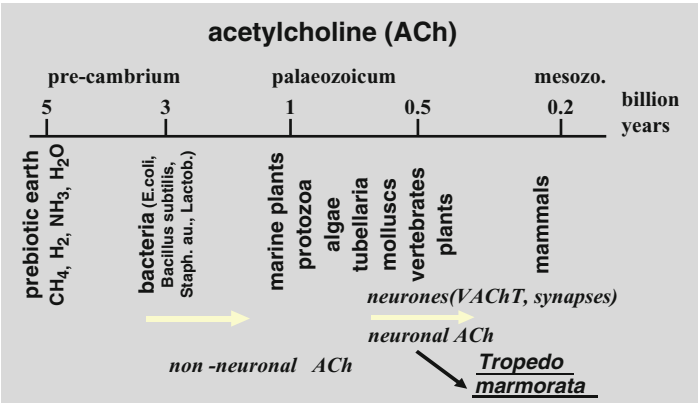

Fig. 1 Timescale of the appearance of acetylcholine during evolution. Organic compounds such as glycine, glutamic acid as well as carboxylic acids (acetic, propionic and succinic acid) have been detected in the Urey–Miller experiment addressed to identify compounds in the prebiotic earth. Hence, the tertiary amine choline may have been generated likewise before the occurrence of living organisms. Acetylation represents one of the most common reactions in nature; thus acetylcholine may have been established shortly after choline. Cholinesterase activity is present in plants and other primitive organisms. Moreover, binding structures comparable to m- and n-AChRs are expressed in plants (see effects of atropine and tubocurarine on *Urtica dioica)*. In conclusion, a complete cholinergic chemical signalling system (acetylcholine, binding structures and esterase) has been generated by evolution very early. When the animal kingdom was established, a more sophisticated communication system was required, i.e. the peripheral and central nervous system. Neurons have taken advantage of the already established cholinergic system and have improved its effectiveness (cellular packing in vesicles, synchronized release, hot spots of receptors and esterase activity) to mediate rapid and repetitive communication (ms timescale) with a very high safety factor. From that period onwards acetylcholine was also acting as neurotransmitter. The electrical organ of some fishes (e.g. Torpedo marmorata) can be regarded as an interesting lateral branch of this evolutionary process

of the cholinergic system in more or less all cells in humans. Thus, the terms "non-neuronal acetylcholine" and "non-neuronal cholinergic system" have been created to discriminate the system from the neuronal one. In the last years, important review articles have been published to illuminate this topic in more detail (Sastry and Sadavongvivad 1979; Grando 1997; Wessler et al. 1998, 1999, 2001a, 2003; Kawashima and Fujii 2000, 2004; Eglen 2006; Grando et al. 2006, 2007; Kurzen et al. 2007; Wessler and Kirkpatrick 2008; Kawashima and Fujii 2008; Kummer et al. 2008).

2 Synthesis of Acetylcholine Outside of Cholinergic Neurones

Synthesis and release of acetylcholine by non-neuronal cells (i.e. cells not innervated by extrinsic or intrinsic cholinergic neurons) represent a "*conditio sine qua non*" to allow the stimulation of m- or n-AChRs by non-neuronal acetylcholine.

Synthesis of acetylcholine is mediated via two enzymes, choline acetyltransferase (ChAT) and carinitine acetyltransferase (CarAT). Both enzymes have been found to mediate the synthesis of non-neuronal acetylcholine in primitive cells such as plants and also in vertebrates (White and Cavallito 1970; White and Wu 1973; Horiuchi et al. 2003; Yamada et al. 2005). Figure 1 roughly summarizes the approximate timescale during the evolutionary process with regard to the generation of acetylcholine. Table 1 gives an overview about acetylcholine synthesis beyond neurons, i.e. by cells or organisms independent of cholinergic innervation. The non-neuronal expression is obvious for all cells, tissues or organisms where neurons do not exist (e.g. plants, bacteria and unicellular organisms). However, in the case of mammals one may argue that demonstration of acetylcholine is caused by contaminating neuronal acetylcholine. This possibility can be definitively ruled out for the following reasons:

(a) Acetylcholine synthesis has been shown in isolated cultured cells (keratinocytes, airway epithelial cells, endothelial cells and cardiomyocytes) and cell lines such as leukemic T-cells, embryonic stem cells, colon or lung cancer cell lines which are free of neurons (Grando et al. 1993, Reinheimer et al. 1996; Fujii et al. 1998; Kawashima and Fujii 2000, 2003; Paraoanu et al. 2007; Cheng et al. 2008; Song et al. 2003; Kakinuma et al. 2009).
(b) Likewise ChAT mRNA and ChAT protein have been demonstrated in most of these cells.
(c) Multiple authors have shown specific labelling of non-neuronal cells by anti-ChAT antibodies (for references, see Table 1 and review articles cited above).
(d) The human placenta, free of cholinergic neurons, synthesizes, stores and releases acetylcholine (Olubadewo and Rama Sastry 1978; Wessler et al. 2001b).
(e) Previously, in vivo release of acetylcholine from human skin has been demonstrated by dermal microdialysis; botulinum toxin known to block neuronal acetylcholine release did not inhibit acetylcholine release from the human skin (Schlereth et al. 2006).

All these findings with cells isolated or generated from mammals indicate the presence of non-neuronal acetylcholine independent of intrinsic or extrinsic cholinergic neurons. This conclusion can be regarded as a mandatory consequence of the evolutionary process, i.e. the extreme early appearance of acetylcholine in bacteria, unicellular organisms and primitive plants. In the majority of these primitive organisms the function of acetylcholine remains to be established. However in the plant, *Urtica dioica,* experimental evidence has been published about the biological role of acetylcholine (Wessler et al. 2001a).

Already in 1949 Emmelin and Feldberg reported the presence of acetylcholine in nettle plants (Emmelin and Feldberg 1949). In *Urtica dioica*, we found a considerable amount of acetylcholine in leaves, stems and particularly in the roots (about 0.5 µmol/g dry weight; Wessler et al. 2001a). It is obvious that such a high synthesis must be linked to biological functions. Figure 2 shows the effect of 1 µM atropine which markedly reduced the intercellular space, the cell vacuole and cell size and induced proliferation of the thylakoid membrane. In conclusion, acetylcholine, at least in nettle plants, is involved in the regulation of water homeostasis and

Table 1 Positive anti-ChAT immunoreactivity or HPLC detection of ACh

Non-mammals	
Bacteria	*Bacillus subtilis, Escherichia coli, Lactobacillus plantarum, Staphylococcus aureus*
Archaea	Hyperthermophiles, Methanogens, Halophiles
Fungi	*Agaricus bisporus*, *Cantharellus cibarius* Yeast *Saccharomyces cerevisiae*, Shiitake mushroom *Lentinus eddoes*, Matsutake mushroom *Tricholoma matsutake*
Protozoa	*Paramecium, Trypanosoma rhodesiense*
Plants	*Amaranthus caudatus, Arum specifcum, Arum maculatum, Brachythecium, Capsella bursa-pastoris, Citrus aurantium, Cucurbita pepo, Equisetum robustum, Equisetum arvense, Fragaria vesca, Helianthus annuus,* moss callus*, Phaseolus vulgaris, Pisum sativum, Senecio vulgaris, Sinapis albam, spinacea oleracea, Urtica dioica,* Anthophyta *Arabidopsis thaliana, Solanum melongena, Phyllostachys bambusoides, Phyllostachys pubescens,* Coniferophyta *Cryptomeria japonica, Pinus thunbergii, Podocarpus macrophyllus,* Pterophyta *Pteridium, Gleichenia glauca, mung bean*
Primitive animals	Chordate, Mollusca, Annelida, Cindaria, Porifera
Mammals (rat, humans)	
Epithelial cells	
Airways	Basal, ciliated, secretory and brush cells
Alimentary tract	Mucosa of oral cavity, oesophagus, stomach(partially), jejunum, ileum, colon, sigmoid, gall bladder
Skin	Keratinocytes, eccrine and sebaceous glands
Kidney	Tubuli
Urogenital tract (mice)	Urothelium, vaginal mucosa, granulosa cells, embryonic stem cells
Placenta	Trophoblast
Glandular tissue	Female breast, thymus
Eye	Cornea
Endothelial cells	Skin, umbilical vein, pulmonary vessels
Immune cells	Mononuclear leukocytes, bone marrow-derived dendritic cells, macrophages, skin mast cells
Mesothelial cells	Pleura, pericardium
Mesenchymal cells	Adipocytes (skin), smooth muscle fibres (skin, airways), fibroblasts (airways [a]), tendon (tenocytes)
Brain	Astrocytes [a]

Data from: Sastry and Sadavongvivad (1979); Grando et al. (1993); Grando (1997); Klapproth et al. (1997); Wessler and Kirkpatrick (2001a, b); Wessler et al. (1998, 1999, 2001a); Kawashima et al. (1998); Kawashima and Fujii (2000, 2004); Horiuchi et al. (2003); Yamada et al. (2005); Danielson et al. (2007); Paraoanu et al. (2007)

[a] Unpublished observation (Wessler and Kirkpatrick 2007)

photosynthesis. Interestingly, tubocurarine caused comparable effects, which may indicate common binding structures as it is already known for the α9 subtype (n-AChRs). Atropine, like tubocurarine, and a variety of other antagonists or agonists at m- and n-AChRs represent alkaloids or are formed as toxins by hunting animals. Also this observation points to the extreme significance of the cholinergic system in

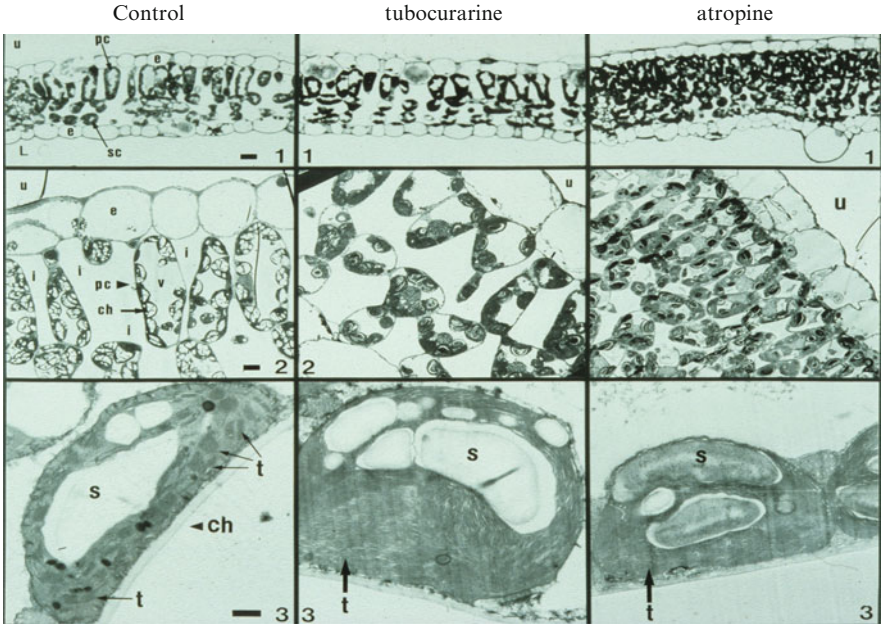

Fig. 2 Effect of tubocurarine and atropine on leaves of *Urtica dioica*. *Urtica dioica* was incubated (48 h) with the stems in water (control) or in water containing 30 μM tubocurarine or 1 μM atropine (*panel 1*: bar represents 14.5 μm, magnification 400×; *panel 2* electron microscopy: *bar* represents 4 μm, magnification 1,300×; *panel 3*: bar represents 0.5 μm, magnification 17,000×). Shown are representative leaves. *Left-hand row*: panel 1 shows the regular morphology with epidermal (e) cells at the upper (u) and lower (L) side and parenchymal cells (*pc* palisade cells; *sc* spongy cells); panel 2 shows the upper epidermal cells and palisade cells with the chloroplasts (ch) and cell vacuole (v) and intercellular space (i); panel 3 shows an individual chloroplast containing the stratified thycaloid membranes (t) and starch (s). Middle row: tubocurarine caused reduction of palisade cell size and proliferation of the thylakoid membrane. *Right-hand row*: atropine changed cell organization, reduced intercellular space, cell vacuole and cell size and increased thylakoid membrane (from Wessler et al. 2001a)

the plant and animal kingdom. Most likely the alkaloids atropine and tubocurarine and related compounds are expressed by plants as part of an endogenous regulatory pathway with acetylcholine or a similar cholinomimetic molecule acting as endogenous agonist.

3 Release of Acetylcholine from Non-neuronal Cells

A unique condition for the stimulation of m-AChRs by non-neuronal acetylcholine is the demonstration of its release from non-neuronal cells. Therefore in completion to Table 1, where ChAT expression and acetylcholine synthesis are shown, some

examples are given in this paragraph to demonstrate the release of non-neuronal acetylcholine from non-neuronal cells.

(a) *Urtica dioica* contains large amounts of acetylcholine verified by HPLC measurement. When *Urtica dioica* with the roots or stems was incubated for 6 h in water, substantial amounts of acetylcholine were found (about 0.1–0.9 μM acetylcholine solution); similar results were observed when isolated leaves were placed in water (Wessler and Kirkpatrick, unpublished observation). It remains to be elucidated whether the acetylcholine in the incubating water represents leakage or active release.

(b) The human placenta is not innervated by extrinsic or intrinsic cholinergic neurons but synthesizes considerable amounts of acetylcholine (Loewi and Navratil 1926). Release of acetylcholine has been measured from isolated villus pieces or in vitro perfused placental cotyleda (Olubadewo and Rama Sastry 1978; Boura et al. 1986; Sastry 1997; Wessler et al. 2001b).

(c) Release of acetylcholine was demonstrated from cultured bovine arterial endothelial cells (Kawashima et al. 1990).

(d) Cultured epithelial cell prepared from bronchi of monkeys releases acetylcholine into the supernatant (Proskocil et al. 2004). It is known that non-neuronal acetylcholine is released via organic cation transporters (OCT; Wessler et al. 2001c). In OCT-knockout mice airway epithelial acetylcholine content was doubled compared to control, which indicates an in vivo release of acetylcholine from these cells (Kummer et al. 2006a, b). Also the inhibitory effect of n-AChRs antagonists on the migration of cultured airway epithelial cells indicates indirectly the release of non-neuronal acetylcholine (Tournier et al. 2006).

(e) Cell lines of small cell lung cancer such as the H82 cell line synthesize and secrete acetylcholine into the supernatant (Song et al. 2003).

(f) Cultured keratinocytes have been shown to synthesize and secrete acetylcholine into the supernatant (Grando et al. 1993). In addition, in vivo release of non-neuronal acetylcholine from human volunteers after local pretreatment with botulinum toxin has been demonstrated by dermal microdialysis (Schlereth et al. 2006).

(g) Acetylcholine synthesis and release into the surrounding culture medium is demonstrated for MOLT-3 cells, a T-cell line (Fujii et al. 1996). Moreover, stimulation of these cells by the mitogen phytohemagglutinin enhanced both synthesis and release of acetylcholine (Fujii et al. 1996, 1998).

(h) Acetylcholine is also found in the plasma of various mammalian species (Kawashima and Fujii 2000), which indicates the release from circulating immune cells and/or endothelial cells. However, it should be mentioned that Okonek and Kilbinger (1974) did not detect acetylcholine in the plasma of healthy volunteers but in that of patients intoxicated with an organophosphorus cholinesterase inhibitor. This is not a surprising finding because specific cholinesterase and butyrylcholinesterase are present in the plasma and in

addition specific cholinesterase in red blood cells. Blocking of cholinesterase will increase the availability of circulating acetylcholine.

(i) Cultured urothelial cells isolated from the rat urinary bladder were labelled with radioactive choline. Enhanced release of radioactivity occurred after exposure to a hypotonic Krebs solution, and atropine facilitated this efflux which was not affected by brefeldin, a compound blocking vesicular acetylcholine release (Hanna-Mitchell et al. 2007).

(j) Several colon cancer cell lines (Caco-2; H508) have been shown to synthesize and release acetylcholine into the culture media (Cheng et al. 2008).

All these findings clearly indicate that acetylcholine is synthesized and released from cells independent of neurons or neuronal input. When non-neuronal acetylcholine is released from these cells, i.e. transported into the extracellular space, it can diffuse in close proximity to its source but also to neighbouring cells, because the expression level of cholinesterase activity is lower in non-innervated than in innervated cells (for example placenta; Sastry and Sadavongvivad 1979). Consequently, non-neuronal acetylcholine can mediate auto- and paracrine effects by stimulating m- and n-AChRs which are ubiquitously expressed in the majority of cells (for muscarinic receptors, see Table 2; for review, see Wessler and Kirkpatrick 2008). However, less is known about the release mechanisms involved.

Release of acetylcholine from neurons represents a complex synchronized mechanism (exo-/endocytosis), in which vesicles highly packed with acetylcholine are activated via a transient increase in free intracellular calcium and fuse with the plasma membrane to release acetylcholine into the synaptic cleft or extracellular space (Katz and Miledi 1965). Thus, a threshold concentration of acetylcholine is established within the innervated effector cells. Neurotoxins (tetanus and botulinum toxin) have been used to identify the key proteins within the nerve terminal required for exocytosis (for review, see Meunier et al. 2002). In contrast to the detailed knowledge about the release machinery for neuronal acetylcholine, we know less about the release of non-neuronal acetylcholine. Acetylcholine at physiological pH represents a permanent cation. Organic cation transporters (OCTs) are widely expressed in different cell types (Koepsell 1998; Dresser et al. 1999). Three organic cation transporters have been cloned which represent high capacity non-neuronal monoamine transporters, OCT subtype 1 (Gründemann et al. 1994; Nagel et al. 1997), subtype 2 (Okuda et al. 1996; Gründemann et al. 1997, 1998) and subtype 3, the latter also being known as extraneuronal monoamine transporter uptake 2 (Gründemann et al. 1998; Kekuda et al. 1998; Wu et al. 1998). One or even all subtypes are expressed by more or less all mammalian cells. In the human placenta it has been demonstrated that non-neuronal acetylcholine is released into the extracellular space via OCT subtypes 1 and 3 (Wessler et al. 2001c) and in the airway epithelial cells subtypes 1 and 2 are involved (Kummer et al. 2006a, b). Also transfected ooyctes showed the release of acetylcholine via OCT subtypes 1 and 2 (Lips et al. 2007). In murine airways epithelial cells OCT subtype 2 is particularly expressed at the luminal side, allowing directed luminal release of non-neuronal acetylcholine (Lips et al. 2005). In human skin, quinine, a strong inhibitor

Table 2 Expression of m-AChRs on non-neuronal cells

Tissue	Cell type	Muscarinic	References
Airways	Surface epithelium	M1 (small airways) M3 (M2; BEAS-2B cell line)	Barnes (1993), Gosens et al. (2006), Gwilt et al. (2007), Mak and Barnes (1989), Lazareno et al. (1990), Matthiesen et al. (2007), Metzen et al. (2003), Proskocil et al. (2004), Ramnarine et al. (1996), Racke et al. (2006), Tsutsumi et al. (1999), and Wessler and Kirkpatrick (2001b)
	Alveolar type 2 cells and alveolar wall	M1, M4 (rabbit)	
	Glands (epithelial cells)	M3 > M1	
	Smooth muscle fibres	M2 > M3	
	Fibroblasts	M2 > M1 > M3 > M4	
Skin	Keratinocytes	M1, M2, M3, M4, (m5 mRNA)	Buchli et al. (1999), Hagforsen (2007), Grando (1997), Grando et al. (2006), Casanova et al. (2006) and Kurzen et al. (2004, 2006)
	Pilosebaceous unit	All subtypes	
	Sweat glands	M2–M5	
	Myoepithelial acinar cells	Aee subtypes	
	Melanocytes	All subtypes	
	Fibroblasts	All subtypes	
Intestine	Surface epithelium	M1, M3	Gautam et al. (2004, 2005, 2006), Haberberger et al. (2006), Hirota and McKay (2006), and Xie et al. (2005)
	Colonic epithelial cell line		
	Glands (salivary cells, gastric cells, pancreatic acinar cells)	M1, M3	
Urogenital tract	Kidney	M1, M3	Bschleipfer et al. (2007), Fritz et al. (2001), Mayerhofer and Fritz (2002), Kang et al. (2003), Mohuczy-Dominiak and Garg (1992), Robey et al. (2001), Takeda et al. (1994), and Zarghooni et al (2007)
	Ovary granulosa cells	M1, M3, M5	
	Urothelium	M1, M2, M3, M4, M5	
	Oocytes	M3, M4	
Vascular tissue	Endothelial cells	M3, M1, (m2 mRNA)	Grueb et al. (2006), Khurana et al. (2004), Tracey and Peach (1992), Walch et al. (2001), and Yamada et al. (2001)
	Aorta/pulmonary vessels		
	Corneal endothelium (human)	M2, M4, M5	
	Brain vessels (mice)	M3, M5	
	Smooth muscle fibres	M3	
Immune system	MNLs (human)	M1–M5 (variable expression)	Kawashima and Fujii (2000, 2003, 2004, 2008) and Reinheimer et al. (2000)
	Macrophages	M2, M3 (human) M1–M5 (mouse)	
	DC cells	M1–M5 (mouse)	
	Mast cells	M1 (human)	
Heart	Cardiomyocytes	M2, M3	Pönicke et al. (2003) and Sheikh Abdul Kadir et al. (2010)

of OCTs, tended to reduce release of non-neuronal acetylcholine and the combination of quinine and carnitine, a substrate for another subtype (known as OCTN2) nearly halved the release of non-neuronal acetylcholine (Schlereth et al. 2006). In conclusion, the role of OCT subtypes in mediating acetylcholine release may differ between different cells. Moreover, also other transporters or transport mechanisms (endosomes) may be involved. It is important to identify the release mechanisms of non-neuronal acetylcholine in more detail because new therapeutical targets can be developed and unwanted side effects of applied drugs may possibly be linked to so far unknown interaction with the non-neuronal cholinergic system and the release of non-neuronal acetylcholine. It would be important to identify such interactions. Finally, it should be considered that acetylcholine does not represent a unique endogenous ligand at m-AChRs. For example, bile acids have been shown to stimulate m-AChRs (Raufman et al. 2003; Sheikh Abdul Kadir et al. 2010).

4 Stimulation of m-AChRs by Non-neuronal Acetylcholine

4.1 Plants

Table 2 gives an overview about the expression of different subtypes of m-AChRs on non-neuronal cells in different mammalian species. However, the first example for the stimulation of a binding site comparable to m-AChRs is given in Sect. 2 and Fig. 2. Atropine at a concentration of 1 μM produced substantial morphological changes in the leaves of *Urtica dioica* comparable to d-tubocurarine. The leaves became dry, although the stems were standing in water; this effect was prevented when 1 μM acetylcholine was added in addition to atropine and tubocurarine (Wessler et al. 2001a). The present observation indicates that even plants make use of the cholinergic signalling system and have developed binding sites for acetylcholine which are recognized by the alkaloid atropine. Blockade of this binding site is followed by severe failure of water homeostasis and a change in photosynthesis. Obviously in the plant kingdom a variety of alkaloids targeting m- and n-AChRs have been established (m-AChRs antagonists: L-hyoscyamine together with its racemate atropine, scopolamine; allosteric m-AChRs antagonists: strychnine and brucine; m-AChRs agonists: muscarine, acetylcholine, pilocarpine and arecoline; n-AChRs antagonists: alcuronium, tubocurarine and toxiferine; n-AChRs agonists: nicotine, acetylcholine, cytosine and arecoline). In addition to these alkaloids several so-called muscarinic toxins have been identified in recent years (Adem et al. 1988; Servent and Fruchart-Gaillard 2009). As already mentioned above the existence of these natural m- and n-AChR agonists/antagonists led us to the conclusion that plants and most likely also other organisms regulate their own cell function via endogenous ligands at m- and n-AChRs.

4.2 Bacteria

Motility of two photosynthetic bacteria (*Rhodospirillum rubrum* and *thiospirillium jenense*), which are endowed with flagella, was stopped by 1 mM atropine. However, it should be considered that physostigmine and other cholinesterase inhibitors reduced motility as well (Faust and Doetsch 1971); probably the system became desensitized in the presence of cholinesterase inhibitors.

4.3 Mussels and Sea Urchin

Mytilus edulis and *anodonta* are marine mussels. The gill plates of these mussels do not contain nerves or muscles (Burn 1956) but show impressive ciliary activity. Atropine at 1.4 μM produced a small increase (12%) in ciliary movement, but 1.4 mM atropine reduced (20%) activity (Burn 1956; Burn and Day 1958). In addition, gill plates contain about 3.8 nmol acetylcholine per gram wet weight and Bülbring concluded already in the 50s of the last century that acetylcholine functions as a local hormone in maintaining the rhythmic movements of cilia on the gill plates (Bülbring et al. 1953). The rather high concentration of atropine required for an inhibitory effect is of course a surprising observation. However, binding properties may differ between rather primitive marine organisms and mammals.

Sperm of non-mammals and mammals contains acetylcholine (Bishop et al. 1976; Wessler et al. 1998), and in bull spermatozoa ChAT activity is particularly concentrated within the tail region (Bishop et al. 1976). The motility of the sperm of marine organisms such as sea urchin *(Arbacia punctulata)* is reduced by 50% in the presence of 100 μM atropine, whereas low concentrations within the range of 1 nM caused a 50% increase (Nelson 1974), indicating inhibitory and facilitatory m-AChR-related mechanisms.

4.4 Mammals

4.4.1 Epithelial Cells

Epithelial cells of the skin, the airways and the digestive tract represent well-characterized cells with respect to the expression of the non-neuronal cholinergic system and the role of muscarinic receptors.

(a) Skin
The pioneering work by Grando on the role of the cholinergic system in keratinocytes is summarized in previous review articles, particularly the effects of m-AChRs on proliferation, differentiation, adhesion and motility of keratinocytes (Grando 2006; Grando et al. 2006; Kurzen et al. 2006, 2007).

Antagonists at m-AChRs caused keratinocyte detachment and increased permeability of a keratinocyte monolayer to a radioactive tracer (Nguyen et al. 2003, 2004). Likewise, Wessler et al. (1999) reported an increase in the mean distance between two neighbouring keratinocytes from 0.78 to 1.06 μm in isolated human skin treated for 30 min with 1 μM atropine. Antagonists of m-AChRs inhibit proliferation of keratinocytes. M3 and M4 receptors produce opposite effects on radon migration as shown in experiments with siRNA in agarose gel outgrowth experiments. Silencing the M4 decreased and that of M3 increased radon migration distances of keratinocytes (Grando et al. 2006). Correspondingly, in M3−/− knockout mice basal epithelization rate was increased, because the start of wound epithelization is mediated by keratinocyte migration, this being facilitated in the absence of M3 receptors (Li et al. 2004; Grando et al. 2006).

Using organotypic cocultures with fibroblasts and keratinocytes to examine the role of the non-neuronal cholinergic system on epidermal physiology it was found that atropine reduced proliferation rate, impaired the epidermal barrier function by the reduction of tight junctions and caused acantholysis by inhibiting the expression of cell adhesion proteins (Kurzen et al. 2006, 2007). These experiments clearly demonstrate the stimulation of m-AChRs by non-neuronal acetylcholine, because under culture conditions any contribution of neuronal acetylcholine can be excluded.

(b) Airways

Ciliary beat frequency is activated by acetylcholine and m-AChR agonists (preferable M3) and reduced by m-AChR antagonists (Wong et al. 1988a, b; Salathe et al. 1997). Also cilia-driven transport or mucociliary clearance is stimulated by m-AChR agonists, most likely via the M3 subtype, at least in mice (Acevedo 1994; Klein et al. 2009). Intensive positive ChAT immunoreactivity has been demonstrated at the apical pole of the cilia (Wessler et al. 1998, Wessler and Kirkpatrick 2001b) and a luminal release of acetylcholine from ciliated cells has been postulated because of the apical localization of OCTs (Lips et al. 2005; Kummer et al. 2008). Thus, it is justified to conclude that non-neuronal acetylcholine released from ciliated and neighbouring epithelial cells stimulates m-AChRs via auto-/paracrine mechanisms to increase ciliary beat frequency and mucociliary clearance. In in vitro experiments with bovine trachea atropine at a high concentration of 100 μM reduced ciliary beat frequency, whereas a concentration of 100 nM produced the opposite effect (Wong et al. 1988b). In the rabbit trachea atropine at a concentration of 1.4 μM reduced ciliary activity by about 55%. Despite these complex observations more evidence in favour of a depressing effect of atropine on ciliary beat frequency has been accumulated (Wanner 1986). Ipratropium bromide, likewise a non-selective m-AChR antagonist, did not produce an inhibitory effect on ciliary activity (Wanner 1986). However, in a study, in which the effect of ipratropium bromide on cough clearance in COPD patients was tested, the antagonist diminished the effectiveness of cough in clearing radiolabelled particles (Bennett et al. 1993). In conclusion, ciliary activity is most likely

modified by non-neuronal acetylcholine stimulating m-AChRs via auto- and paracrine mechanisms. Experiments with gene-deficient mice showed stimulation via M3 and an inhibition via M2 receptors (Klein et al. 2009). This pattern may at least partly explain the opposite effects of m-AChR antagonists described above.

Acetylcholine and m-AChR agonists stimulate the release of prostanoids and inflammatory mediators. For example, the release of PGE_2 from the rat trachea is epithelium-dependent and mediated via M3 receptors (Brunn et al. 1995). Comparably, neutrophil and monocyte chemotatic activity is released from bovine cultured airway epithelial cells via m-AChRs (Koyama et al. 1992). Thus, one can also conclude that the endogenous ligand, non-neuronal acetylcholine, can modulate the release of PGE_2 and other mediators as well.

Applied acetylcholine doubled the proliferation rate of human cultured airway epithelial cells (Metzen et al. 2003). This effect is prevented by the combination of n- and m-AChR antagonists, i.e. by a combination of 30 μM tubocurarine and 1 μM pirenzepine. However, a combination of tubocurarine with 1 μM of either AFDX 116 or hexahydrosiladifenidol did not abolish the facilitatory effect of acetylcholine, indicating an M1 subtype involved (Metzen et al. 2003). In addition, the basal [H]thymidine incorporation was significantly reduced in the presence of tubocurarine together with 1 μM pirenzepine, thus indicating an endogenous, basal activity mediated by non-neuronal acetylcholine (Metzen et al. 2003). This evidence shows that M1 receptors stimulated by non-neuronal acetylcholine are involved in the regulation of the proliferation rate of human airway epithelial cells. Correspondingly, it was found that m-AChR antagonists reduced cell growth of small cell lung carcinoma cell in vitro (culture conditions) and in vivo experiments with tumours in nude mice (Song et al. 2007).

A recent finding indicates a direct coupling of m-AChRs on airway epithelia to caveolin (Schlenz et al. 2010). Caveolae are omega-shaped invaginations of the plasma membrane representing focal points to concentrate G-protein regulated receptors and the respective signal transduction machinery. Disintegration of the caveolae reduced muscarinic bronchoconstriction substantially (Schlenz et al. 2010). Thus, there exists a link between both the functional organization of caveolae and m-AChRs. In immunogold electron microscopy to visualize the subcellular location of ChAT in human placenta we have found strong immunogold deposition within caveolae (Wessler and Kirkpatrick, unpublished observation). Possibly, non-neuronal acetylcholine is synthesized in close proximity to caveolae, suggesting an endogenous tone in the regulation of caveolin-dependent signalling into the cell.

(c) Intestine

Atropine substantially reduces fluid secretion in isolated rabbit intestinal epithelial cells treated with *Shigella dysenteriae* type 1 toxin (Kaur et al. 1995). In addition, it is known that m-AChR antagonists prevent the early-onset diarrhoea induced by irinotecan, which is also known as cholinergic syndrome and at least partly mediated by cholinesterase inhibition (Hecht 1998).

Acetylcholine is synthesized within human intestinal epithelial cells (Klapproth et al. 1997) and it is known that acetylcholine stimulates apical chloride and water secretion. Moreover, intestinal epithelial cells express various m-AChR subtypes (M1, M2 and M3 receptors; Lepor et al. 1990; O'Malley et al. 1995; Haberberger et al. 2006; Jönsson et al. 2007). Under the condition of cholinesterase inhibition or of Shigella toxin-activation epithelial acetylcholine is most likely directly responsible for stimulation of m-AChRs and the induction of profound diarrhoea. Of course, this concept lacks direct experimental evidence in vivo, but the rationale is based on morphological and functional data obtained with respect to the expression of m-AChRs, the local synthesis of acetylcholine and its well-known cellular effects.

Human colon cell cancers express M3 receptors (Frucht et al. 1999; Cheng et al. 2008). In H508 human colon cancer cell lines proliferation rate was increased by cholinesterase inhibitors, whereas atropine or p-fluorohexahydro-sila-difenidol inhibited basal proliferation rate by 40% (Cheng et al. 2008; Shah et al. 2009). These results clearly demonstrate the stimulation of m-AChRs located on epithelial cancer cells by non-neuronal acetylcholine.

4.4.2 Endothelial Cells

In vascular tissue M3 and M1 receptors mediate the release of nitric oxide (NO) and other vasoactive mediators. In addition, it has been demonstrated that endothelial cells synthesize and contain acetylcholine (for references, see Kirkpatrick et al. 2003). Recently it has been demonstrated that gradual hypothermia induces an NO-dependent vasodilatation, which could be prevented by 1 μM pirenzepine (Evora et al. 2007), in canine isolated coronary, femoral and renal arteries. This observation indicates the involvement of endogenous acetylcholine in the intrinsic regulatory loop. Most likely hypothermia induces the release of endothelial acetylcholine which by stimulation of M3 and M1 receptors mediates the generation of NO and vasodilatation. Unfortunately, the authors did not test whether the effect of hypothermia was resistant to the sodium channel blocker tetrodotoxin.

4.4.3 Immune Cells

Activation of T-cells by the T-cell receptor complex via phytohemagglutinin stimulates the synthesis of acetylcholine as well the expression of M5 receptors (Kawashima and Fujii 2000, 2003; Kawashima et al. 2007). Likewise the expression of ChAT was upregulated in mononuclear cells and bone marrow dendritic cells stimulated with Concanavalin A and lipopolysaccharide, respectively (Kawashima et al. 2007). In experiments with isolated naive $CD8^+$ T-cells the so-called mixed lymphocyte reaction was used to measure the transformation into cytolytic T-cells by alloantigens. When in addition either acetylcholinesterase – to remove acetylcholine – or atropine – to block m-AChRs – was added, the

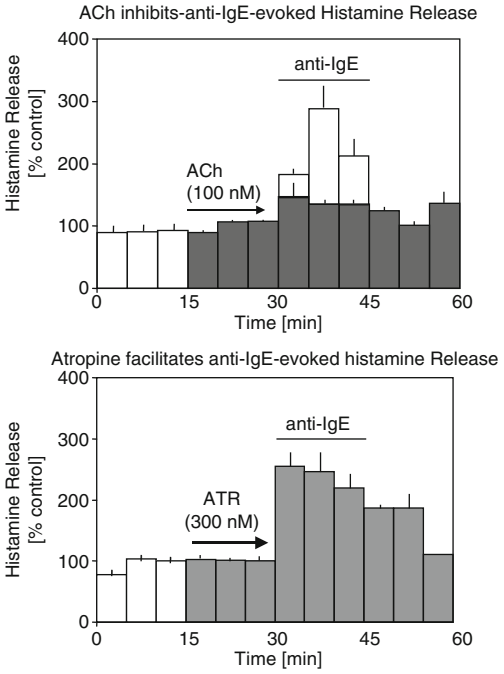

Fig. 3 Effect of acetylcholine and atoprine on histamine release from human bronchi evoked by anti-IgE antibodies (values from Reinheimer et al. 1997). Open columns represent control and filled columns histamine release in the presence of acetylcholine (ACh) or atropine (ATR). The basal histamine release in the presence of acetylcholine or atropine did not differ from control

generation of lytic activity was substantially suppressed (Zimring et al. 2005). These in vitro experiments clearly demonstrate that non-neuronal acetylcholine stimulates m-AChRs via auto-paracrine mechanisms and thus can trigger the generation of cytolytic T-cells. In experiments with M1-receptor knockout mice the generation of cytolytic T-cell activity was also reduced, indicating the M1 subtype involved (Zimring et al. 2005).

Histamine release from human airway mucosal mast cells is strongly inhibited by m-AChRs of the M1 subtype (Reinheimer et al. 1997, 2000; see also Fig. 3). Figure 4 shows the dose–response curve for applied acetylcholine to inhibit the histamine release evoked by the calcium ionophore A23187 from human bronchi. Obviously, acetylcholine was extremely sensitive, and in the absence of cholinesterase inhibitors even at a concentration of 0.1 nM suppressed histamine release by more than 60%; i.e. an IC_{50} value of 0.03 nM was found (Reinheimer et al. 1997). Such an extreme high affinity of m-AChR agonists has never been reported for the classical responses of effector cells innervated by the parasympathetic nervous system. Most likely, m-AChRs of non-neuronal cells are adapted to the considerably lower levels of acetylcholine released from these cells in contrast to the synchronized high threshold release of neuronal acetylcholine. Acetylcholine also suppressed the release evoked by anti-IgE antibodies, i.e. a more physiological stimulus (Fig. 3; Reinheimer et al. 1997). Importantly, atropine 300 nM facilitated the release of histamine indicating an endogenous inhibitory feedback loop, i.e. the stimulation of M1 receptors by non-neuronal acetylcholine even under in vitro experiments with isolated human bronchi (Fig. 3; Reinheimer et al. 1997).

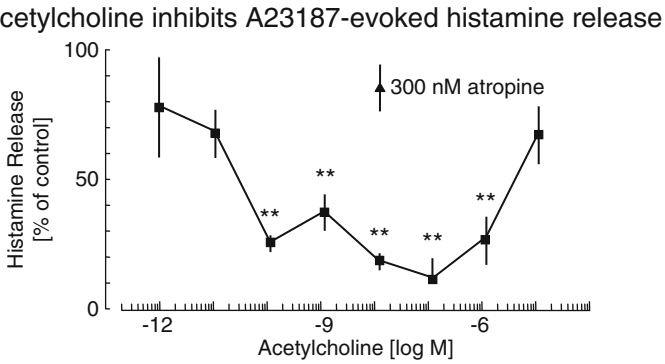

Fig. 4 Concentration–response curve of acetylcholine on the A23187-evoked histamine release from human airways (from Reinheimer et al. 1997)

5 Conclusion

Figure 1 gives an overview about the approximate evolutionary timescale with respect to the first appearance of acetylcholine acting as a signalling molecule. Without any doubt acetylcholine and the cholinergic system has been created in rather primitive organisms such as plants and unicellular organisms. Experiments with classical antagonists of n- and m-AChRs operating in mammalian tissue are also effective in these primitive organisms; i.e. binding proteins recognizing acetylcholine and transmitting a signal are already established in these organisms. The experiments with *Urtica dioica* and the reversibility by applied acetylcholine demonstrate these properties. Thus, it is important to recognize acetylcholine as a ubiquitous cell signalling molecule independent of neuronal communication. This situation explains the following observations, some of them already known for many decades:

- The ubiquitous expression of acetylcholinesterase and unspecific cholinesterase in nature, including humans. This expression pattern prevents the overspill of acetylcholine and allows non-neuronal acetylcholine to act very locally at individual cell level or limited networks of cells for fine-tuning of cell functions.
- The ubiquitous expression of ChAT by non-neuronal cells in mammals, including humans.
- The ubiquitous expression of various subtypes of n- and m-AChRs on non-neuronal cells, i.e. cells not innervated by cholinergic neurons. The existence of all different subtypes of n- and m-AChRs on these cells allows a very sophisticated local regulation of phenotypic cell functions via chemical transmission, i.e. a situation comparable to neurons and the brain.

Whenever agonists or antagonists at m-AChRs are applied therapeutically (for example cholinesterase inhibitors, agonists such as carbachol and bethanchol, and antagonist for the treatment of COPD or travel sickness) it must be considered that

all m-AChRs, i.e. those stimulated by neuronal as well as by non-neuronal acetylcholine, are affected. Antipsychotic drugs with atropine-like properties will interfere with innervated and non-innervated cells. For example the possible change of blood count, skin diseases such as erythema, exanthema and rash or side effects in the heart (arrhythmia) observed after the treatment with phenothiazines or clozapine are so far less well understood. However, it is important to consider whether these effects could be linked to the non-neuronal cholinergic system.

Finally, it is important to analyze the non-neuronal cholinergic system and its contribution to acute or chronic diseases, particularly with respect to acute and chronic inflammation, tumorigenesis and angiogenesis to develop more specific therapeutic targets to minimize side effects and maximize the therapeutic benefit.

References

Acevedo M (1994) Effect of acetyl choline on ion transport in sheep tracheal epithelium. Pflugers Arch 427:543–546

Adem A, Asblom A, Johansson G et al (1988) Toxins from the venom of the green mamba *Dendroaspis angusticeps* that inhibit the binding of quinuclidinyl benzilate to muscarinic acetylcholine receptors. Biochim Biophys Acta 968:340–345

Barnes PJ (1993) Muscarinic receptor subtypes in airways. Life Sci 52:521–527

Bennett MA, Watt PA, Thurston H (1993) Impaired endothelium-dependent relaxation in two-kidney, one clip Goldblatt hypertension: effect of vasoconstrictor prostanoids. J Hypertens Suppl 11:S134–S135

Beyer G, Wense UT (1936) Über den Nachweis von Hormonen in einzelligen Tieren: Cholin und Acetylcholin in Paramecium. Pflügers Arch Gesamte Physiol MenschenTiere 237:417–422

Bishop MR, Sastry BV, Schmidt DE et al (1976) Occurrence of choline acetyltransferase and acetylcholine and other quaternary ammonium compounds in mammalian spermatozoa. Biochem Pharmacol 25:1617–1622

Boura AL, Gude NM, King RG et al (1986) Acetylcholine output and foetal vascular resistance of human perfused placental cotyleda. Br J Pharmacol 88:301–306

Brunn G, Wessler I, Racke K (1995) Mucosa-dependent muscarinic liberation of prostaglandins from rat isolated trachea. Br J Pharmacol 116:1991–1998

Bschleipfer T, Schukowski K, Weidner W et al (2007) Expression and distribution of cholinergic receptors in the human urothelium. Life Sci 80:2303–2307

Buchli R, Ndoye A, Rodriguez JG et al (1999) Human skin fibroblasts express m2, m4, and m5 subtypes of muscarinic acetylcholine receptors. J Cell Biochem 74:264–277

Bülbring E, Burn JH, Shelley HJ (1953) Acetylcholine and ciliary movement in the gill plate of Mytilus edulis. Proc R Soc Ser Biol Sci 141:445–466

Burn JH (1956) Functions of automatic transmitters. Williams and Wilkins, Baltimore

Burn JH, Day M (1958) The action of tubocurarine and acetylcholine on ciliary movement. J Physiol 141:520–526

Casanova M, Furlan C, Sterin-Borda L, et al (2006) Muscarinic cholinoceptor activation modulates DNA synthesis and CD40 expression in fibroblast cells. Auton Autacoid Pharmacol 26:293–301

Cheng K, Samimi R, Xie G et al (2008) Acetylcholine release by human colon cancer cells mediates autocrine stimulation of cell proliferation. Am J Physiol Gastrointest Liver Physiol 295:G591–G597

Comline RS (1946) Synthesis of acetylcholine by non-nervous tissue. J Physiol (Lond) 105:6P–7P

Dale HH (1914) The action of certain esters and ethers of choline and their relation to muscarine. J Pharmacol Exp Ther 6:147–190
Dale HH, Dudley HW (1929) The presence of histamine and acetylcholine in the spleen of the ox and the horse. J Physiol 68:97–123
Danielson P, Andersson G, Alfredson H et al (2007) Extensive expression of markers for acetylcholine synthesis and of M2 receptors in tenocytes in therapy-resistant chronic painful patellar tendon tendinosis – a pilot study. Life Sci 80:2235–2238
Dresser MJ, Zhang L, Giacomini KM (1999) Molecular and functional characteristics of cloned human organic cation transporters. Pharm Biotechnol 12:441–469
Eglen RM (2006) Muscarinic receptor subtypes in neuronal and non-neuronal cholinergic function. Auton Autacoid Pharmacol 26:219–233
Emmelin N, Feldberg W (1949) Distribution of acetylcholine and histamine in nettle plants. New Phytol 48:143
Erzen I, Brzin M (1979) Cholinergic mechanisms in *Planaria torva*. Comp Biochem Physiol 64C:207–216
Evora PR, Cable DG, Chua YL et al (2007) Nitric oxide and prostacyclin-dependent pathways involvement on in vitro induced hypothermia. Cryobiology 54:106–113
Ewins AJ (1914) Acetylcholine, a new active principle of ergot. Biochem J 8:44–49
Faust MA, Doetsch RN (1971) Effect of drugs that alter excitable membranes on the motility of *Rhodospirillum rubrum* and *Thiospirillum jenense*. Can J Microbiol 17:191–196
Fischer H (1971) Vergleichende Pharmakologie von Überträgersubstanzen in tiersystematischer Darstellung. In: Eichler O, Farah A, Herken H, Welch AD (eds) Vergleichende Pharmakologie von Überträgersubstanzen in tiersystematischer Darstellung. Springer, Heidelberg
Fritz S, Wessler I, Breitling R et al (2001) Expression of muscarinic receptor types in the primate ovary and evidence for nonneuronal acetylcholine synthesis. J Clin Endocrinol Metab 86:349–354
Frucht H, Jensen RT, Dexter D et al (1999) Human colon cancer cell proliferation mediated by the M3 muscarinic cholinergic receptor. Clin Cancer Res 5:2532–2539
Fujii T, Tsuchiya T, Yamada S et al (1996) Localization and synthesis of acetylcholine in human leukemic T cell lines. J Neurosci Res 44:66–72
Fujii T, Yamada S, Watanabe Y et al (1998) Induction of choline acetyltransferase mRNA in human mononuclear leukocytes stimulated by phytohemagglutinin, a T-cell activator. J Neuroimmunol 82:101–107
Gautam D, Heard TS, Cui Y et al (2004) Cholinergic stimulation of salivary secretion studied with M1 and M3 muscarinic receptor single- and double-knockout mice. Mol Pharmacol 66:260–267
Gautam D, Han SJ, Heard TS et al (2005) Cholinergic stimulation of amylase secretion from pancreatic acinar cells studied with muscarinic acetylcholine receptor mutant mice. J Pharmacol Exp Ther 313:995–1002
Gautam D, Duttaroy A, Cui Y et al (2006) M1-M3 muscarinic acetylcholine receptor-deficient mice: novel phenotypes. J Mol Neurosci 30:157–160
Gosens R, Zaagsma J, Meurs H et al (2006) Muscarinic receptor signaling in the pathophysiology of asthma and COPD. Respir Res 7:73
Grando SA (1997) Biological functions of keratinocyte cholinergic receptors. J Investig Dermatol Symp Proc 2:41–48
Grando SA (2006) Cholinergic control of epidermal cohesion. Exp Dermatol 15:265–282
Grando SA, Kist DA, Qi M et al (1993) Human keratinocytes synthesize, secrete, and degrade acetylcholine. J Invest Dermatol 101:32–36
Grando SA, Pittelkow MR, Schallreuter KU (2006) Adrenergic and cholinergic control in the biology of epidermis: physiological and clinical significance. J Invest Dermatol 126:1948–1965
Grando SA, Kawashima K, Kirkpatrick CJ et al (2007) Recent progress in understanding the non-neuronal cholinergic system in humans. Life Sci 80:2181–2185

Grueb M, Reinthal E, Rohrbach JM et al (2006) Muscarinic acetylcholine receptor subtypes in human corneal epithelium and endothelium. Graefes Arch Clin Exp Ophthalmol 244:1191–1195

Gründemann D, Gorboulev V, Gambaryan S et al (1994) Drug excretion mediated by a new prototype of polyspecific transporter. Nature 372:549–552

Gründemann D, Babin-Ebell J, Martel F et al (1997) Primary structure and functional expression of the apical organic cation transporter from kidney epithelial LLC-PK1 cells. J Biol Chem 272:10408–10413

Gründemann D, Schechinger B, Rappold GA et al (1998) Molecular identification of the corticosterone-sensitive extraneuronal catecholamine transporter. Nat Neurosci 1:349–351

Gwilt CR, Donnelly LE, Rogers DF (2007) The non-neuronal cholinergic system in the airways: an unappreciated regulatory role in pulmonary inflammation? Pharmacol Ther 115:208–222

Haberberger R, Schultheiss G, Diener M (2006) Epithelial muscarinic M1 receptors contribute to carbachol-induced ion secretion in mouse colon. Eur J Pharmacol 530:229–233

Hagforsen E (2007) The cutaneous non-neuronal cholinergic system and smoking related dermatoses: studies of the psoriasis variant palmoplantar pustulosis. Life Sci 80:2227–2234

Hanna-Mitchell AT, Beckel JM, Barbadora S et al (2007) Non-neuronal acetylcholine and urinary bladder urothelium. Life Sci 80:2298–2302

Hecht JR (1998) Gastrointestinal toxicity or irinotecan. Oncology (Williston Park) 12:72–78

Hirota CL, McKay DM (2006) Cholinergic regulation of epithelial ion transport in the mammalian intestine. Br J Pharmacol 149:463–479

Horiuchi Y, Kimura R, Kato N et al (2003) Evolutional study on acetylcholine expression. Life Sci 72:1745–1756

Jönsson M, Norrgard O, Forsgren S (2007) Presence of a marked nonneuronal cholinergic system in human colon: study of normal colon and colon in ulcerative colitis. Inflamm Bowel Dis 13:1347–1356

Kakinuma Y, Akiyama T, Sato T (2009) Cholinoceptive and cholinergic properties of cardiomyocytes involving an amplification mechanism for vagal efferent effects in sparsely innervated ventricular myocardium. FEBS J 276:5111–5125

Kang D, Park JY, Han J et al (2003) Acetylcholine induces Ca^{2+} oscillations via m3/m4 muscarinic receptors in the mouse oocyte. Pflugers Arch 447:321–327

Katz B, Miledi R (1965) Release of acetylcholine from a nerve terminal by electric pulses of variable strength and duration. Nature 207:1097–1098

Kaur T, Singh S, Gorowara S et al (1995) Role of enteric nervous system in *Shigella dysenteriae* type 1 toxin-induced fluid secretion in rabbit ileum. J Diarrhoeal Dis Res 13:159–165

Kawashima K, Fujii T (2000) Extraneuronal cholinergic system in lymphocytes. Pharmacol Ther 86:29–48

Kawashima K, Fujii T (2003) The lymphocytic cholinergic system and its biological function. Life Sci 72:2101–2109

Kawashima K, Fujii T (2004) Expression of non-neuronal acetylcholine in lymphocytes and its contribution to the regulation of immune function. Front Biosci 9:2063–2085

Kawashima K, Fujii T (2008) Basic and clinical aspects of non-neuronal acetylcholine: overview of non-neuronal cholinergic systems and their biological significance. J Pharmacol Sci 106:167–173

Kawashima K, Watanabe N, Oohata H et al (1990) Synthesis and release of acetylcholine by cultured bovine arterial endothelial cells. Neurosci Lett 119:156–158

Kawashima K, Fujii T, Watanabe Y et al (1998) Acetylcholine synthesis and muscarinic receptor subtype mRNA expression in T-lymphocytes. Life Sci 62:1701–1705

Kawashima K, Yoshikawa K, Fujii YX et al (2007) Expression and function of genes encoding cholinergic components in murine immune cells. Life Sci 80:2314–2319

Kekuda R, Prasad PD, Wu X et al (1998) Cloning and functional characterization of a potential-sensitive, polyspecific organic cation transporter (OCT3) most abundantly expressed in placenta. J Biol Chem 273:15971–15979

Khurana S, Chacon I, Xie G et al (2004) Vasodilatory effects of cholinergic agonists are greatly diminished in aorta from M3R−/− mice. Eur J Pharmacol 493:127–132
Kirkpatrick CJ, Bittinger F, Nozadze K et al (2003) Expression and function of the non-neuronal cholinergic system in endothelial cells. Life Sci 72:2111–2116
Klapproth H, Reinheimer T, Metzen J et al (1997) Non-neuronal acetylcholine, a signalling molecule synthezised by surface cells of rat and man. Naunyn Schmiedebergs Arch Pharmacol 355:515–523
Klein MK, Haberberger RV, Hartmann P et al (2009) Muscarinic receptor subtypes in cilia-driven transport and airway epithelial development. Eur Respir J 33:1113–1121
Koelle GB (1963) The coming of age of therapeutics. Am J Pharm Sci Support Public Health 135:38–44
Koepsell H (1998) Organic cation transporters in intestine, kidney, liver, and brain. Annu Rev Physiol 60:243–266
Koyama S, Rennard SI, Robbins RA (1992) Acetylcholine stimulates bronchial epithelial cells to release neutrophil and monocyte chemotactic activity. Am J Physiol 262:L466–L471
Kummer W, Wiegand S, Akinci S et al (2006a) Role of acetylcholine and muscarinic receptors in serotonin-induced bronchoconstriction in the mouse. J Mol Neurosci 30:67–68
Kummer W, Wiegand S, Akinci S et al (2006b) Role of acetylcholine and polyspecific cation transporters in serotonin-induced bronchoconstriction in the mouse. Respir Res 7:65
Kummer W, Lips KS, Pfeil U (2008) The epithelial cholinergic system of the airways. Histochem Cell Biol 130:219–234
Kurzen H, Berger H, Jager C et al (2004) Phenotypical and molecular profiling of the extraneuronal cholinergic system of the skin. J Invest Dermatol 123:937–949
Kurzen H, Henrich C, Booken D et al (2006) Functional characterization of the epidermal cholinergic system in vitro. J Invest Dermatol 126:2458–2472
Kurzen H, Wessler I, Kirkpatrick CJ et al (2007) The non-neuronal cholinergic system of human skin. Horm Metab Res 39:125–135
Lazareno S, Buckley NJ, Roberts FF (1990) Characterization of muscarinic M4 binding sites in rabbit lung, chicken heart, and NG108-15 cells. Mol Pharmacol 38:805–815
Lentz TL (1966) Intramitochondrial glycogen granules in digestive cells of Hydra. J Cell Biol 29:162–167
Lepor H, Rigaud G, Shapiro E et al (1990) Muscarinic cholinergic and alpha 2-adrenergic receptors in the epithelium and muscularis of the human ileum. Surgery 107:461–467
Li W, Fan J, Chen M et al (2004) Mechanisms of human skin cell motility. Histol Histopathol 19:1311–1324
Lips KS, Volk C, Schmitt BM et al (2005) Polyspecific cation transporters mediate luminal release of acetylcholine from bronchial epithelium. Am J Respir Cell Mol Biol 33:79–88
Lips KS, Luhrmann A, Tschernig T et al (2007) Down-regulation of the non-neuronal acetylcholine synthesis and release machinery in acute allergic airway inflammation of rat and mouse. Life Sci 80:2263–2269
Loewi O (1921) Über humorale Übertragbarkeit der Herznervenwirkung. Plügers Arch 189:239–242
Loewi O, Navratil E (1926) Über humorale Übertragbarkeit der Herznervenwirkung. X. Über das Schicksal des Vagusstoff. Pflugers Arch Ges Physiol 214:678–688
Mak JC, Barnes PJ (1989) Muscarinic receptor subtypes in human and guinea pig lung. Eur J Pharmacol 164:223–230
Matthiesen S, Bahulayan A, Holz O et al (2007) MAPK pathway mediates muscarinic receptor-induced human lung fibroblast proliferation. Life Sci 80:2259–2262
Mayerhofer A, Fritz S (2002) Ovarian acetylcholine and muscarinic receptors: hints of a novel intrinsic ovarian regulatory system. Microsc Res Tech 59:503–508
Metzen J, Bittinger F, Kirkpatrick CJ et al (2003) Proliferative effect of acetylcholine on rat trachea epithelial cells is mediated by nicotinic receptors and muscarinic receptors of the M1-subtype. Life Sci 72:2075–2080

Meunier FA, Schiavo G, Molgo J (2002) Botulinum neurotoxins: from paralysis to recovery of functional neuromuscular transmission. J Physiol Paris 96:105–113
Mohuczy-Dominiak D, Garg LC (1992) Muscarinic receptors in MDCK cells are coupled to multiple messenger systems. Am J Physiol 263:C1289–C1294
Nagel G, Volk C, Friedrich T et al (1997) A reevaluation of substrate specificity of the rat cation transporter rOCT1. J Biol Chem 272:31953–31956
Nelson L (1974) Control of sperm motility: a neurochemical approach. In: Afzelius BA (ed) The functional anatomy of the spermatozoa. Pergamon Press, New York
Nguyen VT, Arredondo J, Chernyavsky AI et al (2003) Keratinocyte acetylcholine receptors regulate cell adhesion. Life Sci 72:2081–2085
Nguyen VT, Chernyavsky AI, Arredondo J et al (2004) Synergistic control of keratinocyte adhesion through muscarinic and nicotinic acetylcholine receptor subtypes. Exp Cell Res 294:534–549
O'Malley KE, Farrell CB, O'Boyle KM et al (1995) Cholinergic activation of Cl^- secretion in rat colonic epithelia. Eur J Pharmacol 275:83–89
Okonek S, Kilbinger H (1974) Determination of acetylcholine, nitrostigmine and acetylcholinesterase activity in four patients with severe nitrostigmine (E 605 forte) intoxication. Arch Toxicol 32:97–108
Okuda M, Saito H, Urakami Y et al (1996) cDNA cloning and functional expression of a novel rat kidney organic cation transporter, OCT2. Biochem Biophys Res Commun 224:500–507
Olubadewo JO, Rama Sastry BV (1978) Human placental cholinergic system: stimulation-secretion coupling for release of acetylcholine from isolated placental villus. J Pharmacol Exp Ther 204:433–445
Paraoanu LE, Steinert G, Koehler A et al (2007) Expression and possible functions of the cholinergic system in a murine embryonic stem cell line. Life Sci 80:2375–2379
Pönicke K, Heinroth-Hoffmann I, Brodde OE (2003) Demonstration of functional M3-muscarinic receptors in ventricular cardiomyocytes of adult rats. Br J Pharmacol 138:156–160
Proskocil BJ, Sekhon HS, Jia Y et al (2004) Acetylcholine is an autocrine or paracrine hormone synthesized and secreted by airway bronchial epithelial cells. Endocrinology 145:2498–2506
Racke K, Juergens UR, Matthiesen S (2006) Control by cholinergic mechanisms. Eur J Pharmacol 533:57–68
Ramnarine SI, Haddad EB, Khawaja AM et al (1996) On muscarinic control of neurogenic mucus secretion in ferret trachea. J Physiol 494:577–586
Raufman JP, Cheng K, Zimniak P (2003) Activation of muscarinic receptor signaling by bile acids: physiological and medical implications. Dig Dis Sci 48:1431–1444
Reinheimer T, Bernedo P, Klapproth H et al (1996) Acetylcholine in isolated airways of rat, guinea pig, and human: species differences in role of airway mucosa. Am J Physiol 270:L722–L728
Reinheimer T, Baumgartner D, Hohle KD et al (1997) Acetylcholine via muscarinic receptors inhibits histamine release from human isolated bronchi. Am J Respir Crit Care Med 156:389–395
Reinheimer T, Mohlig T, Zimmermann S et al (2000) Muscarinic control of histamine release from airways. Inhibitory M1-receptors in human bronchi but absence in rat trachea. Am J Respir Crit Care Med 162:534–538
Robey RB, Ruiz OS, Baniqued J et al (2001) SFKs, Ras, and the classic MAPK pathway couple muscarinic receptor activation to increased Na-HCO(3) cotransport activity in renal epithelial cells. Am J Physiol Renal Physiol 280:F844–F850
Salathe M, Lipson EJ, Ivonnet PI et al (1997) Muscarinic signaling in ciliated tracheal epithelial cells: dual effects on Ca^{2+} and ciliary beating. Am J Physiol 272:L301–L310
Sastry BV (1997) Human placental cholinergic system. Biochem Pharmacol 53:1577–1586
Sastry BV, Sadavongvivad C (1979) Cholinergic systems in non-nervous tissues. Pharmacol Rev 30:65–132

Schlenz H, Kummer W, Jositsch G et al (2010) Muscarinic receptor-mediated bronchoconstriction is coupled to caveolae in murine airways. Am J Physiol Lung Cell Mol Physiol 298: L626–L636

Schlereth T, Birklein F, an Haack K et al (2006) In vivo release of non-neuronal acetylcholine from the human skin as measured by dermal microdialysis: effect of botulinum toxin. Br J Pharmacol 147:183–187

Servent D, Fruchart-Gaillard C (2009) Muscarinic toxins: tools for the study of the pharmacological and functional properties of muscarinic receptors. J Neurochem 109:1193–1202

Shah N, Khurana S, Cheng K et al (2009) Muscarinic receptors and ligands in cancer. Am J Physiol Cell Physiol 296:C221–C232

Sheikh Abdul Kadir SH, Miragoli M, Abu-Hayyeh S et al (2010) Bile acid-induced arrhythmia is mediated by muscarinic M2 receptors in neonatal rat cardiomyocytes. PLoS One 5:e9689

Smallman BN, Maneckjee A (1981) The synthesis of acetylcholine by plants. Biochem J 194:361–364

Song P, Sekhon HS, Jia Y et al (2003) Acetylcholine is synthesized by and acts as an autocrine growth factor for small cell lung carcinoma. Cancer Res 63:214–221

Song P, Sekhon HS, Lu A et al (2007) M3 muscarinic receptor antagonists inhibit small cell lung carcinoma growth and mitogen-activated protein kinase phosphorylation induced by acetylcholine secretion. Cancer Res 67:3936–3944

Stephenson M, Rowatt E (1947) The production of acetylcholine by a strain of *Lactobacillus plantarum*. J Gen Microbiol 1:279–298

Takeda M, Yoshitomi K, Taniguchi J et al (1994) Inhibition of amiloride-sensitive apical Na+ conductance by acetylcholine in rabbit cortical collecting duct perfused in vitro. J Clin Invest 93:2649–2657

Tournier JM, Maouche K, Coraux C et al (2006) α3α5β2-Nicotinic acetylcholine receptor contributes to the wound repair of the respiratory epithelium by modulating intracellular calcium in migrating cells. Am J Pathol 168:55–68

Tracey WR, Peach MJ (1992) Differential muscarinic receptor mRNA expression by freshly isolated and cultured bovine aortic endothelial cells. Circ Res 70:234–240

Tsutsumi H, Ohsaki M, Seki K et al (1999) Respiratory syncytial virus infection of human respiratory epithelial cells enhances both muscarinic and beta2-adrenergic receptor gene expression. Acta Virol 43:267–270

Walch L, Brink C, Norel X (2001) The muscarinic receptor subtypes in human blood vessels. Therapie 56:223–226

Wanner A (1986) Effect of ipratropium bromide on airway mucociliary function. Am J Med 81:23–27

Wessler IK, Kirkpatrick CJ (2001a) The non-neuronal cholinergic system: an emerging drug target in the airways. Pulm Pharmacol Ther 14:423–434

Wessler I, Kirkpatrick CJ (2001b) Role of non-neuronal and neuronal acetylcholine in the airways. In: Zaagsma J, Meurs H, Roffel AF (eds) Muscarinic receptors in the airways. Birkhäuser Verlag, Berlin

Wessler I, Kirkpatrick CJ (2008) Acetylcholine beyond neurons: the non-neuronal cholinergic system in humans. Br J Pharmacol 154:1558–1571

Wessler I, Kirkpatrick CJ, Racke K (1998) Non-neuronal acetylcholine, a locally acting molecule, widely distributed in biological systems: expression and function in humans. Pharmacol Ther 77:59–79

Wessler I, Kirkpatrick CJ, Racke K (1999) The cholinergic 'pitfall': acetylcholine, a universal cell molecule in biological systems, including humans. Clin Exp Pharmacol Physiol 26:198–205

Wessler I, Kilbinger H, Bittinger F et al (2001a) The biological role of non-neuronal acetylcholine in plants and humans. Jpn J Pharmacol 85:2–10

Wessler I, Roth E, Schwarze S et al (2001b) Release of non-neuronal acetylcholine from the human placenta: difference to neuronal acetylcholine. Naunyn Schmiedebergs Arch Pharmacol 364:205–212

Wessler I, Roth E, Deutsch C et al (2001c) Release of non-neuronal acetylcholine from the isolated human placenta is mediated by organic cation transporters. Br J Pharmacol 134:951–956
Wessler I, Kilbinger H, Bittinger F et al (2003) The non-neuronal cholinergic system in humans: expression, function and pathophysiology. Life Sci 72:2055–2061
White HL, Cavallito CJ (1970) Inhibition of bacterial and mammalian choline acetyltransferases by styrylpyridine analogues. J Neurochem 17:1579–1589
White HL, Wu JC (1973) Choline and carnitine acetyltransferases of heart. Biochemistry 12:841–846
Whittaker VP (1963) Identification of acetylcholine and related esters of biological origin. In: Eichler O, Farah A, Koelle GB (eds) Handbuch der experimentellen Pharmakologie. Springer, New York
Wong LB, Miller IF, Yeates DB (1988a) Regulation of ciliary beat frequency by autonomic mechanisms: in vitro. J Appl Physiol 65:1895–1901
Wong LB, Miller IF, Yeates DB (1988b) Stimulation of ciliary beat frequency by autonomic agonists: in vivo. J Appl Physiol 65:971–981
Wu X, Kekuda R, Huang W et al (1998) Identity of the organic cation transporter OCT3 as the extraneuronal monoamine transporter (uptake2) and evidence for the expression of the transporter in the brain. J Biol Chem 273:32776–32786
Xie G, Drachenberg C, Yamada M et al (2005) Cholinergic agonist-induced pepsinogen secretion from murine gastric chief cells is mediated by M1 and M3 muscarinic receptors. Am J Physiol Gastrointest Liver Physiol 289:G521–G529
Yamada M, Lamping KG, Duttaroy A et al (2001) Cholinergic dilation of cerebral blood vessels is abolished in M(5) muscarinic acetylcholine receptor knockout mice. Proc Natl Acad Sci U S A 98:14096–14101
Yamada T, Fujii T, Kanai T et al (2005) Expression of acetylcholine (ACh) and ACh-synthesizing activity in Archaea. Life Sci 77:1935–1944
Zarghooni S, Wunsch J, Bodenbenner M et al (2007) Expression of muscarinic and nicotinic acetylcholine receptors in the mouse urothelium. Life Sci 80:2308–2313
Zimring JC, Kapp LM, Yamada M et al (2005) Regulation of CD8+ cytolytic T lymphocyte differentiation by a cholinergic pathway. J Neuroimmunol 164:66–75

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