Muscarinic Pain Pharmacology: Realizing the Promise of Novel Analgesics by Overcoming Old Challenges

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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

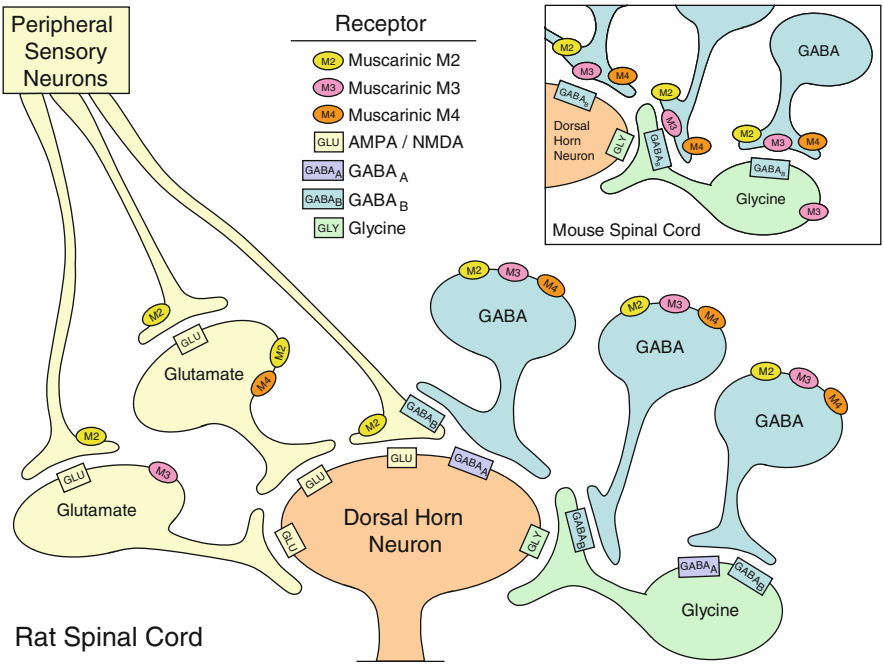

Fig. 1 Representation of the distribution and function of M2, M3, and M4 mAChR subtypes in the modulation of glutamatergic, GABAergic, glycinergic inputs on dorsal horn neurons in rats and mice. In rats, excitatory glutamatergic input to dorsal horn neurons is inhibited by the activation of M2 on primary 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.

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