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

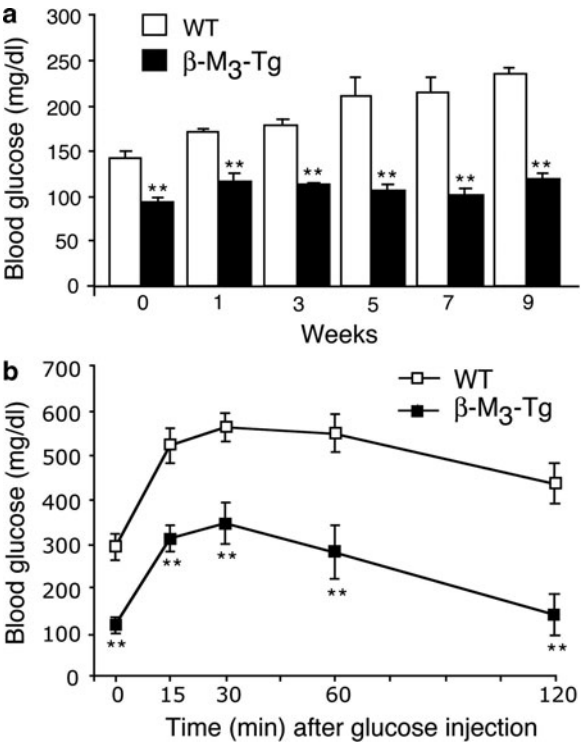

Fig. 1 Improved glucose homeostasis in transgenic mice selectively overexpressing M_3 mAChRs in pancreatic β cells (β-M_3-Tg mice). (**a**) Blood glucose levels of mice maintained on a high-fat diet. β-M_3-Tg mice and WT littermates were maintained on a high-fat diet for 8 weeks. Blood glucose levels 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).

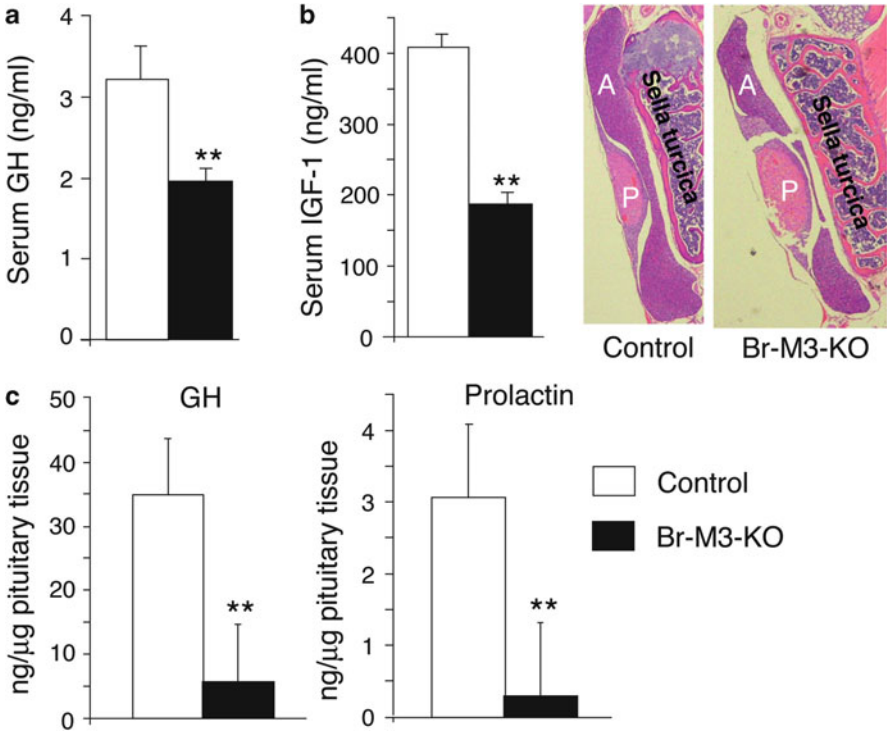

Fig. 2 Brain (Br)-M3-KO mice show reduced levels of hormones critical for somatic growth and hypoplasia of the anterior pituitary gland. (**a**) Reduction in serum GH and IGF-1 levels in Br-M3-KO mice, as compared to control littermates. (**b**) Selective reduction in the size of the anterior pituitary 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

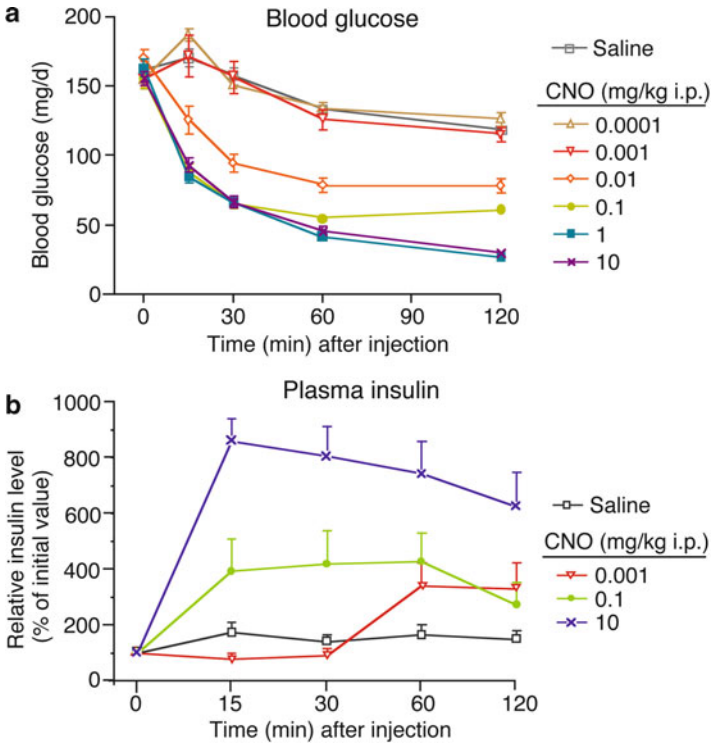

Fig. 3 CNO-induced changes in blood glucose and plasma insulin levels in transgenic mice expressing an M_3 mAChR-based G_q-RASSL in pancreatic β cells only (β-R-q Tg mice). (**a**) CNO-induced decreases in blood glucose levels. β-R-q Tg mice received a single i.p. injection of increasing doses of CNO 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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