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MUSCARINIC ACETYLCHOLINE RECEPTOR KNOCKOUT MICE: Novel Phenotypes and Clinical Implications*

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■ **Abstract** Muscarinic acetylcholine receptors (mAChRs; M_1–M_5) play key roles in regulating the activity of many important functions of the central and peripheral nervous system. Because of the lack of ligands endowed with a high degree of receptor subtype selectivity and the fact that most tissues or cell types express two or more mAChR subtypes, identification of the physiological and pathophysiological roles of the individual mAChR subtypes has proven a difficult task. To circumvent these difficulties, several laboratories recently employed gene-targeting techniques to generate mutant mouse strains deficient in each of the five mAChR subtypes. Phenotyping studies showed that each mutant mouse line displayed characteristic physiological, pharmacological, behavioral, biochemical, or neurochemical deficits. The novel insights gained from these studies should prove instrumental for the development of novel classes of muscarinic drugs.

INTRODUCTION

Acetylcholine (ACh) is a major neurotransmitter in the central and peripheral nervous systems (1, 2). The many important physiological actions of ACh are initiated by its binding to two distinct classes of plasma membrane receptors: the nicotinic (nAChRs) and muscarinic ACh receptors (mAChRs). Whereas the nAChRs function as ACh-gated cation channels, the mAChRs are prototypical members of the superfamily of G protein-coupled receptors (2–4). The muscarinic actions of ACh are mediated by five molecularly distinct mAChR subtypes (M_1–M_5) (2–4).

Based on their ability to activate different classes of heterotrimeric G proteins, the five mAChR subtypes can be subdivided into two major functional classes. The M_2 and M_4 receptors show selectivity for G proteins of the G_i family, whereas the M_1, M_3, and M_5 receptors selectively couple to G proteins of the G_q class (2–4).

Different experimental approaches, including immunohistochemical and mRNA hybridization studies, have shown that mAChRs are present in virtually all organs, tissues, or cell types (2, 5–7). Peripheral mAChRs mediate the classical muscarinic actions of ACh on organs or tissues that are innervated by parasympathetic nerves (1, 2). The most prominent actions mediated by these peripheral mAChRs include reduction of heart rate and stimulation of glandular secretion and smooth muscle contraction (1, 2).

Central mAChRs are involved in regulating an extraordinarily large number of cognitive, behavioral, sensory, motor, and autonomic functions (1, 8–11). Reduced or increased signaling through distinct mAChR subtypes has been implicated in the pathophysiology of several major diseases of the CNS, including Alzheimer's and Parkinson's disease, depression, schizophrenia, and epilepsy (1, 8–11).

During the past two decades, numerous studies have explored the roles of specific mAChR subtypes (M_1–M_5) in mediating the diverse physiological actions of ACh. Such knowledge is considered essential for the development of novel therapeutic approaches aimed at inhibiting or enhancing signaling through specific mAChR subtypes. However, the task of assigning specific physiological functions to distinct mAChR subtypes has proven very challenging, primarily owing to the lack of muscarinic agonists and antagonists that show a high degree of subtype selectivity for the individual mAChR subtypes (2–4, 11). Another complicating factor is that most organs, tissues, or cell types express multiple mAChRs (2, 5–7). These factors have led to conflicting reports regarding the potential physiological and pathophysiological roles of specific mAChR subtypes, particularly as far as the central muscarinic actions of ACh are concerned.

To circumvent these difficulties, several laboratories recently applied gene targeting techniques to generate mutant mouse lines deficient in each of the five mAChR genes (12–22). In all of these studies, individual mAChR genes were disrupted in mouse embryonic stem (ES) cells using specifically designed targeting vectors. Subsequently, standard transgenic and mouse breeding techniques were applied to obtain homozygous mAChR mutant mice (12–22).

Mutant mice lacking M_1, M_2, M_3, M_4, or M_5 mAChRs were viable, fertile, and appeared generally healthy (12–22). Moreover, none of the mutant mouse strains displayed any gross behavioral or morphological abnormalities. However, recent studies employing different physiological, pharmacological, behavioral, biochemical, and neurochemical techniques have revealed that each of the five mAChR mutant mouse lines displays characteristic phenotypical deficits or changes.

Several studies indicate that disruption of one specific mAChR gene does not seem to have major effects on the expression levels of the remaining four mAChRs (12–14, 16, 17, 20), at least not in the limited number of tissues that have been studied so far. This observation suggests that it is unlikely that compensatory

changes in mAChR expression levels have a major impact on the outcome of mouse phenotyping studies.

In this review, I summarize the key findings that have emerged from recent studies carried out with the newly generated M_1–M_5 mAChR mutant mice. In many cases, I also briefly discuss the potential clinical implications of the observed phenotypes. A table summarizing the major results of these studies can be found at the end of this chapter (Table 1).

M_1 mAChR-DEFICIENT MICE ($M1R^{-/-}$ MICE)

M_1 mAChRs are abundantly expressed in all major regions of the forebrain, including cerebral cortex, hippocampus, and striatum (5–7, 23). It is therefore likely that M_1 mAChRs play a role in the many central actions of ACh that involve the activity of forebrain mAChRs. Pharmacological evidence suggests, for example, that M_1 receptors are involved in mediating higher cognitive processes, such as learning and memory (24–26).

Lack of Pilocarpine-Induced Seizure Activity in $M1R^{-/-}$ Mice

The M_1 receptor gene was the first mAChR gene to be inactivated in mice (12). In this initial study, Hamilton et al. (12) examined the ability of pilocarpine, a nonsubtype-selective muscarinic agonist, to induce epileptic seizures in wild-type (WT) and M_1 receptor-deficient ($M1R^{-/-}$) mice. Whereas pilocarpine consistently elicited seizures in WT mice, it was completely devoid of seizure activity in $M1R^{-/-}$ mice. The lack of M_2, M_3, M_4, or M_5 receptors did not interfere with pilocarpine-induced seizure responses (27). These results raise the possibility that increased signaling through central M_1 receptors may play a role in the pathophysiology of at least certain forms of epileptic seizures (12).

Increased Locomotor Activity in $M1R^{-/-}$ Mice

In behavioral studies, $M1R^{-/-}$ mice did not display any significant deficits in sensory-motor gating, nociception, motor coordination, and anxiety-related behaviors (17). However, $M1R^{-/-}$ mice showed a pronounced increase in locomotor activity that was consistently observed in all tests that included locomotor activity measurements (17). Gerber et al. (18) recently reported that the hyperactivity phenotype of the $M1R^{-/-}$ mice is associated with a significant increase (approximately twofold) in extracellular dopamine concentrations in the striatum, most probably owing to an increase in dopamine release. These authors proposed that the lack of stimulatory M_1 receptors present on a subset of inhibitory striatal (striosomal) neurons projecting to the dopamine-containing neurons of the substantia nigra pars compacta may be responsible for the observed increase in striatal dopamine outflow. However, it is also possible that the M_1 receptors mediating inhibition of striatal dopamine release in WT mice are located on extrastriatal (e.g., cortical)

neurons and exert their inhibitory effects through a more complex neuronal network.

Independent of the precise mechanism by which the lack of M_1 receptors leads to an increase in locomotor activity and striatal dopamine release, the findings by Miyakawa et al. (17) and Gerber et al. (18) suggest that centrally active, selective M_1 mAChR antagonists are potentially useful in the treatment of Parkinson's disease, a brain disorder characterized by drastically reduced striatal dopamine levels. Because many studies have shown that schizophrenia is associated with increased dopaminergic transmission in various forebrain areas, improper signaling through M_1 receptors may also contribute to the pathophysiology of certain forms of schizophrenia (18).

Performance of M1R$^{-/-}$ Mice in Learning and Memory Tasks

To study the potential role of M_1 receptors in cognition, Miyakawa et al. (17) subjected M1R$^{-/-}$ mice to several hippocampus-dependent learning and memory tasks. M1R$^{-/-}$ mice performed equally well as their WT littermates in the Morris water maze, a test that is frequently used to assess spatial reference memory in rodents (17). Moreover, M1R$^{-/-}$ mice displayed normal freezing levels during context testing carried out 24 h after fear conditioning, and they did not show any significant cognitive deficits in the eight-arm radial maze test during training with a 30–120 s delay time between individual trials (17). Conversely, M1R$^{-/-}$ mice showed performance deficits in the eight-arm radial maze test during trials without delay and during auditory-cued and context testing carried out 48 h and 4 weeks, respectively, after fear conditioning (17). However, the extent of these behavioral deficits showed a very good correlation with the degree of hyperactivity displayed by the tested M1R$^{-/-}$ mice (17), which suggests that the hyperactivity phenotype exhibited by the M1R$^{-/-}$ mice makes a major contribution to the observed performance deficits. Interestingly, the behavioral pattern displayed by the M1R$^{-/-}$ mice is somewhat reminiscent of human attention deficit/hyperactivity disorder in which hyperactivity is often accompanied by cognitive deficits (28).

In a related study, Anagnostaras et al. (29) recently reported that M1R$^{-/-}$ mice showed a phenotype that included both enhancements as well as impairments of distinct cognitive functions. M1R$^{-/-}$ mice also exhibited a mild reduction in hippocampal long-term potentiation (LTP) in response to theta burst stimulation (Schaffer-CA1 synapse) (29). M1R$^{-/-}$ mice showed normal or enhanced memory for tasks that involved matching-to-sample problems (contextual fear conditioning and Morris water maze). On the other hand, M1R$^{-/-}$ mice displayed significant impairments in nonmatching-to-sample working memory and consolidation (win-shift radial arm and social discrimination learning). Anagnostaras et al. (29) therefore concluded that M_1 receptors are not essential for memory formation or initial stability of memory in the hippocampus, but are most likely involved in processes requiring interactions between cerebral cortex and hippocampus. However, the relationship between the hyperactivity phenotype displayed by the M1R$^{-/-}$ mice

(17, 18) and the observed behavioral deficits needs to be studied more rigorously in the future.

The relatively mild and selective cognitive impairments exhibited by the $M1R^{-/-}$ mice, combined with the observation that scopolamine treatment resulted in comparable cognitive deficits in WT and $M1R^{-/-}$ mice in the Morris water maze test (29), clearly indicate that non-M_1 mAChRs must play critical roles in learning and memory. Because impaired central muscarinic signaling is associated with Alzheimer's disease and normal aging processes (30, 31), identification of these non-M_1 mAChRs contributing to the cognition-enhancing effects of ACh is of considerable therapeutic relevance.

Electrophysiological Deficits of $M1R^{-/-}$ Mice

Electrophysiological studies demonstrated that muscarinic agonist-mediated inhibition of the M current (I_m), a tonically active voltage-dependent K^+ channel (2), was abolished in sympathetic ganglion neurons derived from $M1R^{-/-}$ mice (12). However, mAChR-mediated inhibition of I_m in mouse hippocampal CA1 (32) and CA3 (20) pyramidal cells remained unaffected by the lack of M_1 receptors. Because mAChR-mediated suppression of I_m is known to increase neuronal firing rate, the lack of this activity in $M1R^{-/-}$ mice is predicted to reduce neuronal excitability in response to preganglionic stimulation. In keeping with this notion, systemic administration of McN-A-343, a muscarinic agonist that can activate M_1 mAChRs located on postsynaptic sympathetic ganglion neurons with high efficacy (33), caused stimulatory cardiovascular effects in WT mice but failed to do so in $M1R^{-/-}$ mice (34).

Shapiro et al. (35) demonstrated that the slow, voltage-independent muscarinic inhibition of N- and P/Q-type Ca^{2+} channels was also absent in sympathetic ganglion neurons from $M1R^{-/-}$ mice (35). It should be noted in this context that the fast, voltage-dependent muscarinic inhibition of N- and P/Q-type Ca^{2+} channels remained intact in $M1R^{-/-}$ mice but was lacking in $M2R^{-/-}$ mice (35).

Activation of hippocampal mAChRs triggers oscillatory network activity at γ frequencies (20–80 Hz) in hippocampal preparations from WT mice (20). Such γ oscillations are characterized by the synchronized firing of large ensembles of neurons and can occur in different areas of the brain under various behavioral conditions, including the performance of certain cognitive tasks [see (20) and references cited therein]. Strikingly, muscarine-induced γ oscillations were absent in hippocampi (CA3 area) from $M1R^{-/-}$ mice (20). However, this activity remained unaffected by the lack of M_2–M_5 receptors (20). More detailed electrophysiological studies showed that the muscarine-induced hippocampal γ oscillations were dependent on M_1 receptor-mediated depolarization of hippocampal CA3 pyramidal neurons, involving the activation of the mixed Na^+/K^+ current, I_h, and the Ca^{2+}-dependent nonspecific cation current, I_{cat} (20). The potential cognitive or behavioral deficits, if any, caused by the lack of M_1 mAChR-mediated hippocampal γ oscillations remain to be investigated.

Biochemical Deficits of M1R$^{-/-}$ Mice

Biochemical studies showed that M1R$^{-/-}$ mice also displayed specific signaling defects. Hamilton & Nathanson (36) demonstrated that muscarinic agonist-induced activation of the mitogen-activated protein kinase (MAPK) pathway was virtually abolished in primary cortical cultures from newborn M1R$^{-/-}$ mice. In a related study, Berkeley et al. (37) showed that muscarinic agonist-mediated MAPK activation was also absent in CA1 hippocampal pyramidal neurons from M1R$^{-/-}$ mice. In contrast, this activity remained unaffected by the lack of M_2–M_4 mAChRs (37). In agreement with the results of the MAPK assays, muscarinic agonist-mediated stimulation of phosphatidyl inositol (PI) hydrolysis was reduced by >60% in primary cortical cultures from newborn M1R$^{-/-}$ mice (36). Moreover, in vitro [^{35}S]-GTPγS binding assays showed that muscarinic agonist-induced activation of G proteins of the G_q family was virtually abolished in hippocampal and cortical preparations from M1R$^{-/-}$ mice (38). In contrast, this activity remained essentially intact in the corresponding tissues from M3R$^{-/-}$ mice (38). In agreement with these in vitro studies, pilocarpine-induced in vivo PI hydrolysis was absent in cortical and hippocampal tissues from M1R$^{-/-}$ mice (27). These findings are consistent with the observation that the M_1 receptor is by far the most abundant G_q/G_{11}-coupled mAChR subtype expressed in the forebrain (5, 7).

The PI and MAPK signaling cascades regulate many important neuronal functions, including neuronal plasticity, differentiation, and survival. It therefore remains to be studied whether the biochemical deficits displayed by the M1R$^{-/-}$ mice can be linked to specific developmental, physiological, or behavioral deficits.

M_2 AND M_4 mAChR-DEFICIENT MICE (M2R$^{-/-}$ AND M4R$^{-/-}$ MICE)

The M_2 and M_4 mAChRs are both linked to G proteins of the G_i family and share similar ligand binding properties, which makes it difficult to distinguish between those two receptor subtypes by classical pharmacological tools (2–4). As outlined below, recent studies with M2R$^{-/-}$ and M4R$^{-/-}$ mice have shown that several physiological or pharmacological functions are mediated by a mixture of M_2 and M_4 mAChRs. For this reason, the phenotypes displayed by the M2R$^{-/-}$ and M4R$^{-/-}$ mice are discussed in the same subchapter.

M_2 receptors are widely expressed in the CNS (5–7) and in the body periphery, particularly in the heart and in smooth muscle tissues (2, 39, 40). In contrast, M_4 receptors are preferentially expressed in the CNS, particularly in different areas of the forebrain (5–7).

Role of M_2 Receptors in Cardiac and Smooth Muscle Function

Several recent studies have employed M2R$^{-/-}$ mice to explore the physiological roles of cardiac and smooth muscle M_2 receptors.

HEART Following stimulation of the parasympathetic nervous system, ACh released from cardiac vagal nerve endings interacts with mAChRs located in the sinoatrial node to trigger a reduction in heart beating frequency (2, 40). In vitro studies showed that carbachol-mediated bradycardic responses were completely abolished in isolated spontaneously beating atria prepared from $M2R^{-/-}$ mice (41). Moreover, recent in vivo studies demonstrated that vagally or muscarinic agonist (methacholine)-induced reductions in heart rate were also absent in $M2R^{-/-}$ mice (J.T. Fisher, S.G. Vincent, J. Gomeza, M. Yamada & J. Wess, unpublished observations). These findings indicate, consistent with the observation that the M_2 mAChR is by far the most abundant mAChR subtype expressed in the heart (2, 13, 40), that the negative chronotropic effects following vagal stimulation or administration of muscarinic agonists are exclusively mediated by M_2 receptors. This conclusion is also in agreement with a large body of pharmacological evidence derived from the use of subtype-preferring muscarinic antagonists (1–4). Several laboratories have shown that non-M_2 mAChRs, including the M_1, M_3, M_4, and M_5 receptor subtypes, are also expressed in cardiac tissues (40, 42, 43). However, the data reviewed above indicate that these non-M_2 mAChRs are unlikely to make significant contributions to the regulation of heart rate.

SMOOTH MUSCLE The M_2 receptor is the predominant mAChR subtype expressed by smooth muscle tissues, where it is coexpressed with a smaller population of M_3 receptors (39). Stengel et al. (41) first showed that carbachol was approximately twofold less potent in contracting isolated smooth muscle preparations from stomach fundus, urinary bladder, and trachea from $M2R^{-/-}$ mice (compared to the corresponding preparations from WT mice). In contrast to these relatively mild deficits, carbachol-mediated contractile responses were greatly reduced, but not abolished, in smooth muscle preparations from $M3R^{-/-}$ mice [(15, 44, 45) for additional details, see the section below entitled Role of M_3 Receptors in Smooth Muscle Function). Strikingly, carbachol-mediated contractions were found to be almost completely abolished in ileal and urinary bladder preparations from mice deficient in both M_2 and M_3 mAChRs ($M2R^{-/-}/M3R^{-/-}$ mice) (46). Similar results were obtained with tracheal and stomach fundus smooth muscle strips prepared from $M2R^{-/-}/M3R^{-/-}$ mice (P.W. Stengel, M. Yamada, J. Wess & M.L. Cohen, unpublished observations).

Matsui et al. (47) recently reported that the relaxant effects of forskolin were enhanced in different smooth muscle tissues from $M2R^{-/-}$ mice stimulated with the muscarinic agonist, oxotremorine-M. This observation suggests that stimulation of smooth muscle M_2 receptors can counteract the relaxant effects of agents that increase cAMP levels, probably via M_2 receptor-induced activation of G proteins of the G_i family, which mediate inhibition of adenylyl cyclase. Taken together, these data indicate that M_2 receptors facilitate smooth muscle contractility through both direct and indirect mechanisms.

Role of M_2 Receptors in Muscarinic Agonist-Mediated Tremor, Hypothermia, and Corticosterone Release

The potential involvement of M_2 receptors in muscarinic agonist-mediated tremor, hypothermia, and corticosterone release has also been studied by the use of $M2R^{-/-}$ mice (13, 53).

MUSCARINIC AGONIST-INDUCED TREMOR Systemic administration of oxotremorine or other centrally active muscarinic agonists causes akinesia and whole-body tremor (48, 49), two of the key symptoms of Parkinson's disease. Although the precise mechanisms underlying this syndrome remain unclear, several studies suggest the involvement of striatal mAChRs [see (13) and references therein]. Strikingly, Gomeza et al. (13) found that oxotremorine-mediated akinesia and tremor responses were totally abolished in $M2R^{-/-}$ mice. On the other hand, oxotremorine retained full tremorogenic activity in mice deficient in M_1, M_3, M_4, or M_5 mAChRs (12, 27). In the past, pharmacological antagonism of oxotremorine-induced tremor activity has been used as a model system for identifying new anti-Parkinson drugs. The lack of muscarinic agonist-induced tremor in $M2R^{-/-}$ mice therefore suggests that drugs originating from such screens were selected based on their ability to block central M_2 receptors.

MUSCARINIC AGONIST-MEDIATED HYPOTHERMIA A considerable body of evidence indicates that mAChRs located in thermoregulatory centers of the hypothalamus contribute to the regulation of body temperature (50). Consistent with this concept, systemic administration of oxotremorine or other centrally muscarinic agonists causes pronounced reductions in body temperature (13, 50). Gomeza et al. (13) showed that oxotremorine-induced hypothermic responses were significantly reduced, but not abolished, in $M2R^{-/-}$ mice. This observation suggests that both M_2 and non-M_2 mAChRs may play a role in the regulation of body temperature. The molecular nature of the non-M_2 mAChRs involved in this activity remains to be identified.

MUSCARINIC AGONIST-INDUCED CORTICOSTERONE RELEASE Centrally active muscarinic agonists can stimulate the hypothalamic-pituitary-adrenocortical axis via release of corticotropin-releasing hormone (CRH) (51, 52). In agreement with this observation, a recent study showed that systemic administration of the partial muscarinic agonist, BuTAC ([5R-(exo)]-6-[4-butylthio-1,2,5-thiadiazol-3-yl]-1-azabicyclo-[3.2.1]-octane), led to robust increases in serum corticosterone levels in WT mice (53). Strikingly, this response was abolished in $M2R^{-/-}$ mice (53), which suggests the involvement of M_2 receptors. However, because ACTH (adrenocorticotropic hormone) and CRH levels were not measured in this study, the precise localization of the M_2 receptors involved in mediating BuTAC-induced corticosterone release remains to be determined.

Role of M_2 Receptors in Learning and Memory

Pharmacological evidence suggests that central M_2 receptors play a role in modulating learning and memory processes (24, 54–57). However, behavioral studies using different M_2 receptor-preferring antagonists have led to contradictory results. Whereas some studies suggest that blockade of central M_2 receptors enhances learning and memory (55, 56), other investigators have arrived at the opposite conclusion (54, 57). In agreement with this latter set of studies, Tzavara et al. (58) recently reported that $M2R^{-/-}$ mice showed significant performance deficits in a passive avoidance test. This behavioral deficit was associated with significant changes in pharmacologically and physiologically evoked ACh release in the hippocampus (58), most probably owing to the absence of presynaptic M_2 autoreceptors mediating inhibition of ACh release (see below). $M2R^{-/-}$ mice also displayed significant deficits in behavioral flexibility and working memory in the Barnes circular maze and the T-maze delayed alternation test, respectively (I. Fedorova, T. Seeger, T. Miyakawa, E. Koustova, J. Gomeza, A.S. Basile, C. Alzheimer & J. Wess, unpublished observations). Electrophysiological studies demonstrated that hippocampal LTP (Schaffer-CA1 synapse) was drastically reduced following high-frequency stimulation of hippocampal slices from $M2R^{-/-}$ mice (I. Fedorova, T. Seeger, T. Miyakawa, E. Koustova, J. Gomeza, A.S. Basile, C. Alzheimer & J. Wess, unpublished observations). Gene disruption studies have established a good correlation between certain forms of learning and memory and reduced LTP at the Schaffer-CA1 synapse [for reviews, see (60, 61)]. It is therefore likely that the observed deficits in hippocampal synaptic plasticity contribute to the cognitive deficits displayed by the $M2R^{-/-}$ mice. Because impaired central muscarinic signaling is linked to the cognitive decline associated with Alzheimer's disease and old age (30, 31), these findings should be of considerable clinical relevance.

M_2 and M_4 Receptors Mediate Muscarinic Agonist-Induced Analgesia

Administration of centrally active muscarinic agonists induces robust analgesic effects that are dependent on both spinal and supraspinal mechanisms (62–64). Because the potential use of muscarinic agonists as analgesic drugs may be less likely to lead to tolerance and addiction associated with the use of classical opioid analgesics (64, 65), identification of the mAChR subtype(s) involved in this activity is of considerable therapeutic interest.

Because the M_2 and M_4 receptor subtypes couple to similar G proteins (G_i class) as the opioid receptors that are known to mediate potent analgesic effects, Gomeza et al. (13, 14) first examined whether muscarinic agonist-mediated analgesic responses were altered in $M2R^{-/-}$ and $M4R^{-/-}$ mice. Tail-flick and hot-plate analgesia tests showed that the analgesic potency of oxotremorine, administered systemically (s.c.), was markedly reduced (but not abolished) in $M2R^{-/-}$ mice (13) but remained nearly unchanged in $M4R^{-/-}$ mice (14). A similar pattern was observed after intrathecal (i.t.) or intracerebroventricular (i.c.v.) administration

of oxotremorine (66). Strikingly, oxotremorine was virtually devoid of analgesic activity in mutant mice that lacked both M_2 and M_4 receptors ($M2R^{-/-}/M4R^{-/-}$ mice) (66), independent of the route of application (s.c., i.t., or i.c.v.). Taken together, these data indicate that both M_2 and M_4 receptors are involved in mediating the analgesic effects of muscarinic agonists at the spinal and supraspinal level. However, M_2 receptors clearly play a predominant role in this activity. Most likely, the antinociceptive activity mediated by M_4 receptors remained undetected in $M4R^{-/-}$ mice (14) because of the presence of the predominant M_2 receptor pathway.

Radioligand binding studies carried out with spinal cord tissue from WT and mAChR mutant mice indicated that ~90% of all mAChRs in the spinal cord represent M_2 receptors (66), providing a possible explanation for the predominant role of the M_2 receptor subtype in inhibiting pain impulses at the spinal level. Several lines of evidence suggest that both presynaptic and postsynaptic mechanisms contribute to the mAChR-mediated analgesic responses at the spinal level [discussed in (66)]. However, the mechanisms underlying the analgesic effects mediated by activation of supraspinal (brain) M_2 and M_4 receptors remain to be determined.

Interestingly, Duttaroy et al. (66) recently reported that two novel muscarinic agonists chemically derived from epibatidine, CMI-936 and CMI-1145 (67), displayed reduced analgesic activity in both $M2R^{-/-}$ and $M4R^{-/-}$ mice, independent of the route of application. Radioligand binding studies showed that the two epibatidine derivatives, in contrast to oxotremorine, exhibited significantly higher affinity (~ 6–16-fold) for M_4 than for M_2 receptors, providing a molecular basis for the observed differences in agonist activity profiles. Because M_4 receptors, unlike M_2 receptors, do not seem to mediate important peripheral functions, the development of selective M_4 receptor agonists as novel analgesic agents represents an attractive goal.

Recent studies suggest that activation of mAChRs present on peripheral nociceptors of the skin can also suppress the transmission of pain impulses (68, 69). Electrophysiological and neurochemical studies with skin and skin-saphenous nerve preparations demonstrated that muscarine-induced peripheral antinociception was abolished in $M2R^{-/-}$ mice but not significantly affected in $M4R^{-/-}$ mice (70). Activation of these peripheral M_2 receptors may contribute to the analgesic effects observed after systemic administration of muscarinic agonists (see above). Stimulation of these peripheral M_2 mAChRs is thought to occur by ACh synthesized and released by different cell types of the skin (71, 72).

M_2 and M_4 Receptors Act as Muscarinic Autoreceptors and Heteroreceptors

Several recent studies have used $M2R^{-/-}$ and $M4R^{-/-}$ mutant mice as novel experimental tools to examine the potential roles of the M_2 and M_4 receptor subtypes as muscarinic autoreceptors and heteroreceptors.

MUSCARINIC AUTORECEPTORS ACh, like many other neurotransmitters, can inhibit its own release via stimulation of so-called inhibitory autoreceptors present on cholinergic nerve endings (73). Because autoinhibition of neurotransmitter release is most frequently mediated by receptors coupled to G proteins of the G_i family, Zhang et al. (74) recently examined whether autoinhibition of ACh release was altered in different central tissues (hippocampus, cerebral cortex, and striatum) from $M2R^{-/-}$ and $M4R^{-/-}$ mice. Specifically, these authors measured oxotremorine-mediated inhibition of potassium-stimulated [^{3}H]ACh release using superfused hippocampal, cortical, and striatal slices that had been preincubated with [^{3}H]choline to label cellular ACh pools. These studies showed that autoinhibition of ACh release is mediated predominantly by M_2 receptors in the mouse hippocampus and cerebral cortex, but primarily by M_4 receptors in the mouse striatum (74). Because the proper regulation of ACh release in the hippocampus, cerebral cortex, and striatum is thought to be critically involved in numerous fundamental functions of the CNS, including cognition and locomotor control, these findings should be of high clinical relevance.

By using a similar approach, Zhou et al. (75) recently demonstrated that the mAChRs mediating autoinhibition of ACh release in mouse heart atria represent a mixture of M_4 and non-M_4 (probably M_2) receptors. In the mouse urinary bladder, autoinhibition of ACh release was found to be mediated predominantly by M_4 receptors (75). However, a recent study examining neurotransmitter release from phrenic diaphragm preparations of WT and $M2R^{-/-}$ mice showed that the inhibitory muscarinic autoreceptors present on peripheral cholinergic motor nerves represent M_2 receptors (76).

MUSCARINIC HETERORECEPTORS Activation of mAChRs located on peripheral sympathetic nerve terminals (so-called muscarinic heteroreceptors) results in the inhibition of norepinephrine release (77). Trendelenburg et al. (78) recently used various peripheral preparations from WT and mAChR mutant mice to study the molecular identity of these muscarinic heteroreceptors. Specifically, these investigators determined electrically evoked [^{3}H]norepinephrine release using cardiac (atrial), urinary bladder, and vas deferens tissues from WT, $M2R^{-/-}$, and $M4R^{-/-}$ mice. This analysis showed that the release-inhibitory muscarinic heteroreceptors represent mixtures of M_2 and non-M_2 receptors in all three tissues studied (78). Whereas the non-M_2 heteroreceptors present in the vas deferens are likely to represent primarily M_4 receptors (78), the identity of the non-M_2 heteroreceptors present in heart atria and urinary bladder remains uncertain.

Role of M_4 Receptors in Facilitating Striatal Dopamine Release

A proper balance between striatal muscarinic cholinergic and dopaminergic neurotransmission is required for coordinated locomotor control (79). Several studies have shown that activation of striatal mAChRs can facilitate dopamine release in the striatum (80, 81). Zhang et al. (82) recently showed that oxotremorine-mediated potentiation of potassium-stimulated [^{3}H]dopamine release was absent in striatal

slice preparations from M4R$^{-/-}$ mice. In striatal preparations from WT mice, the oxotremorine-mediated facilitation of dopamine release could be prevented by treatment with tetrodotoxin (82). This observation, together with the known subcellular localization of the M_4 receptor subtype in the striatum (83, 84), suggests that the M_4 receptors regulating striatal dopamine release are probably located on the cell bodies of striatal GABAergic projection neurons (82).

Behavioral Phenotypes Displayed by M4R$^{-/-}$ Mice

In behavioral studies, M4R$^{-/-}$ mice displayed a small but statistically significant increase in basal locomotor activity (14). Moreover, the locomotor stimulation observed after administration of a centrally active D1 dopamine receptor agonist was greatly enhanced in M4R$^{-/-}$ mice (14). In the striatum, M_4 mAChRs are preferentially expressed by striatal projection neurons that express D1 dopamine receptors (79, 85, 86). These neurons give rise to the so-called direct striato-nigral pathway activation of which is predicted to facilitate locomotion (79). The findings by Gomeza et al. (14) therefore support the concept that striatal M_4 receptors exert an inhibitory effect on D1 receptor-stimulated locomotor activity. Because functional interactions between cholinergic and dopaminergic pathways play critical roles in proper striatal function (79), these findings should be of considerable interest for the treatment of Parkinison's disease and related movement disorders.

In a related study, Karasawa et al. (22) assessed the ability of the muscarinic antagonist scopolamine to suppress the cataleptic responses observed after administration of the D2-type dopamine receptor antagonist haloperidol to WT and M4R$^{-/-}$ mice. While scopolamine treatment abolished haloperidol-induced catalepsy in WT mice, it had little effect on haloperidol-mediated cataleptic responses in M4R$^{-/-}$ mice (22). Haloperidol-induced catalepsy is often used as an animal model to mimic the extrapyramidal motor side effects caused by antipsychotic drugs. The findings by Karasawa et al. (22) therefore suggest that selective M_4 receptor antagonists may be of therapeutic benefit in treating the extrapyramidal symptoms in Parkinson's disease and drug-induced parkinsonism.

Behavioral studies with M4R$^{-/-}$ mice also suggest that central M_4 receptors play a role in modulating prepulse inhibition (PPI) of the startle reflex, a measure of attention (87). Specifically, Felder et al. (87) showed that M4R$^{-/-}$ mice displayed a significant increase in sensitivity to the PPI-disrupting effect of the psychomimetic phencyclidine, a noncompetitive NMDA receptor antagonist. Because phencyclidine-mediated disruption of PPI is often used as an animal model of psychosis, central M_4 receptors may represent a novel drug target for the treatment of schizophrenia and related neurological disorders.

M_3 mAChR-DEFICIENT MICE (M3R$^{-/-}$ MICE)

Like the M_2 receptor, the M_3 mAChR subtype is widely expressed in different regions of the brain (88) and in peripheral organs and tissues innervated by

parasympathetic nerves (2, 39). At present, little is known about the physiological roles of the central M_3 receptors. In the periphery, M_3 receptors are predicted to play key roles in ACh-mediated regulation of smooth muscle contractility and glandular function (1–4, 39).

M3R$^{-/-}$ Mice are Hypophagic and Lean

Yamada et al. (16) reported that M3R$^{-/-}$ mice showed a pronounced reduction in body weight (by ~25%) that was associated with a significant decrease (by ~50%) in the mass of peripheral fat pads. The lack of M_3 receptors also led to striking reductions (~5–10-fold) in serum leptin and insulin levels (16), probably primarily owing to the reduction in total body fat mass (89). M3R$^{-/-}$ mice showed normal linear growth (16), indicating that the lack of M_3 receptors did not lead to a generalized growth retardation. Food intake studies indicated that M3R$^{-/-}$ mice consumed considerably less food than their WT littermates (16). M3R$^{-/-}$ mice did not display any abnormalities in several other behavioral tests and showed normal locomotor activity and metabolic rate (16). It is therefore likely that the observed reduction in food intake is the primary cause of the lean phenotype exhibited by the M3R$^{-/-}$ mice.

Yamada et al. (16) also demonstrated that M_3 receptors are expressed at relatively high levels in the hypothalamus, the key control center for the regulation of appetite. Several additional observations suggested that the lack of hypothalamic M_3 receptors may be responsible, at least partially, for the observed reduction of food intake displayed by the M3R$^{-/-}$ mice. First, M3R$^{-/-}$ mice showed greatly reduced expression levels of melanin-concentrating hormone (MCH) (16), an appetite-stimulating peptide synthesized almost exclusively in so-called second-order neurons of the lateral hypothalamus (89). This was a surprising finding because MCH levels are normally increased in fasted mice or under conditions of leptin deficiency (89). Second, i.c.v. administration of the appetite-stimulating peptide agouti-related peptide (AGRP) failed to stimulate food intake in M3R$^{-/-}$ mice (16). AGRP, which is synthesized in so-called first-order hypothalamic neurons of the arcuate nucleus, normally stimulates food intake by modulating the activity of the MCH-containing hypothalamic neurons and other secondary hypothalamic feeding centers (89). MCH-containing neurons express M_3 receptors (16), and muscarinic stimulation increases hypothalamic MCH expression (90). Taken together, these observations suggest that hypothalamic M_3 receptors play a role in stimulating MCH expression and modulating the proper responsiveness of MCH neurons to input from first-order hypothalamic feeding centers (e.g., AGRP-containing neurons). Inactivation of this pathway is likely to be responsible, at least partially, for the hypophagia phenotype of the M3R$^{-/-}$ mice. Pharmacological manipulation of this hypothalamic cholinergic pathway may therefore represent a novel strategy for the control of food intake.

Several studies have shown that mAChR-mediated stimulation of smooth muscle contractility (15, 44, 45) and salivary secretion (15, 16, 27) are reduced in

$M3R^{-/-}$ mice (see below for more details). Thus, an important question is to what extent these peripheral deficits contribute to the reduced body weight and food intake displayed by the $M3R^{-/-}$ mice. In vivo studies indicated that gastrointestinal function, including food transit time, is normal in $M3R^{-/-}$ mice (15, 16), suggesting that potential gastrointestinal complications are unlikely to have a major impact on food intake and weight gain in these mutant animals. Because $M3R^{-/-}$ mice showed improved weight gain when offered a wet paste diet rather than standard dry pellet food, Matsui et al. (15) proposed that impaired salivation may be a major factor responsible for the hypophagia phenotype of the $M3R^{-/-}$ mice. However, these authors did not measure food intake and did not show any growth curves for WT control mice fed with the wet paste diet, making these data difficult to interpret. On the other hand, Yamada et al. (16) demonstrated that $M3R^{-/-}$ mice consumed less food independent of whether they were offered standard dry pellet food or a wet mash diet, suggesting that impaired salivation does not play a major role in causing reduced food intake in the $M3R^{-/-}$ mice. In any case, the relative contribution of central versus peripheral deficits to the hypophagia phenotype displayed by the $M3R^{-/-}$ mice remains to be analyzed in more detail in future studies.

Role of M_3 Receptors in Smooth Muscle Function

Pharmacological studies with subtype-preferring muscarinic antagonists have shown that M_3 receptors play a key role in mediating muscarinic agonist (Ach)-induced smooth muscle contractions (2, 39). In agreement with this concept, in vitro studies demonstrated that smooth muscle tissues (urinary bladder, ileum, stomach fundus, trachea, and gallbladder preparations) derived from $M3R^{-/-}$ mice exhibited significantly reduced maximum contractile responses (E_{max}) following addition of the cholinergic agonist carbachol (15, 44, 45). However, the degree of reduction in E_{max} values differed significantly from tissue to tissue, ranging from only $\sim$40% in tracheal smooth muscle (44) to $>$90% in urinary bladder (15). As discussed above (see Role of M_2 Receptors in Cardiac and Smooth Muscle Function), studies with mutant mice deficient in both M_2 and M_3 mAChRs convincingly demonstrated that the non-M_3 mAChRs responsible for the contractile responses remaining in smooth muscle preparations from $M3R^{-/-}$ mice are M_2 receptors (46).

In vivo studies showed that $M3R^{-/-}$ mice had enlarged pupils (15), confirming previous findings that the tone of the pupillary sphincter muscle is maintained by tonic activation of M_3 receptors. However, $M3R^{-/-}$ mice retained a weak light reflex, and atropine was able to further increase pupil size in $M3R^{-/-}$ mice (15), indicating that non-M_3 mAChRs also contribute to modulating ocular smooth muscle contractility. Male $M3R^{-/-}$ mice also exhibited severely distended urinary bladders (15), consistent with the in vitro studies indicating an important role of M_3 receptors in bladder smooth muscle contractility. However, for reasons that are unclear at present, the degree of bladder distension was much less severe in

female M3R$^{-/-}$ mice (15). Recent in vivo studies also demonstrated that vagally or muscarinic agonist (methacholine)-induced bronchoconstrictor responses were abolished in M3R$^{-/-}$ mice (J.T. Fisher, S.G. Vincent, J. Gomeza, M. Yamada & J. Wess, unpublished observations). This observation is in agreement with the outcome of previous pharmacological studies indicating that the M_3 receptor subtype plays a key role in ACh-mediated increases in airway smooth muscle tone (39, 91).

Role of M_3 Receptors in Salivary Secretion

Pharmacological evidence suggests that the M_3 receptor subtype plays an important role in mAChR-mediated salivation (2). Interestingly, the oral cavity of M3R$^{-/-}$ mice was found to be moist, suggesting that M_3 receptor activity is not essential for basal salivary flow (15, 16). Matusi et al. (15) reported that injection of a single low dose of pilocarpine (1 mg/kg, s.c.) induced salivation in WT mice but failed to do so in M3R$^{-/-}$ mice. In a related study, Yamada et al. (16) injected WT and M3R$^{-/-}$ mice with three different doses of pilocarpine (1, 5, and 15 mg/kg, s.c.). These authors reported (16), in contrast to the findings by Matsui et al. (15), that M3R$^{-/-}$ mice showed a reduction in agonist-induced salivation (by ~50%) only at one of the three pilocarpine doses used (5 mg/kg), suggesting that both M_3 and non-M_3 mAChRs (M_1 receptors?) participate in mAChR-mediated stimulation of salivary secretion. One possible explanation for these discrepant results is that Matsui et al. (15) only examined one single low dose of pilocarpine using an experimental setup that was different from that employed by Yamada et al. (16). In agreement with the concept that both M_3 and non-M_3 mAChRs play a role in muscarinic agonist-induced increases in saliva output, Bymaster et al. (27) recently reported that the salivation responses following administration of two other muscarinic agonists, oxotremorine and oxotremorine-M (each 0.3 mg/kg, s.c.), were significantly reduced, but clearly not abolished, in M3R$^{-/-}$ mice. Additional studies using other mAChR mutant mouse strains suggested that multiple non-M_3 mAChR subtypes, including the M_1 (27), M_4 (27), and M_5 (21) receptors, may contribute to mAChR-mediated salivation. More detailed studies are needed to confirm that all of these receptors are indeed expressed in salivary gland tissues and, if so, to what extent the individual receptors contribute to salivary flow under more physiological conditions.

M_5 mAChR-DEFICIENT MICE (M5R$^{-/-}$ MICE)

Until recently, little was known about the physiological functions of the M_5 receptor, which was the last mAChR subtype to be cloned (92, 93). M_5 receptors are expressed at rather low levels in both neuronal and nonneuronal cells (93). Interestingly, M_5 receptor mRNA represents the only mAChR mRNA that can be detected in dopaminergic neurons of the midbrain (94, 95). Pharmacological

studies indicate that the M_5 receptor shares very similar functional and ligand binding properties with the M_3 receptor (93), making it very difficult to distinguish between these two receptor subtypes by classical pharmacological means.

M_5 Receptors Mediate ACh-Induced Dilation of Cerebral Arteries and Arterioles

It is well known that ACh, by interacting with mAChRs located on the vascular endothelium, is able to dilate most vascular beds (96, 97). Recently, M_5 receptor mRNA has been identified in various peripheral and cerebral blood vessels (98), including human brain microvessels (99). To test the hypothesis that vascular M_5 receptors participate in mediating the vasorelaxing effects of ACh, Yamada et al. (19) examined the effects of ACh on a series of vascular preparations from WT and $M5R^{-/-}$ mice. This analysis showed that the absence of M_5 receptors had no significant effect on ACh-induced relaxation of extracerebral arteries (carotid and coronary arteries). In contrast, ACh virtually lost the ability to dilate cerebral arteries and arterioles prepared from $M5R^{-/-}$ mice, as studied with the basilar artery and pial arterioles as model systems (19). These findings support the concept that the vasorelaxing effects of ACh on cerebral arteries and arterioles are mediated by endothelial M_5 receptors. Preliminary data suggest that ACh-induced relaxation of extracranial arteries is mediated predominantly by the M_3 receptor subtype (K.G. Lamping, J. Wess & F.M. Faraci, unpublished observations).

Neuronally released ACh is known to play a role in the regulation of cerebral vascular resistance and regional blood flow (100, 101). It has also been suggested that deficits in cortical cholinergic vasodilation may play a role in the pathophysiology of Alzheimer's disease (102, 103) and that activation of cholinergic vasodilator fibers can reduce neuronal damage during certain forms of focal cerebral ischemia (101, 104). Vascular M_5 mAChRs may therefore represent an attractive novel therapeutic target for the treatment of a variety of cerebrovascular disorders.

M_5 Receptors Facilitate Dopamine Release in the Striatum and Nucleus Accumbens

Several recent studies have used $M5R^{-/-}$ mice as tools to study the potential role of M_5 receptors in modulating dopamine release from dopaminergic neurons of the midbrain (substantia nigra pars compacta and nucleus accumbens).

DOPAMINE RELEASE IN THE STRIATUM In situ mRNA hybridization studies have shown that the dopamine-containing neurons of the substantia nigra pars compacta exclusively express M_5 receptors (94, 95). Because these neurons provide the major dopaminergic innervation of the striatum and muscarinic agonists can facilitate striatal dopamine release (80, 81), it has been proposed that M_5 receptor activity may play a role in modulating striatal dopamine release (95). To test this

hypothesis, two recent studies (19, 82) examined in vitro [^{3}H]dopamine release in striatal slices prepared from WT and mAChR-deficient mice. Yamada et al. (19) showed that the potency of oxotremorine in enhancing potassium-stimulated striatal [^{3}H]dopamine release was significantly reduced (by ~5–10-fold) in tissues from M5R$^{-/-}$ mice (E_{max} values, however, remained unaffected), suggesting that both M_5 and non-M_5 mAChRs participate in facilitating oxotremorine-mediated striatal dopamine release. The M_5 receptors involved in mediating this effect probably represent muscarinic heteroreceptors located on the terminals of the dopaminergic neurons innervating the striatum (82). As discussed earlier in this review (see Role of M_4 Receptors in Facilitating Striatal Dopamine Release, above), the non-M_5 mAChRs capable of mediating muscarinic agonist-induced increases in striatal dopamine release probably represent M_4 receptors located on the cell bodies of GABA-containing striatal projection neurons (82). It should also be mentioned in this context that activation of striatal M_3 receptors is predicted to inhibit striatal dopamine release, as studied with striatal slices from M3R$^{-/-}$ mice (82). The precise neuronal pathways through which the individual mAChRs exert their modulatory effects on striatal dopamine release remain to be elucidated.

DOPAMINE RELEASE IN THE NUCLEUS ACCUMBENS In situ mRNA hybridization studies (94) have also shown that the M_5 receptor is the predominant mAChR subtype expressed by the dopamine-containing neurons of the ventral tegmental area (VTA). This midbrain region is known to provide the major dopaminergic innervation of the nucleus accumbens (Nac) and other limbic areas (105, 106). A large body of evidence indicates that the activity of this mesolimbic dopaminergic pathway plays an important role in mediating the rewarding effects of opioids and other drugs of abuse (105, 106). Forster et al. (107) recently demonstrated that electrical stimulation of the laterodorsal tegmental nucleus (LDT), a mesopontine nucleus that provides the major source of cholinergic input to the dopamine-containing neurons of the VTA (108), triggers a three-phasic pattern of changes in dopamine efflux in the Nac of WT mice. Strikingly, the long-lasting increase in dopamine levels in the Nac (phase III) was selectively abolished in M5R$^{-/-}$ mice (107). It is likely that this neurochemical deficit is caused by the absence of excitatory M_5 receptors expressed by the dopamine-containing VTA neurons.

M5R$^{-/-}$ Mice Show Reduced Sensitivity to the Rewarding Effects of Morphine and Cocaine

Because activation of mesolimbic M_5 receptors facilitates dopamine release in the Nac (107), Basile et al. (109) recently tested the hypothesis that M5R$^{-/-}$ mice might exhibit changes in drug-seeking behavior. Behavioral studies showed that the rewarding effects of morphine, the prototypical opioid analgesic, were significantly reduced in M5R$^{-/-}$ mice, as studied in the conditioned place preference test (109). This behavioral deficit was associated with distinct biochemical deficits in the

Nac, including a reduction in morphine-stimulated dopamine efflux and Fos-B expression (109). In addition, when morphine-dependent mice were treated with the opioid receptor antagonist, naloxone, the resulting withdrawal symptoms were significantly less severe in $M5R^{-/-}$ than in WT control mice (109). On the other hand, the analgesic efficacy of morphine and the degree of tolerance that mice developed to the analgesic effects of morphine were similar in WT and $M5R^{-/-}$ mice (109). Behavioral studies with $M5R^{-/-}$ mice also suggest that M_5 receptor activity can modulate cocaine-associated reinforcement and withdrawal (110). These findings suggest that centrally active M_5 receptor antagonists may become therapeutically useful for the treatment of drug addiction.

Other Phenotypes Displayed by $M5R^{-/-}$ Mice

Following the administration of pilocarpine (1 mg/kg, s.c.), $M5R^{-/-}$ mice secreted slightly less saliva than WT control mice, specifically during the late phase of the salivation response [(21); for a more detailed discussion of mAChR-mediated salivation, see Role of M_3 Receptors in Salivary Secretion, above]. This observation raises the possibility that activation of glandular M_5 receptors may contribute to ACh-mediated salivary secretion. Takeuchi et al. (21) also reported that the $M5R^{-/-}$ mice drank more than twice as much water as the WT mice following an extended period (18 h) of food and water deprivation. The physiological basis underlying this behavioral phenotype remains to be elucidated.

CONCLUSIONS

In conclusion, the phenotypical analysis of M_1-M_5 mAChR-deficient mice has led to a wealth of new information about the physiological roles of the individual mAChR subtypes. It is likely that the development of mutant mouse lines that lack two or more mAChR subtypes or in which specific mAChR subtypes can be inactivated in a conditional fashion will provide even more powerful research tools. Precise knowledge of the physiological and potential pathophysiological roles of the individual mAChR subtypes should pave the way for the development of novel muscarinic drugs useful in a large variety of pathophysiological conditions.

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TABLE 1 Summary of major phenotypes displayed by M_1–M_5 mAChR-deficient mice

Disrupted mAChR gene	Phenotype	References
M_1	Lack of pilocarpine-mediated seizure activity and loss of muscarinic agonist-mediated M current (I_m) inhibition in sympathetic ganglion neurons	(12)
	Absence of slow, voltage-independent muscarinic inhibition of N- and P/Q- type Ca^{2+} channels in sympathetic ganglion neurons	(35)
	Increased locomotor activity	(17, 18)
	Increased extracellular dopamine levels in the striatum	(18)
	Loss of muscarinic agonist-induced MAPK activation and pronounced reduction of muscarinic agonist-mediated PI hydrolysis in primary cortical cultures	(36)
	Lack of carbachol-mediated MAPK activation in CA1 hippocampal pyramidal neurons	(37)
	Absence of muscarinic agonist-induced GTPγS binding to G proteins of the G_q family in hippocampus and cerebral cortex	(38)
	Lack of muscarine-mediated γ oscillations in area CA3 of the hippocampus	(20)
	Absence of cardiovascular stimulation following systemic administration of McN-A-343	(34)
	Lack of pilocarpine-stimulated in vivo PI hydrolysis in hippocampus and cerebral cortex	(27)
	Selective impairment in nonmatching-to-sample working memory and consolidation	(29)
M_2	Absence of oxotremorine-mediated tremor and reduced oxotremorine-mediated hypothermia	(13)
	Reduced muscarinic agonist-mediated analgesic responses	(13, 66)

(*Continued*)

TABLE 1 (*Continued*)

Disrupted mAChR gene	Phenotype	References
	Loss of fast, voltage-dependent muscarinic inhibition of N- and P/Q-type Ca^{2+} channels in sympathetic ganglion neurons	(35)
	Lack of carbachol-mediated bradycardia in isolated heart atria	(41)
	Slight reduction in the potency of carbachol in mediating contractions of different smooth preparations (in vitro)	(41, 46)
	Lack of oxotremorine-mediated inhibition of [^{3}H]ACh release from K^+-depolarized hippocampal and cortical slices	(74)
	Failure of a partial muscarinic agonist (BuTAC) to trigger increases in serum corticosterone levels	(53)
	Absence of muscarine-mediated desensitization of peripheral nociceptors	(70)
	Impairment in carbachol-mediated inhibition of electrically stimulated [^{3}H]norepinephrine release from heart atria, urinary bladder, and vas deferens	(78)
	Increased relaxant effects of forskolin and isoproterenol on oxotremorine-M-mediated contractions of different smooth muscle tissues	(47)
	Altered time course of evoked ACh release at the neuromuscular junction	(76)
	Lack of carbachol-mediated contractions of ileal and bladder smooth muscle preparations in mice lacking both M_2 and M_3 mAChRs (in vitro)	(46)
	Impaired performance in the passive avoidance test and enhanced ACh efflux in the hippocampus (in vivo)	(60)
M_3	Increase in pupil size and urinary bladder distension (in vivo)	(15)
	Pronounced impairments in carbachol-mediated contractions of different smooth muscle preparations (in vitro)	(15, 44, 45)

(*Continued*)

TABLE 1 (*Continued*)

Disrupted mAChR gene	Phenotype	References
	Reductions in body weight, mass of peripheral fat deposits, and food intake	(16)
	Impairments in muscarinic agonist-induced salivation	(15, 16, 27)
	Increased oxotremorine-stimulated [^{3}H]dopamine outflow from K^+-depolarized striatal slices	(82)
M_4	Increased locomotor activity under basal conditions and after administration of a D1 dopamine receptor agonist	(14)
	Increased sensitivity to phencyclidine-mediated disruptions in prepulse inhibition	(87)
	Lack of oxotremorine-mediated inhibition of [^{3}H]ACh release from K^+-depolarized striatal slices	(74)
	Absence of oxotremorine-stimulated [^{3}H]dopamine outflow from K^+-depolarized striatal slices	(82)
	Reduced carbachol-mediated inhibition of electrically stimulated [^{3}H]norepinephrine release from vas deferens	(78)
	Reduced autoinhibiton of [^{3}H]ACh release in heart atria and urinary bladder	(75)
	Lack of muscarinic agonist-mediated analgesic responses in mice lacking both M_2 and M_4 mAChRs	(66)
	Failure of scopolamine to antagonize haloperidol-mediated cataleptic effects	(22)
	Impaired migration of epidermal keratinocytes	(111)
	Increased basal ACh efflux in the hippocampus (in vivo)	(58)
M_5	Lack of ACh-mediated dilation of cerebral arteries and arterioles	(19)
	Reduced oxotremorine-stimulated [^{3}H]dopamine outflow from K^+-depolarized striatal slices	(19)

(*Continued*)

TABLE 1 (*Continued*)

Disrupted mAChR gene	Phenotype	References
	Lack of sustained increase in dopamine levels in the nucleus accumbens triggered by electrical stimulation of the laterodorsal tegmental nucleus	(107)
	Reduced sensitivity to the rewarding effects of morphine and cocaine and reduced severity of drug withdrawal symptoms	(109, 110)
	Slightly impaired pilocarpine-induced salivation and increased water intake after an extended period of food and water deprivation	(21)

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