Control of Glycinergic Input to Spinal Dorsal Horn Neurons by Distinct Muscarinic Receptor Subtypes Revealed Using Knockout Mice

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List of abbreviations:

CNQX: 6-cyano-7-nitroquinoxaline-2,3-dione;
4-DAMP: 4-diphenylacetoxy-N-methylpiperidine methiodide;  GABA: γ-aminobutyric acid;
GDP-β-S: guanosine 5’-O-(2-thiodiphosphate);  mAChRs: muscarinic acetylcholine receptors;
mIPSCs: miniature inhibitory postsynaptic currents;        NMDA, N-methyl-D-aspartic acid;
sIPSCs: spontaneous inhibitory postsynaptic currents

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Abstract

Muscarinic acetylcholine receptors (mAChRs) play an important role in the tonic regulation of nociceptive transmission in the spinal cord. However, how mAChR subtypes contribute to the regulation of synaptic glycine release is unknown. To determine their role, glycinergic spontaneous inhibitory postsynaptic currents (sIPSCs) were recorded in lamina II neurons by using whole-cell recordings in spinal cord slices of wild-type (WT) and mAChR subtype knockout (KO) mice. In WT mice, the mAChR agonist oxotremorine-M dose dependently decreased the frequency of sIPSCs in most neurons but had variable effects in other neurons. In contrast, in M3-KO mice, oxotremorine-M consistently decreased the glycinergic sIPSC frequency in all neurons tested, and in M2/M4 double-KO mice, it always increased the sIPSC frequency. In M2/M4 double-KO mice, the potentiating effect of oxotremorine-M was attenuated by higher concentrations in some neurons through activation of GABA B receptors. In pertussis toxin-treated WT mice, oxotremorine-M also consistently increased the sIPSC frequency. In M2-KO and M4-KO mice, the effect of oxotremorine-M on sIPSCs was divergent because of the opposing functions of the M3 subtype and the M2 and M4 subtypes. This study demonstrates that stimulation of the M2 and M4 subtypes inhibits glycinergic inputs to spinal dorsal horn neurons of mice, whereas stimulation of the M3 subtype potentiates synaptic glycine release. Furthermore, GABA B receptors are involved in the feedback regulation of glycinergic synaptic transmission in the spinal cord. This study revealed distinct functions of mAChR subtypes in controlling glycinergic input to spinal dorsal horn neurons.
Introduction

The cholinergic system and muscarinic acetylcholine receptors (mAChRs) are important for the regulation of nociceptive transmission in the spinal cord. In this regard, blocking of mAChRs in the spinal cord causes a large decrease in the nociceptive threshold (Zhuo and Gebhart, 1991). Intrathecal administration of mAChR agonists or acetylcholinesterase inhibitors produces a potent analgesic effect in many species, including rats, mice, and humans (Iwamoto and Marion, 1993; Naguib and Yaksh, 1994; Hood et al., 1997; Ellis et al., 1999; Duttaroy et al., 2002; Chen and Pan, 2003). Molecular cloning studies have revealed five molecularly distinct mAChRs (Caulfield, 1993; Wess, 1996). The odd-numbered subtypes (M1, M3, and M5) are selectively linked to Gq/11 proteins, and the even-numbered subtypes (M2 and M4) are preferentially coupled to the pertussis toxin (PTX)-sensitive Gi/o proteins (Felder, 1995; Wess, 1996; Caulfield and Birdsall, 1998). The highest density of spinal mAChRs is located in the superficial laminae in both rats and humans (Yamamura et al., 1983; Scatton et al., 1984; Villiger and Faull, 1985; Hoglund and Baghdoyan, 1997; Li et al., 2002). Previous studies have documented that M2, M3, and M4 mAChR subtypes are present in the spinal dorsal horn and that the M2 and M3 subtypes are particularly concentrated in the superficial laminae (Hoglund and Baghdoyan, 1997; Duttaroy et al., 2002; Chen et al., 2005). Although the role of the M2 and M4 subtypes in mAChR agonist-induced analgesia has been established (Ellis et al., 1999; Gomeza et al., 1999a; Duttaroy et al., 2002; Li et al., 2002), the mechanisms by which each mAChR subtype contributes to the regulation of nociceptive transmission in the spinal cord are not clear.

The spinal lamina II neurons receive nociceptive inflow from primary afferent neurons
and synaptic input from glutamatergic excitatory and GABAergic/glycinergic interneurons in rats and mice (Yoshimura and Nishi, 1995; Pan and Pan, 2004; Zhang et al., 2005; Wang et al., 2006; Zhang et al., 2006). Glycine is an important inhibitory neurotransmitter in the spinal cord, and blockade of glycine receptors in the spinal cord leads to hypersensitivity of dorsal horn neurons and allodynia (Yaksh, 1989; Cronin et al., 2004). Glycine-like immunoreactive axons, dendrites and cell bodies are present in the spinal superficial dorsal horn (Todd, 1990; Todd et al., 1996). In the rat spinal cord, the M₃ subtype is mainly responsible for muscarinic potentiation of synaptic glycine release (Wang et al., 2006). However, because highly selective agonists and antagonists for specific mAChR subtypes are not available, the definitive function of the individual mAChR subtypes in the regulation of glycinergic input to spinal dorsal horn neurons have yet to be established. In the present study, we used mAChR subtype-knockout (KO) mice to determine the role of the M₂, M₃ and M₄ subtypes in the control of glycinergic input to spinal lamina II neurons. This study provides unambiguous evidence about the specific function of these three mAChR subtypes in the control of glycinergic synaptic transmission in the spinal dorsal horn.
Materials and Methods

Animals. All the wild-type (WT) and mAChR subtype single- and double-KO mice (5-6 weeks old) were obtained from the National Institute of Diabetes and Digestive and Kidney Diseases (J.W.). The genetic background of M2-WT and M2-KO mice is 129J1 x CF1, that of M4-WT, M4-KO and M3-KO is 129SvEv x CF1, and that of the M2/M4-WT and M2/M4 double-KO mice is 129J1 (25%) x 129SvEv (25%) x CF1 (50%). The generation and breeding of M2 KO, M3 KO, M4 KO, and M2/M4 double-KO mice have been described elsewhere (Gomeza et al., 1999a; Gomeza et al., 1999b; Yamada et al., 2001; Duttaroy et al., 2002; Fukudome et al., 2004). Mouse genotyping was carried out by Southern blotting and polymerase chain reaction analysis of mouse-tail DNA, as described previously (Gomeza et al., 1999a; Gomeza et al., 1999b; Yamada et al., 2001; Duttaroy et al., 2002). The experimental protocols and procedures were approved by the Animal Care and Use Committee of the University of Texas M. D. Anderson Cancer Center and conformed to the guidelines of the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

Intrathecal PTX treatment. Mice were anesthetized with 2% isoflurane in O2 via a nose cone. The lumbar region was shaved and prepared with Betadine solution, and the intervertebral spaces were widened by placing the animal on a Plexiglas tube. The animals were then injected at the L5-L6 level by using a 30-gauge needle connected to a Hamilton syringe filled with 1 µg PTX dissolved in 4 µl of water. Correct subarachnoid positioning of the tip of the needle was verified by a tail-flick reflex (Hylden and Wilcox, 1980). Animals were allowed
to recover for 3 days in their home cage before the final electrophysiological experiments.

**Spinal cord slice preparation.** Mice were anesthetized with 2% isoflurane in O₂ and the lumbar segment of the spinal cord was rapidly removed through laminectomy. The mice were then killed by inhalation of 5% isoflurane. The spinal cord segment was immediately placed in an ice-cold sucrose artificial cerebrospinal fluid (aCSF) pre-saturated with 95% O₂ and 5% CO₂. The sucrose aCSF contained (in mM) sucrose, 234; KCl, 3.6; MgCl₂, 1.2; CaCl₂, 2.5; NaH₂PO₄, 1.2; glucose, 12.0; and NaHCO₃, 25.0. The tissue was then placed in a shallow groove formed in a gelatin block and glued onto the stage of a vibratome (Technical Product International, St. Louis, MO). Transverse spinal cord slices (350 µm) were cut in the ice-cold sucrose aCSF and pre-incubated in Krebs solution oxygenated with 95% O₂ and 5% CO₂ at 34 °C for at least 1 h before they were transferred to the recording chamber. The Krebs solution contained (in mM) NaCl, 117.0; KCl, 3.6; MgCl₂, 1.2; CaCl₂, 2.5; NaH₂PO₄, 1.2; glucose, 11.0; and NaHCO₃, 25.0. Each slice was placed in a glass-bottomed chamber (Warner Instruments, Hamden, CT) and fixed with parallel nylon threads supported by a U-shaped stainless steel weight. The slice was continuously perfused with Krebs solution at 5.0 ml/min at 34 °C maintained by an inline solution heater and a temperature controller (TC-324, Warner Instruments).

**Electrophysiological recordings.** Recordings of postsynaptic currents were performed using the whole-cell voltage-clamp method, as we described previously (Li et al., 2002; Zhang et al., 2005). The lamina II has a distinct translucent appearance and can easily be distinguished under the microscope. The neurons located in the lamina II in the spinal slice were identified
under a fixed stage microscope (BX51WI, Olympus, Tokyo, Japan) with differential interference contrast/infrared illumination. The electrode for the whole-cell recordings was triple pulled from borosilicate glass capillaries with a puller (P-97, Sutter Instruments, Novato, CA). The impedance of the pipette was 4-7 M\(\Omega\) when filled with internal solution containing (in mM) \(\text{Cs}_2\text{SO}_4, 110.0; \text{KCl}, 5.0; \text{MgCl}_2, 2.0; \text{CaCl}_2, 0.5; \text{HEPES}, 5.0; \text{EGTA}, 5.0; \text{ATP-Mg}, 5.0; \text{Na-GTP}, 0.5; \text{guanosine 5-O-(2-thiodiphosphate)} \times \text{(GDP-\(\beta\)-S), 1; and QX314, 10.0; adjusted to pH 7.2-7.4 with 1 M \text{CsOH} (290-320 \text{mOsm}). GDP-\(\beta\)-S was added to the internal solution to block the possible postsynaptic effect mediated by mAChR agonists through G proteins (Li et al., 2002; Zhang et al., 2005). QX314, a sodium channel blocker, was added to the internal solution to suppress the action potential generation from the recorded cell.

Recordings of postsynaptic currents began about 5 min after whole-cell access was established and the current reached a steady state. The input resistance was monitored, and the recording was abandoned if the resistance changed more than 15%. Signals were recorded using an amplifier (MultiClamp700A, Axon Instruments, Foster City, CA) at a holding potential of 0 mV, filtered at 1-2 kHz, digitized at 10 kHz, and stored in a Pentium computer with pCLAMP 9.0 (Axon Instruments). All spontaneous inhibitory postsynaptic currents (sIPSCs) were recorded in the presence of 10 \(\mu\text{M}\) bicuculline, a \(\text{GABA}_\text{A}\) receptor antagonist, and 20 \(\mu\text{M}\) 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX), a specific glutamate non-N-methyl-D-aspartic acid (NMDA) antagonist. To record the miniature inhibitory postsynaptic currents (mIPSCs), 1 \(\mu\text{M}\) tetrodotoxin was added in the perfusion solution. The conditions for measuring sIPSCs and mIPSCs were the same except that tetrodotoxin was used during the recording of mIPSCs.

Oxotremorine-M, 4-diphenylacetoxy-N-methylpiperidine methiodide (4-DAMP),
himbacine, GDP-β-S, strychnine, and CNQX were obtained from Sigma (St. Louis, MO). Tetrodotoxin and QX314 were obtained from Alomone Labs (Jerusalem, Israel). AFDX-116, CGP55845, and bicuculline were purchased from Tocris (Ellisville, MO). Muscarinic toxin 3 (MT-3) was purchased from Peptide Institute (Osaka, Japan). Drugs were dissolved in Krebs solution and perfused into the tissue chamber with the use of syringe pumps.

**Data analysis.** Data are presented as means ± S.E.M. The sIPSCs and mIPSCs were analyzed off-line with a peak detection program (MiniAnalysis, Synaptosoft, Decatur, GA). Measurements of the amplitude and frequency of sIPSCs and mIPSCs were performed for at least 2 min during control, drug application, and washout recovery. The sIPSCs and mIPSCs were detected by the fast rise time of the signal over an amplitude threshold above the background noise (Zhang et al., 2005; Zhang et al., 2006). The amplitude detection threshold was typically 6-8 pA. We manually excluded an event when the noise was erroneously identified as sIPSCs or mIPSCs by the computer program. The background noise level was typically constant throughout the recording in a given neuron. The cumulative probabilities of the amplitude and inter-event interval of sIPSCs and mIPSCs were compared using the Komogorov-Smirnov test. The effects of oxotremorine-M on the frequency and amplitude of sIPSCs and mIPSCs were determined using paired two-tailed Student's *t*-test or one-way analysis of variance. The Komogorov-Smirnov test was used to determine whether the drug effect on sIPSCs and mIPSCs was significantly different. *P* < 0.05 was considered statistically significant.
Results

**Role of mAChRs in the control of glycine release in WT mice**

To determine the concentration-dependent effect of oxotremorine-M on glycinergic sIPSCs of lamina II neurons in WT mice, 1, 3, 5, and 10 µM oxotremorine-M, a non-selective agonist for all mAChR subtypes, was perfused into the tissue chamber. Each concentration was applied for 3 min. The effect of oxotremorine-M on glycinergic sIPSCs was similar among three WT groups of mice with different genetic backgrounds. Therefore, the data obtained from the three groups were pooled. The baseline frequency of sIPSCs ranged from 0.28 to 2.65 Hz in 27 lamina II neurons recorded. Oxotremorine-M at 3-10 µM significantly decreased the frequency of sIPSCs in 15 of 27 (55.6%) neurons in a concentration-dependent manner (Fig. 1, A-C). Oxotremorine-M had no significant effect on sIPSCs in 8 of 27 (29.6%, Fig. 1C) neurons but had a variable effect in 4 of 27 (14.8%) neurons (Table 1). The sIPSCs were completely blocked by 2 µM strychnine, a specific glycine receptor antagonist (Fig. 1A).

In another 9 lamina II neurons in which 5 µM oxotremorine-M significantly decreased the frequency of sIPSCs (from 0.86 ± 0.19 Hz to 0.57 ± 0.17 Hz), the effect of this agent on mIPSCs was tested. In the presence of 1 µM tetrodotoxin, oxotremorine-M significantly decreased the frequency of mIPSCs (from 0.84 ± 0.16 Hz to 0.60 ± 0.14 Hz; Fig. 1D). These results suggest that stimulation of mAChRs primarily reduces synaptic glycine release and that the mAChRs involved in this activity are present on the presynaptic terminals of glycinergic interneurons in the mouse spinal dorsal horn.
Effect of oxotremorine-M on glycinergic sIPSCs in M3-KO mice

To assess the role of the M2 and M4 mAChR subtypes in the inhibitory effect of oxotremorine-M on synaptic glycine release, we tested the effect of oxotremorine-M on glycinergic sIPSCs in M3-KO mice. Oxotremorine-M (1-10 µM) decreased the frequency of sIPSCs in a concentration-dependent manner, and this effect was observed in all 12 neurons tested (Fig. 2). These data strongly suggest that activation of the M2 and M4 subtypes decreases inhibitory glycinergic input to spinal dorsal horn neurons.

We further determined the potential role the M2 and M4 subtypes in the inhibitory effect of the mAChR agonist on synaptic glycine release to spinal dorsal horn neurons in M3-KO mice. In 14 other lamina II neurons from the M3-KO mice, the effect of 5 µM oxotremorine-M on the frequency of sIPSCs was partially blocked by 10 µM AFDX-116 (Fig. 3, A and B), an M2 receptor-preferring antagonist (Coelho et al., 2000; Douglas et al., 2001; Wang et al., 2006). In 12 additional neurons from M3-KO mice, the effect of oxotremorine-M was completely blocked by 2 µM himbacine (Fig. 3C), an M2/M4-preferring antagonist (Dorje et al., 1991; Miller et al., 1992; Doller et al., 1999; Zhang et al., 2005). These data provide further evidence that the M2 and M4 subtypes contribute to the inhibition of spinal glycine release by stimulation of mAChRs in mice.

Effect of oxotremorine-M on glycinergic sIPSCs and mIPSCs in M2/M4 double-KO mice

To determine the role of the M3 subtype in muscarinic regulation of synaptic glycine release to lamina II neurons, we examined the effect of oxotremorine-M on the glycinergic sIPSCs and mIPSCs in M2/M4 double-KO mice. In a total of 18 neurons tested, 1-10 µM
oxotremorine-M dose dependently increased the frequency but not the amplitude of sIPSCs in 10 of 18 (55.6%) neurons (Fig. 4, A and B). In the remaining 8 (44.4%) neurons, oxotremorine-M at lower concentrations (1 and 3 µM) increased the frequency of sIPSCs. Surprisingly, the potentiating effect of oxotremorine-M on the frequency of sIPSCs was attenuated at higher concentrations (5 and 10 µM) (Fig. 4, A-C).

Synaptic GABA release is profoundly increased by oxotremorine-M because it stimulates the M3 subtype expressed in GABAergic interneurons in M2/M4 double-KO mice (Zhang et al., 2006). Thus, the increased GABA release could spillover and activate presynaptic GABAB receptors on glycinergic neurons to inhibit glycine release. To test this hypothesis, we used the GABAB receptor antagonist CGP55845 (Li et al., 2002; Wang et al., 2006). In the presence of 1 µM CGP55845, oxotremorine-M significantly increased the frequency of sIPSCs in a concentration-dependent fashion in all 12 neurons studied (Fig. 4D). These findings indicate that activation of the M3 subtype contributes to muscarinic potentiation of spinal glycine release and that spinal glycine release by the M3 subtype is influenced by concurrent M3 receptor-mediated stimulation of GABAergic interneurons, which evokes spinal GABA release and activate presynaptic GABAB receptors on glycinergic interneurons to limit the further release of glycine.

It has been documented that M2, M3, and M4 mAChR subtypes are present in the spinal dorsal horn (Hoglund and Baghdoyan, 1997; Duttaroy et al., 2002; Chen et al., 2005). To confirm the role of the M3 subtype in the potentiating effect of oxotremorine-M on synaptic glycine release in M2/M4 double-KO mice, we further tested the effect of 5 µM oxotremorine-M on glycinergic sIPSCs in the presence of 50 nM 4-DAMP, an M3 subtype-preferring mAChR antagonist (Cembala et al., 1998; Moriya et al., 1999; Zhang et al., 2005). The potentiating
effect of 5 µM oxotremorine-M on sIPSCs was completely blocked by subsequent application of 50 nM 4-DAMP in all 7 neurons examined (Fig. 5A).

Additionally, in another 11 lamina II neurons from M₂/M₄ double-KO mice, 5 µM oxotremorine-M significantly increased the frequency, but not the amplitude, of mIPSCs in the presence of 1 µM CGP55845 (Fig. 5B). However, compared with the initial effect of oxotremorine-M on the sIPSCs, the effect of oxotremorine-M on the mIPSCs was significantly attenuated. These data suggest that the M₃ subtype is located on the presynaptic terminals and somatodendritic sites of glycinergic interneurons in the spinal cord.

**Effect of oxotremorine-M on sIPSCs in PTX-treated WT mice**

To further define the role of the M₃ subtype in the potentiating effect of oxotremorine-M on sIPSCs, a group of WT mice were treated intrathecally with 1 µg of PTX to inactivate G_{i/o} proteins that are coupled to M₂/M₄ subtypes (Zhang et al., 2005). In all 14 neurons from PTX-treated mice, oxotremorine-M consistently increased the frequency of sIPSCs in a dose-dependent manner (Fig. 6). These data are similar to those obtained from M₂/M₄ double-KO mice in the presence of the GABAB receptor antagonist CGP55845 and are consistent with the notion that the M₂/M₄ subtypes and GABAB receptors are coupled to PTX-sensitive G_{i/o} proteins.

**Effect of oxotremorine-M on sIPSCs in M₂-KO mice**

To delineate the relative role of the M₃ and M₄ subtypes in the action of oxotremorine-M on glycinergic sIPSCs, the effect of oxotremorine-M on sIPSCs was tested in M₂-KO mice. In this protocol, 1 µM CGP55845 was used to eliminate the influence of GABAB receptors on the
effect of oxotremorine-M on spinal glycine release. In 19 of 32 (59.4%) neurons, 3-10 µM oxotremorine-M dose-dependently increased the glycinergic sIPSCs (Fig. 7A). Oxotremorine-M had no significant effect on the frequency of glycinergic sIPSCs in the remaining 13 neurons (40.6%).

To determine whether the lack of effect of oxotremorine-M on sIPSCs in lamina II neurons was due to the opposing effects of the M3 and M4 subtypes, we selected another 10 neurons in which the initial application of 5 µM oxotremorine-M had no significant effect on sIPSCs. After bath perfusion of 100 nM MT-3 toxin, a selective M4 subtype antagonist (Jolkkonen et al., 1994; Ellis et al., 1999), oxotremorine-M significantly increased the frequency of glycinergic sIPSCs in all neurons tested (Fig. 7B). These findings suggest that in the absence of the M2 subtype, the M3 subtype plays a major role in the control of spinal glycine release. Furthermore, the effect of the M3 subtype on glycinergic input was suppressed by the M4 subtype in a population of dorsal horn neurons.

**Effect of oxotremorine-M on sIPSCs in M4-KO mice**

To assess the relative role of the M2 and M3 subtypes, we examined the effect of oxotremorine-M in M4-KO mice. CGP55845 (1 µM) was also used to remove the influence of GABA_B receptors on oxotremorine-M-induced glycine release. In 13 of 33 (39.4%) neurons from M4 single-KO mice, oxotremorine-M dose dependently increased the frequency of sIPSCs (Fig. 8). In contrast, oxotremorine-M decreased the frequency of sIPSCs in another 13 (39.4%) neurons and had no effect on the frequency of sIPSCs in the remaining 7 (21.2%) neurons. Together with the above results, these data suggest that in the absence of the M4 subtype, the M2...
and M\textsubscript{3} subtypes have a similar important role in the regulation of spinal glycine release.
Discussion

In this study, we used mACHR KO mice to define the contribution of individual mACHR subtypes to the regulation of glycinergetic input to spinal dorsal horn neurons. We found that oxotremorine-M decreased the frequency of glycinergetic sIPSCs and mIPSCs in most spinal lamina II neurons in WT mice. This agonist consistently and significantly decreased the frequency of sIPSCs in all neurons tested in M3 KO mice but significantly increased the frequency of sIPSCs in all neurons tested in M2/M4 double-KO mice. We found it interesting that this potentiating effect was attenuated by higher concentrations of oxotremorine-M in some neurons. When the GABA\textsubscript{B} receptor was blocked, oxotremorine-M increased the frequency of sIPSCs in a concentration-dependent manner in all neurons tested in M2/M4 double-KO mice. Furthermore, oxotremorine-M consistently increased the frequency of sIPSCs in PTX-treated WT mice. This study provides the important new information that activation of the M2 and M4 subtypes inhibits glycinergetic input to the spinal dorsal horn neurons, whereas stimulation of the M3 subtype potentiates synaptic glycine release in the spinal dorsal horn. GABA\textsubscript{B} receptors are also involved in the feedback regulation of glycinergetic synaptic transmission when GABAergic interneurons are stimulated in the spinal cord.

Stimulation of mACHRs in the spinal cord inhibits nociceptive transmission. In this regard, application of mACHR agonists to the spinal cord suppresses the response of dorsal horn neurons to nociceptive stimuli (Chen and Pan, 2004). Three mACHR subtypes (M2, M3, and M4) are present in the spinal cord dorsal horn (Hoglund and Baghdoyan, 1997; Duttaroy et al., 2002; Chen et al., 2005). The mACHR agonist-induced antinociception is abolished in M2/M4 double-
KO mice (Duttaroy et al., 2002). We found that oxotremorine-M significantly inhibited the frequency but not the amplitude of mIPSCs and sIPSCs in WT mice; this observation suggested that the inhibitory mAChRs are probably located on the presynaptic terminals of glycinergic interneurons. We also determined the functional role of the M\(_2\)/M\(_4\) subtypes in the control of synaptic glycine release in M\(_3\) single-KO mice: compared with its effect in WT mice, oxotremorine-M consistently decreased the frequency of sIPSCs in all tested neurons in M\(_3\)-KO mice. The inhibitory action of the M\(_2\)/M\(_4\) subtypes is further supported by our finding that the M\(_2\)/M\(_4\) subtype-preferring antagonist himbacine abolished the inhibitory effect of oxotremorine-M in M\(_3\) KO mice. Because the M\(_2\) subtype-preferring antagonist AFDX-116 only partially blocked the inhibitory effect of oxotremorine-M, both the M\(_2\) and M\(_4\) subtypes appear to contribute to the inhibition of synaptic glycine release in the spinal cord by mAChRs.

Despite the findings from our current study, the physiological function of the M\(_3\) subtype in the control of spinal nociceptive transmission remains largely unknown. We found that in M\(_2\)/M\(_4\) double-KO mice, the overall effect of oxotremorine-M was to increase glycinergic sIPSCs. Our observation that the M\(_3\) subtype-preferring mAChR antagonist 4-DAMP (Cembala et al., 1998; Moriya et al., 1999; Zhang et al., 2005) abolished the potentiating effect of oxotremorine-M on sIPSCs in M\(_2\)/M\(_4\) double-KO mice provides further support for our conclusion that the M\(_3\) subtype contributes to the potentiation of spinal glycine release. In M\(_2\)/M\(_4\) double-KO mice, oxotremorine-M significantly increased the frequency of mIPSCs, but significantly less than it increased the frequency of sIPSCs. Thus, the M\(_3\) subtype is likely located on the presynaptic terminals as well as on somatodendritic sites of glycinergic interneurons in the mouse spinal cord. We were surprised that the potentiating effect of oxotremorine-M on the frequency of sIPSCs was
attenuated at higher concentrations. Because oxotremorine-M evokes GABA release through the M₃ subtype to dorsal horn neurons in M₂/M₄ double-KO mice (Zhang et al., 2006), we hypothesized that the increased GABA release could activate GABAₐ receptors to limit synaptic glycine release in the spinal cord of M₂/M₄ double-KO mice. We found that when the GABAₐ receptor antagonist CGP55845 was used, oxotremorine-M at higher concentrations produced a consistent increase in glycinergic sIPSCs in M₂/M₄ double-KO mice. Thus, GABAₐ receptors present on glycinergic interneurons function as important heteroreceptors in the dynamic feedback control of glycine release following concurrent activation of the M₃ subtype on GABAergic interneurons in the spinal dorsal horn. This conclusion is supported by our finding that in PTX-treated WT mice, oxotremorine-M dose dependently increased the frequency of sIPSCs and mimicked the effect of oxotremorine-M in M₂/M₄ double-KO mice in the presence of GABAₐ receptor antagonist. These findings are consistent with the notion that the M₂ and M₄ subtypes and the GABAₐ receptors are all coupled to PTX-sensitive Gᵢₒ proteins. By using M₂/M₄ double-KO mice, we have discovered a close interaction between GABAergic and glycinergic interneurons through GABAₐ receptors in the spinal dorsal horn.

Because of the opposing functions of the M₃ subtype and the M₂ and M₄ subtypes in regulating spinal glycine release (increased versus decreased glycine release, respectively), oxotremorine-M may increase or decrease synaptic glycine release to spinal dorsal horn neurons, depending on the distribution and the relative density of these three subtypes on the glycinergic interneurons that make synaptic contact with the postsynaptic neuron. This notion is clearly supported by our finding that blocking the M₄ subtype with MT-3 toxin revealed a potentiating effect of the M₃ subtype in M₂-KO mice. Because the major effect of oxotremorine-M on
glycinergic sIPSCs of dorsal horn neurons is inhibitory in WT mice, the role of the M3 subtype in the modulation of glycine release appears to be masked by the dominant action of the M2 and M4 subtypes in the spinal dorsal horn. Thus, the M2 and M4 subtypes play a more important role than the M3 subtype in mAChR regulation of synaptic glycine release in the spinal dorsal horn of mice. In M2-KO mice, oxotremorine-M (by activating M3 and M4 subtypes) increased the frequency of sIPSCs in about 60% of the neurons. Since this agent did not inhibits the glycinergic sIPSCs in these mice, the function of the M4 subtype on glycinergic interneurons is likely masked by the presence of the M3 subtype. We also assessed the relative roles of the M2 (to decrease glycine release) and the M3 (to increase glycine release) subtypes in M4-KO mice. We found that oxotremorine-M decreased the frequency of sIPSCs in about 40% of the neurons but increased the frequency of sIPSCs in approximately 40% of neurons and had no effect on sIPSCs in about 20% neurons in M4-KO mice. These data suggest that the M2 and M3 subtypes are equally important in the regulation of spinal glycine release. Thus, our findings provide important evidence that the three mAChR subtypes in the spinal cord contribute to a different extent (i.e., M2 = M3 > M4) to the muscarinic modulation of glycinergic input to dorsal horn neurons.

Results from our previous studies using relatively selective mAChR antagonists suggested that the activation of mAChRs potentiates spinal glycine release in rats (Wang et al., 2006). In the present study, however, stimulation of mAChRs primarily inhibited glycine release in the mouse spinal cord. Hence, there exist important species differences regarding the function and subcellular distribution of mAChR subtypes in the regulation of spinal glycine release. For example, activation of the M2 subtype seems to increase spinal glycine release in rats (Wang et
al., 2006), whereas in the mouse spinal cord the function of the M₂ subtype is to inhibit glycine release. Despite this difference, activation of the M₂ and M₄ subtypes in the spinal cord has a potent antinociceptive action in both species (Ellis et al., 1999; Gomeza et al., 1999a; Duttaroy et al., 2002; Li et al., 2002). It is not yet clear how the reduction of glycine release by spinal M₂ and M₄ subtypes contributes to the analgesic effect of mAChR agonists in mice. Because the recorded lamina II neurons are likely interneurons, it is possible that reduced glycinergic input may lead to disinhibition of inhibitory neurons in spinal dorsal horn to reduce nociceptive transmission (Zhang et al., 2006). Furthermore, glycine is a co-agonist for the glycine binding site of NMDA receptors. Decreased synaptic glycine release may reduce nociceptive transmission by decreasing NMDA receptor activity in the superficial dorsal horn.

In summary, our study using subtype selective KO mice provides unequivocal evidence that activation of the M₂ and M₄ mAChR subtypes inhibits glycine release and that activation of the M₃ subtype potentiates glycine release in the spinal dorsal horn of mice (Fig. 9). Another interesting finding is that the GABA₉ receptor is involved in the regulation of spinal glycine release through activation of the M₃ subtype expressed on GABAergic interneurons (Fig. 9). These findings provide new insights into the distinct functions of the M₂, M₃ and M₄ subtypes in the regulation of glycinergic input to spinal dorsal horn neurons. This information has broad implications in the study of spinal dorsal horn circuitry involved in the regulation of nociceptive transmission because the activity of glycinergic interneurons in the spinal cord might be modified through the use of different mAChR subtype-KO mice.
References


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Footnotes

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Legends for Figures

Figure 1. Effect of oxotremorine-M on glycinergic sIPSCs and mIPSCs of spinal lamina II neurons in WT mice. A, original traces of glycinergic sIPSCs during control, application of 3, 5 and 10 µM oxotremorine-M (oxo) in one neuron. Note that the sIPSCs were abolished by 2 µM strychnine. B, cumulative plot of sIPSCs of the same neuron in A showing the distribution of the inter-event interval and amplitude of sIPSCs during control, application of 5 µM oxotremorine-M, and washout. The average baseline frequency of sIPSCs during control was 1.90 Hz. C, group data showing the effect of oxotremorine-M on the sIPSCs in WT mice. D, summary data showing the effect of oxotremorine-M on the frequency of mIPSCs in WT mice (n = 9 neurons). Data are presented as means ± S.E.M. *, P < 0.05 compared with the control.

Figure 2. Effect of oxotremorine-M on sIPSCs in M3 KO mice. A, representative traces showing sIPSCs during control, application of 3, 5 and 10 µM oxotremorine-M (oxo) and washout in one neuron. B, cumulative probability plot analysis of sIPSCs of the same neuron in A showing the distribution of the inter-event interval and amplitude of sIPSCs during control, application of 5 µM oxotremorine-M, and washout. The baseline frequency of sIPSCs was 1.08 Hz during the control. C, summary data showing the effect of oxotremorine-M on the frequency of sIPSCs in M3 double-KO mice (n = 12 neurons). Data are presented as means ± S.E.M. *, P < 0.05 compared with the control.
Figure 3. Effect of oxotremorine-M on sIPSCs in the presence of himbacine or AFDX-116 or himbacine in M3-KO mice. A, original traces of sIPSCs during control, initial application of 5 μM oxotremorine-M, 10 μM AFDX-116 alone, and 10 μM AFDX-116 plus 5 μM oxotremorine-M in one lamina II neuron. B, summary data showing the effect of 5 μM oxotremorine-M on the frequency of sIPSCs before and after application of 10 μM AFDX-116 (n = 14 neurons). C, summary data showing the effect of 5 μM oxotremorine-M on the frequency of sIPSCs before and after application of 2 μM himbacine (n = 12 neurons). Data are presented as means ± S.E.M. *, P < 0.05 compared with the control. #, P < 0.05 compared with the initial effect of oxotremorine-M.

Figure 4. Effect of oxotremorine-M on sIPSCs in M2/M4 double-KO mice. A, raw tracings of sIPSCs during control, application of 3, 5, 10 μM oxotremorine-M (oxo) in one cell without the GABA_B receptor antagonist CGP55845 (1 μM). B, cumulative probability plot of sIPSCs of the same neuron in A showing the distribution of the inter-event interval and amplitude of sIPSCs during control, application of 3 and 5 μM oxotremorine-M. C, group data showing the effect of oxotremorine-M on sIPSCs without 1 μM CGP55845. D, summary data showing the effect of oxotremorine-M on the frequency of sIPSCs in the presence of 1 μM CGP55845 (n = 12 neurons). Data are presented as means ± S.E.M. *, P < 0.05 compared with the control.

Figure 5. Effect of oxotremorine-M on sIPSCs and mIPSCs in M2/M4 double-KO mice. A, group data showing the effect of 50 nM 4-DAMP on the 5 μM oxotremorine-M-induced increase in the
frequency of sIPSCs in M2/M4 double-KO mice (n = 7 neurons). B, summary data showing the
effect of 5 µM oxotremorine-M on the frequency of mIPSCs in M2/M4 double-KO mice (n = 11
neurons). Data are presented as means ± S.E.M. *, P < 0.05 compared with the control.

Figure 6. Effect of oxotremorine-M on sIPSCs in PTX (1 µg)-treated WT mice. A, original traces
of sIPSCs during application of 1-10 µM oxotremorine-M and washout. B, cumulative
probability plot analysis of sIPSCs of the same neuron in A showing the distribution of the inter-
event interval and amplitude of sIPSCs during control, application of 5 µM oxotremorine-M, and
washout. C, summary data showing the effect of oxotremorine-M on the frequency of sIPSCs (n
= 14 neurons). Data are presented as means ± S.E.M. *, P < 0.05 compared with the control.

Figure 7. Effect of oxotremorine-M on sIPSCs in M2-KO mice. A, group data showing the effect
of oxotremorine-M on the frequency of sIPSCs in 32 neurons. B, effect of 100 nM MT-3 toxin
on the frequency of sIPSCs in 10 neurons that did not respond to initial application of 5 µM
oxotremorine-M in M2 KO mice. Data are presented as means ± S.E.M. *, P < 0.05 compared
with the control.

Figure 8. Effects of oxotremorine-M on the frequency of sIPSCs in M4-KO mice (n = 33
neurons). Data are presented as means ± S.E.M. *, P < 0.05 compared with respective controls.

Figure 9. Schematic drawing highlighting the distinct function of M2, M3, and M4 mAChR
subtypes in the control of glycinergic input to dorsal horn neurons of mice. The relationship
between the GABA_B receptor and mAChRs in the control of synaptic glycine release is also shown. Note that the M_2, M_3, and M_4 mAChR subtypes may be located on the same or different glycineric neurons and presynaptic terminals. -, inhibition; +, potentiation.
Table 1. Variable effects of oxotremorine-M (1-10 µM) on the frequency (Hz) of glycinergic sIPSCs in 4 of 27 neurons in WT mice.

<table>
<thead>
<tr>
<th>neuron</th>
<th>control</th>
<th>1 µM</th>
<th>3 µM</th>
<th>5 µM</th>
<th>10 µM</th>
<th>Washout</th>
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<tr>
<td>1</td>
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<td>1.73</td>
<td>2.09</td>
<td>4.88</td>
<td>1.84</td>
</tr>
</tbody>
</table>
A

WT

control

oxo 3 \mu M

oxo 5 \mu M

oxo 10 \mu M

strychnine

B

Cumulative probability

inter-event interval (ms)

0.0
0.2
0.4
0.6
0.8
1.0

oxo 0.5 \mu M

washout

control

Amplitude (pA)

0
20
40
60
80

oxo 5 \mu M

washout

control

C

Frequency (Hz)

control

1
3
5
10
washout

no change, n=8

decrease, n=15

D

Frequency (Hz)

control

oxo

wash

TTX

oxo

washout

*
Figure 2

A

M3 KO

control

oxo 3 μM

oxo 5 μM

oxo 10 μM

washout

B

Cumulative probability

Inter-event interval (ms)

0.0 0.2 0.4 0.6 0.8 1.0

0 1000 2000 3000 4000 5000

control

oxo 5 μM

washout

C

Frequency (Hz)

control 1 3 5 10 washout

n=12

Oxotremorine-M (μM)
WT with PTX treatment

control
oxo 1 µM
oxo 3 µM
oxo 5 µM
oxo 10 µM
washout

B
Cumulative probability

Inter-event interval (ms)

Amplitude (pA)

C
Frequency (Hz)

Oxotremorine-M (µM)

n=14

*
M4 KO

Frequency (Hz)

control 1 3 5 10 washout

Oxotremorine-M (µM)

n=13

n=13

n=7

*