M2, M3 and M4 Receptor Subtypes Contribute to Muscarinic
Potentiation of GABAergic Inputs to Spinal Dorsal Horn Neurons

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List of abbreviations:

sIPSCs: spontaneous inhibitory postsynaptic currents; GABA: γ-aminobutyric acid;
mIPSCs: miniature inhibitory postsynaptic currents;
GDP-β-S: guanosine 5′-O-(2-thiodiphosphate);
CNQX: 6-cyano-7-nitroquinoxaline-2,3-dione;
4-DAMP: 4-diphenylacetoxy-N-methylpiperidine methiodide

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Abstract

The spinal cholinergic system and muscarinic receptors are important for regulation of nociception. Activation of spinal muscarinic receptors produces analgesia and inhibits dorsal horn neurons through potentiation of GABAergic inputs. To determine the role of receptor subtypes in the muscarinic agonist-induced synaptic GABA release, spontaneous inhibitory postsynaptic currents (sIPSCs) were recorded in lamina II neurons using whole-cell voltage-clamp recordings in rat spinal cord slices. The muscarinic receptor agonist oxotremorine-M dose-dependently (1-10 µM) increased GABAergic sIPSCs but not miniature IPSCs. The potentiating effect of oxotremorine-M on sIPSCs was completely blocked by atropine. In rats pretreated with intrathecal pertussis toxin to inactive inhibitory G_{i/o} proteins, 3 µM oxotremorine-M had no significant effect on sIPSCs in 31 of 55 (56%) neurons tested. In the remaining 24 (44%) neurons in pertussis toxin-treated rats, oxotremorine-M caused a small increase in sIPSCs, and this effect was completely abolished by subsequent application of 25 nM 4-DAMP, a relatively selective M_{3} subtype antagonist. Furthermore, himbacine (1 µM), a relatively specific antagonist for M_{2} and M_{4} subtypes, produced a large reduction in the stimulatory effect of oxotremorine-M on sIPSCs, and the remaining effect was abolished by 4-DAMP. Additionally, the M_{4} receptor antagonist MT-3 toxin (100 nM) significantly attenuated the effect of oxotremorine-M on sIPSCs. Collectively, these data suggest that M_{2} and M_{4} receptor subtypes play a predominant role in muscarinic potentiation of synaptic GABA release in the spinal cord. The M_{3} subtype also contributes to increased GABAergic tone in spinal...
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dorsal horn by muscarinic agonists.
Introduction

The spinal dorsal horn is an important site for nociceptive transmission as well as modulation. Intrathecal administration of muscarinic agonists or acetylcholinesterase inhibitors produces potent analgesia in rodents and humans (Iwamoto and Marion, 1993; Naguib and Yaksh, 1994; Hood et al., 1997; Ma et al., 2001; Duttaroy et al., 2002). The analgesic effect is mediated by muscarinic receptors, since the muscarinic antagonist atropine blocks the antinociceptive effects produced by both muscarinic agonists or acetylcholinesterase inhibitors (Zhuo and Gebhart, 1991; Naguib and Yaksh, 1994). Receptor autoradiography and immunocytochemistry studies have shown that the highest density of muscarinic receptors in the spinal cord is located in the superficial lamina in both rats and humans (Yamamura et al., 1983; Scatton et al., 1984; Villiger and Faull, 1985; Li et al., 2002). Molecular cloning studies have revealed five molecularly distinct muscarinic acetylcholine receptors referred to as M₁-M₅ (Caulfield, 1993; Wess, 1996). The five muscarinic receptor subtypes are all linked to different types of G proteins. The odd-numbered muscarinic subtypes (M₁, M₃, and M₅) are selectively linked to G₉/₁₁ proteins, while the even-numbered subtypes (M₂ and M₄) are preferentially coupled to the pertussis toxin (PTX)-sensitive G₁₁₀ family (Felder, 1995; Wess, 1996; Caulfield and Birdsall, 1998). Although previous studies have shown that M₂, M₃, and M₄ muscarinic receptor subtypes are present in the spinal cord dorsal horn (Hoglund and Baghdoyan, 1997; Yung and Lo, 1997; Duttaroy et al., 2002), the possible physiological functions of these individual subtypes are not fully understood.
GABA is the most important inhibitory neurotransmitter in the mammalian central nervous system. Stimulation of spinal GABA release and GABA_{B} receptors is an important analgesic mechanism of muscarinic agonists in spinal dorsal horn (Baba et al., 1998; Li et al., 2002; Chen and Pan, 2003). The spinal lamina II neurons are under the tonic control of glutamatergic excitatory and GABAergic/glycinergic inhibitory inputs. Glutamate released from primary afferents is a major excitatory neurotransmitter, which conveys nociceptive information to neurons in the superficial lamina (Yoshimura and Jessell, 1990). Muscarinic receptor activation increases GABA release in spinal dorsal horn (Baba et al., 1998). We have shown that acetylcholine can inhibit glutamate release, and inhibition of glutamate release is at least partly mediated by spinal GABA release (Li et al., 2002). Also, muscarinic agents inhibit dorsal horn projection neurons through GABA_{B} receptors (Chen and Pan, 2004). However, the subtypes of muscarinic receptors in potentiation of synaptic GABA release in the spinal cord are not clear. In the present study, we used different pharmacological interventions to determine possible muscarinic receptor subtypes that are involved in the spinal cord GABA release.
Methods

All the surgical preparations and experimental protocols were approved by the Animal Care and Use Committee of the Pennsylvania State University College of Medicine. Sprague-Dawley rats (3-4 weeks old; Harlan, Indianapolis, IN) were used in this study.

**Intrathecal treatment with PTX.** To determine the involvement of M₂ and M₄ subtypes in the stimulatory effect of oxotremorine-M on synaptic GABA release, a group of rats was pretreated with intrathecal PTX to inactivate inhibitory Gᵢ₀ proteins (Wong et al., 1992; Fields and Casey, 1997; Chen and Pan, 2004). Intrathecal catheters were inserted in rats anesthetized using 2% halothane. The catheters (polyethylene-10 tubing) were inserted through an incision in the cisternal membrane and advanced 4.5 cm caudal so that the tip of each catheter was positioned at the lumbar spinal level. Rats were injected with intrathecal 2 μg of PTX 5-7 days before the final electrophysiology experiments.

**Spinal cord slice preparations.** Rats were anesthetized with 2% halothane in O₂ and the lumbar segment of the spinal cord was rapidly removed through a limited laminectomy. The rats then were killed by inhalation of 5% halothane. The segment of lumbar spinal cord was immediately placed in an ice-cold sucrose artificial cerebrospinal fluid (aCSF) presaturated with 95% O₂ and 5% CO₂. The sucrose aCSF contained (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 on 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 then preincubated 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 (mM): NaCl, 117.0; KCl, 3.6; MgCl₂, 1.2; CaCl₂, 2.5; NaH₂PO₄, 1.2; glucose, 11.0; and NaHCO₃, 25.0. Recordings of postsynaptic currents were performed using the whole-cell voltage-clamp method, as we described previously (Li et al., 2002; Pan et al., 2002). The lamina II has a distinct translucent appearance and can easily be distinguished under the microscope. In this study, efforts were made to record neurons in the outer zone of lamina II (Li et al., 2002; Pan and Pan, 2004). The neurons located in the dorsal one-third of lamina II in the spinal slice were identified under a fixed stage microscope (BX50WI, 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 Instrument, Novato, CA). The impedance of the pipette was 4-7 MΩ when filled with internal solution containing (mM): Cs₂SO₄, 110; KCl, 5; MgCl₂, 2.0; CaCl₂, 0.5; HEPES, 5.0; EGTA, 5.0; ATP-Mg, 5.0; Na-GTP, 0.5; and guanosine 5’-O-(2-thiodiphosphate) (GDP-β-S), 1; QX314 10; adjusted to pH 7.2-7.4 with 1 M CsOH (290-320 mOsm). GDP-β-S was added to the internal solution to block the possible postsynaptic effect mediated by muscarinic agonists through G proteins (Li et al., 2002; Pan et al., 2002). QX314 was added to the internal solution to suppress the action potential generation from the recorded cell. The 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 began 5 min later, after whole-cell access was established and the current reached a steady state. The input resistance was monitored and the recording was abandoned if it changed more than 15% (Li et al., 2002; Pan et al., 2002). 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 into a Pentium computer with pCLAMP 9.0 (Axon Instruments). All spontaneous inhibitory postsynaptic currents (sIPSCs) were recorded in the presence of 2 µM strychnine, a glycine receptor antagonist, and 20 µM 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX). To record the miniature inhibitory postsynaptic currents (mIPSCs), 1 µM tetrodotoxin (TTX) was added in the perfusion solution.

Oxotremorine-M, himbacine, MT-3 toxin, 4-diphenylacetoxy-N-methylpiperidine methiodide (4-DAMP), GDP-β-S, atropine, strychnine, CNQX, and bicuculline were obtained from Sigma-Aldrich. PTX was purchased from List Biological Laboratories (San Jose, CA). TTX and QX314 were obtained from Alomone Labs (Jerusalem, Israel). Drugs were dissolved in Krebs solution and perfused into the slice chamber using syringe pumps.

**Data analysis.** Data are presented as means ± SE. The sIPSCs and mIPSCs were analyzed off-line with a peak detection program (MiniAnalysis, Synaptosoft, Leonia, NJ). Measurements of the amplitude and frequency of sIPSCs and mIPSCs were performed over a period of at least 1 min during control, drug application, and recovery. For each analysis, 300-1500 events were included. The mIPSCs and sIPSCs were detected by the fast rise time of the
signal over an amplitude threshold above the background noise. The amplitude detection threshold was typically 5-6 pA. We manually excluded the event when the noise was erroneously identified as the mIPSCs or sIPSCs by the program. The background noise level was typically constant throughout the recording of a single neuron. The cumulative probability of the amplitude and interevent interval of mIPSCs/sIPSCs was compared using the Kolmogorov-Smirnov test, which estimates the probability that two cumulative distributions are similar. The effects of oxotremorine-M on the amplitude and frequency of sIPSCs and mIPSCs were determined by paired two-tailed Student's t-test or one-way ANOVA. Neurons were considered to be responsive to oxotremorine-M if the frequency of sIPSCs was altered > 20%. P < 0.05 was considered to be statistically significant.
Results

All lamina II neurons tested exhibited spontaneous IPSCs at a holding potential of 0 mV. The sIPSCs and mIPSCs were recorded in the presence of 2 µM strychnine and 20 µM CNQX to isolate GABAergic IPSCs.

Effect of oxotremorine-M on sIPSCs and mIPSCs. The baseline frequency of sIPSCs ranged from 0.41 to 3.07 Hz (1.47 ± 0.23 pA), while the amplitude of sIPSCs during control was 12.31 ± 0.87 pA (from 8.15 to 22.47 pA, n = 16). To determine the concentration-dependent effect of oxotremorine-M on sIPSCs, 1, 3, 5 and 10 µM oxotremorine-M, a general specific muscarinic receptor agonist, was perfused in an accumulative fashion to the tissue chamber. Each concentration was applied for a duration of 3 min. In a total of 20 lamina II neurons, oxotremorine-M at the concentration of 1 µM had no significant effect on sIPSCs. Oxotremorine-M increased sIPSCs in a concentration-dependent manner (Fig. 1). At 3 µM, oxotremorine-M significantly increased the frequency (ranging from 1.86 to 7.33 Hz; 5.58 ± 0.43 pA) of sIPSCs in all 16 neurons examined. Among the 16 neurons tested, 3 µM oxotremorine-M also significantly increased the amplitude of sIPSCs from 12.87 ± 1.56 to 17.13 ± 2.12 pA in 8 cells. In another 8 neurons, 3 µM oxotremorine-M did not significantly increase the amplitude of sIPSCs (11.75 ± 0.83 to 11.42 ± 1.07 pA). The sIPSCs were completely blocked by 20 µM bicuculline, indicating that they were mediated by GABA_A receptors (Fig. 1A).

The stimulatory effect of 3 µM oxotremorine-M on sIPSCs were completely blocked by
the specific muscarinic antagonist atropine (2 μM, n = 9, Fig. 2, A and B). Atropine was applied 3-4 min before applying the agonist. In 6 separate lamina II neurons, we determined if application of the same concentration of oxotremorine-M produces a reproducible effect on sIPSC. The initial effect of oxotremorine-M on sIPSCs can be completely washed out in 10 min. Repeat application of 3 μM oxotremorine-M caused a similar increase in the amplitude and frequency of sIPSCs (Fig. 2C).

In another 10 lamina II neurons, the effect of 3 μM oxotremorine-M on mIPSCs was tested in the presence of 1 μM TTX. Oxotremorine-M did not significantly alter the frequency and amplitude of mIPSCs in all 10 cells tested (Fig. 3).

**Effect of oxotremorine-M on sIPSCs in PTX-treated rats.** To determine the contribution of M₂ and M₄ subtypes to the effect of oxotremorine-M on synaptic GABA release to lamina II neurons, rats were pretreated with intrathecal 2 μg PTX to inactivate inhibitory Gᵢ/o proteins 5-7 days before final electrophysiology experiments. In 5 rats pretreated with PTX, a total of 27 lamina II neurons was tested. Oxotremorine-M (1-10 μM) had no significant effect on the frequency of sIPSCs in 19 of 27 cells (Fig. 4). Furthermore, although 3-10 μM oxotremorine-M significantly increased the frequency of sIPSCs in concentration-dependent manner in the remaining 8 neurons (Fig. 4), its potentiating effect was significantly reduced compared to that in untreated rats. Oxotremorine-M only slightly increased the amplitude (from 12.92 ± 0.73 to 18.58 ± 1.36 pA, P > 0.05) of sIPSCs in only 3 of 8 neurons tested.

In another 4 rats pretreated with PTX, we determined if the remaining effect of oxotremorine-M on sIPSCs is mediated by the M₃ subtype in the spinal cord. In 12 of 28 lamina
II neurons tested, 3 µM oxotremorine-M had no significant effect on both the frequency and amplitude of sIPSCs. In the remaining 16 neurons, 3 µM oxotremorine-M significantly increased the frequency of sIPSCs (P < 0.05, Fig. 5). Among these 16 cells tested, oxotremorine-M significantly increased the amplitude (from 11.09 ± 0.63 to 17.76 ± 2.84 pA) of sIPSCs in 5 neurons. This effect was completely blocked by subsequent application of 25 nM 4-DAMP (Fig. 5), a relatively selective M3 receptor antagonist (Ehlert, 1996; Yigit et al., 2003). In the preliminary study, we found that 25 nM 4-DAMP is the lowest concentration that completely blocked the stimulatory effect of 3 µM oxotremorine-M on sIPSCs of lamina II cells in M2/M4 double-knockout mice (Zhang et al., unpublished data).

**Effect of himbacine on the stimulatory action of oxotremorine-M on sIPSCs.** To further determine the role of M2 and M4 receptor subtypes in the effect of oxotremorine-M on sIPSCs, a relatively selective M2 and M4 antagonist, himbacine (Dorje et al., 1991; Miller et al., 1992; Doller et al., 1999), was used. Himbacine was applied 3-4 min before applying the agonist. In 15 of 30 (50%) cells tested, the effect of 3 µM oxotremorine-M on the frequency of sIPSCs was completely blocked by 1 µM himbacine (Fig. 6C). In another 15 (50%) cells, the effect of 3 µM oxotremorine-M on the frequency of sIPSCs was significantly attenuated by 1 µM himbacine (Fig. 6, A and D). In the above 30 neurons tested, 3 µM oxotremorine-M significantly increased the amplitude (from 13.74 ± 0.82 to 25.69 ± 2.19 pA) of sIPSCs in 13 cells. Following application of 1 µM himbacine, oxotremorine-M increased the amplitude (from 12.47 ± 1.17 to 16.97 ± 1.45 pA, P < 0.5) of sIPSCs in 7 of 13 cells examined. Furthermore, we determined if the remaining effect of oxotremorine-M on sIPSCs in the presence of 1 µM
himbacine is mediated by the M₃ subtype in some cells. In all 6 lamina II neurons tested, the effect of oxotremorine-M on sIPSCs was blocked completely by subsequent application of 25 nM 4-DAMP (Fig. 6, A and D).

**Effect of MT-3 toxin on the stimulatory action of oxotremorine-M on sIPSCs.** To further delineate the role of the M₄ receptor subtype in the effect of oxotremorine-M on sIPSCs, MT-3 toxin, a selective M₄ receptor antagonist (Jolkkonen et al., 1994; Ellis et al., 1999), was used. MT-3 toxin (100 nM) was perfused for 4 min followed by 3 μM oxotremorine-M. In 12 of 14 cells tested, MT-3 significantly reduced the effect of 3 μM oxotremorine-M on the frequency of sIPSCs (Fig. 7, A and B). Among these 12 cells, the unblocked effect of oxotremorine-M was completely abolished by further perfusion of 1 μM himbacine in 6 (50%) cells (Fig. 7C). In another 6 (50%) cells, the stimulatory effect of oxotremorine-M on the frequency of sIPSCs was only partially attenuated by 1 μM himbacine but was completely eliminated by further application of 25 nM 4-DAMP (Fig. 7D). In the remaining 2 of 14 cells tested, the effect of 3 μM oxotremorine-M on the frequency of sIPSCs was completely blocked by 100 nM MT-3 (from 1.08 to 1.01 Hz in one cell, and from 1.46 to 1.55 Hz in another cell). Among the 14 cells studied above, 3 μM oxotremorine-M significantly increased the amplitude of sIPSCs from 16.98 ± 2.61 to 25.08 ± 3.99 pA in 10 neurons. Following application of 100 nM MT-3, 3 μM oxotremorine-M only significantly increased the amplitude of sIPSCs from 13.76 ± 1.42 to 19.59 ± 3.17 pA in 4 of 10 neurons.
Discussion

The antinociceptive effect of spinally administered muscarinic agonists is mediated partially through increased synaptic GABA release. In the present study, we investigated the muscarinic receptor subtypes involved in the spinal GABA release using a proper combination of muscarinic subtype antagonists, PTX, and MT-3 toxin. The specific muscarinic receptor agonist oxotremorine-M increased GABAergic sIPSCs in a concentration-dependent manner, and this effect was abolished by the muscarinic receptor antagonist atropine. In rats pretreated with intrathecal PTX, oxotremorine-M only increased sIPSCs in 44% cells tested. Also, a relatively selective M₂ and M₄ subtype antagonist, himbacine, caused a large reduction in the stimulatory effect of oxotremorine-M on sIPSCs. Furthermore, bath application of 4-DAMP, a relatively selective M₃ subtype antagonist, completely abolished both the remaining effect of oxotremorine-M on sIPSCs in PTX-pretreated rats and the residual effect of oxotremorine-M on sIPSCs in the presence of himbacine. Additionally, the M₄ receptor antagonist MT-3 toxin significantly reduced the effect of oxotremorine-M on sIPSCs. Therefore, this study provides new information that M₂ and M₄ receptor subtypes play a critical role in potentiation of GABAergic inputs to spinal dorsal horn neurons. The M₃ subtype also contributes, to a lesser extent, to the stimulatory effect of muscarinic agonists on synaptic GABA release in the spinal cord.

Although the spinal muscarinic receptors in antinociception are well established, the subtypes mediating this effect remain unclear. Previous behavioral studies suggest that
muscarinic analgesia is mediated through M\textsubscript{1} and M\textsubscript{2} receptors (Iwamoto and Marion, 1993) or M\textsubscript{1} and M\textsubscript{3} receptors in the spinal cord (Naguib and Yaksh, 1997). However, the highly M\textsubscript{1}-selective agonist xanomeline does not produce any analgesic effect (Sheardown et al., 1997). Using muscarinic subtype knockout mice, it has been shown that the M\textsubscript{2} and M\textsubscript{4} subtypes are responsible for the analgesic effect of muscarinic receptor agonists (Gomez et al., 1999; Duttaroy et al., 2002). Furthermore, radioligand binding experiments fail to demonstrate the presence of the M\textsubscript{1} receptor in the rat spinal cord (Hoglund and Baghdoyan, 1997). Thus, only M\textsubscript{2}, M\textsubscript{3}, and M\textsubscript{4} muscarinic receptor subtypes have been documented in spinal cord dorsal horn, and the M\textsubscript{2} and M\textsubscript{3} subtypes are particularly concentrated in the superficial laminae of the spinal cord (Hoglund and Baghdoyan, 1997; Yung and Lo, 1997; Li et al., 2002). Nevertheless, the functional roles of these three muscarinic receptor subtypes in synaptic transmission and muscarinic analgesia remain to be established.

The potential cellular mechanisms of muscarinic analgesia in the spinal cord have been shown in recent studies. For example, carbachol can increase the GABA release to spinal lamina II neurons (Baba et al., 1998). Glutamate released from primary afferents to spinal dorsal horn neurons is important for transmission of nociceptive information (Yoshimura and Jessell, 1990; Pan and Pan, 2004). The excitatory inputs from the primary afferents to spinal dorsal horn neurons are tonically controlled by GABAergic interneurons (Barber et al., 1978; Lu and Perl, 2003; Pan and Pan, 2004). We have shown that acetylcholine inhibits spinal glutamate release, and this inhibitory action is partially mediated by GABA release and activation of presynaptic GABA\textsubscript{b} receptors in the spinal cord (Li et al., 2002). Furthermore, the
antinociceptive effect produced by intrathecal administration of muscarinic agonists is significantly attenuated by the specific GABA\textsubscript{B} receptor antagonist CGP55845 (Li et al., 2002). We also found that muscarinic inhibition of dorsal horn projection neurons is mediated by GABA\textsubscript{B} receptors, and this effect is through PTX-sensitive \( G_{i/o} \) proteins (Chen and Pan, 2004). Thus, the \( M_2 \) and \( M_4 \) are the most important subtypes for the inhibitory effect of muscarinic agents on nociception and dorsal horn neurons. In the present study, oxotremorine-M significantly increased the frequency of sIPSCs but not mIPSCs, indicating that oxotremorine-M-induced GABA release is through activation of muscarinic receptors located at somatodendritic sites but not on the presynaptic terminals of GABAergic interneurons in the spinal cord. To determine the role of \( M_2 \) and \( M_4 \) subtypes in oxotremorine-M-induced synaptic GABA release, we examined if inactivation of \( G_{i/o} \) proteins with intrathecal pretreatment of PTX altered the effect of oxotremorine-M on sIPSCs. In rats pretreated with PTX, 56\% of lamina II neurons failed to respond to oxotremorine-M. Also, in the remaining 44\% neurons, the effect of oxotremorine-M on the frequency of sIPSCs was significantly attenuated compared with that in untreated rats. Furthermore, the relatively selective \( M_2 \) and \( M_4 \) subtype antagonist himbacine (Miller et al., 1992; Doller et al., 1999) produced a similar inhibitory effect on oxotremorine-M-induced increase in GABAergic sIPSCs of lamina II neurons. These data are consistent with the behavioral data about the critical role of \( M_2 \) and \( M_4 \) receptors in muscarinic analgesia using \( M_2/M_4 \) subtype knockout mice (Duttaroy et al., 2002). Therefore, the spinal \( M_2 \) and \( M_4 \) subtypes play a predominant role in increased GABAergic input to spinal dorsal horn neurons by muscarinic agonists.
Although the M₂ receptor appears to be the most predominant subtype in the spinal cord (Yung and Lo, 1997; Duttaroy et al., 2002), there is a small but functionally significant M₄ subtype in the spinal cord (Hoglund and Baghdoyan, 1997; Ellis et al., 1999; Duttaroy et al., 2002; Mulugeta et al., 2003). Also, the spinal cord M₄ receptors have been shown to be important in the muscarinic analgesia in mice (Ellis et al., 1999). We used MT-3 toxin to further assess the contribution of the M₄ subtype to muscarinic agonist-induced synaptic GABA release to spinal dorsal horn neurons. MT-3 has a 40-fold selectivity for the M₄ subtype over the M₁ subtype and a greater than 500-fold selectivity for the M₄ receptor over M₂, M₃, and M₅ receptors (Jolkkonen et al., 1994; Liang et al., 1996). We found that MT-3 significantly reduced the effect of oxotremorine-M on sIPSCs in most cells tested. This suggests that the M₄ subtype plays an important role in muscarinic agonists-induced increase in the GABAergic tone in the spinal cord. The signaling mechanisms underlying the potentiating effect of M₂ and M₄ subtypes on synaptic GABA release in the spinal cord are not clear. The stimulatory action of M₂ and M₄ muscarinic subtypes on synaptic GABA release is probably due to their coupling to specific types of inhibitory Gᵢ proteins and associated adenylyl cyclase isozymes. For example, activation of the M₄ muscarinic receptor inhibits the activity of adenylyl cyclase isozymes I, V, VI, and VIII, but stimulates the activity of adenylyl cyclase isozymes II, IV, and VII (Nevo et al., 1998). It has been shown that the M₄ receptor could stimulate cAMP production through activation of adenylyl cyclase II that is coupled to G₁₁ or G₂ proteins (Liu et al., 1999). Therefore, modulation of GABAergic transmission by M₂ and M₄ receptors is different in the brain and spinal cord.
Another important finding of this study is that the M₃ receptor subtype also plays a role in potentiation of GABAergic inputs to spinal dorsal horn neurons by the muscarinic agonist. The presence of M₃ receptor subtype in the rat spinal cord has been shown in a radioligand binding study (Hoglund and Baghdoyan, 1997). In the present study, we found that oxotremorine-M still increased sIPSCs in 42% of lamina II neurons in rats pretreated with intrathecal PTX. This effect was completely blocked by the relatively selective M₃ antagonist 4-DAMP (Felder, 1995; Ehlert, 1996; Yigit et al., 2003). Similarly, the potentiating effect of oxotremorine-M on GABAergic sIPSCs in the presence of himbacine was also abolished by 4-DAMP. Thus, these data strongly suggest that the M₃ subtype also plays a significant role in increased GABAergic inputs to the spinal dorsal horn neurons produced by muscarinic agonists. Further studies are warranted to elucidate the specific role of the spinal M₃ subtype in muscarinic analgesia.

In summary, we determined the muscarinic receptor subtypes in mediating the increased spinal GABAergic inputs to rat dorsal horn neurons in the present study. We found that PTX pretreatment and the M₂ and M₄ receptor antagonist himbacine blocked most of the effect of oxotremorine-M on sIPSCs in the spinal cord. The remaining effect of oxotremorine-M on sIPSCs was abolished by an M₃ subtype antagonist 4-DAMP. This converging evidence strongly suggests that M₂ and M₄ as well as M₃ receptor subtypes are all involved in the augmentation of synaptic GABA release to spinal dorsal horn neurons by muscarinic agonists. Therefore, these three muscarinic receptor subtypes likely act synergistically in potentiation of the spinal inhibitory tone and mediate the antinociceptive actions of muscarinic receptor.
agonists. This new information is important for our understanding of the pharmacological mechanisms underlying muscarinic analgesia in the spinal cord. It should be acknowledged that GABA and glycine are often co-released to most but not all lamina II neurons (Pan and Pan, 2004). We did not study glycineric IPSCs in this particular work because strychnine was used to isolate GABAergic IPSCs throughout the study. The effect of muscarinic modulation of spinal glycine release and its implication in the analgesic action of muscarinic agonists should be systematically investigated in future studies.
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Figure legends

Figure 1. Effect of oxotremorine-M on sIPSCs of spinal lamina II neurons. A, Original tracings of sIPSCs during control, application of 3 and 5 μM oxotremorine-M (Oxo), and washout in one lamina II cell. The effect of 3 and 10 μM oxotremorine-M on the frequency of sIPSCs is significantly different. Note that the sIPSCs were abolished by 20 μM bicuculline. B, Cumulative probability plot analysis of sIPSCs of the same neuron in A showing the distributions of the amplitude and inter-event interval during control, application of 3 μM oxotremorine-M, and washout. C, Summary data showing that oxotremorine-M concentration-dependently increased the sIPSCs in 16 cells. Data presented as means ± S.E.M. *, P < 0.05 compared with the pre-drug control.

Figure 2. Effect of oxotremorine-M on sIPSCs was blocked by atropine. A, Representative tracings showing sIPSCs during control, application of 3 μM oxotremorine-M (Oxo), atropine alone, and 2 μM atropine plus oxotremorine-M. B, Summary data showing the effect of 3 μM oxotremorine-M on sIPSCs before and after 2 μM atropine (n = 9). C, Summary data showing that repeat perfusion of 3 μM oxotremorine-M (Oxo-1 and Oxo-2) had a similar effect on the frequency of sIPSCs (n = 6). Data presented as means ± S.E.M. *, P < 0.05 compared with control.

Figure 3. Comparison of the effect of oxotremorine-M on sIPSCs and mIPSCs. A, Original
tracings showing sIPSCs during control, application of 3 μM oxotremorine-M (Oxo), TTX alone, and 3 μM oxotremorine-M in the presence of 1 μM TTX. B, Summary data showing that oxotremorine-M increased the frequency of sIPSCs but not mIPSCs (n = 10). Data presented as means ± S.E.M. *, P < 0.05 compared with control.

Figure 4. Effect of oxotremorine-M on sIPSCs of spinal lamina II neurons in PTX-pretreated rats. A, Raw tracings of sIPSCs during control, application of 3 and 5 μM oxotremorine-M (Oxo), and washout in one oxotremorine-M-responsive cell. B, Summary data showing that oxotremorine-M had no effect on sIPSCs in 19 cells but concentration-dependently increased the frequency of sIPSCs in another 8 cells in PTX-pretreated rats. Note that the data in Fig. 1C (untreated rats) were re-plotted for comparison. Data presented as means ± S.E.M. *, P < 0.05 compared with the pre-drug control.

Figure 5. Effect of oxotremorine-M and 4-DAMP on sIPSCs of lamina II neurons recorded from in PTX-pretreated rats. A, Original tracings of sIPSCs during control, application of 3 μM oxotremorine-M (Oxo), 25 nM 4-DAMP alone, and application of oxotremorine-M plus 4-DAMP. B, Cumulative probability plot analysis of sIPSCs of the same neuron in A showing the distributions of the amplitude and inter-event interval during control, application of 3 μM oxotremorine-M, and oxotremorine-M plus 25 nM 4-DAMP. C, Summary data showing the effect of 3 μM oxotremorine-M on sIPSCs in 16 cells before and after application of 25 nM 4-DAMP. Data presented as means ± S.E.M. *, P < 0.05 compared with control.
Figure 6. Effect of himbacine and 4-DAMP on the stimulatory action of oxotremorine-M on the frequency of sIPSCs. A, Original tracings of sIPSCs during control, application of 3 μM oxotremorine-M (Oxo), 1 μM himbacine (Him) alone, and oxotremorine-M plus 1 μM himbacine and 25 nM 4-DAMP in one lamina II neuron. B, Cumulative probability plot analysis of sIPSCs of the same neuron in A showing the distributions of the amplitude and inter-event interval during control, application of 3 μM oxotremorine-M, and oxotremorine-M plus 1 μM himbacine. C, Summary data showing that 1 μM himbacine completely blocked the effect of 3 μM oxotremorine-M on the frequency of sIPSCs in 15 neurons. D, Group data showing that 25 nM 4-DAMP abolished the remaining effect of 3 μM oxotremorine-M on sIPSCs in the presence of 1 μM himbacine (n = 6). Data presented as means ± S.E.M. *, P < 0.05 compared with control.

Figure 7. Effect of MT-3 toxin, himbacine, and 4-DAMP on the stimulatory action of oxotremorine-M on sIPSCs of lamina II neurons. A, Representative tracings of sIPSCs during control, application of 3 μM oxotremorine-M (Oxo), 100 nM MT-3 alone, and oxotremorine-M plus MT-3 toxin in one cell. B, Summary data showing the effect of 3 μM oxotremorine-M on sIPSCs before and after 100 nM MT-3 in 12 neurons. C, Group data showing the effect of oxotremorine-M on the frequency of sIPSCs before and after 100 nM MT-3 and 1 μM himbacine (Him) in 6 cells. D, Group data showing the effect of oxotremorine-M before and after perfusion of MT-3 toxin, himbacine, and 25 nM 4-DAMP in another 6 neurons. Data presented as means ± S.E.M. *, P < 0.05 compared with respective controls.
Fig. 1.

A

Control

Oxo 3 μM

Oxo 5 μM

Washout

Bicuculline

B

Cumulative Probability

Amplitude (pA)

50 pA

2 s

Cumulative probability

Inter-event Interval (ms)

C

Frequency (Hz)

control

Oxotremorine-M (μM)

*
A

Control

Oxo 3 μM

Atropine

Atropine + oxo

B

![Graph showing frequency (Hz) vs. time](image)

- **Initial Oxo**
- **Atropine + Oxo**

C

![Graph showing frequency (Hz) vs. concentration](image)

- **Oxo-1**
- **Oxo-2**

Fig. 2.
Fig. 3.
Fig. 4.
Fig. 5.
Fig. 6.
Fig. 7.