

Opposing Functions of Spinal M₂, M₃, and M₄ Receptor Subtypes in Regulation of GABAergic Inputs to Dorsal Horn Neurons Revealed by Muscarinic Receptor Knockout Mice

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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;

mAChRs: muscarinic acetylcholine receptors

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Abstract

Spinal muscarinic acetylcholine receptors (mAChRs) play an important role in regulation of nociception. To determine the role of individual mAChR subtypes in control of synaptic GABA release, spontaneous inhibitory postsynaptic currents (sIPSCs) and miniature IPSCs (mIPSCs) were recorded in lamina II neurons using whole-cell recordings in spinal cord slices of wild-type and mAChR subtype knockout (KO) mice. The mAChR agonist oxotremorine-M (3–10 μ M) dose-dependently decreased the frequency of GABAergic sIPSCs and mIPSCs in wild-type mice. However, in the presence of the M_2 and M_4 subtype-preferring antagonist himbacine, oxotremorine-M caused a large increase in the sIPSC frequency. In M_3 KO and M_1/M_3 double-KO mice, oxotremorine-M produced a consistent decrease in the frequency of sIPSCs, and this effect was abolished by himbacine. Surprisingly, in M_2/M_4 double-KO mice, oxotremorine-M consistently increased the frequency of sIPSCs and mIPSCs in all neurons tested, and this effect was completely abolished by 4-DAMP, an M_3 subtype-preferring antagonist. In M_2 or M_4 single-KO mice, oxotremorine-M produced a variable effect on sIPSCs; it increased the frequency of sIPSCs in some cells but decreased the sIPSC frequency in other neurons. Collectively, this study strongly suggests that activation of the M_3 subtype increases synaptic GABA release in the spinal dorsal horn of mice. In contrast, stimulation of presynaptic M_2 and M_4 subtypes predominantly attenuates GABAergic inputs to dorsal horn neurons in mice, an action that is opposite to the role of M_2 and M_4 subtypes in the spinal cord of rats.

Introduction

Muscarinic acetylcholine receptor (mAChRs) in the dorsal horn of the spinal cord regulate different physiological functions including nociception. Intrathecal administration of muscarinic agonists or acetylcholinesterase inhibitors produces a potent analgesic effect in many different 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, 2004). Molecular cloning studies have revealed five molecularly distinct mAChRs referred to as M₁-M₅ (Caulfield, 1993; Wess, 1996). The five mAChR subtypes are all linked to different types of G proteins. The odd-numbered muscarinic subtypes (M₁, M₃, and M₅) are selectively linked to G_{q/11} proteins, while the even-numbered subtypes (M₂ and M₄) are preferentially coupled to the pertussis toxin-sensitive G_{i/o} proteins (Felder, 1995; Wess, 1996; Caulfield and Birdsall, 1998). Receptor autoradiography and immunocytochemistry studies have shown that the highest density of muscarinic receptors in the spinal cord 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 muscarinic receptor subtypes are present in the spinal cord dorsal horn (Hoglund and Baghdoyan, 1997; Yung and Lo, 1997; Duttaroy et al., 2002; Chen et al., 2005). Although the role of the M₂ and M₄ receptor subtypes in the muscarinic agonist-induced analgesia has been established (Ellis et al., 1999; Gomeza et al., 1999; Duttaroy et al., 2002; Li et al., 2002), the mechanisms by which each mAChR subtype contributes to the muscarinic analgesia in the spinal cord are not well understood.

The spinal lamina II neurons are tonically controlled by glutamatergic excitatory and GABAergic/glycinergic inhibitory inputs. Glutamate released from primary afferent nerves is a major excitatory neurotransmitter, which conveys nociceptive information to the superficial lamina neurons (Yoshimura and Jessell, 1990). Our previous study in rats has shown that stimulation of mAChRs inhibits glutamate release, and inhibition of glutamate release is indirectly mediated by increased GABA release and activation of presynaptic GABA_B receptors (Li et al., 2002). Also, muscarinic agonists likely produce analgesia through inhibition of deep dorsal horn projection neurons of rats through GABA_B receptors in rats (Chen and Pan, 2004). Thus, stimulation of spinal GABA release and GABA_B receptors is considered an important mechanism by which muscarinic agonists exert their analgesic effects (Li et al., 2002; Chen and Pan, 2004).

Using a proper combination of mAChR subtype-preferring antagonists and the muscarinic toxin, we have recently found that the M₂, M₃, and M₄ receptor subtypes all contribute to potentiation of GABAergic tone in the spinal cord of rats (Zhang et al., 2005). However, the currently available 'selective' muscarinic antagonists are endowed with only a relatively small degree of subtype specificity (Wess, 1996; Caulfield and Birdsall, 1998). Therefore, it is essential to further substantiate the role of mAChR subtypes in regulation of GABAergic inputs to spinal dorsal horn neurons using other approaches, such as mAChR subtype knockout (KO) mice (Wess, 2004). In the present study, we determined the role of individual mAChR subtypes in regulation of synaptic GABA release to spinal lamina II neurons using mAChR KO mice. This study revealed an unexpected species difference in the specific role of individual mAChR subtypes in regulating GABAergic inputs to spinal dorsal horn neurons.

Materials and Methods

Animals. All wild-type and mAChR subtype single- and double-KO mice were obtained from University of Tokyo (M.M.) and the National Institute of Diabetes and Digestive and Kidney Diseases (J.W.). The generation and breeding of M₂ KO, M₄ KO, M₃ KO, M₁/M₃ double-KO, and M₂/M₄ double-KO mice have been described elsewhere (Gomez et al., 1999; Duttaroy et al., 2002; Matsui et al., 2002; Fukudome et al., 2004). All the KO mice of M.M. originated from 129/SvJ ES cells and were backcrossed onto the C57BL/6Jcl background (CLEA, Japan) for more than seven generations. Some of the M₃ KO and M₂/M₄ double KO mice were obtained from J.W. The M₃ KO mice of J.W. were backcrossed for ten generations onto the C57BL/6NTac background. The M₂/M₄ double-KO mice of J.W. were maintained on a 129J1/CF1 mixed genetic background (Duttaroy et al., 2002). Age- (5-7 weeks old) and sex-matched descendants derived from the wild-type mice resulting from intercrossing of these mutants were used in this study. Because the effects of oxotremorine-M on sIPSCs and mIPSCs were identical in the two strains of wild-type, M₃ KO, and M₂/M₄ double KO mice, the electrophysiology data from M₃ KO and M₂/M₄ double KO mice were pooled. Mouse genotyping was carried out by Southern blotting and polymerase chain reaction analysis of mouse-tail DNA, as described previously (Duttaroy et al., 2002; Fukudome et al., 2004). The experimental protocols and procedures were approved by the Animal Care and Use Committee of the Pennsylvania State University College of Medicine and conformed to the guidelines of the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

Spinal cord slice preparations. Mice were anesthetized with 2% halothane in O₂ and the lumbar segment of the spinal cord was rapidly removed through a limited laminectomy. Mice were then killed by inhalation of 5% halothane. The segment of the lumbar spinal cord was immediately placed in an ice-cold sucrose artificial cerebrospinal fluid (aCSF) pre-saturated 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. 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.

The lamina II has a distinct translucent appearance and can be identified easily under the microscope. In this study, neurons in the outer zone of lamina II were selected for recording (Li et al., 2002; Pan et al., 2002). The neurons located in the dorsal one-third of lamina II in the spinal slice were used for recording under a fixed stage microscope (BX51WI, Olympus, Tokyo, Japan) with differential interference contrast/infrared illumination. The electrode for the whole-cell recordings was pulled from borosilicate glass capillaries using 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; 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. 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 were performed using the whole-cell voltage-clamp method, as we described previously (Li et al., 2002; Zhang et al., 2005). 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%. 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 2 μ M strychnine, a glycine receptor antagonist, and 20 μ M 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX), a specific glutamate non-NMDA antagonist. To record the miniature inhibitory postsynaptic currents (mIPSCs), 1 μ M tetrodotoxin (TTX) was added in the perfusion solution.

Oxotremorine-M, 4-diphenylacetoxy-N-methylpiperidine methiodide (4-DAMP), GDP- β -S, atropine, himbacine, strychnine, CNQX, and bicuculline were obtained from Sigma (St. Louis, MO). TTX and QX314 were obtained from Alomone Labs (Jerusalem, Israel). Drugs were dissolved in Krebs solution and perfused into the tissue chamber using syringe pumps.

Data analysis. Data are presented as means \pm SEM. 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 over a period of at least 1 min during control, drug application, and recovery. For each analysis, at least 300 events were included. The sIPSCs and mIPSCs were detected by the fast rise time of the signal over an amplitude threshold above the background noise. Neurons were considered to be responsive to oxotremorine-M if the frequency or amplitude of sIPSCs and mIPSCs was altered > 20%. The cumulative probability of the amplitude and inter-event interval of sIPSCs and mIPSCs was compared using the Komogorov-Smirnov test, which estimates the probability that two cumulative distributions are similar. The effects of oxotremorine-M on the frequency and amplitude of sIPSCs and mIPSCs were determined by paired two-tailed Student's *t*-test or repeated measures ANOVA. $P < 0.05$ was considered to be statistically significant.

Results

All lamina II neurons tested exhibited spontaneous IPSCs at a holding potential of 0 mV. There were no significant differences in the input resistance and the baseline frequency and amplitude of sIPSCs in different types of mAChR KO and wild-type mice (Table 1).

Effect of oxotremorine-M on sIPSCs and mIPSCs in wild-type mice. To determine the concentration-dependent effect of oxotremorine-M on sIPSCs in spinal cord lamina II neurons of wild-type mice, 1, 3, 5, and 10 μ M oxotremorine-M, a non-selective agonist for all mAChR subtypes, was perfused to the tissue chamber. Each concentration was applied for a duration of 3 min. In a total of 15 lamina II neurons, 1 μ M oxotremorine-M had no significant effect on the frequency and amplitude of sIPSCs. Oxotremorine-M from 3-10 μ M significantly decreased the frequency of sIPSCs in 12 of 15 (80%) neurons in a concentration-dependent manner (Fig. 1, A, B, and C). Oxotremorine-M decreased the amplitude of sIPSCs in 7 of the above 12 (58.3%) cells (from 15.76 ± 3.78 to 10.11 ± 1.37 pA at the concentration of 5 μ M, $P < 0.05$), but did not significantly alter the IPSC amplitude in the remaining 5 cells (from 12.46 ± 1.12 to 11.44 ± 0.96 pA at the concentration of 5 μ M). Oxotremorine-M had no significant effect on sIPSCs in the remaining 3 (20%) neurons. The sIPSCs were completely eliminated by 10 μ M bicuculline, a specific GABA_A receptor antagonist (Fig. 1A).

In another 17 lamina II neurons, the effect of 5 μ M oxotremorine-M on mIPSCs was tested. Oxotremorine-M significantly decreased the frequency but not the amplitude of mIPSCs in 14 of 17 cells tested (Fig. 1D). Oxotremorine-M had no significant effect on the frequency (from 0.91 ± 0.18 to 0.88 ± 0.14 Hz) and amplitude (from 16.81 ± 1.65 to 15.80 ± 1.18 pA) of

mIPSCs in the remaining 3 cells.

In 11 additional lamina II neurons, we tested the effect of the M₂ and M₄ subtype-preferring antagonist himbacine (Dorje et al., 1991; Miller et al., 1992; Doller et al., 1999) on oxotremorine-M-induced decreases in the frequency of sIPSCs in wild-type mice. In all 11 cells tested, 3 μ M oxotremorine-M initially decreased the frequency of sIPSCs (Fig. 1E). Interestingly, oxotremorine-M induced a large increase in the frequency of sIPSCs in the presence of 2 μ M himbacine (Fig. 1E).

Effect of oxotremorine-M on sIPSCs and mIPSCs in M₁/M₃ double-KO and M₃ single-KO mice. To assess the role of the M₂ and M₄ mAChR subtypes in the inhibitory effect of oxotremorine-M on synaptic GABA release, we tested the effect of oxotremorine-M on sIPSCs in M₁/M₃ double-KO and M₃ single-KO mice. In M₁/M₃ double-KO mice, 3-10 μ M oxotremorine-M significantly decreased the frequency of sIPSCs in a concentration-dependent manner in all 12 cells tested (Fig. 2, A). Oxotremorine-M decreased the amplitude of sIPSCs in 6 of 12 cells (from 23.99 ± 4.45 to 16.32 ± 3.88 pA at the concentration of 5 μ M, $P < 0.05$), but did not significantly alter the IPSC amplitude in the remaining 6 cells (from 16.68 ± 3.69 to 16.05 ± 4.19 pA at the concentration of 5 μ M, $P > 0.05$).

Additionally, we tested the effect of himbacine on oxotremorine-M-induced decreases in the frequency of sIPSCs in M₁/M₃ double-KO mice. In 6 cells tested, 3 μ M oxotremorine-M initially decreased the frequency of sIPSCs (Fig. 2B). In the presence of 2 μ M himbacine, oxotremorine-M had no significant effect on the frequency of sIPSCs (Fig. 2B).

Previous pharmacological studies have suggested the presence of the M₁ subtype in the spinal dorsal horn (Iwamoto and Marion, 1993; Naguib and Yaksh, 1997). Thus, both M₃ KO

and M₁/M₃ double-KO mice were used to determine if the M₁ subtype plays a role in the effect of oxotremorine-M on synaptic GABA release in the spinal cord. In M₃ single-KO mice, oxotremorine-M also decreased the frequency of sIPSCs in a concentration-dependent manner in all 15 cells tested (Fig. 2C). Oxotremorine-M decreased the amplitude of sIPSCs in 6 of 15 cells (from 22.68 ± 3.40 to 14.91 ± 2.52 pA at the concentration of 5 μ M, $P < 0.05$), but had no effect on another 9 cells (from 10.98 ± 1.65 to 9.82 ± 1.58 pA at the concentration of 5 μ M, $P > 0.05$). Application of 5 μ M oxotremorine-M significantly decreased the frequency (from 1.15 ± 0.27 to 0.51 ± 0.11 Hz) but not the amplitude (11.82 ± 0.53 versus 11.28 ± 0.45 pA) of mIPSCs in 11 of 13 cells. Oxotremorine-M had no significant effect on the frequency (from 1.22 to 1.20 Hz in one cell and from 1.26 to 1.31 Hz in another cell) of mIPSCs in the remaining 2 cells in M₃ single-KO mice.

Effect of oxotremorine-M on sIPSCs and mIPSCs in M₂/M₄ double-KO mice. To determine the role of the M₃ subtype in muscarinic regulation of synaptic GABA release to lamina II neurons, we then examined the effects of oxotremorine-M on the sIPSCs and mIPSCs in M₂/M₄ double-KO mice. Strikingly, oxotremorine-M (3-10 μ M) significantly increased the frequency of sIPSCs in a concentration-dependent fashion in 15 of 17 (88%) neurons tested (Fig. 3, A, B, and C). Oxotremorine-M had no effect on the frequency of sIPSCs in the remaining 2 of 17 neurons (from 1.06 Hz to 1.16 Hz and from 1.83 to 1.95 Hz at concentration of 5 μ M). Oxotremorine-M also increased the amplitude of sIPSCs in 5 of 17 cells (from 17.62 ± 2.63 to 29.40 ± 6.71 Hz at the concentration of 5 μ M, $P < 0.05$), but had no significant effect on the amplitude of sIPSCs in the remaining 12 cells (from 23.18 ± 2.78 to 22.12 ± 2.65 Hz at the concentration of 5 μ M, $P > 0.05$). The sIPSCs were completely abolished by bath application of

10 μ M bicuculline.

To determine if the M_3 subtype mediates the stimulating effect of oxotremorine-M on synaptic GABA release in M_2/M_4 double-KO mice, we further tested the effect of 3 μ M oxotremorine-M on sIPSCs in the presence of 25 nM 4-DAMP, an M_3 subtype-preferring muscarinic antagonist (Ehlert, 1996; Yigit et al., 2003; Zhang et al., 2005). The effect of 3 μ M oxotremorine-M on sIPSCs was completely blocked by subsequent application of 25 nM 4-DAMP in all 6 cells examined (Fig. 3D).

In another 15 lamina II neurons from M_2/M_4 double-KO mice, 3 μ M oxotremorine-M significantly increased the frequency but not the amplitude of mIPSCs in 13 neurons ($P < 0.05$, Fig. 4, A-C). This effect was also blocked by 25 nM 4-DAMP in all 8 cells tested (Fig. 4D). Oxotremorine-M had no effect on the frequency (from 0.74 to 0.78 Hz and from 1.88 to 2.01 Hz) and amplitude (from 12.56 to 12.28 pA and from 27.16 to 30.94 pA) of mIPSCs in the remaining 2 cells.

Effect of oxotremorine-M on sIPSCs in M_2 single-KO and M_4 single-KO mice. To further delineate the role of the M_2 and M_4 receptor subtypes in the inhibitory action of oxotremorine-M on sIPSCs, the effect of oxotremorine-M on sIPSCs was tested in M_2 single-KO and M_4 single-KO mice, respectively. In M_2 single-KO mice, 3-10 μ M oxotremorine-M significantly increased the frequency of sIPSCs in a concentration-dependent manner in 16 of 24 (66.6%) neurons (Fig. 5, A-C). In contrast, oxotremorine-M had no significant effect on the frequency of sIPSCs in 4 of 24 (16.6%) neurons and significantly decreased the frequency of sIPSCs in 4 of 24 (16.6%) neurons. Oxotremorine-M increased the amplitude in 4 of the 24 cells (Fig. 5C), but had no significant effect on the amplitude of sIPSCs in 18 cells and even decreased

the IPSC amplitude of 2 cells.

On the other hand, in M₄ single-KO mice, 3-10 μ M oxotremorine-M significantly increased the frequency of sIPSCs in only 4 of 17 (23.5%) neurons (Fig. 6). Oxotremorine-M had no significant effect on the frequency and amplitude of sIPSCs in 3 of 17 (17.7%, Fig. 6) neurons and significantly decreased the frequency and amplitude of sIPSCs in the remaining 10 of 17 neurons (58.8%, Fig. 6B).

Discussion

In this study, we used mAChR KO mice to define the role of individual mAChR subtypes in regulation of GABAergic inputs to spinal dorsal horn neurons. We found, unexpectedly, that oxotremorine-M decreased the frequency of GABAergic sIPSCs and mIPSCs in wild-type mice. Further studies showed that in M₁/M₃ double-KO and M₃ single-KO mice, oxotremorine-M significantly decreased the frequency of sIPSCs and mIPSCs. Strikingly, oxotremorine-M significantly increased the frequency of sIPSCs and mIPSCs in M₂/M₄ double-KO mice. These results indicate that the M₂ and M₄ subtypes are responsible for the muscarinic inhibition of spinal GABA release in mice. On the other hand, stimulation of the M₃ subtype is predicted to potentiate GABAergic tone in the spinal cord dorsal horn of mice. Therefore, this study provides new information that activation of the M₂ and M₄ subtypes inhibits GABAergic inputs to spinal dorsal horn neurons in mice, which is opposite to the functional role of M₂ and M₄ mAChRs in the control of spinal GABA release in rats (Zhang et al., 2005). Nevertheless, stimulation of the M₃ subtype potentiates synaptic GABA release in the spinal dorsal horn in both mice and rats.

The mAChRs in the dorsal horn of the spinal cord are important for regulation of nociception. In this regard, intrathecal administration of muscarinic agonists or acetylcholinesterase inhibitors produces a potent analgesic effect in many different 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, 2004). Three mAChR subtypes, M₂, M₃, and M₄ receptors, are present in the spinal cord dorsal horn, and the M₂ and M₃ subtypes are particularly concentrated in the superficial laminae of the spinal cord (Hoglund and

Baghdoyan, 1997; Yung and Lo, 1997; Li et al., 2002). Due to lack of highly specific agents for the individual mAChR subtypes, the receptor subtypes that mediate the analgesic effect of muscarinic agonists were not established until recently. The role of the M₂ and M₄ receptor subtypes in mediating the analgesic effect of mAChR agonists has been documented using M₂ KO and M₂/M₄ double-KO mice (Gomez et al., 1999; Duttaroy et al., 2002). The frequency of sIPSCs and mIPSCs is the most important measure of synaptic GABA release in this study. The change in the sIPSC amplitude by oxotremorine-M may reflect various pools of GABA-containing vesicles released from GABAergic terminals. We found in this study that oxotremorine-M inhibited sIPSCs and mIPSCs of dorsal horn neurons in wild-type mice, which is in contrast to the potentiating effect of oxotremorine-M on GABAergic IPSCs of dorsal horn neurons in rats (Zhang et al., 2005). In M₃ single-KO and M₁/M₃ double-KO mice, oxotremorine-M consistently decreased the frequency of sIPSCs. Oxotremorine-M also significantly decreased the frequency, but not the amplitude, of mIPSCs in M₃ KO mice, suggesting that the M₂ and M₄ subtypes are present on the presynaptic terminals of GABAergic interneurons in the mouse spinal cord. The role of M₂ and M₄ subtypes in inhibition of synaptic GABA release in the mouse spinal dorsal horn is further supported by our finding that the M₂ and M₄ subtype-preferring antagonist himbacine reversed the inhibitory effect of oxotremorine-M on sIPSCs in wild-type mice and abolished the inhibitory effect of oxotremorine-M on sIPSCs in M₁/M₃ double-KO mice. Because oxotremorine-M consistently decreased the frequency of GABAergic sIPSCs and mIPSCs of dorsal horn neurons in wild-type mice, M₂/M₄ subtypes play a critical role in muscarinic inhibition of synaptic GABA release in the spinal dorsal horn of mice.

Contrary to the inhibitory effect of oxotremorine-M on IPSCs in wild-type and M₃ KO

mice, oxotremorine-M consistently increased the frequency of GABAergic sIPSCs in M₂/M₄ double-KO mice. Furthermore, the M₃ subtype-preferring muscarinic antagonist, 4-DAMP, completely blocked the effect of oxotremorine-M on sIPSCs in M₂/M₄ double-KO mice. These data strongly suggest that activation of the M₃ subtype potentiates synaptic GABA release in the spinal dorsal horn of mice. Interestingly, in wild-type mice, the role of the M₃ subtype in muscarinic modulation of GABA release appears to be overwhelmed by the dominant action of the M₂/M₄ subtypes in the spinal dorsal horn of mice. This may explain why the potential role of the M₃ subtype in muscarinic potentiation of spinal GABA release is only revealed by the use of M₂/M₄ double-KO mice and himbacine. Because oxotremorine-M significantly increased the frequency but not amplitude of mIPSCs in M₂/M₄ double-KO mice, the M₃ subtype is also likely located on the presynaptic terminals of GABAergic interneurons in the mouse spinal cord. The presence of the M₃ receptor subtype in the rat spinal cord has been suggested in a radioligand binding study (Hoglund and Baghdoyan, 1997). We have shown that the M₃ subtype plays a role in potentiation of spinal GABA release by oxotremorine-M in rats (Zhang et al., 2005). However, the physiological function of the M₃ subtype in the spinal cord remains largely unknown, and its functional role in the spinal analgesic effect of muscarinic receptor agonists needs to be further defined in M₃ KO mice. Although previous pharmacological studies have suggested the presence of the M₁ subtype in the spinal dorsal horn (Iwamoto and Marion, 1993; Naguib and Yaksh, 1997), we found no difference in the inhibitory effect of oxotremorine-M on sIPSCs between the M₃ single-KO and M₁/M₃ double-KO mice. Hence, these data suggest that the M₁ subtype is not involved in muscarinic regulation of synaptic GABA release to spinal dorsal horn neurons.

The variable effect of oxotremorine-M on sIPSCs in the M₂ and M₄ single-KO mice is

most likely due to the fact that the recorded neurons receive different afferent inputs with distinct presynaptic mAChR subtypes. It is unlikely that this variability can be explained by the presence of different types of lamina II neurons since the effect of oxotremorine-M on sIPSCs is consistent in M₁/M₃ double-KO, M₃ single-KO, and M₂/M₄ double-KO mice. This notion is further supported by our finding that following blockade of the M₂ and M₄ subtypes with himbacine, oxotremorine-M consistently increased (but not decreased) the frequency of sIPSCs in wild-type mice. Because of the opposing functions of the M₃ and M₂/M₄ subtypes in regulating spinal GABA release in mice (increase versus decrease in GABA release, respectively), the net effect of oxotremorine-M on synaptic GABA release depends on the distribution and the relative density of the M₃ and M₂/M₄ subtypes on the nerve terminals that synapse with the recorded neuron. We observed that oxotremorine-M increased the frequency of sIPSCs in most (66.6%) lamina II neurons but decreased the IPSC frequency in some (16.6%) cells in M₂ single-KO mice. These results suggest that there may be more M₃ than M₄ mAChRs on the presynaptic terminals of GABAergic neurons in the mouse spinal dorsal horn. On the other hand, oxotremorine-M can stimulate both M₂ (to decrease GABA release) and M₃ (to increase GABA release) subtypes in M₄ single-KO mice. We found that oxotremorine-M decreased the frequency of sIPSCs in 58.8% cells while it increased the IPSC frequency in 23.5% of the dorsal horn neurons tested in M₄ single-KO mice. These data suggest that the M₂ subtype plays a greater role than the M₃ subtype in muscarinic regulation of spinal GABA release. Therefore, these mAChR subtypes contribute to a different extent (i.e., M₂ > M₃ > M₄) to the muscarinic modulation of spinal GABA release in mice. These electrophysiological data are consistent with the receptor binding and behavioral studies showing that M₂ is the predominant mAChR subtype in the mouse spinal cord (Gomez et

al., 1999; Duttaroy et al., 2002; Chen et al., 2005; Oki et al., 2005).

GABA is the most important inhibitory neurotransmitter in the central nervous system. Stimulation of spinal GABA release is an important mechanism by which muscarinic agonists produce their analgesic effects in the spinal dorsal horn of rats (Li et al., 2002; Chen and Pan, 2004). Nevertheless, we found that oxotremorine-M inhibits synaptic GABA release to dorsal horn neurons through activation of M₂ and M₄ mAChRs in wild-type mice. Although it is uncertain how this effect (disinhibition) contributes to the mechanisms of spinal muscarinic analgesia in mice, it is possible that the location of mAChRs and spinal dorsal horn circuitry are organized differently in rats than in mice. As illustrated in Fig. 7, the mAChR agonists may inhibit dorsal horn projection neurons through at least two indirect mechanisms in mice. Assuming that the recorded lamina II neuron is an inhibitory interneuron (presumably GABAergic neurons), decreased GABA release following activation of the M₂ and M₄ subtypes could increase the excitability of the inhibitory interneuron. Consequently, it can potentiate the release of inhibitory neurotransmitters to dorsal horn projection neurons resulting in inhibition of nociceptive transmission. Alternatively, if the recorded lamina II neuron is an excitatory interneuron (supposedly glutamatergic, Fig. 7), decreased synaptic GABA release by mAChR agonists could increase the excitability of the excitatory interneuron. As a result, the increased synaptic glutamate release to the inhibitory interneuron that synapses directly with the dorsal horn projection neuron could lead to increased inhibitory tone to the dorsal horn projection neuron and suppression of nociceptive transmission in the mouse spinal cord. Our findings indicate that the pharmacological mechanisms through which mAChR subtypes regulate synaptic transmission and nociception at the spinal cord level are likely complex. For example, glutamate released from

primary afferents to spinal dorsal horn neurons is important for transmission of nociceptive information (Yoshimura and Jessell, 1990; Li et al., 2002). The M₂ and M₄ subtypes may also be involved in inhibition of glutamate release from primary afferent nerves to dorsal horn neurons (Li et al., 2002). The specific role of mAChR subtypes in regulating spinal glutamate release has not been examined in mice and will be determined in future studies.

In summary, the most salient finding of the present study is that activation of M₂ and M₄ mAChR subtypes in the mouse spinal cord inhibits GABA release (Fig. 8), which is opposite to the potentiating effect of the M₂ and M₄ subtypes on spinal GABAergic tone in rats (Zhang et al., 2005). Furthermore, unlike the rat spinal cord in which the M₂, M₃, and M₄ mAChRs are located on somatodendritic sites of GABAergic interneurons (Zhang et al., 2005), the mAChR subtypes (M₂, M₃, and M₄) appear located on the presynaptic terminals of GABAergic interneurons in the spinal cord of mice (Fig. 8). The different subcellular location of the M₂/M₄ subtypes may be the basis for distinct functions and signaling mechanisms of these mAChR subtypes in regulating GABAergic synaptic transmission in the spinal dorsal horn of rats and mice. A species difference in the mAChR subtype signaling between rats and mice has been reported in the superior cervical ganglionic neurons (Shapiro et al., 1999; Shapiro et al., 2001). In this regard, the fast inhibition of N- and P/Q-type Ca²⁺ channels is mediated by the M₄ subtype in the rat but by the M₂ subtype in the mouse (Shapiro et al., 1999; Shapiro et al., 2001). The present study provides important new information about the species difference in regulation of GABAergic transmission by mAChR subtypes and the potential mechanisms of muscarinic analgesia in the spinal cord.

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Footnotes

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Figure legends

Figure 1. Effect of oxotremorine-M on sIPSCs and mIPSCs of spinal lamina II neurons in wild-type mice. A, Original tracings of sIPSCs during control, application of 3 and 5 μ M oxotremorine-M (Oxo), and washout in one lamina II cell. Note that the sIPSCs were abolished by 20 μ M bicuculline. B, Cumulative plot of sIPSCs of the same neuron in A showing the distribution of the inter-event interval and amplitude during control, application of 5 μ M oxotremorine-M, and washout. C, Summary data showing that the effect of oxotremorine-M on the sIPSCs in 12 cells in wild-type mice. D, Summary data showing the effect of oxotremorine-M on the frequency of mIPSCs in wild-type mice ($n = 14$). E, Group data showing the effect of 3 μ M oxotremorine-M on sIPSCs before and during application of 2 μ M himbacine (Him) in 11 lamina II neurons. Data presented as means \pm S.E.M. *, $P < 0.05$ compared with the control.

Figure 2. Effect of oxotremorine-M on sIPSCs in M_1/M_3 double-KO and M_3 single-KO mice. A, Summary data showing the dose-response effect of oxotremorine-M on the frequency of sIPSCs in M_1/M_3 double-KO mice. B, Group data showing the effect of 3 μ M oxotremorine-M on sIPSCs before and during application of 2 μ M himbacine (Him) in 6 lamina II neurons from M_1/M_3 double-KO mice. C, Summary data showing the dose-response effect of oxotremorine-M on the frequency of sIPSCs in M_3 single-KO mice. Data presented as means \pm S.E.M. *, $P < 0.05$ compared with respective controls.

Figure 3. Effect of oxotremorine-M on sIPSCs in M₂/M₄ double-KO mice. A, Raw tracings of sIPSCs during control, application of 1, 3, and 5 μ M oxotremorine-M (Oxo), and washout in one cell. Note that the sIPSCs were abolished by 20 μ M bicuculline. 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 5 μ M oxotremorine-M, and washout. C, Summary data showing the dose-response effect of oxotremorine-M on sIPSCs in 15 cells. D, Effect of 3 μ M oxotremorine-M on the frequency of sIPSCs in 6 cells before and after application of 25 nM 4-DAMP. Data presented as means \pm S.E.M. *, $P < 0.05$ compared with control.

Figure 4. Effect of oxotremorine-M (Oxo) on mIPSCs in M₂/M₄ double-KO mice. A, Representative tracings of mIPSCs during control, application of 3 μ M oxotremorine-M (Oxo), and washout in one cell. B, Cumulative probability plot of mIPSCs of the same neuron in A showing the distribution of the inter-event interval and amplitude of mIPSCs during control, during application of 3 μ M oxotremorine-M, and washout. C, Effect of 3 μ M oxotremorine-M on the frequency of mIPSCs in 13 cells from M₂/M₄ double-KO mice. D, Effect of 3 μ M oxotremorine-M on the frequency of mIPSCs before and after application of 25 nM 4-DAMP. These 8 neurons were part of 13 cells shown in C. Data presented as means \pm S.E.M. *, $P < 0.05$ compared with the control.

Figure 5. Effect of oxotremorine-M on the frequency of sIPSCs in M₂ KO mice. A, Original tracings of sIPSCs during control, application of 3 and 5 μ M oxotremorine-M (Oxo), and washout in one lamina II neuron. B, Cumulative probability plot of sIPSCs of the same neuron in A showing the distributions of the inter-event interval and amplitude during control, application of 5

μ M oxotremorine-M, and washout. C, Group data showing differential effects of 5 μ M oxotremorine-M on the frequency of sIPSCs in 24 cells. Data presented as means \pm S.E.M. *, $P < 0.05$ compared with control.

Figure 6. Effect of oxotremorine-M on the frequency of sIPSCs in M_4 KO mice. A, Representative tracings of sIPSCs during control, application of 5 μ M oxotremorine-M (Oxo), and washout in one cell. B, Group data showing differential effects of oxotremorine-M on the frequency of sIPSCs increased in 17 cells. Data presented as means \pm S.E.M. *, $P < 0.05$ compared with respective controls.

Figure 7. Diagram illustrating two possible mechanisms of inhibition of dorsal horn projection neurons due to decreased synaptic GABA release to lamina II interneurons by activation of M_2 and M_4 mAChR subtypes in the mouse spinal cord. See Discussion for details.

Figure 8. Schematic drawing highlighting the major differences in the subcellular location and physiological function of M_2 , M_3 , and M_4 mAChR subtypes in control of synaptic GABA release to lamina II neurons in the spinal cord dorsal horn of mice (A) and rats (B). Note that M_2 , M_3 , and M_4 mAChR subtypes may be located on the same or different GABAergic neurons and terminals. AC, adenylyl cyclase; -, inhibition; +, potentiation; oxo, oxotremorine-M.

Table 1. Comparison of the input resistance, and the basal frequency and amplitude of sIPSCs in spinal lamina II neurons from the wild-type and different mAChR subtype KO mice.

	Input Resistance (MΩ)	Basal Frequency of sIPSCs (Hz)	Basal Amplitude of sIPSCs (pA)
Wild-type (n = 39)	468.1 ± 34.4	1.35 ± 0.33	18.97 ± 2.04
M ₁ /M ₃ KO (n = 18)	486.8 ± 58.2	1.48 ± 0.30	21.88 ± 3.21
M ₃ KO (n = 28)	457.8 ± 31.8	1.27 ± 0.27	22.37 ± 2.16
M ₂ /M ₄ KO (n = 34)	516.5 ± 28.6	1.59 ± 0.36	17.41 ± 1.08
M ₂ KO (n = 24)	496.9 ± 46.5	1.15 ± 0.27	14.82 ± 1.54
M ₄ KO (n = 17)	448.7 ± 47.1	1.24 ± 0.37	15.10 ± 1.28

A wild-type

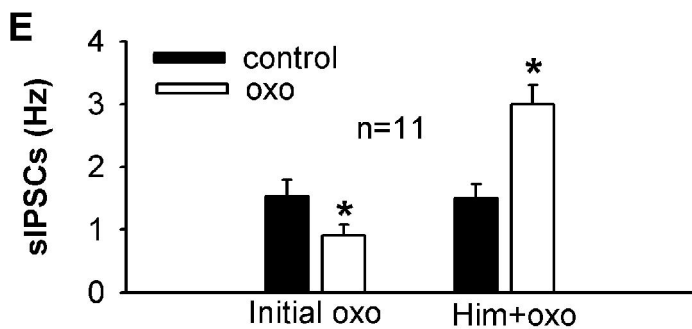
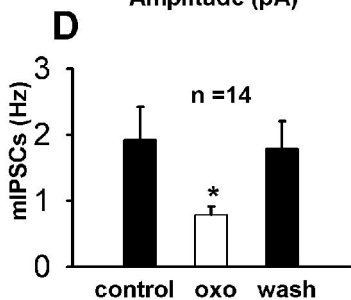
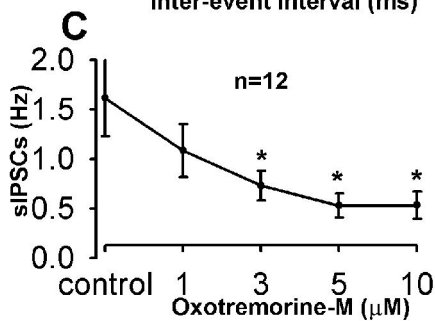
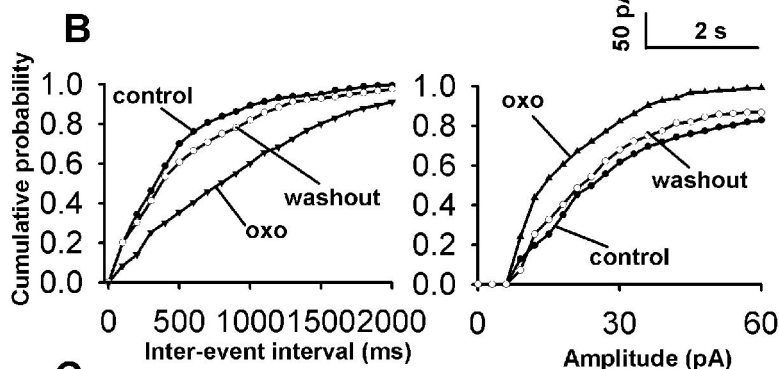


Fig1

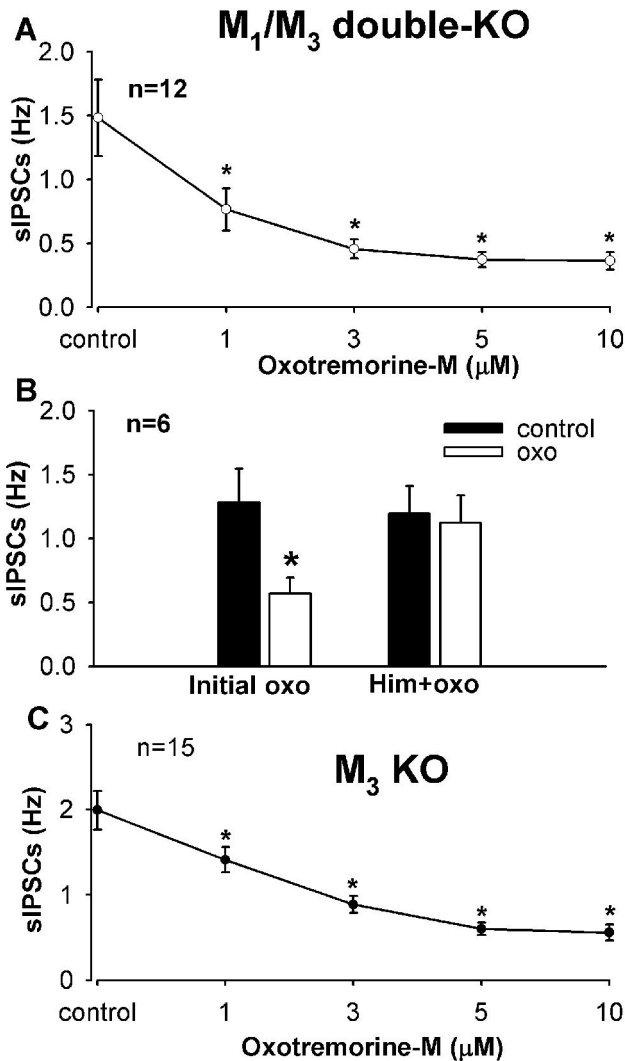


Fig2

M_2/M_4 double-KO

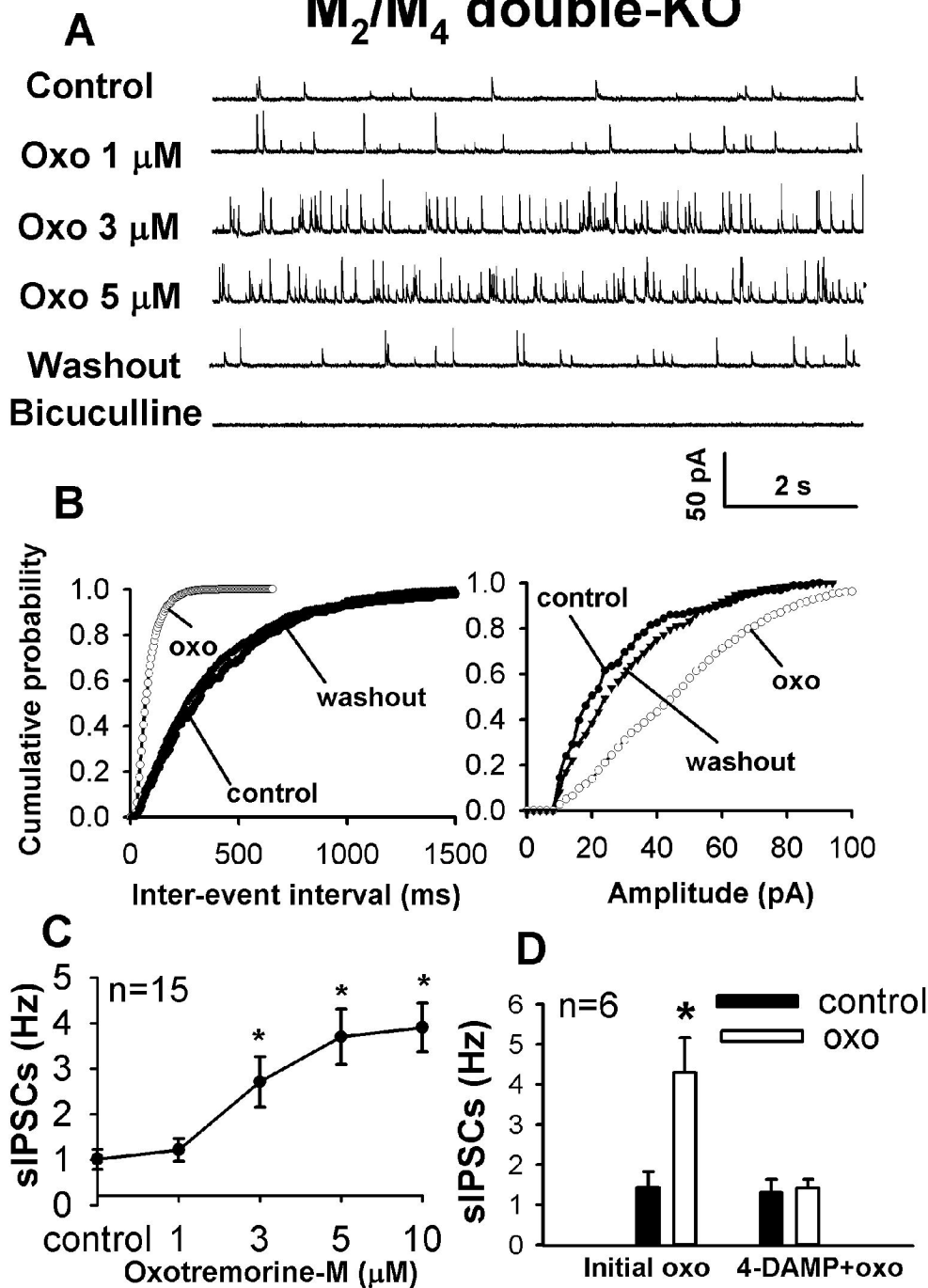


Fig.3

M_2/M_4 double-KO

A

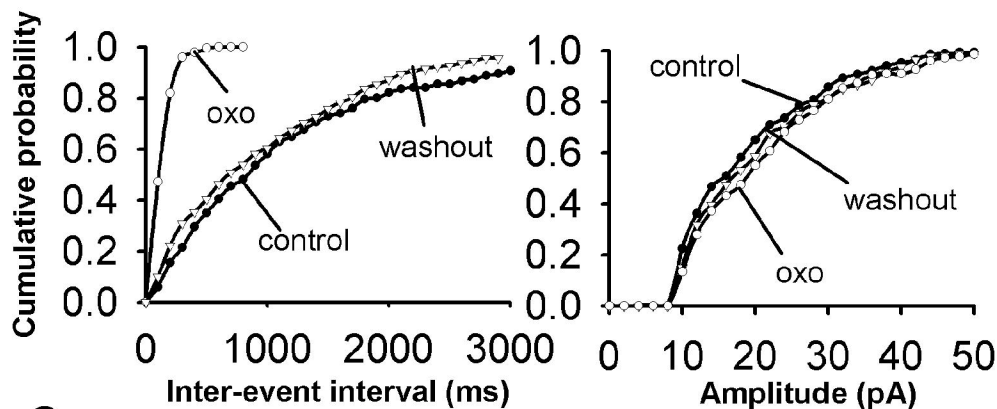
Control

Oxo 3 μ M

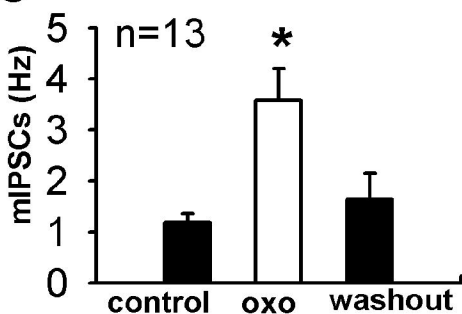
Washout

50 pA
2 s

B



C



D

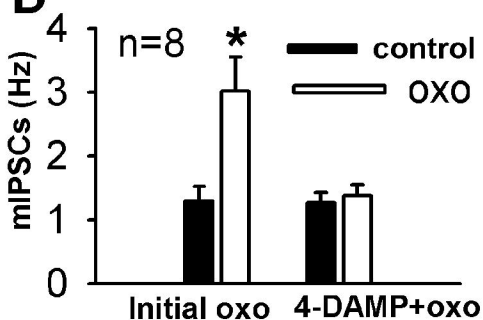
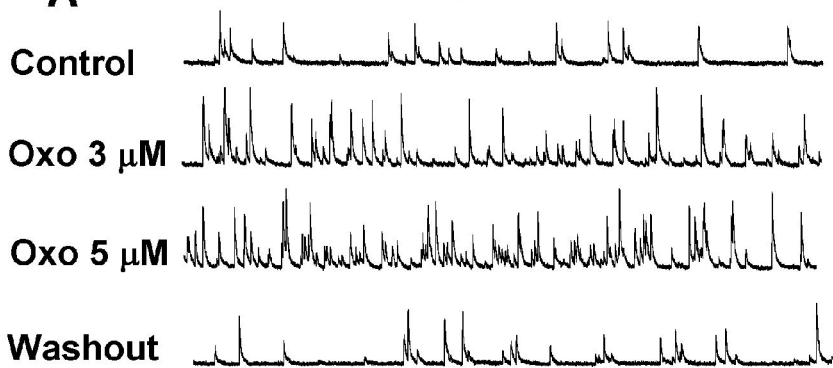


Fig4

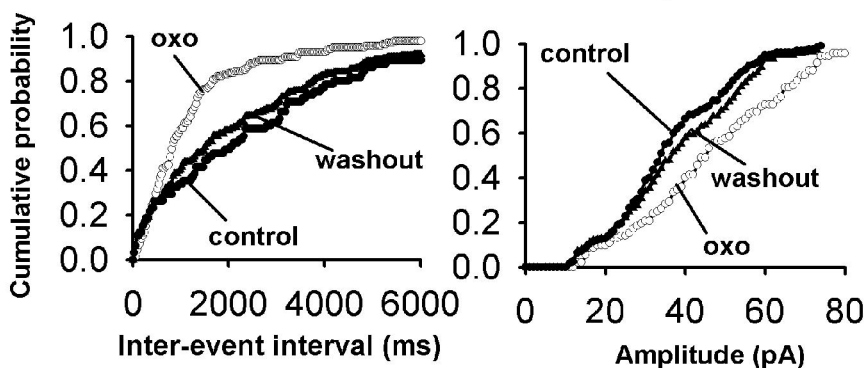
M₂ KO

A



100 pA
2 s

B



C

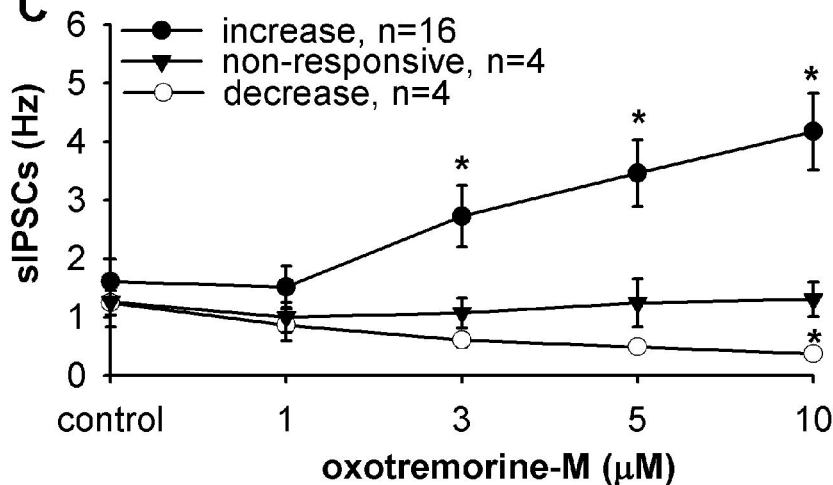


Fig5

M₄ KO

A

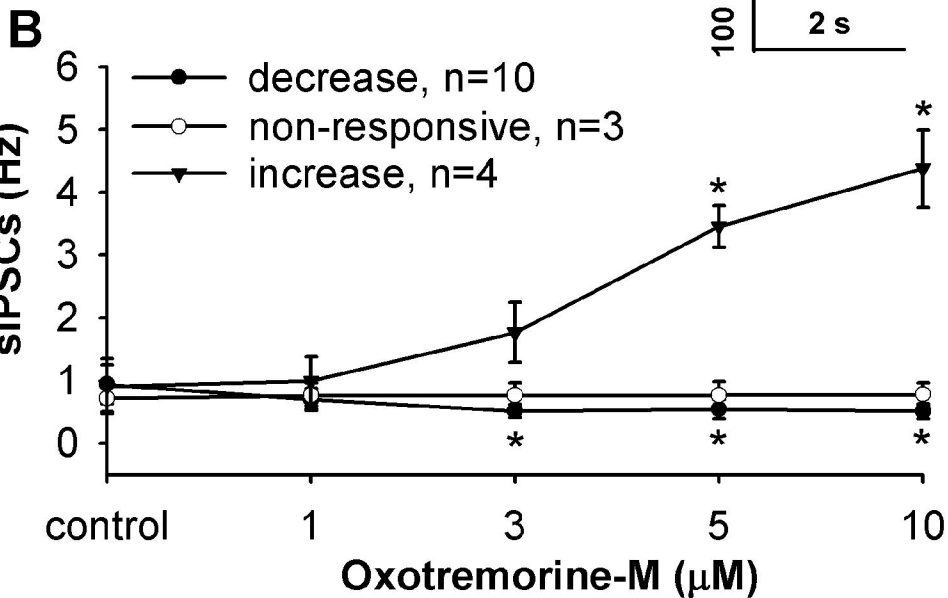
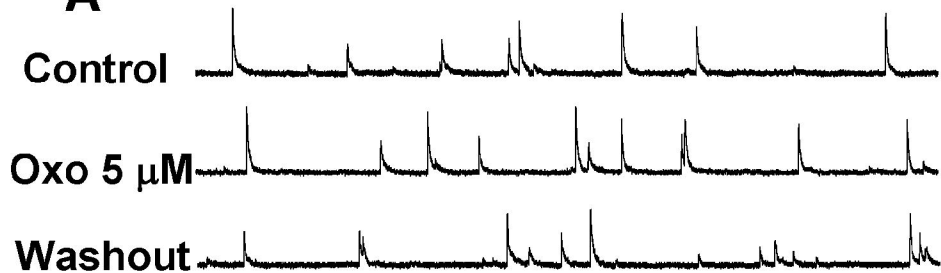


Fig6

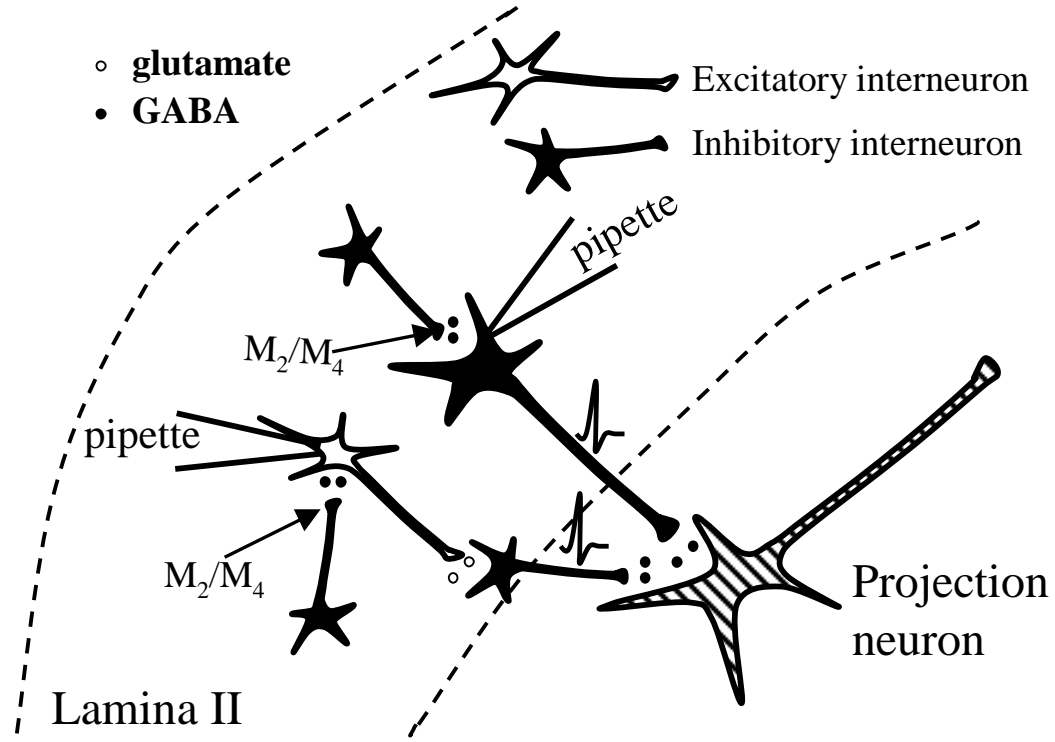


Fig. 7

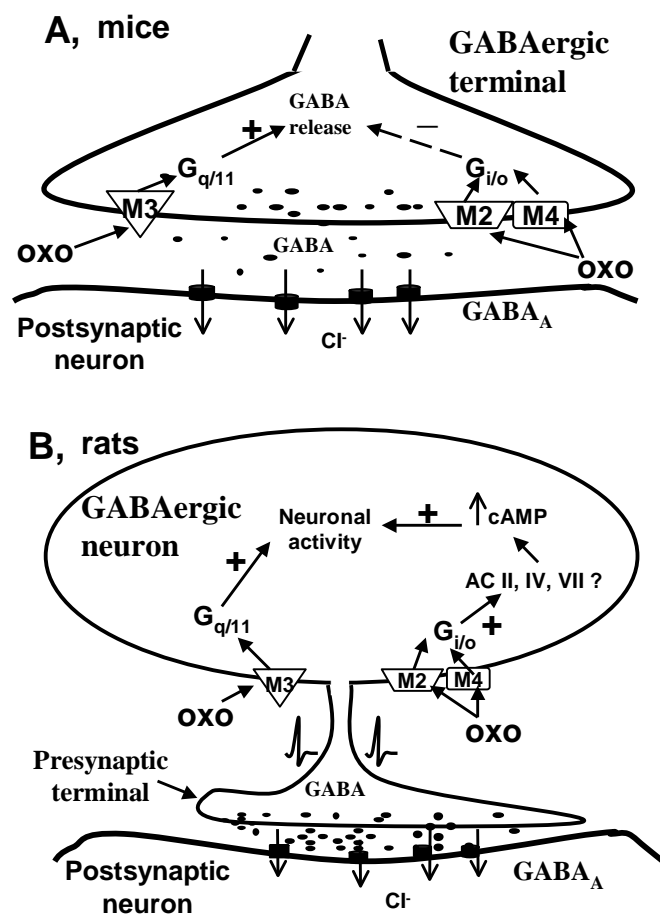


Fig.8