

# Dynamic Control of Glutamatergic Synaptic Input in the Spinal Cord by Muscarinic Receptor Subtypes Defined Using Knockout Mice\*

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Activation of muscarinic acetylcholine receptors (mAChRs) in the spinal cord inhibits pain transmission. At least three mAChR subtypes ( $M_2$ ,  $M_3$ , and  $M_4$ ) are present in the spinal dorsal horn. However, it is not clear how each mAChR subtype contributes to the regulation of glutamatergic input to dorsal horn neurons. We recorded spontaneous excitatory postsynaptic currents (sEPSCs) from lamina II neurons in spinal cord slices from wild-type (WT) and mAChR subtype knock-out (KO) mice. The mAChR agonist oxotremorine-M increased the frequency of glutamatergic sEPSCs in 68.2% neurons from WT mice and decreased the sEPSC frequency in 21.2% neurons. Oxotremorine-M also increased the sEPSC frequency in ~50% neurons from  $M_3$ -single KO and  $M_1/M_3$  double-KO mice. In addition, the  $M_3$  antagonist J104129 did not block the stimulatory effect of oxotremorine-M in the majority of neurons from WT mice. Strikingly, in  $M_5$ -single KO mice, oxotremorine-M increased sEPSCs in only 26.3% neurons, and J104129 abolished this effect. In  $M_2/M_4$  double-KO mice, but not  $M_2$ - or  $M_4$ -single KO mice, oxotremorine-M inhibited sEPSCs in significantly fewer neurons compared with WT mice, and blocking group II/III metabotropic glutamate receptors abolished this effect. The  $M_2/M_4$  antagonist himbacine either attenuated the inhibitory effect of oxotremorine-M or potentiated the stimulatory effect of oxotremorine-M in WT mice. Our study demonstrates that activation of the  $M_2$  and  $M_4$  receptor subtypes inhibits synaptic glutamate release to dorsal horn neurons.  $M_5$  is the predominant receptor subtype that potentiates glutamatergic synaptic transmission in the spinal cord.

The spinal cholinergic system and muscarinic acetylcholine receptors (mAChRs)<sup>2</sup> are important for the control of noci-

ceptive transmission. For example, neurons and nerve terminals expressing choline acetyltransferase and acetylcholinesterase (enzymes for acetylcholine synthesis and degradation, respectively) are located in the spinal dorsal horn (1, 2). The superficial laminae contain the highest density of mAChRs in the spinal dorsal horn (3–5). Stimulation of mAChRs attenuates the responses of dorsal horn neurons to noxious stimuli (6), whereas blocking spinal mAChRs with atropine causes a large increase in pain sensitivity (7). Furthermore, spinally administered mAChR agonists or acetylcholinesterase inhibitors produce potent analgesia in both animals and humans (8–11). Because agonists and antagonists that are highly selective for all mAChR subtypes are still lacking at this time, it is difficult to rely on pharmacological approaches alone to define which individual mAChR subtypes are involved in the regulation of synaptic and nociceptive transmission at the spinal level.

Molecular cloning studies have revealed the existence of five molecularly distinct mAChR subtypes ( $M_1$ – $M_5$ ) (12). The odd-numbered subtypes ( $M_1$ ,  $M_3$ , and  $M_5$ ) couple efficiently through the  $G_{q/11}$  class of G proteins to activate phospholipase C, which leads to inositol triphosphate-mediated calcium release from the endoplasmic reticulum and diacylglycerol-mediated activation of protein kinase C. The even-numbered mAChRs ( $M_2$  and  $M_4$ ) inhibit adenylyl cyclase activity through activation of the  $G_{i/o}$  class of G proteins (12, 13). In the spinal dorsal horn,  $M_2$  is the major mAChR subtype, and the  $M_3$  and  $M_4$  subtypes represent only a fraction of the total mAChRs at the spinal level (11, 14–16). Using mAChR subtype knock-out (KO) mice and an siRNA approach, we have shown that both the  $M_2$  and  $M_4$  subtypes mediate the analgesic effect of mAChR agonists in both rats and mice (11, 14, 17). In addition, using mAChR subtype-KO mice, we have demonstrated that the  $M_2$ ,  $M_3$ , and  $M_4$  subtypes are differentially involved in the control of GABAergic and glycinergic inhibitory synaptic transmission in the spinal dorsal horn (18, 19). Glutamate is the predominant excitatory neurotransmitter involved in nociceptive transmission in the spinal dorsal horn. However, it remains unclear how individ-

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<sup>2</sup> The abbreviations used are: mAChR, muscarinic cholinergic receptor; KO, knockout; aCSF, artificial cerebrospinal fluid; sEPSCs, spontaneous excitatory postsynaptic currents; mEPSCs, miniature excitatory postsynaptic currents; GDP- $\beta$ -S, guanosine 5'-O-(2-thiodiphosphate); CNQX, 6-cyano-7-nitroquinoxaline-2,3-dione; mGluRs, metabotropic glutamate receptors; J104129, ( $\alpha R$ )- $\alpha$ -cyclopentyl- $\alpha$ -hydroxy-N-[1-(4-methyl-3-pentenyl)-

4-piperidinyl]benzeneacetamide fumarate; TTX, tetrodotoxin; CPPG, (R,S)- $\alpha$ -cyclopropyl-4-phosphonopentanoic acid; LY341495, (2S)-2-amino-2-[(1S,2S)-2-carboxycycloprop-1-yl]-3-(xanth-9-yl) propanoic acid; Oxo, oxotremorine-M; HMB, himbacine.

ual mAChR subtypes contribute to the regulation of glutamatergic input to dorsal horn neurons.

Therefore, in this study, we recorded spontaneous excitatory postsynaptic currents (sEPSCs) from lamina II neurons in spinal cord slices from wild-type and mAChR subtype-KO mice to define the role of the individual mAChR subtypes in the regulation of synaptic glutamate release to the superficial dorsal horn neurons. In addition to providing unequivocal evidence showing differential regulation of glutamatergic input by the presynaptic  $M_2$ ,  $M_3$ , and  $M_4$  subtypes, we found, unexpectedly, that  $M_5$  is the predominant subtype that potentiates glutamatergic transmission in the spinal dorsal horn. Our study also revealed a reciprocal interaction between the  $M_2/M_4$  and  $M_3/M_5$  subtypes in the control of glutamatergic synaptic transmission in the spinal cord. Furthermore, we found that group II/III metabotropic glutamate receptors (mGluRs) are involved in the  $M_3/M_5$ -mediated feedback loop that regulates glutamatergic input in the spinal cord. This new information is important for our understanding of the complex interactions among mAChR subtypes in the dynamic control of glutamatergic transmission in the spinal cord. Clearly, our findings are crucial for the development of novel mAChR subtype-selective analgesic drugs endowed with increased efficacy and reduced side effects.

### EXPERIMENTAL PROCEDURES

**Animals**—All WT and mAChR subtype single and double KO mice (6–9 weeks old) used in this study were obtained from the National Institute of Diabetes and Digestive and Kidney Diseases (National Institutes of Health, Bethesda, MD). The genetic background of the  $M_2$ -KO,  $M_4$ -KO, and  $M_2/M_4$  double-KO mice was C57/BL6 and that of the  $M_3$ -KO,  $M_5$ -KO, and  $M_1/M_3$  double-KO mice was 129SvEv/CF1. The genetic background of the  $M_2$ -KO,  $M_4$ -KO, and  $M_2/M_4$  double-KO mice was C57/BL6. In studies using these mutant mice, C57/BL6 WT mice (Taconic) were used as controls. The  $M_3$ -KO,  $M_5$ -KO, and  $M_1/M_3$  double-KO mice were maintained on a mixed 129SvEv (50%)/CF1 (50%) background. In studies using these mutant mice, WT mice with the same mixed genetic background served as controls. The generation and breeding of the  $M_2$ -KO,  $M_3$ -KO,  $M_4$ -KO,  $M_5$ -KO,  $M_1/M_3$  double-KO, and  $M_2/M_4$  double-KO mice have been described previously (11, 17, 20–23). The mouse genotyping was carried out by Southern blotting and polymerase chain reaction analysis of mouse-tail DNA. The experimental protocols and procedures were approved by the Animal Care and Use Committee at The University of Texas MD Anderson Cancer Center and conformed to the National Institutes of Health guidelines for the ethical use of animals.

**Spinal Cord Slice Preparation**—Mice were anesthetized with 2% isoflurane, 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) presaturated with 95%  $O_2$  and 5%  $CO_2$ . The sucrose aCSF contained (in mM) 206 sucrose, 2.8 KCl, 1.0  $MgCl_2$ , 1.0  $CaCl_2$ , 1.2  $NaH_2PO_4$ , 25.0 glucose, and 26.0  $NaHCO_3$ . The tissue was then placed in a shallow groove

formed in a gelatin block and glued onto the stage of a vibratome. Transverse spinal cord slices (350  $\mu m$ ) were cut in the ice-cold sucrose aCSF and preincubated in Krebs' solution oxygenated with 95%  $O_2$  and 5%  $CO_2$  at 34°C for at least 1 h before they were transferred to the recording chamber. The Krebs' solution contained (in mM) 117.0 NaCl, 3.6 KCl, 1.2  $MgCl_2$ , 2.5  $CaCl_2$ , 1.2  $NaH_2PO_4$ , 11.0 glucose, and 25.0  $NaHCO_3$ . Each slice was placed in a glass-bottomed chamber and fixed with parallel nylon threads supported by a stainless steel weight. The slice was continuously perfused with Krebs' solution at 5.0 ml/min at 34°C, which was maintained by an inline solution heater and a temperature controller.

**Electrophysiological Recordings**—Recordings of postsynaptic currents were performed in lamina II neurons using the whole-cell voltage clamp method, as we described previously (18, 19, 24). The neurons located in the lamina II were identified under a fixed-stage microscope (BX51WI; Olympus, Tokyo, Japan). The electrode was pulled from borosilicate glass capillaries. Patch electrodes with a resistance of 5–10 M $\Omega$  were filled with an internal solution containing (in mM) 135.0 potassium gluconate, 5.0 KCl, 2.0  $MgCl_2$ , 0.5  $CaCl_2$ , 5.0 HEPES, 5.0 EGTA, 5.0 ATP-Mg, 0.5 Na-GTP, 1.0 GDP- $\beta$ -S, and 10.0 QX314, adjusted to pH 7.2–7.4 with 1 M KOH (290–300 mOsm). sEPSCs were recorded at a holding potential of –60 mV. To record the miniature EPSCs (mEPSCs), 0.5  $\mu M$  tetrodotoxin (TTX) was added to the perfusion solution. The input resistance was continuously monitored, and the recording was abandoned if it changed more than 15%. Signals were processed with an amplifier (MultiClamp 700A; Axon Instruments, Union City, CA), filtered at 1–2 kHz, digitized at 10 kHz, and stored in a computer with pCLAMP 9.0 (Axon Instruments).

Oxotremorine-M, himbacine, and GDP- $\beta$ -S were obtained from Sigma-Aldrich. TTX and QX314 were obtained from Alomone Labs (Jerusalem, Israel). (RS)- $\alpha$ -cyclopropyl-4-phosphonopentanoic acid (CPPG), (2S)-2-amino-2-[(1S,2S)-2-carboxycycloprop-1-yl]-3-(xanth-9-yl)propanoic acid (LY341495), and ( $\alpha$ R)- $\alpha$ -cyclopentyl- $\alpha$ -hydroxy-N-[1-(4-methyl-3-pentenyl)-4-piperidinyl]benzeneacetamide fumarate (J104129) were purchased from Tocris Cookson Inc. (Ellisville, MO). All the drugs were dissolved in Krebs' solution freshly and perfused using syringe pumps.

**Data Analysis**—Data are presented as means  $\pm$  S.E. The amplitudes and frequencies of sEPSCs and mEPSCs were analyzed off-line using a peak detection program (MiniAnalysis; Synaptosoft, Decatur, GA). The detection of events was accomplished by setting a threshold above the noise level. The sEPSCs and mEPSCs were detected by the fast rise time of the signal over an amplitude threshold (typically 6–10 pA) above the background noise. We manually excluded the event when the noise was erroneously identified as a sEPSC by the software program. The cumulative probability of the amplitude and inter-event interval of the sEPSCs and mEPSCs was compared using the Kolmogorov-Smirnov test, which estimates the probability that two distributions are similar. This test was used first to determine whether the effect of oxotremorine-M on the sEPSCs and mEPSCs was significantly different in individual neurons. The effect of oxotremorine-M on the

frequency and amplitude of sEPSCs and mEPSCs was determined by one-way ANOVA using Dunnett's or Tukey's post hoc test.  $p < 0.05$  was considered to be statistically significant.

## RESULTS

**Effects of Oxotremorine-M on sEPSCs and mEPSCs of Lamina II Neurons in WT Mice**—To determine the role of mAChRs in the control of synaptic glutamate release to lamina II neurons, we first examined the effect of oxotremorine-M, a specific agonist that stimulates all mAChR subtypes, on glutamatergic sEPSCs in WT mice. The effect of oxotremorine-M on glutamatergic sEPSCs was similar among the two groups of WT mice (C57/BL6 or 129SvEv/CF1 genetic background). Specifically, there were no significant differences in the proportion of neurons in which oxotremorine-M increased (70.5% versus 63.6%) or decreased (20.5% versus 22.7%) the frequency of sEPSCs between C57/BL6-WT mice and 129SvEv/CF1-WT mice. Therefore, the data obtained from the two groups of WT mice were pooled. To examine the concentration-dependent effect of oxotremorine-M, the drug was perfused in a cumulative fashion (1, 3, 5, and 10  $\mu\text{M}$ ; each concentration applied for 3 min) onto the slice chamber. Oxotremorine-M significantly increased the frequency, but not the amplitude, of the sEPSCs in 21 neurons in a concentration-dependent manner (Fig. 1, A–C). The cumulative probability analysis of glutamatergic sEPSCs revealed that the distribution pattern of the inter-event interval of sEPSCs was shifted toward the left in response to oxotremorine-M (Fig. 1B). In another 13 neurons, oxotremorine-M decreased the frequency, but not the amplitude, of the sEPSCs in a concentration-dependent fashion (Fig. 1D).

In a total of 66 neurons randomly recorded from WT mice, bath application of 3  $\mu\text{M}$  oxotremorine-M for 3 min significantly increased the frequency of sEPSCs in 45 (68.2%) neurons (Fig. 1E). In 14 of 66 (21.2%) neurons, 3  $\mu\text{M}$  oxotremorine-M significantly decreased the frequency of sEPSCs (Fig. 1E). In general, it took 10–15 min to wash out the stimulatory or inhibitory effect of oxotremorine-M. In the remaining 7 (10.6%) neurons, oxotremorine-M had no significant effect on the frequency of sEPSCs. The sEPSCs were blocked by 20  $\mu\text{M}$  CNQX, a non-*N*-methyl-D-aspartate glutamate receptor antagonist, in all neurons tested (data not shown). Because oxotremorine-M had no significant effect on the amplitude of sEPSCs, only the data showing the effects of the drug on the frequency of sEPSCs are presented in the sections below.

We next used himbacine, an  $M_2/M_4$  subtype-preferring antagonist (18, 19, 25–27), to determine the potential role of the  $M_2/M_4$  subtypes in the inhibitory effect of oxotremorine-M on sEPSCs in WT mice. The effective concentration of himbacine has been determined in our previous studies (18, 19, 25). Oxotremorine-M (3  $\mu\text{M}$ ) initially increased the frequency of sEPSCs in 14 neurons. In these 14 neurons, subsequent application of 2  $\mu\text{M}$  himbacine significantly potentiated the stimulatory effect of oxotremorine-M on the frequency of sEPSCs (Fig. 2, A and B). Interestingly, in another 9 neurons in which initial application of oxotremorine-M significantly inhibited the sEPSCs, the drug significantly increased the fre-

quency of sEPSCs in the presence of 2  $\mu\text{M}$  himbacine (Fig. 2C). These data suggest that the  $M_2/M_4$  subtypes mediate the inhibitory effect of the mAChR agonist on synaptic glutamate release to spinal dorsal horn neurons.

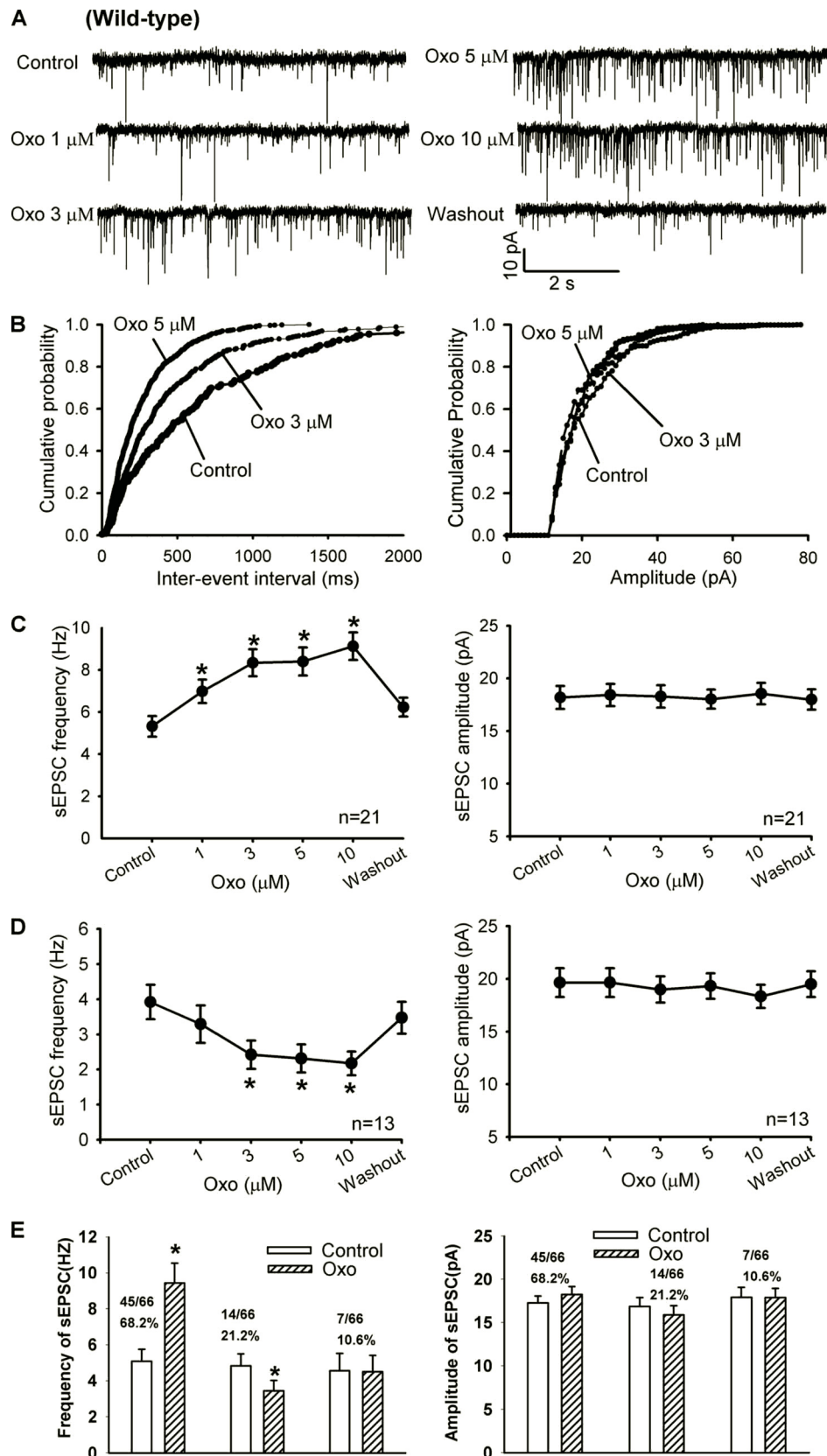
To determine whether the  $M_3$  subtype is involved in the stimulatory effect of oxotremorine-M on glutamate release in the spinal cord of WT mice, we tested the effect of J104129, an  $M_3$  subtype-preferring antagonist (28). In the preliminary experiments, we confirmed that 50 nM J104129 did not alter the inhibitory or excitatory effects of 3  $\mu\text{M}$  oxotremorine-M on sEPSCs in  $M_3$ -KO mice. In 6 of 21 neurons in which oxotremorine-M caused a small increase in the frequency of sEPSCs, 50 nM J104129 completely blocked the excitatory effect of oxotremorine-M on the sEPSCs (Fig. 2D). However, in the remaining 15 neurons in which oxotremorine-M produced a large increase in the frequency of sEPSCs, J104129 did not significantly alter its excitatory effect (Fig. 2E). These results suggest that although the  $M_3$  subtype contributes to the increased glutamate release induced by mAChR activation in a subpopulation of neurons, a non- $M_3$  subtype seems to be involved in the potentiation of glutamatergic input in the majority of dorsal horn neurons.

We next examined the possible subcellular location (*i.e.* presynaptic terminals versus somatodendritic sites) of the mAChR subtypes in the spinal dorsal horn. If the mAChR subtypes are present on the somatodendritic site of the glutamatergic neurons, oxotremorine-M would be expected to have little effect on the mEPSCs (*i.e.* EPSCs recorded in the presence of 0.5  $\mu\text{M}$  TTX). Bath application of 3  $\mu\text{M}$  oxotremorine-M either increased or decreased the frequency of the mEPSCs in a similar manner as the sEPSCs, *i.e.* in neurons where oxotremorine-M increased the sEPSCs, the mEPSCs were also increased and *vice versa* (Fig. 3). These data suggest that the mAChRs that modulate glutamatergic transmission in the spinal dorsal horn are primarily present on presynaptic terminals.

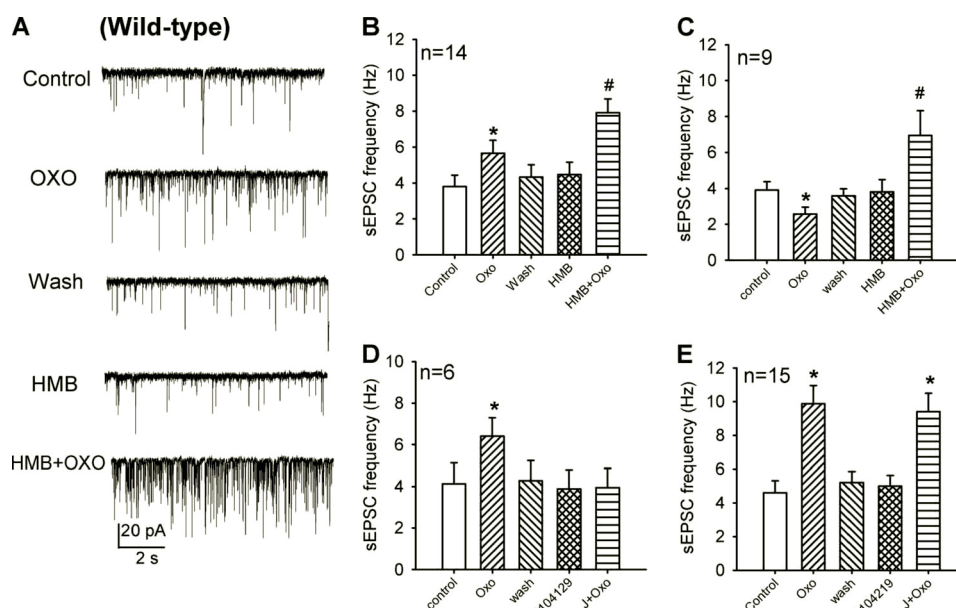
**Effects of Oxotremorine-M on sEPSCs and mEPSCs of Lamina II Neurons in  $M_1/M_3$  Double-KO mice**—In a total of 50 neurons tested from  $M_1/M_3$  double-KO mice, 3  $\mu\text{M}$  oxotremorine-M significantly increased the frequency of sEPSCs in 24 (48.0%) neurons (Fig. 4A). Oxotremorine-M increased the sEPSC frequency in significantly fewer neurons from  $M_1/M_3$  double-KO mice than from WT mice (48.0% versus 68.2%;  $p < 0.05$ , Fisher's exact test). Oxotremorine-M had no significant effect on the frequency and amplitude of sEPSCs in 12 of 50 (24.0%) neurons and significantly decreased the frequency of sEPSCs in the remaining 14 (28.0%) neurons (Fig. 4A).

In 17 neurons in which oxotremorine-M initially increased frequency of sEPSCs, subsequent application of 2  $\mu\text{M}$  himbacine further increased the stimulatory effect of 3  $\mu\text{M}$  oxotremorine-M on the frequency of sEPSCs (Fig. 4, B and C). In another 7 neurons from  $M_1/M_3$  double-KO mice, himbacine converted the initial inhibitory effect of oxotremorine-M to an excitatory effect (Fig. 4D). In an additional 11 neurons, 3  $\mu\text{M}$  oxotremorine-M produced a similar stimulatory effect on the frequency of sEPSCs and mEPSCs (Fig. 4E). Because the oxotremorine-M-induced increases in the fre-

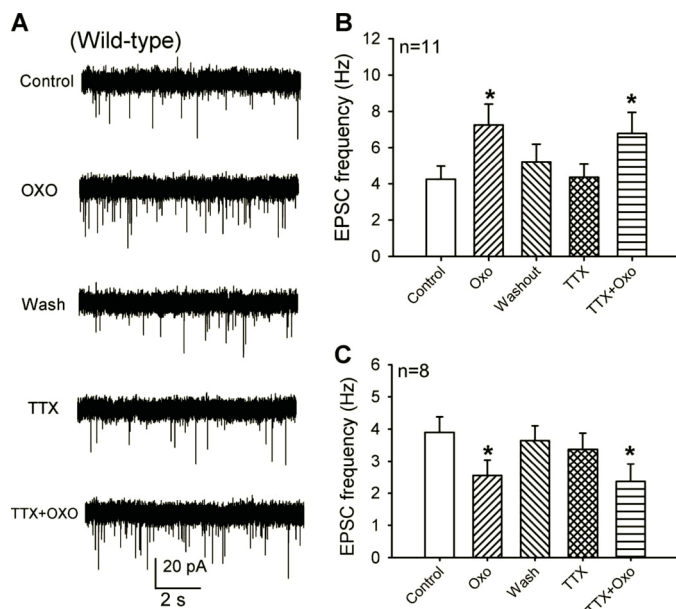




**FIGURE 1. Diverse effects of oxotremorine-M on glutamatergic sEPSCs in lamina II neurons from WT mice.** *A*, original traces of sEPSCs during baseline control, during application of 1, 3, 5, and 10  $\mu\text{M}$  oxotremorine-M, and upon washout in one lamina II neuron. *B*, cumulative probability plots of the same neuron in *A* show the distribution of inter-event interval and amplitude of sEPSCs at baseline and during perfusion of 3 and 5  $\mu\text{M}$  oxotremorine-M. *C*, summary data from 21 neurons showing the concentration-dependent stimulatory effect of oxotremorine-M on the frequency and amplitude of sEPSCs. *D*, summary data from another 13 neurons showing the concentration-dependent inhibitory effect of oxotremorine-M on the frequency and amplitude of sEPSCs. *E*, summary data show three types of neurons in which 3  $\mu\text{M}$  oxotremorine-M had distinct effects on sEPSCs. Data are presented as means  $\pm$  S.E. \*,  $p < 0.05$  compared with the control.



**FIGURE 2. Effects of himbacine or J104129 on the oxotremorine-M-induced changes in sEPSCs of lamina II neurons from WT mice.** *A*, original traces of sEPSCs during control, during application of 3  $\mu$ M oxotremorine-M with and without 2  $\mu$ M himbacine in one lamina II neuron. *B*, summary data from 14 neurons showing that himbacine had no effect on oxotremorine-M-induced increases in the sEPSC frequency. *C*, summary data from another 9 neurons showing that himbacine converted the initial inhibitory effect of oxotremorine-M on the sEPSC frequency to an excitatory effect. *D*, summary data from 6 neurons showing that 50 nM J104129 blocked the stimulatory effect of oxotremorine-M on the sEPSC frequency. *E*, summary data from 15 neurons showing that 50 nM J104129 had no effect on oxotremorine-M-induced increases in the sEPSC frequency. Data are presented as means  $\pm$  S.E. \*,  $p < 0.05$  compared with the control. #,  $p < 0.05$  compared with the initial effect of oxotremorine-M.

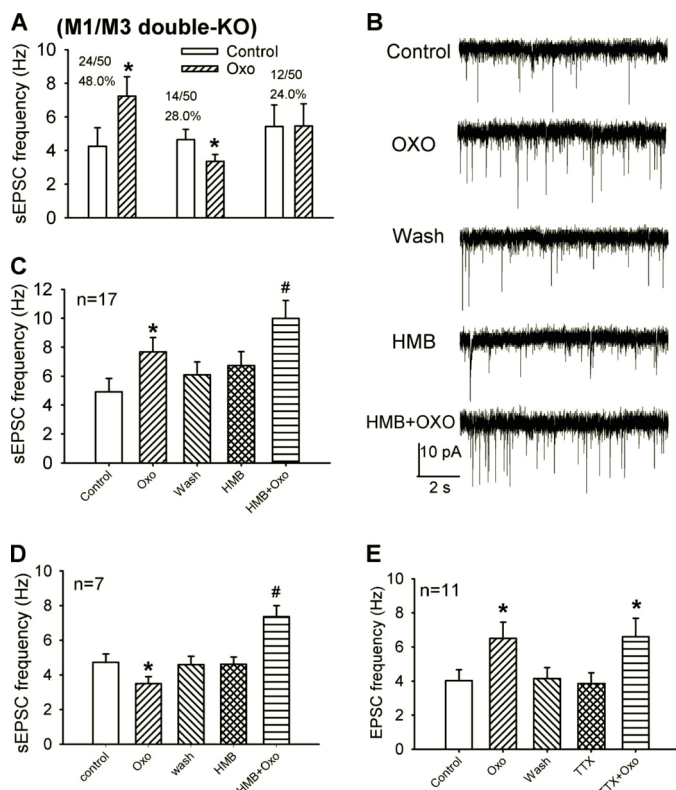


**FIGURE 3. Effects of oxotremorine-M on glutamatergic mEPSCs of lamina II neurons from WT mice.** *A*, representative traces of sEPSCs during control, during application of 3  $\mu$ M oxotremorine-M with and without 0.5  $\mu$ M TTX in one lamina II neuron. *B*, summary data from 11 neurons showing that oxotremorine-M similarly increased the frequency of sEPSCs and mEPSCs (with TTX). *C*, summary data from 8 neurons showing that oxotremorine-M inhibited the frequency of sEPSCs and mEPSCs (with TTX) in a similar manner. Data are presented as means  $\pm$  S.E. \*,  $p < 0.05$  compared with the control.

quency of sEPSCs were not blocked in the majority of neurons from the  $M_1/M_3$  double-KO mice, we considered it possible that the  $M_5$  subtype may be more important for the potentiation of synaptic glutamate release by mAChR activation in the spinal dorsal horn.

**Effects of Oxotremorine-M on sEPSCs and mEPSCs of Lamina II Neurons in  $M_5$ -KO Mice**—Because the stimulatory effect of oxotremorine-M on sEPSCs was still present in the majority of neurons in the  $M_1/M_3$  double-KO mice, we used  $M_5$ -KO mice to determine whether the  $M_5$  subtype contributed to the mAChR activation-induced synaptic glutamate release in the spinal cord. The baseline frequencies of sEPSCs was significantly lower in the neurons from the  $M_5$ -KO mice than in those from all other groups of mice (Fig. 5A). In the majority of neurons (39 of 76, 51.3%) from the  $M_5$ -KO mice, 3  $\mu$ M oxotremorine-M significantly inhibited the sEPSC frequency (Fig. 5A). The stimulatory effect of 3  $\mu$ M oxotremorine-M on sEPSCs was observed in only 20 of 76 (26.3%) neurons from  $M_5$ -KO mice. Oxotremorine-M increased the frequency of sEPSCs in significantly fewer neurons from the  $M_5$ -KO mice than the WT mice (26.3% versus 68.2%;  $p < 0.05$ ; Fisher's exact test). In addition, the magnitude of the increase in the sEPSC frequency induced by 3  $\mu$ M oxotremorine-M was significantly smaller in the neurons from the  $M_5$ -KO mice than in those from all other groups of mice (Fig. 5A). Notably, the potentiating effect of oxotremorine-M was readily washed out within 5 min after cessation of the bath application.

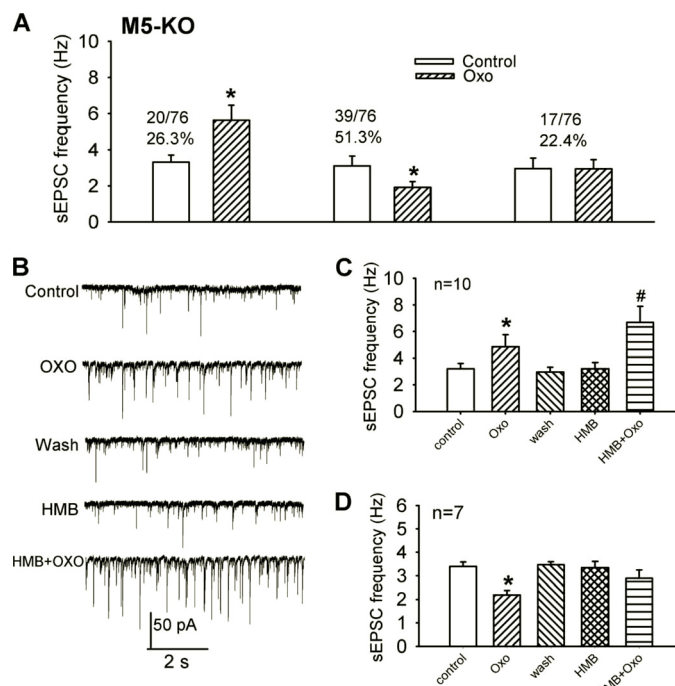
In 10 neurons from  $M_5$ -KO mice, bath application of 2  $\mu$ M himbacine significantly potentiated the initial stimulatory effect of oxotremorine-M on the sEPSCs (Fig. 5, B and C). In another 7 neurons, himbacine blocked the inhibitory effects of oxotremorine-M on the sEPSCs (Fig. 5D). In 9 additional neurons in which 3  $\mu$ M oxotremorine-M initially increased the frequency of sEPSCs, 50 nM J104129 converted the stimulatory effect of oxotremorine-M on the sEPSCs to an inhibitory effect (Fig. 6, A and B). Furthermore, 3  $\mu$ M oxotremo-



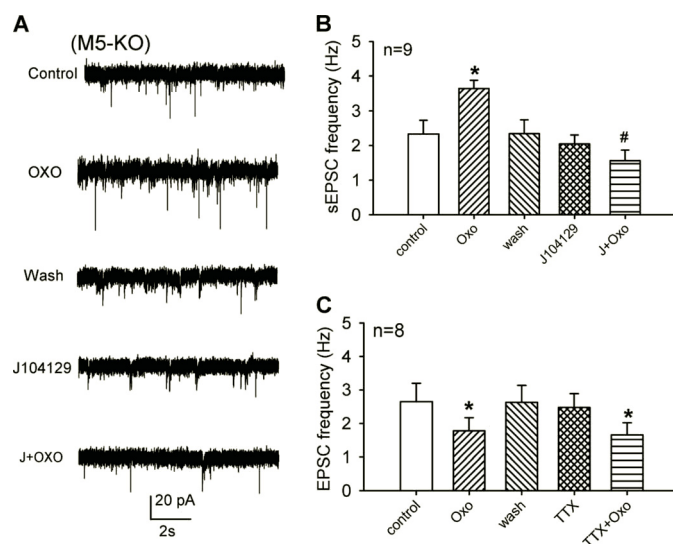
**FIGURE 4. Effects of oxotremorine-M on glutamatergic sEPSCs of lamina II neurons from M<sub>1</sub>/M<sub>3</sub> double-KO mice.** A, summary data show three types of neurons in which 3  $\mu$ M oxotremorine-M had distinct effects on the frequency of sEPSCs. B, original traces of sEPSCs during control, during application of 3  $\mu$ M oxotremorine-M with and without 2  $\mu$ M himbacine in one lamina II neuron. C, summary data from 17 neurons showing that himbacine potentiated the stimulatory effect of oxotremorine-M on the sEPSC frequency. D, summary data from another 7 neurons showing that himbacine converted the initial inhibitory effect of oxotremorine-M on the sEPSC frequency to a stimulatory effect. E, summary data from 11 neurons show that oxotremorine-M similarly increased the frequency of sEPSCs and mEPSCs (with TTX). Data are presented as means  $\pm$  S.E. \*,  $p < 0.05$  compared with the control. #,  $p < 0.05$  compared with the initial effect of oxotremorine-M.

rine-M significantly inhibited the frequency of both sEPSCs and mEPSCs in 8 neurons tested (Fig. 6C). These results strongly suggest that stimulation of mAChRs potentiates synaptic glutamate release primarily through the M<sub>5</sub> subtype in the spinal dorsal horn.

**Effects of Oxotremorine-M on sEPSCs and mEPSCs of Lamina II Neurons in M<sub>3</sub>-KO Mice**—In 30 of 60 (50.0%) lamina II neurons from M<sub>3</sub>-KO mice, 3  $\mu$ M oxotremorine-M significantly increased the frequency of sEPSCs (Fig. 7A). Oxotremorine-M increased the sEPSCs frequency in significantly fewer neurons from M<sub>3</sub>-KO mice than from WT mice (50.0% versus 68.2%;  $p < 0.05$ , Fisher's exact test). Oxotremorine-M significantly reduced the frequency of sEPSCs in 17 of 60 (28.3%) neurons and had no effect on the frequency of sEPSCs in the remaining 13 (21.7%) neurons (Fig. 7A). In 11 neurons in which oxotremorine-M increased the frequency of sEPSCs, 2  $\mu$ M himbacine further increased the frequency of sEPSCs (Fig. 7B). In another 11 neurons from M<sub>3</sub>-KO mice in which oxotremorine-M had an inhibitory effect on the sEPSCs, himbacine abolished the inhibitory effect (Fig. 7C).

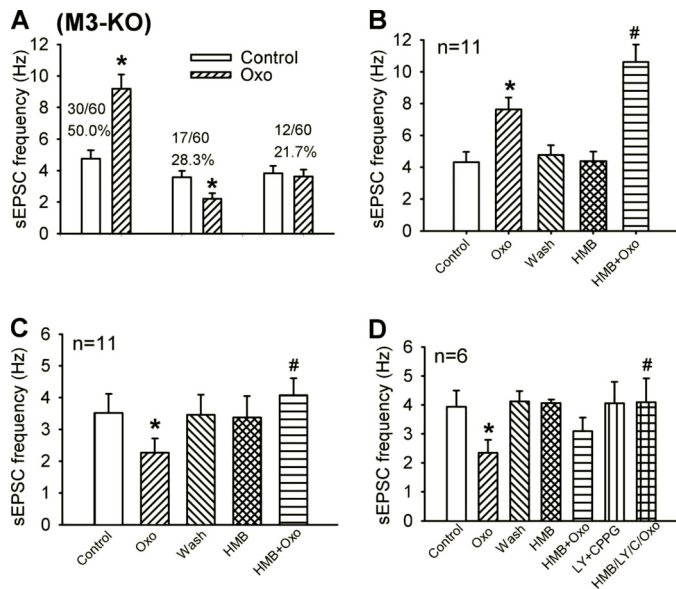


**FIGURE 5. Effects of oxotremorine-M on glutamatergic sEPSCs of lamina II neurons from M<sub>5</sub>-KO mice.** A, summary data show three types of neurons in which 3  $\mu$ M oxotremorine-M had distinct effects on the frequency of sEPSCs. B, representative traces of sEPSCs during control, during application of 3  $\mu$ M oxotremorine-M with and without 2  $\mu$ M himbacine in one lamina II neuron. C, summary data from 10 neurons showing that himbacine potentiated the stimulatory effect of oxotremorine-M on the sEPSC frequency. D, summary data from 7 neurons showing that himbacine blocked the inhibitory effect of oxotremorine-M on the sEPSC frequency. Data are presented as means  $\pm$  S.E. \*,  $p < 0.05$  compared with the control. #,  $p < 0.05$  compared with the initial effect of oxotremorine-M.



**FIGURE 6. Effects of J104129 or TTX on the oxotremorine-M-induced changes in sEPSCs of lamina II neurons in M<sub>5</sub>-KO mice.** A, original traces of sEPSCs during control, during application of 3  $\mu$ M oxotremorine-M with and without 50 nM J104129 in one lamina II neuron. B, summary data from 9 neurons showing J104129 converted the initial stimulatory effect of oxotremorine-M on the sEPSC frequency to inhibitory. C, summary data from 8 neurons showing that oxotremorine-M inhibited the frequency of sEPSCs and mEPSCs (with TTX) in a similar manner. Data are presented as means  $\pm$  S.E. \*,  $p < 0.05$  compared with the control. #,  $p < 0.05$  compared with the initial effect of oxotremorine-M.



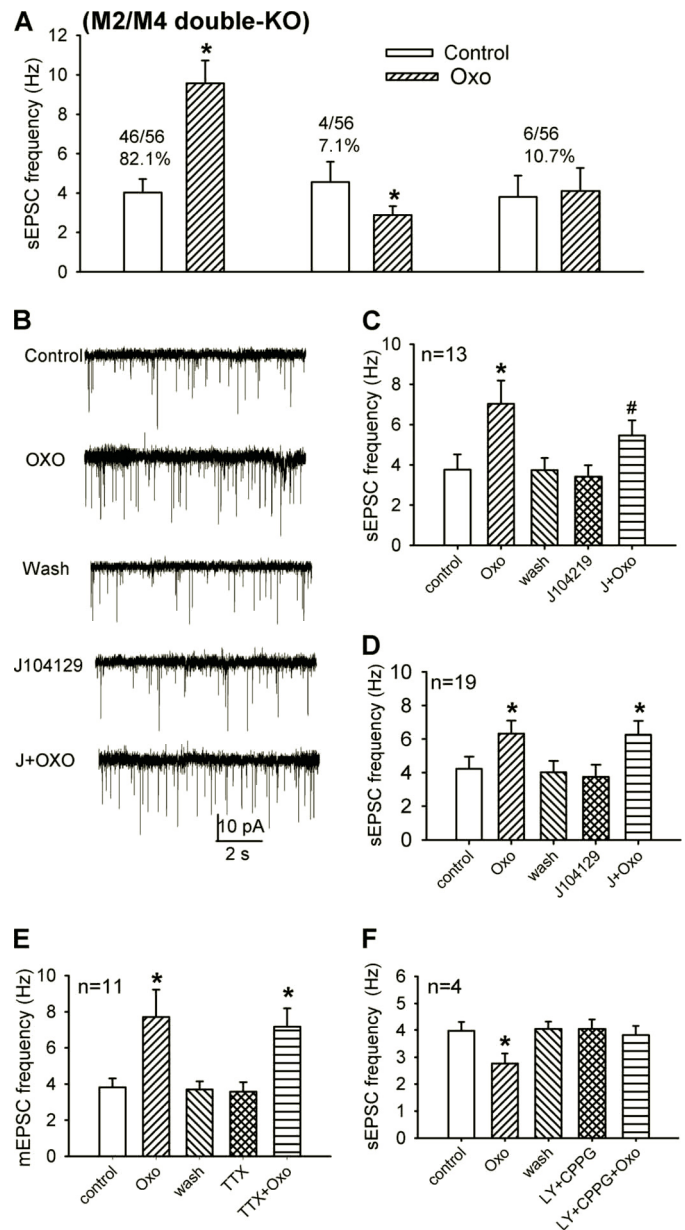


**FIGURE 7. Effects of oxotremorine-M on glutamatergic sEPSCs of lamina II neurons from  $M_3$ -KO mice.** A, summary data show three types of neurons in which  $3 \mu\text{M}$  oxotremorine-M had distinct effects on the frequency of sEPSCs. B, summary data from 11 neurons showing that himbacin enhanced the stimulatory effect of oxotremorine-M on the sEPSC frequency. C, summary data from another 11 neurons showing that himbacin converted the initial inhibitory effect of oxotremorine-M on the sEPSC frequency to a stimulatory effect. D, summary data from 6 neurons showing that himbacin completely blocked the inhibitory effect of oxotremorine-M on sEPSCs in the presence of  $100 \text{ nM}$  LY341495 and  $200 \mu\text{M}$  CPPG. Data are presented as means  $\pm$  S.E. \*,  $p < 0.05$  compared with the control. #,  $p < 0.05$  compared with the initial effect of oxotremorine-M.

In 6 neurons from  $M_3$ -KO mice, himbacin only partially reduced the inhibitory effect of oxotremorine-M on the sEPSCs. We speculated that the mAChR agonist may stimulate  $M_5$  in the  $M_3$ -KO mice to cause excessive glutamate release, which could activate the group II/III mGluRs (29) to subsequently inhibit synaptic glutamate release in this subpopulation of neurons. We therefore further tested the effect of oxotremorine-M in the presence of  $100 \text{ nM}$  LY341495 and  $200 \mu\text{M}$  CPPG, which are selective antagonists for group II and III mGluRs, respectively (29–32). In these 6 neurons, LY341495 and CPPG completely blocked the inhibitory effect of oxotremorine-M (Fig. 7D).

**Effects of Oxotremorine-M on sEPSCs and mEPSCs of Lamina II Neurons in  $M_2/M_4$  Double-KO Mice**—To delineate the role of the  $M_2$  and  $M_4$  subtypes in the inhibition of synaptic glutamate release in the spinal cord, we tested the effect of oxotremorine-M in  $M_2/M_4$  double-KO mice. In 45 of 56 (82.1%) lamina II neurons,  $3 \mu\text{M}$  oxotremorine-M significantly increased the frequency of sEPSCs (Fig. 8A). In contrast to the results seen in the other groups of mice, oxotremorine-M significantly reduced the frequency of sEPSCs in only 4 of 56 (7.1%) neurons (Fig. 8A). Oxotremorine-M inhibited the frequency of sEPSCs in significantly fewer neurons from  $M_2/M_4$  double-KO mice than from WT mice (7.1% versus 21.2%;  $p < 0.05$ ; Fisher's exact test). Oxotremorine-M had no significant effect on the sEPSCs in the remaining 6 (10.7%) neurons.

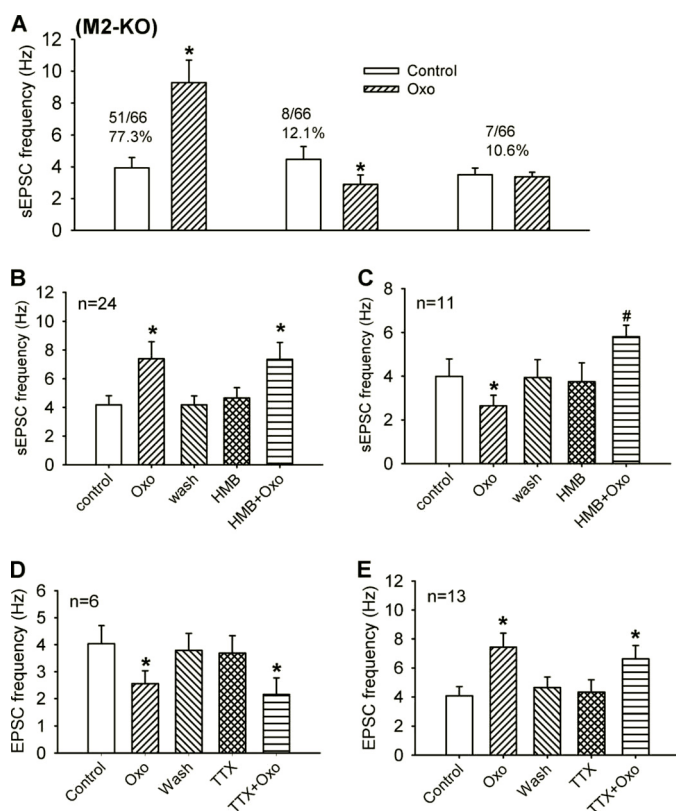
In 13 neurons from  $M_2/M_4$  double-KO mice, blocking the  $M_3$  subtype with  $50 \text{ nM}$  J104129 significantly attenuated the excitatory effect of  $3 \mu\text{M}$  oxotremorine-M on the sEPSC fre-



**FIGURE 8. Effects of oxotremorine-M on glutamatergic sEPSCs of lamina II neurons from  $M_2/M_4$  double-KO mice.** A, summary data show three types of neurons in which  $3 \mu\text{M}$  oxotremorine-M had distinct effects on the frequency of sEPSCs. B, original traces of sEPSCs during control, during application of  $3 \mu\text{M}$  oxotremorine-M with and without  $50 \text{ nM}$  J104129 in one lamina II neuron. C, summary data from 13 neurons showing that J104129 attenuated the stimulatory effect of oxotremorine-M on the sEPSC frequency. D, summary data from 19 neurons showing that J104129 had no effect on oxotremorine-M-induced increases in the sEPSC frequency. E, summary data from 11 neurons showing that oxotremorine-M similarly increased the frequency of sEPSCs and mEPSCs (with TTX). F, summary data show that  $100 \text{ nM}$  LY341495 and  $200 \mu\text{M}$  CPPG blocked the inhibitory effect of oxotremorine-M on sEPSCs in 4 neurons. Data are presented as means  $\pm$  S.E. \*,  $p < 0.05$  compared with the control. #,  $p < 0.05$  compared with the initial effect of oxotremorine-M.

quency (Fig. 8, B and C). However, in another 19 neurons, J104129 did not significantly alter the oxotremorine-M-induced increases in the sEPSC frequency (Fig. 8D). In 11 additional neurons, oxotremorine-M increased the frequencies of the both sEPSC and mEPSCs in a similar manner (Fig. 8E).

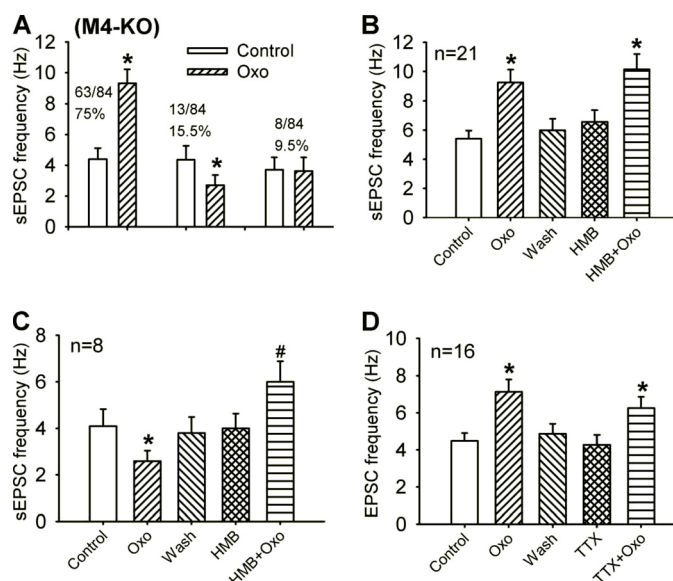
We noticed that even in the  $M_2/M_4$  double-KO mice,  $3 \mu\text{M}$  oxotremorine-M still increased the sEPSC frequency in a



**FIGURE 9. Effects of oxotremorine-M on glutamatergic sEPSCs of lamina II neurons from M<sub>2</sub>-KO mice.** A, summary data show three types of neurons in which 3  $\mu$ M oxotremorine-M had distinct effects on the frequency of sEPSCs. B, summary data from 24 neurons showing that himbacine had no effect on oxotremorine-M-induced increases in the sEPSC frequency. C, summary data from another 11 neurons showing that himbacine converted the initial inhibitory effect of oxotremorine-M on the sEPSC frequency to stimulatory. D, summary data from 6 neurons showing that oxotremorine-M similarly inhibited the frequency of sEPSCs and mEPSCs (with TTX). E, summary data from 13 neurons showing that oxotremorine-M increased the frequency of sEPSCs and mEPSCs (with TTX) in a similar manner. Data are presented as means  $\pm$  S.E. \*,  $p < 0.05$  compared with the control. #,  $p < 0.05$  compared with the initial effect of oxotremorine-M.

small population (7.1%) of lamina II neurons. Because stimulation of the M<sub>3</sub>/M<sub>5</sub> subtypes in M<sub>2</sub>/M<sub>4</sub> double-KO mice could result in a large increase in synaptic glutamate release, it is possible that the overflow of glutamate can access and stimulate presynaptic group II and III mGluRs to subsequently reduce the glutamatergic input to some lamina II neurons. In 4 neurons in which the frequency of sEPSCs was initially inhibited by 3  $\mu$ M oxotremorine-M, bath application of 100 nM LY341495 and 200  $\mu$ M CPPG completely blocked the inhibitory effect of oxotremorine-M (Fig. 8F).

**Effects of Oxotremorine-M on sEPSCs and mEPSCs of Lamina II Neurons in M<sub>2</sub>-KO Mice**—In 51 of 66 (77.3%) neurons recorded from M<sub>2</sub>-KO mice, 3  $\mu$ M oxotremorine-M significantly increased the frequency of sEPSCs (Fig. 9A). Oxotremorine-M significantly inhibited the frequency of sEPSCs in 8 of 66 (12.1%) neurons (Fig. 9A). The percentage of neurons in which oxotremorine-M inhibited sEPSCs was not significantly different between the M<sub>2</sub>-KO mice and WT mice (12.1% versus 21.2%;  $p > 0.05$ , Fisher's exact test).



**FIGURE 10. Effects of oxotremorine-M on glutamatergic sEPSCs of lamina II neurons from M<sub>4</sub>-KO mice.** A, summary data show three types of neurons in which 3  $\mu$ M oxotremorine-M had distinct effects on the frequency of sEPSCs. B, summary data from 21 neurons showing that himbacine potentiated the effect of oxotremorine-M on the sEPSC frequency. C, summary data from another 8 neurons showing that himbacine converted the initial inhibitory effect of oxotremorine-M on the sEPSC frequency to a stimulatory effect. D, summary data from 16 neurons showing that oxotremorine-M stimulated the frequency of sEPSCs and mEPSCs (with TTX) in a similar manner. Data are presented as means  $\pm$  S.E. \*,  $p < 0.05$  compared with the control. #,  $p < 0.05$  compared with the initial effect of oxotremorine-M.

Bath application of 2  $\mu$ M himbacine did not significantly alter the stimulating effect of 3  $\mu$ M oxotremorine-M on sEPSCs in 24 neurons tested (Fig. 9B). However, in another 11 neurons, himbacine converted the initial inhibitory effect of oxotremorine-M to an excitatory effect (Fig. 9C). In addition, 3  $\mu$ M oxotremorine-M either increased or decreased the frequency of sEPSC and mEPSCs in neurons from M<sub>2</sub>-KO mice in a similar manner (Fig. 9, D and E).

**Effects of Oxotremorine-M on sEPSCs and mEPSCs of Lamina II Neurons in M<sub>4</sub>-KO Mice**—In M<sub>4</sub>-KO mice, 3  $\mu$ M oxotremorine-M significantly increased the frequency of sEPSCs in 63 of 84 (75.0%) neurons (Fig. 10A). In 13 of 84 (15.5%) neurons, oxotremorine-M significantly inhibited the frequency of sEPSCs (Fig. 10A). The percentage of neurons in which oxotremorine-M inhibited sEPSCs was not significantly different between M<sub>4</sub>-KO mice and WT mice (15.5% versus 21.2%;  $p > 0.05$ , Fisher's exact test).

In 21 neurons from the M<sub>4</sub>-KO mice, 2  $\mu$ M himbacine did not significantly alter the stimulatory effect of oxotremorine-M on the frequency of sEPSCs (Fig. 10B). In another 8 neurons, himbacine converted the inhibitory effect of oxotremorine-M on sEPSCs to an excitatory effect (Fig. 10C). In an additional 16 neurons, 3  $\mu$ M oxotremorine-M significantly increased the frequency of both the sEPSCs and mEPSCs (Fig. 10D). Collectively, the electrophysiological data obtained from M<sub>2</sub>/M<sub>4</sub> double-KO and M<sub>2</sub> and M<sub>4</sub> single-KO mice suggest that the M<sub>2</sub> and M<sub>4</sub> subtypes are mainly present on the presynaptic terminals to inhibit synaptic glutamate release in the spinal dorsal horn.



## DISCUSSION

In the present study, we used genetic and electrophysiological approaches to determine the functional activity of individual mAChR subtypes in the control of spinal glutamatergic transmission. We found that the mAChR agonist oxotremorine-M significantly increased the frequency of the glutamatergic sEPSCs in the majority of lamina II neurons but reduced the sEPSC frequency in a subpopulation of neurons in the spinal cord of WT mice. The inhibitory effect of oxotremorine-M on sEPSCs in WT mice was largely blocked by the  $M_2/M_4$ -preferring antagonist himbacine or in  $M_2/M_4$  double-KO mice. However, the stimulatory effect of oxotremorine-M on the frequency of sEPSCs was still present in ~50% neurons from the  $M_3$ -KO or  $M_1/M_3$  double-KO mice. Surprisingly, we found that the stimulatory effect of oxotremorine-M on the sEPSCs was profoundly reduced in  $M_5$ -KO mice and that blocking the  $M_3$  subtype with J104129 abolished the stimulatory effect of oxotremorine-M on the sEPSCs in neurons from these mice. Furthermore, in neurons from WT,  $M_2$ -KO,  $M_3$ -KO,  $M_4$ -KO, and  $M_5$ -KO mice, the  $M_2/M_4$  antagonist himbacine either enhanced the stimulating effect of oxotremorine-M on the sEPSCs or converted its inhibitory effect to an excitatory effect. Collectively, our study provides unambiguous evidence that the presynaptic  $M_2$  and  $M_4$  subtypes contribute to the muscarinic inhibition of synaptic glutamate release in the spinal cord. Our findings also support the novel concept that presynaptic  $M_5$  and to a lesser extent  $M_3$  receptors are important for the potentiation of excitatory glutamatergic input to dorsal horn neurons.

Both the  $M_2$  and  $M_4$  subtypes in the spinal cord are critically involved in the inhibition of nociceptive transmission by stimulation of mAChRs in mice (11, 17) and in rats (14). Previous studies have shown that the  $M_2$  subtype, representing the majority (~90%) of spinal cord mAChRs, is particularly expressed in the superficial dorsal horn (11, 14, 16, 24, 33). In addition, the  $M_4$  subtype is expressed at low levels in the spinal cord (11, 14, 34). We found that the percentage of neurons in which oxotremorine-M inhibited the sEPSC frequency was greatly reduced in  $M_2/M_4$ -double-KO mice. Furthermore, blocking the  $M_2$  and  $M_4$  subtypes with himbacine not only attenuated the inhibitory effect of oxotremorine-M on sEPSCs but also converted the effect of oxotremorine-M from an excitatory to an inhibitory one in many neurons tested from WT,  $M_2$ -KO,  $M_4$ -KO,  $M_3$ -KO,  $M_5$ -KO, and  $M_1/M_3$  double-KO mice. These data clearly suggest that both  $M_2$  and  $M_4$  subtypes contribute to the inhibition of synaptic glutamate release induced by activation of mAChRs in the spinal cord. In addition, the inhibitory effect of oxotremorine-M on the frequency of sEPSCs was not significantly attenuated by TTX in  $M_2$ -KO,  $M_4$ -KO, and WT mice, suggesting that these two inhibitory mAChR subtypes are primarily located on the presynaptic terminals. Furthermore, we found that the percentage of neurons in which oxotremorine-M inhibited sEPSCs was not significantly reduced in  $M_2$  and  $M_4$  single-KO mice. This is likely due to the fact that the  $M_2$  and  $M_4$  both play a significant role in the attenuation of glutamatergic transmission by the mAChR agonist. This notion is further supported

by our finding that himbacine significantly attenuated the inhibitory effect of oxotremorine-M on sEPSCs in the  $M_2$  and  $M_4$  single-KO mice.

In this study, we found that in neurons from WT mice, oxotremorine-M significantly increased the frequency of glutamatergic sEPSCs in the majority (68.2%) of neurons, while it inhibited the sEPSCs in 26.7% of neurons. These data are distinctly different from what we found in the rat spinal cord, where oxotremorine-M only inhibited the sEPSC frequency in lamina II neurons (35). These distinct effects likely reflect a species difference between rats and mice. For example, we have shown that oxotremorine-M increases synaptic GABA release in the rat spinal cord but primarily reduces GABA release in the mouse spinal cord (18, 25). Because the excitatory effect of oxotremorine-M persisted in the presence of the voltage-gated  $Na^+$  channel blocker TTX (*i.e.* mEPSCs) in WT and  $M_2/M_4$  double-KO mice, our data suggest that the mAChR subtypes that stimulate synaptic glutamate release are primarily present at the presynaptic terminals in the mouse spinal cord.

Previous studies have shown that the  $M_2$ ,  $M_3$ , and  $M_4$  subtypes are all involved in the control of spinal synaptic transmission (18, 19, 25, 35, 36). Although the  $M_5$  subtype has been shown to be present in the mouse spinal cord (37, 38), the important function of  $M_5$  in the spinal cord has not been recognized previously. We found that oxotremorine-M still increased the frequency of sEPSCs in about 50% neurons from the  $M_3$ -KO and  $M_1/M_3$  double-KO mice. In addition, the  $M_3$ -preferring antagonist J104219 did not abolish the excitatory effect of oxotremorine-M on sEPSCs in WT mice. Thus, we hypothesized that the  $M_5$  subtype may be critically involved in potentiating synaptic glutamate release in the spinal cord. Consistent with this hypothesis, we observed that both the baseline frequency of sEPSCs and the magnitude of the increases in the sEPSC frequency induced by oxotremorine-M were significantly less in the neurons from  $M_5$ -KO mice than in those from all other groups of mice. Furthermore, we found that the percentage of neurons in which oxotremorine-M increased the sEPSC frequency (26.3%) was markedly smaller in the  $M_5$ -KO mice than in the WT mice. Our findings provide the first evidence showing that the  $M_5$  subtype plays a major role in the increased synaptic glutamate release induced by mAChR activation in the spinal dorsal horn. Nevertheless, the potential role of  $M_5$  in the control of spinal nociceptive transmission remains to be defined in more detail.

Unlike the  $M_2$  and  $M_4$  subtypes, knockdown of  $M_3$  in the spinal cord with specific siRNA does not significantly affect the analgesic effect of mAChR agonists in rats (14). Because of the dominance of  $M_5$  in the potentiation of synaptic glutamate release, the functional activity of  $M_3$  is probably masked by the presence of  $M_5$  in the spinal cord. In  $M_5$ -KO mice, we found that blocking the  $M_3$  subtype with J104219 completely blocked the remaining stimulatory effect of oxotremorine-M on the sEPSC frequency. We also found that the percentage of neurons in which oxotremorine-M increased the frequency of sEPSCs was significantly less in the neurons from the  $M_3$ -KO and  $M_1/M_3$  double-KO mice than in those from the WT mice. Furthermore, in both WT and  $M_2/M_4$  double-KO mice,

J104219 significantly attenuated the stimulatory effect of oxotremorine-M on sEPSCs in a subpopulation of lamina II neurons. Therefore, these data suggest that the potentiation of synaptic glutamate release induced by mAChR activation is mediated by both the  $M_3$  and  $M_5$  subtypes in the spinal cord. We obtained no evidence for the role of  $M_1$  subtype in the control of glutamatergic synaptic transmission in the spinal dorsal horn. This conclusion is based on (1) the effect of oxotremorine-M on sEPSCs in  $M_3$  single-KO mice was nearly identical to that in  $M_1/M_3$  double-KO mice and (2) the stimulatory effect of oxotremorine-M on sEPSCs in  $M_5$  KO mice was completely blocked by the  $M_3$ -preferring antagonist J104219.

Interestingly, we found that oxotremorine-M still inhibited the sEPSCs in a subpopulation of neurons in  $M_2/M_4$  double-KO mice. In these neurons, blocking group II/III mGluRs with LY341495 and CPPG completely abolished the inhibitory effect of oxotremorine-M. These data clearly suggest that the increased glutamate release owing to stimulation of the  $M_3$  and  $M_5$  subtypes can access and activate presynaptic group II and III mGluRs (39) to subsequently reduce glutamatergic input in these neurons. Thus, group II/III mGluRs are indirectly involved in the feedback regulation of glutamatergic input by the  $M_3$  and  $M_5$  subtypes in the spinal cord.

Another interesting finding of our study is the complex function and dynamic interactions between four mAChR subtypes in the control of synaptic glutamatergic transmission in the spinal cord. For example, we found that himbacine further potentiated the excitatory effects of oxotremorine-M on the frequency of sEPSCs in WT,  $M_2$ -KO,  $M_3$ -KO,  $M_4$ -KO,  $M_5$ -KO, and  $M_1/M_3$  double-KO mice. In addition, in a subpopulation of dorsal horn neurons from WT,  $M_2$ -KO,  $M_3$ -KO,  $M_4$ -KO,  $M_5$ -KO, and  $M_1/M_3$  double-KO mice, himbacine converted the inhibitory effect of oxotremorine-M on the sEPSCs to a stimulatory effect. Furthermore, in neurons from  $M_5$ -KO mice, J104219 converted the stimulatory effect of oxotremorine-M on the sEPSCs to an inhibitory effect. These results suggest a reciprocal interaction among the inhibitory  $M_2/M_4$  and the excitatory  $M_3/M_5$  subtypes present in dorsal horn neurons. The glutamatergic synaptic terminals expressing these four mAChR subtypes are likely intermingled in the superficial spinal dorsal horn. Therefore, the  $M_2/M_4$  and  $M_3/M_5$  subtypes, located either on the same or separate presynaptic glutamatergic terminals, can greatly influence the amount of synaptic glutamate release to a given dorsal horn neuron by activation of mAChRs in the spinal cord. Because  $M_2$  and  $M_4$  are coupled to  $G_{i/o}$  proteins, stimulation of these two subtypes can inhibit synaptic glutamate release through inhibition of voltage-gated calcium channels (40–42). On the other hand,  $M_3$  and  $M_5$  are coupled to  $G_{q/11}$  proteins, and activation of these two subtypes could increase presynaptic glutamate release through stimulation of phospholipase C-inositol triphosphate, which increase the intracellular calcium level (43, 44). It would be interesting to determine whether  $M_3/M_5$  stimulation “antagonizes” the muscarinic analgesic effect at the spinal level by comparing the effects of mAChR agonists on nociception in WT,  $M_3$ -KO, and  $M_5$ -KO mice.

In summary, our study using mAChR subtype-KO mice provides unequivocal evidence that the  $M_5$ , and to a lesser extent,  $M_3$  subtypes contribute to the potentiation of glutamatergic input to spinal dorsal horn neurons in mice. Also, we demonstrated that the  $M_2$  and  $M_4$  subtypes mediate the inhibition of synaptic glutamate release induced by mAChR activation in the spinal dorsal horn. Furthermore, the reciprocal interactions between the inhibitory  $M_2$  and  $M_4$  subtypes and the stimulatory  $M_3$  and  $M_5$  subtypes are involved in the dynamic regulation of glutamatergic synaptic transmission in the spinal cord. Finally, we found that the group II/III mGluRs are involved in the  $M_3/M_5$ -mediated feedback loop that regulates glutamatergic input in the spinal cord. The diverse functions and interactions among different mAChR subtypes are important for our understanding of the complex actions produced by spinally administered mAChR agonists or acetylcholinesterase inhibitors. Our findings are critical in guiding the development of mAChR subtype-selective analgesic drugs endowed with increased efficacy and reduced side effects.

## REFERENCES

- Ribeiro-da-Silva, A., and Cuello, A. C. (1990) *J. Comp. Neurol.* **295**, 370–384
- Wetts, R., and Vaughn, J. E. (1994) *Neuroscience* **63**, 1117–1124
- Scatton, B., Dubois, A., Javoy-Agid, F., and Camus, A. (1984) *Neurosci. Lett.* **49**, 239–245
- Yamamura, H. I., Wamsley, J. K., Deshmukh, P., and Roeske, W. R. (1983) *Eur. J. Pharmacol.* **91**, 147–149
- Villiger, J. W., and Faull, R. L. (1985) *Brain Res.* **345**, 196–199
- Chen, S. R., and Pan, H. L. (2004) *Neuroscience* **125**, 141–148
- Zhuo, M., and Gebhart, G. F. (1991) *Pain* **46**, 211–222
- Chen, S. R., and Pan, H. L. (2003) *J. Pharmacol. Exp. Ther.* **307**, 676–681
- Hood, D. D., Mallak, K. A., James, R. L., Tuttle, R., and Eisenach, J. C. (1997) *J. Pharmacol. Exp. Ther.* **282**, 86–92
- Nakayama, M., Ichinose, H., Nakabayashi, K., Satoh, O., Yamamoto, S., and Namiki, A. (2001) *J. Clin. Anesth.* **13**, 86–89
- Duttaroy, A., Gomez, J., Gan, J. W., Siddiqui, N., Basile, A. S., Harman, W. D., Smith, P. L., Felder, C. C., Levey, A. I., and Wess, J. (2002) *Mol. Pharmacol.* **62**, 1084–1093
- Wess, J. (1996) *Crit. Rev. Neurobiol.* **10**, 69–99
- McKinney, M. (1993) *Prog. Brain Res.* **98**, 333–340
- Cai, Y. Q., Chen, S. R., Han, H. D., Sood, A. K., Lopez-Berestein, G., and Pan, H. L. (2009) *J. Neurochem.* **111**, 1000–1010
- Chen, Y. P., Chen, S. R., and Pan, H. L. (2005) *J. Pharmacol. Exp. Ther.* **315**, 696–703
- Höglund, A. U., and Baghdoyan, H. A. (1997) *J. Pharmacol. Exp. Ther.* **281**, 470–477
- Gomez, J., Shannon, H., Kostenis, E., Felder, C., Zhang, L., Brodtkin, J., Grinberg, A., Sheng, H., and Wess, J. (1999) *Proc. Natl. Acad. Sci. U.S.A.* **96**, 1692–1697
- Zhang, H. M., Chen, S. R., Matsui, M., Gautam, D., Wess, J., and Pan, H. L. (2006) *Mol. Pharmacol.* **69**, 1048–1055
- Zhang, H. M., Zhou, H. Y., Chen, S. R., Gautam, D., Wess, J., and Pan, H. L. (2007) *J. Pharmacol. Exp. Ther.* **323**, 963–971
- Yamada, M., Miyakawa, T., Duttaroy, A., Yamanaka, A., Moriguchi, T., Makita, R., Ogawa, M., Chou, C. J., Xia, B., Crawley, J. N., Felder, C. C., Deng, C. X., and Wess, J. (2001) *Nature* **410**, 207–212
- Fisahn, A., Yamada, M., Duttaroy, A., Gan, J. W., Deng, C. X., McBain, C. J., and Wess, J. (2002) *Neuron* **33**, 615–624
- Gautam, D., Heard, T. S., Cui, Y., Miller, G., Bloodworth, L., and Wess, J. (2004) *Mol. Pharmacol.* **66**, 260–267
- Gomez, J., Zhang, L., Kostenis, E., Felder, C., Bymaster, F., Brodtkin, J., Shannon, H., Xia, B., Deng, C., and Wess, J. (1999) *Proc. Natl. Acad. Sci. U.S.A.* **96**, 10483–10488

24. Li, D. P., Chen, S. R., Pan, Y. Z., Levey, A. I., and Pan, H. L. (2002) *J. Physiol.* **543**, 807–818
25. Zhang, H. M., Li, D. P., Chen, S. R., and Pan, H. L. (2005) *J. Pharmacol. Exp. Ther.* **313**, 697–704
26. Doller, D., Chackalamannil, S., Czarniecki, M., McQuade, R., and Rupert, V. (1999) *Bioorg. Med. Chem. Lett.* **9**, 901–906
27. Miller, J. H., Aagaard, P. J., Gibson, V. A., and McKinney, M. (1992) *J. Pharmacol. Exp. Ther.* **263**, 663–667
28. Mitsuya, M., Mase, T., Tsuchiya, Y., Kawakami, K., Hattori, H., Kobayashi, K., Ogino, Y., Fujikawa, T., Satoh, A., Kimura, T., Noguchi, K., Ohtake, N., and Tomimoto, K. (1999) *Bioorg. Med. Chem.* **7**, 2555–2567
29. Zhou, H. Y., Zhang, H. M., Chen, S. R., and Pan, H. L. (2007) *J. Neurophysiol.* **97**, 871–882
30. Conn, P. J., and Pin, J. P. (1997) *Annu. Rev. Pharmacol. Toxicol.* **37**, 205–237
31. Schoppa, N. E., and Westbrook, G. L. (1997) *J. Neurophysiol.* **78**, 1468–1475
32. Zhang, H. M., Chen, S. R., and Pan, H. L. (2009) *Neuroscience* **158**, 875–884
33. Yung, K. K., and Lo, Y. L. (1997) *Neurosci. Lett.* **229**, 81–84
34. Chen, S. R., Wess, J., and Pan, H. L. (2005) *J. Pharmacol. Exp. Ther.* **313**, 765–770
35. Zhang, H. M., Chen, S. R., and Pan, H. L. (2007) *J. Neurophysiol.* **97**, 102–109
36. Wang, X. L., Zhang, H. M., Li, D. P., Chen, S. R., and Pan, H. L. (2006) *J. Physiol.* **571**, 403–413
37. Wei, J., Walton, E. A., Milici, A., and Buccafusco, J. J. (1994) *J. Neurochem.* **63**, 815–821
38. Oki, T., Takagi, Y., Inagaki, S., Taketo, M. M., Manabe, T., Matsui, M., and Yamada, S. (2005) *Brain Res. Mol. Brain Res.* **133**, 6–11
39. Zhou, H. Y., Zhang, H. M., Chen, S. R., and Pan, H. L. (2008) *J. Pharmacol. Exp. Ther.* **324**, 1000–1010
40. Pan, H. L., Wu, Z. Z., Zhou, H. Y., Chen, S. R., Zhang, H. M., and Li, D. P. (2008) *Pharmacol. Ther.* **117**, 141–161
41. Wanke, E., Bianchi, L., Mantegazza, M., Guatteo, E., Mancinelli, E., and Ferroni, A. (1994) *Eur. J. Neurosci.* **6**, 381–391
42. Allen, T. G., and Brown, D. A. (1993) *J. Physiol.* **466**, 173–189
43. Billups, D., Billups, B., Challiss, R. A., and Nahorski, S. R. (2006) *J. Neurosci.* **26**, 9983–9995
44. Patterson, R. L., van Rossum, D. B., Ford, D. L., Hurt, K. J., Bae, S. S., Suh, P. G., Kurosaki, T., Snyder, S. H., and Gill, D. L. (2002) *Cell* **111**, 529–541