THE JOURNAL OF BIOLOGICAL CHEMISTRY VOL. 289, NO. 20, pp. 14321–14330, May 16, 2014
Published in the U.S.A.

Differential Regulation of Primary Afferent Input to Spinal Cord by Muscarinic Receptor Subtypes Delineated Using Knockout Mice*

Received for publication, January 22, 2014, and in revised form, March 19, 2014 Published, JBC Papers in Press, April 2, 2014, DOI 10.1074/jbc.M114.550384

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Background: Activation of muscarinic receptors reduces pain at the spinal level.
Results: M_2 and M_4 subtypes mediate muscarinic inhibition of sensory nerve input. M_5 subtype stimulation directly increases sensory input or indirectly inhibits it through metabotropic glutamate receptors.
Conclusion: M_2, M_4, and M_5 subtypes differentially regulate nociceptive input to the spinal cord.
Significance: This study demonstrates how individual muscarinic receptor subtypes control pain transmission.

Stimulation of muscarinic acetylcholine receptors (mAChRs) inhibits nociceptive transmission at the spinal level. However, it is unclear how each mAChR subtype regulates excitatory synaptic input from primary afferents. Here we examined excitatory postsynaptic currents (EPSCs) of dorsal horn neurons evoked by dorsal root stimulation in spinal cord slices from wild-type and mAChR subtype knock-out (KO) mice. In wild-type mice, mAChR activation with oxotremorine-M decreased the amplitude of monosynaptic EPSCs in ~67% of neurons but increased it in ~10% of neurons. The inhibitory effect of oxotremorine-M was attenuated by the M_2/M_4 antagonist himbacine in the majority of neurons, and the remaining inhibition was abolished by group II/III metabotropic glutamate receptor (mGluR) antagonists in wild-type mice. In M_2/M_4 double-KO mice, oxotremorine-M inhibited monosynaptic EPSCs in significantly fewer neurons (~26%) and increased EPSCs in significantly more neurons (33%) compared with wild-type mice. Blocking group II/III mGluRs eliminated the inhibitory effect of oxotremorine-M in M_2/M_4 double-KO mice. In M_2 single-KO and M_4 single-KO mice, himbacine still significantly reduced the inhibitory effect of oxotremorine-M. However, the inhibitory and potentiating effects of oxotremorine-M on EPSCs in M_3 single-KO and M_1/M_3 double-KO mice were similar to those in wild-type mice. In M_5 single-KO mice, oxotremorine-M failed to potentiate evoked EPSCs, and its inhibitory effect was abolished by himbacine. These findings indicate that activation of presynaptic M_2 and M_4 subtypes reduces glutamate release from primary afferents. Activation of the M_5 subtype either directly increases primary afferent input or inhibits it through indirectly stimulating group II/III mGluRs.

The muscarinic acetylcholine receptors (mAChRs)[3] are closely involved in the regulation of nociceptive transmission at the spinal cord level. It has been demonstrated that spinally administered mAChR agonists or acetylcholinesterase inhibitors reduce pain in both animals and humans (1–5). Conversely, blocking spinal mAChRs causes pain hypersensitivity (6). Among the five mAChR subtypes (M_1–M_5), the odd-numbered subtypes (M_1, M_3, and M_5) couple to $G_{q/11}$ proteins to activate phospholipase C, which leads to inositol trisphosphate-mediated intracellular calcium release and to diacylglycerol-mediated protein kinase C activation. On the other hand, stimulation of even-numbered mAChRs (M_2 and M_4) typically inhibits adenylyl cyclase activity and voltage-activated calcium channels through activation of $G_{i/o}$ proteins (7–9). In both rats and humans, mAChRs in the spinal cord are densest in the superficial dorsal horn (10–12). M_2 is the major mAChR subtype expressed in the spinal cord, whereas M_3 and M_4 subtypes represent only a fraction of the total mAChRs in the spinal cord (5, 13–15). Using mAChR subtype-knock-out (KO) mice and siRNA approaches, we have shown that M_2 and M_4 subtypes in the spinal cord are essential for the analgesic effects produced by mAChR agonists in both rats and mice (5, 14, 16).

Neurons in the superficial dorsal horn, especially the lamina II (substantia gelatinosa), primarily receive sensory information from nociceptive primary afferent nerves (17, 18). Glutamate is the major excitatory neurotransmitter released from central terminals of primary afferent nerves (19, 20). The close contact between primary afferent terminals and second-order sensory neurons in the spinal dorsal horn represents the first synapse for transmission and regulation of sensory input. Inhibition of glutamate release from primary afferents represents a critical mechanism underlying the analgesic effects of activation of

* This work was supported, in whole or in part, by National Institutes of Health Grants NS073935 and NS045602. This work was also supported by a grant from the N. G. and Helen T. Hawkins Endowment (to H.-L. P.).
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[3] The abbreviations used are: mAChR, muscarinic acetylcholine receptor; CPPG, (*RS*)-α-cyclopropyl-4-phosphonophenylglycine; EPSC, excitatory postsynaptic current; eEPSC, evoked EPSC; J104129, (αR)-α-cyclopentyl-α-hydroxy-*N*-[1-(4-methyl-3-pentenyl)-4-piperidinyl]benzeneacetamide fumarate; GDPβS, guanosine 5′-*O*-(2-thiodiphosphate); LY341495, (2*S*)-2-amino-2-[(1*S*,2*S*)-2-carboxycycloprop-1-yl]-3-(xanth-9-yl) propanoic acid; mGluR, metabotropic glutamate receptor.

many $G_{i/o}$-coupled receptors (21). Interestingly, stimulation of mAChRs potentiates glutamate release in the majority of spinal dorsal horn neurons in mice (22), which makes it difficult to explain the analgesic effect of mAChR agonists and acetylcholinesterase inhibitors. Therefore, it is important to determine how each mAChR subtype specifically regulates primary afferent input to second-order dorsal horn neurons in the spinal cord.

In the present study, we took advantage of available mAChR subtype single- and double-KO mice to define the function of individual mAChR subtypes involved in the regulation of primary afferent input to dorsal horn neurons. Our study provides unambiguous evidence that activation of the M_2 and M_4 subtypes directly inhibits primary afferent input to spinal dorsal horn neurons. Our study also reveals a complex role of the M_5 subtype in regulating primary afferent input; activation of the M_5 subtype expressed at primary afferent terminals potentiates primary afferent input, whereas stimulation of the M_5 subtype can also indirectly inhibit primary afferent input through increased glutamate release from spinal interneurons and subsequent activation of group II/III metabotropic glutamate receptors (mGluRs). This new information advances our understanding of how individual mAChR subtypes control nociceptive input and will help in the designing of effective strategies for pain control using mAChR subtype-selective analgesics.

EXPERIMENTAL PROCEDURES

Animals—Wild-type (WT) and mAChR subtype single- and double-KO mice (male, 6–8 weeks old) were obtained from NIDDK, National Institutes of Health (Bethesda, MD). All experimental protocols and procedures were approved by the Animal Care and Use Committee of the University of Texas M.D. Anderson Cancer Center and conformed to the National Institutes of Health guidelines on the ethical use of animals. The genetic background of the M_2-KO, M_4-KO, and M_2/M_4 double-KO mice was C57/BL6. 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. WT mice with the same mixed genetic background served as controls for mutant mice. The generation 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 was described previously (14, 16, 23–26). Mouse genotyping was carried out using polymerase chain reaction analysis of tail DNA (14, 16, 23, 24).

Spinal Cord Slice Preparation—The lumbar segment of the spinal cord was rapidly removed through laminectomy after the mice were anesthetized with 2–3% isoflurane. The animals were then euthanized by inhalation of 5% isoflurane. The spinal tissue was immediately placed in ice-cold sucrose artificial cerebrospinal fluid presaturated with 95% O_2 and 5% CO_2. The sucrose artificial cerebrospinal fluid contained 206 mM sucrose, 2.8 mM KCl, 1.0 mM $MgCl_2$, 1.0 mM $CaCl_2$, 1.2 mM NaH_2PO_4, 25.0 mM glucose, and 26.0 mM $NaHCO_3$. Transverse spinal cord slices (350 μm thick) were cut using a vibratome in the ice-cold sucrose artificial cerebrospinal fluid 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 117.0 mM NaCl, 3.6 mM KCl, 1.2 mM $MgCl_2$, 2.5 mM $CaCl_2$, 1.2 mM NaH_2PO_4, 11.0 mM glucose, and 25.0 mM $NaHCO_3$. Each slice was placed in a glass-bottomed chamber and was continuously perfused with Krebs' solution at 5.0 ml/min at 34 °C.

Electrophysiological Recordings—Recordings of excitatory postsynaptic currents (EPSCs) were performed using the whole-cell voltage clamp method, as we described previously (22, 27, 28). Neurons located in the outer zone of lamina II were identified using infrared and differential interference contrast optics. These neurons were selected for recording because they predominantly receive direct input from nociceptive primary afferents (20, 27, 29). Recording electrodes with a resistance of 5–10 megaohms were pulled from borosilicate glass capillaries and filled with internal solution containing 135.0 mM potassium gluconate, 5.0 mM KCl, 2.0 mM $MgCl_2$, 0.5 mM $CaCl_2$, 5.0 mM HEPES, 5.0 mM EGTA, 5.0 mM ATP-Mg, 0.5 mM Na-GTP, 1.0 mM GDPβS, and 10.0 mM lidocaine *N*-ethyl bromide, adjusted to pH 7.2–7.4 with 1 M KOH (290–300 mosM). GDPβS, a general G protein inhibitor, was added to the internal solution to block the postsynaptic effect mediated by mAChR agonists through G proteins (27, 30).

EPSCs were elicited by electrical stimulation (0.2 ms, 0.3–0.6 mA, 0.2 Hz) through a bipolar tungsten electrode placed on the dorsal root, which stimulates both Aδ- and C-fibers (27, 30). EPSCs were recorded at a holding potential of −60 mV in the presence of 2 μM strychnine (a glycine receptor antagonist) and 10 μM bicuculline (a $GABA_A$ receptor antagonist). Monosynaptic EPSCs were identified on the basis of their constant latency and the absence of conduction failure of evoked EPSCs in response to a 20-Hz electrical stimulation (27, 31). Recordings of EPSCs began 6–7 min after whole-cell access was established and the current reached a steady state. In some neurons, a paired pulse protocol (29) was used to confirm the presynaptic effect of oxotremorine-M. The paired pulse ratio was expressed as the ratio of the amplitude of the second synaptic response to the amplitude of the first synaptic response. The input resistance was monitored, and the recording was abandoned if it changed more than 15%. The signals were processed with an amplifier (MultiClamp700A; Axon Instruments, Union City, CA), filtered at 1–2 kHz, digitized at 10 kHz, and stored in a computer.

Oxotremorine-M and himbacine were obtained from Sigma-Aldrich. (*RS*)-C-cyclopropyl-4-phosphonophenylglycine (CPPG), (αR)-α-cyclopentyl-α-hydroxy-*N*-[1-(4-methyl-3-pentenyl)-4-piperidinyl]benzeneacetamide fumarate (J104129), and (2*S*,1′*S*,2′*S*)-2-(2-carboxycyclopropyl)-2-(9*H*-xanthen-9-yl) glycine (LY341495) were purchased from Tocris (Ellisville, MO). All of the drugs were dissolved in Krebs' solution immediately before the experiment was started and were perfused using syringe pumps.

Data Analysis—Data are presented as means ± S.E. The amplitude of evoked EPSCs was analyzed using Clampfit (Axon Instruments). The effect of drugs on EPSCs was analyzed over a period of 3 min during drug application. Neurons were considered to be responsive to oxotremorine-M if the amplitude of evoked EPSCs was altered by >15%. The drug effect on the amplitude of EPSCs was determined by using repeated measures analysis of variance with Dunnett's or Tukey's post hoc test. The difference in the percentage of neurons responsive to oxotremorine-M between WT and mAChR subtype-KO mice

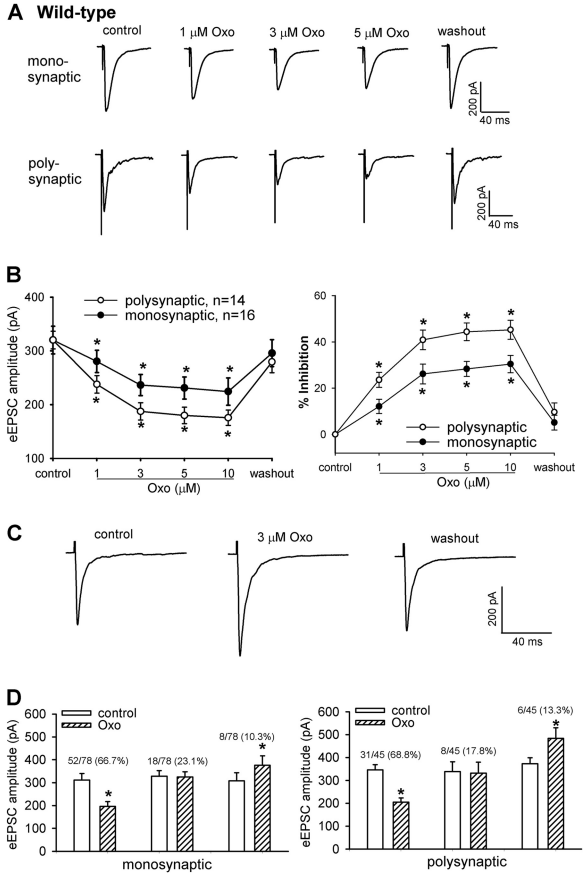

FIGURE 1. **Effects of oxotremorine-M on glutamatergic EPSCs of spinal dorsal horn neurons evoked from the dorsal root in WT mice.** *A*, original traces of EPSCs show the inhibitory effect of 1–5 μM oxotremorine-M on monosynaptic and polysynaptic EPSCs recorded from two separate neurons in one WT mouse. *B*, group data show the concentration-dependent inhibitory effect of 1–10 μM oxotremorine-M on the amplitude of evoked EPSCs (eEPSCs) recorded from dorsal neurons in WT mice. *C*, original traces show the stimulating effect of 3 μM oxotremorine-M on evoked monosynaptic EPSCs in one dorsal horn neuron of a WT mouse. *D*, three types of neurons for which 3 μM oxotremorine-M had distinct effects on monosynaptic and polysynaptic EPSCs of dorsal horn neurons in WT mice. Data are presented as means ± S.E. (*error bars*). *, $p < 0.05$ compared with the control.

was determined using Fisher's exact test. $p < 0.05$ was considered to be statistically significant.

RESULTS

Effect of Oxotremorine-M on Primary Afferent Stimulation-evoked Glutamatergic EPSCs in WT Mice—To determine the effect of oxotremorine-M on glutamate release from primary afferents to lamina II neurons, the effect of this agent on identified monosynaptic EPSCs in WT mice was studied. Glutamatergic EPSCs were elicited by electrical stimulation of the dorsal root. Oxotremorine-M (1, 3, 5, and 10 μM), a specific mAChR agonist that acts on all five mAChR subtypes, was perfused in a cumulative fashion onto the slice chamber. Each concentration was applied for 3 min. Because the effect of oxotremorine-M on glutamatergic EPSCs was similar among two the WT groups of mice (C57/BL6 and 129SvEv/CF1 genetic backgrounds) (22), data obtained from the two WT control groups were pooled. Oxotremorine-M at 1–10 μM produced a concentration-dependent reduction in the peak amplitude of monosynaptic EPSCs in 16 neurons examined (Fig. 1, *A* and *B*). Also, oxotremorine-M concentration-dependently reduced the amplitude of polysynaptic EPSCs (reflecting glutamate release from both primary afferent terminals and interneurons) in another 14 neurons tested. The inhibitory effect of oxotremorine-M was significantly greater in polysynaptic EPSCs than in monosynaptic EPSCs in lamina II neurons (Fig. 1, *A* and *B*).

In lamina II neurons recorded from WT mice, oxotremorine-M at 3 μM significantly inhibited the amplitude of monosynaptic EPSCs in 52 of 78 (66.7%) neurons (Fig. 1*D*). Similarly, oxotremorine-M significantly reduced the amplitude of polysynaptic EPSCs in 31 of 45 (68.8%) neurons. Bath application of 3 μM oxotremorine-M either had no effect or significantly increased the amplitude of monosynaptic and polysynaptic EPSCs in a small population of lamina II neurons (Fig. 1, *C* and *D*).

To determine the role of M_2 and M_4 subtypes in the inhibitory effect of oxotremorine-M on synaptic glutamate release from primary afferents in WT mice, we used himbacine, a selective antagonist for M_2 and M_4 subtypes (28, 32–34). After the initial effect of 3 μM oxotremorine-M on monosynaptic EPSCs was tested, 2 μM himbacine was bath-perfused for 3 min before reapplication of oxotremorine-M. The effective concentration of himbacine has been determined previously (28, 35). In 12 of 19 neurons in which initial application of oxotremorine-M significantly reduced the amplitude of monosynaptic EPSCs, himbacine abolished the inhibitory effect of oxotremorine-M on the amplitude of EPSCs (Fig. 2*A*). In the remaining seven neurons, himbacine only partially blocked the inhibitory effect of oxotremorine-M. To determine whether group II/III mGluRs are involved in the inhibitory effect of oxotremorine-M on glutamate release from primary afferents, we used LY341495 and CPPG, which are group II and group III mGluR antagonists, respectively (36, 37). The remaining inhibitory effect of oxotremorine-M on monosynaptic EPSCs was completely blocked by subsequent treatment with 100 nM LY341495 plus 200 μM CPPG in these seven neurons (Fig. 2*A*).

In 15 lamina II neurons recorded from WT mice, the inhibitory effect of 3 μM oxotremorine-M on the amplitude of polysynaptic EPSCs was completely blocked by 2 μM himbacine in nine neurons (Fig. 2*B*). In the remaining six neurons, himbacine did not fully block the inhibitory effect of oxotremorine-M on polysynaptic EPSCs. Subsequent application of 100 nM LY341495 plus 200 μM CPPG abolished the remaining inhibitory effect of oxotremorine-M on polysynaptic EPSCs in these six neurons (Fig. 2*B*).

In an additional nine neurons, bath application of oxotremorine-M significantly increased the paired pulse ratio of monosynaptic EPSCs evoked from the dorsal root (Fig. 2*C*), suggesting a presynaptic effect of oxotremorine-M. We also determined the role of group II/III mGluRs in muscarinic inhibition of monosynaptic EPSCs in the absence of himbacine. Treatment with LY341495 and CPPG abolished the inhibitory effect of oxotremorine-M on monosynaptic EPSCs in 8 of 20 neurons and partially inhibited the oxotremorine-M effect in the remaining 12 neurons (Fig. 2*D*).

Effect of Oxotremorine-M on Primary Afferent Stimulation-evoked EPSCs in M_2/M_4 Double-KO Mice—To delineate the role of both M_2 and M_4 subtypes in the action of oxotremorine-M on glutamate release from primary afferents, we tested

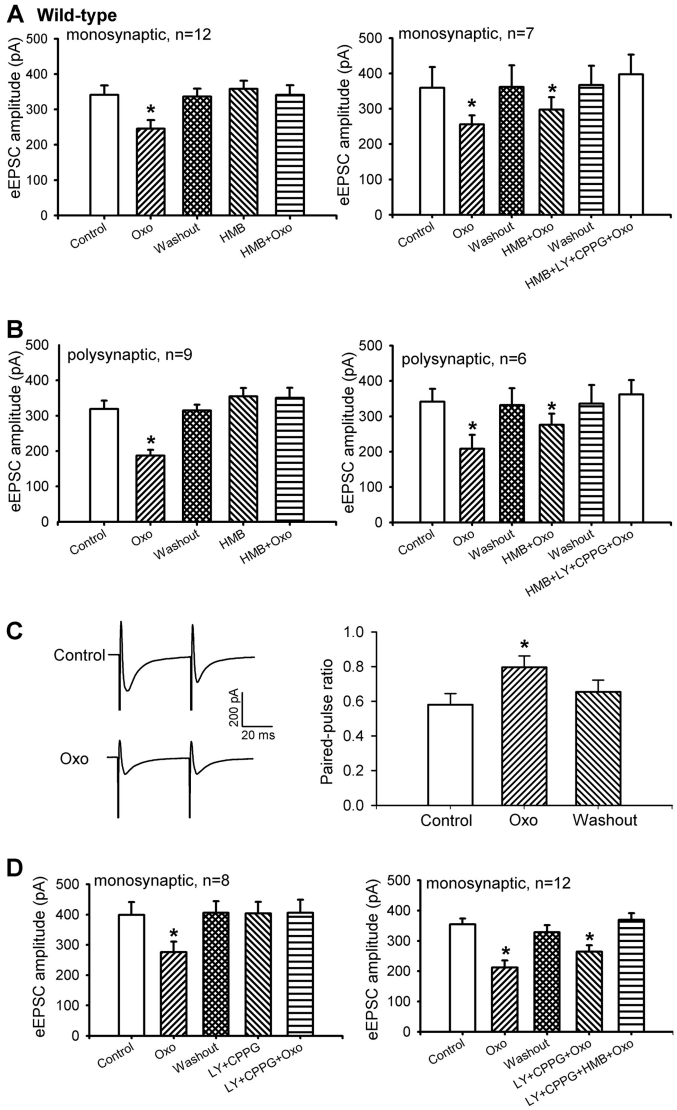

FIGURE 2. **Role of M_2/M_4 subtypes and group II/III mGluRs in the inhibitory effect of oxotremorine-M on monosynaptic and polysynaptic EPSCs of dorsal horn neurons in WT mice.** *A*, summary data show that himbacine at least partially blocked the inhibitory effect of oxotremorine-M and that subsequent LY341495 plus CPPG further blocked the oxotremorine-M effect on monosynaptic EPSCs of dorsal neurons in WT mice. *B*, same as *A* but with polysynaptic EPSCs. *C*, raw recording traces and mean data show the effect of 3 μM oxotremorine-M on the paired pulse ratio of evoked monosynaptic EPSCs in nine neurons. *D*, group data show that LY341495 plus CPPG either abolished or partially inhibited the inhibitory effect of oxotremorine-M on monosynaptic EPSCs in lamina II neurons. Data are presented as means ± S.E. (*error bars*). *, $p < 0.05$ compared with the baseline control.

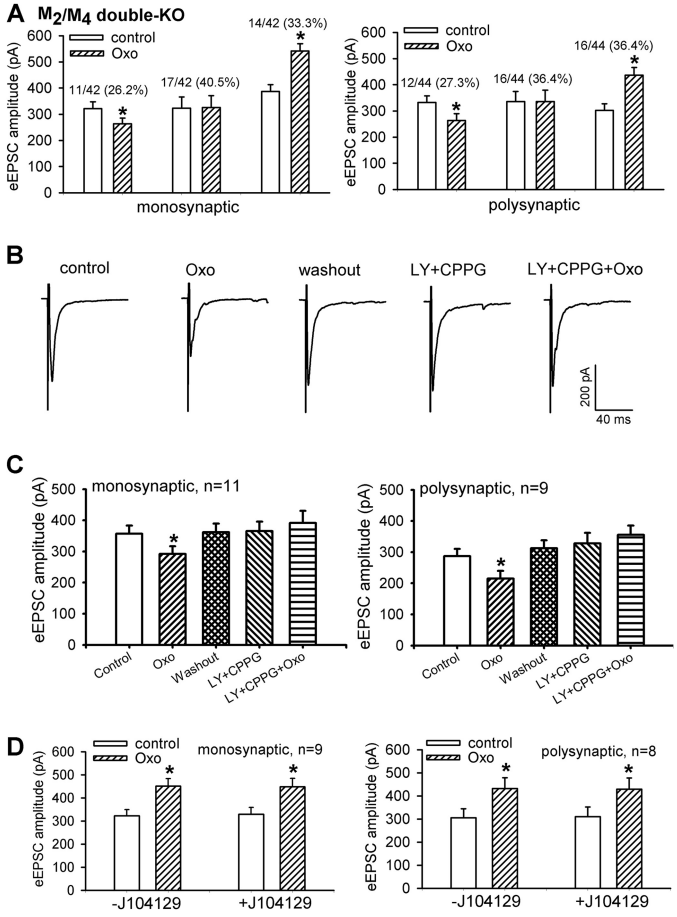

FIGURE 3. **Effects of oxotremorine-M on monosynaptic and polysynaptic EPSCs of dorsal neurons evoked from the dorsal root in M_2/M_4 double-KO mice.** *A*, summary data show three types of neurons in which 3 μM oxotremorine-M had distinct effects on monosynaptic and polysynaptic EPSCs of dorsal horn neurons in M_2/M_4 double-KO mice. *B* and *C*, representative traces and group data show that LY341495 plus CPPG blocked the inhibitory effect of oxotremorine-M on monosynaptic and polysynaptic EPSCs in dorsal neurons recorded from M_2/M_4 double-KO mice. *D*, summary data show the potentiating effect of 3 μM oxotremorine-M on monosynaptic and polysynaptic EPSCs before and during application of J104129 in dorsal neurons from M_2/M_4 double-KO mice. Data are presented as means ± S.E. (*error bars*). *, $p < 0.05$ compared with the respective controls.

the effect of oxotremorine-M on monosynaptic EPSCs in M_2/M_4 double-KO mice. Bath application of 3 μM oxotremorine-M significantly decreased the peak amplitude of monosynaptic EPSCs in only 11 of 42 neurons (26.2%; Fig. 3*A*). The inhibitory effect of oxotremorine-M on monosynaptic EPSCs occurred in significantly fewer neurons in M_2/M_4 double-KO mice than in WT mice (66.7%). In another 14 of 42 neurons (33.3%), oxotremorine-M significantly increased the amplitude of monosynaptic EPSCs evoked from the dorsal root (Fig. 3*A*), which was significantly greater than that (10.3%) in WT mice. Similarly, oxotremorine-M inhibited the amplitude of polysynaptic EPSCs in significantly fewer (27.3% *versus* 68.8%) lamina II neurons and increased the amplitude of polysynaptic EPSCs in significantly more (36.4% *versus* 13.3%) neurons in M_2/M_4 double-KO mice (Fig. 3*A*), compared with WT mice. Furthermore, the degree of inhibition of monosynaptic and polysynaptic EPSCs produced by 3 μM oxotremorine-M was significantly lower in M_2/M_4 double-KO mice than in WT mice (Figs. 1*D* and 3*A*).

We then determined whether group II/III mGluRs contribute to the inhibitory effect of oxotremorine-M on monosynaptic and polysynaptic EPSCs evoked from primary afferents in M_2/M_4 double-KO mice. In 11 lamina II neurons in which initial application of 3 μM oxotremorine-M reduced the amplitude of monosynaptic EPSCs, bath application of 100 nM LY341495 plus 200 μM CPPG completely blocked the inhibitory effect of oxotremorine-M in all neurons tested (Fig. 3, *B* and *C*). Also, in another nine neurons recorded from M_2/M_4 double-KO mice, oxotremorine-M failed to reduce the amplitude of polysynaptic EPSCs in the presence of LY341495 and CPPG (Fig. 3*C*). In all six neurons from the M_2/M_4 double-KO mice, himbacine did

not alter the inhibitory effect of oxotremorine-M on evoked EPSCs.

In addition, we determined whether the M_3 subtype is involved in the potentiating effect of oxotremorine-M on evoked EPSCs in the spinal cord slices of M_2/M_4 double-KO mice. We used J104129, an M_3 subtype-preferring antagonist (22, 38). Bath application of 50 nM J104129 did not significantly alter the potentiating effect of oxotremorine-M on the peak amplitude of monosynaptic EPSCs or polysynaptic EPSCs in all lamina II neurons tested (Fig. 3*D*). These results indicate that the M_2/M_4 subtypes contribute to mAChR activation-induced reduction in glutamatergic input from primary afferents and that group II/III mGluRs mediate the inhibition of primary afferent input by mAChR activation in the spinal dorsal horn.

Effect of Oxotremorine-M on Primary Afferent Stimulation-evoked EPSCs in M_2-KO Mice—To further determine the role of the M_2 subtype in the mAChR inhibition of glutamate release from primary afferents, we examined the effect of oxotremorine-M on monosynaptic EPSCs in M_2-KO mice. In 17 of 44 (38.6%) lamina II neurons from M_2-KO mice, 3 μM oxotremorine-M significantly decreased the amplitude of monosynaptic EPSCs. Oxotremorine-M significantly increased the amplitude of monosynaptic EPSCs in 14 of 44 (31.8%) neurons (Fig. 4*A*). Compared with WT mice, oxotremorine-M inhibited monosynaptic EPSCs in significantly fewer neurons and potentiated monosynaptic EPSCs in significantly more neurons in M_2-KO mice.

In 11 of 19 neurons from M_2-KO mice in which 3 μM oxotremorine-M initially inhibited the amplitude of monosynaptic EPSCs, 2 μM himbacine abolished the inhibitory effect of oxotremorine-M (Fig. 4*B*). In the remaining eight neurons, himbacine only partially reduced the effect of oxotremorine-M. Further treatment with 100 nM LY341495 and 200 μM CPPG completely blocked the inhibitory effect of oxotremorine-M in these eight neurons (Fig. 4*C*).

Effect of Oxotremorine-M on Primary Afferent Stimulation-evoked EPSCs in M_4-KO Mice—Next, we assessed the role of the M_4 subtype in the mAChR inhibition of primary afferent input using M_4-KO mice. In a total of 46 lamina II neurons recorded from M_4-KO mice, bath application of 3 μM oxotremorine-M significantly decreased the amplitude of monosynaptic EPSCs in 21 neurons (45.7%; Fig. 5*A*). In 12 of 46 (26.1%) neurons, oxotremorine-M significantly increased the amplitude of monosynaptic EPSCs (Fig. 5*A*). Compared with WT mice, oxotremorine-M inhibited monosynaptic EPSCs in significantly fewer neurons and potentiated monosynaptic EPSCs in significantly more neurons in M_4-KO mice.

In 10 of 17 lamina II neurons recorded from M_4-KO mice, himbacine completely blocked the inhibitory effect of 3 μM oxotremorine-M on the amplitude of monosynaptic EPSCs (Fig. 5*B*). In another seven neurons, himbacine did not fully block the inhibitory effect of oxotremorine-M on monosynaptic EPSCs, but this remaining inhibitory effect was abolished by subsequent treatment with LY341495 and CPPG (Fig. 5*C*). The above data indicate that M_2 and M_4 subtypes as well as group II/III mGluRs are involved in inhibition of glutamatergic input from primary afferents to dorsal horn neurons by mAChR activation.

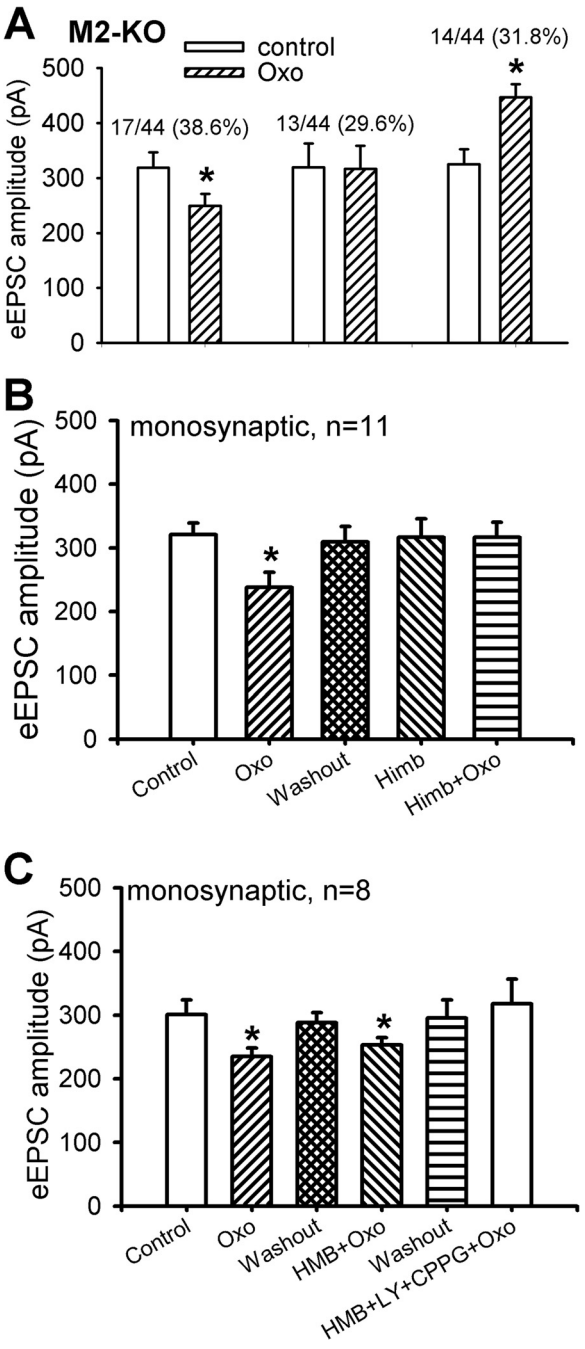

FIGURE 4. **Effects of oxotremorine-M on evoked monosynaptic EPSCs of dorsal horn neurons from M_2 single-KO mice.** *A*, group data show three types of neurons in which 3 μM oxotremorine-M had distinct effects on monosynaptic EPSCs of dorsal horn neurons in M_2-KO mice. *B*, summary data show the inhibitory effect of oxotremorine-M on the amplitude of monosynaptic EPSCs of dorsal horn neurons before and during himbacine application. *C*, summary data show that LY341495 and CPPG blocked the inhibitory effect of oxotremorine-M on monosynaptic EPSCs of dorsal horn neurons in the presence of himbacine. Data are presented as mean ± S.E. (*error bars*). *, $p < 0.05$ compared with the control.

Effect of Oxotremorine-M on Primary Afferent Stimulation-evoked EPSCs in M_1/M_3 Double-KO Mice—To examine whether M_1 and M_3 subtypes are involved in the effect of oxotremorine-M on primary afferent input, we examined the effect of oxotremorine-M on monosynaptic EPSCs in M_1/M_3 double-KO mice. In a total of 32 lamina II neurons recorded

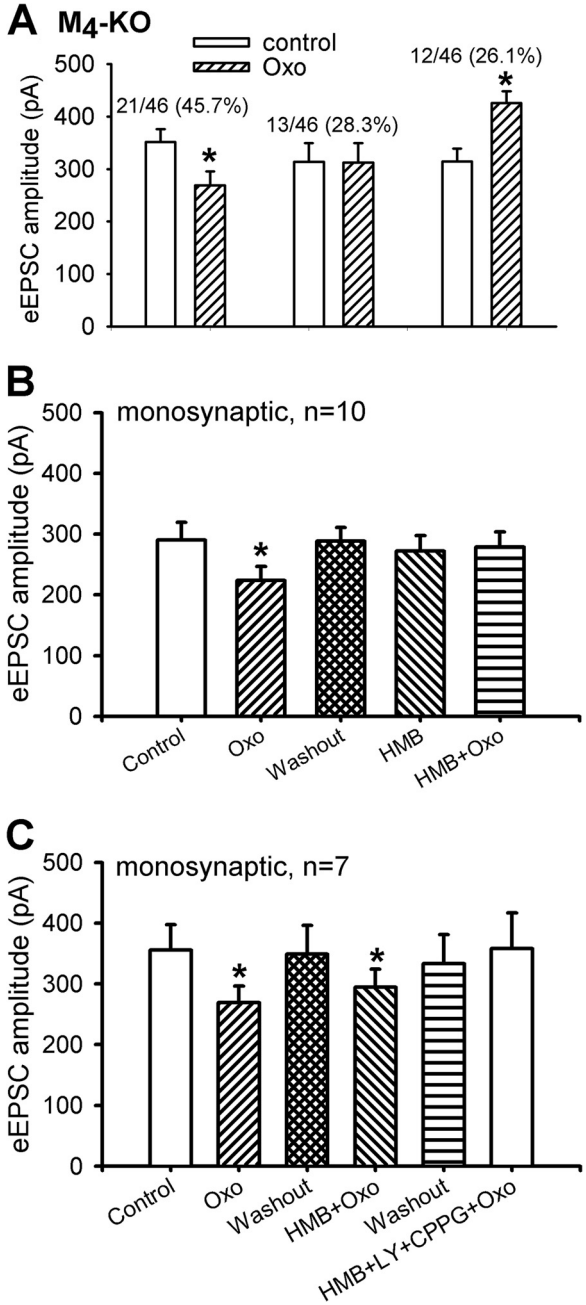

FIGURE 5. **Effects of oxotremorine-M on monosynaptic EPSCs of dorsal horn neurons evoked from the dorsal root in M_4 single-KO mice.** *A*, summary data show three types of neurons in which oxotremorine-M had distinct effects on the amplitude of monosynaptic EPSCs of dorsal horn neurons. *B*, group data show that himbacine blocked the inhibitory effect of oxotremorine-M on the amplitude of monosynaptic EPSCs of dorsal horn neurons in M_4-KO mice. *C*, summary data show that LY341495 and CPPG abolished the inhibitory effect of oxotremorine-M on monosynaptic EPSCs of dorsal horn neurons in the presence of himbacine. Data are presented as mean ± S.E. (*error bars*). *, $p < 0.05$ compared with the control.

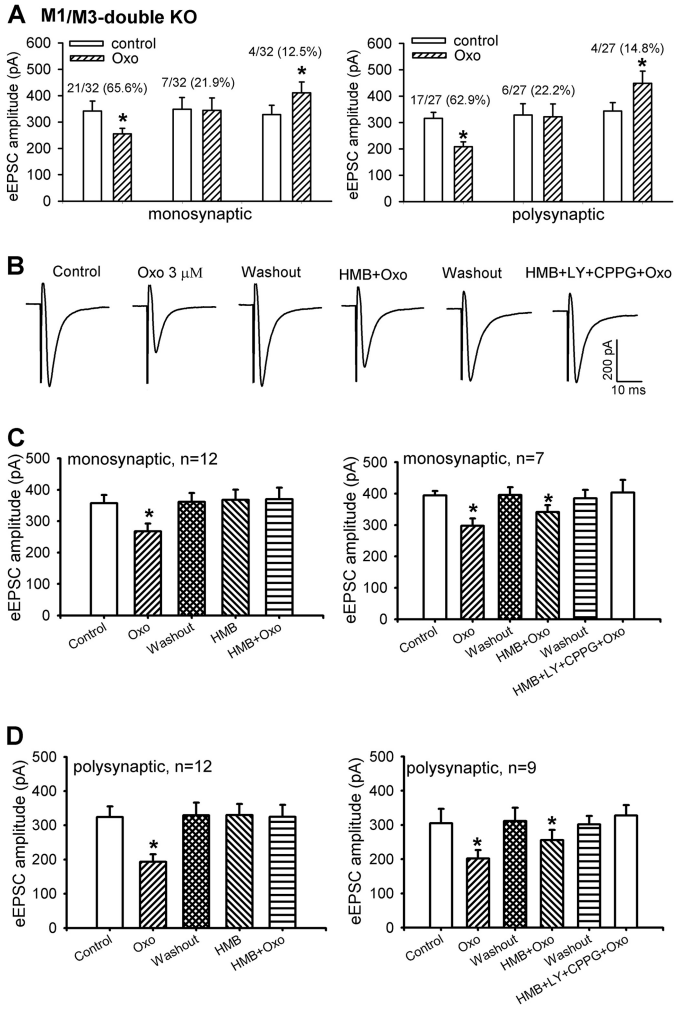

FIGURE 6. **Effects of oxotremorine-M on monosynaptic and polysynaptic EPSCs of dorsal horn neurons in M_1/M_3 double-KO mice.** *A*, summary data show three types of neurons in which 3 μM oxotremorine-M had distinct effects on monosynaptic and polysynaptic EPSCs of dorsal horn neurons in M_1/M_3 double-KO mice. *B*, original traces show that LY341495 and CPPG abolished the inhibitory effect of oxotremorine-M on monosynaptic EPSCs of one neuron in the presence of himbacine. *C*, *bar graphs* show that himbacine alone abolished the inhibitory effect of oxotremorine-M on monosynaptic EPSCs of dorsal horn neurons recorded from M_1/M_3 double-KO mice. *D*, summary data show that LY341495 and CPPG abolished the inhibitory effect of oxotremorine-M on monosynaptic EPSCs in the presence of himbacine in dorsal horn neurons recorded from M_1/M_3 double-KO mice. Data are presented as mean ± S.E. (*error bars*). *, $p < 0.05$ compared with the respective controls.

from M_1/M_3 double-KO mice, 3 μM oxotremorine-M significantly decreased the amplitude of monosynaptic EPSCs in 21 (65.6%) neurons. In 4 of 32 (12.5%) neurons, oxotremorine-M significantly increased the amplitude of monosynaptic EPSCs (Fig. 6*A*). Furthermore, in a total of 27 lamina II neurons with polysynaptic EPSCs from M_1/M_3 double-KO mice, oxotremorine-M significantly decreased the EPSC amplitude in 17 (62.9%) neurons. Oxotremorine-M significantly increased the amplitude of polysynaptic EPSCs in 4 of 27 (14.8%) neurons recorded from M_1/M_3 double-KO mice (Fig. 6*A*).

In 12 of 19 lamina II neurons from M_1/M_3 double-KO mice in which initial oxotremorine-M inhibited the amplitude of monosynaptic EPSCs, bath application of 2 μM himbacine abolished the effect of oxotremorine (Fig. 6, *B* and *C*). In the rest of the seven neurons, himbacine did not fully block the inhibitory effect of oxotremorine-M on the amplitude of monosynaptic EPSCs. This remaining inhibitory effect was completely blocked by subsequent application of 100 nM LY341495 and 200 μM CPPG (Fig. 6*C*).

In 12 of 21 lamina II neurons with polysynaptic EPSCs recorded from M_1/M_3 double-KO mice, himbacine abolished

the inhibitory effect of oxotremorine-M. In the remaining nine neurons, himbacine only partially reduced the inhibitory effect of oxotremorine-M. The residual effect of oxotremorine-M on polysynaptic EPSCs was completely blocked by further treatment with LY341495 and CPPG in these nine neurons (Fig. 6*D*).

Effect of Oxotremorine-M on Primary Afferent Stimulation-evoked EPSCs in M_3-KO Mice—We also explored whether the M_3 subtype could be involved in the control of primary afferent input by mAChRs. In a total of 32 lamina II neurons from M_3-KO mice, 3 μM oxotremorine-M significantly decreased the amplitude of monosynaptic EPSCs in 21 (65.6%) neurons. In another 4 of 32 (12.5%) neurons, oxotremorine-M significantly increased the amplitude of monosynaptic EPSCs (Fig. 7*A*).

In 12 of 20 lamina II neurons recorded from M_3-KO mice, himbacine abolished oxotremorine-M-induced inhibition of the amplitude of monosynaptic EPSCs (Fig. 7*B*). In the remaining eight neurons, oxotremorine-M still significantly attenuated the amplitude of monosynaptic EPSCs in the presence 2 μM himbacine. This remaining inhibitory effect was abolished by subsequent application of 100 nM LY341495 and 200 μM CPPG (Fig. 7*C*). Because the inhibitory and potentiating effects of oxotremorine-M on monosynaptic EPSCs in M_1/M_3 double-KO mice and M_3-KO mice were similar to those in WT mice, we conclude that M_1 and M_3 subtypes do not contribute significantly to the regulation of primary afferent input by mAChRs.

Effect of Oxotremorine-M on Primary Afferent Stimulation-evoked EPSCs in M_5-KO Mice—In addition, we determined whether the M_5 subtype contributes to the regulation of primary afferent input by mAChRs using M_5-KO mice. In 23 of 37 (62.2%) lamina II neurons from M_5-KO mice, 3 μM oxotremorine-M significantly inhibited the peak amplitude of monosynaptic EPSCs. Oxotremorine-M had no significant effect on the amplitude of monosynaptic EPSCs in the rest of 14 (37.8%) neurons (Fig. 8*A*). The proportion of neurons with different responses to oxotremorine-M (*i.e.* by decreasing or increasing the amplitude of monosynaptic EPSCs) in WT and various mAChR subtype-KO mice is summarized in Fig. 9. For polysynaptic EPSCs evoked from the dorsal root, 3 μM oxotremorine-M significantly reduced the amplitude of EPSCs in 22 of 36 (61.1%) neurons but had no effect on polysynaptic EPSCs in the remaining 14 (39.9%) neurons from M_5-KO mice (Fig. 8*A*).

Furthermore, in lamina II neurons in which oxotremorine-M initially reduced the amplitude of monosynaptic and polysynaptic EPSCs recorded from M_5-KO mice, himbacine alone completely blocked the inhibitory effect of oxotremorine-M on monosynaptic and polysynaptic EPSCs in all neurons tested (Fig. 8, *B* and *C*). These data indicate that direct activation of the M_5 subtype located on primary afferent terminals potentiates primary afferent input. Also, the M_5 subtype is indirectly involved in the inhibition of primary afferent input by group II/III mGluRs following mAChR stimulation in the spinal cord.

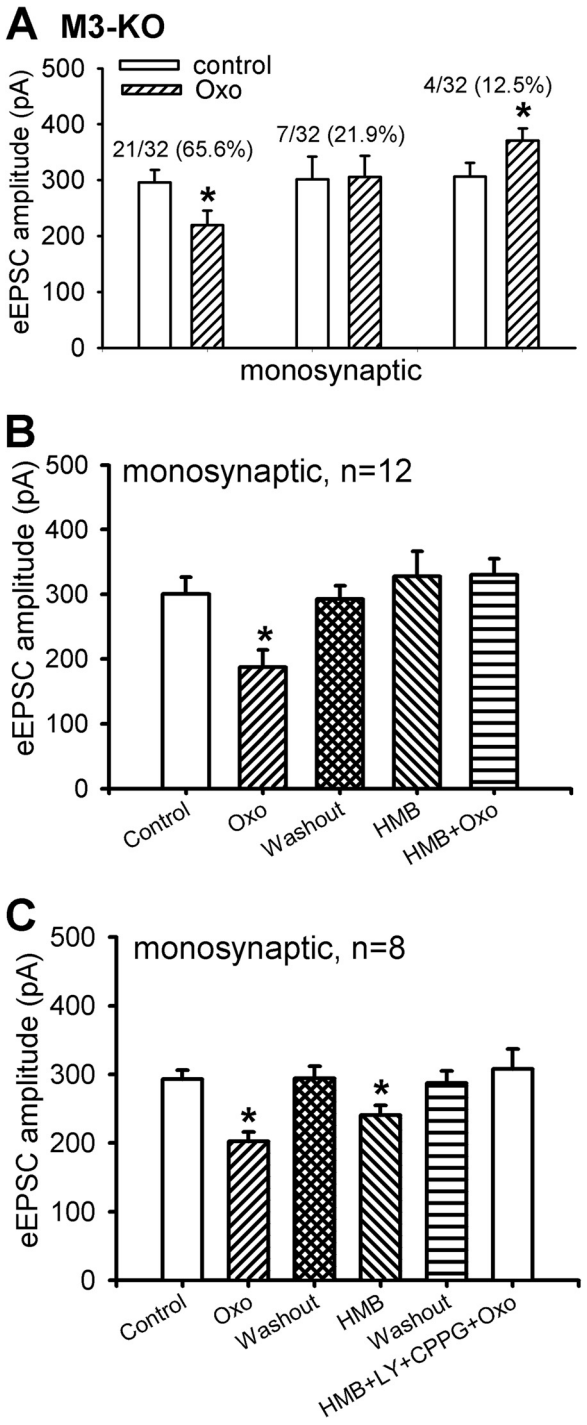

FIGURE 7. **Effects of oxotremorine-M on monosynaptic EPSCs of dorsal horn neurons recorded from M_3 single-KO mice.** *A*, *bar graph* shows three types of neurons in which 3 μM oxotremorine-M had distinct effects on monosynaptic EPSCs of dorsal horn neurons in M_3-KO mice. *B*, summary data show the inhibitory effect of oxotremorine-M on the amplitude of monosynaptic EPSCs of dorsal horn neurons before and during himbacine application. *C*, group data show that LY341495 and CPPG blocked the inhibitory effect of oxotremorine-M on monosynaptic EPSCs of dorsal horn neurons in the presence of himbacine. Data are presented as mean ± S.E. (*error bars*). *, $p < 0.05$ compared with the control.

DISCUSSION

The major objective of this study was to combine genetic and electrophysiological approaches to define the functional role of individual mAChR subtypes in the regulation of primary afferent input to spinal dorsal horn neurons. Because there are no highly selective mAChR subtype agonists and antagonists, it is difficult to rely on pharmacological agents alone to delineate the specific role of individual mAChR subtypes in the regulation of nociceptive transmission at the spinal level. The use of

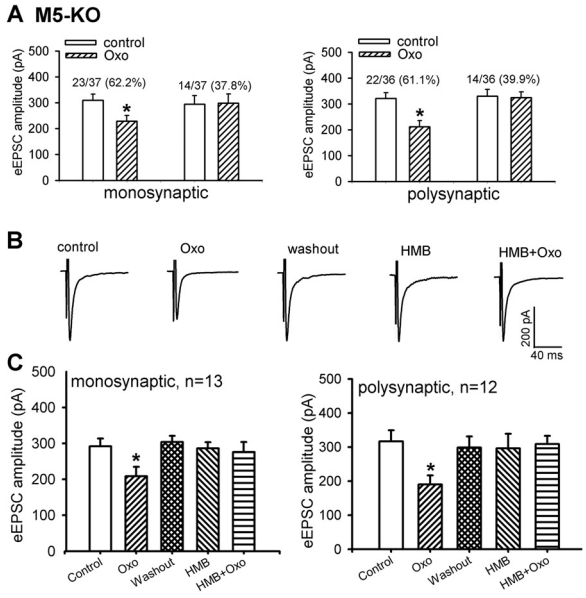

FIGURE 8. **Effects of oxotremorine-M on monosynaptic and polysynaptic EPSCs of dorsal horn neurons in M_5-KO mice.** *A*, group data show that 3 μM oxotremorine-M either inhibited or had no effect on monosynaptic and polysynaptic EPSCs of dorsal horn neurons recorded from M_5-KO mice. *B*, original traces show that himbacine alone abolished the inhibitory effect of oxotremorine-M on monosynaptic EPSCs of one dorsal neuron from a M_5-KO mouse. *C*, *bar graphs* show that himbacine alone completely blocked the inhibitory effect of oxotremorine-M on monosynaptic and polysynaptic EPSCs of dorsal horn neurons recorded from M_5-KO mice. Data are presented as mean ± S.E. (*error bars*). *, $p < 0.05$ compared with the respective controls.

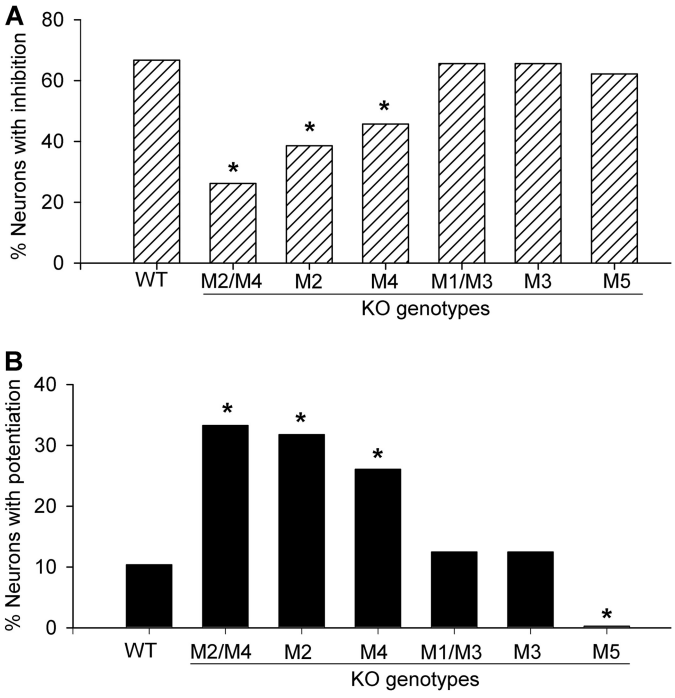

FIGURE 9. **Distinct effects of oxotremorine-M on glutamate release from primary afferent terminals to dorsal horn neurons in WT and various mAChR subtype-KO mice.** *A* and *B*, histograms show the proportion of neurons with different responses to oxotremorine-M (*i.e.* by decreasing or increasing the amplitude of monosynaptic EPSCs) in WT and various mAChR subtype-KO mice. *, $p < 0.05$ compared with the WT group.

spinal cord slice preparation is appropriate for this study, because it preserves the intrinsic connection between primary afferent terminals and second-order neurons, where native mAChR subtypes are expressed. We reported previously that the mAChR agonist oxotremorine-M increased the frequency of spontaneous EPSCs in two-thirds of lamina II neurons in WT mice (22). However, increased glutamatergic transmission in the spinal cord by mAChR activation is inconsistent with the potent inhibitory effects of mAChR agonists on nociception and the excitability of spinal dorsal horn neurons *in vivo* (5, 14, 39). Also, recordings of spontaneous EPSCs alone cannot identify the sources of glutamate release, especially glutamatergic input from primary afferent nerves. Therefore, in this study, we specifically determined how each mAChR subtype regulates primary afferent input by recording monosynaptic EPSCs evoked from the dorsal root. We found that oxotremorine-M significantly decreased the amplitude of monosynaptic and polysynaptic EPSCs in about two-thirds of lamina II neurons in WT mice. Furthermore, blocking M_2 and M_4 subtypes with himbacine (32, 33) either completely or partially blocked the inhibitory effect of oxotremorine-M in all laminar II neurons examined in WT mice. Interestingly, oxotremorine-M inhibited polysynaptic EPSCs more than monosynaptic EPSCs in dorsal horn neurons in WT mice. This result is probably due to the presence of additional M_2/M_4 subtypes and group II/II mGluRs on spinal cord interneurons involved in the polysynaptic transmission. These data suggest that activation of presynaptic M_2 and M_4 subtypes inhibits excitatory glutamatergic input from primary afferents in the majority of dorsal horn neurons.

Our study provides unambiguous evidence showing that both M_2 and M_4 subtypes are expressed at primary afferent terminals to inhibit their input to dorsal horn neurons. In support of this conclusion, we found that the inhibitory effect of oxotremorine-M on monosynaptic and polysynaptic EPSCs evoked by dorsal root stimulation was significantly reduced and occurred in significantly fewer dorsal horn neurons in M_2/M_4 double-KO mice than in WT mice. Because himbacine still significantly attenuated the inhibitory effect of oxotremorine-M on monosynaptic EPSCs in M_2 single-KO and M_4 single-KO mice, these data indicate that both M_2 and M_4 subtypes contribute to muscarinic inhibition of primary afferent input. In the spinal cord, the M_2 subtype represents ~90% of mAChRs, which are mainly distributed in the superficial dorsal horn (13, 14, 40). Stimulation of the M_2 and M_4 subtypes can inhibit the activity of voltage-gated calcium channels in primary sensory neurons (9), which can explain their inhibitory effect on monosynaptic and polysynaptic EPSCs evoked from the dorsal root. Although the spinal M_4 subtype is expressed at low levels (14, 15), its expression level is significantly increased in primary sensory neurons in painful diabetic neuropathy (9). Thus, the M_4 subtype remains an attractive therapeutic target for neuropathic pain control. Our electrophysiological results are consistent with animal behavioral data regarding the critical role of the M_2 and M_4 subtypes involved in muscarinic analgesia at the spinal level (5, 14).

Another salient finding of our study is that the M_5 subtype plays a complex role in regulating primary afferent input. We found that in WT mice, oxotremorine-M significantly increased the amplitude of monosynaptic and polysynaptic EPSCs in a small population (10–13%) of dorsal horn neurons. This potentiating effect became more pronounced in many

dorsal horn neurons recorded from M_2/M_4 double-KO mice. We found that blocking the M_3 subtype did not significantly alter the stimulating effect of oxotremorine-M on evoked EPSCs in M_2/M_4 double-KO mice. Furthermore, the stimulating effect of oxotremorine-M on monosynaptic and polysynaptic EPSCs was not altered in M_3-KO and M_1/M_3 double-KO mice. Thus, the M_1 and M_3 subtypes are not significantly involved in the muscarinic control of primary afferent input. Strikingly, the potentiating effect of oxotremorine-M on evoked EPSCs was completely absent in M_5-KO mice. These findings indicate that the M_5 subtype is expressed at the primary afferent terminals and that its stimulation can increase primary sensory input in a subpopulation of dorsal horn neurons.

We previously showed that the M_5 subtype is mainly responsible for the increased frequency of spontaneous glutamatergic EPSCs and indirect activation of group II/III mGluRs in the spinal cord induced by oxotremorine-M (22). Group II/III mGluRs are $G_{i/o}$-protein-coupled receptors, which can inhibit voltage-gated calcium channels (41, 42) and attenuate glutamatergic transmission in the spinal dorsal horn (43, 44). When spinal glutamate release is potentiated by oxotremorine-M, glutamate can spill over to activate group II/III mGluRs present at adjacent primary afferent terminals, where they provide negative feedback regulation of glutamate release to dorsal horn neurons (37, 43). We found in this study that the inhibitory effect of oxotremorine-M on monosynaptic and polysynaptic EPSCs was still present in M_2/M_4 double-KO mice and in the presence of himbacine in WT mice. Blocking group II/III mGluRs with LY341495 and CPPG abolished the remaining inhibitory effect of oxotremorine-M on the amplitude of monosynaptic and polysynaptic EPSCs in WT mice and in M_2/M_4 double-KO mice. Importantly, the inhibitory effect of oxotremorine-M on monosynaptic and polysynaptic EPSCs was completely blocked by himbacine alone in all dorsal horn neurons from M_5-KO mice. Therefore, in addition to its potentiating effect on glutamate release from primary afferents, the M_5 subtype is critically involved in subsequent activation of group II/III mGluRs to restrain excessive primary afferent input to the spinal cord.

Our study uncovers a complex and reciprocal interaction between the M_2/M_4 subtypes, the M_5 subtype, and group II/III mGluRs at the same or different primary afferent terminals (Fig. 10). On the one hand, the M_5 subtype is present on a small subset of primary afferent terminals, and its activation can cause increased glutamate release. This potentiating effect of the M_5 subtype was especially evident in the absence of M_2 and M_4 subtypes, because oxotremorine-M increased monosynaptic and polysynaptic EPSCs in significantly more dorsal horn neurons in M_2-KO, M_4-KO, and M_2/M_4 double-KO mice than in WT mice. On the other hand, stimulation of the M_5 subtype on glutamatergic interneurons potentiates glutamate release, leading to inhibition of primary afferent input via group II/III mGluRs. The latter action may consequently reduce nociceptive transmission, and it is possible that blocking group II/III mGluRs could attenuate the analgesic effect of spinally administered mAChR agonists. The fact that the M_5 subtype is involved in both potentiation of primary afferent input and attenuation of primary afferent input (indirectly via group II/III mGluRs) could explain why the inhibitory effect of oxotremorine-M on monosynaptic and polysynaptic EPSCs was not significantly potentiated or reduced in M_5-KO mice. It will be interesting to determine how the muscarinic analgesic effect is altered in M_5-KO mice.

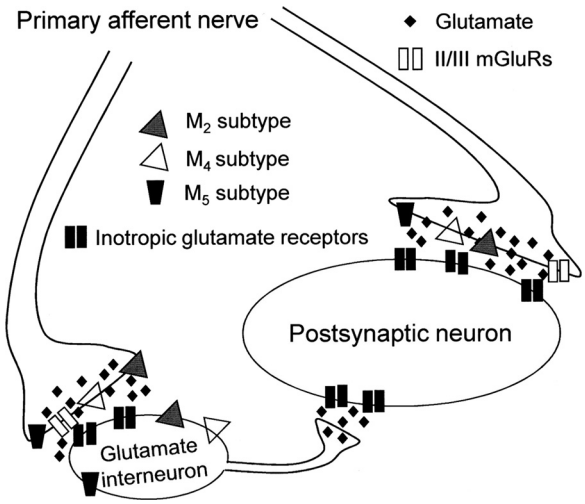

FIGURE 10. **Schematic drawing showing the distribution of the three mAChR subtypes on primary afferent terminals and glutamatergic interneurons in the mouse spinal dorsal horn.** In this diagram, the primary afferent nerve terminal contacts the recorded postsynaptic neuron either directly (monosynaptic) or indirectly through the glutamatergic interneuron (polysynaptic). The functional interaction between the M_2, M_4, and M_5 subtypes and group II/III mGluRs in the control of glutamate release from primary afferent nerves is described in detail under "Discussion."

In summary, our study using mAChR subtype-KO mice revealed that M_2, M_4, and M_5 subtypes are present at primary afferent terminals and differentially regulate nociceptive transmission in the spinal cord. Our study provides unequivocal evidence that mAChR agonist-induced inhibition of glutamatergic input from primary afferents is largely mediated by M_2 and M_4 subtypes. Stimulation of the M_5 subtype can directly increase primary afferent input and indirectly lead to activation of group II/III mGluRs, which participate in the negative feedback control of glutamate release from primary afferents. This new information is critical to our understanding of how individual mAChR subtypes regulate nociceptive input to the spinal dorsal horn. Our findings are also important for the development of novel subtype-specific mAChR analgesics with improved efficacy and reduced side effects.

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