# Dynamic regulation of glycinergic input to spinal dorsal horn neurones by muscarinic receptor subtypes in rats

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**Activation of spinal muscarinic acetylcholine receptors (mAChRs) inhibits nociception. However, the cellular mechanisms of this action are not fully known. In this study, we determined the role of mAChR subtypes in regulation of synaptic glycine release in the spinal cord. Whole-cell voltage-clamp recordings were performed on lamina II neurones in the rat spinal cord slices. The mAChR agonist oxotremorine-M significantly increased the frequency of glycinergic sIPSCs but not mIPSCs. Surprisingly, the effect of oxotremorine-M on sIPSCs was largely attenuated at a higher concentration. On the other hand, 1–10 $\mu$M oxotremorine-M dose-dependently increased the frequency of sIPSCs in rats pretreated with intrathecal pertussis toxin. Furthermore, oxotremorine-M also dose-dependently increased the frequency of sIPSCs in the presence of himbacine (an $M_2/M_4$ mAChR antagonist) or AF-DX116 (an $M_2$ mAChR antagonist). The $M_3$ mAChR antagonist 4-DAMP abolished the stimulatory effect of oxotremorine-M on sIPSCs. Interestingly, the $GABA_B$ receptor antagonist CGP55845 potentiated the stimulatory effect of oxotremorine-M on sIPSCs. In the presence of CGP55845, both himbacine and AF-DX116 similarly reduced the potentiating effect of oxotremorine-M on sIPSCs. Collectively, these data suggest that the $M_3$ subtype is present on the somatodendritic site of glycinergic neurones and is mainly responsible for muscarinic potentiation of glycinergic input to spinal dorsal horn neurones. Concurrent stimulation of mAChRs on adjacent GABAergic interneurones attenuates synaptic glycine release through presynaptic $GABA_B$ receptors on glycinergic interneurones. This study illustrates a complex dynamic interaction between GABAergic and glycinergic synapses in the spinal cord dorsal horn.**

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The spinal cord cholinergic system and muscarinic acetylcholine receptors (mAChRs) are important for regulation of nociception. In this regard, stimulation of mAChRs in the spinal cord suppresses the response of dorsal horn neurones to nociceptive stimuli (Chen & Pan, 2004). Furthermore, intrathecal administration of mAChR agonists or acetylcholinesterase inhibitors produces potent analgesia in both animals and humans (Iwamoto & Marion, 1993; Naguib & Yaksh, 1994; Hood *et al.* 1997; Duttaroy *et al.* 2002). Receptor autoradiography and immunocytochemistry studies have shown that the highest density of mAChRs in the spinal cord is concentrated in the superficial laminae in both rats and humans (Yamamura *et al.* 1983; Scatton *et al.* 1984; Villiger & Faull, 1985; Hoglund & Baghdoyan, 1997; Li *et al.* 2002). However, the precise cellular mechanisms involved in the muscarinic analgesia are not clear. Molecular cloning studies have identified five molecularly distinct mAChRs referred to as $M_1$–$M_5$ (Caulfield, 1993; Wess, 1996). The five mAChR subtypes are all linked to different families of G proteins. $M_1$, $M_3$ and $M_5$ are selectively linked to $G_{q/11}$ proteins, while $M_2$ and $M_4$ are preferentially coupled to the pertussis toxin (PTX)-sensitive $G_{i/o}$ family (Fields & Casey, 1997; Caulfield & Birdsall, 1998). Recent studies performed in mAChR knockout mice provide further evidence that $M_2$ and $M_4$ subtypes play an essential role in muscarinic analgesia (Gomeza *et al.* 1999; Duttaroy *et al.* 2002). Although previous studies have shown that $M_2$, $M_3$ and $M_4$ mAChR subtypes are present in the spinal cord dorsal horn (Hoglund & Baghdoyan, 1997; Yung & Lo, 1997; Duttaroy *et al.* 2002; Chen *et al.* 2005*a*), it remains uncertain how these individual mAChR subtypes participate in the muscarinic analgesic action in the spinal cord.

We have shown that stimulation of spinal GABA release and $GABA_B$ receptors is an important analgesic

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mechanism of mAChR agonists in the spinal dorsal horn (Li *et al.* 2002; Chen & Pan, 2003). Also, $M_2$, $M_3$ and $M_4$ subtypes all contribute to increased GABAergic tone in the rat spinal cord (Zhang *et al.* 2005). In addition to GABA, glycine is an important inhibitory neurotransmitter in the spinal cord (Yoshimura & Nishi, 1995; Pan & Pan, 2004). For example, intrathecal administration of strychnine, a glycine receptor antagonist, induces hypersensitivity of spinal dorsal horn neurones and allodynia (Yaksh, 1989; Cronin *et al.* 2004). It has been shown that GABA and glycine are often coreleased from the same synaptic terminals to putative motor neurones in the spinal cord (Jonas *et al.* 1998). However, the mechanisms of regulation of glycinergic transmission in the spinal dorsal horn remain largely unknown. In this study, we determined the potential function of mAChR subtypes in the control of glycinergic inputs to dorsal horn neurones. This study provides new information about muscarinic regulation of nociception and extends our current understanding of the complex mechanisms involved in the control of glycinergic and GABAergic transmission in the spinal cord dorsal horn.

## Methods

### Animals

Male Sprague-Dawley rats (3–4 weeks old; Harlan, Indianapolis, IN, USA) were used in this study. All the surgical preparations and experimental protocols were approved by the Animal Care and Use Committee of the Pennsylvania State University College of Medicine. Some rats were pretreated with intrathecal pertussis toxin (PTX) to inactivate inhibitory $G_{i/o}$ proteins (Wong *et al.* 1992; Fields & Casey, 1997; Zhang *et al.* 2005). Intrathecal catheters were inserted in rats anaesthetized using 2% halothane. The catheters (polyethylene-10 tubing) were inserted through an incision in the cisternal membrane and advanced 4.5 cm caudal so that the tip of each catheter was positioned at the lumbar spinal level. It has been shown that intrathecal 0.125–2 $\mu$g PTX dose-dependently attenuated the antinociceptive effect of a $\mu$ opioid agonist in rats (Wong *et al.* 1992). We thus used two high doses of intrathecal PTX (1 and 2 $\mu$g) in our study to ensure a maximal effect of PTX in the spinal cord. All the rats included in the present study were used for final electrophysiological experiments 5–7 days following PTX injection. During this period, we did not observed any neurological damage and impairment or symptoms suggestive of spontaneous chronic pain in these rats.

### Spinal cord slice preparations

Rats were anaesthetized with 2% halothane in $O_2$ and the lumbar segment of the spinal cord was rapidly removed through a limited laminectomy. The rats then were killed by inhalation of 5% halothane. The segment of the lumbar spinal cord was immediately placed in ice-cold sucrose artificial cerebrospinal fluid (aCSF) presaturated with 95% $O_2$ and 5% $CO_2$. The sucrose aCSF contained (mM): 234 mM sucrose, 3.6 mM KCl, 1.2 mM $MgCl_2$, 2.5 mM $CaCl_2$, 1.2 mM $NaH_2PO_4$, 12.0 mM glucose, and 25.0 mM $NaHCO_3$. The tissue block was then placed in a shallow groove formed in a gelatin block and glued on the stage of a vibratome (Technical Product International, St Louis, MO, USA). Transverse spinal cord slices (350 $\mu$m) were cut in the ice-cold sucrose aCSF and then preincubated in Krebs solution continuously gassed 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 $NaHCO_3$. The lamina II has a distinct translucent appearance and can easily be distinguished under the microscope (Zhang *et al.* 2005). The neurones located in lamina II of the spinal cord slice were identified under a fixed-stage microscope (BX50WI, Olympus, Tokyo, Japan) with differential interference contrast/infrared illumination. The electrode for the whole-cell recordings was pulled from borosilicate glass capillaries with a puller (P-97, Sutter Instrument Co., Novato, CA, USA). The impedance of the pipette was 3–5 MΩ when filled with internal solution containing: 110 mM $Cs_2SO_4$, 5 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, and 1 mM guanosine 5′-*O*-(2-thiodiphosphate) (GDP-$\beta$-S), 10 mM QX314, adjusted to pH 7.2–7.4 with 1 M CsOH (290–320 mosmol $l^{-1}$). GDP-$\beta$-S was added to the internal pipette solution to block the possible postsynaptic effect mediated by muscarinic agonists through G proteins (Li *et al.* 2002; Zhang *et al.* 2005). QX314 was added to the internal solution to suppress the action potential generation from the recorded cell. The slice was placed in a glass-bottomed chamber (Warner Instruments, Hamden, CT, USA) and fixed with parallel nylon threads supported by a U-shaped stainless steel weight. The slice was continuously perfused with Krebs solution at 5.0 ml $min^{-1}$ at 34°C maintained by an inline solution heater and a temperature controller (TC-324; Warner Instruments).

### Electrophysiological recordings

Recordings of postsynaptic currents were performed using the whole-cell voltage-clamp method, as we previously described (Li *et al.* 2002; Pan *et al.* 2002; Zhang *et al.* 2005). Recordings of postsynaptic currents began 5 min after whole-cell access was established and the current reached a steady state. The input resistance was monitored and the recording was abandoned if it changed more than 15%. Signals were recorded using an amplifier (MultiClamp

700A, Axon Instruments, Union City, CA, USA) at a holding potential of 0 mV, filtered at 1–2 kHz, digitized at 10 kHz, and stored in a Pentium computer with pCLAMP 9.0 (Axon Instruments). All spontaneous inhibitory postsynaptic currents (sIPSCs) were recorded in the presence of 10 $\mu$M bicuculline, a $GABA_A$ receptor antagonist, and 20 $\mu$M 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX), a non-NMDA antagonist. To record the miniature inhibitory postsynaptic currents (mIPSCs), 1 $\mu$M tetrodotoxin (TTX) was added in the perfusion solution.

Oxotremorine-M (Oxo-M), himbacine, AF-DX116, CGP55845, 4-diphenylacetoxy-*N*-methylpiperidine methiodide (4-DAMP), GDP-$\beta$-S, atropine, strychnine, CNQX and bicuculline were obtained from Sigma-Aldrich (St Louis, MO, USA). PTX was purchased from List Biological Laboratories (San Jose, CA, USA). TTX and QX314 were obtained from Alomone Laboratories (Jerusalem, Israel). Drugs were dissolved in Krebs solution and perfused into the slice chamber at final concentrations.

### Data analysis

Data are presented as means ± S.E.M. The sIPSCs and mIPSCs were analysed off-line with a peak detection program (MiniAnalysis, Synaptosoft, Decatur, GA, USA). Measurements of the amplitude and frequency of sIPSCs and mIPSCs were performed over a period of at least 1 min during control, drug application and recovery. For each analysis, 300–1500 events were included. The mIPSCs and sIPSCs were detected by the fast rise time of the signal over an amplitude threshold above the background noise. We manually excluded the event when the noise was erroneously identified as the mIPSCs or sIPSCs by the program. The background noise level was typically constant throughout the recording of the single neurone. The cumulative probability of the amplitude and interevent interval of mIPSCs/sIPSCs was compared using the Kolmogorov-Smirnov test, which estimates the probability that two cumulative distributions are similar. The effect of Oxo-M on the amplitude and frequency of sIPSCs and mIPSCs was determined by either two-tailed Student's *t* test or repeated measures ANOVA. Neurones were considered to be responsive to Oxo-M if the frequency of sIPSCs was altered > 20%. $P < 0.05$ was considered to be statistically significant.

## Results

### Effect of Oxo-M on glycinergic sIPSCS and mIPSCS

To determine the effect of Oxo-M on glycinergic sIPSCs, the non-selective mAChR agonist Oxo-M was applied through bath perfusion. The effect of Oxo-M on sIPSCs was examined at the concentration of 1, 3, 5 and 10 $\mu$M. In a total of 22 lamina II neurones tested, Oxo-M at the concentration of 3 $\mu$M significantly increased the frequency of sIPSCs in 16 of 22 neurones (72.73%, Fig. 1*A*–*C*). Interestingly, when the concentration of Oxo-M was increased to 5 and 10 $\mu$M, the potentiating effect of Oxo-M on the frequency of sIPSCs was attenuated compared with that of 3 $\mu$M Oxo-M in the above 16 neurones (Fig. 1*C*). Oxo-M at the concentration of 1–10 $\mu$M did not significantly alter the amplitude of sIPSCs (Fig. 1*D*). The sIPSCs were completely abolished by 2 $\mu$M strychnine (Fig. 1*A*). In the remaining six cells (27.27%), 1–10 $\mu$M Oxo-M failed to alter significantly the frequency and amplitude of sIPSCs.

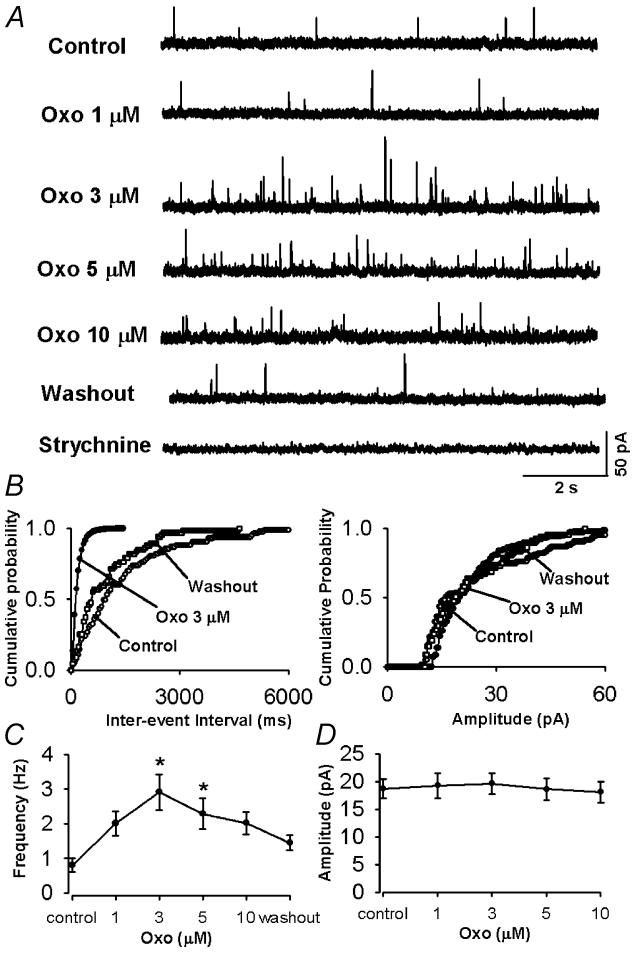


**Figure 1. Effect of oxotremorine-M on glycinergic sIPSCs of spinal lamina II neurones**
*A*, original tracings of sIPSCs during control, during application of 1, 3, 5 and 10 $\mu$M oxotremorine-M (Oxo), and washout in one lamina II cell. Note that the sIPSCs were abolished by 2 $\mu$M strychnine. *B*, cumulative probability plot of sIPSCs of the same neurone as in *A* showing the distribution of the amplitude and interevent interval of sIPSCs for control, during application of 3 $\mu$M oxotremorine-M, and after washout. *C* and *D*, summary data showing that oxotremorine-M increased the frequency but not the amplitude of sIPSCs in 16 cells. Data presented as means ± S.E.M. *$P < 0.05$ compared with the control.

To ensure that the effect of Oxo-M on glycinergic sIPSCs is through mAChRs, the specific mAChR antagonist atropine (2 $\mu$M) was used. Atropine completely blocked the effect of Oxo-M on the frequency of sIPSCs in all nine neurones tested (Fig. 2*A*). In seven separate lamina II neurones, the initial effect of Oxo-M on sIPSCs was completely washed out in 10 min. Repeated application of 3 $\mu$M Oxo-M caused a similar increase in the frequency of sIPSCs (Fig. 2*B*).

Oxo-M may increase synaptic glycine release through mAChRs located on either somatodendritic sites or terminals of glycinergic neurones. We therefore determined the effect of Oxo-M on glycinergic mIPSCs, which represent action potential-independent synaptic release of glycine. In nine additional lamina II neurones, 3 $\mu$M Oxo-M significantly increased the frequency of sIPSCs (Fig. 2*C*). The effect of 3 $\mu$M Oxo-M on mIPSCs then was tested in the presence of 1 $\mu$M TTX. Oxo-M did not significantly alter the frequency and amplitude of mIPSCs in all nine cells examined (Fig. 2*C*). Together, these data suggest that activation of mAChRs on the somatodendritic site of glycinergic interneurones increases the synaptic glycine release to spinal dorsal horn neurones.

### Effect of Oxo-M on glycinergic sIPSCs and mIPSCs in PTX-treated rats

$M_2$ and $M_4$ mAChR subtypes are preferentially coupled to the PTX-sensitive $G_{i/o}$ family (Felder, 1995; Wess, 1996; Caulfield & Birdsall, 1998). To determine the contribution of $M_2$ and $M_4$ subtypes to the effect of Oxo-M on glycinergic synaptic inputs to lamina II neurones, we pretreated rats with intrathecal 1 or 2 $\mu$g PTX (Wong *et al.* 1992; Fields & Casey, 1997; Zhang *et al.* 2005) to inactivate $G_{i/o}$ proteins 5–7 days before the final electrophysiological experiments.

In 15 lamina II neurones obtained from rats pretreated with intrathecal 2 $\mu$g PTX, 3–10 $\mu$M Oxo-M significantly increased the frequency of glycinergic sIPSCs in a dose-dependent manner (Fig. 3*A*–*C*). A similar effect was observed in 10 lamina II neurones from rats

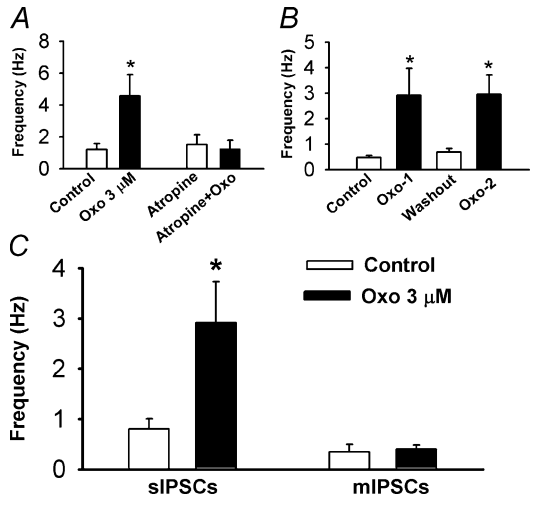


**Figure 2. Effect of oxotremorine-M (Oxo) on glycinergic sIPSCs was blocked by atropine**
*A*, summary data showing the effect of 3 $\mu$M oxotremorine-M on sIPSCs before and after application of 2 $\mu$M atropine ($n = 9$). *B*, summary data showing that repeated perfusion of 3 $\mu$M oxotremorine-M (Oxo-1 and Oxo-2) produced a similar effect on the frequency of sIPSCs ($n = 7$). *C*, summary data showing that oxotremorine-M increased the frequency of sIPSCs but not of mIPSCs ($n = 9$). Data presented as means ± S.E.M. $^*P < 0.05$ compared with the respective control.

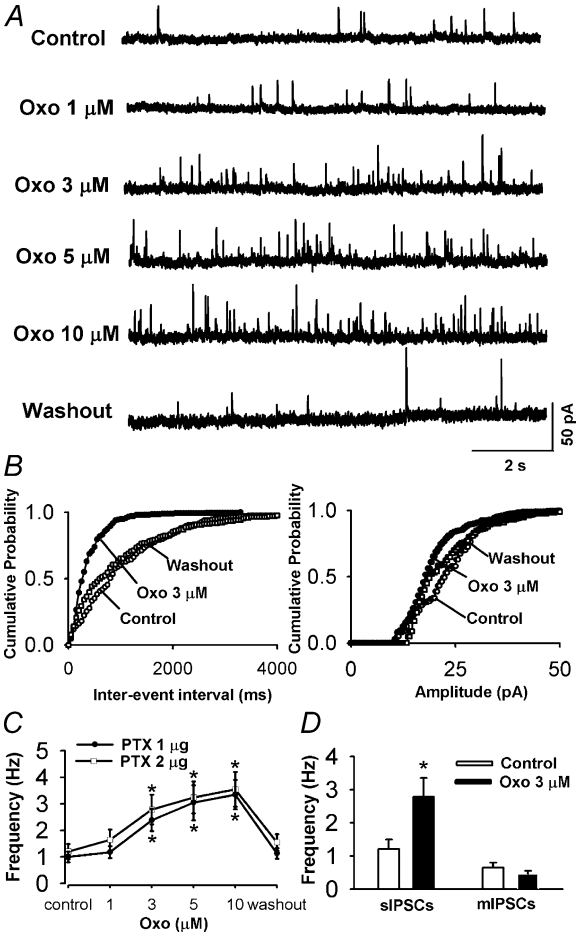


**Figure 3. Effect of oxotremorine-M on glycinergic sIPSCs of spinal lamina II neurones in PTX-pretreated rats**
*A*, raw tracings of sIPSCs for control, during application of 1, 3, 5 and 10 $\mu$M oxotremorine-M (Oxo), and after washout in one cell. *B*, cumulative probability plot of sIPSCs of the same neurone as in *A* showing the distribution of the amplitude and interevent interval for control, during application of 3 $\mu$M oxotremorine-M, and after washout. *C*, summary data showing that oxotremorine-M concentration-dependently increased the frequency of sIPSCs in rats pretreated with 1 $\mu$g ($n = 15$ cells) or 2 $\mu$g ($n = 10$ cells) PTX. *D*, summary data showing that oxotremorine-M increased the frequency of sIPSCs but not mIPSCs ($n = 8$) in pertussis toxin-treated rats. $^*P < 0.05$ compared with the control.

pretreated with intrathecal 1 $\mu$g PTX. The dose–response effect of Oxo-M on glycinergic IPSCs was not significantly different between rats pretreated with 1 and 2 $\mu$g PTX (Fig. 3*C*). In another eight lamina II neurones recorded from PTX (2 $\mu$g)-treated rats, 3 $\mu$M Oxo-M initially induced a significant increase in the frequency of sIPSCs (Fig. 3*D*). However, in the presence of 1 $\mu$M TTX, 3 $\mu$M Oxo-M had no significant effect on the frequency and amplitude of mIPSCs in all eight cells tested (Fig. 3*D*). Thus, it appears that $G_{i/o}$ proteins are not required for the presynaptic effect of the mAChR agonist in the spinal cord. These data also suggest that inactivation of $G_{i/o}$ proteins with PTX increases the effectiveness of the mAChR agonist at higher concentrations.

### Role of $M_3$ mAChR subtype in Oxo-M-induced increase in glycinergic sipscs

The $M_3$ mAChR subtype is present in the spinal dorsal horn (Hoglund & Baghdoyan, 1997; Zhang *et al.* 2005). Since the $M_3$ subtype is coupled to $G_{q/11}$, but not $G_{i/o}$, proteins, we next determined if the effect of Oxo-M on potentiation of glycinergic sIPSCs is mediated by the $M_3$ subtype in the spinal cord. In these experiments, 4-DAMP, an $M_3$-preferring mAChR antagonist (Ehlert, 1996; Yigit *et al.* 2003; Zhang *et al.* 2005), was employed. Initial application of 3 $\mu$M Oxo-M significantly increased the frequency of glycinergic sIPSCs in nine cells (Fig. 4*A* and *B*). In the presence of 25 nM 4-DAMP, 3 $\mu$M Oxo-M failed to increase the frequency of sIPSCs in all nine cells examined (Fig. 4*C*).

In the spinal cord from rats pretreated with intrathecal PTX, 3 $\mu$M Oxo-M also significantly increased the frequency of sIPSCs in eight neurones (Fig. 4*D*). Bath application of 25 nM 4-DAMP completely blocked the potentiating effect of 3 $\mu$M Oxo-M on the frequency of sIPSCs in these eight cells tested (Fig. 4*D*). These data suggest that the $M_3$ mAChR subtype plays a predominant role in potentiation of glycinergic inhibitory tone in the spinal cord by the mAChR agonist.

### Role of $M_2$ and $M_4$ mAChR subtypes in Oxo-M-induced increase in glycinergic sIPSCs

The effect of $M_2$/$M_4$ mAChR subtypes often opposes that of the $M_3$ subtype due to their coupling to different types of G proteins (Murthy & Makhlouf, 1997; Alfonzo *et al.* 1998). Because Oxo-M exhibited a 'typical' dose–response effect on sIPSCs in PTX-treated rats, we reasoned if $M_2$ and $M_4$ subtypes are responsible for the attenuated effect of Oxo-M on glycinergic IPSCs at a higher concentration. To assess the role of $M_2$ and $M_4$ subtypes in the effect of Oxo-M on synaptic glycine release to lamina II neurones, himbacine, an $M_2$- and $M_4$-preferring mAChR antagonist (Dorje *et al.* 1991; Miller *et al.* 1992; Doller *et al.* 1999), was used. In 17 neurones tested, 3–10 $\mu$M Oxo-M caused a concentration-dependent increase in the frequency of sIPSCs in the presence of 1 $\mu$M himbacine (Fig. 5*A*–*C*).

We also used AF-DX116, an $M_2$-preferring mAChR antagonist (Coelho *et al.* 2000; Douglas *et al.* 2001), to further delineate the role of the $M_2$ mAChR subtype in the effect of Oxo-M on glycinergic sIPSCs. In the presence of 10 $\mu$M AF-DX116, 3–10 $\mu$M Oxo-M concentration-dependently increased the frequency of sIPSCs in 15 cells (Fig. 5*D*). The potentiating effect of Oxo-M on sIPSCs in the presence of himbacine or AF-DX116 was distinctly different from the 'bell-shaped' dose–response effect of Oxo-M on sIPSCs without $M_2$/$M_4$ mAChR antagonists (Fig. 1*C*). These data seem to suggest that $M_2$ and $M_4$ mAChR subtypes are not involved in the potentiating effect of Oxo-M on synaptic glycine release to lamina II neurones. Furthermore, blocking of the $M_2$ and

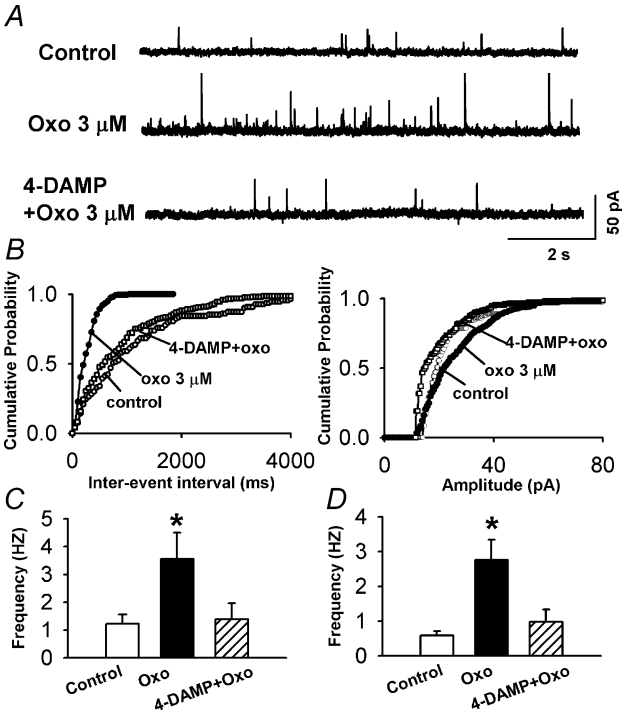


**Figure 4. Effect of 4-DAMP on oxotremorine-M-induced increase in sIPSCs of lamina II neurones**
*A*, original tracings of sIPSCs during control, during application of 3 $\mu$M oxotremorine-M (Oxo), and application of oxotremorine-M plus 25 nM 4-DAMP in one cell. *B*, cumulative probability plot of sIPSCs of the same neurone as in *A* showing the distribution of the amplitude and interevent interval for control and during application of 3 $\mu$M oxotremorine-M and oxotremorine-M plus 25 nM 4-DAMP. *C*, summary data showing the effect of 3 $\mu$M oxotremorine-M on sIPSCs before and after application of 25 nM 4-DAMP in 9 cells in untreated rats. *D*, summary data showing the effect of 3 $\mu$M oxotremorine-M on sIPSCs before and after application of 25 nM 4-DAMP in 8 cells recorded from pertussis toxin-treated rats. Data presented as means ± S.E.M. $^*P < 0.05$ compared with control.

$M_4$ subtypes augments the effect of mAChR agonists on synaptic glycine release at higher concentrations.

### Influence of $GABA_B$ receptors on the effect of Oxo-M on glycinergic sIPSCs

$GABA_B$ receptors are also coupled to PTX-sensitive $G_{i/o}$ proteins (Blalock *et al.* 1999), and the $GABA_B$ effect may be also eliminated by PTX treatment. We have shown that activation of mAChRs dose-dependently increases synaptic GABA release in the rat spinal cord (Zhang *et al.* 2005). Thus, it is possible that stimulation of mAChRs can increase synaptic GABA release, which indirectly inhibits glycine release through presynaptic $GABA_B$ receptors. To determine if $GABA_B$ receptors are involved in the attenuated effect of Oxo-M at higher concentrations on glycinergic sIPSCs, CGP55845, a specific $GABA_B$ receptor antagonist (Riley *et al.* 2001; Li *et al.* 2002), was utilized. In the presence of 1 $\mu$M CGP55845, Oxo-M significantly increased the frequency of sIPSCs in a dose-dependent manner in 13 neurones (Fig. 6*A*–*C*). Notably, the effect of Oxo-M on the frequency of sIPSCs reached the maximum at 3 $\mu$M, and this effect was sustained at 5 and 10 $\mu$M (Fig. 6*C*). These data show that the effect of Oxo-M on synaptic glycine release is influenced by $GABA_B$ receptors, probably through increased GABA release due to concurrent stimulation of mAChRs on adjacent GABAergic interneurones in the lamina II.

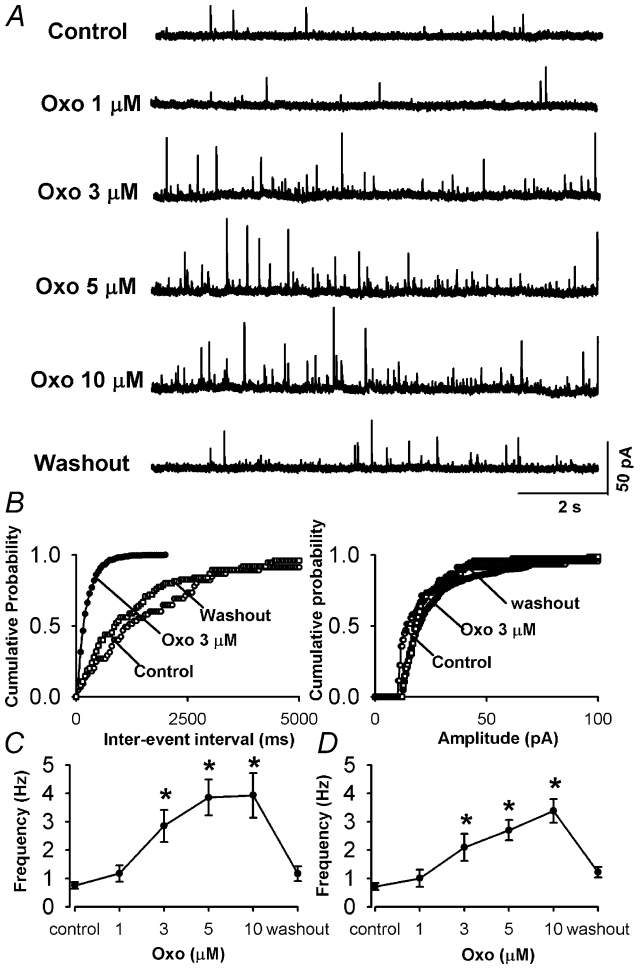


**Figure 5. Effect of himbacine and AF-DX116 on the potentiating effect of oxotremorine-M on the frequency of sIPSCs**
*A*, original tracings of sIPSCs for control, during application of 1, 3, 5 and 10 $\mu$M oxotremorine-M (Oxo), and after washout in the presence of 1 $\mu$M himbacine in one lamina II neurone. *B*, cumulative probability plot of sIPSCs of the same neurone as in *A* showing the distribution of the amplitude and interevent interval for control, during application of 3 $\mu$M oxotremorine-M, and after washout. *C*, summary data showing that oxotremorine-M concentration-dependently increased the frequency of sIPSCs in the presence of 1 $\mu$M himbacine in 17 cells. *D*, summary data showing that oxotremorine-M increased the frequency of sIPSCs in a concentration-dependent manner in the presence of 10 $\mu$M AF-DX116 ($n = 15$). Data presented as means ± S.E.M. $^*P < 0.05$ compared with the respective control.

### Role of $M_2$ and $M_4$ mAChR subtypes on glycinergic neurones in the potentiating effect of Oxo-M on glycinergic sIPSCs

The $M_2$ and $M_4$ mAChR subtypes are present on GABAergic interneurones in the spinal dorsal horn (Zhang *et al.* 2005). Bath application of Oxo-M can stimulate mAChRs present on both GABAergic and glycinergic interneurones. We thus further studied the respective role of $M_2$ and $M_4$ subtypes on glycinergic neurones in the effect of Oxo-M on synaptic glycine release. We first blocked $GABA_B$ receptors (to remove the indirect effect of Oxo-M on GABAergic neurones) and then tested the effect of Oxo-M on glycinergic IPSCs before and after blocking $M_2/M_4$ (himbacine) or $M_2$ (AF-DX116) mAChRs. In 15 lamina II neurones, initial application of 5 $\mu$M Oxo-M significantly increased the frequency of sIPSCs in the presence of 1 $\mu$M CGP55845 (Fig. 6*D*). Himbacine (1 $\mu$M) alone did not have significant effect on sIPSCs, but significantly reduced the potentiating effect of 5 $\mu$M Oxo-M on the frequency of sIPSCs in the above 15 cells when 1 $\mu$M CGP55845 was co-administered (Fig. 6*D*).

In another 16 cells tested, 10 $\mu$M AF-DX116 also significantly decreased the stimulating effect of 5 $\mu$M Oxo-M on the frequency of glycinergic sIPSCs in the presence of 1 $\mu$M CGP55845 (Fig. 6*E*). Bath application of AF-DX116 alone had no significant effect on sIPSCs in all the neurones tested (Fig. 6*E*). These results suggest that stimulation of $M_2$ and $M_4$ mAChR subtypes on glycinergic interneurones increases synaptic glycine release to spinal lamina II neurones.

## Discussion

This study determed the role of mAChR subtypes in regulating glycinergic inputs to spinal lamina II neurones.

We found that the mAChR agonist Oxo-M significantly increased glycinergic sIPSCs at a lower concentration (3 $\mu$M). Unexpectedly, such an effect was largely attenuated by a higher concentration of Oxo-M (5–10 $\mu$M). In PTX-treated rats or in the presence of himbacine and AF-DX116, a dose-dependent effect of Oxo-M on the frequency of sIPSCs was revealed. Furthermore, the potentiating effect of Oxo-M on glycinergic sIPSCs was abolished by 4-DAMP, an $M_3$ subtype-preferring mAChR antagonist. These data suggest that the $M_3$ mAChR subtype is mainly responsible for the potentiating effect of mAChR agonists on synaptic glycine release in the spinal dorsal horn. Importantly, we demonstrated that the $GABA_B$ receptor antagonist CGP55845 increased the potentiating effect of Oxo-M on glycinergic sIPSCs. Thus, stimulation of mAChRs on GABAergic interneurones attenuates glycine release through presynaptic $GABA_B$ receptors present on glycinergic interneurones. This study provides new information about the complex mechanisms involved in dynamic regulation of glycinergic inhibitory tone in the spinal cord by mAChR subtypes.

GABA and glycine are the two major inhibitory neurotransmitters that mediate the IPSCs in the spinal dorsal horn (Yoshimura & Nishi, 1995; Pan & Pan, 2004; Zhang *et al.* 2005). It has been documented that $M_2$, $M_3$ and $M_4$ mAChR subtypes are present in the rodent spinal dorsal horn (Hoglund & Baghdoyan, 1997; Duttaroy *et al.* 2002; Chen *et al.* 2005*a*). Increased GABAergic inhibitory tone by muscarinic agonists is mediated by $M_2$, $M_3$ and $M_4$ mAChR subtypes (Zhang *et al.* 2005). Although glycine is another well known inhibitory neurotransmitter in the spinal cord, little information is available about the functional role of mAChRs in the control of glycinergic synaptic transmission. The nociceptive inputs from the primary afferents to spinal dorsal horn neurones are tonically controlled by GABAergic and glycinergic interneurones (Yaksh, 1989; Cronin *et al.* 2004; Pan & Pan, 2004; Chen *et al.* 2005*b*). Unlike the effect of Oxo-M on GABA release in the spinal cord (Zhang *et al.* 2005), we found that the potentiating effect of Oxo-M on glycinergic sIPSCs was attenuated at the higher concentrations. Because the effect of Oxo-M on sIPSCs was not attenuated in PTX-treated rats, stimulation of mAChRs may increase glycinergic sIPSCs through mAChR subtypes that are not coupled to PTX-sensitive $G_{i/o}$ proteins. In the present study, the stimulating effect of Oxo-M on glycinergic sIPSCs was completely abolished by 4-DAMP. Thus, the $M_3$ subtype is responsible for increased synaptic glycine release by activation of mAChRs. In support of this conclusion, blocking of $M_2$ and $M_2/M_4$ subtypes with AF-DX116 and himbacine, respectively, had little effect on Oxo-M-induced increases in glycinergic sIPSCs. The radioligand binding study has shown that the $M_3$ mAChR is present in the rat spinal cord (Hoglund & Baghdoyan, 1997), which plays a role in Oxo-M-induced synaptic

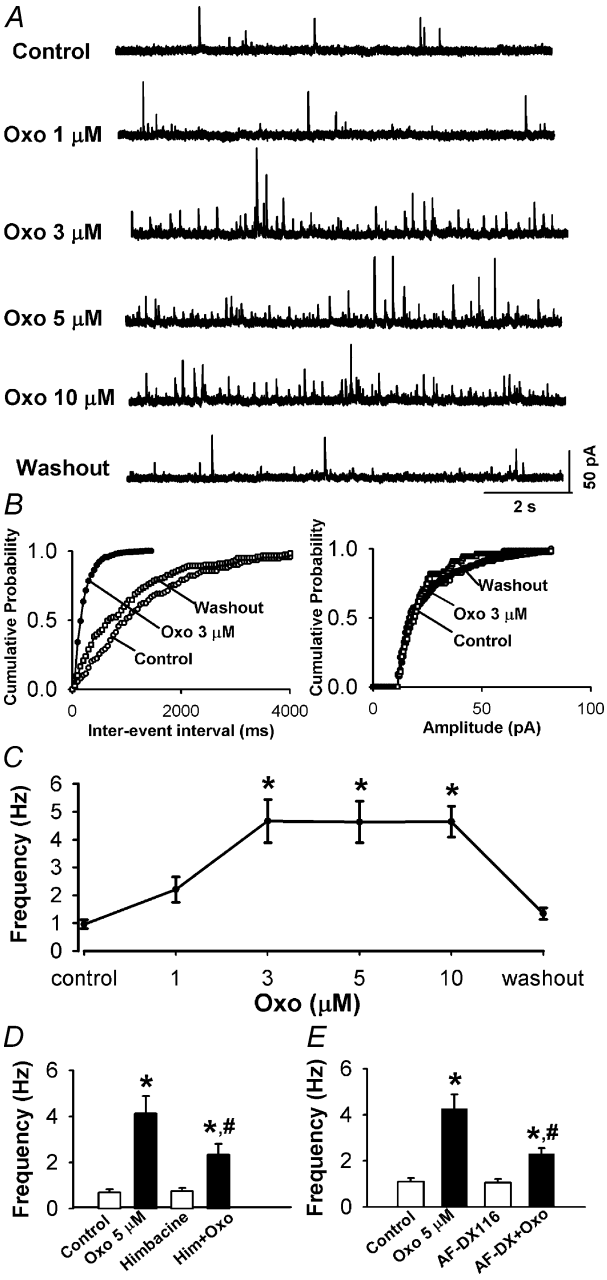


**Figure 6. Effect of CGP55845 on the potentiating effect of oxotremorine-M on sIPSCs in lamina II neurones**
*A*, original tracings of sIPSCs during control, during application of 1, 3, 5 and 10 $\mu$M oxotremorine-M (Oxo) in the presence of 1 $\mu$M CGP55845 in one lamina II neurone. *B*, cumulative probability plot of sIPSCs of the same neurone as in *A* showing the distribution of the amplitude and interevent interval for control, during application of 3 $\mu$M oxotremorine-M, and after washout. *C*, summary data showing the effect of oxotremorine-M (3–10 $\mu$M) on the frequency of sIPSCs in the presence of 1 $\mu$M CGP55845 in 13 cells. *D*, summary data showing the effect of 5 $\mu$M oxotremorine-M on the frequency of sIPSCs before and after application of 1 $\mu$M himbacine in the presence of 1 $\mu$M CGP55845 in 15 neurones. *E*, summary data showing the effect of 5 $\mu$M oxotremorine-M on sIPSCs before and after application of 10 $\mu$M AF-DX116 in the presence of 1 $\mu$M CGP55845 in another 16 cells. Data presented as means ± S.E.M. $^*P < 0.05$ compared with the control; #$P < 0.05$ compared with the initial effect of oxotremorine-M.

GABA release to dorsal horn neurones (Zhang *et al.* 2005). Nevertheless, the physiological function of the $M_3$ subtype in the spinal cord remains largely unknown, and its functional role in the spinal analgesic effect of mAChR agonists needs to be further defined. Because Oxo-M had no significant effect on the frequency and amplitude of mIPSCs, the $M_3$ mAChR subtype is likely to be located at the somatodentritic site of glycinergic interneurones. Therefore, data from this electrophysiologcal study suggest that the $M_3$ mAChR subtype on the glycinergic neurones plays a major role in potentiation of the glycinergic inhibitory tone in the spinal cord by mAChR agonists.

The attenuated effect of Oxo-M on glycinergic sIPSCs at higher concentrations is unexpected. The effect of $M_2/M_4$ mAChR subtypes often antagonizes that of the $M_3$ subtype by coupling to different families of G proteins (Murthy & Makhlouf, 1997; Alfonzo *et al.* 1998). We found that in the spinal cord pretreated with PTX to inactive $G_{i/o}$ proteins (Felder, 1995; Wess, 1996; Caulfield & Birdsall, 1998), the potentiating effect of Oxo-M on glycinergic sIPSCs evidently became dose dependent. Therefore, we initially hypothesized that Oxo-M at a higher concentration may activate $M_2$ and $M_4$ mAChR subtypes to counteract the effect produced by the $M_3$ subtype. In this regard, blockade of the $M_2$ subtype with AF-DX116 or $M_2/M_4$ subtypes with himbacine indeed revealed a concentration-dependent increase in the frequency of glycinergic sIPSCs by Oxo-M. Thus, it seems that $M_2$ and $M_4$ mAChR subtypes may be activated by Oxo-M at a higher concentration to reduce the potentiating effect of the $M_3$ subtype on glycinergic sIPSCs. However, these experiments do not distinguish if the $M_2$ and $M_4$ subtypes are present on glycinergic interneurones or other neuronal types in the spinal cord. Although himbacine and AF-DX116 eliminated the attenuating effect of higher concentrations of Oxo-M on glycinergic sIPSCs, this also could be explained by blockade of $M_2$ and $M_4$ subtypes on GABAergic interneurones (Zhang *et al.* 2005), leading to attenuation of synaptic GABA release (and $GABA_B$ receptor activation) by the mAchR agonist.

Because both $M_2/M_4$ mAChR subtypes and $GABA_B$ receptors are preferentially coupled to the PTX-sensitive $G_{i/o}$ family (Felder, 1995; Wess, 1996; Caulfield & Birdsall, 1998; Blalock *et al.* 1999), Oxo-M at a higher concentration may reduce synaptic glycine release through activation of either $M_2/M_4$ subtypes or, alternatively, presynaptic $GABA_B$ receptors on glycinergic interneurones. The $M_2/M_4$ subtypes are located on GABAergic interneurones (Zhang *et al.* 2005), and these subtypes also may be present on glycinergic interneurones in the spinal dorsal horn. Hence, PTX treatment could have inactivated the signalling of $M_2$ and $M_4$ subtypes on both types of interneurones. Previous studies have shown that the GABAergic and glycinergic synapses are

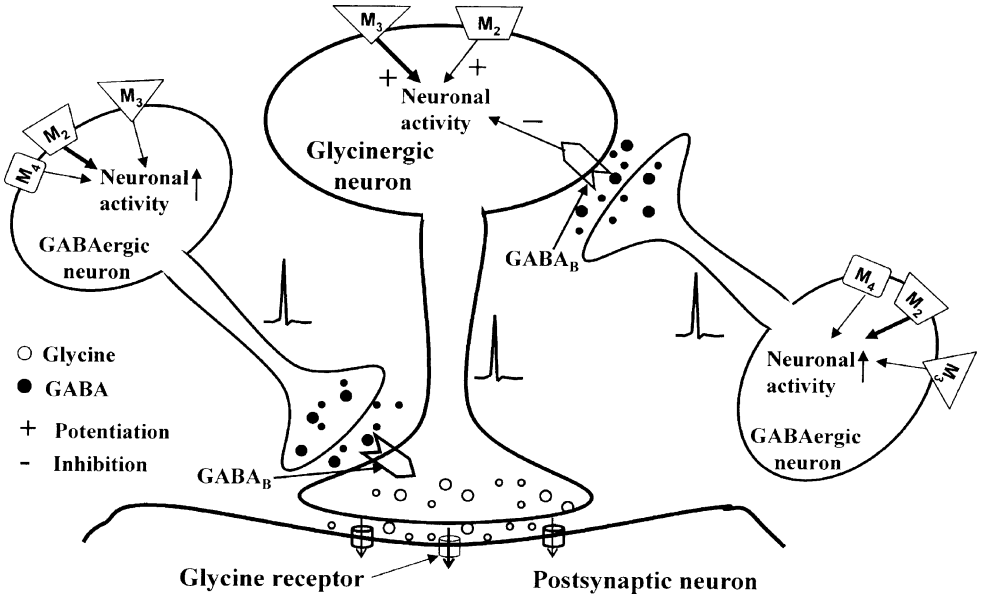


**Figure 7. Schematic drawing highlighting the interaction between GABAergic and glycinergic synapses in the spinal dorsal horn due to activation of mAChRs**

The mAChR subtypes are present on the somatodendritic site of GABAergic and glycinergic interneurones. The $M_3$ subtype on the glycinergic neurone is mainly responsible for the potentiating effect of mAChR agonists on synaptic glycine release. Concurrent stimulation of mAChR subtypes (primarily $M_2$) on the neighbouring GABAergic neurone evokes synaptic GABA release. The increased release of GABA can activate presynaptic $GABA_B$ receptors present on the somatodendritic sites or terminals of glycinergic neurones in the vicinity, which consequently counteracts the potentiating effect of mAChR agonists on synaptic glycine release to lamina II neurones.

closely intermingled in lamina II of the spinal cord (Todd *et al.* 1996; Pan & Pan, 2004). It is possible that at the higher concentration, the mAChR agonists may stimulate mAChRs ($M_2$, $M_3$ and $M_4$) on GABAergic neurones to increase their excitability to augment synaptic GABA release, which could attenuate glycine release through presynaptic $GABA_B$ receptors present on the glycinergic interneurones in the vicinity. This hypothesis is supported by our finding that when CGP55845 was used to block the $GABA_B$ receptors, Oxo-M caused a large increase in the frequency of glycinergic sIPSCs in a dose-dependent manner. In fact, in the presence of CGP55845, Oxo-M produced a maximal effect at a lower concentration, and such an effect was maintained at the higher concentration. Therefore, this study provides novel information that $GABA_B$ receptors present on glycinergic interneurones (at either the somatodendritic sites or terminals) function as important heteroreceptors in the dynamic feedback control of glycine release following activation of mAChRs in the spinal dorsal horn (Fig. 7).

It has been suggested that glycine and GABA are often co-released from the same synaptic terminals in the spinal cord (Jonas *et al.* 1998). However, our study suggests that a different and more complex mechanism exists for regulation of the synaptic release of these two inhibitory neurotransmitters in the spinal dorsal horn. In this regard, our data suggest that the potentiating effect (mainly via the $M_3$ subtype) of the mAChR agonist on synaptic glycine release is indirectly affected by activation of $GABA_B$ receptors due to simultaneous stimulation of mAChRs (mainly via the $M_2$ subtype) on GABAergic interneurones. Nevertheless, some important technical differences exist between these two studies. The study by Jonas *et al.* (1998) used synaptically coupled pairs of interneurones and putative motor neurones in the deep spinal laminae, while in our present study, the recording was performed on interneurones located in the spinal lamina II. We have shown that the mAChR agonist stimulates $M_2$, $M_3$ and $M_4$ subtypes on GABAergic neurones to elicit synaptic GABA release (Zhang *et al.* 2005), and increased GABA can spread to neighbouring glutamatergic synapses to activate $GABA_B$ receptors to indirectly reduce glutamate release in the spinal dorsal horn (Li *et al.* 2002; Chen & Pan, 2003). The present study reveals a close interaction between GABAergic and glycinergic interneurones through $GABA_B$ receptors upon activation of mAChRs in the spinal dorsal horn.

To further assess the contribution of $M_2$ and $M_4$ subtypes to the mAChR agonist-induced synaptic glycine release, we used the $GABA_B$ receptor antagonist CGP55845 to eliminate the influence of GABAergic interneurones stimulated by mAChR agonists. Using this approach, we were able to investigate the role of respective $M_2$ and $M_4$ subtypes that are present on glycinergic neurones in the effect of Oxo-M on sIPSCs. We found that the $M_2/M_4$-preferring antagonist himbacine and the $M_2$-preferring antagonist AF-DX116 caused a similar attenuation of the potentiating effect of Oxo-M on glycinergic sIPSCs. Thus, stimulation of the $M_2$ subtype on the glycinergic neurones appears to increase synaptic glycine release onto lamina II neurones when coincidental activation of $GABA_B$ receptors is prevented. Importantly, the functional role of the $M_2$ subtype on glycinergic interneurones in the spinal dorsal horn seems to be overwhelmed due to concurrent activation of mAChRs on GABAergic neurones by mAChR agonists. We observed that the potentiating effect of Oxo-M on glycinergic IPSCs was more pronounced in the presence of CGP55845 than that in the presence of AF-DX116 or himbacine. This is likely to be due to the potentiating effect of the $M_2$ mAChR subtype on glycinergic neurones being blocked, while blockade of $GABA_B$ receptors eliminated the indirect effect of mAChR subtypes on GABAergic neurones (Fig. 7).

In summary, our study provides novel information that mAChR subtypes are differentially involved in the dynamic regulation of glycinergic inputs to spinal dorsal horn neurones. As illustrated in Fig. 7, the $M_3$ subtype located on the somatodendritic site of glycinergic neurones is primarily responsible for the stimulatory effect of the mAChR agonist on glycinergic sIPSCs. Interestingly, the mAChR agonist also activates mAChRs on GABAergic interneurones, and through $GABA_B$ receptors on the nearby glycinergic interneurones, to attenuate this potentiating effect on synaptic glycine release. We also have demonstrated that, after elimination of the effect of mAChR agonists on GABAergic interneurones, activation of the $M_2$ subtype on glycinergic neurones appears to contribute to increased glycine release (Fig. 7). Thus, the mAChRs on the GABAergic neurones and the $GABA_B$ receptor on glycinergic neurones are both involved in the dynamic regulation of glycinergic synaptic input to spinal dorsal horn neurones. This study provides new insights into the mechanisms of the analgesic action of mAChR agonists in the spinal cord. In this regard, activation of mAChRs stimulates both GABAergic and glycinergic interneurones to increase the inhibitory tone to suppress the nociceptive transmission in the spinal cord. Furthermore, this study provides new functional evidence showing that GABA and glycine are released from separate synaptic terminals in the spinal dorsal horn following stimulation of mAChRs. GABAergic interneurones and $GABA_B$ receptors play an important role in feedback regulation of glycinergic synaptic transmission in the spinal cord.

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