Presynaptic $GABA_A$ receptors facilitate GABAergic transmission to dopaminergic neurons in the ventral tegmental area of young rats

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γ-Aminobutyric acid A receptor ($GABA_AR$)-mediated postsynaptic currents (IPSCs) were recorded from dopaminergic neurons of the ventral tegmental area of young rats in acute brain slices and from mechanically dissociated neurons. Low concentrations (0.1–0.3 μM) of muscimol, a selective $GABA_AR$ agonist, increased the amplitude, and reduced the paired pulse ratio of evoked IPSCs. Moreover, muscimol increased the frequency but not the amplitude of spontaneous IPSCs (sIPSCs). These data point to a presynaptic locus of muscimol action. It is interesting that 1 μM muscimol caused an inhibition of sIPSCs, which was reversed to potentiation by the $GABA_B$ receptor antagonist CGP52432. Isoguvacine, a selective $GABA_AR$ agonist that belongs to a different class, mimicked the effects of muscimol on sIPSCs: it increased them at low (≤ 0.5 μM), and decreased them at a higher concentration (1 μM). Hence, the activation of presynaptic $GABA_ARs$ facilitates GABA release, which is limited by presynaptic $GABA_BRs$. Furthermore, facilitation of sIPSCs by muscimol was eliminated in a medium containing tetrodotoxin or cadmium. It is noteworthy that sIPSC frequency was greatly increased by 4,5,6,7-tetrahydroisoxazolo[5,4-c]pyridin-3-ol(gaboxadol, or THIP), an agonist with preferential effects on extrasynaptic $GABA_ARs$ containing $\alpha4\beta\delta$ subunits, or by guvacine, a GABA transport blocker, which increases ambient GABA levels. In addition, sIPSC frequency was attenuated by furosemide, a selective antagonist of $\alpha6$ subunits. Thus, the presynaptic $GABA_ARs$ may be situated at extrasynaptic sites and may contain $\alpha4/6\beta\delta$ subunits. Given the marked sensitivity of extrasynaptic $GABA_ARs$ to ambient GABA, alcohols and anaesthetics, these receptors may present a critical site for regulating synaptic function in the developing brain in both physiological and pathological situations.

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γ-aminobutyric acid (GABA) is the main inhibitory neurotransmitter in the adult mammalian brain. In immature neurons, however, GABA is the primary excitatory neurotransmitter, has a depolarizing action, and is pivotal in control of neuronal firing, intracellular calcium signalling and neuronal development (Ben-Ari *et al.* 1997; Delpire, 2000; Ben-Ari, 2002; Owens & Kriegstein, 2002). The ventral tegmental area (VTA), one of the key brain regions for reward, is rich in dopaminergic (DA) neurons that project to the nucleus accumbens and prefrontal cortex (Beckstead *et al.* 1979). The DA neurons in the midbrain receive GABAergic inputs from local GABAergic neurons (Spanagel & Weiss, 1999; Diana & Tepper, 2002), medium spiny GABAergic projection neurons in nucleus accumbens (Waddington & Cross, 1978), and GABAergic neurons in ventral pallidum (Groenewegen *et al.* 1993).

Many neurotransmitter and peptide receptors are expressed in GABAergic axon terminals which synapse onto VTA DA neurons (Mansour *et al.* 1991; Cameron & Williams, 1993; Wu *et al.* 1995; Lu *et al.* 1997; Bonci & Malenka, 1999; Mansvelder & McGehee, 2000; Grillner *et al.* 2000; Svingos *et al.* 2001; Bergevin *et al.* 2002; Garzon & Pickel, 2002; Zheng *et al.* 2002; Szabo *et al.* 2002). The activation of these receptors modulates GABA release. Recent work from this laboratory demonstrated the existence of glycine receptors on the GABAergic terminals synapsing onto VTA DA neurons, and showed that activation of these receptors increased GABA release in young rats (Ye *et al.* 2004). Presynaptic $GABA_ARs$

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were found to modulate neurotransmitter release in both peripheral and central synapses. Classic studies of the neuromuscular junction (Dudel & Kuffler, 1961; Takeuchi & Takeuchi, 1966) and spinal motor neurons (Eccles, 1964) indicate that the activation of presynaptic $GABA_A$Rs inhibits transmitter release. Nevertheless, recent studies in rats showed that the activation of presynaptic $GABA_A$Rs increases the release of glutamate or glycine (Jang *et al.* 2002, 2005, 2006; Koga *et al.* 2005). However, it remains unknown whether GABA receptors exist on GABAergic axon terminals in the VTA, and how these presynaptic $GABA_A$ and $GABA_B$ receptors regulate GABAergic transmission.

In the present study, using a combination of pharmacological and electrophysiological approaches, we have provided evidence indicating that functional $GABA_A$ and $GABA_B$ receptors are located on the GABAergic axon terminals which synapse onto VTA DA neurons. Activation of these $GABA_A$ or $GABA_B$ receptors, respectively, facilitates or suppresses GABAergic transmission in the immature neurons. We have also provided evidence supporting an extrasynaptic locus for these presynaptic $GABA_A$Rs. In view of the marked sensitivity of the extrasynaptic $GABA_A$Rs to ambient GABA, alcohols and anaesthetics, these receptors are likely to play a critical role in the regulation of synaptic function in the developing brain in physiological and pathological situations. Some of these results have been reported previously in abstract form (Xiao & Ye, 2005; Ye *et al.* 2006*a*).

Methods

Preparations

All procedures abided by the UK Animals (Scientific Procedures) Act 1986. The care and use of animals and the experimental protocol of this study were approved by the Institutional Animal Care and Use Committee of the University of Medicine and Dentistry of New Jersey. All efforts were made to minimize animal suffering, to reduce the number of animal used, and to utilize alternatives to *in vivo* techniques. All experiments were done on brains from young Sprague–Dawley rats (at postnatal day (P) 4–10). Most experiments were performed using midbrain slices, which were prepared as previously described (Ye *et al.* 2004, 2006*b*). In brief, rats were anaesthetized with ketamine and xylazine (80 and 10 mg kg^{-1}, respectively, administered intraperitoneally) and then killed by decapitation. The midbrain was isolated and sliced in the coronal plane (250–300 μm) with VF-100 or VF-200 slicers (Precisionary Instruments, Greenville, NC, USA), while kept in ice-cold modified glycerol-based artificial cerebral spinal fluid (GACSF) saturated with 95% O_2–5% CO_2 (carbogen) containing (mM): glycerol 250, KCl 2.5, NaH_2PO_4 1.2, $MgCl_2$ 1.2, $CaCl_2$ 2.4, $NaHCO_3$ 26 and glucose 11. Slices (two per animal) were allowed to recover at 31°C for at least 1 h in a holding chamber, before they were placed in the recording chamber and superfused (1.5–2.0 ml min^{-1}) with carbogen-saturated ACSF. The recovering bath was filled with standard carbogenated ACSF containing (mM): NaCl 125, KCl 2.5, NaH_2PO_4 1.2, $MgCl_2$ 1.2, $CaCl_2$ 2.4, $NaHCO_3$ 26 and glucose 11. Some experiments were done on single neurons isolated from VTA slices using an enzyme-free mechanically dissociation procedure, as previously described (Ye *et al.* 2004). Briefly, midbrain slices (300 μm thick) were kept in carbogenated ACSF at room temperature (21–24°C) for at least 1 h before mechanical dissociation. The slices were then transferred to a 35 mm culture dish (Primaria 3801; Becton Dickinson, Rutherford, NJ, USA). These dishes were filled with standard external solution containing (mM): NaCl 140, KCl 5, $CaCl_2$ 2, $MgCl_2$ 2, Hepes 10 and glucose 10; 320 mosmol l^{-1}, pH adjusted to 7.4 with NaOH. Under an inverted microscope (Nikon, Tokyo, Japan), the VTA was identified medial to the accessory optic tract and lateral to the fasciculus retroflexus. A heavily fire-polished glass pipette, lightly touching the surface of the VTA, was vibrated horizontally at 20 Hz for 2 min using a homemade device. The slice was then removed. The isolated neurons adhered to the bottom of the dish within 20 min and were then ready for electrophysiological experiments.

Electrophysiological recording

Whole-cell and loose-patch cell-attached configurations were used to record electrical activity with MultiClamp 700A or Axopatch 200B amplifiers (Axon Instruments, Molecular Devices, Union City, CA, USA), Digidata 1320A or 1322A analog-to-digital converters (Axon Instruments), and pClamp 9.2 software (Axon Instruments). Data were filtered at 2 kHz and sampled at 10 kHz. Analyses were limited to data from cells having initial series resistance of 15–25 MΩ and the changes of which were less than 20% during recording.

The patch electrodes had a resistance of 3–5 MΩ, when filled with (mM): CsF 135, CsCl 5, EGTA 5, $CaCl_2$ 0.5, Hepes 10, Mg-ATP 2 and GTP 0.1 (for brain slice recording, Turecek & Trussell, 2001); or CsCl 140, $MgCl_2$ 2, EGTA 4, $CaCl_2$ 0.4, Hepes 10, Mg-ATP 2 and GTP 0.1 (for recordings from mechanically dissociated neurons); pH was adjusted to 7.2 with Tris base, and the osmolarity to 300 mOsmol l^{-1} with sucrose. Throughout the experiments, the bath was continuously perfused with the standard external solution (for the isolated neurons) or ACSF (for brain slices). All recordings were made in these solutions at an ambient temperature of 20–23°C.

In brain slices, cells were visualized with an upright microscope (E600FN; Nikon) and near infrared illumination. To evoke monosynaptic IPSCs, a glass

stimulation electrode with a tip of 10 μm in diameter was placed 50–100 μm away from the recording site in the VTA. Paired stimuli (100–200 μs depolarizing pulses, 50 ms apart) were given at the rate of 0.05 Hz.

Chemicals and applications

Most of the chemicals including D(–)-2-amino-5-phospho-pentanoate (AP5), 6,7-dinitroquinoxaline-2, 3-dione (DNQX), bicuculline (BIC), cadmium chloride, isoguvacine, furosemide, 4,5,6,7-tetrahydroisoxazolo [5, 4-c]pyridine-3-ol (gaboxadol, or THIP), guvacine hydrochloride, Tyr-D-Ala-Gly-N-Me-Phe-Gly-ol enkephalin (DAMGO) and tetrodotoxin (TTX), were obtained from Sigma (St Louis, MO, USA). Muscimol and CGP52432 were from Tocris (Ellisville, MO, USA). The solutions, prepared before the experiment, were applied to a dissociated neuron with a Y-tube. This exchanged the external solution surrounding the neurons within 40 ms (Zhou *et al.* 2006). In experiments on brain slices, chemicals were added in known concentrations to the superfusate. The fact that 10 μM bicuculline blocked most IPSCs (in slices) within 90 s is an indication of the effective bath exchange time.

Data analysis

Spontaneous discharges and inhibitory postsynaptic currents (sIPSCs) were counted and analysed with Clampfit 9.2 (Axon Instruments); sIPSCs were screened automatically (5 pA amplitude threshold), checked visually and accepted or rejected according to their rise and decay times. The frequency and amplitude of all events, during and after drug applications, were normalized to the mean of the values observed during the initial control period. Cumulative probability plots of the incidence of various inter-event intervals and amplitudes (for 100–1500 sIPSCs), recorded under different conditions from the same neuron, were compared with the Kolmogorov–Smirnov (K-S) test. For other plots, data obtained over a 1–2 min period at the peak of a drug response were normalized to the average values of the frequency and amplitude of sIPSCs or miniature IPSCs (mIPSCs) during the initial control period (4–5 min). The amplitude of eIPSCs was measured with Clampfit 9.2. For each experimental condition, we averaged the amplitudes of eIPSCs or paired pulse ratio ($PPR = IPSC_2/IPSC_1$) from 10 to 20 trials. $IPSC_1$ and $IPSC_2$ are the IPSCs in response to the first and second stimulus of the paired pulses, respectively. In the figures, single eIPSCs or paired eIPSCs are averages of > 10 successive traces. Data were expressed as means ($\pm$ S.E.M.). The statistical significance of drug effects was assessed by Student's paired two-tailed t test. Values of $P < 0.05$ were considered significant.

Results

Identification of DA neurons

Experiments were done in acute midbrain slices (Fig. 1*A*) or mechanically dissociated neurons (Fig. 1*B*). All recordings were obtained from putative DA neurons identified by their pharmacological and physiological properties. Specifically, spontaneous firing of VTA neurons was first recorded with the loose-patch cell-attached configuration (Fig. 1*C*). The depression of spontaneous firing by 0.2 μM quinpirole (QP), a dopamine D_2/D_3 receptor agonist (Fig. 1*Ca*) (in slices and isolated neurons), and facilitation by 1 μM DAMGO, a μ-opioid receptor agonist (Fig. 1*Cb*) (in slices), is probably due to DAMGO-induced disinhibition; this is a characteristic feature of VTA DA neurons (Margolis *et al.* 2006). We then changed the recording into the whole-cell configuration by applying further suction. The existence of a prominent inward current (I_h) activated by hyperpolarizing voltage steps (between -60 and -160 mV) recorded under voltage clamp (Fig. 1*Da*), or a voltage-sag in response to a hyperpolarizing current pulse (-100 pA) recorded under current clamp (Fig. 1*Db*) (Lacey *et al.* 1989), was used to further identify the putative VTA DA neurons (in slices and isolated neurons). I_h is present in 70% of VTA DA neurons, and it is well documented that GABAergic neurons do not have I_h (Cameron *et al.* 1997; Jones & Kauer, 1999; Neuhoff *et al.* 2002; Margolis *et al.* 2006; Schilstrom *et al.* 2006). Although I_h currents are not present exclusively in the DA cells, it is unlikely that the I_h-positive tyrosine hydroxylase-negative cells made a significant contribution to our observations.

Muscimol potentiates GABAergic evoked IPSCs (eIPSCs)

In midbrain slices, eIPSCs were recorded from VTA DA neurons at a holding potential (V_H) of 0 mV in the presence of AP5 (50 μM) and DNQX (20 μM). These eIPSCs were completely eliminated by 10 μM bicuculline (BIC), a specific $GABA_AR$ antagonist, indicating that they were mediated by $GABA_ARs$ (Fig. 2*Aa*). As illustrated in Fig. 2*Aa* and *Ab*, 0.3 μM muscimol (Mus), a specific agonist for $GABA_ARs$, prominently potentiated the amplitude of eIPSCs. The potentiation was reversible; eIPSCs recovered to control level after washout of muscimol. In seven experiments, 0.3 μM muscimol significantly enhanced eIPSCs by 35 ± 7% (control, 76 ± 6; muscimol, 103 ± 11 pA, $P = 0.02$; Fig. 2*C*).

Muscimol-induced enhancement of IPSCs could be due to a facilitation of GABA release, a potentiation of postsynaptic $GABA_A$ receptors, or a combination of the two. To determine whether a presynaptic mechanism is involved, we compared the response to paired pulse

stimulation (at 50 ms interval), a measure that changes in a highly predictable fashion with changes in the probability of transmitter release (Dobrunz & Stevens, 1997). As shown in Fig 2*Ba*, 0.3 μM muscimol prominently increased the amplitude of the first IPSC ($eIPSC_1$), but only slightly increased the second eIPSC ($eIPSC_2$), and thus significantly decreased the paired pulse ratio ($PPR = eIPSC_2/eIPSC_1$). Figure 2*Bb* shows that 0.3 μM muscimol reversibly decreased the PPR of seven cells. On average, 0.3 μM muscimol changed the PPR by $20 \pm 1\%$ (muscimol, 0.95 ± 0.05; control, 1.19 ± 0.08, $n = 7$, $P = 0.001$, Fig. 2*C*). These results indicate that muscimol increases presynaptic GABA release.

Muscimol presynaptically modifies sIPSCs

To further test this possibility, we compared sIPSCs recorded from slices in the absence and presence of muscimol. In the presence of AP5 (50 μM) and DNQX (20 μM), the application of 10 μM bicuculline completely eliminated all sIPSCs (data not shown), indicating that they were mediated by $GABA_A$Rs. Muscimol (0.3 μM) prominently increased sIPSC frequency (Fig. 3*A*) by $28 \pm 8\%$ (muscimol, 1.62 ± 0.38; control, 1.28 ± 0.31 Hz, $n = 6$, $P < 0.01$; Fig. 3*C*). This was further illustrated by cumulative plots of the incidence of various intervals between successive sIPSCs (Fig. 3*B*): 0.3 μM muscimol induced a marked shift towards shorter intervals (Fig. 3*Ba*, K-S test, $P < 0.01$), but no significant change in amplitude (Fig. 3*Bb*, K-S test, $P > 0.05$), and in the mean values ($101 \pm 6\%$ of control: muscimol, 17 ± 3; control, 17 ± 3 pA, $n = 6$, $P = 0.49$; Fig. 3*Cb*). These data further support a presynaptic locus of action of muscimol. Figure 3*Ca* illustrates the dose dependence of the action of muscimol on sIPSC frequency. While 0.1 μM muscimol enhanced sIPSC frequency by $35 \pm 7\%$ (muscimol, 1.84 ± 0.40; control, 1.39 ± 0.30 Hz, $n = 8$, $P < 0.01$), unexpectedly, 1 μM muscimol decreased sIPSC frequency by $40 \pm 12\%$ (muscimol, 0.82 ± 0.09; control, 1.60 ± 0.30 Hz, $n = 5$, $P < 0.05$; Fig. 3*Ca*; also see Fig. 4*A*). On the other hand,

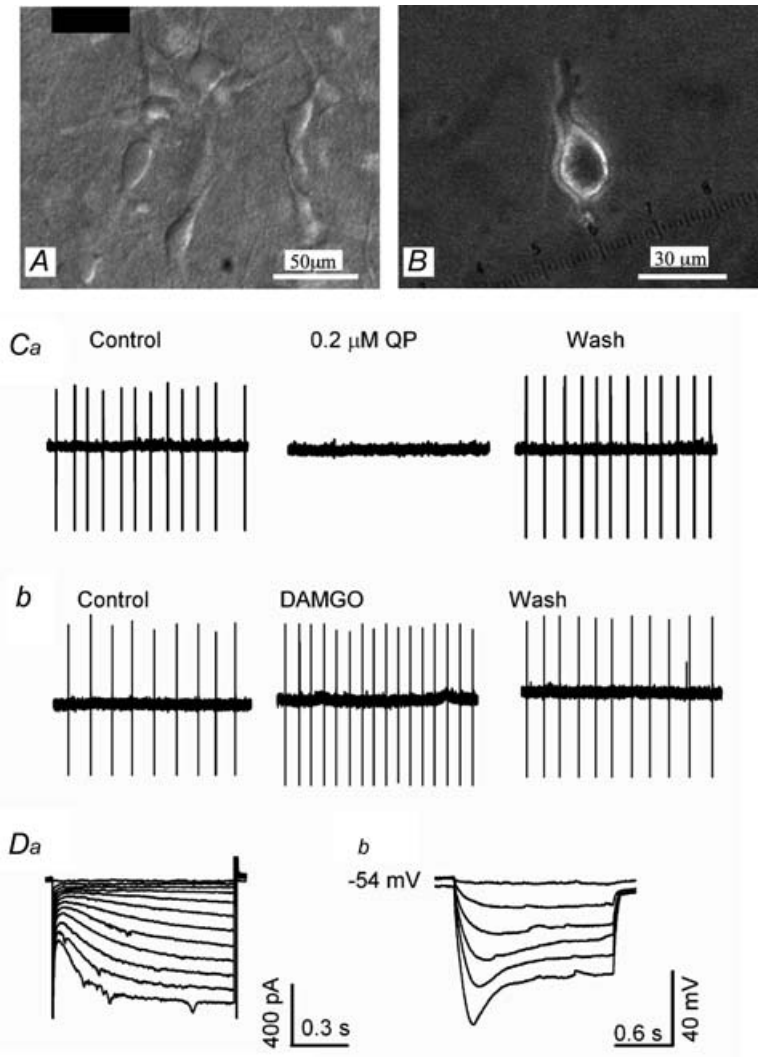

Figure 1. Electrophysiological and pharmacological properties of DA neurons in VTA
A, photomicrograph of neurons in a midbrain slice, obtained by a Nikon E600FN upright microscope, under differential interference contrast illumination, with the aid of a near-infrared CCD camera (40 ×, water immersion objective). *B*, a mechanically dissociated putative DA neuron from the VTA. *C*, spontaneous firings (5 s) of a DA neuron recorded in a slice were reversibly inhibited by quinpirole (0.2 μM), and facilitated by DAMGO (1 μM). *D*, this putative DA neuron has a large I_h induced by hyperpolarizing voltage (*Da*), and a prominent time-dependent 'sag' in membrane potential in response to −100 pA current, which was applied as 25 pA steps from 0 pA (*Db*).

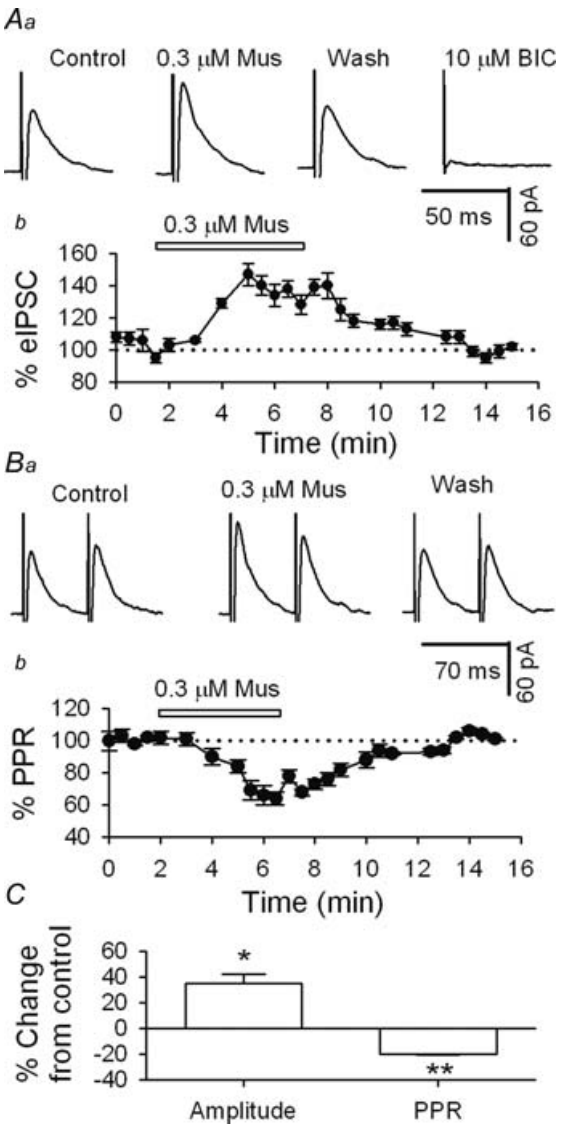

Figure 2. Muscimol enhances evoked IPSCs (eIPSCs) in putative DA neurons of VTA in midbrain slices
Aa, the $GABA_AR$ agonist muscimol (Mus, 0.3 μM) enhanced the amplitude of IPSCs evoked by pulse stimulation within VTA and recorded from a putative DA neuron, which was completely blocked by bicuculline (BIC, 10 μM). *Ab*, time course of change in eIPSC amplitude (mean ± S.E.M.) induced by 0.3 μM muscimol ($n = 7$). Before plotting, the peak amplitude of eIPSCs was normalized to the value obtained just before the application of muscimol. *Ba*, responses to paired pulse stimulation (at 50 ms interval) showed clear facilitation, which was significantly reduced by 0.3 μM muscimol. Data are averages of 10 traces. *Bb*, time course (mean ± S.E.M.) of 0.3 μM muscimol-induced reduction of percentage paired pulse ratio ($PPR = IPSC_2/IPSC_1$, $n = 7$). $IPSC_1$ and $IPSC_2$ are the IPSCs in response to the first and second stimulus of the paired pulses, respectively. *C*, summary of the effects of 0.3 μM muscimol ($n = 7$). Change of amplitude was calculated as $[(IPSC_{Mus}/IPSC_{control}) - 1] \times 100$. $IPSC_{mus}$ and $IPSC_{control}$ are the IPSCs recorded in the presence and absence of muscimol, respectively. Change of PPR was calculated as $[(PPR_{Mus}/PPR_{control}) - 1] \times 100$. PPR_{Mus} and $PPR_{control}$ are the paired pulse ratios obtained in the presence and absence of muscimol, respectively. $P < 0.05$, $P < 0.01$, paired *t* test for muscimol application *versus* pre-muscimol control. All IPSCs were recorded from VTA DA neurons of young rats (postnatal day 4–10). All IPSCs were recorded in the presence of AP5 (50 μM) and DNQX (20 μM), at a holding potential (V_H) of 0 mV (when recorded in brain slices) or of −50 mV (when recorded from isolated neurons with CsCl-based pipette solution (see Methods).

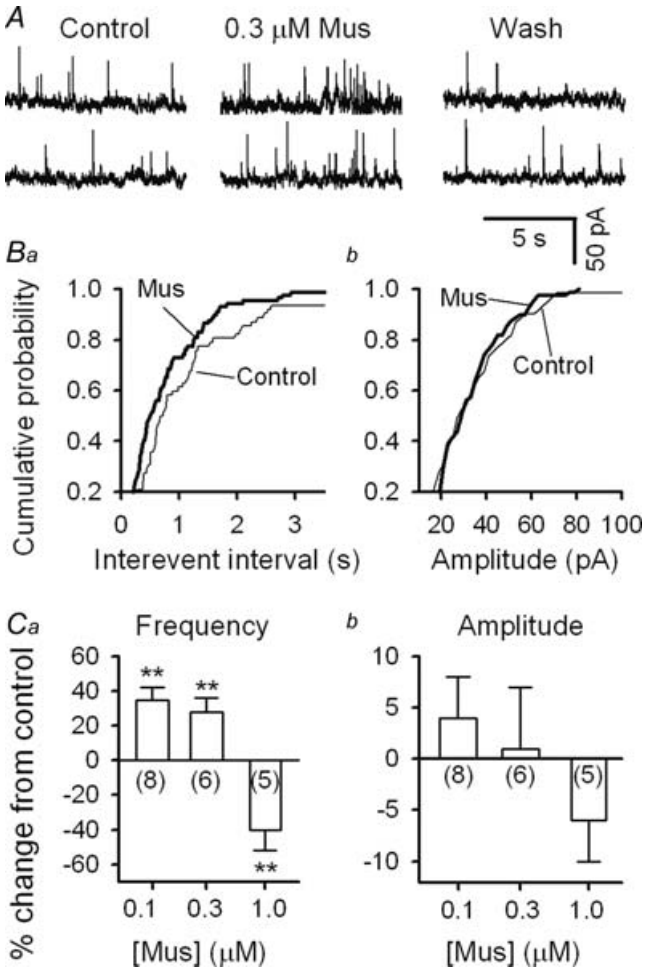

Figure 3. Muscimol modulates the frequency of spontaneous IPSCs (sIPSCs) in putative DA neurons of VTA in slices
All sIPSCs were recorded at a holding potential (V_H) of 0 mV, in the presence of AP5 (50 μM) and DNQX (20 μM). *A*, application of 0.3 μM muscimol greatly (and reversibly) increased the frequency of sIPSCs. *B*, representative cumulative probability plots show increased incidence of short intervals (*Ba*, K-S test, $P < 0.01$), but no change in the amplitudes (*Bb*, K-S test, $P > 0.5$). *C*, muscimol (0.1, 0.3 and 1 μM) showed dose-dependent effects of on the frequency (*Ca*), but no effect on amplitude (*Cb*) of sIPSCs (mean ± S.E.M.). Change of sIPSCs was calculated as $[IPSC_{Mus}/IPSC_{control}) - 1] \times 100$. Number of neurons in each group is indicated in parentheses. $^{**}P < 0.01$, paired *t* test for muscimol application *versus* pre-muscimol control.

at all these concentrations, muscimol did not alter sIPSC amplitude, as shown by the histogram in Fig. 3*Cb* (0.1 μM: muscimol, 18 ± 3; control, 17 ± 3 pA, $n = 8$, $P = 0.19$; 1 μM: muscimol, 13 ± 1; control, 15 ± 2 pA, $n = 5$, $P = 0.11$). These results are consistent with a presynaptic mechanism of muscimol action.

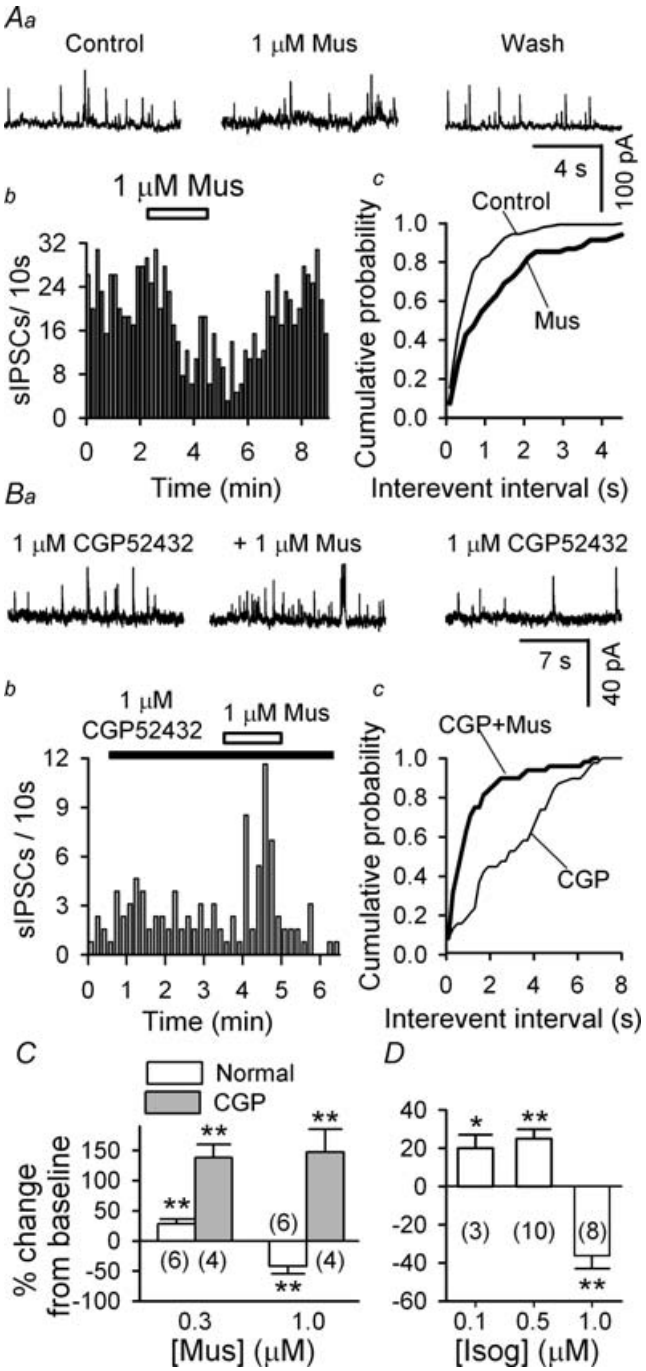

Figure 4. A $GABA_B$ receptor antagonist enhances the increase in spontaneous IPSC (sIPSC) frequency induced by muscimol in midbrain slices

All sIPSCs were recorded at a holding potential (V_H) of 0 mV, in the presence of AP5 (50 μM) and DNQX (20 μM). *Aa* and *Ba*, traces recorded from putative DA neurons show that the application of 1 μM muscimol (Mus) reversibly reduced the frequency of sIPSCs, but in the presence of 1 μM CGP52432, a $GABA_B$ receptor antagonist, the application of 1 μM muscimol reversibly increased the frequency of sIPSCs (*Ba*). *Ab* and *Bb*, time course of the effect of 1 μM muscimol on sIPSC frequency in the absence (*Ab*) or presence (*Bb*) of 1 μM CGP52432. *Ac* and *Bc*, cumulative probability plot of inter-event intervals before and during the application of 1 μM muscimol in the absence (*Ac*, rightward shift, K-S test, $P < 0.01$) and presence (*Bc*, leftward shift, K-S test, $P < 0.01$) of 1 μM CGP52432. *C*, summary of effects of muscimol on sIPSC frequency in the absence (□) and presence (▨) of 1 μM CGP52432. *D*, summary of the effects of isoguvacine (Isog, 0.1, 0.5 and 1.0 μM) on the frequency of sIPSCs. Change of sIPSCs was calculated as $[IPSC_{isog}/IPSC_{control}) - 1] \times 100$. $IPSC_{isog}$ and $IPSC_{control}$ are the IPSCs recorded in the presence and absence of isoguvacine, respectively. Number of neurons in each group is indicated in parentheses. $^*P < 0.05$, $^{**}P < 0.01$, paired *t* test for muscimol (or isoguvacine) *versus* pre-muscimol (or isoguvacine) control.

Activation of $GABA_B$ receptors limits muscimol-induced facilitation of GABAergic IPSCs

Most GABAergic synapses contain presynaptic $GABA_B$Rs (Misgeld *et al.* 1995). These presynaptic $GABA_B$Rs act as autoreceptors, and they limit transmitter release by reducing Ca^{2+} entry (Misgeld *et al.* 1995). To assess an involvement of $GABA_B$Rs, we compared the effect of muscimol on sIPSCs, recorded from slices, in the absence and presence of CGP52432 (1 μM), a selective $GABA_B$R antagonist. Figure 4*Aa–c* shows that when applied alone, 1 μM muscimol prominently suppressed sIPSC frequency, and induced significant rightward shift of the cumulative probability plot of inter-event intervals (K-S test, $P < 0.01$). By contrast, in the presence of 1 μM CGP52432, 1 μM muscimol prominently increased sIPSC frequency (Fig. 4*Ba* and *Bb*), by 147 ± 38% (muscimol, 0.49 ± 0.18; control, 0.20 ± 0.07 Hz, $n = 4$, $P < 0.01$; Fig. 4*C*), and induced significant leftward shift of the cumulative probability plot of inter-event intervals (K-S test, $P < 0.01$, Fig. 4*Bc*). CGP52432 also enhanced the action of muscimol at lower concentrations. In the presence of 1 μM CGP52432, 0.3 μM muscimol increased sIPSC frequency by 138 ± 24% (muscimol, 0.47 ± 0.16; control, 0.19 ± 0.03 Hz, $n = 4$, $P < 0.01$), which was significantly greater than that in the absence of CGP52432 (28 ± 8%: muscimol, 1.62 ± 0.38; control, 1.28 ± 0.31 Hz, $n = 6$, $P < 0.01$; Fig. 4*C*). These data indicate the existence of presynaptic $GABA_B$Rs on the GABAergic terminals synapsing onto VTA DA neurons, and that activation of these $GABA_B$Rs inhibits GABA release.

Muscimol (10 μM) was found to inhibit synaptic transmission through the activation of presynaptic $GABA_B$Rs in a pontine auditory nucleus (Yamauchi *et al.* 2000). To determine whether 1 μM muscimol-induced inhibition of sIPSCs in VTA is due to muscimol-dependent activation of presynaptic $GABA_B$Rs, we examined the effects of isoguvacine, a selective $GABA_A$R agonist from a different class, on sIPSCs. Note that a direct action of isoguvacine on $GABA_B$Rs has not been reported. As can be seen in Fig. 4*D*, 0.1 and 0.5 μM isoguvacine, respectively, increased sIPSC frequency by 20 ± 7% (control, 1.35 ± 0.36; 0.1 μM isoguvacine, 1.62 ± 0.30 Hz, $n = 3$, $P < 0.05$), and by 25 ± 5% (control, 1.33 ± 0.30; 0.5 μM isoguvacine,

1.67 ± 0.39 Hz, $n = 10$, $P < 0.01$), whereas, 1 μM isoguvacine decreased sIPSC frequency by $36 \pm 7\%$ (control, 2.05 ± 0.32; 1 μM isoguvacine, 1.47 ± 0.26 Hz, $n = 8$, $P < 0.01$). Thus, it is unlikely that a direct activation of $GABA_B$Rs by muscimol plays a major role in 1 μM muscimol-induced inhibition of sIPSCs.

Muscimol acts in a tetrodotoxin- and cadmium-sensitive manner

We then studied the mechanism underlying muscimol-induced enhancement of sIPSC frequency on VTA DA neurons freshly isolated using an enzyme-free procedure. These isolated neurons retain attached functional excitatory and inhibitory synaptic boutons (Ye *et al.* 2004; Zhou *et al.* 2006).

The sIPSCs were recorded at a V_H of -60 mV with CsCl-containing pipette solution (see Method) in the presence of AP5 (50 μM) and DNQX (20 μM). Bicuculline (10 μM) completely blocked these sIPSCs, indicating that they were mediated by $GABA_A$ receptors (data not shown). Muscimol (0.3 μM) robustly enhanced sIPSC frequency and induced significant leftward shift of the cumulative probability plot of inter-event intervals (K-S test: $P < 0.01$; Fig. 5*Aa* and *Ab*). The enhancement (mean ± s.e.m.) from eight cells was $82 \pm 22\%$ (muscimol, 3.49 ± 0.96; control, 1.93 ± 0.53 Hz, $n = 8$, $P = 0.004$; Fig. 5*Da*). Note that the enhancement induced by 0.3 μM muscimol in the isolated neurons is larger than that observed in slices (also see Fig. 6). The mechanism underlying this difference warrants further study. The simplest interpretation is that the solution around an isolated neuron was much better controlled. Muscimol (0.3 μM) did not change the frequency of the sIPSCs either in the presence of a sodium channel blocker, 0.5 μM tetrodotoxin (TTX) ($99 \pm 3\%$ of that before muscimol application, from 0.65 ± 0.18 Hz in TTX to 0.64 ± 0.18 Hz in TTX + muscimol, $n = 6$, $P = 0.40$, Fig. 5*Ba*, *Bb* and *Da*), or when voltage-gated calcium channels (VGCCs) were blocked by 100 μM $CdCl_2$ ($99 \pm 6\%$ of that before muscimol application, from 0.3 ± 0.1 Hz in $CdCl_2$ to 0.3 ± 0.1 Hz in $CdCl_2$ + muscimol, $n = 7$, $P = 0.33$; Fig. 5*Ca*, *Cb* and *Da*). In addition, muscimol did not alter the mean amplitude of the sIPSCs in the absence ($96 \pm 4\%$ of that before muscimol application, from 57 ± 9 pA in control to 54 ± 8 pA, $n = 8$, $P = 0.18$) and presence of TTX ($103 \pm 6\%$ of that before muscimol application, from 23 ± 4 pA in TTX to 28 ± 5 pA in TTX + muscimol, $n = 6$, $P = 0.33$) or $CdCl_2$ ($101 \pm 5\%$ of that before muscimol application, from 14 ± 3 pA in $CdCl_2$ to 14 ± 2 pA in $CdCl_2$ + muscimol, $n = 7$, $P = 0.39$) (Fig. 5*Db*). These results suggest that enhancement of GABA release by muscimol depends on both Na^+ channels and VGCCs.

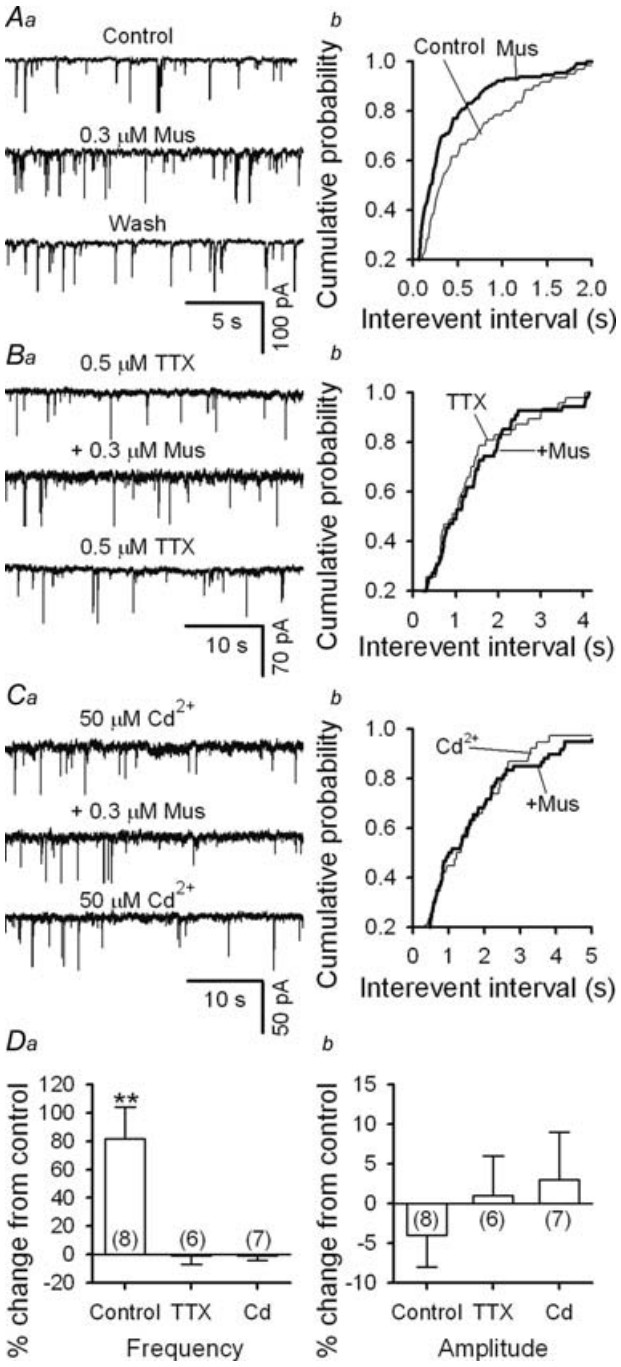

Figure 5. Tetrodotoxin or cadmium eliminated muscimol-induced increase in the frequency of spontaneous IPSCs (sIPSCs) recorded from mechanically dissociated VTA DA neurons
All sIPSCs were recorded at a holding potential (V_H) of -50 mV, in the presence of AP5 (50 μM) and DNQX (20 μM) with CsCl-based pipette solution. *Aa–Ca*, the application of 0.3 μM muscimol greatly increased the frequency of spontaneous IPSCs in the absence (*Aa*), but not in the presence of 0.5 μM TTX (*Ba*) or 50 μM $CdCl_2$ (*Ca*). *Ab–Cb*, cumulative probability plots of the inter-event intervals of sIPSCs in the absence (*Ab*), and presence of 0.5 μM TTX (*Bb*) or 50 μM $CdCl_2$ (*Cb*). *Da* and *Db*, summary of effects of 0.3 μM muscimol on the frequency (*Da*) and amplitude (*Db*) of sIPSCs in conditions indicated. $P < 0.01$, paired *t* test for muscimol application *versus* pre-muscimol control.

Muscimol modulates GABA release via $GABA_A$ receptors containing $\alpha 4/6\beta\delta$ subunits

To identify the subunits of presynaptic $GABA_A$Rs that mediate muscimol-induced facilitation of GABAergic transmission, we conducted experiments on VTA DA neurons freshly isolated using an enzyme-free procedure, as previously mentioned.

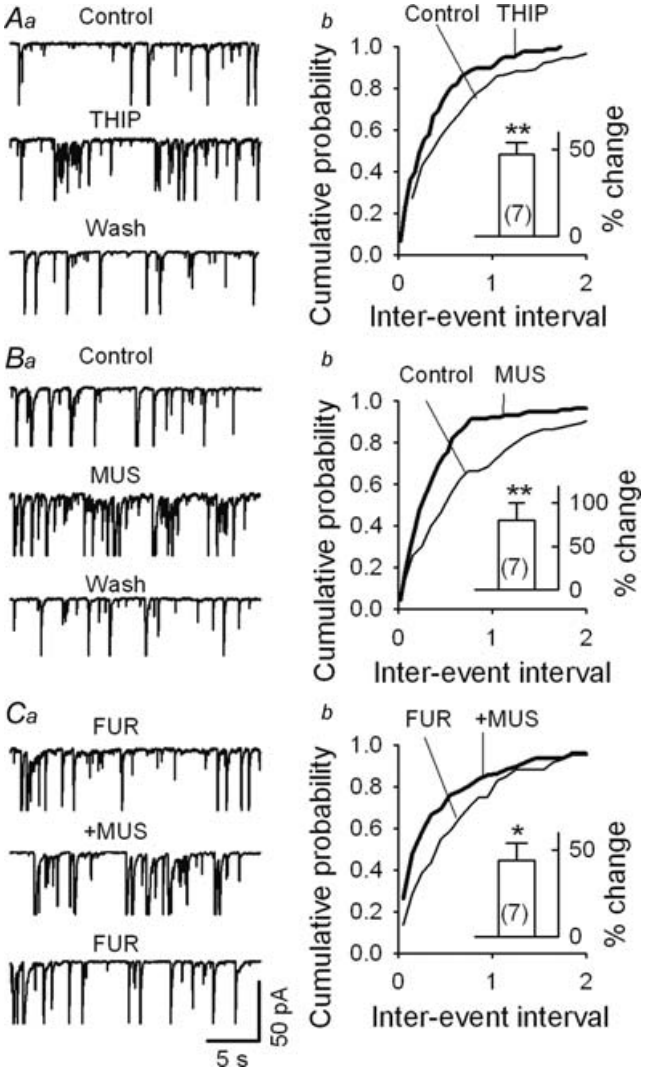

Figure 6. Multi-subunits of $GABA_A$ receptors mediate muscimol-induced enhancement of spontaneous IPSCs (sIPSCs)
All sIPSCs were recorded from mechanically dissociated VTA DA neurons at a holding potential (V_H) of −50 mV, in the presence of AP5 (50 μM) and DNQX (20 μM) with CsCl-based pipette solution. Gabaxadol (THIP, 1 μM, *Aa*), an agonist with selective effects on extrasynaptic $GABA_A$Rs containing $\alpha 4\beta\delta$ subunits, induced an increase in sIPSC frequency, and a leftward shift of cumulative probability plots of inter-event interval of sIPSCs (*Ab*). Muscimol (MUS, 0.3 μM, *Ba*) increased sIPSC frequency, and induced a leftward shift of cumulative probability plots of inter-event interval of sIPSCs (*Bb*). In the presence of furosemide (FUR, 100 μM), a selective antagonist of $GABA_A$Rs containing $\alpha 6$ subunit, the effect of muscimol (0.3 μM) was significantly smaller (*Cb*). Insets are summaries of increases in sIPSC frequency induced by gabazadol (*Ab*), muscimol alone (*Bb*), and muscimol in the presence of furosemide (*Cb*). Number of cells in each group is indicated in parentheses. $P < 0.01$, paired *t* test for gabaxedol application *versus* pre-gaboxedol control (*Ab*), or for muscimol application *versus* pre-muscimol control (*Bb* and *Cb*).

The application of 1 μM gaboxadol, a selective agonist for extrasynaptic $GABA_A$Rs containing $\alpha 4\beta\delta$ subunits (Brown *et al.* 2002), induced a prominent increase in sIPSC frequency by $47 \pm 7\%$ (control, 1.32 ± 0.05; gaboxadol, 1.90 ± 0.33 Hz, $n = 7$, $P < 0.01$), and a significant leftward shift of cumulative probability plot of inter-event intervals of sIPSCs (K-S test, $P < 0.001$; Fig. 6*Aa* and *Ab*).

Next, we compared muscimol-induced facilitation in the absence and presence of furosemide, a selective blocker for $GABA_A$Rs containing the $\alpha 6$ subunit (Hamann *et al.* 2002). As described earlier, when muscimol (0.3 μM) was applied alone, it enhanced sIPSC frequency robustly, by $82 \pm 18\%$ ($n = 7$, $P < 0.01$, Fig. 6*Ba*), and induced a significant leftward shift of cumulative probability plot (K-S test, $P < 0.001$ Fig. 6*Bb*). By contrast, furosemide (100 μM) significantly decreased sIPSC frequency by $29 \pm 6\%$ ($n = 7$, $P < 0.01$, data not illustrated). After the response to furosemide had stabilized, a mixture of 100 μM furosemide and 0.3 μM muscimol was applied. As illustrated in Fig. 6*Ca–Cb*, under this condition, 0.3 μM muscimol increased sIPSC frequency by only $44 \pm 10\%$ ($n = 7$, $P < 0.01$), which is significantly smaller than the muscimol-induced facilitation observed in the absence of furosemide ($82 \pm 18\%$, $n = 7$, $P < 0.05$, paired *t* test). These results indicate that enhancement of GABA release by muscimol might be mediated by $GABA_A$Rs containing $\alpha 4\beta\delta$ or $\alpha 6$ subunits. Nevertheless, furosemide is a blocker of some Cl^- transporters, such as the K^+ Cl^- cotransporter (KCC2). By blocking KCC2, furosemide could induce a shift of Cl^- reversal potential towards less negative potentials. In immature neurons, it will increase the driven force for Cl^- efflux (Zhu *et al.* 2005), and enhance presynaptic $GABA_A$R function. This will counteract the inhibitory effect of furosemide on GABA release. If this were the case, furosemide-induced inhibition of GABA release observed in our experiments would be underestimated.

Increasing ambient GABA increases sIPSC frequency in VTA DA neurons in brain slices

According to the localization, $GABA_A$Rs are classified as synaptic and extrasynaptic receptors. These two kinds of receptors seem different in subunit composition (Farrant & Nusser, 2005; but see Santhakumar *et al.* 2006). $GABA_A$Rs containing δ, $\alpha 4$, $\alpha 5$ or $\alpha 6$ subunits appear to be predominantly extrasynaptic. Therefore, our data suggest that muscimol might enhance GABA release by the activation of extrasynaptic $GABA_A$Rs expressed on GABAergic axon terminals.

Because extrasynaptic $GABA_A$Rs can be activated by GABA escaping from the synaptic cleft, we tested whether

an increase in ambient GABA levels could enhance sIPSC frequency on VTA DA neurons in brain slices. In this series of experiments, we recorded sIPSCs on VTA DA neurons (V_H, −50 mV) in the presence of 50 μM AP5, 20 μM DNQX and 1 μM CGP52432, using CsCl-based pipette solution.

The application of guvacine (20 μM), a GABA transporter blocker, induced a robust and reversible increase in the frequency of sIPSCs recorded in slices (Fig. 7*A* and *C*) without inducing a significant postsynaptic current (5 ± 1 pA). Guvacine (20 μM) induced a large leftward shift in the cumulative probability plot of inter-event intervals between successive sIPSCs (K-S test, $P < 0.001$; upper panel in Fig. 7*B*) and an increase of $72 \pm 14\%$ ($n = 7$, $P = 0.001$, upper panel inset in Fig. 7*B*) in sIPSC frequency, while it did not alter sIPSC amplitude (K-S test, $P > 0.5$, lower panel in Fig. 7*B*; $0 \pm 3\%$, $n = 7$, $P = 0.46$, lower panel inset in Fig. 7*B*). These results suggest that an increase in ambient GABA level enhances GABAergic sIPSCs via a presynaptic mechanism in the developing brain.

Discussion

We firstly demonstrated the existence of the presynaptic $GABA_A$ and $GABA_B$ receptors on GABAergic axon terminals that synapse onto the DA neurons in the VTA. The presynaptic $GABA_A$Rs, which may be situated at extrasynaptic sites, may contain $\alpha 4/6\beta\delta$ subunits. In the immature neurons, the activation of these $GABA_A$Rs enhances GABA release, which is normally limited by the presynaptic $GABA_B$R activity. The interaction between these two presynaptic GABA receptors may play a critical role in the regulation of VTA DA neuronal excitability in the developing brain.

Presynaptic $GABA_A$ receptors facilitate GABA release onto VTA DA neurons of young rats

Low concentrations of muscimol enhanced GABAergic synaptic transmission in the VTA, as evidenced by the increase in the amplitude, the decrease in the paired pulse ratio of eIPSCs, and the increase in the frequency but not the amplitude of spontaneous IPSCs. These results lend support for a presynaptic locus of muscimol action. Muscimol enhances also the frequency of sIPSCs recorded from mechanically dissociated neurons. This finding indicates that functioning $GABA_A$Rs exist on, or near, GABAergic axon terminals on VTA DA neurons. Several mechanisms may underlie presynaptic $GABA_A$R modulation of neurotransmitter release (Vitten & Isaacson, 2001). In developing and injured neurons, nerve terminals contain high intracellular Cl^- levels (Ben-Ari, 2002; Nabekura *et al.* 2002). Activation of $GABA_A$Rs causes depolarization, which elevates the intracellular calcium concentration via activation of VGCCs, and increases neurotransmitter release.

Our previous studies showed that intracellular Cl^- concentration is high in VTA neurons of young rats, and activation of glycine receptors, opening also a Cl^- channel, induces membrane depolarization (Ye, 2000; Wang *et al.* 2005). Furthermore, activation of presynaptic glycine receptors increases GABA release in neonatal rats (Ye *et al.* 2004). In keeping with these findings, we showed here that several $GABA_A$R agonists enhanced GABA release onto VTA DA neurons of neonatal rats.

In the VTA, a low concentration of muscimol enhanced sIPSC frequency. The enhancement was not seen in the presence of tetrodotoxin or cadmium. These data indicate that enhancement by muscimol depends on Na^+ channel and VGCCs. In nerve terminals of CNS, N- and P/Q-type VGCCs mediate most of the calcium-dependent

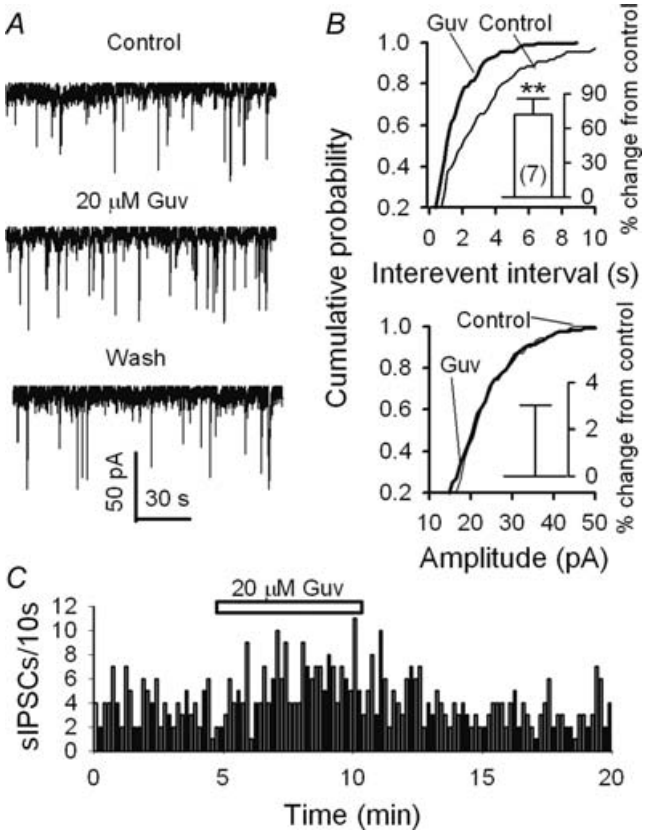

Figure 7. An increase in ambient GABA levels increases the frequency of spontaneous IPSCs (sIPSCs), recorded from VTA DA neurons in slices

The holding potential was −50 mV. *A*, guvacine (Guv, 20 μM), a GABA transport blocker, increased sIPSC frequency. *B*, cumulative probability plots show that the application of guvacine increased incidence of shorter intervals between sIPSCs (upper panel), but there was no associated change in sIPSC amplitude (lower panel). Inset, pooled data from seven cells show that guvacine increased sIPSC frequency but not the amplitude. *C*, time course of guvacine-induced increase in sIPSC frequency.

transmitter release (Takahashi & Momiyama, 1993; Poncer *et al.* 1997). Based on our data, we postulate that at low concentrations, muscimol induces a slight depolarization, which is enough to activate Na^+ channels, but not the VGCCs. Following the activation of Na^+ channels, VGCCs are activated, leading to an increase in intraterminal calcium concentration, and an increase in transmitter release. Similar mechanisms have been implicated in presynaptic glycine receptor activation-induced enhancement of GABA release (Ye *et al.* 2004), and also in presynaptic $GABA_A$R activation-induced enhancement of glutamate release (Koga *et al.* 2005; Jang *et al.* 2006).

Nevertheless, in the VTA, 1 μM muscimol (or isoguvacine) reduced sIPSC frequency. Several possible mechanisms could account for this observation. First, the opening of the presynaptic $GABA_A$Rs decreases the input resistance of the terminal and acts as an electrical shunt, which leads to a reduction in the amplitude of the action potential as it invades the nerve ending. This results in a reduction of neurotransmitter release (Vitten & Isaacson, 2001). Second, strong depolarization can inactivate Na^+ channels, causing inhibition of invasion of action potentials (Segev, 1990; Stuart & Redman, 1992). Third, a higher concentration of muscimol (and isoguvacine) may indirectly activate presynaptic $GABA_B$Rs, which results in a reduction of GABA release. The results shown in Fig. 4*A*–*C* support the latter mechanism as a major contributing factor (see below).

Interaction between presynaptic $GABA_A$ and $GABA_B$ receptors

In the VTA, muscimol-induced enhancement of GABA release was observed only at low concentrations (0.1 and 0.3 μM). At a higher concentration (1 μM), muscimol suppressed GABA release. However, application of the $GABA_B$R antagonist CGP52432 reversed the effect of 1 μM muscimol, from inhibition to potentiation. Furthermore, CGP52432 potentiated 0.3 μM muscimol-induced enhancement. These data suggest the existence of both $GABA_A$ and $GABA_B$ receptors on the GABAergic axon terminals, which synapse onto the DA neurons in the VTA. The activation of presynaptic $GABA_A$ and $GABA_B$ receptors increases and decreases GABA release, respectively. The net effect of muscimol is determined by the balance between the activities of these two types of receptors.

It is possible that at high concentrations, the release of GABA induced by muscimol (and isoguvacine) is high enough to activate presynaptic $GABA_B$Rs and therefore opposes the effect of presynaptic $GABA_A$Rs. The higher EC_{50} value of muscimol (and isoguvacine) for presynaptic $GABA_B$Rs may be due to their extrasynaptic location. Recent evidence indicates that $GABA_B$Rs are located extrasynaptically, and that both pre- and postsynaptic $GABA_B$Rs are located some distance from the site of release. This means that to recruit $GABA_B$Rs, the GABA concentration must be high, so that more GABA can escape from the synaptic cleft (Nicoll, 2004). In response to low concentrations of muscimol (and isoguvacine), the escaped GABA may not be enough to activate presynaptic $GABA_B$Rs. Only at much higher concentrations, can muscimol (and isoguvacine) induce enough spillover of GABA to activate $GABA_B$Rs, overcome the effects of $GABA_A$Rs, and consequently inhibit GABA release. In a recent study in hippocampal neurons, it was found that potentiation by ethanol of GABAergic synaptic transmission is limited by presynaptic $GABA_B$Rs (Ariwodola & Weiner, 2004). It will be interesting to determine how the interaction between the presynaptic $GABA_A$ and $GABA_B$ receptors contributes to the effects of ethanol in the VTA.

Presynaptic $GABA_A$ receptors on GABAergic terminals on VTA DA neurons may be at extrasynaptic sites and may contain $\alpha 4/6\beta\delta$ subunits

As mentioned above, VTA DA neurons receive abundant GABAergic inputs from several brain areas. Previous histological studies indicated expression of various subunits of $GABA_A$Rs in these areas of adult rats. The $\alpha 1$, $\alpha 3$, $\beta 2$, $\gamma 1$ and $\gamma 3$ subunits were found in medium-sized neurons in nucleus accumbens. Neurons in ventral pallidum express $\alpha 1$, $\alpha 3$, $\alpha 5$, $\beta 1$–3, $\gamma 1$, $\gamma 3$ and δ subunits. In the VTA, the $\alpha 1$–3, $\alpha 5$, $\beta 1$, $\gamma 3$ and δ subunits were found in the neurons, while the $\alpha 1$–3, $\beta 1$–3, $\gamma 2$ and δ subunits were found in the processes (Pirker *et al.* 2000; Schwarzer *et al.* 2001). Using a combination of single-cell PCR and *in situ* hybridization, Okada *et al.* (2004) found that $\alpha 2$–4, $\beta 1$, $\beta 3$ and $\gamma 2$ subunits are expressed on VTA DA neurons. These studies have not examined expression of the $\alpha 6$ subunit.

In the VTA, we observed that a low concentration of gaboxadol induced a robust increase of sIPSC frequency and furosemide attenuated enhancement by muscimol of sIPSC frequency. These findings suggest that functional $GABA_A$Rs containing $\alpha 4\beta\delta$ or $\alpha 6$ subunits exist on GABAergic axon terminals on VTA DA neurons. To the best of our knowledge, this is the first evidence showing the possibility of the existence of functional $\alpha 4$ and $\alpha 6$ subunits in VTA GABAergic axon terminals.

Extrasynaptic $GABA_A$Rs were found in several cell types, such as cerebellar granule cells, hippocampal cells and forebrain neurons. The δ subunit forms extrasynaptic receptors with $\alpha 6$ and $\beta 2/3$ in cerebellar granule cells, and with the $\alpha 4$ and $\beta 1$–3 subunits in thalamus, neostriatum and dentate gyrus. In hippocampal pyramidal cells, the $\alpha 5$ subunit forms extrasynaptic receptors probably with $\beta 3$

and $\gamma 2$ subunits. In view of the accumulating evidence that $GABA_A$Rs containing δ, $\alpha 4$, $\alpha 5$ or $\alpha 6$ subunits seem to be predominantly extrasynaptic (Farrant & Nusser, 2005; but see Santhakumar *et al.* 2006), we postulated that some of the presynaptic $GABA_A$Rs may be situated at extrasynaptic sites on or near the GABAergic axon terminal. This notion was supported by our observation that increasing ambient GABA levels by application of guvacine, a GABA transport blocker, increased the frequency of sIPSCs.

Physiological role of the presynaptic $GABA_A$ receptors in the VTA of young rats

Our finding that functional GABA receptors exist at GABAergic terminals on VTA DA neurons provides a new mechanism for the regulation of the excitability of these neurons. In young rats, GABA induces membrane depolarization and an increase in calcium concentration, which is critical for normal growth of neurons and their synaptic connections. However, this action must be balanced by other mechanisms. Our findings indicate that release of GABA facilitates further GABA release through the activation of presynaptic $GABA_A$Rs, which is attenuated by presynaptic $GABA_B$Rs. This integrated action will ensure the accuracy of signal transmission and may protect neurons from over-excitation and neuroexcitotoxicity.

Based on our data, we further propose that the presynaptic $GABA_A$Rs are situated probably, at least in part, at extrasynaptic sites. Accumulating evidence indicates that extrasynaptic $GABA_A$Rs are highly sensitive to, and tonically activated by, ambient GABA levels. Hence, by activating these receptors, ambient GABA levels might alter the presynaptic resting potential, which has been shown recently to be a powerful means for regulating synaptic function (Awatramani *et al.* 2005). Furthermore, in view of emerging evidence that extrasynaptic $GABA_A$Rs are particularly sensitive to alcohols and anaesthetics (Orser *et al.* 2002; Carta *et al.* 2004; Hanchar *et al.* 2005; Wallner *et al.* 2006), these receptors may represent a critical site in the regulation of synaptic function in the developing brain in both physiological and pathological situations.

References

Ariwodola OJ & Weiner JL (2004). Ethanol potentiation of GABAergic synaptic transmission may be self-limiting: role of presynaptic $GABA_B$ receptors. *J Neurosci* **24**, 10679–10686.

Awatramani GB, Price GD & Trussell LO (2005). Modulation of transmitter release by presynaptic resting potential and background calcium levels. *Neuron* **48**, 109–121.

Beckstead RM, Domesick VB & Nauta WJ (1979). Efferent connections of the substantia nigra and ventral tegmental area in the rat. *Brain Res* **175**, 191–217.

Ben-Ari Y (2002). Excitatory actions of GABA during development: the nature of the nurture. *Nat Rev Neurosci* **3**, 728–739.

Ben-Ari Y, Khazipov R, Leinekugel X, Caillard O & Gaiarsa JL (1997). $GABA_A$, NMDA and AMPA receptors: a developmentally regulated 'Menage a trois'. *Trends Neurosci* **20**, 523–529.

Bergevin A, Girardot D, Bourque MJ & Trudeau LE (2002). Presynaptic mu-opioid receptors regulate a late step of the secretory process in rat ventral tegmental area GABAergic neurons. *Neuropharmacology* **42**, 1065–1078.

Bonci A & Malenka RC (1999). Properties and plasticity of excitatory synapses on dopaminergic and GABAergic cells in the ventral tegmental area. *J Neurosci* **19**, 3723–3730.

Brown N, Kerby J, Bonnert TP, Whiting PJ & Wafford KA (2002). Pharmacological characterization of a novel cell line expressing human $\alpha_4\beta_3\delta$ $GABA_A$ receptors. *Br J Pharmacol* **136**, 965–974.

Cameron DL, Wessendorf MW & Williams JT (1997). A subset of ventral tegmental area neurons is inhibited by dopamine, 5-hydroxytryptamine and opioids. *Neuroscience* **77**, 155–166.

Cameron DL & Williams JT (1993). Dopamine D1 receptors facilitate transmitter release. *Nature* **366**, 344–347.

Carta M, Mameli M & Valenzuela CF (2004). Alcohol enhances GABAergic transmission to cerebellar granule cells via an increase in Golgi cell excitability. *J Neurosci* **24**, 3746–3751.

Delpire E (2000). Cation-chloride cotranspoters in neuronal communication. *News Physiol Sci* **15**, 344–347.

Diana M & Tepper JM (2002). Electrophysiological pharmacology of mesencephalic dopaminergic neurons. In *Handbook of Experimental Pharmacology*, ed. Di Chiara G, pp. 1–61. Springer-Verlag, Berlin Heidelberg.

Dobrunz LE & Stevens CF (1997). Heterogeneity of release probability, facilitation, and depletion at central synapses. *Neuron* **18**, 995–1008.

Dudel J & Kuffler SW (1961). Presynaptic inhibition at the crayfish neuromuscular junction. *J Physiol* **155**, 543–562.

Eccles JC (1964). Presynaptic inhibition in the spinal cord. *Prog Brain Res* **12**, 65–91.

Farrant M & Nusser Z (2005). Variations on an inhibitory theme: phasic and tonic activation of $GABA_A$ receptors. *Nat Rev Neurosci* **6**, 215–229.

Garzon M & Pickel VM (2002). Ultrastructural localization of enkephalin and mu-opioid receptors in the rat ventral tegmental area. *Neuroscience* **114**, 461–474.

Grillner P, Berretta N, Bernardi G, Svensson TH & Mercuri NB (2000). Muscarinic receptors depress GABAergic synaptic transmission in rat midbrain dopamine neurons. *Neuroscience* **96**, 299–307.

Groenewegen HJ, Berendse HW & Haber SN (1993). Organization of the output of the ventral striatopallidal system in the rat: ventral pallidal efferents. *Neuroscience* **57**, 113–142.

Hamann M, Rossi DJ & Attwell D (2002). Tonic and spillover inhibition of granule cells control information flow through cerebellar cortex. *Neuron* **33**, 625–633.

Hanchar HJ, Dodson PD, Olsen RW, Otis TS & Wallner M (2005). Alcohol-induced motor impairment caused by increased extrasynaptic $GABA_A$ receptor activity. *Nat Neurosci* **8**, 339–345.

Jang IS, Ito Y & Akaike N (2005). Feed-forward facilitation of glutamate release by presynaptic $GABA_A$ receptors. *Neuroscience* **135**, 737–748.

Jang IS, Jeong HJ, Katsurabayashi S & Akaike N (2002). Functional roles of presynaptic $GABA_A$ receptors on glycinergic nerve terminals in the rat spinal cord. *J Physiol* **541**, 423–434.

Jang IS, Nakamura M, Ito Y & Akaike N (2006). Presynaptic $GABA_A$ receptors facilitate spontaneous glutamate release from presynaptic terminals on mechanically dissociated rat CA3 pyramidal neurons. *Neurosci* **138**, 25–35.

Jones S & Kauer JA (1999). Amphetamine depresses excitatory synaptic transmission via serotonin receptors in the ventral tegmental area. *J Neurosci* **19**, 9780–9787.

Koga H, Ishibashi H, Shimada H, Jang IS, Nakamura TY & Nabekura J (2005). Activation of presynaptic $GABA_A$ receptors increases spontaneous glutamate release onto noradrenergic neurons of the rat locus coeruleus. *Brain Res* **1046**, 24–31.

Lacey MG, Mercuri NB & North RA (1989). Two cell types in rat substantia nigra zona compacta distinguished by membrane properties and the actions of dopamine and opioids. *J Neurosci* **9**, 1233–1241.

Lu XY, Churchill L & Kalivas PW (1997). Expression of D1 receptor mRNA in projections from the forebrain to the ventral tegmental area. *Synapse* **25**, 205–214.

Mansour A, Meador-Woodruff JH, Zhou QY, Civelli O, Akil H & Watson SJ (1991). A comparison of D1 receptor binding and mRNA in rat brain using receptor autoradiographic and in situ hybridization techniques. *Neuroscience* **45**, 359–371.

Mansvelder HD & McGehee DS (2000). Long-term potentiation of excitatory inputs to brain reward areas by nicotine. *Neuron* **27**, 349–357.

Margolis EB, Lock H, Hjelmstad GO & Fields HL (2006). The ventral tegmental area revisited: is there an electrophysiological marker for dopaminergic neurons. *J Physiol* **577**, 907–924.

Misgeld U, Bijak M & Jarolimek W (1995). A physiological role for $GABA_B$ receptors and the effects of baclofen in the mammalian central nervous system. *Prog Neurobiol* **46**, 423–462.

Nabekura J, Ueno T, Okabe A, Furuta A, Iwaki T, Shimizu-Okabe C, Fukuda A & Akaike N (2002). Reduction of KCC2 expression and GABAA receptor-mediated excitation after in vivo axonal injury. *J Neurosci* **22**, 4412–4417.

Neuhoff H, Neu A, Liss B & Roeper J (2002). I_h channels contribute to the different functional properties of identified dopaminergic subpopulations in the midbrain. *J Neurosci* **22**, 1290–1302.

Nicoll RA (2004). My close encounter with $GABA_B$ receptors. *Biochem Pharmacol* **68**, 1667–1674.

Okada H, Matsushita N, Kobayashi K & Kobayashi K (2004). Identification of GABAA receptor subunit variants in midbrain dopaminergic neurons. *J Neurochem* **89**, 7–14.

Orser BA, Canning KJ & Macdonald JF (2002). Mechanisms of general anesthesia. *Curr Opin Anaesthesiol* **15**, 427–433.

Owens DF & Kriegstein AR (2002). Is there more to GABA than synaptic inhibition? *Nat Rev Neurosci* **3**, 715–727.

Pirker S, Schwarzer C, Wieselthaler A, Sieghart W & Sperk G (2000). GABAA receptors: immunocytochemical distribution of 13 subunits in the adult rat brain. *Neurosci* **101**, 815–850.

Poncer JC, McKinney RA, Gähwiler BH & Thompson SM (1997). Either N- or P-type calcium channels mediate GABA release at distinct hippocampal inhibitory synapses. *Neuron* **18**, 463–472.

Santhakumar V, Hanchar HJ, Wallner M, Olsen RW & Otis TS (2006). Contributions of the GABAA receptor alpha6 subunit to phasic and tonic inhibition revealed by a naturally occurring polymorphism in the alpha6 gene. *J Neurosci* **26**, 3357–3364.

Schilstrom B, Yaka R, Argilli E, Suvarna N, Schumann J, Chen BT, Carman M, Singh V, Mailliard WS, Ron D & Bonci A (2006). Cocaine enhances NMDA receptor-mediated currents in ventral tegmental area cells via dopamine D5 receptor-dependent redistribution of NMDA receptors. *J Neurosci* **26**, 8549–8558.

Schwarzer C, Berresheim U, Pirker S, Wieselthaler A, Fuchs K, Sieghart W & Sperk G (2001). Distribution of the major gamma-aminobutyric $acid_A$ receptor subunits in the basal ganglia and associated limbic brain areas of the adult rat. *J Comp Neurol* **433**, 526–549.

Segev I (1990). Computer study of presynaptic inhibition controlling the spread of action potentials into axon terminals. *J Neurophysiol* **63**, 987–998.

Spanagel R & Weiss F (1999). The dopamine hypothesis of reward: past and current status. *Trends Neurosci* **22**, 521–527.

Stuart GJ & Redman SJ (1992). The role of $GABA_A$ and $GABA_B$ receptors in presynaptic inhibition of Ia EPSPs in cat spinal motoneurones. *J Physiol* **449**, 551–572.

Svingos AL, Garzon M, Colago EE & Pickel VM (2001). Mu-opioid receptors in the ventral tegmental area are targeted to presynaptically and directly modulate mesocortical projection neurons. *Synapse* **41**, 221–229.

Szabo B, Siemes S & Wallmichrath I (2002). Inhibition of GABAergic neurotransmission in the ventral tegmental area by cannabinoids. *Eur J Neurosci* **15**, 2057–2061.

Takahashi T & Momiyama A (1993). Different types of calcium channels mediate central synaptic transmission. *Nature* **366**, 156–158.

Takeuchi A & Takeuchi N (1966). A study of the inhibitory action of gamma- aminobutyric acid on neuromuscular transmission in the crayfish. *J Physiol* **183**, 418–432.

Turecek R & Trussell LO (2001). Presynaptic glycine receptors enhance transmitter release at a mammalian central synapse. *Nature* **411**, 587–590.

Vitten H & Isaacson JS (2001). Synaptic transmission: exciting times for presynaptic receptors. *Curr Biol* **11**, R695–R697.

Waddington JL & Cross AJ (1978). Neurochemical changes following kainic acid lesions of the nucleus accumbens: implications for a GABAergic accumbal-ventral tegmental pathway. *Life Sci* **22**, 1011–1014.

Wallner M, Hanchar HJ & Olsen RW (2006). Low dose acute alcohol effects on GABAA receptor subtypes. *Pharmacol Ther* **112**, 513–528.

Wang F, Xiao C & Ye JH (2005). Taurine activates excitatory non-synaptic glycine receptors on dopamine neurones in ventral tegmental area of young rats. *J Physiol* **565**, 503–516.

Wu YN, Mercuri NB & Johnson SW (1995). Presynaptic inhibition of gamma-aminobutyric $acid_B$-mediated synaptic current by adenosine recorded in vitro in midbrain dopamine neurons. *J Pharmacol Exp Ther* **273**, 576–581.

Xiao C & Ye JH (2005). GABA-induced potentiation of GABAergic synaptic transmission is limited by presynaptic GABAB receptors. *Abstr Soc Neurosci* 40.13.

Yamauchi T, Hori T & Takahashi T (2000). Presynaptic inhibition by muscimol through GABAB receptors. *Eur J Neurosci* **12**, 3433–3436.

Ye J (2000). Physiology and pharmacology of native glycine receptors in developing rat ventral tegmental area neurons. *Brain Res* **862**, 74–82.

Ye J, Wang F, Krnjevic K, Wang W, Xiong ZG & Zhang J (2004). Presynaptic glycine receptors on GABAergic terminals facilitate discharge of dopaminergic neurons in ventral tegmental area. *J Neurosci* **24**, 8961–8974.

Ye J, Xiao C & Zhou C (2006*a*). Presynaptic GABAA receptors regulate GABAergic transmission of dopamine neurons in the ventral tegmental area. *Abstr Soc Neurosci* 328.10.

Ye JH, Zhang J, Xiao C & Kong JQ (2006*b*). Patch-clamp studies in the CNS illustrate a simple new method for obtaining viable neurons in rat brain slices: glycerol replacement of NaCl protects CNS neurons. *J Neurosci Methods* **158**, 251–259.

Zheng F, Grandy DK & Johnson SW (2002). Actions of orphanin FQ/nociceptin on rat ventral tegmental area neurons in vitro. *Br J Pharmacol* **136**, 1065–1071.

Zhou C, Xiao C, McArdle JJ & Ye JH (2006). Mefloquine enhances nigral gamma-aminobutyric acid release via inhibition of cholinesterase. *J Pharmacol Exp Ther* **317**, 1155–1160.

Zhu L, Lovinger D & Delpire E (2005). Cortical neurons lacking KCC2 expression show impaired regulation of intracellular chloride. *J Neurophysiol* **93**, 1557–1568.

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