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# Ethanol Facilitates Glutamatergic Transmission to Dopamine Neurons in the Ventral Tegmental Area

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The cellular mechanisms underlying alcohol addiction are poorly understood. In several brain areas, ethanol depresses glutamatergic excitatory transmission, but how it affects excitatory synapses on dopamine neurons of the ventral tegmental area (VTA), a crucial site for the development of drug addiction, is not known. We report here that in midbrain slices from rats, clinically relevant concentrations of ethanol (10–80 mM) increase the amplitude of evoked EPSCs and reduce their paired-pulse ratio in dopamine neurons in the VTA. The EPSCs were mediated by glutamate α-amino-3-hydroxy-5-methylisoxazole-4-propionic acid (AMPA) receptors. In addition, ethanol increases the frequency but not the amplitude of spontaneous EPSCs. Furthermore, ethanol increases extracellular glutamate levels in the VTA of midbrain slices. The effects of ethanol are mimicked by SKF 38393, a dopamine $D_1$ receptor agonist, and by GBR 12935, a dopamine reuptake inhibitor, and they are blocked by SKF 83566, a $D_1$ antagonist, or by reserpine, which depletes dopamine stores. The enhancement of sEPSC frequency reaches a peak with 40 mM ethanol and declines with concentrations $\geqslant$80 mM ethanol, which is quite likely a result of $D_2$ receptor activation as raclopride, a $D_2$ receptor blocker, significantly enhanced 80 mM ethanol-induced enhancement of sEPSCs. Finally, 6, 7-dinitroquinoxaline-2, 3-dione (DNQX), an AMPA receptor antagonist, attenuates ethanol-induced excitation of VTA DA neurons. We therefore conclude that, acting via presynaptic $D_1$ receptors, ethanol at low concentrations increases glutamate release in the VTA, thus raising somatodendritic dopamine release, which further activates the presynaptic $D_1$ receptors. Enhancement of this positive feedback loop may significantly contribute to the development of alcohol addiction.
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## INTRODUCTION

Numerous studies have associated the mesolimbic dopaminergic (DA) system, originating from the ventral tegmental area (VTA), with ethanol addiction. For instance, (1) ethanol consumption is reduced by the administration of dopamine antagonists (Samson *et al*, 1993; Price and Middaugh, 2004) or agonists (Ng and George, 1994), or by the genetic deletion of dopamine $D_1$ receptors ($D_1$R) (El-Ghundi *et al*, 1998); (2) pharmacological manipulations of VTA DA neuron activity alter ethanol consumption (Rassnick *et al*, 1993; Nowak *et al*, 1998); (3) ethanol increases dopamine release, from both the synaptic terminals (in nucleus accumbens (NAcc)) and the somatodendritic region in the VTA (Imperato and Di Chiara, 1986; Campbell *et al*, 1996; Hodge *et al*, 1997; Ikemoto *et al*, 1997; Koob *et al*, 1998; Doyon *et al*, 2003, 2005); and (4) ethanol stimulates the spontaneous activity of VTA DA neurons (Imperato and Di Chiara, 1986; Brodie *et al*, 1990, 1999a, b; Doyon *et al*, 2003, 2005; Xiao *et al*, 2007). However, the specific mechanism by which ethanol excites VTA DA neurons has not been fully clarified.

Glutamate, the main excitatory neurotransmitter in the brain, excites VTA DA neurons (Suaud-Chagny *et al*, 1992). Glutamate release in the VTA can be regulated by many agents. For instance, it is increased by activating $D_1$ (Kalivas and Duffy, 1995) and nicotinic acetylcholine receptors (Erhardt *et al*, 2002), as well as receptors for corticotropin-releasing factor (Wang *et al*, 2005), or decreased by activating $GABA_B$, adenosine $A_1$ (Bonci and Malenka, 1999), κ and μ opioid (Margolis *et al*, 2005), $CB_1$

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cannabinoid (Melis *et al*, 2004), metabotropic glutamate, and $M_3$ muscarinic acetylcholine (Zheng and Johnson, 2003) receptors.

Glutamatergic (GLUergic) transmission is an important mediator of behavioral changes caused by ethanol (Eckardt *et al*, 1998; Krystal *et al*, 2003). In several brain regions, ethanol inhibits NMDA and non-NMDA glutamate receptors, as well as glutamate release (Siggins *et al*, 2005), but in some situations, ethanol increases glutamate release (Selim and Bradberry, 1996; Dahchour *et al*, 2000; Roberto *et al*, 2004; Zhu *et al*, 2007). For instance, systemic administration of ethanol increases glutamate release in NAcc of low-alcohol-sensitive rats (Dahchour *et al*, 2000) and addiction-prone Lewis rats (Selim and Bradberry, 1996). In recent studies on the central nucleus of the amygdala, acute ethanol increased glutamate release only in rats receiving chronic ethanol treatment (Roberto *et al*, 2004; Zhu *et al*, 2007). However, no information is available about the effects of ethanol on GLUergic transmission to VTA DA neurons.

Bearing in mind that genetic deletion of $D_1$Rs reduces ethanol consumption (El-Ghundi *et al*, 1998) and that ethanol stimulates somatodendritic dopamine release, which may activate $D_1$Rs expressed on GLUergic axons synapsing onto VTA DA neurons (Lu *et al*, 1997), we hypothesized that ethanol indirectly activates $D_1$Rs to potentiate GLUergic transmission in this region. To examine this possibility, we analyzed the effects of acute ethanol on EPSCs and extracellular glutamate levels in the VTA in midbrain slices.

## MATERIALS AND METHODS

All procedures were approved by the Institutional Animal Care and Use Committee of the University of Medicine and Dentistry of New Jersey, in accordance with the guidelines of the National Institutes of Health (Guide for the Care and Use of Laboratory Animals), minimizing the number of animals and their suffering. Experiments were done on slices from adolescent Sprague–Dawley rats (at postnatal days 22–32).

### Slice Preparation

The midbrain slices were prepared as described earlier (Ye *et al*, 2004, 2006). Rats were anesthetized with ketamine/xylazine and then decapitated. Coronal midbrain slices (250 μm thick) were cut with a VF-200 slicer (Precisionary Instruments Inc., Greenville, NC) in ice-cold glycerol-based artificial cerebrospinal fluid containing (in mM) 250 glycerol, 1.6 KCl, 1.2 $NaH_2PO_4$, 1.2 $MgCl_2$, 2.4 $CaCl_2$, 25 $NaHCO_3$, and 11 glucose, and saturated with 95%$O_2$/5%$CO_2$ (carbogen) (Ye *et al*, 2006). Two slices per animal were allowed to recover for at least 1 h in a holding chamber at 31°C in carbogen-saturated standard ACSF, which has the same composition as glycerol-based artificial cerebrospinal fluid, except that glycerol was replaced by 125 mM NaCl.

### Electrophysiological Recording

Electrical signals were obtained in whole-cell and loose-patch cell-attached patch clamp configurations with MultiClamp 700 A amplifiers (Molecular Devices Co., Union City, CA, USA), a Digidata 1320 A A/D converter (Molecular Devices Co.) and pCLAMP 9.2 software (Molecular Devices Co.). Data were filtered at 1 kHz and sampled at 5 kHz. The patch electrodes had a resistance of 4–6 MΩ when filled with a pipette solution containing (in mM): 135 K gluconate, 5 KCl, 2 $MgCl_2$, 10 HEPES, 2 Mg ATP, 0.2 GTP, and 2 QX-314. The pH was adjusted to 7.2 with Tris base. A single slice was transferred to a 0.4 ml recording chamber, where it was held down by a platinum ring. Throughout the experiments, the bath was continually perfused with carbogenated ACSF (1.5–2.0 ml/min). Cells were visualized with an upright microscope (E600FN, Nikon) and near-infrared illumination.

All recordings were obtained from putative DA neurons identified by their pharmacological, physiological, and cytochemical properties. Specifically, spontaneous firing of VTA neurons was first recorded with the loose-patch cell-attached configuration. The depression of spontaneous firing by 0.2 μM quinpirole, a dopamine $D_2/D_3$ receptor agonist, and facilitation by 1 μM DAMGO, a μ-opioid receptor agonist (as a result of DAMGO-induced disinhibition of inhibitory GABAergic neurons in the VTA, Figure 1c), are characteristic features of VTA DA neurons (Johnson *et al*, 1992; Johnson and North, 1992; Margolis *et al*, 2003). Further suction changed the recording to the whole-cell configuration. A prominent inward current ($I_h$) activated by hyperpolarizing voltage steps (between −60 and −140 mV, Figure 1d, left panel) or a corresponding voltage sag in response to a hyperpolarizing current pulse (between −100 and 0 pA, Figure 1d, right panel) (Lacey *et al*, 1989) confirmed the identity of putative DA neurons.

To evoke monosynaptic EPSCs (eEPSCs), the tip of a glass-stimulating pipette, filled with 1 M NaCl, was placed 50–100 μm from the recorded VTA neuron. Electrical stimuli (100–200 μs in duration) were applied at the rate of 0.05 Hz. Near the start of the recording, an input/output curve was obtained, and the stimulation was then set to 20–30% of the maximum, an intensity that evoked stable responses with no failures. Paired eEPSCs were elicited with a pair of identical stimuli separated by an interval of 50 ms. Series resistance was checked before and after the experiments by series resistance compensation. To measure input resistance, we applied a 5 mV, 400 ms hyperpolarizing pulse and divided 5 mV by the final value of the evoked current. The data were discarded if series resistance (15–30 MΩ) or input resistance (300–500 MΩ) changed by more than 20% during the whole-cell recording. All these experiments were done at a temperature of 33 ± 1°C, maintained by an automatic temperature controller (Warner Instruments, Hamden, CT).

### Immunocytochemistry

The identification of VTA DA neuron by the responses to quinpirole and the expression of $I_h$ has been challenged (Margolis *et al*, 2006). Therefore, to validate the electrophysiological and pharmacological identification of DA neurons, a subset of neurons were examined immunocytochemically for tyrosine hydroxylase (TH). In these experiments, we included Alexa Fluor® 555 dextran (0.05%, w/v, Invitrogen Corporation, Carlsbad, CA) in

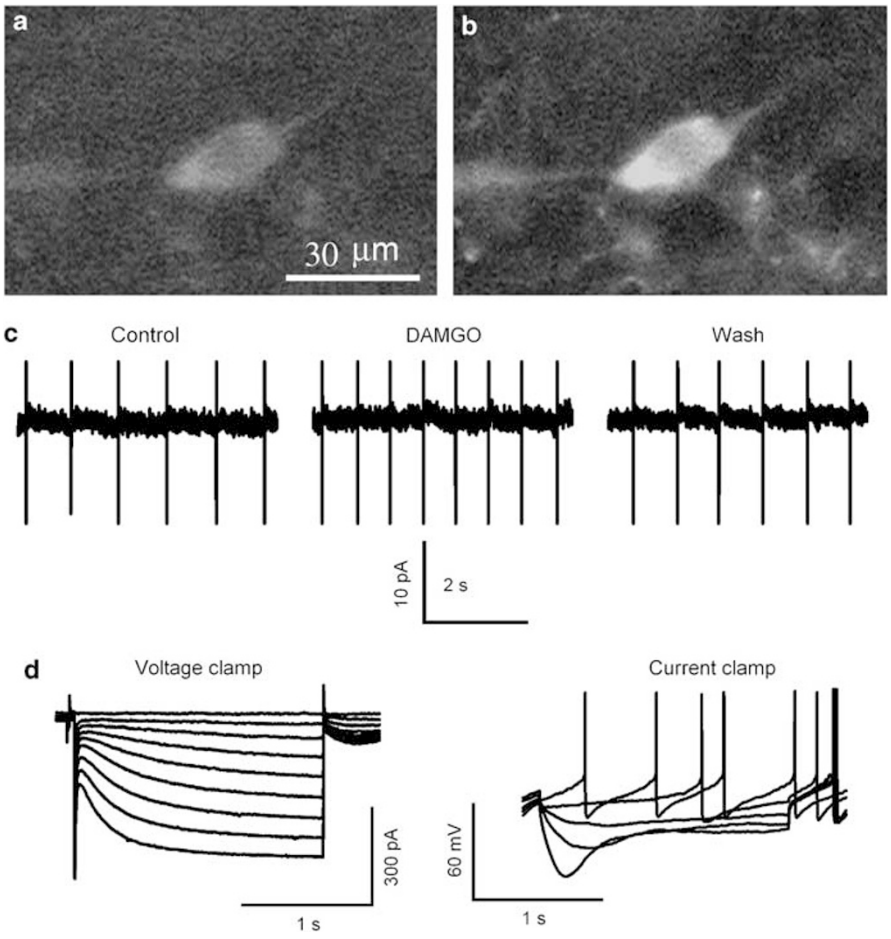


**Figure 1** Identification of recorded neuron as VTA DA neuron. (a) Electrically recorded cell was labeled with Alexa FluoR 555 Dextran (0.05%) released from recording pipette, yielding red fluorescence. (b) Subsequent cytochemical treatment showed that it was also TH-positive (green fluorescence. (c) Spontaneous discharge recorded in cell-attached mode was accelerated by 2 μM DAMGO. (d) Under voltage clamp, the currents induced by a series of hyperpolarizing voltage pulses show the large time-dependent inward currents ($I_h$, left panel). Under current clamp, this neuron has prominent time-dependent decreases in voltage during application of rectangular hyperpolarizing currents (right panel).

the intra-pipette solution to label the recorded neurons (red fluorescence in Figure 1a). After electrical recording, to stain for tyrosine hydroxylase, the slice was fixed for 2 h in 4% paraformaldehyde in PBS at 4°C, washed two times with cold PBS, and stored overnight at 4°C in PBS/0.1% sodium azide. It was then permeabilized for 20 min at 4°C in TBS/0.5% Triton X-100, blocked for 1 h at 4°C in 5% donkey serum in TBS/0.1% Triton X-100, and incubated overnight at 4°C with a primary antibody rabbit anti-TH (Pel-Freez Biologicals, Rogers, AR, 1:100 dilution) in TBS/0.1% Triton X-100 containing 5% donkey serum. The slice was then washed three times (5 min each) in TBS/0.1% Triton X-100, incubated for 1 h in secondary antibody at room temperature (Alexa Fluor 488 goat anti-rabbit IgG, Invitrogen Corporation, Carlsbad, CA, 1:500 dilution) in TBS/0.1% Triton X-100 containing 5% donkey serum, washed three times (10 min each) in TBS/0.1% Triton X-100, and then covered with Vectashield. Slides were immediately examined on a three-color immunofluorescence microscope (Nikon Instruments Inc., Melvelle, NY). TH-positive neurons were stained green, Figure 1b).

Among 16 recorded and red fluorescence-labeled neurons, 12 (75%) were also TH-positive and 10 (63%) were $I_h$-positive (not significantly different: by Fisher's exact test, $p = 0.70$). $I_h$ was expressed in 9/12 (75%) TH-positive neurons but not in four TH-negative neurons (significantly different: by Fisher's exact test, $p = 0.019$). Among 10 $I_h$-positive neurons, nine (90%) were TH-positive and six (60%) were disinhibited by DAMGO (not significantly different: by Fisher's exact test, $p = 0.30$). All neurons (6/6) that were $I_h$-positive and disinhibited by DAMGO were TH-positive and thus highly likely to be DA neurons. This supports the notion that $I_h$ is expressed in a substantial portion, if not all, of VTA DA neurons (Johnson *et al*, 1992; Johnson and North, 1992; Margolis *et al*, 2006). In agreement with previous studies, the neurons expressing $I_h$ and disinhibited by μ-opioid agonist were therefore very likely to be dopaminergic, because, (1) VTA GABAergic neurons have no prominent $I_h$ (Jones and Kauer, 1999) and they are inhibited by μ-opioid receptor agonists (Margolis *et al*, 2003), and (2) in the VTA, DAMGO uniquely excites DA neurons by a mechanism of disinhibition (Margolis *et al*, 2003; Xiao *et al*, 2007).

### Measurement of Glutamate in Midbrain Slices

We measured extracellular glutamate in the VTA with a new method developed by Shao and Feldman (2007). A midbrain slice was transferred to a 1.2 ml chamber and perfused with carbogenated ACSF (at a rate of 2 ml/min). The VTA was visualized with an upright microscope (Axioskop 2FS, Zeiss). The tip of the sampling pipette—its inner diameter (450–550 μm) approximately covering the VTA in young rats—was pressed against the surface of the slice over the VTA. Ten microliters of sample fluid was collected at the rate of 2 μl/min by a micro-syringe pump (UltraMicroPump II, World Precision Instruments, Sarasota, FL). A small piece of filter paper in the tip prevented entry of brain tissue into the pipette. The collected fluid was immediately frozen and kept at −20°C. After the samples were thawed, glutamate was measured by high performance liquid chromatography (Olive *et al*, 2000).

### Chemicals and Applications

Bicuculline, DL-2-amino-5-phosphono-valeric acid (DL-APV), 6, 7-dinitroquinoxaline-2, 3-dione (DNQX), QX-314, reserpine hydrochloride, tetrodotoxin (TTX), [D-Ala$^2$, N-Me-Phe$^4$, Gly$^5$-ol] enkephalin (DAMGO) were obtained from Sigma-Aldrich Chemical Company (St Louis, MO, USA). (−)-Quinpirole hydrochloride (QP), GBR 12935, N-allyl-(±)-SKF 38393, (±)-SKF 83566 were from TOCRIS Bioscience (Ellisville, MO, USA). The 1000× stock solutions were aliquoted and then stored at −20, 4°C, or room temperature according to the catalog recommendations. Reserpine (10 mM) was dissolved in acetic acid (1/1000), APV (50 mM), and DNQX (20 mM) in ~0.1 N NaOH, and other agents in deionized water. Ethanol (95%, prepared from grain) was from Pharmco (Brookfield, CT, USA) and stored in glass bottles. Drugs at final concentration were added to the superfusate.

### Data Analysis

Spontaneous excitatory postsynaptic currents (sEPSCs) and miniature EPSCs (mEPSCs) were counted and analyzed with Clampfit 9.2 (Molecular Devices Co.). They were screened automatically by 'template search.' The template was selected visually according to the rise and decay phases of sEPSCs, and the amplitude threshold was set to 5 pA. The height of evoked eEPSCs (eEPSCs) was measured with Clampfit 9.2 and was used to calculate the paired pulse ratio (PPR = $EPSC_2/EPSC_1$), where $EPSC_1$ and $EPSC_2$ were evoked by the first and second stimuli. The eEPSC amplitude, PPR, and the frequency of sEPSCs and mEPSCs during and after drug applications were normalized to their mean value during the initial control period (>2 min). These data were used to depict summarized time courses (10–30 s per/bin). The baseline mean values were obtained during the initial control period and the mean values, during drug application over a 1–2 min period at the peak of a drug response. Drug effects were expressed as % change (mean ± SEM) from pre-drug control baseline. Paired or unpaired two-tailed *t*-test evaluated the statistical significance of various drug effects (% change from control baseline). Cumulative probability plots of the incidence of various inter-event intervals and amplitudes (for 100–1500 sEPSCs and mEPSCs), recorded in control conditions and during drug applications to the same neuron, were analyzed with the Kolmogorov–Smirnov (K–S) test. In the figures, single eEPSCs or paired eEPSCs are averages of >10 successive traces. Values of $p<0.05$ were considered significant.

## RESULTS

### Ethanol Enhances Evoked EPSCs in Putative DA Neurons in VTA

In the presence of 10 μM bicuculline and at a holding potential ($V_H$) of −70 mV, EPSCs were evoked by a local stimulating electrode. These EPSCs were completely blocked by 20 μM DNQX, indicating that they were mediated by α-amino-3-hydroxy-5-methylisoxazole-4-propionic acid (AMPA) receptors (Figure 2b). Unexpectedly, 40 mM ethanol enhanced the amplitude of such eEPSCs (Figure $2a_{1-2}$) by 33 ± 5% (from 70 ± 14 to 90 ± 17 pA, $n=9$; $p<0.01$, by paired *t*-test). Next, we applied pairs of stimuli (at 50 ms interval) to evoke paired EPSCs. As shown in Figure $2c_{1-2}$, ethanol (40 mM) increased the amplitude of the first EPSC of each pair but not the second, and thus decreased the paired-pulse ratio (PPR = $EPSC_2/EPSC_1$) by 22 ± 3%, from 1.5 ± 0.2 before to 1.2 ± 0.1 after ethanol ($n=9$, $p<0.01$, paired *t*-test). Changes in transmitter release generally affect the PPR (Bonci and Williams, 1997; Ye *et al*, 2004). These results suggest that ethanol increases presynaptic glutamate release.

### Ethanol Increases the Frequency but not the Amplitude of Spontaneous EPSCs (sEPSCs) in Putative DA Neurons in VTA

We then examined sEPSCs in 10 μM bicuculline at a $V_H$ of −70 mV. Their suppression by 20 μM DNQX (Figure 3a) indicated that they were indeed GLUergic sEPSCs mediated by AMPA receptors. Ethanol (40 mM) increased their frequency (Figure 3b and c) by 61 ± 11% (from 1.1 ± 0.5 to 1.6 ± 0.7 Hz, $n=9$, $p<0.01$, by paired *t*-test, Figure $3e_1$). This is further shown in Figure $3d_1$ by the sharp increase in the probability of shorter intervals between successive sEPSCs (K–S test, $p<0.01$). After washout of ethanol, the sEPSC frequency returned to control levels (Figure 3b and c). This effect of ethanol was reproducible after washout for >10 min (data not shown). However, 40 mM ethanol did not alter sEPSC amplitudes (K–S test: $p>0.5$, Figure $3d_2$). Figure $3e_1$ illustrates the dose dependence of ethanol-induced increases in sEPSC frequency: for ethanol concentrations up to 40 mM, sEPSC frequency increased progressively (10 mM: 15 ± 3%, $n=7$, $p<0.01$; 20 mM: 37 ± 4%, $n=9$, $p<0.01$; 40 mM: 61 ± 11%, $n=9$, $p<0.01$) but when the concentration was doubled again to 80 mM, the increase in frequency diminished to 41 ± 5% ($n=6$, $p<0.01$; Figure $3e_1$). On the other hand, at all these concentrations, ethanol did not significantly alter sEPSC amplitudes, as shown by the histogram in Figure $3e_2$ (10 mM: 5 ± 6%, $n=7$, $p=0.3$; 20 mM: 7 ± 6%, $n=9$, $p=0.18$; 40 mM: 9 ± 9%, $n=9$, $p=0.17$; 80 mM: 2 ± 5%, $n=6$, $p=0.47$). These results are consistent with a presynaptic mechanism of

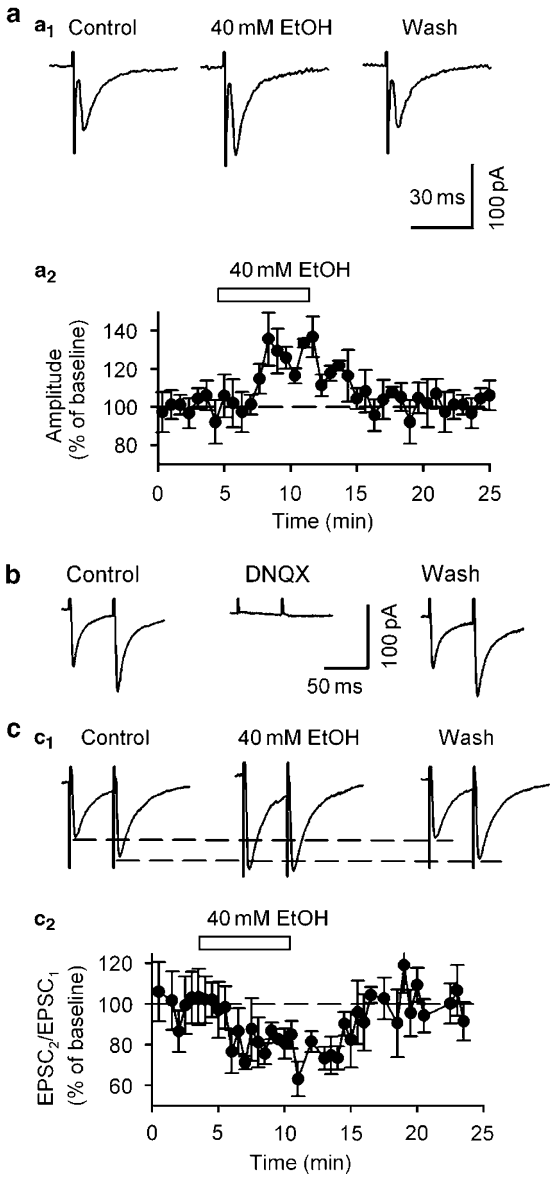


**Figure 2** Ethanol enhances EPSCs evoked in putative DA neurons in midbrain slices. (a) EPSCs evoked by electrical stimulation within the VTA ($a_1$). ($a_2$) Time course of change in eEPSC amplitude (mean ± SEM) induced by 40 mM ethanol ($n = 5$). (b) Responses to paired-pulse stimulation (at 50 ms interval), which were completely blocked by AMPA-type glutamate antagonist DNQX (20 μM). ($c_1$) Ethanol significantly reduced the paired-pulse ratio. Data are averages of 10 traces. ($c_2$) Time course of ethanol-induced reduction of paired-pulse facilitation (mean ± SEM; $n = 5$). All EPSCs were recorded at a $V_H$ of −70 mV and in the presence of 10 μM bicuculline.

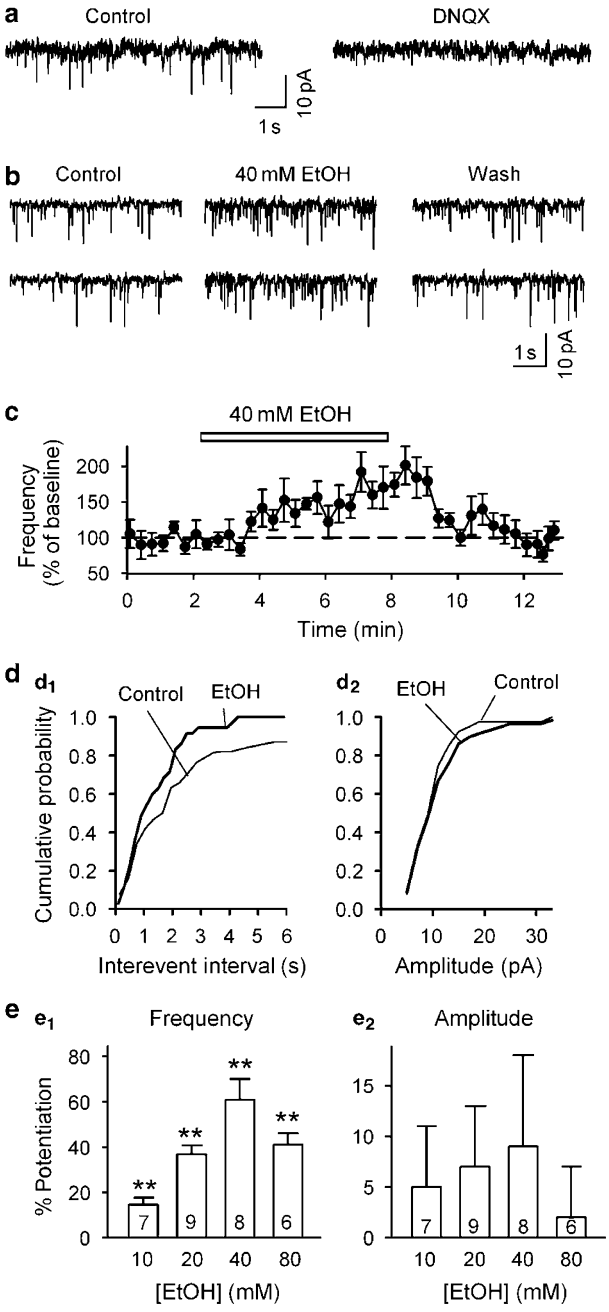


**Figure 3** Ethanol increases the frequency of GLUergic spontaneous EPSCs (sEPSCs) recorded in putative DA neurons in midbrain slices. (a) Recorded at $V_H$ −70 mV and in bicuculline (10 μM), these sEPSCs were suppressed by 20 μM DNQX. (b) 40 mM ethanol greatly (but reversibly) increased sEPSC frequency. (c) Time course of mean increase in sEPSC frequency induced by 40 mM ethanol ($n = 6$). (d) Representative cumulative probability plots show increased incidence of short intervals between sEPSCs ($d_1$), but no change in the amplitude of sEPSCs ($d_2$). (e) Dose-dependent potentiation of sEPSC frequency ($e_1$), but unaltered amplitude ($e_2$) (means ± SEM). (Number of cells in each group is indicated). $^{**}p < 0.01$, paired *t*-test for ethanol application *vs* pre-ethanol control.

ethanol action, enhancing GLUergic transmission by increasing glutamate release.

## Ethanol does not Alter Miniature EPSCs (mEPSCs) in Putative DA Neurons in VTA

Next, we recorded mEPSCs in the presence of tetrodotoxin (TTX, 1 μM) and bicuculline (10 μM). Applications of TTX reduced the frequency of the spontaneous events only slightly, albeit significantly (1.4 ± 0.4 Hz in control, 1.1 ± 0.4 Hz in TTX, $p < 0.05$, $n = 5$), but not their mean amplitude (17.9 ± 1.7 pA in control, 17.1 ± 1.8 pA in TTX, $p = 0.26$, $n = 5$). Ethanol (40 mM) did not significantly alter either the frequency or the amplitude of such mEPSCs (Figure 4a–b): the frequency changed by only 4 ± 3% ($n = 7$, $p = 0.7$, control, 0.7 ± 0.2; ethanol, 0.8 ± 0.2 Hz) and the

amplitudes by $1 \pm 5\%$ ($n = 7$, $p = 1$, control, $6.7 \pm 0.6$ pA; ethanol, $6.7 \pm 0.4$ pA). These results indicate that ethanol's action is linked to TTX-sensitive $Na^+$ channels.

### Ethanol-Induced Increase in Glutamatergic Activity is Eliminated by a $D_1R$ Antagonist

Systemic administration of ethanol increases somatodendritic dopamine release from the VTA *in vivo* (Campbell *et al*, 1996). In the VTA, $D_1$Rs are expressed on GLUergic axons (Lu *et al*, 1997) but not on the soma of DA neurons (Mansour *et al*, 1992; Lu *et al*, 1997). The activation of $D_1$Rs increases glutamate levels in the VTA (Kalivas and Duffy, 1995) as well as glutamatergic transmission in the globus pallidus (Hernandez *et al*, 2007). To test for a possible involvement of $D_1$Rs in ethanol-induced enhancement of GLUergic transmission, we compared the effect of ethanol on sEPSC frequency in the absence and presence of 10 μM SKF83566, a $D_1$R antagonist. All sEPSCs were recorded in the presence of 10 μM bicuculline. As illustrated in Figure 5a, 40 mM ethanol produced the usual increase in sEPSC frequency. Though SKF83566 (10 μM) itself did not alter baseline sEPSC frequency, it suppressed the effect of ethanol (40 mM). On five cells, ethanol (40 mM) alone increased sEPSC frequency by $53.0 \pm 12.3\%$ ($p < 0.01$), but it did not significantly change sEPSC frequency in the presence of SKF83566 ($2.3 \pm 15.3\%$, $p > 0.5$). These results show that $D_1$Rs are essential for the action of ethanol on GLUergic transmission. This would be in line with evidence that dopamine $D_1$Rs increase intra-terminal $Ca^{2+}$ levels through $Na^+$ channel-mediated membrane depolarization (Wu *et al*, 2006). Also relevant may be the finding that TTX reduces somatodendritic dopamine release (Chen *et al*, 1991; Kalivas and Duffy, 1991; Chen and Reith, 1994; Cragg and Greenfield, 1997; Reith *et al*, 1997).

For another index of ongoing glutamate release, we measured extracellular glutamate levels in the VTA in acute midbrain slices. After collecting samples from slices in standard ACSF, we applied ethanol in the bath. Samples were collected again between 1 and 6 min after the start of ethanol superfusion. Ethanol (40 mM) sharply increased glutamate levels in the samples by $150 \pm 49\%$ (from $69 \pm 27$ nM in control to $144 \pm 35$ nM in 40 mM ethanol, $n = 6$ slices, $p < 0.01$, Figure 5b, left column). Although the $D_1$R antagonist SKF83566 (5 μM) alone had no significant effect on glutamate levels, in the presence of SKF83566 (5 μM), 40 mM ethanol was ineffective (sampled glutamate

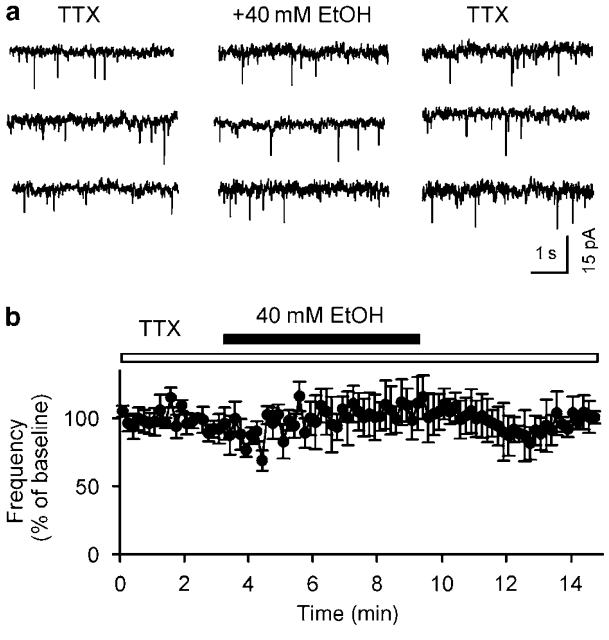


**Figure 4** Ethanol has no effect on miniature EPSCs (mEPSCs). The mEPSCs were recorded in VTA DA neurons at $V_H$ -70 mV, in the presence of bicuculline (10 μM) and tetrodotoxin (TTX, 1 μM). (a) 40 mM ethanol did not change the incidence of mEPSCs. (b) Plot of mean (± SEM) frequency of mEPSCs for seven cells shows no change in 40 mM ethanol.

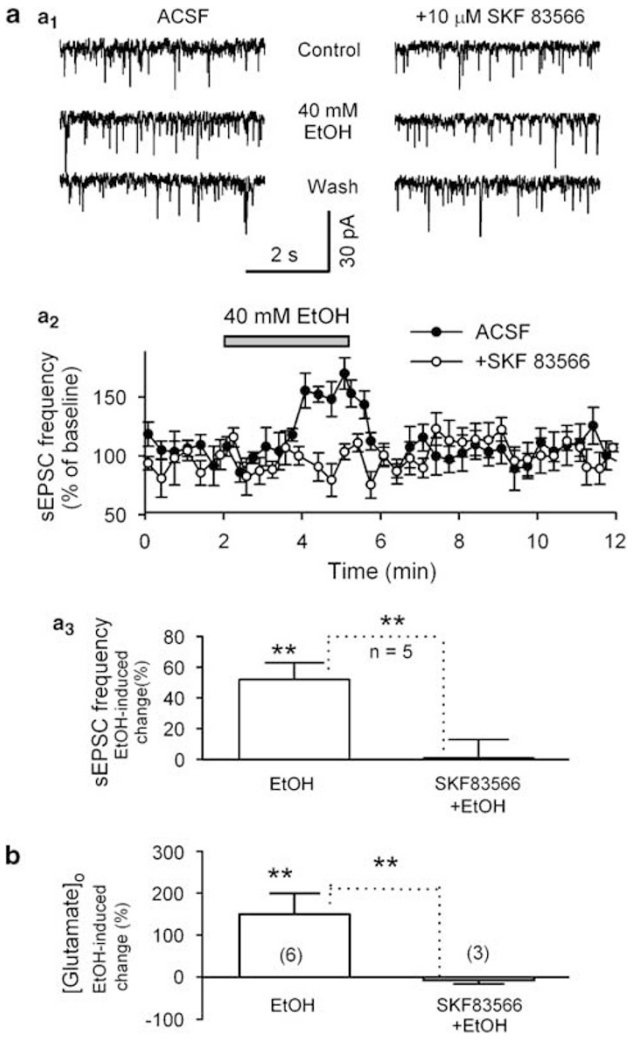


**Figure 5** Dopamine $D_1$R antagonist eliminates ethanol-induced facilitation of glutamate release. ($a_1$) Traces recorded from a putative DA neuron show that ethanol reversibly increased the frequency of sEPSCs in the absence (left panel), but not in the presence (right panel), of 10 μM SKF83566 ($D_1$R antagonist). ($a_2$) Time course of ethanol effect on sEPSC frequency in the absence (ACSF) or presence of 10 μM SKF83566. ($a_3$) Summary of ethanol-induced changes in sEPSC frequency (mean + SEM from five cells). (b) Ethanol-induced increase in glutamate concentration in fluid samples collected from the VTA in midbrain slices was blocked by SKF83566. Summary of % changes in (glutamate) caused by 40 mM ethanol alone (data from six slices). $^{**}p < 0.01$, paired *t*-test for drug application *vs* pre-drug control.

changed by $-8 \pm 8\%$: from $106 \pm 17$ nM in SKF 83566 to $99 \pm 20$ nM in SKF 83566 + 40 mM ethanol, $n = 3$, $p > 0.5$, Figure 5b, right column).

### Effects of Ethanol and $D_1R$ Agonist on sEPSC Frequency

In further tests of the involvement of $D_1Rs$, we applied SKF38393, a $D_1R$ agonist. As illustrated in Figure $6a_{1-3}$, 100 nM SKF38393 significantly increased sEPSC frequency. This effect was fully reversible, the sEPSC frequency soon returning to its control level after washout (Figure $6a_2$, and more details in the upper panels of Figure $6b_1$). However, there was no significant change in sEPSC amplitudes (data not shown). As illustrated in Figure $6a_3$, the effects of SKF38393 were dose-dependent: 10, 100, 1000, and 3000 nM SKF38393 increased sEPSC frequency by $38 \pm 3\%$ ($n = 9$, $p < 0.01$), $68 \pm 13\%$ ($n = 7$, $p < 0.001$), $121 \pm 28\%$ ($n = 7$, $p = 0.001$), and $102 \pm 20\%$ ($n = 4$, $p < 0.01$), respectively. There were no corresponding changes in sEPSC amplitude: $5 \pm 4\%$ ($n = 9$, $p = 0.31$); $10 \pm 9\%$ ($n = 7$, $p = 0.22$), $0 \pm 8\%$ ($n = 7$, $p = 0.24$), and $-3 \pm 10\%$ ($n = 4$, $p = 0.84$), respectively, (data not shown). These results, which closely parallel the effects of ethanol, provide additional evidence for the presence of functional $D_1Rs$ that modulate synaptic glutamate release.

Bearing in mind that (1) ethanol enhanced sEPSCs through the $D_1R$-related pathway, and that (2) $D_1R$ activation mimicked ethanol effects, we tested the effect of ethanol on $D_1R$ using submaximal concentrations of ethanol and $D_1R$ agonist, to avoid occlusion resulting from maximal activation of $D_1Rs$ by either agent. We compared the changes in sEPSC frequency produced by the $D_1R$ agonist SKF 38393 (10 nM) in the absence and presence of 20 mM ethanol (Figure $6b_{1-2}$). When applied alone, 10 nM SKF38393 increased sEPSC frequency by $47 \pm 9\%$ ($n = 5$, $p < 0.01$, in comparison with the pre-drug control, Figure $6b_1$ upper panels, and $6b_2$). The sEPSC frequency almost completely recovered after 10 min washout of SKF38393. Then, we preincubated the slice with 20 mM ethanol for $>10$ min, which increased the sEPSC frequency (Figure $6b_1$). On the higher but stable baseline frequency recorded in the presence of ethanol, the addition of 10 nM SKF38393 induced a much greater rise in the sEPSC frequency (by $87 \pm 11\%$, $n = 5$, $p < 0.01$, Figure $6b_{1-2}$) ($n = 5$, $p < 0.01$). This suggests that ethanol enhances $D_1R$ function.

### Are Dopamine Levels in the VTA Crucial for Ethanol's Action?

Although susceptible to block by the $D_1R$ antagonist, ethanol's action could be independent of the dopamine level. In that case, ethanol should be effective even when the dopamine level is decreased. To test this possibility, we applied ethanol to midbrain slices in which dopamine was depleted by reserpine. Because reserpine was dissolved in acetic acid (final concentration 1/5000 v/v), control tests were done on slices pretreated for $>90$ min with this concentration of the vehicle alone. Under these conditions, ethanol (40 mM) elicited the usual increase in sEPSC

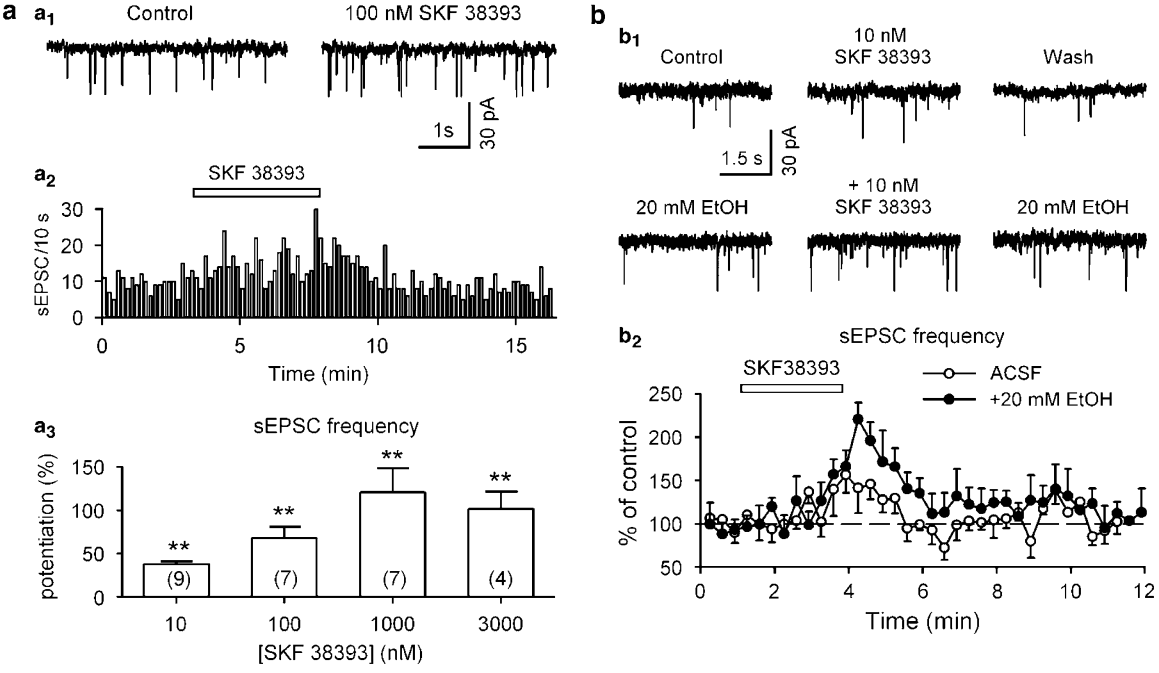


**Figure 6** $D_1R$ agonist mimics the effects of ethanol on sEPSC frequency. ($a_{1-2}$) the $D_1R$ agonist SKF38393 (100 nM) increased sEPSC frequency in putative VTA DA neurons. ($a_3$) Dose–response relation for SKF38393-induced increase in sEPSC frequency. ($b_1$) SKF38393 (10 nM) reversibly enhanced sEPSC frequency (upper traces). EtOH (20 mM) also increased sEPSC frequency (lower left trace). In the presence of 20 mM EtOH, SKF38393 (10 nM) further increased sEPSC frequency (lower middle trace). ($b_2$) Average time course of changes in sEPSC frequency induced by 10 nM SKF38393 in the absence (ACSF) and presence of 20 mM ethanol (+20 mM EtOH; means ± SEM; data from five cells). Open bar indicates the duration of SKF38393 application; baseline (100%) represents pre-SKF38393 frequency in the presence of ACSF or 20 mM EtOH. $^{**}p < 0.01$, paired *t*-test for SKF38393 application *vs* pre-SKF38393.

frequency (by 109 ± 46%; from 1.7 ± 0.6 to 2.8 ± 0.7 Hz, $p < 0.01$, $n = 5$, Figure 7a$_1$ and the left column in Figure 7a$_3$). By contrast, 40 mM ethanol was ineffective when applied to slices pretreated for similar periods with 10 μM reserpine (Figure 7a$_2$), sEPSC frequency changing by −14 ± 10% of control (from 1.2 ± 0.3 to 1.0 ± 0.3 Hz, $n = 5$; Figure 7a$_3$). This result indicates that the release of dopamine is necessary for ethanol's action.

An important mechanism is suggested by previous evidence that acute administration of ethanol (i.p.) elevates extracellular dopamine levels in the VTA *in vivo* (Campbell *et al*, 1996; Yan *et al*, 2005). To elicit a similar rise in local dopamine levels in slices, we applied GBR 12935, a selective blocker of dopamine transport. As illustrated in Figure 7b, 20 nM GBR12935 significantly and reversibly increased the frequency of sEPSCs recorded in VTA DA neurons (by 48 ± 11%, $n = 6$, $p = 0.01$). Similar to ethanol, GBR12935 was ineffective in the presence of the $D_1$R antagonist SKF83566 (10 μM) (the sEPSC frequency changed by 2 ± 4%, $n = 6$, $p = 0.16$, Figure 7b$_2$). This finding shows that a rise in the endogenous dopamine level also mimics the action of ethanol.

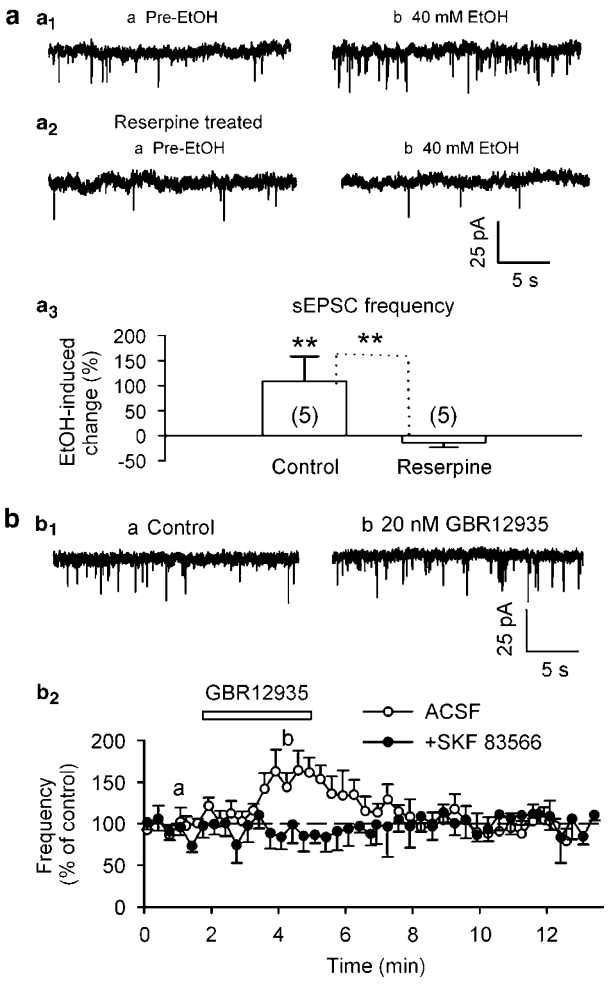


**Figure 7** Effects of ethanol on sEPSC frequency are suppressed by dopamine depletion and mimicked by block of dopamine transport. (a$_{1-2}$) Although 40 mM ethanol induced typical increase in sEPSC frequency in slice pretreated for >90 min with acetic acid (v/v = 1/5000, the vehicle for reserpine) (a$_1$), there was no such effect in slice pretreated with 10 μM reserpine for >90 min (a$_2$). (a$_3$) Summary of effects of reserpine (data from five cells). $^{**}p < 0.01$, paired *t*-test for ethanol application *vs* pre-ethanol control or, as indicated, nonpaired *t*-test for control (acetic acid) group *vs* reserpine group. (b$_1$) Increase in sEPSC frequency produced by GBR12935 (20 nM, a dopamine transport blocker). (b$_2$) The effect of GBR12935 is blocked by the $D_1$R antagonist SKF83566 (10 μM, $n = 6$).

As ethanol-induced enhancement of sEPSCs depends on dopamine release, the smaller effect of ethanol at 80 mM than at 40 mM could result from the activation of $D_2$-like dopamine receptors ($D_2$R); it is known that dopamine and other agonists at high concentrations can activate $D_2$Rs, which inhibit glutamate release (Koga and Momiyama, 2000). To test this possibility, we compared the effects of ethanol (20 and 80 mM) in the absence and presence of 10 μM raclopride, a $D_2$R antagonist: 80 mM ethanol alone increased sEPSC frequency by 38 ± 10% ($n = 5$, $p = 0.02$); in the presence of raclopride, 80 mM ethanol induced a much greater increase in frequency, by 173 ± 33% ($n = 5$, $p < 0.01$). This difference is significant ($n = 5$, $p < 0.05$, paired *t*-test, ethanol *vs* ethanol + raclopride). Nevertheless, 20 mM ethanol similarly increased sEPSC frequency in the absence (37 ± 7%, $n = 4$, $p = 0.006$) and the presence of raclopride (43 ± 10%, $n = 4$, $p = 0.01$) ($p = 0.4$, paired *t*-test). These results support the idea that the reduced efficacy of ethanol at high concentrations—and the consequent bell-shaped ethanol dose–response relation—are probably caused by the activation of $D_2$Rs.

### Effects of an AMPA Receptor Antagonist on Ethanol-Induced Excitation of VTA Dopamine Neurons

To assess the contribution of glutamatergic transmission to the overall facilitating action of ethanol, we examined the effect of 10 μM DNQX (an AMPA/KA-type glutamate antagonist) on the ethanol-induced increase in spontaneous firing of putative DA neurons. On average, 40 mM ethanol alone increased the firing rate by 19.2 ± 6.0% ($n = 10$, $p < 0.01$). In the presence of DNQX, 40 mM ethanol increased the firing rate by 12.4 ± 3.2% ($n = 10$, $p < 0.01$), indicating a ~35% reduction of ethanol's effect ($n = 10$, $p < 0.01$). Thus, ethanol facilitation of GLUergic transmission contributes significantly to the excitation of VTA DA neurons.

## DISCUSSION

Our major finding is that clinically relevant concentrations of ethanol increase glutamate release onto VTA DA neurons. Moreover, this intriguing action of ethanol is mediated by presynaptic $D_1$Rs. These effects were produced by concentrations of ethanol close to the blood alcohol levels observed in rats after voluntary ethanol consumption (Robinson *et al*, 2000; Doyon *et al*, 2003, 2005). Even more relevant is the virtually identical bell-shaped concentration-dependence of the effects of ethanol in our experiments and in those of Rodd *et al* (Rodd *et al*, 2004), who measured ethanol self-injected by rats directly into VTA. As glutamate has a major role in mediating the behavioral manifestations of ethanol consumption (Eckardt *et al*, 1998), increased glutamate release in VTA could be an important aspect of alcohol addiction.

### Ethanol Enhances Glutamatergic Transmission to VTA DA Neurons by Activation of Presynaptic $D_1$Rs

Several lines of evidence indicate that ethanol increases synaptic glutamate release in VTA. Ethanol enhanced the amplitude but decreased the paired-pulse ratio of evoked EPSCs. It increased sEPSC frequency without affecting their amplitude. Furthermore, it raised the extracellular glutamate level in VTA. The absence of any change in mEPSC frequency is clear evidence of the involvement of TTX-sensitive $Na^+$ channels. Ethanol did not alter the amplitude of both sEPSCs and mEPSCs, indicating that ethanol has no effect on postsynaptic AMPA receptors. This is probably because (1) ethanol's effects on AMPARs are small at relatively low concentrations ($<80$ mM); (2) synaptic AMPARs in juvenile neurons are more resistant to ethanol (Mameli *et al*, 2005); (3) ethanol could activate some unknown internal signaling pathway in VTA DA neurons, which counteracts direct inhibition of AMPARs.

Dopamine receptors consist of $D_1$-like ($D_1$ and $D_5$ receptors) and $D_2$-like ($D_2$, $D_3$ and $D_4$ receptors) families. Dopamine receptors of both the $D_2$ family (in particular $D_2$R) (Blum *et al*, 1990) and $D_1$R have been implicated in the mechanisms of drug dependence and abuse: for example, disruption of $D_1$R gene expression (El-Ghundi *et al*, 1998) or administration of $D_1$R antagonist (Liu and Weiss, 2002) attenuates or prevents alcohol-seeking behavior.

In our experiments, a $D_1$R antagonist eliminated ethanol facilitation of sEPSC frequency. That ethanol enhances glutamate release by $D_1$R activation was confirmed by our *in vitro* assays: ethanol raised extracellular glutamate levels, and this effect was eliminated by a $D_1$R antagonist. Moreover, like ethanol, a $D_1$R agonist increased sEPSC frequency without affecting their amplitude. These results suggest that ethanol enhances glutamate release by activating $D_1$Rs. In the midbrain, a $D_1$R agonist modulates action potential-dependent glutamate release (Kalivas and Duffy, 1995), and, in the globus pallidus, it increases the frequency but not the amplitude of sEPSCs (Hernandez *et al*, 2007). Our observation that ethanol did not change mEPSCs suggests that ethanol may not directly activate $D_1$Rs.

### How Does Ethanol Activate Presynaptic $D_1$Rs?

In reserpine-treated slices, in which dopamine is depleted, ethanol failed to alter sEPSC frequency. This further excludes ethanol's direct activation of presynaptic $D_1$Rs. Our finding that ethanol enhanced the action of a $D_1$R agonist on sEPSCs supports the possibility that ethanol potentiates dopamine's action on presynaptic $D_1$Rs. But the similar effects of ethanol and dopamine reuptake blocker rather point to a rise in extracellular dopamine concentration, which could result from ethanol-induced somatodendritic dopamine release and/or block of dopamine reuptake, in keeping with previous evidence that systemically administered ethanol (1–2 g/kg) raises extracellular dopamine in VTA (Campbell *et al*, 1996; Yan *et al*, 2005). Therefore, in addition to the potentiation of $D_1$R function, the enhancement of dopamine release would activate a positive feedback pathway through which ethanol facilitates both glutamate and dopamine release. This hypothesis is supported by previous studies showing that ethanol stimulates DA neurons (Brodie *et al*, 1990, 1999a, b; Xiao *et al*, 2007); and also somatodendritic dopamine release (Campbell *et al*, 1996; Yan *et al*, 2005) in VTA. The lack of effect of ethanol on mEPSCs provides additional support, as dopamine release in VTA is TTX sensitive (Kalivas and Duffy, 1991; Chen and Reith, 1994; Cragg and Greenfield, 1997). Note that ethanol significantly excites VTA DA neurons only at relatively high concentrations ($\geqslant 80$ mM). Thus, ethanol may enhance glutamate release by potentiation of $D_1$R function at low concentrations ($\leqslant 40$ mM), and potentiate both $D_1$R function and dopamine release at higher concentrations.

A rise in local dopamine levels is also suggested by the bell-shaped dose-dependence of ethanol's action, both in our experiments and in the *in vivo* experiments of Rodd *et al* (2004). This may result from opposite effects of dopamine (the nonselective endogenous agonist) acting on different receptors. At low concentration, dopamine appears to bind predominantly to $D_1$Rs and at higher concentrations to $D_2$Rs. Thus, Trantham-Davidson *et al* (2004) found that low concentrations of dopamine ($<0.5\,\mu$M) enhanced IPSCs by activating mainly $D_1$Rs, whereas higher concentrations of dopamine ($>1\,\mu$M) reduced IPSCs by predominantly activating $D_2$Rs. In comparable experiments on VTA and globus pallidus, Koga and Momiyama (2000) and Hernandez *et al* (2007) found that dopamine or a $D_2$ agonist inhibits EPSCs in DA neurons through presynaptic $D_2$Rs (in VTA, with a high $IC_{50}$ of 16 $\mu$M for dopamine). That the bell-shaped concentration dependence of ethanol's action in VTA slices is due to opposite presynaptic actions through $D_1$Rs and then $D_2$Rs is strongly supported by our finding that the facilitation of sEPSCs by 80 mM ethanol was greatly potentiated when $D_2$Rs were blocked.

In the present study, we found that acute ethanol enhances glutamate release at synapses on VTA DA neurons. This is quite likely a result of the unique anatomical organization of VTA and its responses to ethanol. In VTA, dopamine released somatodendritically appears to modulate glutamate release by activating both $D_1$Rs and $D_2$Rs on adjacent GLUergic terminals. Ethanol could stimulate glutamate release through either potentiation of the presynaptic $D_1$Rs or potentiation of these $D_1$Rs and dopamine release. In previous studies, ethanol inhibited glutamate release in the hippocampus by inhibiting N-, P/Q-type voltage-dependent calcium channels (VDCCs) (Maldve *et al*, 2004; Mameli *et al*, 2005), in NAcc by stimulating opioid peptide release (Nie *et al*, 1993, 1994), or by activating $GABA_B$ receptors (Steffensen *et al*, 2000). Anatomically, these pathways may be relevant for the effect of ethanol on glutamate release in VTA. However, $D_1$Rs drive the activation of protein kinase A, which is known to antagonize ethanol inhibition of N- and P/Q-type VDCCs (Solem *et al*, 1997). Although $\mu$-opioid receptors are sparsely expressed on GLUergic terminals (Garzon and Pickel, 2001), their activation also disinhibits VTA DA neurons (Johnson *et al*, 1992; Xiao *et al*, 2007), resulting in increased dopamine release. Hence, the inhibition of glutamate release may be counteracted by $D_1$R activation. In the NAcc and hippocampus,

ethanol increases GABA release, which activates $GABA_B$ receptors (Siggins *et al*, 2005); whereas we and others demonstrated that ethanol reduces GABA release in VTA (Gallegos *et al*, 1999; Stobbs *et al*, 2004; Xiao *et al*, 2007, Xiao and Ye, 2008). Therefore, in VTA, ethanol may not inhibit glutamate release by activating $GABA_B$ receptors. Although there may be other pathways involved in ethanol's effect on glutamate release in some other brain areas, our data suggest that ethanol affects these pathways in a different manner in VTA and that dopamine receptor activation alleviates their impact.

### Enhancement of Glutamate Release as an Indirect Mechanism of Ethanol Excitation of VTA DA Neurons

Previous studies have shown that ethanol can directly excite DA neurons in VTA *in vivo* (Gessa *et al*, 1985) and in slices (Brodie *et al*, 1990; Okamoto *et al*, 2006; Xiao *et al*, 2007), as well as enzymatically dissociated DA neurons (Brodie *et al*, 1999a). Such direct effects were minimal with ethanol concentrations of 40 mM (at which ethanol produced its maximum effect in the current experiments) and were prominent only with ethanol at $\geqslant$80 mM. Major direct actions on DA neurons were therefore unlikely in our experiments.

VTA DA neurons receive numerous inputs that can modulate their eventual output. Integration of the synaptic inputs and the intrinsic properties sets the frequency and pattern of firing (Mereu *et al*, 1997; Kitai *et al*, 1999; Johnson and Wu, 2004). Our finding that blockade of GLUergic transmission substantially attenuated but did not abolish ethanol's (40 mM) effect on DA cell firing, indicates that potentiation of GLUergic transmission is not the sole mechanism of ethanol action in VTA. Other mechanisms include disinhibition (Xiao *et al*, 2007), as well as direct excitation of VTA DA neurons—though the latter may become a major factor only when (ethanol) rises $>$40 mM (Brodie *et al*, 1990).

In conclusion, the present study shows that ethanol-induced facilitation of glutamatergic transmission increases firing of VTA DA neurons. This effect is mediated by somatodendritically released dopamine, which activates $D_1$Rs on GLUergic terminals, causing an increase in glutamate release. The resulting acceleration in DA neuronal firing leads to further increase in dopamine release. By potentiating dopamine's action on presynaptic $D_1$Rs and thus augmenting dopamine release, ethanol reinforces a positive feedback loop, that may constitute the neuronal substrate of ethanol addiction.

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## DISCLOSURE

The authors declare no conflict of interest.

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