

Alcohol Inhibits NR2B-Containing NMDA Receptors in the Ventral Bed Nucleus of the Stria Terminalis

Thomas L Kash¹, Robert T Matthews¹ and Danny G Winder^{*,1,2,3}

¹Department of Molecular Physiology and Biophysics, Vanderbilt University School of Medicine, Nashville, TN, USA; ²Center for Molecular Neuroscience, Vanderbilt University School of Medicine, Nashville, TN, USA; ³JF Kennedy Center for Research on Human Development, Vanderbilt University School of Medicine, Nashville, TN, USA

Components of the mesolimbic dopamine system, in particular dopaminergic cells in the ventral tegmental area (VTA), have been implicated in the acute reinforcing actions of ethanol. The ventral bed nucleus of the stria terminalis (vBNST) potentially regulates dopaminergic cell firing in the VTA, and has been implicated in the behavioral actions of ethanol. The *N*-methyl-D-aspartate receptor (NMDAR) is a major molecular target of ethanol, however, current evidence suggests that ethanol regulation of NMDAR function is widely variable and likely depends on a number of factors. Thus, it is critical to investigate ethanol regulation of NMDAR function at synapses relevant to ethanol-regulated behaviors, such as in the vBNST. Here we show, using multiple techniques, that ethanol inhibits NMDAR function in vBNST neurons in a postsynaptic fashion. Further, we demonstrate the functional presence of both NR2A and NR2B-containing NMDARs in the vBNST. While genetic removal of NR2A did not alter the magnitude of ethanol inhibition, pharmacological blockade of NR2B rendered synaptically activated NMDARs insensitive to ethanol inhibition. Finally, we demonstrate that ethanol inhibits NMDARs in cells in the vBNST that project to the VTA, providing a direct means by which ethanol in the vBNST can modulate the dopaminergic system.

Neuropsychopharmacology (2008) **33**, 1379–1390; doi:10.1038/sj.npp.1301504; published online 11 July 2007

Keywords: anxiety; withdrawal; plasticity; addiction; stress; postsynaptic

INTRODUCTION

The motivational and rewarding effects of ethanol are thought to be mediated, at least in part, via activation of the mesolimbic dopamine system, in particular dopaminergic cells within the ventral tegmental area (VTA) (Brodie *et al*, 1999; Melendez *et al*, 2002). Excitability of VTA dopaminergic neurons is regulated in part by afferents from a number of brain regions (Georges and Aston-Jones, 2002; Grillner and Mercuri, 2002). Of particular interest, the VTA receives a large projection from the ventral bed nucleus of the stria terminalis (vBNST) (Georges and Aston-Jones, 2002). This projection is thought to be excitatory, as stimulation in the vBNST can increase both VTA DA cell firing and bursting (Georges and Aston-Jones, 2002). The BNST is a structure within the extended amygdala that has been implicated both in rodents and primates as a key mediator of stress and reward interactions (Walker *et al*, 2003; Burow *et al*, 2005; Choi *et al*, 2007; Harris and Aston-

Jones, 2007), which are thought to be important determinants of ethanol intake.

Pharmacological manipulations in the BNST, such as infusion of GABA receptor antagonists (Hyytia and Koob, 1995; Eiler and June, 2007) or D1 receptor antagonists (Eiler *et al*, 2003) can disrupt ethanol-seeking behaviors, suggesting that the BNST plays an important role in behavioral responses to ethanol. Little is known, however, about the effects that acute ethanol administration has on neuronal function in this region. The *N*-methyl-D-aspartate receptor (NMDAR) has been heavily implicated in the acute actions of ethanol in both behavioral (Boyce-Rustay and Holmes, 2005) and molecular studies (Roberto *et al*, 2004). NMDARs in the BNST have been specifically implicated in the regulation of stress and anxiety (Matys *et al*, 2005; Pawlak *et al*, 2005), which have been suggested to be important in regulating ethanol-seeking behavior in humans and in animal models of alcohol drinking. Further, we have previously shown that NMDARs in the dorsal BNST are ethanol sensitive (Weitlauf *et al*, 2004). However, ethanol sensitivity of NMDARs on neurons in the vBNST has not been examined.

NMDARs are tetrameric complexes generally composed of two NR1 subunits and two NR2 subunits. The NR2 subunits have drawn particular interest as they confer unique biophysical and signaling properties to NMDARs

*Correspondence: Dr DG Winder, Department of Molecular Physiology and Biophysics, Vanderbilt University School of Medicine, 23rd and Pierce Ave S, Room 724B, RRB, Nashville, TN 37232-0615, USA, Tel: +1 615 322 1144, Fax: +1 615 322 1462, E-mail: danny.winder@vanderbilt.edu
Received 30 April 2007; revised 31 May 2007; accepted 8 June 2007

(Cull-Candy and Leszkiewicz, 2004). To date, studies examining the subunit specificity of NMDAR ethanol sensitivity have reached divergent results. Several early studies suggested that ethanol targets NR2B-containing NMDARs (Lovinger, 1995; Fink and Gothert, 1996), while different results were obtained in the cerebellum (Popp *et al*, 1999, also see Engblom *et al*, 1997). More recently it was suggested that NR2A-containing NMDARs are specifically ethanol sensitive in the hippocampus (Suvarna *et al*, 2005). However, another study, also in the hippocampus, demonstrated that NR2B-containing NMDARs are the target of ethanol (Izumi *et al*, 2005). This lack of consistent results is echoed in studies examining the ethanol sensitivity of NMDARs in heterologous expression systems. A number of studies have demonstrated that NR2A and NR2B-containing NMDARs are more sensitive than NR2C-containing NMDARs (Chu *et al*, 1995; Mirshahi and Woodward, 1995), although a recent study indicates that this sensitivity may be modulated by NR1 splice variant expression (Jin and Woodward, 2006). Further complicating the interpretation of heterologous studies, it has been shown that these differences can depend on the expression system utilized (Smothers *et al*, 2001). The study of the precise mechanisms of ethanol inhibition of NMDARs has provided mixed results as well. While much of the literature supports a postsynaptic mechanism (Lovinger *et al*, 1989) for ethanol inhibition of NMDA-excitatory postsynaptic currents (EPSCs), recent studies in brain slices have suggested an additional presynaptic component (Hendricson *et al*, 2004; Zhang *et al*, 2005; Zhu *et al*, 2007).

Taken together, the above findings suggest that the mechanisms of action of ethanol at NMDARs vary across different regions of the brain. Thus, it is critical to examine the ethanol sensitivity of NMDAR-mediated synaptic transmission in brain regions that are critical to the behavioral actions of ethanol. Here, we have examined ethanol sensitivity of NMDARs in the vBNST. Using multiple techniques, we examined both the mechanism and subunit specificity of ethanol inhibition of NMDARs in the vBNST. We show that ethanol inhibits NMDARs postsynaptically. Additionally, we provide evidence that both NR2A and NR2B subunits are present in synaptic NMDARs in the vBNST, and that an NR2B antagonist can abolish ethanol sensitivity. Finally, we show that ethanol can inhibit NMDARs in vBNST cells that project to the vBNST.

MATERIALS AND METHODS

Brain Slice Preparation

All procedures were performed according to Vanderbilt University Institutional Animal Care and Use Committee-approved procedures. Male C57Bl/6J mice (6–8 weeks old, Jackson Laboratories) were decapitated under anesthesia (isoflurane). The brains were quickly removed and placed in ice-cold sucrose-artificial cerebrospinal fluid (ACSF): (in mM) 194 sucrose, 20 NaCl, 4.4 KCl, 2 CaCl₂, 1 MgCl₂, 1.2 NaH₂PO₄, 10.0 glucose, and 26.0 NaHCO₃ saturated with 95% O₂/5% CO₂. Slices 300 μ m in thickness were prepared using a Tissue Slicer (Leica). Rostral slices containing anterior portions of BNST (bregma 0.26–0.02 mm) (Frank-

lin and Paxinos, 1997) were identified using the internal capsule, anterior commissure, fornix, and stria terminalis as landmarks. Slices were then stored in a heated (approximately 28°C), oxygenated (95% O₂/5% CO₂) holding chamber containing 'normal' ACSF (ACSF: (in mM) 124 NaCl, 4.4 KCl, 2 CaCl₂, 1.2 MgSO₄, 1 NaH₂PO₄, 10.0 glucose, and 26.0 NaHCO₃) or transferred to a submerged recording chamber where they were perfused with heated (26°C, unless otherwise noted), oxygenated ACSF at a rate of about 2 ml/min. Slices were allowed to equilibrate in normal ACSF for 1 h before experiments began.

Whole-Cell Voltage-Clamp Recordings

Slices were placed in a submerged chamber (Warner Instruments) and neurons of the vBNST were directly visualized with infrared video microscopy (Olympus). Recording electrodes (3–6 M Ω) were pulled on a Flaming–Brown Micropipette Puller (Sutter Instruments) using thin-walled borosilicate glass capillaries. NMDA-EPSCs were evoked by local fiber stimulation with bipolar nichrome electrodes. Stimulating electrodes were placed in the vBNST, 100–500 μ m medial from the recorded neuron, and electrical stimuli (5–40 V with a 100–150 μ s duration) were applied at 0.1 Hz while recording NMDA-EPSCs and 0.2 Hz while recording AMPA-EPSCs. NMDA-EPSCs were recorded from a holding potential of –70 mV and pharmacologically isolated by adding 25 μ M picrotoxin and either 20 μ M 6-cyano-7-nitroquinoline-2,3-dione (CNQX) or 10 μ M NBQX in a zero Mg²⁺ ACSF (in mM: 124 NaCl, 4.4 KCl, 3.7 CaCl₂, 1 NaH₂PO₄, 10 glucose, and 26 NaHCO₃, pH 7.2–7.4; 290–310 mOsmol). Recording electrodes were filled with (in mM) K⁺-gluconate (135), NaCl (5), HEPES (10), EGTA (0.6), ATP (4), GTP (0.4), and biocytin (0.1%) pH 7.2, 290–295 mOsmol. AMPA-EPSCs were isolated by adding 25 μ M picrotoxin and recording at a holding potential of –70 mV in normal ACSF. At this holding potential in the presence of picrotoxin in normal ACSF, application of either CNQX or NBQX completely abolished the postsynaptic current. Signals were acquired via a Multiclamp 700B amplifier (Axon Instruments), digitized and analyzed via pClamp 9.2 software (Axon Instruments).

For experiments in which NMDA was exogenously applied, the following protocol was used. A 1.0 mM NMDA solution was prepared in fresh ACSF and loaded into a thin-walled borosilicate glass capillary pulled using a Flaming–Brown Micropipette Puller (Sutter Instruments). The capillary tube was then mounted in a pipette holder connected to a Picospritzer II (Parker) delivery system. This pipette was then placed within approximately 10 μ m of a cell to be recorded using video microscopy guidance. At this point, an independent patch electrode was positioned next to the cell and a whole-cell voltage-clamp configuration was obtained. Following break-in, the cell was allowed to rest for 5 min, and then the NMDA was applied using pressure ejection with pressure settings of 30–40 p.s.i. and pulse length of 3–15 ms. During the course of the experiment, NMDA was applied every 10 s to allow complete return to baseline holding current. The ACSF in these experiments was identical to those used for evoked NMDA-EPSC experiments.

Input resistance, holding current, and series resistance were all monitored continuously throughout the duration

of experiments. Experiments in which changes in series resistance were greater than 20% were not included in the data analysis. Experiments were analyzed by measuring the parameter of interest; peak amplitude or area of the synaptic response which was normalized to the baseline period. The baseline period is defined as the 5 min period immediately preceding application of the drug. NMDA-EPSC decay was fitted with two exponentials using Clampfit 9.2 for averages of several traces from baseline and 'drug' values as done previously (Fu *et al*, 2005). To allow for direct comparison of decay times between experimental conditions, the two decay time components, τ_1 and τ_2 , were combined into a weighted time constant, τ_w , using the equation: $\tau_w = (\tau_1 a_1) + (\tau_2 a_2)$, where a_1 and a_2 are the relative amplitudes of the two exponential components.

Statistical Analyses

Statistical analyses were performed using Microsoft Excel, Graphpad Prism and Microcal Origin. Specifically, when determining if a compound had a significant effect (for example, 50 mM ethanol), a Student's paired *t*-test was used, comparing the baseline value to the experimental value. For the ethanol experiments, the baseline value was the average value obtained from the recordings obtained in the 2 min immediately preceding application of ethanol, and the experimental value was determined by averaging the recordings obtained 4–6 min following removal of ethanol. In order to compare the effects of different concentrations of ethanol to one another, a one-way ANOVA was used, followed by a Tukey posttest to determine the significance of specific comparisons. When comparing the effects of multiple treatments on ethanol's effect, a one-way ANOVA followed by Dunnett's posttest was used to determine the significance of the differences between the groups. All values given for drug effects throughout the paper are presented as average \pm SEM. For results given in figures, significance is noted in the figure legend. For results not included in figures, significance is noted in the text.

Pharmacology

Picrotoxin, CNQX, NMDA, Ro 25–6981, Ifenprodil, and DL-2-amino-5-phosphonopentanoic acid (DL-APV) were purchased from Sigma-Aldrich (St Louis, MO). NBQX was purchased from Ascent (England). Ethanol (95%) was purchased from Aaper Alcohol and Chemical (Shelbyville, KY). DMSO (0.02%) was used as a vehicle for picrotoxin. For experiments where the acute effects of a drug were examined on NMDA-EPSCs, only one cell per slice was recorded.

Knockout Animals

NR2A knockout (KO) breeder animals extensively backcrossed onto C57Bl6/j were obtained from Dr David Lovinger and were generated as previously reported (Kiyama *et al*, 1998). Animals for this study were generated by the breeding of homozygous KO males and females.

Retrograde Labeling of BNST Neurons

C57Bl6/j male mice, 6–8 weeks old, were anesthetized with tribromoethanol (2.5% solution in saline, 0.19 ml/10 g, i.p.).

Ophthalmic ointment (Puralube Vet) was applied to the eyes to prevent corneal damage. The fur on the top of the head was shaved off and the exposed skin was scrubbed sequentially with 70% ethanol, 10% povidone iodine, and 70% ethanol. The animal was mounted in a stereotaxic apparatus (Kopf Instruments) and placed on a warming pad kept at 39°C. The skin over the skull was cut and deflected enough to expose the bregma and lambda sutures. Local anesthetic cream containing benzocaine was applied to the wound. The head was leveled with respect to the intersections of bregma and lambda with the midline suture. Holes were drilled in the skull directly above the VTA according to the atlas of Franklin and Paxinos (3.3 mm posterior to bregma and 0.5 mm lateral to the midline). The 32-gauge needle of a 0.5 μ l Hamilton zero dead volume syringe was heat sterilized immediately before backfilling with 30 nl of an undiluted suspension of fluorescent microspheres (Molecular Probes, 0.04 μ m diameter, 488/560 nm excitation/emission). The dura was cut and the syringe needle lowered into the VTA (4.7 mm below surface of the skull). Tracer microspheres were slowly injected over 3–4 min and the needle left in place for an additional 10 min. Most animals received bilateral tracer injections. After withdrawing the needle, the wound was closed with sutures and the animal was hydrated with a saline injection (1 ml, s.c.) followed by freshly dissolved ampicillin (0.45 mg/0.1 ml per animal, s.c.). The animal was kept warm during recovery from anesthesia and was given an analgesic when ambulatory (buprenorphine, 0.1 mg/kg, s.c.). Postsurgery, animals were housed singly and weighed and injected with analgesic twice per day for up to 6 days or until the animal gained weight for 2 consecutive days. Animals that lost more than 20% of their body weight or showed signs of uncontrolled pain, stress, or dehydration were euthanized. Healthy animals were used for electrophysiological experiments 5–14 days after surgery.

Histology

The hindbrain of all tracer-injected mice used for whole-cell recordings were immersion fixed in 4% paraformaldehyde/PBS, cryoprotected with 20% sucrose, and sectioned on a cryostat. Sections were mounted on slides and viewed with a fluorescence microscope. All animals with retrograde-labeled BNST neurons were confirmed to have tracer injection sites in the VTA.

RESULTS

Whole-cell recordings were made from neurons in the vBNST in coronal brain slices from adult male C57Bl6 mice. We isolated NMDA-EPSCs by using an Mg^{2+} -free ACSF containing picrotoxin (25 μ M) and CNQX (20 μ M) or NBQX (10 μ M). Local afferent stimulation under these conditions evoked slowly decaying responses at a holding potential of -70 mV. These responses were completely blocked by DL-APV (100 μ M), indicating their mediation by NMDARs (data not shown). Further, in this same Mg^{2+} -free ACSF, we found that brief exogenous application of NMDA (see Materials and Methods) resulted in an inward current at -70 mV that was also completely blocked by DL-APV (100 μ M; data not shown).

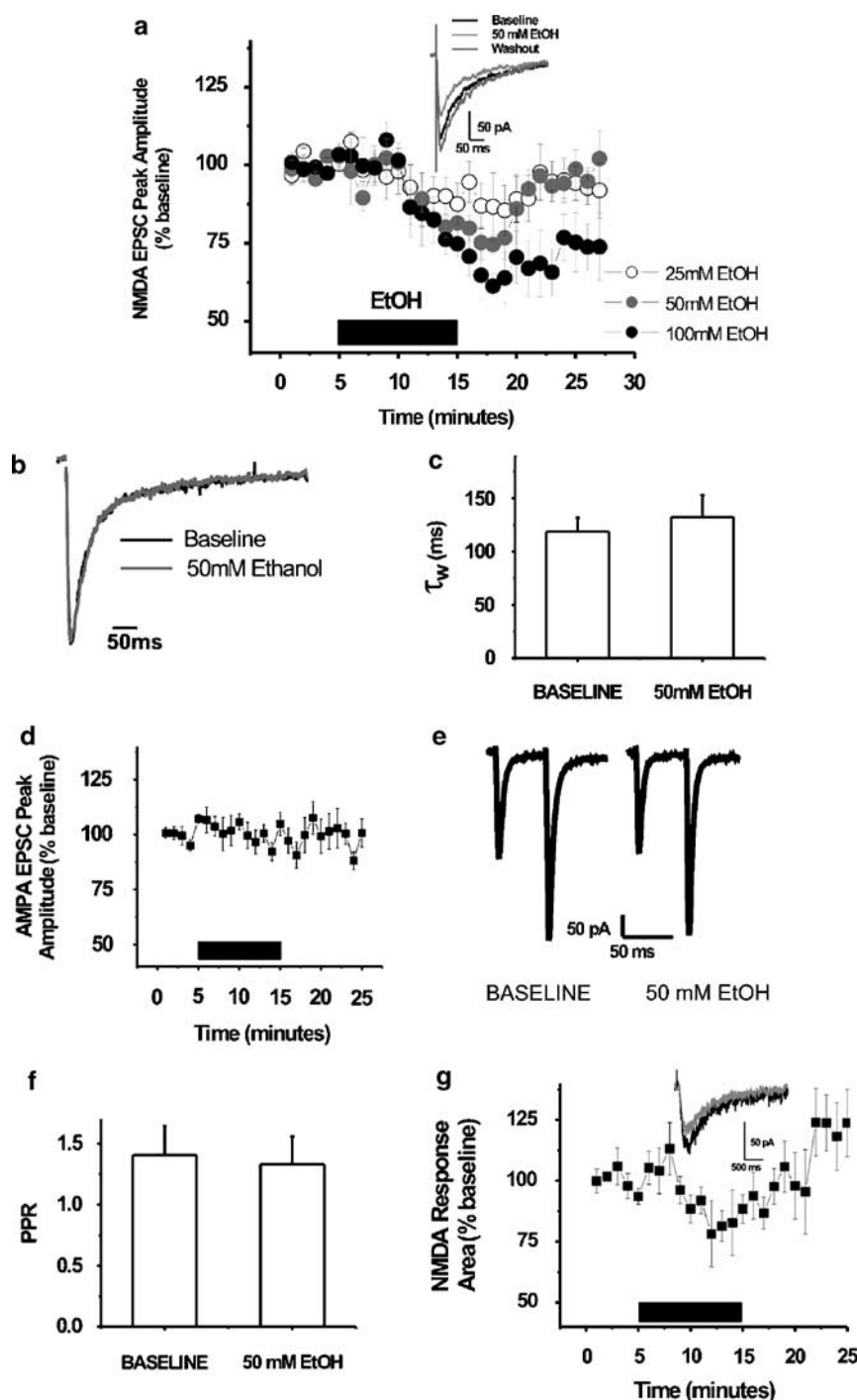


Figure 1 Ethanol inhibits NMDA currents in vBNST in a concentration-dependent and reversible fashion via a postsynaptic mechanism. (a) Ethanol inhibited NMDA-EPSCs in a concentration-dependent fashion. (Inset) Representative traces from an experiment demonstrating the effect of 50 mM ethanol on NMDA-EPSCs and reversal during washout (25 mM, $n = 5$; 50 mM, $n = 5$; 100 mM, $n = 5$). (b) A total of 50 mM of ethanol did not alter the kinetics of the NMDA-EPSC, as shown in representative normalized traces. (c) Average decay time, shown here as the weighted τ , demonstrates the lack of effect of 50 mM ethanol on the decay kinetics of the evoked NMDA-EPSC ($n = 5$). (d) Application of 50 mM ethanol had no effect on electrically evoked AMPA receptor-mediated EPSCs ($n = 6$). (e) Representative traces demonstrating the lack of an effect of 50 mM ethanol on the paired pulse ratio of AMPA EPSCs. (f) Pooled data demonstrating the lack of an effect of 50 mM ethanol on the paired pulse ratio of AMPA-EPSCs. (g) A total of 50 mM ethanol inhibited currents evoked by exogenous application of NMDA ($n = 5$). (Inset) Representative traces demonstrating the inhibitory effect of 50 mM ethanol on exogenously applied NMDA.

Ethanol Inhibits NMDA-EPSCs in a Concentration-Dependent Fashion

While previous studies from this laboratory have shown that ethanol can inhibit NMDAR function in a concentra-

tion-dependent and reversible fashion, these experiments were conducted on NMDAR-mediated field potentials and NMDA-EPSCs in the dorsal BNST (Weitlauf *et al*, 2004). Here, we extended these experiments to the whole-cell voltage-clamp configuration in the vBNST, as the vBNST

Table 1 Ethanol Effects on Evoked NMDA EPSC Kinetics

Condition	Baseline				Drug			
	τ_w (ms)	τ_{fast} (ms)	τ_{slow} (ms)	% fast	τ_w (ms)	τ_{fast} (ms)	τ_{slow} (ms)	% fast
50 mM EtOH (in 20 μ M CNQX)	118 \pm 13 (5)	40 \pm 4 (5)	254 \pm 24 (5)	64 \pm 4 (5)	132 \pm 21 (5)	43 \pm 8 (5)	240 \pm 28 (5)	56 \pm 6 (5)
50 mM EtOH (in 10 μ M NBQX)	188 \pm 34 (7)	67 \pm 11 (7)	435 \pm 96 (7)	48 \pm 4 (7)	166 \pm 26 (7)	56 \pm 9 (7)	304 \pm 32 (7)	55 \pm 6 (7)
100 mM EtOH (in 20 μ M CNQX)	153 \pm 29 (5)	56 \pm 10 (5)	262 \pm 45 (5)	55 \pm 5 (5)	127 \pm 27 (5)	52 \pm 13 (5)	200 \pm 22 (5)	57 \pm 11 (5)

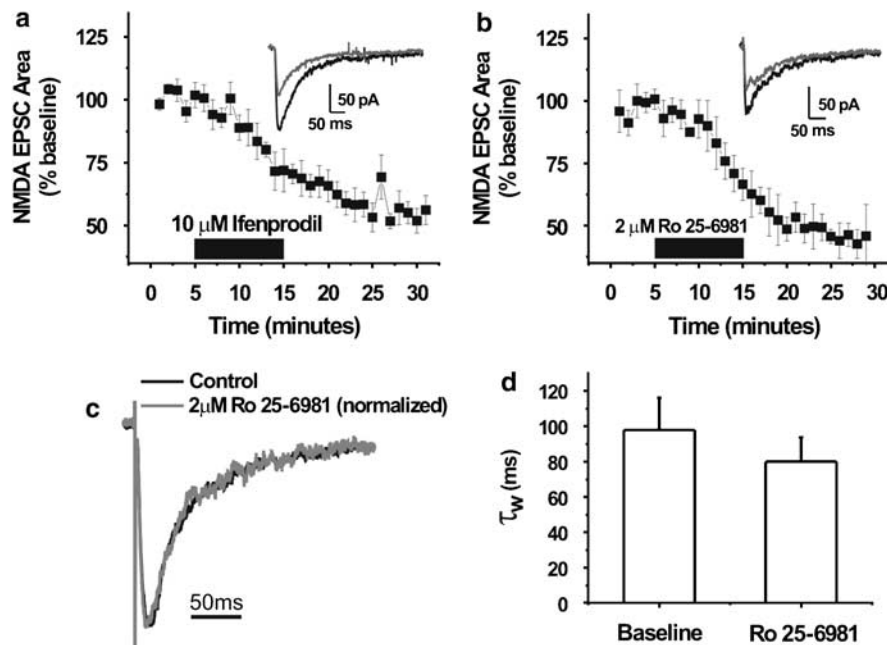


Figure 2 Synaptic NMDARs in the vBNST contain the NR2B subunit. (a) Bath application of the NR2B-selective antagonists, ifenprodil ($n = 5$), and Ro 25-6981 ($n = 5$) (b) inhibited evoked NMDA currents in the vBNST. (c) Ro 25-6981 did not alter the kinetics of the NMDA-EPSC, as demonstrated in the representative normalized traces. (d) Average decay time, shown here as weighted τ , demonstrates the lack of effect of Ro-25-6981 on the decay kinetics of the evoked NMDA-EPSC ($n = 5$).

has been specifically shown to be critical to modulation of VTA activity. Ethanol inhibited NMDA-EPSCs in a concentration-dependent fashion (25 mM ethanol, $85 \pm 7\%$ of baseline area, $n = 5$, $p < 0.01$; 50 mM ethanol, $75 \pm 10\%$ of baseline area, $n = 5$, $p < 0.01$; 100 mM ethanol, $48 \pm 5\%$ of baseline area, $n = 5$, $p < 0.01$; effects on peak amplitude are shown in Figure 1a). A comparison between the effects of multiple concentrations of ethanol reveals a significant difference between 25 mM and 100 mM ((ANOVA ($F(2, 12) = 5.457$), $p < 0.05$) $p < 0.05$, Tukey posttest)). This effect was clearly reversible at lower concentrations of ethanol (Figure 1a). A similar level of ethanol inhibition (50 mM ethanol, $79 \pm 4\%$ of baseline peak amplitude, $n = 6$, $p < 0.01$) of the NMDA-EPSC was observed with NBQX (10 μ M) in place of CNQX. Further, we have examined the effect of 50 mM ethanol on NMDA-EPSCs at 30–31 $^{\circ}$ C and have found the magnitude of inhibition to be similar (data not shown).

While ethanol reduced both the peak amplitude and area of NMDA-EPSCs, we found that ethanol did not alter the decay time of the NMDA-EPSC as shown by the lack of effect on both the weighted τ (Figures 1b and c) and the

individual components of the decay (Table 1). It is important to note that both 25 and 50 mM ethanol are within the behaviorally relevant range of doses that can be achieved during a single episode of alcohol drinking (approximately equivalent to blood alcohol concentrations of 0.11 and 0.22 mg/dl). For this reason, we used 50 mM ethanol for the experiments in which we wanted to examine the site (presynaptic vs postsynaptic) of action of ethanol.

Ethanol Inhibits NMDA-EPSCs in the vBNST through a Postsynaptic Mechanism

While the bulk of the literature demonstrates that ethanol inhibits NMDAR function via a postsynaptic mechanism, several recent studies in the nucleus accumbens and central nucleus of the amygdala (CeA) suggest a presynaptic component (Hendricson *et al*, 2004; Zhang *et al*, 2005; Zhu *et al*, 2007). In order to evaluate the effect of ethanol on presynaptic function at glutamatergic synapses in the vBNST, we examined the ability of ethanol to inhibit AMPA receptor-mediated synaptic transmission. If ethanol inhibits

Table 2 Effects of NR2 Subunit Manipulations on Evoked NMDA EPSC Kinetics

Condition	Baseline				Drug			
	τ_w (ms)	τ_{fast} (ms)	τ_{slow} (ms)	% fast	τ_w (ms)	τ_{fast} (ms)	τ_{slow} (ms)	% fast
2 μ M Ro 25-6981 (in 20 μ M CNQX)	98 \pm 18 (5)	39 \pm 9 (5)	239 \pm 67 (5)	66 \pm 6 (5)	80 \pm 13 (5)	41 \pm 10 (5)	163 \pm 16 (5)	70 \pm 9 (5)
2 μ M Ro 25-6981 (in 10 μ M NBQX)	168 \pm 33 (5)	45 \pm 13 (5)	312 \pm 112 (5)	66 \pm 13 (5)	126 \pm 22 (5)	55 \pm 11 (5)	212 \pm 54 (5)	45 \pm 11 (5)
10 μ M Ifenprodil (in 20 μ M CNQX)	165 \pm 20 (5)	56 \pm 4 (5)	337 \pm 40 (5)	61 \pm 6 (5)	175 \pm 35 (5)	56 \pm 7 (5)	303 \pm 57 (5)	58 \pm 10 (5)
NR2A KO (in 20 μ M CNQX)	310 \pm 49 (10)*	75 \pm 8* (10)	420 \pm 125 (10)	48 \pm 9 (10)	—	—	—	—

* $p < 0.05$ from values obtained in wild-type animals.

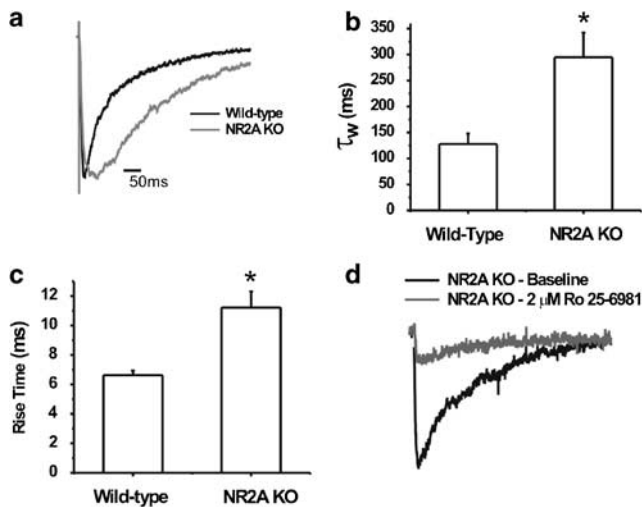


Figure 3 Synaptic NMDARs in the vBNST contain the NR2A subunit. (a) Representative amplitude-normalized NMDA-EPSC from NR2A knockout and wild-type demonstrating alterations in the kinetic profile. (b) There was a significant increase in both the decay and (c) rise time when compared to NMDA-EPSCs from wild-type animals (NR2A knockout, $n = 12$; wild type, $n = 10$). (d) Representative traces demonstrating the robust inhibition of NMDA-EPSCs by the NR2B-selective antagonist, Ro 25-6981, in the NR2A knockout animal. * $p < 0.05$ using Student's t -test.

NMDAR function via a reduction in glutamate release, then a corresponding reduction in AMPA-EPSCs should be observed. However, ethanol (50 mM) had no effect on AMPA-EPSCs in the vBNST ($95 \pm 7\%$ of baseline peak amplitude, $n = 5$, Figure 1d). Additionally, we examined the effect of 50 mM ethanol on the paired pulse ratio of AMPA-EPSCs, a measure that is sensitive to alterations in probability of glutamate release, and found no significant difference (Figures 1f and g). These findings are consistent with a lack of effect of ethanol on glutamate release in the vBNST. To evaluate the postsynaptic actions of ethanol on NMDARs, we examined the ability of ethanol to inhibit currents evoked by exogenous application of NMDA, isolating the postsynaptic component of synaptic transmission. Brief pulses of NMDA produced inward currents with a stable peak amplitude over time. We found that application of 50 mM ethanol resulted in a reduction in the amplitude of these currents elicited by exogenously applied NMDA ($80 \pm 9\%$ of peak amplitude, $n = 5$, $p < 0.01$, effects on area shown in Figure 1e). The time course and magnitude of inhibition were similar to that observed on

synaptically evoked NMDA-EPSCs. Taken together, these findings strongly suggest that ethanol inhibits NMDAR function in the vBNST via a postsynaptic mechanism.

NR2A and NR2B Subunits Contribute to Synaptic NMDARs in the vBNST

Having established that ethanol is acting postsynaptically in the vBNST, we next sought to determine if ethanol had NR2 subtype selectivity, using a combination of pharmacological and genetic manipulations. Determining the selectivity of the actions of ethanol is critical to understanding how it modulates activity in the brain, as different NR2 subunit containing NMDARs can have distinct signaling pathways. We first determined the contribution of individual NR2 subunits to the synaptic response. In order to evaluate the functional synaptic presence of the NR2B subunit, we utilized two NR2B-selective antagonists, ifenprodil and Ro 25-6981. Bath application of 10 μ M ifenprodil caused nonreversible but stable reduction of both the area ($55 \pm 4\%$ of baseline area, $n = 5$, $p < 0.01$, Figure 2a) and peak amplitude ($68 \pm 6\%$ of baseline peak amplitude, $n = 5$, $p < 0.01$) of the NMDA-EPSC. A total of 2 μ M of Ro 25-6981 caused a similar reduction of both the area ($49 \pm 9\%$ of baseline area, $n = 5$, $p < 0.01$, Figure 2b) and peak amplitude ($50 \pm 4\%$ of baseline peak amplitude, $n = 5$, $p < 0.01$) of the NMDA-EPSC. Similar results were obtained using 2 μ M of Ro 25-6981 in the presence of NBQX (10 μ M) in place of CNQX ($42 \pm 9\%$ of baseline peak amplitude, $n = 5$, $p < 0.01$, $43 \pm 6\%$ of baseline area, $n = 5$, $p < 0.01$). Rise (data not shown) and decay times of NMDA-EPSCs (representative normalized traces shown in Figure 2c) were unaltered following application of both Ro 25-6981 (Figure 2d, Table 2) and ifenprodil (Table 2). Although ifenprodil and Ro 25-6981 have been shown to substantially alter the decay kinetics at synapses where diheteromeric NR2B NMDARs make significant contributions (Bartlett et al, 2006), at other synapses a similar lack of effects on kinetics has been observed (Lopez de Armentia and Sah, 2003).

Given the lack of an effective pharmacological tool to explore the role of the NR2A subunit (see Berberich et al, 2005; Weitauf et al, 2005; Frizelle et al, 2006; Neyton and Paoletti, 2006; Kash and Winder, 2007), we utilized NR2A KO mice to determine the functional synaptic presence of the NR2A subunit in the vBNST. While NMDA-EPSCs were still readily elicited in vBNST neurons from NR2A KO mice, there was a significant alteration in the kinetics of the response

(Figures 3a–c), consistent with what has been demonstrated in other brain regions (Fu *et al*, 2005; Lu *et al*, 2006; Philpot *et al*, 2007). Both the decay time (294 ± 47 ms for the NR2A KO, $n = 12$; 128 ± 20 ms for the wild type, $n = 10$; $p < 0.01$; Figure 3b) and the rise time (11 ± 1 ms for the NR2A KO, $n = 12$; 6.6 ± 0.3 ms for the wild type, $n = 10$; $p < 0.01$; Figure 3c) were significantly slower when compared to wild-type animals. Data from recombinant systems indicate that the kinetics of diheteromeric NR1/NR2A NMDARs are faster than other di- and triheteromeric NMDAR configurations, thus these data are what would be predicted if 'faster' NR2A subunits contribute to the synaptic NMDA-EPSC in the wild-type vBNST glutamate synapses (Vicini *et al*, 1998; Erreger *et al*, 2005). Similar observations have been made for synaptic NMDA-EPSCs in cultured cerebellar granule cells from NR2A KO mice (Fu *et al*, 2005). As in the wild-type mice, we assessed NR2B contributions to the NMDA-EPSC through the use of NR2B ligands in neurons from NR2A KO mice. Ro 25-6981 ($2 \mu\text{M}$) in the NR2A KO nearly completely blocked the NMDA-EPSC ($27 \pm 6\%$ of baseline peak amplitude, $n = 4$, $p < 0.01$, Figure 3d), confirming that vBNST

NMDA-EPSCs in NR2A KO mice are mediated predominantly by diheteromeric NR2B NMDARs.

NR2A is not Required for Ethanol Inhibition of NMDA-EPSCs In vBNST

It has been suggested that ethanol can selectively target NMDARs containing specific subunits (Suvarna *et al*, 2005, but see Jin and Woodward, 2006). A recent study suggested that the NR2A subunit plays an important role in regulating NMDA-EPSC ethanol sensitivity in the hippocampus (Suvarna *et al*, 2005). Given the lack of an effective NR2A-selective antagonist (Frizelle *et al*, 2006), we utilized brain slices obtained from NR2A KO mice to evaluate the role of NR2A-containing NMDARs in ethanol inhibition. We found that 100 mM ethanol inhibited NMDA-EPSCs in vBNST neurons in the NR2A KO ($66 \pm 4\%$ of baseline peak amplitude, $n = 5$, $p < 0.01$, Figure 4a) to the same degree as in wild-type animals (Figure 4f), demonstrating that the presence of the NR2A subunit is not required for ethanol inhibition of NMDA-EPSCs in the vBNST.

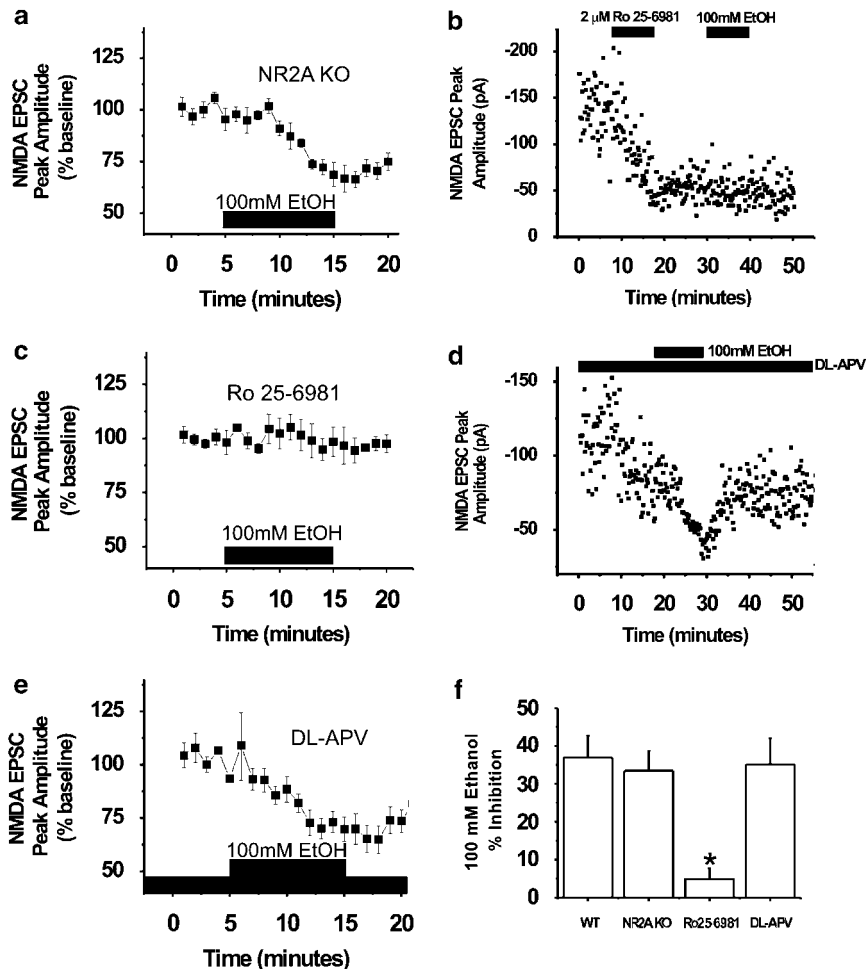


Figure 4 Ethanol inhibits NMDA-EPSCs in an NR2B-dependent fashion. (a) The ability of 100 mM ethanol to inhibit NMDA-EPSCs was intact in NR2A knockout mice, $n = 5$. (b) A representative experiment showing the reduced effect of 100 mM ethanol following application of $2 \mu\text{M}$ Ro 25-6981. (c) Pooled data demonstrating the impaired ability of 100 mM ethanol to inhibit NMDA-EPSCs following application of Ro 25-6981, $n = 5$. (d) A representative experiment showing the effect of 100 mM ethanol following application of $10 \mu\text{M}$ DL-APV, $n = 4$. (e) Pooled data demonstrating the ability of 100 mM ethanol to inhibit NMDA-EPSCs in the presence of $10 \mu\text{M}$ DL-APV. (f) The inhibitory effect of 100 mM ethanol is altered following treatment of Ro 25-6981, but not following treatment of DL-APV or in the NR2A knockout when compared to wild-type animals.

At some synapses, it has been suggested that NR2B-containing NMDARs are specifically targeted by ethanol (Roberto *et al*, 2004; Izumi *et al*, 2005). We utilized a pharmacological approach to test the role of NR2B subunits in ethanol sensitivity of the NMDA-EPSC. As previously shown (Figure 2b), application of 2 μ M Ro 25-6981 produced an approximately 50% reduction in the peak amplitude and area of the NMDA-EPSC. Interestingly, we found that both a 10 min preapplication (as shown in Figure 4b) and a continuous application (included in pooled results in Figure 4c) of Ro 25-6981 to slices prepared from wild-type mice occluded the subsequent ability of ethanol to inhibit NMDA-EPSCs ($97 \pm 4\%$ of baseline peak amplitude, $n = 5$, Figures 4b, c, f). In order to rule out the possibility that this effect of Ro 25-6981 was a result of utilizing a smaller NMDA-EPSC for analysis, we examined the effects of ethanol under conditions of similar impairment in the presence of a subsaturating concentration of the nonselective NMDAR antagonist DL-APV (10 μ M). We found that the effect of ethanol was still apparent following a similar reduction ($49 \pm 12\%$ of baseline peak amplitude, $n = 4$, $p < 0.01$) of NMDAR function due to continuous application of DL-APV (10 μ M) (Figures 4d–f). These results suggest that ethanol inhibition of NMDA-EPSCs in the vBNST is due

to inhibition of NR2B-containing NMDARs, as has been suggested in the CeA (Roberto *et al*, 2004) and the hippocampus (Izumi *et al*, 2005).

Ethanol Inhibits NMDA-EPSCs in vBNST Cells that Project to the VTA

It has been reported by both our group (Egli and Winder, 2003) and others (Rainnie, 1999; Dumont and Williams, 2004) that there are electrophysiologically distinct populations of neurons in the vBNST. Specifically, a population of neurons in the vBNST that projects to the VTA was identified in the rat using a retrograde labeling technique (Dumont and Williams, 2004). Interestingly, these projection neurons have been shown to be differentially modulated by norepinephrine (Dumont and Williams, 2004) as well as altered following cocaine self-administration (Dumont *et al*, 2005). In order to more completely understand the ability of ethanol to modulate the important interactions between the vBNST and the VTA, we wanted to specifically determine if vBNST neurons that project to the VTA were similarly sensitive to ethanol. Fluorescent microspheres injected into the VTA were retrogradely transported to the vBNST (representative injection site image shown in Figures 5a and b). Labeled neurons were visualized

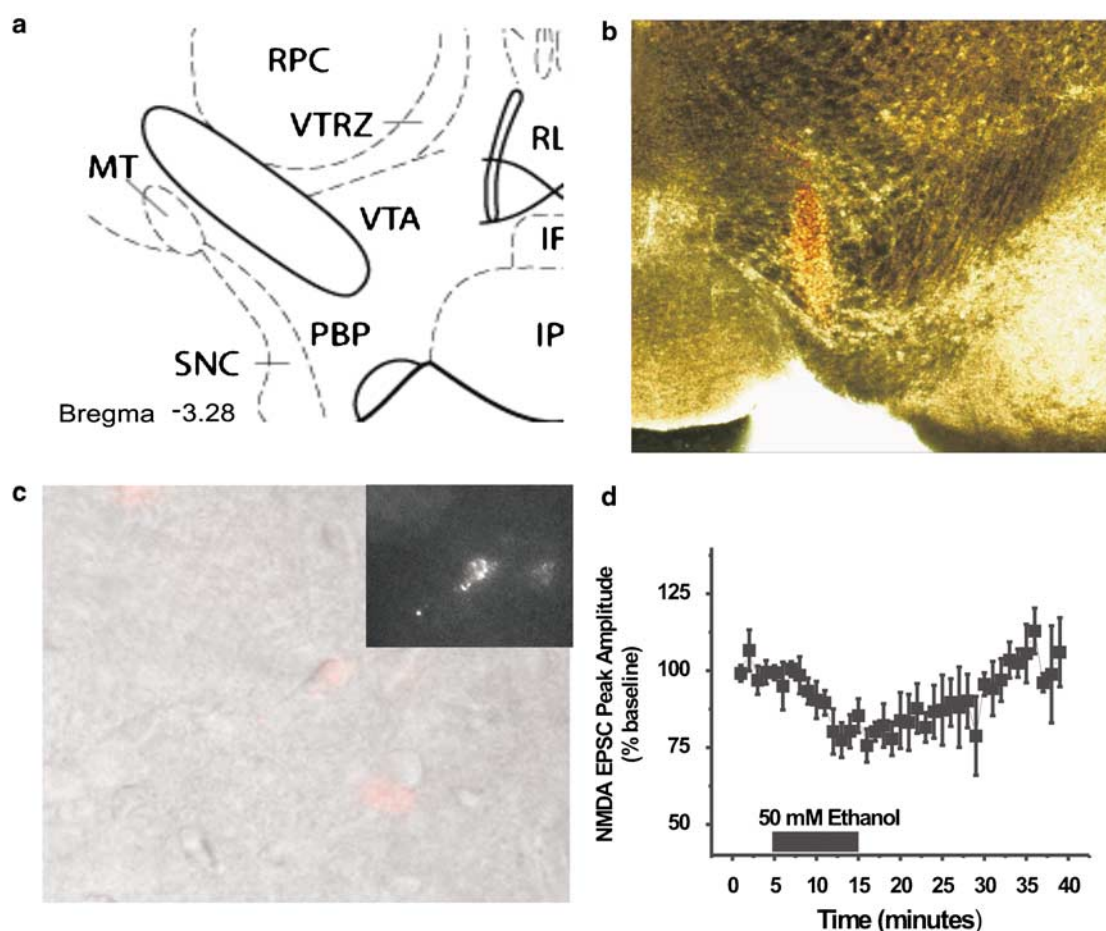


Figure 5 Ethanol inhibits NMDA-EPSCs in vBNST projection neurons. (a) Diagram adapted from mouse brain atlas showing coronal section at AP level of the VTA. (b) Bright field image of a typical VTA microsphere injection site at the same level as A. (c) Merged fluorescent and IR-DIC images of vBNST cells demonstrating the presence of labeled fluorescent microspheres following injection of microspheres into the VTA. The tracer-labeled cell in the center was patched and tested for effects of ethanol. Fluorescent channel of the tracer-filled cell (inset). (d) A total of 50 mM ethanol inhibited NMDA-EPSCs in vBNST cells that project to the VTA ($n = 7$).

in brain slices using fluorescent microscopy and examined using whole-cell voltage clamp (Figure 5c). These neurons displayed characteristic resting membrane properties similar to what had been reported previously in rat; low capacitance and high resistance (Dumont and Williams, 2004). We examined the ability of ethanol (50 mM) to inhibit NMDA-EPSCs in the presence of NBQX in both labeled cells ($n=5$) and cells that had similar membrane properties ($n=2$), and found a similar level of inhibition as seen previously in the unbiased sample of cells ($79 \pm 4\%$ of baseline peak amplitude, $n=7$, $p<0.01$, effects on area shown in Figure 5d). These data demonstrate that ethanol can inhibit NMDARs on vBNST neurons that project to the VTA.

DISCUSSION

In this study we show that acute ethanol application reduces NMDA-EPSCs in neurons of the vBNST through a postsynaptic mechanism. We also demonstrate that both NR2A and NR2B subunits contribute to NMDA-EPSCs in the vBNST. While the NR2A subunit is not required for ethanol regulation, application of an NR2B-selective antagonist removes ethanol sensitivity. Finally, we show specifically that ethanol inhibits NMDA-EPSCs in vBNST cells that project to the VTA.

Ethanol Inhibits NMDA-EPSCs in vBNST through a Postsynaptic Mechanism

As previously reported in dBNST (Weitlauf *et al*, 2004), acute ethanol application inhibits NMDA-EPSCs in the vBNST in a concentration-dependent and reversible (at low concentrations) fashion. Interestingly, the highest concentration of ethanol applied to the slices in this study, 100 mM, produced inhibition of the NMDA-EPSC that did not reverse upon removal of ethanol. It is currently unclear what this finding reflects, a lack of washout, or a form of plasticity of NMDAR. This intriguing possibility will be examined in future studies. Recent studies have suggested a presynaptic contribution to ethanol's actions on NMDA-EPSCs in some brain regions, including the ethanol-dependent rat CeA (Roberto *et al*, 2004) as well as the ethanol naive rat CeA (Zhu *et al*, 2007). In the vBNST, we found that ethanol did not inhibit AMPA-EPSCs, yet inhibits currents elicited by exogenous application of NMDA; suggesting that ethanol acts solely via a postsynaptic mechanism to inhibit NMDA-EPSCs. One potential caveat to the exogenously applied NMDA experiments is that NMDA applied to the slice could activate presynaptic NMDARs leading to alterations in neurotransmitter and neuromodulator release. The differences between the present results and the studies discussed above could be due to species differences or subtle variants in procedure. However, another more interesting possibility is that they could be due to fundamental region-specific differences in ethanol regulation of glutamatergic transmission. Curiously, when examining the effect of ethanol on exogenously applied NMDA currents, we noted a trend toward an increase in the area of the NMDA-induced current following the washout of 50 mM ethanol, however this did not reach significance ($p=0.065$). Further, we did not note a trend

toward a similar rebound effect when examining the effect of 50 mM ethanol on NMDA-EPSCs.

NMDAR Composition in the vBNST

In order to determine the subunit composition of synaptic NMDARs in neurons of the vBNST, we used a combination of genetic and pharmacological approaches. The NR2B-selective antagonists, Ro 25-6981 and ifenprodil, reduced evoked NMDA-EPSCs by $\sim 50\%$, indicating the presence of NR2B subunits. In the NR2A KO mouse, the kinetics of synaptic NMDA-EPSCs in vBNST were considerably slower, suggesting the presence of NR2A-containing NMDARs in the wild-type animal, as was seen in other brain regions (Lu *et al*, 2006). These results suggest that both NR2A and NR2B subunits contribute to NMDA-EPSCs during normal synaptic stimulation at glutamate synapses to vBNST neurons in wild-type animals. These contributions could occur through the synaptic presence of diheteromeric NR1/NR2A and NR1/NR2B NMDARs, triheteromeric NR1/NR2A/NR2B NMDARs, or some combination of these. The current data set is most

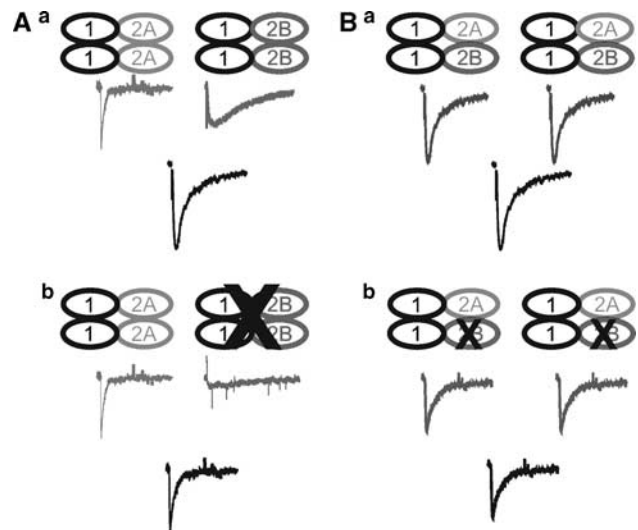


Figure 6 Model illustrating potential effects of NR2B antagonists on diheteromeric and triheteromeric NMDARs. (Aa) Schematic representation of a synapse containing a combination of diheteromeric NR2A and NR2B NMDARs. The individual kinetic profiles are shown color-coded below the receptor diagrams. The total NMDA-EPSC profile is in black. The NMDA-EPSC from the NR2A diheteromers are shown with a rapid decay, as has been noted previously. The NMDA-EPSC from the NR2B diheteromers are shown with a slower decay, as was seen in the NR2A knockout. (Ab) A schematic representation of the effects of an NR2B antagonist (denoted simply by the black X) on a mixed population of diheteromeric NMDARs. The resulting synaptic trace (in black) has a faster decay compared to the no-drug condition, as has been shown in the hippocampus in young rat hippocampus. (Ba) Schematic representation of a synapse containing triheteromeric NMDARs containing NR2A and NR2B subunits. (Bb) A schematic representation of the effects of an NR2B antagonist on triheteromeric receptors. Recent evidence (Hatton and Paoletti, 2005) demonstrated that NR2B antagonists can inhibit triheteromeric receptors, albeit with reduced efficacy, as demonstrated with the reduced amplitude of the traces as compared to those in b1. However, it is important to note the lack of alteration in the decay kinetics, reflecting the results obtained in the vBNST. Our data are most consistent with the hypothesis that the majority of NMDARs in the vBNST neurons are of this type. It is important to note that the traces shown in this figure are illustrations.

consistent with a preponderance of triheteromeric synaptic NMDARs mixed with a smaller population of NR1/NR2A and NR1/NR2B diheteromeric receptors at these synapses. Supporting this idea is the finding that while both Ro 25-6981 and ifenprodil produce a roughly 50% inhibition of the NMDA-EPSC in wild-type neurons, they do not alter the NMDA-EPSC kinetics, as was seen in the CeA (Lopez de Armentia and Sah, 2003). If the NMDA-EPSC were mediated by receptors composed of two distinct diheteromeric populations, then removal of the slower NR1/NR2B NMDAR current, using either Ro 25-6981 or ifenprodil, would result in an alteration of kinetics, as seen in the hippocampus from 2-week-old rat hippocampus (Bartlett *et al*, 2006). In support of this, recent experiments demonstrated that ifenprodil has a much reduced efficacy, but similar potency, of inhibition of triheteromeric *vs* diheteromeric NMDARs, consistent with our observed incomplete inhibition of the NMDA-EPSC by Ro 25-6981 and ifenprodil (Hatton and Paoletti, 2005). When extrapolated to a synaptic response, if the receptors were primarily triheteromeric, the application of an NR2B-selective antagonist would inhibit all receptors and thus would not selectively remove any one component, resulting in no change in kinetics (illustrated in Figure 6B). The presence of triheteromeric NMDARs is consistent with findings obtained from other brain regions using similar approaches (Luo *et al*, 1997; Tovar and Westbrook, 1999; Thomas *et al*, 2006).

NMDAR Subtype Selectivity of Ethanol Inhibition

Our data suggest that ethanol selectively inhibits NR2B-containing NMDARs in the vBNST. However, the idea that ethanol effects NMDARs in a subtype-selective fashion is not without controversy. An early study demonstrated a correlation between ethanol and ifenprodil sensitivity in cultured cortical neurons (Lovinger, 1995). However, no correlation between ifenprodil sensitivity and ethanol inhibition was noted in striatal or cerebellar cultures (Popp *et al*, 1998, 1999), suggesting region-specific selectivity of ethanol on NMDARs. This possibility is supported by findings that either NR2B, in the CeA, cortex, and the striatum (Fink and Gothert, 1996; Roberto *et al*, 2004), or NR2A, in the hippocampus (Suvana *et al*, 2005) (also see Izumi *et al*, 2005), underlie the ethanol-sensitive population of NMDARs.

In the vBNST, we found that the effects of ethanol on the NMDA-EPSC persisted in slices obtained from NR2A KO mice, indicating that the presence of NR2A subunits is not required for ethanol-inhibition of NMDARs. Interestingly, the magnitude of ethanol inhibition in the NR2A KO was similar to the wild-type animals. One potential explanation is that in the vBNST, only a single NR2B subunit is required for maximal ethanol inhibition. This would be in agreement with our findings that the NMDAR in wild-type animals are primarily triheteromeric containing a single NR2B subunit and the NR2A KO which likely contains only NR2B. However, as we did not examine a range of ethanol concentrations, we cannot rule out that NR2A subunits play a role in determining ethanol potency. We found that pretreatment with the NR2B antagonist, Ro 25-6981, prevented the inhibitory actions of ethanol. These findings suggest that ethanol-sensitivity of NMDA-EPSCs in the vBNST is governed by NR2B subunits, in general agreement with

several studies (Lovinger, 1995; Roberto *et al*, 2004; Izumi *et al*, 2005). Interestingly, we did not see any alteration in the kinetics of decay in the presence of ethanol, suggesting that ethanol is targeting triheteromeric rather than diheteromeric NR2B-containing NMDARs. We cannot rule out the possibility that diheteromeric NR2B-containing NMDARs are also present. Indeed, the maintained ethanol sensitivity we observe in the NR2A KO suggests that this is possible.

There are several mechanisms by which the NR2B antagonist could regulate ethanol sensitivity, and we cannot distinguish between them with the present data set. One explanation is that, in triheteromeric NMDARs, Ro 25-6981 can prevent further inhibition by ethanol via an allosteric interaction specific to these receptors, however due to the difficulty in isolating triheteromeric NMDARs, this has not been examined. A second possibility is that NR2B-containing receptors may maintain other NMDARs in an ethanol-sensitive state through a signaling mechanism. If this were the case, this blockade would alter the phosphorylation or protein-protein interactions of NMDARs and that would alter the ethanol sensitivity. Indeed, the ability of Ro 25-6981 to enhance NMDAR function in the hippocampus can be blocked by phosphatase inhibition (Mallon *et al*, 2005). In support of this, evidence suggests that ethanol sensitivity of NMDARs and other channels may be regulated by posttranslational modifications (Malve *et al*, 2002, but see Xu and Woodward, 2006). Finally, it is possible that the presence of NR2B specifically imparts ethanol sensitivity on synaptically activated NMDARs in the vBNST.

Implications for Ethanol's Actions in the vBNST

Whereas we provide both a potential mechanistic explanation and molecular target of the acute actions of ethanol in the vBNST, how this effect modulates behavior remains to be determined. Using a retrograde labeling technique, we found that NMDA-EPSCs in vBNST cells that project to the VTA are inhibited by ethanol. A series of elegant studies by Georges and Aston-Jones (2001, 2002) examined the role of the vBNST on VTA dopaminergic cell activity. They found evidence for three forms of modulation: short-latency excitation, short-latency inhibition, and a long-latency excitation likely due to a polysynaptic pathway. Interestingly, they found that antagonism of NMDARs abolished the short-latency excitation and attenuated the long-latency excitation, but had no effect on the short-latency inhibition. This suggests that NMDARs in the vBNST are key regulators of excitability of VTA neurons, both for direct and indirect excitatory projections. The ability of ethanol to inhibit NMDAR function in the vBNST suggests that ethanol can alter the ability of this region to regulate dopaminergic cells in the VTA. In support of this possibility, a recent study in rat found that inhibition of GABA_A receptors in the BNST can attenuate the ability of a D2 antagonist applied locally in the VTA to impair ethanol self-administration (Eiler and June, 2007). We suggest that this is evidence for a long feedback loop from VTA target regions (such as the BNST) back to the VTA that can regulate dopamine release. One interesting possibility is that the effect of ethanol in the vBNST could be a mechanistic component of this proposed long feedback loop. Another distinct possibility is that ethanol is blocking all forms of NMDAR-dependent plasti-

city, long-term potentiation, as well as long-term depression, which could result in a net increase in glutamatergic transmission to the VTA. In addition to the effects on the mesolimbic pathway, several studies have suggested that the vBNST can also regulate recruitment of the paraventricular nucleus of the hypothalamus during acute stress (Crane *et al*, 2003; Spencer *et al*, 2005), suggesting that ethanol in the vBNST may contribute to the effects of ethanol on anxiety.

In summary, we provide evidence that ethanol inhibits NMDARs postsynaptically in an NR2B subunit-controlled fashion in an important anatomic target for ethanol, the vBNST. Further, we directly demonstrate that ethanol can impair NMDAR function in cells that project to the VTA, possibly altering signaling in an important regulatory feedback loop.

ACKNOWLEDGEMENTS

This work was funded by INIA-STRESS (NIAAA) and an NRSA F32 award (NIAAA). We thank Dr Roger Colbran for critical comments on a previous version of this manuscript.

DISCLOSURE/CONFLICT OF INTEREST

Dr Kash declares that, except for income received from primary employer, no financial support or compensation has been received from any individual or corporate entity over the past 3 years for research or professional service. Dr Matthews received compensation from Meharry Medical College. Dr Winder received a distribution from Columbia University for the licensing of transgenic mouse technology to Memory Pharmaceuticals and received a consultancy fee from MEDAcrop. All of the authors declare that there are no personal financial holdings that could be perceived as constituting a potential conflict of interest.

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