High-Frequency Stimulation Induces Ethanol-Sensitive Long-Term Potentiation at Glutamatergic Synapses in the Dorsolateral Bed Nucleus of the Stria Terminalis

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Anatomical and functional data support a critical role for the bed nucleus of the stria terminalis (BNST) in the interaction between stress and alcohol/substance abuse. We report here that neurons of the dorsal anterolateral BNST respond to glutamatergic synaptic input in a synchronized way, such that an interpretable extracellular synaptic field potential can be readily measured. High-frequency stimulation of these glutamatergic inputs evoked NMDA receptor (NMDAR)-dependent long-term potentiation (LTP). We found that an early portion of this LTP is reduced by acute exposure to ethanol in a GABA_A receptor-dependent manner. This effect of ethanol is accompanied by a significant and reversible dose-dependent attenuation of isolated NMDAR signaling and is mimicked by incomplete NMDAR blockade.

Key words: alcohol; alcoholism; synaptic plasticity; stress; anxiety; addiction

Introduction

Understanding the neural mechanisms underlying anxiety and its relationship with responses to alcohol and drugs of abuse is critical to the development of effective treatments of both anxiety disorders and stress-induced relapse to alcohol and drug intake. A component of the “extended amygdala,” the bed nucleus of the stria terminalis (BNST) receives stress-related information from a variety of brain centers and sends output to both stress and reward pathways. Consistent with this anatomy, the BNST plays a critical role in anxiety and fear (Fendt et al., 2003; Walker et al., 2003; Dong and Swanson, 2004), as well as the interaction between stress and substance abuse (Aston-Jones et al., 1999; Erb and Stewart, 1999).

Information concerning “processive” stressors enters the BNST from the ventral subiculum, limbic cortex, and basolateral amygdala in the form of primarily glutamatergic afferents (Walker et al., 2003). Indeed, stimulation of these afferents in an *in vitro* slice preparation evokes short latency EPSPs on neurons within the BNST that contain both AMPA receptor (AMPA)- and NMDA receptor (NMDAR)-dependent components (Egli and Winder, 2003). Blockade of glutamatergic transmission in the BNST disrupts augmentation of fear-potentiated startle, suggesting that these synapses may play a role in the progression

from acute stress responses to anxiety (Walker and Davis, 1997). A growing literature suggests that the BNST plays a critical and specific role in many lasting stress/anxiety responses to nonspecific and unconditioned stressors (Fendt et al., 2003; Walker et al., 2003).

Synaptic plasticity at glutamatergic synapses in specific brain regions has been suggested to play an important role in the neuroadaptations that occur after substance abuse (Hyman and Malenka, 2001) and persistent anxiety (Sapolsky, 2003). Moreover, much literature suggests that stressors and drugs of abuse can regulate synaptic plasticity at glutamatergic synapses that support it (Blitzer et al., 1990; Winder et al., 2002; Maroun and Richter-Levin, 2003). Thus, synaptic plasticity at synapses within the BNST could play a key role in the development of anxiety and stress-induced reinstatement of substance of abuse intake. Indeed, the BNST is a target for both the acute as well as the long-term effects of ethanol exposure on the brain (Chang et al., 1995; Carboni et al., 2000; Le et al., 2000; Olive et al., 2002).

Currently, little is known of the synaptic and excitable properties of neurons of the BNST (Sawada et al., 1980; Sawada and Yamamoto, 1981; Rainnie, 1999; Egli and Winder, 2003), nor of the effects of ethanol on these properties. Here, we demonstrate in acutely prepared brain slices of adult mouse BNST that neurons within this region respond in a coordinated manner to afferent glutamatergic input, and that these inputs are capable of undergoing dependent long-term potentiation (LTP). We found that this potentiation is NMDAR dependent and L-type voltage-gated calcium channel (VGCC) independent. Furthermore, we found that an early component of this LTP is attenuated by acute application of ethanol in a GABA_A receptor (GABA_AR)-dependent manner. This effect of ethanol is accompanied by a modest attenuation of NMDAR signaling while having little direct impact on synaptic GABA_AR signaling.

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Materials and Methods

Animals

All animals were housed in groups of two to five. Food and water were available *ad libitum*. All procedures were approved by the Animal Care and Use Committee at Vanderbilt University. Mice were housed individually for 1 hr in a quiet room before being killed.

Slice preparation

Male C57BL/6J mice (6–8 wk of age; Jackson Laboratories, Bar Harbor, ME) were decapitated under isoflurane. For field potential and sharp electrode recordings, the brains were removed quickly and placed in ice-cold artificial CSF (ACSF) (in mM: 124 NaCl, 4.4 KCl, 2 CaCl₂, 1.2 MgSO₄, 1 NaH₂PO₄, 10 glucose, and 26 NaHCO₃, pH 7.2–7.4; 290–310 mOsm). Hemisected coronal slices (300 μ m) were prepared with a Vibratome (Pelco). For whole-cell patch-clamp recordings, brains were placed in ice-cold low-sodium ACSF (in mM: 194 sucrose, 30 NaCl, 4.5 KCl, 1.2 MgCl₂, 1.2 NaH₂PO₄, 10 glucose, and 26 NaHCO₃, pH 7.2–7.4; 290–310 mOsm), and slices were prepared with a vibroslicer (Leica, Nussloch, Germany). Slices containing anterior portions of the dorsal anterolateral BNST (dlBNST; bregma, 0.26–0.02 mm) (Franklin and Paxinos, 1997) were selected using the internal capsule, anterior commissure, and stria terminalis as landmarks (Fig. 1A).

Electrophysiology

Field potential recordings. After dissection, slices were transferred to an interface recording chamber where they were perfused with heated ($\sim 29^{\circ}\text{C}$), oxygenated (95% O₂–5% CO₂) ACSF at a rate of ~ 2 ml/min. Slices were allowed to equilibrate in ACSF for at least 1.5 hr before experiments began. A bipolar stainless steel stimulating electrode and a borosilicate glass recording electrode filled with ACSF were placed in the dlBNST to elicit and record an extracellular field response. Baseline responses to a stimulus (50 μ sec) at an intensity that produced $\sim 40\%$ of the maximum response were recorded for no more than 20 min at a rate of 0.05 Hz. Drugs were bath applied, where indicated. To elicit LTP, two trains of 100 Hz, 1 sec tetanus were delivered with a 20 sec intertrain interval at the same intensity as baseline test pulses. Experiments in which the N1 (Fig. 1B) changed by $>20\%$ were discarded. If the N1 could not be accurately estimated, input–output curves taken at the beginning and end of experiments were used to determine whether the N1 at higher stimulation intensities changed. Some experiments were performed in picrotoxin (25 μ M), where indicated.

To isolate NMDA field potential responses, normal ACSF was switched with a modified low magnesium 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 mOsm) containing CNQX (50 μ M) and picrotoxin (25 μ M) midway through the 1.5 hr incubation period. Responses were elicited at a rate of 0.0167 Hz with an intensity of 10–20 V and duration of 100–200 μ sec.

Intracellular sharp electrode recordings. Sharp electrode recordings were as described (Egli and Winder, 2003). Briefly, slices were transferred to an interface recording chamber. Recording electrodes were pulled on a Flaming–Brown micropipette puller (Sutter Instruments, Novato, CA) using thick-walled filament-containing borosilicate glass capillaries. Electrodes were filled with 2 M potassium acetate and were of ~ 120 – 170 M Ω resistance. An input–output curve of synaptic responses was generated by stimulating with a range of input voltages with stimulus durations from 50 to 100 μ s, starting at 3 V and increasing the stimulus in 2 V increments until the cell fired an action potential. If the cell did not fire,

Figure 1. LTP of AMPAR-mediated signaling in the dlBNST. *A*, Schematic of hemisected coronal slice for electrophysiology. IC, Internal capsule; AC, anterior commissure; Ctx, cortex; Str, striatum. *B*, Sharp intracellular LTP in the dlBNST. Inset, Representative traces before and 25 min after tetanus. Calibration: 10 msec, 2 mV. *C*, Attenuation of the N2 portion of the dlBNST field potential with CNQX (10 μ M) and N1 with TTX (1 μ M). Calibration: 5 msec, 0.5 mV. *D*, Extracellular field LTP in the dlBNST. Inset, Representative traces before and 1 hr after tetanus. Calibration: 5 msec, 0.5 mV. $\blacktriangle$, Time of tetanus [two trains (20 sec interstimulus interval); 100 Hz, 1 sec].

the maximum stimulus intensity administered was 35 V. To elicit LTP, two trains of 100 Hz, 1 sec tetanus were delivered with a 20 sec intertrain interval at the same intensity as baseline test pulses. Input resistance was monitored throughout the experiment.

Patch-clamp recordings. Slices were placed in a submerged chamber (Warner Instruments, Hamden, CT), and neurons of the dlBNST were directly visualized with an infrared-differential interference contrast video microscope (Olympus, New Hyde Park, NY). Recording electrodes (3–6 M Ω) were pulled on a Flaming–Brown micropipette puller (Sutter Instruments) using thin-walled borosilicate glass capillaries. For analysis of IPSCs, electrodes were filled with (in mM) 135 K⁺ gluconate, 5 NaCl, 10 HEPES, 0.6 EGTA, 4 ATP, 0.4 GTP, and 0.1% biocytin. For analysis of NMDAR-mediated EPSCs, electrodes were filled with (in mM) 117 Cs²⁺ gluconate, 20 HEPES, 0.4 EGTA, 5 TEA, 2 MgCl₂, 4 ATP, and 0.3 GTP, pH 7.2 (286 mOsm). Signals were acquired via a Multiclamp or Axopatch-1d amplifier (Axon Instruments, Foster City, CA), digitized, and analyzed via pClamp 9.0 software (Axon Instruments). Input resistance (140–800 M Ω) and series resistance (20–40 M Ω) were monitored continuously during the experiment.

Pharmacology

Picrotoxin, CNQX, TTX, and DL-AP-5 were purchased from Sigma-Aldrich (St. Louis, MO). Nimodipine was purchased from Tocris (Ellisville, MO). Ethanol (95%) was purchased from Aaper Alcohol and Chemical (Shelbyville, KY). DMSO (0.05%) was used as a vehicle for picrotoxin. We alternated between control and treatment experiments reported in each figure to account for potential day-to-day as well as time-of-day differences.

Results

Characterization of dlBNST field potentials

Local stimulation in the dlBNST elicits an extracellular field response composed of a stimulus artifact and two prominent neg-

Figure 2. LTP in the dBNST is NMDAR dependent. *A*, Blockade of dBNST LTP with 20 min pretreatment with DL-AP-5 (100 μ M) compared with interleaved controls. *B*, No effect of Nimodipine (10 μ M) on dBNST LTP compared with interleaved controls. $\blacktriangle$, Time of tetanus [two trains (20 sec interstimulus interval); 100 Hz, 1 sec].

active deflections (Sawada et al., 1980); the first we refer to as N1 and the second as N2. The N2, but not the N1, portion of the trace is eliminated by CNQX (10 μ M) (Fig. 1C), suggesting that this portion of the response is primarily AMPAR mediated. The subsequent addition of TTX (1 μ M) (Fig. 1C) eliminates the remaining N1, suggesting that this portion of the response is a measure of sodium channel-dependent firing reminiscent of hippocampal fiber volleys and striatal N1 potentials recorded under similar conditions. The addition of TTX without CNQX eliminates both the N2 and N1 (data not shown). Thus, this dBNST field potential resembles that observed in striatal structures.

LTP in the dBNST

To begin to determine whether glutamatergic afferents into the dBNST support synaptic plasticity, we stimulated these afferents with a moderate intensity tetanus protocol (two 100 Hz, 1 sec trains with a 20 sec intertrain interval). This protocol elicited significant LTP of the N2 ($121.4 \pm 3.5\%$; $n = 22$; $F = 36.291$; $p < 0.001$) but was without effect on the N1 ($92.5 \pm 3.3\%$; $n = 20$; $F = 2.827$; $p = 0.11$), lasting 60 min after tetanus (Fig. 1D). To verify that this LTP represents enhancement of the EPSP, we also examined the effect of this tetanus protocol on the slope of EPSPs from single dBNST neurons using sharp microelectrode recordings. Similar to field recordings, we observed a significant potentiation lasting beyond 25 min post-tetanus ($132.5 \pm 10.8\%$; $n = 5$; $F = 9.404$; $p < 0.05$) (Fig. 1B).

Induction signals for dBNST LTP

To begin to determine the mechanisms underlying induction of LTP in the dBNST, we added the NMDAR antagonist DL-AP-5 (100 μ M) 20 min before tetanization. Although having no effect on the basal synaptic response, DL-AP-5 greatly reduced the potentiation seen after tetanus [$104.98 \pm 3.5\%$ ($n = 20$) vs $125.02 \pm 8.3\%$ ($n = 11$); $F = 6.829$; $p < 0.05$] (Fig. 2A). The same degree of inhibition was observed regardless of whether experiments were done in the presence or absence of picrotoxin (25 μ M; data not shown), so experiments were pooled. Because of the known role of L-type VGCCs in LTP and long-term memory in the lateral amygdala (Bauer et al., 2002), we tested the importance of these channels in BNST LTP. The L-type VGCC blocker nimodipine (10 μ M) applied 1 hr before tetanus had no significant effect on the amount of LTP compared with controls ($122.1 \pm 6.7\%$; $n = 10$; $F = 17.340$; $p < 0.005$) (Fig. 2B).

Acute effect of alcohol on an early component of dBNST LTP

Because ethanol suppresses the induction of LTP in some brain regions, and because of the potential role of dBNST glutamatergic transmission in behaviors mediated by ethanol, we examined the effects of ethanol on dBNST LTP. Adding ethanol (100 mM) to the ACSF had no significant effect on basal transmission within 20 min of application [$95.7 \pm 3.6\%$ ($n = 9$) vs $99.2 \pm 0.7\%$ ($n = 9$); $F = 1.258$; $p = 0.29$] (Fig. 3A). Ethanol did, however, attenuate an early component of dBNST LTP [0–5 min post-tetanus: $133.1 \pm 7.2\%$ ($n = 9$) vs $160.6 \pm 8.6\%$ ($n = 10$); $F = 5.905$; $p < 0.05$] (Figs. 3A, C, 4A). A similar time course of inhibition was observed in a separate set of studies when ethanol was applied for the duration of the experiment,

suggesting that ethanol specifically regulates an early component of LTP [0–5 min post-tetanus: $133.8 \pm 1.9\%$ ($n = 6$) vs $164.3 \pm 9.5\%$ ($n = 7$); $F = 8.405$; $p < 0.05$] (Figs. 3B, C, 4A). Once again, ethanol did not have a significant effect on baseline responses in this set of experiments [$94.9 \pm 2.3\%$ ($n = 6$) vs $100.3 \pm 0.6\%$ ($n = 6$); $F = 4.986$; $p = 0.08$]. To test for the effects of ethanol specifically on LTP maintenance, rather than induction, we added ethanol (100 mM) to the ACSF 2 min before tetanus so that ethanol would reach the chamber immediately (<1 min) after tetanus. Unlike experiments with a 20 min pre-application of the same dose, we saw no significant effect on LTP compared with interleaved controls [0–5 min: $165.61 \pm 11.72\%$ ($n = 6$) vs $161.97 \pm 17.49\%$ ($n = 5$); $F = 0.041$; $p = 0.85$] (Figs. 3D, 4A), suggesting that the effect of ethanol on the early component of LTP was attributable to attenuation of induction and not maintenance. Further consistent with this idea, whereas 20 min preincubation of slices with DL-AP-5 reduced both early and late components of LTP, 10 min preincubation incompletely blocked NMDAR function (data not shown) and selectively attenuated the early component in a manner very similar to ethanol [0–5 min post-tetanus: $133.1 \pm 7.2\%$ ($n = 9$) vs $144.6 \pm 10.1\%$ ($n = 7$); $F = 0.768$; $p = 0.40$] (Figs. 3E, 4A). These data suggest that NMDAR activation produces two distinct LTP components in the dBNST and ethanol selectively regulates the earlier component.

Acute effect of alcohol on GABA_AR signaling in the dBNST

Curiously, we observed no effect of ethanol on LTP when experiments were performed in the presence of picrotoxin [25 μ M; 0–5 min post-tetanus: $163.2 \pm 13.4\%$ ($n = 9$) vs $168.3 \pm 13.9\%$ ($n = 11$); $F = 0.067$; $p = 0.80$] (Figs. 3F, 4A), suggesting that intact GABAergic inhibition is necessary for ethanol regulation of LTP in the dBNST. The regulation occurs despite the fact that LTP magnitude during this time period was unaffected by the inclusion of picrotoxin in the ACSF ($F = 0.212$; $p = 0.65$) (Fig. 4A). Also interesting to note is that, in the presence of picrotoxin, ethanol caused a significant attenuation of baseline responses [$93.3 \pm 2.5\%$ ($n = 9$) vs $100.4 \pm 0.9\%$ ($n = 9$); $F = 6.549$; $p < 0.05$].

That ethanol suppression of LTP was only observed in the presence of intact GABAergic transmission suggests the possibility that modulation of GABAergic function could play a role. Indeed, the GABA_AR is a major target of ethanol, and IPSPs are

enhanced by ethanol in other brain regions, including the central nucleus of the amygdala (Roberto et al., 2003). The dlBNST field response has an additional component that consists of an upward deflection following the rising portion of the N2 that is not observed in the presence of picrotoxin and is acutely inhibited by bicuculline (20 μ M) (Fig. 5A, inset). This upward deflection is also abolished (along with the N2) in the presence of CNQX (10 μ M; data not shown), suggesting that this portion of the trace is driven by glutamatergic signaling. For this reason, this upward deflection was taken as an estimate of GABAergic signaling by local circuit interneurons. To test the hypothesis that ethanol-induced attenuation of NMDAR-dependent plasticity was occurring indirectly through modulation of GABA_ARs, the size of the upward deflection was analyzed during the 20 min of ethanol exposure before tetanus. However, this portion of the trace was unaffected by acute ethanol exposure ($103.7 \pm 4.1\%$; $n = 11$; $F = 0.003$; $p = 0.96$) (Fig. 5A).

We also obtained whole-cell patch-clamp records from dlBNST neurons to more directly measure the impact of ethanol exposure on GABA_AR signaling. To isolate GABA IPSCs, we included CNQX (10 μ M) and DL-AP-5 (100 μ M) in the ACSF to block AMPAR and NMDAR currents, respectively. Bicuculline-sensitive IPSCs (Fig. 5B, left inset) with a reversal potential of ~ -60 mV were readily elicited in these cells. As shown in Figure 5B, ethanol administration had no effect on the peak amplitude of the IPSC ($93.83 \pm 12.78\%$; $n = 7$; $F = 0.045$; $p = 0.84$).

Previous studies have suggested that neurons in the dlBNST may be under tonic inhibition by extrasynaptic GABA_ARs (Egli and Winder, 2003), and a recent study has suggested that, depending on subunit composition, extrasynaptic receptors may be more ethanol sensitive than synaptic receptors (Waller et al., 2003). However, we did not observe a consistent effect of ethanol on input resistance ($n = 6$; $F = 0.418$; $p = 0.55$) or holding current ($F = 0.145$; $N = 7$; $p = 0.72$) in our IPSC analysis (data not shown), suggesting this is unlikely to play a major role in the LTP modulation we observe.

Acute effect of alcohol on NMDAR signaling in the dlBNST

The NMDAR is another major CNS target of ethanol responsible for many ethanol-driven behaviors (Lovinger et al., 1989). Knowing that acute ethanol application attenuates NMDAR-dependent LTP in the dlBNST, we hypothesized that this effect was through the inhibition of NMDAR-mediated currents. To test this, we isolated an NMDAR-mediated dlBNST field response through the use of non-Mg²⁺-containing ACSF in the presence of picrotoxin (25 μ M) and CNQX (50 μ M) to block GABA_AR-mediated and AMPAR-mediated signaling, respec-

Figure 3. Ethanol attenuates early LTP in the dlBNST. *A*, Attenuation of an early phase of dlBNST LTP by a 20 min pre-application of ethanol (100 mM). *B*, Prolonged application of ethanol (100 mM) still shows lack of effect on the late phase of dlBNST LTP. *C*, Dataset from *A* and *B* with an expanded x-axis to emphasize the effect on early LTP. *D*, Comparison of LTP attenuation by acute application of ethanol (100 mM) and 10 min application of DL-AP-5 (100 μ M). *E*, No effect on LTP is seen when ethanol (100 mM) is added after tetanus. *F*, Block of ethanol (100 mM) attenuation by picrotoxin (25 μ M). $\blacktriangle$, Time of tetanus [two trains (20 sec interstimulus interval); 100 Hz, 1 sec].

tively. Under these conditions, an N1 spike was followed by an N2 spike and a more slowly developing and decaying potential. Both of these components of the N2 were virtually abolished by bath application of DL-AP-5 (200 μ M) (Fig. 6A, inset). We found that bath application of ethanol produced a dose-dependent, reversible depression of the N2 portion of this NMDAR-mediated field potential when comparing the average of the last 10 min of ethanol application to the average of the 5 min preceding ethanol application [10 mM: $98.1 \pm 1.3\%$ vs $100.9 \pm 0.7\%$ ($n = 6$), $p = 0.14$; 50 mM: $91.1 \pm 2.7\%$ vs $100.9 \pm 0.8\%$ ($N = 6$), $p < 0.05$; 100 mM: $83.0 \pm 2.9\%$ vs $101.2 \pm 0.9\%$ ($n = 7$), $p < 0.005$; 200 mM: 69.4 ± 5.6 vs 102.7 ± 1.0 ($n = 5$), $p < 0.01$] (Fig. 6A), suggesting that inhibition of the early component of LTP by ethanol may be in part through depression of NMDAR function.

To further test the hypothesis that ethanol regulates NMDAR function in the dlBNST, we used whole-cell patch clamping in BNST neurons. We isolated NMDAR currents by including

Figure 4. Quantification of effects on dIBNST LTP. Quantification of potentiation 0–5 min (*A*) and 55–60 min (*B*) after tetanus for each condition of ethanol and DL-AP-5 application. * $p < 0.05$; + $p < 0.01$.

Figure 5. Ethanol does not regulate LTP via modulation of synaptic GABA_AR signaling in the dIBNST. *A*, A GABA_AR-dependent upward deflection in the dIBNST field potential is not affected by acute ethanol application. Inset, Representative traces before and after bicuculline (20 μ M) application. Calibration: 5 msec, 0.2 mV. *B*, Acute ethanol application (100 μ M) has no effect on the amplitude of GABAergic IPSCs in the dIBNST. Left inset, Representative traces (dual stimulus) before and after bicuculline (30 μ M) application. Calibration: 50 msec, 50 pA. Right inset, Representative traces (dual stimulus) before and after ethanol (100 mM) application. Calibration: 50 msec, 50 pA. The average holding current was -39 ± 8 pA.

CNQX (10 μ M) and picrotoxin (25 μ M) in normal ACSF to block AMPAR- and GABA_AR-mediated currents, respectively, and by voltage clamping the cell at +40 mV to relieve Mg²⁺ blockade of the receptor. The resulting EPSC had a slow decay time and was abolished by 100 μ M DL-AP-5, consistent with mediation by NMDARs (Fig. 6*B*, inset). We found that NMDA EPSCs re-

coded in this manner were relatively stable over a 30 min time period and that they were reduced in amplitude 15–20 min after bath application of ethanol [100 mM, 10 min; $64.2 \pm 5.7\%$ ($n = 13$) vs $90.6 \pm 6.8\%$ ($n = 7$); $F = 8.206$; $p < 0.05$] (Fig. 6*B*).

Discussion

A growing literature suggests that the BNST plays a critical role in the development of anxiety and in stress/anxiety and alcohol/substance abuse interactions. Here, we show that neurons within this structure respond in a concerted manner to afferent inputs such that an interpretable extracellular waveform is readily obtained *in vitro*. In addition, using high-frequency stimulation, we demonstrate that LTP of glutamatergic input to the dIBNST is readily elicited and that this LTP is NMDAR dependent and L-type VGCC independent. Furthermore, we find this plasticity to be modulated by ethanol in a manner that depends on intact GABAergic inhibition. However, acute ethanol reduces evoked synaptic NMDAR function but not GABA_AR function in the BNST.

Potential impact of LTP at dIBNST synapses on behavior

Previous studies have shown that *in vivo* administration of a number of drugs of abuse increases the AMPA/NMDA ratio at synapses onto dopaminergic neurons in the ventral tegmental area (VTA), an effect that is mimicked by cold stress (Saal et al., 2003). Because the BNST provides excitatory input to the VTA (Georges and Aston-Jones, 2001, 2002), the LTP we describe here could result in increased excitatory transmission along this pathway. That we see modulation of this plasticity by ethanol further implicates this synaptic enhancement as a potential connection between stress and reward pathways. Long-term plasticity in the dIBNST could also serve as a mechanism for the modification of anxiety pathways in much the same way as plasticity in the amygdala (Bauer et al., 2002). For example, plasticity of glutamatergic signaling could serve as a potential mechanism underlying the role of the dIBNST in augmentation of fear-potentiated startle by diffuse, longer duration stimuli.

Ethanol attenuation of an early component of LTP and NMDAR signaling in the dIBNST

Systemic ethanol exposure activates BNST neurons (Chang et al., 1995; Carboni et al., 2000), whereas withdrawal from chronic ethanol exposure increases levels of CRF in the BNST, with subsequent exposure to ethanol reducing this elevation (Olive et al.,

2002). Systemic injection of CRF antagonists has been shown to block stress-induced relapse to ethanol seeking (Le et al., 2000). Because neurons of the BNST are targeted by ethanol, we hypothesized that exposure of the BNST to ethanol would result in the modulation of transmission and plasticity at these synapses. Indeed, we found that acute ethanol application attenuates an early portion of dIBNST LTP. In the hippocampus, reduction of LTP by acute ethanol exposure is also seen (Sinclair and Lo, 1986; Blitzer et al., 1990). A curious difference, however, is that ethanol more predominantly regulates an early component of LTP in the dIBNST. This finding, coupled with the results of the brief DL-AP-5 application experiments, suggest that two distinct NMDAR-dependent components comprise dIBNST LTP. Moreover, these data demonstrate an interesting property of NMDAR-dependent LTP in this region. In other brain regions, reduction of NMDAR function is associated with either parallel decreases in early and late components of LTP or more profound inhibition of later phases of LTP. Here, we find the opposite: modest NMDAR inhibition preferentially removes an early component. In future studies, it will be important to determine the mechanisms underlying these two components.

The effects of ethanol on the hippocampus are thought to be mediated predominantly via ethanol-induced attenuation of NMDAR signaling (Lovinger et al., 1989; Pyapali et al., 1999). Here, we report ethanol attenuation of NMDAR signaling in the dIBNST, both in isolated NMDAR-mediated fields as well as whole-cell NMDA currents. Although the attenuation is clearly reversible in extracellular recordings, we did not observe consistent reversal in whole-cell recordings. Incomplete reversal of the effects of ethanol on NMDAR-mediated currents has been reported previously (Ariwodola et al., 2003; Roberto et al., 2004) and likely reflects the washout of vital factors required for recovery from the cell via the patch electrode. Consistent with attenuation of NMDAR signaling by ethanol, Western blotting analysis from dBNST punches indicates the presence of two NR2 subunits (NR2A and NR2B; C. Weitlauf and D. G. Winder, unpublished observations) thought to be regulated by ethanol.

Ethanol and GABAergic transmission in the dIBNST

Surprisingly, we did not observe modulation of GABAergic IPSCs in individual dIBNST cells in response to acute ethanol exposure, nor did we observe enhancement of an indirect, extracellularly recorded measure of GABA_AR function. It is still possible, however, that under specific contexts, ethanol could regulate GABA_AR function in the dIBNST. For example, analysis of spontaneous rather than evoked IPSPs could reveal a separate, ethanol-sensitive population of GABAergic neurons in the BNST. It is also important to note that, in some reports, ethanol enhances GABA_ARs only in the presence of GABA_BR antagonists (Wan et al., 1996). However, because such antagonism was not required for the observed ethanol effects on LTP, it does not likely underlie the actions of ethanol reported here. Finally, it should also be noted that although picrotoxin completely blocked the IPSP (Egli and Winder, 2003), bicuculline only partially blocked

Figure 6. Ethanol attenuates NMDAR signaling in the dIBNST. *A*, Attenuation of the NMDA field response after acute application of ethanol (100 mM). Inset, Representative traces before and after DL-AP-5 application. Calibration: 25 msec, 0.5 mV. *B*, Attenuation of isolated NMDA EPSCs by acute application of ethanol (100 μ M). Inset, Representative traces before and after ethanol or DL-AP-5 application. Calibration: 200 msec, 50 pA.

the IPSC (Fig. 5*B*, left inset), suggesting that additional receptors such as the GABA_C or glycine receptors may contribute to fast inhibitory transmission here.

Why, then, does ethanol regulation of LTP require intact GABAergic inhibition, whereas ethanol appears to directly regulate NMDAR rather than GABA_AR function? The most parsimonious interpretation is that in the presence of GABA_AR antagonists, supersaturating levels of NMDAR activation occur during the tetanus, such that the modest inhibition of NMDAR function seen by ethanol application is insufficient to regulate LTP induction. In contrast, in the absence of GABA_AR antagonists, IPSPs generated via high-frequency stimulation likely dampen depolarization-induced recruitment of NMDAR activation to a level closer to subsaturation. In this context, ethanol-induced attenuation of NMDAR function can now decrease NMDAR recruitment to below saturation and reduction of an early component of LTP is observed. Consistent with this model, we found that brief (10 min) preincubation of slices with DL-AP-5, which produces incomplete antagonism of NMDAR under our conditions, produced an inhibition of the early component of LTP with a time course virtually identical to that elicited by ethanol. Furthermore, previous studies have provided evidence that ethanol can regulate NMDAR function in a GABA_AR-dependent manner in the hippocampus (Schummers and Browning, 2001).

It should be noted, however, that the NMDAR is but one identified target of ethanol in the dIBNST. In future studies, it will be important to determine whether other targets are also regulated by ethanol in the dIBNST. Furthermore, it will be critical to determine not only the acute effects of ethanol within this region but also the effects of more chronic, behaviorally relevant ethanol exposure. Nonetheless, our data presented here suggest that ethanol at a concentration achieved during periods of heavy inebriation is capable of regulating both NMDAR-dependent synaptic plasticity and NMDAR signaling.

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