Ethanol Potentiates GABAergic Synaptic Transmission in a Postsynaptic Neuron/Synaptic Bouton Preparation From Basolateral Amygdala

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Zhu, Ping Jun and David M. Lovinger. Ethanol potentiates GABAergic synaptic transmission in a postsynaptic neuron/synaptic bouton preparation from basolateral amygdala. J Neurophysiol 96: 433–441, 2006. First published April 19, 2006; doi:10.1152/jn.01380.2005. Interactions between ethanol and synaptic transmission mediated by $\gamma$-amino-$N$-butyric acid (GABA) have been suggested to contribute to alcohol intoxication. Ethanol effects on postsynaptic GABA$_A$ receptors have been the major focus of this line of research. There is increasing evidence that ethanol potentiation of GABAergic transmission involves increased GABA release from presynaptic terminals. In the present study, a mechanically isolated neuron/bouton preparation from the basolateral amygdala was used to examine the effects of ethanol on spontaneous GABAergic synaptic currents elicited by GABA release from the presynaptic terminals. We found that ethanol application produced a rapid increase in the frequency of spontaneous GABAergic synaptic currents. An acute tolerance to ethanol was also observed, and this tolerance involved GABA$_B$ receptor activation. The ethanol-induced potentiation did not involve alterations in the function of postsynaptic GABA$_A$ receptors and was independent of presynaptic action potential firing. These findings indicate that ethanol potentiates GABA release, most likely via a direct action on presynaptic boutons.

INTRODUCTION

Alcohol produces acute intoxication through actions on the CNS at ethanol concentration in the millimolar range. Mild to moderate intoxication involves neurobehavioral changes including euphoria, reduction in anxiety as well as cognitive and motor impairment (Katzung 2001). Many cellular and molecular targets for ethanol actions in the CNS have been characterized. Prominent among these are interactions between ethanol and synaptic transmission mediated by $\gamma$-amino-$N$-butyric acid (GABA), the major inhibitory neurotransmitter in brain (Krnievic 2004; Thompson 1994). Indeed, there is strong evidence that interactions between ethanol and GABAergic synaptic transmission contribute to intoxication (Grobin et al. 1998; Mihic 1999). The major focus of past research has been on the actions of ethanol at the GABA$_A$-type receptors, and there is good evidence that these receptors play a role in intoxication.

Recently, evidence that ethanol potentiates presynaptic GABA release has begun to emerge (Nie et al. 2004; Roberto et al. 2003). Ethanol-induced increases in GABAergic transmission have been observed in brain slices of rodent amygdala, cerebellum, hippocampus, and nucleus accumbens (Ariwodola and Weiner 2004; Carta et al. 2004; Nie et al. 2000). Analysis of changes in transmission during alcohol exposure supports the idea that potentiation by ethanol involves increased GABA release (Ariwodola and Weiner 2004; Roberto et al. 2004). However, it is not yet clear if alcohol acts directly on presynaptic terminals or produces potentiation via a less direct mechanism (e.g., altering presynaptic excitability or releasing a neuromodulator from adjacent cells that then acts on the terminal). For instance, studies in the central amygdala indicate that the ethanol-mediated potentiation of GABA release involves stimulation of CRF release that then acts on the GABAergic terminal (Nie et al. 2004). Furthermore, the ethanol-induced increase in GABA release in the cerebellum is due largely to increased action potential frequency in the cell bodies of interneurons (Carta et al. 2004). Thus it is remain to be established whether or not ethanol can directly potentiate GABA release via actions on the terminal.

In the present study, we employed a simple preparation consisting of mechanically isolated neurons that retain attached presynaptic terminals to examine ethanol effects on synaptic transmission. We observed potentiation of GABAergic synaptic responses during acute application of ethanol at concentrations that occur in brain during intoxication. The potentiation was associated with increased frequency of spontaneous GABAergic IPSCs with no change in postsynaptic responses to GABA, and potentiation was independent of action potential firing. We also observed a form of rapid tolerance to alcohol actions that appeared to involve GABA$_B$ receptor activation. These findings indicate that ethanol can potentiate GABAergic transmission by directly acting on presynaptic terminals.

METHODS

Slice preparation

Brain slices (400 µm) containing the basolateral amygdala (BLA) were obtained from Sprague-Dawley rats (P13–15). Sections were cut with a Vibratome (TPI, St. Louis, MO) in cold buffer oxygenated with 95% O$_2$-5% CO$_2$. The composition of the external bathing solution was (in mM) 124 NaCl, 3KCl, 1.3 Mg$_2$SO$_4$, 2 CaCl$_2$, 1.2 NaH$_2$PO$_4$, 25 NaHCO$_3$, and 10 d-glucose. Slices were allowed to sit in a carbogen-bubbled chamber at room temperature for ≥1 h before recording.

Mechanical dissociation

Single neurons were isolated from BLA slices using an enzyme-free mechanical dissociation procedure, as described by Akaike and

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Moorhouse (2003). Briefly, BLA slices were transferred to a 35-mm dish (coated with poly-D-lysine) containing an external buffer with the following composition (in mM) 140 NaCl, 5 KCl, 10 HEPES, 2 MgCl₂, 2 CaCl₂, 10 glucose, 0.005 2.3-dioxo-6-nitro-1,2,3-tetrahydrobenzo[f]quinoxaline-7-sulfonamide disodium (NBQX), and 0.025 d-2-amino-5-phosphonopentanoic acid (AP5), with pH set to 7.4 using NaOH and osmolarity of ~300 mosM. A fire-polished glass micropipette was placed on the surface of the BLA. The tip of the pipette was vibrated horizontally over a distance of 100–200 μM at 6–8 Hz for ~2 min. The slice was removed from the dish, and isolated neurons were allowed to settle to the bottom of the dish for 10–15 min.

Electrophysiology

All recordings were made at room temperature. Patch pipettes were made using a two-stage microelectrode puller (PC-10, Narishige) and had resistances of 4–6 MΩ after filling with solution containing (in mM) 140 CsCl, 10 HEPES, 0.2 or 20 BAPTA, 2.0 MgCl₂, 2.0 Mg-ATP, and 0.3 GTP. This CsCl-based internal solution was used in all experiments examining spontaneous IPSCs to facilitate detection of synaptic responses at a holding potential of –60 mV. In a few neurons, we examined input resistance using a solution in which CsCl was replaced by KMeSO₃.

Whole cell recordings in brain slices were performed on an upright microscope (Axioskop 2FS, Carl Zeiss, Thornwood, NY), where, the slices were constantly superfused with the same buffer used for slice preparation at room temperature (23°C). Neurons in slices were visualized with a ×40 water-immersion objective, and Differential Interference Contrast (DIC) optics. Spontaneous GABAergic IPSCs (sIPSCs) were recorded in the presence of NBQX and AP5 (5/25 μM). Whole cell recordings from mechanically isolated neurons were performed in culture dishes mounted on an inverted microscope at room temperature. Neurons were visualized using a ×10 objective and Modulation Contrast optics. Whole cell current was measured from a holding potential of ~60 mV using conventional patch-clamp techniques. Data were amplified and filtered using an Axopatch 200B amplifier (Axon Instruments, Foster City, CA), and data were stored using pClamp8 software, and digitized using a Digidata 1200 interface (Axon Instruments). Spontaneous synaptic currents were initially detected using MiniAnalysis Software (Synaptosoft, Decatur, GA) with a threshold criterion of 20 pA; smaller events (down to 10 pA) were manually detected during visual inspection of traces. The amplitude of ion current activated by GABA application was measured with a threshold criterion of 20 pA; smaller events (down to 10 pA) were manually detected using MiniAnalysis Software (Synaptosoft, Decatur, GA) and Modulation Contrast optics. Whole cell current was measured using an Axopatch 200B amplifier (Axon Instruments, Foster City, CA), and data were stored using pClamp8 software, and digitized using a Digidata 1200 interface (Axon Instruments). Spontaneous synaptic currents were initially detected using MiniAnalysis Software (Synaptosoft, Decatur, GA) with a threshold criterion of 20 pA; smaller events (down to 10 pA) were manually detected during visual inspection of traces. The amplitude of ion current activated by GABA application was measured using cursor-based analysis using pClamp8 software.

The sIPSCs were sampled for 5 min before ethanol application to estimate baseline transmission, and measurements over a 5-min period were made during ethanol application and subsequent removal of ethanol from the slice preparation and isolated neurons.

Pharmacology

For brain slice experiments, all drugs were bath-applied. For dissociated neurons, external solution exchange was achieved by rapid lateral movement of three-port square glass pipettes driven by a stepper motor using the Warner Instruments (Hamden, CT) Perfusion Fast-Step system. The tip of the drug applicator was placed ~150 μm away from the cell recorded. Complete solution exchange occurred within ~200 ms in this system. AP5, bicuculline (BIC), GABA, and tetrodotoxin (TTX) were purchased from Sigma. Baclofen, NBQX, and SCH50911 were from Tocris (Ellisville, MO). Grain alcohol (190 proof) was from Pharmco (Brookfield, CT).

Statistics

Averaged data are expressed as means ± SE. Statistical significance was analyzed by paired t-test unless otherwise indicated, and the Kolmogorov-Smirnov two-sample test (K-S test) was used to examine changes in the cumulative distributions of spontaneous inhibitory postsynaptic currents (sIPSCs). The significance of the differences in the “tolerance index” was tested using the unpaired t-test. The criterion for statistical significance was P < 0.05. For the isolated neuron experiments several animals were used in each experiment (as indicated in the figure legends). For a given neuron or slice, ethanol was only applied once. No more than two neurons from the same animal were used for this study.

RESULTS

The large pyramidal projection neurons constitute the major neuronal subtype in BLA (>85% of the neurons) (McDonald 1982; Washburn and Moises 1992; Zhu et al. 2005). Smaller-sized neurons are mainly GABAergic interneurons (McDonald 1985). These two types of neurons can be easily distinguished using DIC (BLA slice) or modulation contrast (isolated neurons) optics. In the present experiments, we only examined large pyramidal neurons in both the slice and dissociated neuron preparations. We first examined the effects of EtOH on spontaneous IPSCs in BLA slices. Bath application of EtOH 100 mM increased the frequency and amplitude of sIPSCs (Fig. 1A). Plots of cumulative distribution of sIPSCs indicated that EtOH increased the amplitude of sIPSCs and decreased sIPSC event interval as shown in Fig. 1, B and C. Figure 1, D and E, shows the time course of EtOH potentiation of sIPSC frequency and amplitude in the BLA slice preparation. On average, ethanol caused a 30 ± 5% increase in the frequency of sIPSCs (P < 0.01, paired t-test, n = 6). Potentiation developed over the first two minutes of EtOH application and persisted at a consistent magnitude throughout the duration of alcohol application (Fig. 1C). The sIPSC frequency returned to preEtOH baseline levels after removal of the drug from the superfusion bath.

To determine if a similar effect of ethanol could be observed in a preparation containing only presynaptic terminals attached to postsynaptic neurons, we examined synaptic transmission in mechanically dissociated neurons from BLA slices. Freshly isolated BLA neurons retained short processes (Fig. 2A) and exhibited resting potentials that averaged ~51 ± 3 mV (n = 6) when the recordings were made with KMeSO₃-based internal solution, and spontaneous action potential firing was seen in all cells examined (Fig. 2B). The input resistance averaged 980 ± 45 MΩ (n = 10) for recordings made with the KMeSO₃-based internal solution, and 960 ± 50 MΩ for recordings made using the CsCl-based internal solution (n = 11). When examining EtOH effects on GABAergic transmission, whole cell patch recordings were made with a CsCl-based internal solution.

As we have previously demonstrated, mechanically dissociated BLA neurons exhibit robust GABAergic inhibitory synaptic transmission (Zhu and Lovinger 2005). The transmission can be measured and quantified by recording the frequency and amplitude of sIPSCs. We have previously reported that these sIPSCs are completely blocked in the presence of bicuculline, indicating that the responses are mediated by GABA_A receptors (Zhu and Lovinger 2005).

We took advantage of the rapid exchange of external solution possible in this preparation to examine in detail the time course of ethanol effects on GABA release (Fig. 2C). In the majority of neurons, EtOH induced a rapid increase in sIPSC
frequency within 6–15 s after solution exchange, indicating that EtOH potentiates GABAergic transmission in the isolated neuron preparation, as it does in BLA slices. Ethanol application elicited an increase in the frequency and the amplitude of sIPSCs, and these effects were reversible in mechanically isolated BLA neurons (Fig. 3, A and B). The magnitude of potentiation was dependent on EtOH concentration. On average, at concentrations of 100, 20, and 10 mM, EtOH caused a 166 ± 36% ($P < 0.01$, $n = 6$), 100 ± 28% ($P < 0.05$, $n = 7$), and 8 ± 24% ($P > 0.05$, $n = 7$) increase in the frequency of sIPSCs from control levels (96 ± 15, 130 ± 7, and 125 ± 12 events/min, for the 10, 20, and 100 mM groups, respectively).

At the 100 and 20 mM concentrations, EtOH produced 21 ± 4 pA ($P < 0.01$) and 19 ± 6 pA ($P < 0.01$) peak increases in sIPSC amplitude from control levels of 75 ± 6 and 66 ± 9 pA. There was no significant difference in EtOH effects on sIPSC frequency within 6–15 s after solution exchange, indicating that EtOH potentiates GABAergic transmission in the isolated neuron preparation, as it does in BLA slices. Ethanol application elicited an increase in the frequency and the amplitude of sIPSCs, and these effects were reversible in mechanically isolated BLA neurons (Fig. 3, A and B). The magnitude of potentiation was dependent on EtOH concentration. On average, at concentrations of 100, 20, and 10 mM, EtOH caused a 166 ± 36% ($P < 0.01$, $n = 6$), 100 ± 28% ($P < 0.05$, $n = 7$), and 8 ± 24% ($P > 0.05$, $n = 7$) increase in the frequency of sIPSCs from control levels (96 ± 15, 130 ± 7, and 125 ± 12 events/min, for the 10, 20, and 100 mM groups, respectively).

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amplitude between these two concentrations ($P > 0.05$, ANOVA). At the 10 mM concentration, EtOH only caused a $2 \pm 6$ pA increase in sIPSC amplitude ($P > 0.05$). The time
course of EtOH effects on sIPSC amplitude is plotted in Fig. 4
with 10-s bins. EtOH 100 mM produced a $51 \pm 5\%$ ($P < 0.01$,
$n = 6$) increase in sIPSC amplitude. It should be noted that the
effect of 20 mM sIPSC amplitude is small and is not sustained
throughout the application of this concentration of EtOH.

We also recorded from three cells with a high concentration
of BAPTA in the patch pipette after BAPTA (20 mM). In these
cells 100 mM EtOH produced a $73 \pm 7.6\%$ ($n = 3$, $P < 0.02$,
K-S test) increase in sIPSC frequency. Together with the
observations in neurons filled with our standard internal solu-
tion, these findings indicate that EtOH produces a rapid-onset,
concentration-dependent increase in sIPSC frequency in the
neuron/bouton preparation.

In addition to the EtOH-induced increase in sIPSC fre-
quency, we also observed evidence of an acute tolerance to the
effect of EtOH on sIPSC frequency (Fig. 3, C and D). For
example, when 100 mM ethanol was applied to the preparation,
sIPSC frequency initially increased and then reached a stable
potentiated level within 1–2 min after the onset of EtOH
application. To quantify acute tolerance, we averaged six
consecutive 10-s bins of data during the peak increase in sIPSC
frequency and another six at the very end of the EtOH appli-
cation. The acute tolerance index was assessed by calculating
the ratio of sIPSC frequency at the peak increase over the
frequency just at the end of the EtOH application. The index
for acute tolerance of the ethanol-induced GABA release was
$1.90 \pm 0.2$ for 100 mM EtOH. However, it should be noted that
sIPSC frequency at the end of the EtOH application period
remained at a level greater than the preEtOH control period,
and thus tolerance did not completely eliminate potentiation

FIG. 4. The time course of ethanol effect on sIPSC amplitude. The ampli-
ditude is plotted in 10-s bins. Top: 100 mM ethanol caused a significant increase
in sIPSC amplitude ($P < 0.01$, $n = 7$), whereas 20 and 10 mM ethanol had
little to no effect (middle and bottom). Data were from 12 rats.
(18 $\pm$ 5.4% increase, $P < 0.05$, t-test). The acute tolerance was even more striking during application of 20 mM EtOH (tolerance index $= 2.05 \pm 0.2$) as sIPSC frequency was significantly increased for only the first minute after application of this concentration of EtOH. Although there was no significant difference in tolerance index between 20 and 100 mM EtOH ($P > 0.05$, group t-test), the potentiation of the sIPSC frequency by 20 mM EtOH only lasted for a short period. At the end of 20 mM EtOH application, sIPSC frequency was 89 $\pm$ 11% of control, which is significantly different from sIPSC frequency at the same time during 100 mM EtOH application ($P < 0.05$, unpaired t-test). When EtOH was washed away from the preparation sIPSC frequency decreased to baseline levels for 100 mM EtOH, and remained at or near baseline levels for 20 mM EtOH (Fig. 3, C and D).

Activation of GABA$_B$ receptors on presynaptic boutons may limit ethanol potentiation of GABAergic transmission, as suggested in recent studies (Ariwodola and Weiner 2004; Yang et al. 2000). We have observed that the GABA$_B$ receptor agonist baclofen (5 $\mu$M) inhibits GABAergic transmission in the mechanically isolated neuron-bouton preparation from the BLA (Fig. 5A; 78 $\pm$ 3% decrease in sIPSC frequency and 13 $\pm$ 3 pA reduction in sIPSC amplitude, $n = 4$ cells). This finding indicates that GABA$_B$ receptors are present at synapses in this preparation. The GABA$_B$ antagonist SCH50911 was applied to the isolated neurons to determine if blockade of these receptors alters the response to EtOH. Application of SCH50911 alone did not have any significant effect on sIPSC frequency (Fig. 5B; $\Delta = 1 \pm 4$% from the control level of 128 $\pm$ 11 events/min, $n = 7$, $P > 0.7$, paired t-test). However, when EtOH was applied in the presence of SCH50911, the EtOH-induced tolerance was greatly attenuated (Fig. 5C). On average, 100 mM ethanol caused a 100 $\pm$ 9% ($P < 0.01$, $n = 7$) peak increase in the frequency of sIPSCs, and a 62 $\pm$ 10% increase was observed at the end of ethanol application (average of 6 consecutive bins). The index for acute tolerance produced by 100 mM EtOH was only 1.23 $\pm$ 0.1 in the presence of SCH50911, significantly smaller than the value observed in the absence of antagonist ($P < 0.01$, unpaired t-test, compared with 100 mM EtOH data in Fig. 3B).

The ethanol-induced increase in sIPSC amplitude could be explained by direct enhancement of the function of postsynaptic GABA$_A$ receptors. To examine this possibility, we applied GABA directly to the mechanically isolated neurons in the absence and presence of EtOH using rapid drug superfusion. We applied GABA at a concentration of 0.3 $\mu$M ($<$EC$_{10}$) because EtOH potentiation of GABA receptors has generally been observed only at low agonist concentrations. As shown in Fig. 6, EtOH did not significantly affect the postsynaptic current activated by 0.3 $\mu$M GABA. Peak amplitude of GABA-evoked currents in the presence of EtOH (20, 100 mM) averaged 110 $\pm$ 9% ($P > 0.05$, $n = 12$) and 105 $\pm$ 17% ($P > 0.05$, $n = 7$) of preEtOH baseline values. Thus, under these experimental conditions, we did not observe an EtOH-induced increase in GABA$_A$ receptor function.

We have previously reported that GABA release in the mechanically isolated BLA neurons is partially dependent on presynaptic action potential firing that leads to synchronous neurotransmitter release (Zhu and Lovinger 2005). It is possible that EtOH could alter GABA release by enhancing action potential firing in presynaptic elements of this preparation. We therefore applied TTX, a blocker of voltage-dependent sodium channels to prevent presynaptic action potentials in the absence of EtOH using rapid drug superfusion. Thus, applied TTX, a blocker of voltage-dependent sodium channels to prevent presynaptic action potentials. The sIPSC frequency was 107 $\pm$ 11 ($n = 12$) per min in the absence, and 20 $\pm$ 2 ($n = 10$) per minute in the presence of TTX ($P < 0.001$, t-test), consistent with our previous findings (Zhu and Lovinger 2005). In the presence of 1 $\mu$M TTX, ethanol (100 mM) still produced an increase in sIPSC frequency to 154 $\pm$ 45% above baseline levels ($P < 0.05$, $n = 6$, t-test, Fig. 7A). However, under this condition, ethanol did not significantly affect the mean amplitude of GABAergic synaptic currents as indicated in Fig. 7B. The mean amplitude of action potential independent sIPSCs was 95 $\pm$ 7 pA in the control and 97 $\pm$ 9 pA in ethanol (100 mM). TTX did not abolish ethanol-induced acute tolerance of GABA release as indicated in Fig. 7C (tolerance index = 1.65 $\pm$ 0.2). These observations indicate that EtOH potentiation of GABAergic transmission is unlikely
to occur through increased presynaptic action potential firing and that it is most likely due to a presynaptic mechanism because sIPSC frequency, but not amplitude, is enhanced in the presence of TTX.

DISCUSSION

In the present study, we observed that EtOH potentiates GABAergic synaptic transmission in the mechanically isolated neuron/bouton preparation, and this effect minimally requires a presynaptic terminal and a postsynaptic neuron. Thus ethanol effects due to paracrine actions of substances released from nearby neuronal or glial cells are not necessary for this potentiation. These possibilities cannot be ruled out in studies using the slice preparation, and thus our findings greatly narrow the possible cellular targets of presynaptic EtOH effects on GABAergic transmission. The observed increase in sIPSC frequency, with no change in amplitude in the presence of TTX, supports the idea that EtOH increases presynaptic GABA release by directly acting on GABAergic presynaptic terminals.

Recordings in the isolated neuron condition generally yielded higher input resistances than those observed when recording from neurons in BLA slices (input resistance = 94 ± 11 MΩ, n = 7 neurons in the slice preparation). Furthermore, the sIPSCs measured in the isolated neurons arise from transmission at synapses on the soma and proximal dendrites, whereas sIPSCs in neurons in the slice can come from several places on the extensive dendritic tree of the intact neuron. Thus sIPSCs are more easily detected in the isolated neuron preparation, facilitating detection of EtOH-induced increases in sIPSCs in the neuron/bouton preparation. This is one experimental factor that may account for the larger effect of EtOH in the isolated neurons. However, we do not believe that the increase in sIPSC frequency is secondary to changes in sIPSC amplitude in the neuron/bouton preparation. In a previous study using this preparation, we found that the detection of events is not facilitated even at a holding potential of −90 mV (Zhu and Lovinger 2005). Thus it is unlikely that the improved detection of small-amplitude events contributes to ethanol-induced increase in sIPSC frequency.

The ethanol-induced increase in frequency of GABAergic sIPSCs is not limited to the reduced preparation as we also observed increased sIPSC frequency in the BLA slice preparation. Increases in sIPSC frequency produced by ethanol have also been reported in variety of preparations (Marszalec et al. 2006).

FIG. 6. Ethanol does not alter postsynaptic current activated by a low GABA concentration in isolated BLA neurons. Top: traces were recorded in the presence of 1 μM TTX. The postsynaptic currents were elicited by 0.3 μM GABA in the absence or presence of 100 mM ethanol. Bottom: summary of effects of 100 mM (P > 0.05, n = 12) and 20 mM (P > 0.05, n = 7) ethanol on GABA-elicited currents. Neurons were preexposed to ethanol for 20–30 s before applying GABA. Nine rats were used for testing effects of ethanol on GABA-activated current.

FIG. 7. Ethanol-induced increase in GABA release is action potential independent in the isolated BLA neurons. A–C, TTX, a blocker of voltage-gated sodium channels, did not prevent the EtOH-induced increase in sIPSC frequency. B: 1-min recording traces are from a representative experiment; left: 15 and 31 individual sIPSCs are superimposed from control and in the presence of 100 mM EtOH, respectively; right: average of these sIPSCs. Note the lack of change in mIPSC amplitude in the presence of EtOH. The rise and decay time were 1.7 ± 0.2 and 5.2 ± 0.3 ms for control, and 1.8 ± 0.2 and 5.2 ± 0.3 ms in the presence of ethanol, and these values do not differ across conditions (P > 0.05, group t-test). Four rats were used for testing TTX effects.
BAPTA experiments. This finding suggests that Ca\(^{2+}\) appear to be reduced in magnitude in the high postsynaptic EtOH potentiation. Potentiation of GABA release by EtOH did indicate that this sort of retrograde signal is not necessary for retrograde messengers, such as the endocannabinoid release. These conditions retard the production of GABA to determine if ethanol alters postsynaptic function under these experimental conditions. Similar results also indicate that the presynaptic ethanol effect on GABAergic transmission takes place in a more intact preparation. Thus this presynaptic effect is not a consequence of use of the neuron/bouton preparation. Increasing interneuron excitability contributes to the ethanol enhancement of GABA release in cerebellar slices (Carta et al. 2004), but direct presynaptic effects of ethanol might underlie the enhancement seen in other brain regions. In contrast, ethanol decreases excitatory transmission in part via presynaptic mechanisms, and ethanol also reduces excitability in glutamatergic neurons (Cheng et al. 1999; Marszalek et al. 1998). The overall effect of ethanol is thus to promote synaptic inhibition and reduce synaptic excitation, which ultimately can reduce neuronal network activity (cf. ethanol effects in neonatal rats reported by Galindo et al. 2005).

The ethanol-mediated potentiation could involve a retrograde messenger released from the postsynaptic neuron. However, the potentiation occurred when the postsynaptic membrane was held at −60 mV and when postsynaptic neurons were filled with high BAPTA concentrations to buffer intracellular calcium. These conditions retard the production of retrograde messengers, such as the endocannabinoid release from postsynaptic neurons previously characterized in this neuron/bouton preparation (Zhu and Loving 2005), and indicate that this sort of retrograde signal is not necessary for EtOH potentiation. Potentiation of GABA release by EtOH did appear to be reduced in magnitude in the high postsynaptic BAPTA experiments. This finding suggests that Ca\(^{2+}\)-dependent signal transduction may contribute, at least in part, to the EtOH-induced increase in sIPSC frequency. Thus we cannot conclusively rule out any role for a retrograde signal in EtOH potentiation. However, the basic phenomenon of potentiation is most likely due to a direct action of ethanol on the presynaptic terminal.

Taking advantage of the neuron/bouton preparation, we examined currents activated by application of a low concentration of GABA to determine if ethanol alters postsynaptic receptor function. Ethanol did not significantly alter the amplitude of GABA-activated current in this preparation. This finding is consistent with the lack of effect of ethanol on the amplitude of sIPSCs in the presence of TTX, suggesting that acute ethanol does not alter postsynaptic GABA\(_\text{x}\) receptor function under these experimental conditions. Similar results have been observed in enzymatically isolated BLA neurons (Floyd et al. 2004; McCool et al. 2003). However, we do not wish to overgeneralize the present findings as there is certainly evidence that ethanol can alter GABA\(_\text{x}\) receptor function in neurons (Poelchen et al. 2000; Roberto et al. 2003; Siggins et al. 1987) and heterologous expression systems (Hanchar et al. 2005; Wallner et al. 2003). A preliminary report indicates that EtOH potentiation of GABA\(_\text{x}\) receptor function is attenuated in the presence of high intracellular chloride as we used in the present study (Silberman et al. 2005). Thus ethanol effects on postsynaptic GABA\(_\text{x}\) receptor function may vary with different experimental conditions, adding to our caution about overinterpretation of our negative findings.

Ethanol effects on action potential firing in presynaptic neurons are not necessary for the EtOH-induced potentiation because this potentiation is maintained in the presence of TTX. The magnitude of the increase in sIPSC frequency in the presence of TTX is comparable to that observed in the absence of the sodium channel blocker, suggesting that action potential-independent effects of EtOH account for most, if not all, of the potentiation of GABA release. It should be noted that a recent study using hippocampal slices indicates that the sensitivity of sIPSC frequency (but not mIPSC frequency) to ethanol increases with development (Li et al. 2006). This may reflect an increase in ethanol effects on interneuron excitability with the net result that TTX-sensitive potentiation of IPSCs becomes more prominent. We are unable to test this hypothesis in the BLA neuron/bouton preparation at the present time because it is very difficult to obtain viable mechanically dissociated BLA neurons beyond −P21.

The presynaptic release of GABA also appears to modulate ethanol-induced potentiation. We observed a pronounced acute tolerance during ethanol application, and this tolerance was reduced in the presence of a GABA\(_B\) receptor antagonist. This observation is consistent with recent findings by Ariwodola and Weiner (2004), who observed that activation of GABA\(_B\) receptors produces feedback inhibition that limits ethanol potentiation of GABA release in hippocampal slices. It is not yet clear if the acute, rapid tolerance we observed is simply due to activation of presynaptic GABA\(_B\) receptors by GABA release or if ethanol potentiates GABA\(_B\) receptor function. However, this tolerance can clearly influence the time course and magnitude of ethanol potentiation.

The influence of tolerance most likely accounts for different temporal characteristics of ethanol potentiation in the isolated neuron/bouton and slice preparations. When the ethanol concentration rises very rapidly, as in the isolated neuron/bouton preparation, the potentiation appears to have a very rapid onset (6–15 s), and tolerance only develops after tens of seconds of ethanol exposure. Because solution exchange occurs on a sub-second time scale in this preparation, the time courses of the observed potentiation and tolerance likely reflect the time courses of the molecular mechanisms underlying the processes, independent of any effects of rate of drug application. In contrast, ethanol levels will rise gradually on a time scale of minutes in the BLA slice preparation. In the slice preparation, potentiation will develop slowly as the ethanol concentration gradually climbs. This will allow sufficient time for tolerance to begin to develop. Thus the magnitude of the increase in transmission that is observed will reflect a balance between potentiation and rapid, acute tolerance. The lack of an observable time-dependent loss of potentiation during ethanol application in the slice preparation may reflect the achievement of a steady-state balance between potentiation and tolerance during the initial rising phase of alcohol concentration. This is consistent with the observations of Ariwodola and Weiner (2004) in hippocampal slices, where the magnitude of ethanol potentiation of GABAergic transmission is limited by GABA\(_B\) receptor activation even within the first few minutes of ethanol application. These findings provide a potential mechanistic explanation for differing patterns of intoxication related to the rate of delivery of alcohol to the brain. One can easily imagine that potentiation will dominate with rapid alcohol delivery (i.e., with rapid intake or alcohol inhalation), whereas tolerance may have a greater influence with slower modes of alcohol intake. It will be of interest to determine if behaviors involving the...
BLA show alterations that are dependent on rate of alcohol delivery.

The mechanism(s) underlying the ethanol-induced increase in GABA release is not yet clear. Ethanol stimulates cAMP-mediated signal transduction (Asher et al. 2002; Kumada et al. 2006), which can lead to increased GABA release (Capogna et al. 1995). However, this effect may only come into play after chronic ethanol exposure. Alternatively, ethanol may have more direct effects on vesicle release. These are potential mechanisms that will have to be explored in future studies.

Although increased osmolarity can enhance neurotransmitter release, we do not believe that hyperosmolarity contributes to the EtOH-induced increase in GABA release. Ethanol is amphiphilic and can rapidly reach equilibrium across cellular membranes. It has been demonstrated elegantly in glial cells that EtOH has little osmotic effect under normal conditions and can only produce effects when the ionic environment is fixed such that transmembrane EtOH equilibration brings about a hyperosmotic state (Kimelberg et al. 1993). Furthermore, the persistence of the effect of ethanol on sIPSC frequency in the presence of the GABA_B antagonist suggests that the increase in IPSC frequency is not dependent on a change in osmolarity. Examination of excitatory transmission also suggests that EtOH does not produce a large osmolarity-driven effect on transmission as EtOH decreases sEPSC frequency (Cheng et al. 1999; Marszalec et al. 1998), and it is known that sEPSCs are potentiated under hyperosmolar conditions. For these reasons, we do not believe that increased osmolarity plays much, if any, role in the potentiation by EtOH.

A recent study (Sanna et al. 2004) suggests that ethanol increases IPSC amplitude in hippocampus via two different mechanisms. An early effect of ethanol involves a presynaptic mechanism, whereas a later phase of potentiation is indirect and dependent on neurosteroid synthesis. This late effect may be very important in mediating specific central effects of ethanol. We have not yet explored possible roles of neurosteroids in the potentiation by EtOH in BLA. However, we deem it unlikely that neurosteroids are involved in the present study as these agents act postsynaptically at GABAergic synapses, and we did not observe evidence of a postsynaptic alcohol action. Moreover, in the single-cell preparation with the constant superfusion that we have used, any neurosteroids released from cells would likely be washed away rapidly, diminishing the chance of observing a steroid-mediated component of potentiation.

Stimuli that induce fear responses lead to decreased GABAergic tone in the amygdala (Stork et al. 2002) and persistent reductions in GABA_A receptor binding (Bremner et al. 2000; Chhatwal et al. 2005). The suppression of GABAergic transmission blocks the development and extinction of aversive memories (Harris and Westbrook 1998). Ethanol is known to produce anxiolytic effects during acute intoxication, effects that are shared by almost all substances that increase GABAergic transmission (Ashton et al. 2005; Blanchard and Blanchard 1999; Cowley 1992). Furthermore, the BLA regulates anxiety and related affective states (LeDoux 2000). Thus ethanol potentiation of GABAergic transmission in the BLA is a good candidate mechanism for involvement in the anxiolytic effects observed during acute intoxication. In the present study, ethanol potentiation of GABA release was observed at a concentration near the legal intoxication level (20 mM). Anxiolytic effects of ethanol would be expected at this brain ethanol concentration. In addition to anxiety, the BLA has prominent roles in fear and fear conditioning (Wilensky et al. 1999), and GABAergic BLA synapses have also been implicated in these processes (Gonzalez et al. 1996; Sanders and Shekhar 1995; Wilensky et al. 1999). GABAergic potentiation by ethanol may also contribute to ethanol effects on these brain functions (Papadeas et al. 2001; Roberts et al. 1996).

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GRANTS

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