Ethanol Modulates Synaptic and Extrasynaptic GABA<sub>A</sub> Receptors in the Thalamus

AUTHORS:

Fan Jia, Dev Chandra, Gregg E. Homanics, Neil L. Harrison

ADDRESSES:

C.V. Starr Laboratory for Molecular Neuropharmacology, Dept. of Anesthesiology (FJ, NLH), Weill Cornell Medical College, 1300 York Avenue, New York, NY 10065 and Departments of Anesthesiology and Pharmacology (DC, GEH), University of Pittsburgh, Pittsburgh, PA, 15261
RUNNING TITLE PAGE

Running Title: Ethanol and Thalamic GABA_A receptors

Corresponding Author:

Neil Harrison, PHD

Dept. of Anesthesiology

Weill Cornell Medical College

1300 York Avenue, Room A-1050

New York, NY 10065

Telephone: (212) 746 – 5325

Fax: (212) 746 – 4879

Email: neh2001@med.cornell.edu

Text Pages: 31

Tables: 0

Figures: 6

References: 62

Abstract: 245 words

Introduction: 611 words

Discussion: 1346 words

Nonstandard Abbreviations: GABA_A-R(s), GABA_A receptor(s); IPSC, inhibitory postsynaptic current; VB, ventrobasal thalamic nucleus

Section: Neuropharmacology
Abstract

Drinking alcohol is associated with the disturbance of normal sleep rhythms, and insomnia is a major factor in alcoholic relapse. The thalamus is a brain structure that plays a pivotal role in sleep regulation and rhythmicity. A number of studies have implicated GABA_A receptors (GABA_A-Rs) in the anxiolytic, amnestic, sedative and anesthetic effects of ethanol. In the present study, we examined the effects of ethanol on both synaptic and extrasynaptic GABA_A-Rs of relay neurons in the thalamus. We found that ethanol (≥ 50 mM) elicits a sustained current in thalamocortical relay neurons from the mouse ventrobasal (VB) thalamus, and this current is associated with a decrease in neuronal excitability and firing rate in response to depolarization. The steady current induced by ethanol was totally abolished by gabazine, and was absent in relay neurons from GABA_A-R α_4 subunit knockout mice, indicating that the effect of ethanol is to enhance tonic GABA-mediated inhibition. 50 mM ethanol enhanced the amplitude of tonic inhibition by nearly 50%. On the other hand, ethanol had no effect on spontaneous or evoked inhibitory postsynaptic currents (IPSCs) at 50 mM, but did prolong IPSCs at 100 mM. Ethanol had no effect on the paired-pulse depression ratio, suggesting that the release of GABA from presynaptic terminals is insensitive to ethanol. We conclude that ethanol, at moderate (50mM) but not low (10mM), concentrations can inhibit thalamocortical relay neurons, and that this occurs mainly via the actions of ethanol at extrasynaptic GABA_A-Rs containing GABA_A-R α_4 subunits.
Introduction

Drinking alcohol can promote the onset of sleep, but also disrupts the normal sleep pattern, increases nocturnal awakenings and reduces sleep quality (Drummond et al., 1998). Sleep disturbance caused by chronic alcohol can play a role in the progression of alcoholism, and poor sleep quality is often cited as a factor in alcoholic relapse (Brower et al., 1998; Brower, 2001). Inhibition in the thalamus plays an important role in the normal regulation of sleep cycles (Steriade, 2000; Huguenard and McCormick, 2007; Jia et al., 2007), and may therefore be involved in both the sedative effects of acute alcohol and in the development of alcoholism.

The inhibitory neurotransmitter, γ-aminobutyric acid (GABA) has long been implicated in the anxiolytic, amnestic, sedative and anesthetic effects of alcohol. A large number of studies have investigated the interactions of alcohol with GABA_A receptors (GABA_A-Rs). The standard forms of recombinant GABA_A-Rs that are found at GABAergic synapses (α1βγ2 and α2βγ2 subtypes) are modulated only by > 60 mM ethanol (Sigel et al., 1993; Mihic et al., 1997). Most investigators have failed to observe direct postsynaptic actions of alcohol (< 60 mM) on GABA-mediated inhibitory postsynaptic currents (IPSCs) in brain slices, except at high levels (Ariwodola and Weiner, 2004; Weiner and Valenzuela, 2006). In several brain areas, however, ethanol has been shown to facilitate synaptic inhibition by a presynaptic mechanism, for example in the amygdala (Roberto et al., 2003; Roberto et al., 2004; Roberto and Siggins, 2006; Zhu and Lovinger, 2006), cerebellum (Carta et al., 2004; Hanchar et al., 2005; Ming et al., 2006; Kelm et al., 2007), hippocampus (Ariwodola and Weiner, 2004; Sanna et al., 2004; Galindo et al., 2005) and nucleus accumbens (Nie et al., 2000; Crowder et al., 2002).

A novel form of “tonic inhibition” has also been described in the CNS, which is generated by the persistent activation of extrasynaptic GABA_A-Rs (Semyanov et al., 2004;
Farrant and Nusser, 2005; Mody, 2005). GABAergic tonic inhibition has been shown to regulate the excitability of individual neurons and the behavior of neural networks. Tonic inhibition is most often generated by activation of GABA_A-Rs that contain δ subunits, which are normally located at extrasynaptic or perisynaptic sites (Nusser et al., 1998; Wei et al., 2003). Several laboratories have reported that extrasynaptic GABA_A-Rs containing δ subunits are sensitive to low concentrations (≤ 30 mM) of alcohol (Sundstrom-Poromaa et al., 2002; Wallner et al., 2003; Wei et al., 2004; Hanchar et al., 2005; Hanchar et al., 2006; Santhakumar et al., 2006; Wallner et al., 2006; Glykys et al., 2007); but other laboratories have reported contradictory results (Carta et al., 2004; Borghese et al., 2006; Botta et al., 2007; Korpi et al., 2007).

Tonic inhibition also occurs in the relay neurons of the thalamus (Porcello et al., 2003; Belelli et al., 2005; Cope et al., 2005; Jia et al., 2005; Chandra et al., 2006; Bright et al., 2007; Jia et al., 2008b; Peden et al., 2008), where the extrasynaptic GABA_A-Rs contain α_4, β_2 and δ subunits (Belelli et al., 2005; Chandra et al., 2006). Thalamic extrasynaptic GABA_A-Rs have distinct pharmacological properties that differentiate them from synaptic GABA_A-Rs, consisting mainly of α_1, β_2, and γ_2 subunits (Pirker et al., 2000; Browne et al., 2001; Jia et al., 2005). Several studies show that hypnotics and anesthetics are much more potent at thalamic extrasynaptic GABA_A-Rs than at synaptic receptors (Belelli et al., 2005; Cope et al., 2005; Jia et al., 2005; Chandra et al., 2006). Investigating the effects of alcohol on both synaptic and extrasynaptic inhibition in the thalamus should enhance our understanding of the mechanisms underlying the interaction between alcohol and sleep. We therefore examined the actions of ethanol on the function of GABA_A-Rs of thalamocortical relay neurons in the mouse ventrobasal (VB) thalamus.
Methods

Electrophysiological recordings in brain slices

Experiments were performed in accordance with institutional and federal guidelines, using mice between 23 and 60 days old (C57BL/6, Gabra4+/+ and Gabra4−/−) by methods we have described previously (Jia et al., 2005). The knockout (Gabra4−/−) and wild-type (Gabra4+/+) littermates used were age-matched and on the same genetic background (129X1/S1 × C57BL/6J hybrid; F2-F4 generations) (Chandra et al., 2006). The experimenters were blind to genotype.

Animals were anesthetized with halothane and brains were removed and placed in ice-cold slicing solution, which contained (in mM): 2.5 KCl, 26 NaHCO3, 1.25 NaH2PO4, 220 sucrose, 11 glucose, 10 MgSO4 and 0.5 CaCl2, before horizontal slices (300 µm thick) were cut on a microslicer (VT 1000S, Leica, Wetzlar, Germany). Slices were perfused with carbogenated artificial cerebrospinal fluid (aCSF), which contained (in mM): 124 NaCl, 2.5 KCl, 2 MgSO4, 2 CaCl2, 26 NaHCO3, 1.25 NaH2PO4, and 10 glucose. Whole-cell patch clamp recordings from visually identified thalamic neurons were performed at room temperature as previously described (Jia et al., 2005). Intracellular solution for most voltage-clamp recordings contained (in mM): 140 CsCl, 4 NaCl, 1 MgCl2, 0.05 EGTA, 2 ATP-Mg2+, 0.3 GTP-Na+ and 10 HEPES; pH was adjusted to 7.25 with CsOH. For voltage-clamp recordings involving acamprosate, intracellular solution contained (in mM): 130 CsCH3SO3, 8.3 NaCH3SO3, 1.7 NaCl, 1 CaCl2, 10 EGTA, 2 ATP-Mg2+, 0.3 GTP-Na+, 10 HEPES; pH was adjusted to 7.2 with CsOH. Intracellular solution for current-clamp recordings contained (in mM) 130 K+-gluconate, 5 NaCl, 2 MgCl2, 10 HEPES, 0.5 EGTA, 2 ATP-K+, and 0.3 GTP-Na+, pH adjusted to 7.25 with KOH.

Spontaneous inhibitory postsynaptic currents were recorded at -65 mV and isolated by bath application of 3-5 mM kynurenic acid (Jia et al., 2005), and evoked IPSCs were elicited by
electrical stimulation (50 μs; 2 x threshold) with a bipolar metal electrode (FHC, Bowdoin, ME), located in the RTN. The interval between successive stimuli was long (>15 seconds) in order to prevent cumulative synaptic depression. Access resistance was monitored throughout the recording period, and was less than 20 MΩ throughout.

**Drugs and Data analysis**

Gabazine (4-[6-imino-3-(4-methoxyphenyl) pyridazin-1-yl] butanoic acid hydrobromide), kynurenic acid (4-oxo-1H-quinoline-2-carboxylic acid), baclofen (4-amino-3-(4-chlorophenyl)-butanoic acid) and ethanol were purchased from Sigma (St. Louis, MO). Acamprosate calcium (3-Acetamidopropane-1-sulfonic acid calcium salt) was obtained from Toronto Research Chemicals Inc. (North York, ON, Canada). Off-line analysis was performed using MiniAnalysis 5.5 (Synaptosoft, Decatur, GA), SigmaPlot 6.0 (SPSS, Chicago, IL) and Excel 2000 (Microsoft, Redmond, WA), as described in our previous publications. Tonic currents were measured as illustrated in Figure 2C. The holding current was calculated by averaging an IPSC-free 5 ms section from every 100 ms of record. The all points histograms were fitted with a Gaussian curve. The difference between the peaks of these Gaussian curves in the presence and absence of drug were calculated to determine the change of holding current. Spontaneous IPSCs were detected and analyzed using MiniAnalysis as previously described (Jia et al., 2005). Numeric data are expressed as mean ± SEM, except where indicated. The statistical significance of results was assessed using Student's t test or one-way ANOVA, and a level of p < 0.05 was considered significant.
Results

*Ethanol decreases excitability in thalamic relay neurons via GABA<sub>A</sub>-Rs*

We began by investigating whether ethanol modulates tonic firing of action potentials in depolarized thalamocortical relay neurons (Sherman, 2001). The membrane potentials of VB neurons were maintained at around –60 mV by constant current injection. At this membrane potential, VB neurons were generally silent but displayed sustained AP firing in response to depolarizing current steps. 50 mM ethanol decreased the excitability of VB relay neurons, (Figure 1A) and shifted the input-output curve to the right. This effect of alcohol to inhibit neuronal excitability is dependent on GABA<sub>A</sub>-Rs, since in the presence of gabazine (20 µM), a specific GABA<sub>A</sub>-R antagonist, ethanol had no effect on the input-output relationship (Figure 1B).

In order to facilitate the comparison of firing rates, the amplitude of the current step (500 ms duration) was adjusted in each neuron to induce ~10 APs, corresponding to a firing frequency of ~20 Hz. We then compared the number of APs evoked by depolarizing current steps before and after ethanol application. 20 mM ethanol failed to decrease firing rate (9.6 ± 1.5 vs. 9.4 ± 1.6; p > 0.05, n = 5; Figure 1C). In contrast, 50 mM ethanol significantly reduced the number of evoked APs, from 10.9 ± 1.4 to 8.5 ± 1.4 (p < 0.01, n = 6; Figure 1D). Pre-application of gabazine completely prevented the inhibitory effects of 50 mM ethanol on firing (p > 0.05, n = 5; Figure 1E). These results demonstrate that a sedative-hypnotic concentration (50 mM) of ethanol can reduce the excitability of depolarized VB relay neurons mainly through a GABA<sub>A</sub>-R mechanism.
Ethanol enhances tonic inhibition in VB neurons

We made whole-cell voltage-clamp recordings in order to explore the modulation by ethanol of synaptic and extrasynaptic GABA_A-Rs. Firstly, we examined whether ethanol induces any change of the tonic current mediated by extrasynaptic GABA_A-Rs. At low concentrations (10-30 mM), ethanol induced no significant change of holding current (10 mM: 0.8 ± 0.3 pA, n = 12; 20 mM: 1.8 ± 0.7 pA, n = 18; 30 mM: 2.4 ± 1.2 pA, n = 10), but at higher concentrations (50-100 mM) ethanol elicited a steady and sustained current shift (50 mM: 9.5 ± 1.5 pA, n = 19; 100 mM: 19.0 ± 3.6 pA, n = 12; Figure 2A and B). In some experiments, we applied 20 µM gabazine following the application of 50 mM ethanol. Gabazine not only blocked the ethanol-induced current-shift, but also revealed the underlying tonic inhibition, indicating that the sustained currents induced by ethanol were due to enhancement of tonic inhibition mediated by GABA_A-Rs. We measured the tonic currents before and after ethanol perfusion, as shown in Figure 2C, and the pooled data from all experiments (n = 12) were well fitted by a straight line (R = 0.98), with a slope of 1.50, indicating that 50 mM ethanol enhanced tonic currents by 50%.

Ethanol-evoked currents are absent in VB neurons from Gabra4^-/- mice

We have previously demonstrated that extrasynaptic GABA_A-Rs are absent from the thalamus of Gabra4^-/- mice (Chandra et al., 2006), and we therefore investigated the actions of ethanol in VB neurons from Gabra4^-/- mice and their wild-type littermates (Figure 3). 50 mM ethanol failed to induce any shift in baseline current in VB neurons from α4 knockout mice (1.7 ± 0.9 pA, n = 10). In contrast, wild-type neurons showed measurable current shifts in response to 50mM ethanol (8.6 ± 2.6 pA, n = 6), which were comparable to ethanol-induced currents recorded from C57BL/6 mice. This difference between the genotypes was highly significant (p <
0.01). These results are consistent with the idea that low concentrations of ethanol selectively enhance the activity of extrasynaptic GABA$_A$-Rs in VB neurons that contain $\alpha_4/\delta$ subunits (Jia et al., 2005; Chandra et al., 2006).

**Acamprosate enhances tonic inhibition in VB neurons only at high concentrations**

Our recent work has shown that taurine is also a potent agonist for thalamic extrasynaptic GABA$_A$-Rs (Jia et al., 2008b). Acamprosate (3-acetamidopropane-1-sulfonic acid) is a taurine analog that has been used to treat alcohol abuse and alcoholism (De Witte et al., 2005; Gupta et al., 2005), although its mechanism of action is not yet fully understood. Since neurons become hyperexcitable during alcohol withdrawal, we wondered whether acamprosate might reduce excitability and inhibit the neurons via extrasynaptic GABA$_A$-Rs in a similar way to taurine. Our recordings showed that 1 $\mu$M acamprosate was unable to induce any change in the tonic current in VB neurons (0.1 ± 1.6 pA, n = 6). Tonic currents were also insensitive to 10 and 100 $\mu$M acamprosate (0.3 ± 1.8 pA, n = 5 and 2.8 ± 2.0 pA, n = 6 respectively). At higher concentrations, 200 and 500 $\mu$M acamprosate did elicit modest currents (14.7 ± 3.8 pA, p < 0.05, n=6 and 42.5 ± 6.8 pA, p < 0.01, n=6 respectively; Figure 4). These results are consistent with a recent report (Reilly et al., 2008), which shows that acamprosate at low, clinically relevant concentrations has no effect on $\alpha_4\beta_3\delta$ GABA$_A$-Rs expressed in *Xenopus* oocytes.

**High concentrations of ethanol prolong IPSC decay time**

We next investigated whether ethanol changes the properties of IPSCs. Spontaneous inhibitory synaptic currents are readily observed in VB neurons and can be blocked by gabazine (data not shown), indicating that they are mediated by synaptic GABA$_A$-Rs. The averaged data
from this set of experiments (Figure 5C) show that, at all three concentrations we tested (20, 50 and 100 mM), ethanol induced no significant change in the frequency (percentage change: 20 mM: -3.2 ± 3.6 %, n = 9; 50 mM: 3.8 ± 3.1 %, n = 16; 100 mM: 7.0 ± 6.6 %, n = 8) or the amplitude of spontaneous IPSCs (percentage change: 20 mM: -1.0 ± 2.3 %, n = 9; 50 mM: -1.2 ± 2.4 %, n = 16; 100 mM: 1.5 ± 4.5 %, n = 8). At 100 mM, ethanol did significantly increase the decay time of spontaneous IPSCs by 8.5 ± 2.0 % (p < 0.01, n = 8).

We also compared evoked inhibitory synaptic currents before and after ethanol applications. Gabazine completely blocked evoked IPSCs (data not shown), suggesting that they were mediated by GABA\(_A\)-Rs, and ethanol (20 – 100 mM) did not enhance the amplitude of evoked IPSCs (percentage change: 20 mM: 1.7 ± 1.4 %, n = 7; 50 mM: 2.3 ± 3.1 %, n = 8; 100 mM: 0.3 ± 2.5 %, n = 13); only 100 mM ethanol increased the decay time of evoked IPSCs (percentage change: 20 mM: -0.5 ± 1.5 %, n = 7; 50 mM: 5.8 ± 2.6 %, n = 8; 100 mM: 13.6 ± 2.5 %, p < 0.001, n = 13; Figure 6A and B). Ethanol (≤ 50 mM) does not appear to modulate the function of synaptic GABA\(_A\)-Rs in VB neurons.

**Ethanol has no presynaptic effect on IPSCs in VB neurons**

In many parts of the CNS, alcohol alters synaptic inhibition via a presynaptic mechanism, often by an increase in frequency. These presynaptic effects have been reported in the amygdala and cerebellum, for example (Siggins et al., 2005; Breese et al., 2006; Roberto et al., 2006; Weiner and Valenzuela, 2006). As mentioned above, we recorded evoked IPSCs of VB neurons, and used the well-described paired-pulse stimulation protocol (Zalutsky and Nicoll, 1990), which is widely used to detect a change in transmitter release from presynaptic terminals. In response to paired stimuli (separated by 150 ms), IPSCs in VB relay neurons show substantial paired-pulse
depression. At all concentrations we tested (20 – 100 mM), ethanol had no effect on the paired-pulse ratio. In contrast, baclofen (5 \( \mu \)M), a GABA\(_B\) receptor agonist that acts by decreasing GABA release from the pre-synaptic terminal, significantly increased the paired-pulse ratio from 0.59 ± 0.04 to 0.97 ± 0.17 (p < 0.05, n = 7). These results suggest that ethanol does not modulate synaptic GABA release in the thalamus.
Discussion

Alcohol is one of the most widely abused drugs. Blood alcohol levels between 5-20 mM reduce anxiety and produce mild sedation - these levels are commonly associated with light to moderate intoxication associated with social drinking. 20–50 mM blood ethanol typically elicits profound sedation, cognitive impairment, amnesia and loss of motor coordination. Higher concentrations of ethanol (100mM) in normal individuals cause general anesthesia, decreased ventilation and risk of death (Deitrich and Harris, 1996; Little, 1999). All of these effects are less pronounced in chronic alcoholics, who routinely tolerate extraordinary high levels of the drug. Many of the pharmacological properties of ethanol are shared by drugs such as the benzodiazepines and barbiturates that have long been known to achieve their effects via regulation of the GABA_A-Rs, and a large body of evidence implicates GABA_A-R as an important target for ethanol in the CNS (Martz et al., 1983; Grobin et al., 1998). However, the mechanisms by which ethanol enhances GABAergic transmission are unclear, and may vary substantially among brain regions (Weiner and Valenzuela, 2006).

The main findings of this study are: (i) 50 mM ethanol, but not 20 mM ethanol, reduces firing rate of depolarized VB neurons via GABA_A-Rs; (ii) ethanol (≥ 50 mM) enhances tonic inhibition mediated by extrasynaptic GABA_A-Rs; (iii) 100 mM ethanol, but not 20-50 mM ethanol, exerts a postsynaptic action to prolong IPSCs on VB neurons; (iv) enhancement of tonic currents by ethanol is absent in VB relay neurons from α4 subunit knockout mice; and (v) ethanol has no presynaptic action at inhibitory synapses made by RTN neurons on to VB neurons.

In the present study, we demonstrate that ethanol (≤ 50 mM) does not change the amplitude or decay time of spontaneous IPSCs or evoked IPSCs in VB relay neurons. In contrast, 100 mM ethanol significantly prolongs both spontaneous IPSCs and evoked IPSCs.
This finding is consistent with most previous studies on recombinant “synaptic” GABA\(_\text{A}\)-Rs (\(\alpha_1\beta_\gamma\)2 and \(\alpha_2\beta_\gamma\)2 subtypes) heterologously expressed in cultured cells (Sigel et al., 1993; Mihic et al., 1997) and native synaptic GABA\(_\text{A}\)-Rs in slices of different brain regions (Weiner and Valenzuela, 2006), which suggests that synaptic GABA\(_\text{A}\)-Rs may not act as the direct target of ethanol at sub-anesthetic concentrations (\(\leq 50\text{ mM}\)).

An indirect action of ethanol on GABA\(_\text{A}\)-Rs via presynaptic sites has been observed in many brain regions, including: the amygdala (Roberto et al., 2003; Roberto et al., 2004; Roberto and Siggins, 2006; Zhu and Lovinger, 2006), the cerebellum (Carta et al., 2004; Hanchar et al., 2005; Ming et al., 2006; Kelm et al., 2007), the hippocampus (Ariwodola and Weiner, 2004; Sanna et al., 2004; Galindo et al., 2005) and the nucleus accumbens (Nie et al., 2000; Crowder et al., 2002). However, we were unable to detect any change of spontaneous IPSC frequency or paired-pulse ratio of evoked IPSCs by ethanol (20-100 mM) in VB relay neurons, which suggests that presynaptic GABA release in the thalamus is insensitive to ethanol.

Recently, GABA\(_\text{A}\)-Rs have been shown to be present at extrasynaptic sites as well as subsynaptic sites (Farrant and Nusser, 2005; Mody, 2005). Several groups have begun to look at the potential for alcohol action at extra-synaptic GABA\(_\text{A}\)-Rs. First of all, two groups reported that recombinant \(\alpha_4\beta_2\delta\) (Sundstrom-Poromaa et al., 2002) and \(\alpha_{4/6}\beta_3\delta\) (Wallner et al., 2003) GABA\(_\text{A}\)-Rs are extremely sensitive to alcohol, with enhancement of function noted at alcohol concentrations as low as 3 mM. However, the data in these two studies are inconsistent in terms of the dose-dependent ethanol response at \(\alpha_4\beta_2\delta\) subtype; and two other studies failed to observe the low concentration ethanol effects at recombinant \(\alpha_4\beta\delta\) GABA\(_\text{A}\)-Rs (Borghese et al., 2006; Yamashita et al., 2006). Enhancement of tonic currents mediated presumably by \(\alpha_4\beta\delta\), \(\alpha_4\beta\delta\) or \(\alpha_1\beta\delta\) GABA\(_\text{A}\)-Rs has also been observed in hippocampal or cerebellar brain slices (Wei et al.,
2004; Hanchar et al., 2005; Glykys et al., 2007). These alcohol effects on \( \alpha_6\beta\delta \) GABA\(_A\)-Rs are further exaggerated by a mutation (R100Q) in the \( \alpha_6 \) subunit of the GABA\(_A\)-Rs (Hanchar et al., 2005). Contrasting observations on native extrasynaptic GABA\(_A\)-Rs have been reported by other groups (Carta et al., 2004; Valenzuela et al., 2005; Borghese et al., 2006; Botta et al., 2007; Korpi et al., 2007). The reasons for these discrepancies are still elusive.

In thalamocortical relay neurons and dentate granule cells, \( \alpha_4\beta_2\delta \) GABA\(_A\)-Rs have been shown to be located at extrasynaptic sites and to mediate tonic inhibition (Belelli et al., 2005; Jia et al., 2005; Chandra et al., 2006; Herd et al., 2008). Small, but measurable, tonic currents have been recorded from \( \beta_2 \) knockout mice (Belelli et al., 2005; Herd et al., 2008). The residual tonic currents suggest the possible contribution of the \( \alpha_4\beta_3\delta \) subtype or an unknown subunit compensation induced by gene knockout. Thalamic extrasynaptic GABA\(_A\)-Rs have also been implicated in the action of hypnotics and anesthetics (Belelli et al., 2005; Cope et al., 2005; Chandra et al., 2006; Jia et al., 2008a).

In this study, we found that tonic currents are not significantly enhanced by ethanol at low concentrations (10-30 mM) associated with social alcohol drinking. It seems that extrasynaptic GABA\(_A\)-Rs (mainly \( \alpha_4\beta_2\delta \) subtype) expressed in VB relay neurons are not as sensitive to ethanol as recombinant GABA\(_A\)-Rs expressed in \textit{Xenopus} oocytes (Sundstrom-Poromaa et al., 2002; Wallner et al., 2003), or the extrasynaptic GABA\(_A\)-Rs of dentate granule cells (Wei et al., 2004). However, VB relay neurons are sensitive to sedative or anesthetic concentrations of ethanol (\( \geq 50 \) mM). At 50 mM, ethanol enhances tonic currents by about 50% and decreases the excitability of the relay neurons. The reasons for the discrepancies in this literature are still elusive. They may arise from methodological issues; the differences in expression systems, tissue preparation or recording temperatures, for example, and one must
also consider the possibility that ethanol might indirectly modulate the activity of extrasynaptic GABA_A-Rs, for example: ethanol might enhance tonic inhibition by stimulating the non-vesicular release of taurine (Olive, 2002), a potent agonist of these receptors (Jia et al., 2008b).

We have shown previously that gabazine, an antagonist of GABA_A-Rs, has little effect on the holding current in VB relay neurons from Gabra4^/- mice (Chandra et al., 2006), which is consistent with a loss of these extrasynaptic receptors. Similarly, 50 mM ethanol failed to evoke any significant current shift in VB neurons from Gabra4^/- mice. At this concentration, ethanol does not change the properties of IPSCs from wildtype and Gabra4^/- mice either. Therefore, ethanol (~ 50 mM) selectively enhances the activity of extrasynaptic GABA_A-Rs containing α4 subunits. Similar ablation of ethanol-induced tonic currents has also been shown in dentate gyrus neurons from Gabra4^/- mice (Liang et al., 2008).

Tonic inhibition mediated by extrasynaptic GABA_A-Rs plays a crucial role in regulating excitability at the level of individual neurons and within neuronal networks (Semyanov et al., 2004). In the present study, we show that the tonic firing in VB relay neurons was decreased by 50 mM ethanol but not 20 mM. In addition, pre-applied gabazine occluded the inhibition of tonic firing by 50 mM ethanol, which indicates that GABA_A-Rs are critical for the inhibitory effects of ethanol in the thalamus.

Alcohol not only has sedative effects that can promote sleep onset, but also causes abnormal sleep patterns (Kubota et al., 2002). After drinking alcohol, the time spent in deep (slow-wave) sleep is increased, whereas the time spent in the dreaming state (rapid eye movement sleep) is decreased. There can be little argument concerning the pivotal role of the thalamus in controlling the sleep-wake transition and in sensory transmission. The thalamocortical circuit represents an important potential target for alcohol, as reflected in the
EEG changes that occur during drinking. A previous *in vivo* study demonstrates that THIP, a hypnotic, works through extrasynaptic GABA$_A$-Rs that contain the δ subunits (Winsky-Sommerer et al., 2007). Our results indicate that extrasynaptic GABA$_A$-Rs may also play a role in the action of sedative concentrations of ethanol (~50 mM) in the thalamus. Given that sleep disturbances have been suggested to play a reciprocal role in the progression of alcoholism (Brower et al., 1998; Brower, 2001), the extrasynaptic GABA$_A$-Rs in the thalamus may be a potential therapeutic target for the treatment of alcoholism.
ACKNOWLEDGEMENTS:

We thank Carolyn Ferguson for expert assistance. We thank Dr. Angelo Keramidas and Lindsay Tannenholz for the critical reading for the manuscript. We also thank Felix Wolf (Research Animal Resource Center - RARC, Weill Medical College, Cornell University), and the RARC staff for their assistance.
REFERENCES


Botta P, Mameli M, Floyd KL, Radcliffe RA and Valenzuela CF (2007) Ethanol sensitivity of GABAergic currents in cerebellar granule neurons is not increased by a single amino acid change (R100Q) in the α6 GABA_A receptor subunit. *J Pharmacol Exp Ther* **323**:684-691.


Jia F, Yue M, Chandra D, Homanics GE, Goldstein PA and Harrison NL (2008a) Isoflurane is a potent modulator of extrasynaptic GABA<sub>A</sub> receptors in the thalamus. *J Pharmacol Exp Ther* **324**:1127-1135.


Footnotes:

The work was supported by grants from the NIH (AA 16393 to NLH, AA 13004 and GM 47818 to GEH).
Legends for Figure

Figure 1. Ethanol (50 mM) decreases the excitability of VB neurons via GABA\(_A\)-Rs.

A, Representative current clamp traces demonstrate action potential (AP) firing evoked by current steps (40-160 pA, duration 500 ms) in a VB neuron. After ethanol (50 mM) perfusion, AP firing decreased, and the input-output curve shifted rightwards.

B, Representative current clamp traces demonstrating AP firing evoked by current steps in the presence of gabazine (20 µM) in another VB neuron. Ethanol (50 mM) perfusion failed to change the input-output curves when GABA\(_A\)-Rs were blocked.

C, Exemplar current trace demonstrating that 20 mM ethanol induced no change on firing rate evoked by depolarized current steps. On average, ethanol (20 mM) also makes no change on the numbers of APs (9.6 ± 1.5 vs. 9.4 ± 1.6, p > 0.05, n = 5). N.S.: not significant.

D, Pooled data show that 50 mM ethanol significantly reduced the number of evoked action potentials, from 10.9 ± 1.4 to 8.5 ± 1.4 (**: p < 0.01, n = 6).

E, When GABA\(_A\)-Rs are blocked by gabazine, 50 mM ethanol fails to inhibit tonic action potential firing (12.5 ± 1.7 to 12.4 ± 1.9, p > 0.05, n = 5).

Figure 2. Ethanol (≥ 50 mM) enhances tonic currents mediated by extrasynaptic GABA\(_A\)-Rs.

A, Typical voltage-clamp recordings of four VB neurons in response to the applications of different concentrations (10-100 mM) of ethanol. Ethanol (≥ 50 mM) induced substantial current-shifts.
The averaged current-shifts elicited by ethanol are dose-dependent (10 mM: 0.8 ± 0.3 pA, n = 12; 20 mM: 1.8 ± 0.7 pA, n = 18; 30 mM: 2.4 ± 1.2 pA, n = 10; 50 mM: 9.5 ± 1.5 pA, n = 19; 100 mM: 19.0 ± 3.6 pA, n = 12).

20 µM gabazine occluded the enhancement of tonic currents by 50 mM ethanol, and revealed the background tonic current. The dotted trace and Gaussian fittings were made from the raw trace as described in the Method. Tonic currents before and after ethanol application in this case are 43.4 and 62.2 pA respectively.

Each point corresponds to the tonic currents before (x-axis) and after (y-axis) ethanol application from individual experiment similar to the one shown in C. The points analyzed from twelve experiments were fitted by a straight line pretty well (R = 0.98). The slope of the fitted line is 1.50, well above the unitary line (y=x, the gray dashed line).

Figure 3. Ethanol-induced current-shift is absent in VB neurons from mice lacking the GABA$_A$-R $\alpha_4$ subunit.

Ethanol (50 mM) evoked a holding current shift (~10 pA) in a VB neuron from a wild-type mouse. In contrast, ethanol produced no current shift in a VB neuron from a Gabra4$^{-/-}$ mouse. WT: wild-type; KO: knockout.

Bar graph demonstrates that ethanol (50 mM) induced current shifts in wild-type, but not $\alpha_4$ knockout, VB neurons (knockout: 1.7 ± 0.9 pA, n = 10; wild-type: 8.6 ± 2.6 pA, n = 6; **: p < 0.01).

Figure 4. The tonic currents evoked by acamprosate at high concentrations.

A typical recording of a VB neuron in response to 1 µM acamprosate.
B. Pooled data show that 200 and 500 µM, but not 1-100 µM, acamprosate elicits significant current-shift in VB neurons.

**Figure 5. The effects of ethanol on spontaneous IPSCs.**

A. A typical recording of spontaneous IPSCs in a VB neuron in the absence and presence of 50 mM ethanol. Averaged spontaneous IPSC traces before (black) and after (gray) ethanol application are superimposed to illustrate the similarity in amplitude and decay time.

B. A representative experiment shows that the frequency, amplitude and decay time of spontaneous IPSCs did not change during the ethanol application for more than 20 minutes.

C. Pooled data demonstrate that spontaneous IPSCs are largely insensitive to ethanol. Only 100 mM ethanol significantly increases the decay time of spontaneous IPSCs (**: p < 0.01).

**Figure 6. The effects of ethanol on evoked IPSCs and paired-pulse depression.**

A. Exemplar evoked IPSC traces demonstrating that 100 mM ethanol increase the decay time, but not the amplitude of evoked IPSCs.

B. Average data show that evoked IPSCs are insensitive to ethanol less than 100 mM. Only 100 mM ethanol increased the decay time of evoked IPSCs significantly (****: p < 0.001).

C. Sample traces showing paired-pulse responses before (in dark) and after 100 mM ethanol (in gray) application. The superimposed traces clearly show the similar degree of paired-pulse depression.

D. Bar graph demonstrates that paired-pulse ratio is insensitive to ethanol (20-100 mM), which indicates that the presynaptic GABA release probability is not modified by ethanol.
Figure 1

(A) Control vs. 50 mM Ethanol

(B) Gabazine vs. 50 mM Ethanol + Gabazine

(C) Control vs. 20 mM Ethanol

(D) Control vs. 50 mM Ethanol

(E) Gabazine vs. 50 mM Ethanol + Gabazine
Figure 2
Figure 3

A

WT

KO

50 mM Ethanol

50 mM Ethanol

100 s

20 pA

B

Ethanol-induced Current (pA)

WT

KO

*
Figure 4
Figure 5

(A) Control, 50 mM Ethanol, Superimposed

(B) 50 mM Ethanol

- Frequency (Hz)
- Amplitude (pA)
- Decay time (ms)

(C) Percentage Change (%)

- 20 mM Ethanol
- 50 mM Ethanol
- 100 mM Ethanol

Decay time, Amplitude, Frequency