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Ethanol effects on GABA-gated current in a model of increased $\alpha 4\beta\delta$ GABA_A receptor expression depend on time course and preexposure to low concentrations of the drug

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Abstract

Several recent studies have suggested that $\alpha\beta\delta$ subtypes of γ -aminobutyric acid type A (GABA_A) receptors (δ -GABAR) are a target for low dose ethanol (< 30 mM). However, there are also conflicting reports suggesting that only high doses of the drug (100 mM) modulate these receptors. In addition, the studies which have demonstrated a clear effect of low dose ethanol on δ -GABAR find different effective concentrations for this effect. Here, we test the hypothesis that the apparent disparity in effective concentration is due to time-course effects when low (1–3 mM) dose ethanol is preapplied. To this end, we tested ethanol effects on native GABAR in CA1 hippocampus in a model of increased $\alpha 4\beta\delta$ GABAR expression following 48 h administration of the GABA-modulatory steroid THP (3 α -OH-5 β -pregnan-20-one) to adult, female rats. GABA(EC₂₀)-gated current was recorded with whole-cell patch clamp procedures from acutely isolated pyramidal cells. We assessed ethanol's effect on GABA-gated current using either (1) 2–5 min application of ethanol in increasing concentrations (0.1–30 mM) or (2) coadministration of ethanol with GABA. Two minute application of 1–3 mM ethanol produced optimal potentiation of GABA-gated current following steroid treatment, with higher concentrations less effective. In contrast, 30 mM ethanol produced optimal effects when ethanol was not preapplied. However, following preapplication of 1 mM ethanol, 30 mM ethanol decreased the peak GABA-gated current. These findings suggest that ethanol may act at multiple interacting sites to affect GABAR efficacy and desensitization. These data also suggest that ethanol effects on GABA-gated current are affected by the time course of exposure and previous exposure to low concentrations of the drug.

Keywords

Delta subunit; GABA_A receptor; GABA; Ethanol; Alcohol; Acute tolerance; Allopregnanolone

Introduction

Evidence of low dose ethanol effects on the γ -aminobutyric acid type A (GABA_A) receptor (GABAR) was initially provided by pharmacological and behavioral findings. First, the effects of ethanol mirror those of traditional GABA-modulatory drugs such as benzodiazepines and barbiturates (Luddens and Korpi, 1995). That is, low doses of ethanol reduce anxiety, but higher doses of the drug are sedative and can be anesthetic (Ueno et al., 2001). Secondly, chronic intake of ethanol and its withdrawal alter GABAR subunit composition (Devaud et al., 1997; Mahmoudi et al., 1997), with corresponding changes in receptor pharmacology, the level of

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tonic inhibition, and kinetics of synaptic current (Liang et al., 2004). These changes, in turn are tightly correlated with withdrawal properties, which are similar to those of GABA-modulatory benzodiazepines, that is, generating anxiety and seizures (Ueno et al., 2001). Furthermore, GABA agonists effectively substitute for ethanol in drug discrimination tasks (Engel et al., 2001) and exhibit cross-tolerance with other GABA-modulatory drugs (Buck and Harris, 1990), suggesting a common underlying reward mechanism. In addition, GABAR antagonists and negative modulators decrease ethanol consumption (Koob et al., 1988), while blocking GABA reuptake eliminates ethanol reward (Nguyen et al., 2005), indicating that effects of low dose ethanol on the GABAR mediate some of the reinforcing properties of ethanol.

It is clearly evident from clinical and behavioral studies in humans, as well as anecdotal reports, that low concentrations of ethanol (3–30 mM), as would be produced by social drinking, cause perceptible changes in mood (Doty et al., 1997). Gender differences have also been described (Nyberg et al., 2004b). In fact, an intravenous infusion producing a 3 mM ethanol blood concentration (~1/2 glass of wine in a 120 lb woman (Kalant et al., 1975)) has been shown to produce “relaxation” in women but not in men (Nyberg et al., 2004b). This effect was further verified by a slowing of eye saccade velocity, an objective measure of GABAergic tone, following the ethanol infusion (Nyberg et al., 2004b). Yet despite these indirect behavioral indices of low dose ethanol effects on GABAergic systems, until recently, there has been little or no evidence that low dose ethanol can influence the GABAR directly, although a number of studies have reported effects of low dose ethanol on native GABAR (Roberto et al., 2003; Wan et al., 1996), which cannot always be definitive measures of a postsynaptic action. In contrast, there is a body of evidence demonstrating that high, anesthetic doses of the drug (100–200 mM) potentiate GABA-gated current via a well-defined site within TM2–TM3, which is also a binding pocket for certain volatile anesthetics (Mihic et al., 1997).

Recently, two laboratories (Sundstrom-Poromaa et al., 2002; Wallner et al., 2003) demonstrated that GABAR containing the $\alpha 4$ and δ subunits are sensitive to low concentrations of ethanol (< 30 mM). The experiments were conducted using either $\alpha 4\beta 2\delta$ and/or $\alpha 4\beta 3\delta$ GABAR expressed in oocytes. In both cases, the increases in GABA-gated current produced by low dose ethanol at $\alpha 4\beta \delta$ GABAR were not seen in either $\alpha 4\beta 2$ or $\alpha 4\beta 3$ heteromers, suggesting that inclusion of the δ subunit is essential for this effect. In both cases, expression of $\alpha 4\beta \delta$ was verified by an increased responsiveness to gaboxadol (4,5,6,7-Tetrahydroisoxazolo[5,4-c]pyridin-3-ol or THIP, Brown et al., 2002), a GABA agonist that has increased efficacy at receptors containing the δ subunit.

Further evidence that $\alpha 4\beta \delta$ GABAR are indeed a target for low dose ethanol is provided by the high sensitivity of native $\alpha 4\beta \delta$ GABAR (Sundstrom-Poromaa et al., 2002), which increase expression following withdrawal from the GABA-modulatory steroid THP (3 α -OH-5 β -pregnan-20-one), in a rat model of premenstrual syndrome (Smith et al., 1998). In this model, $\alpha 4$ and δ subunits were seen to coexpress, as determined using coimmunoprecipitation techniques (Sundstrom-Poromaa et al., 2002). The expression of $\alpha 4\beta \delta$ GABAR was also verified pharmacologically by an increased responsiveness of acutely isolated pyramidal cells to gaboxadol compared to GABA (Sundstrom-Poromaa et al., 2002). Under these conditions, low dose ethanol (1–3 mM) enhanced GABA-gated current, assessed using whole cell patch clamp techniques on pyramidal neurons acutely isolated from CA1 hippocampus. In addition, this increased responsiveness to ethanol was reflected behaviorally, where low 0.2–0.4 g/kg doses of ethanol decreased anxiety following progesterone withdrawal, assessed using the acoustic startle response as a model of anxiety behavior (Smith et al., 2004).

The GABAR is a pentamer, which is normally comprised of 2 α , 2 β , and 1 γ subunits (Chang et al., 1990). However, δ can substitute for the γ subunit, yielding a receptor with a high

sensitivity to GABA (Sundstrom-Poromaa et al., 2002) and which does not readily desensitize (Brown et al., 2002; Haas and Macdonald, 1999). As such, it is perfectly suited for the extrasynaptic sites where it has been localized (Wei et al., 2003). These receptors have relatively low expression in the brain (Wisden et al., 1992), but express at high levels in the granule cells of the dentate gyrus, where they generate a tonic current (Stell et al., 2003; Stell and Mody, 2002). Recent studies have demonstrated that low dose (30 mM) ethanol can enhance this tonic current in dentate gyrus (Wei et al., 2004), but not in the CA1 hippocampus, where tonic inhibition is mediated by $\alpha 5\beta 3\gamma 2$ GABAR (Caraiscos et al., 2004), a receptor relatively insensitive to modulation by ethanol (Sundstrom-Poromaa et al., 2002). A more recent study (Glykys et al., 2007) has also shown that $\alpha 1\beta \delta$ GABAR, which express on the GABAergic interneurons in the dentate gyrus, are also responsive to low dose ethanol. Here too, 30 mM ethanol enhanced the tonic current without altering the synaptic current. Thus, these results suggest that low dose ethanol can affect multiple components of the dentate gyrus circuit, where the net effect of the drug may be difficult to predict.

Low dose ethanol effects on $\alpha 4\beta 3\delta$ GABAR are prevented by the benzodiazepine partial inverse agonist RO15-4513 (Wallner et al., 2006), a compound that has been suggested as an alcohol antagonist (Suzdak et al., 1986a). Another study (Hancher et al., 2006) showed that low concentrations of ethanol displace binding of RO15-4513 from the immunopurified δ subunit as well as recombinant $\alpha 4\beta 3\delta$ GABAR, suggesting that these drugs bind to the same site on the receptor. Taken together, these findings provide compelling evidence that $\alpha 4\beta \delta$ and $\alpha 1\beta \delta$ GABAR are a target for low dose ethanol.

However, several recent studies have failed to confirm that low dose ethanol affects $\alpha 4\beta \delta$ GABAR (Borghese et al., 2006; Yamashita et al., 2007). Two separate studies using heterologous expression systems (Borghese et al., 2006; Yamashita et al., 2007) have been unable to detect any effect of ethanol at doses < 100 mM. The reasons for this discrepancy are not clear, but they may due to the possibility that the GABA-gated current recorded reflected mixed populations of $\alpha 4\beta \delta$ with $\alpha \beta$ heteromers, which are insensitive to ethanol (Sundstrom-Poromaa et al., 2002; Wallner et al., 2003). Although both groups of studies verified an increased gaboxadol sensitivity to indicate inclusion of the δ subunit, variations in the amplitude of the baseline current across these studies may reflect heterogeneous populations of receptors (Borghese et al., 2006). Other possibilities include variations in phosphorylation state (Choi et al., 2002) or other intracellular messengers, which are known to influence ethanol responses.

Several recent studies have also failed to observe effects of low dose ethanol on the tonic GABAergic current recorded from regions which express high levels of δ -containing GABAR, such as dentate gyrus (Borghese et al., 2006) and cerebellar granule cells (Carta et al., 2004; Yamashita et al., 2007). The conditions under which these studies were conducted, in fact, differed from those that observed ethanol effects (Wei et al., 2004). First, younger animals were used which may not have achieved high levels of δ subunit expression (Borghese et al., 2006), because these receptors are developmentally regulated (Laurie et al., 1992). Secondly, levels of tonic inhibition may not have been sufficient under recording conditions where tetrodotoxin (TTX) was used (Carta et al., 2004). In sum, for both the recombinant and the native receptor studies, the lack of ethanol effect may be directly related to a lack of δ -GABARs or insufficient levels of GABAR activation.

The effects of ethanol on GABAR function are complex, however, because the studies that revealed significant ethanol modulation of $\alpha 4\beta \delta$ GABAR found a different effective concentration range for this effect (Sundstrom-Poromaa et al., 2002; Wallner et al., 2003). In our initial report (Sundstrom-Poromaa et al., 2002), recombinant $\alpha 4\beta 2\delta$ GABAR were responsive to very low concentrations of ethanol (1–3 mM), but less responsive to higher (>

10 mM) concentrations of the drug. In contrast, Wallner et al. (2003) have shown that $\alpha 4\beta 2\delta$ respond to higher (30 mM) concentrations, while $\alpha 4\beta 3\delta$ begin to show an effect at 3 mM, but this does not decrease with increasing concentrations of ethanol. In attempting to resolve these issues in the present study, we tested the possibility that these differences might be due to the time course of exposure required for ethanol effects and possible effects of prolonged ethanol exposure on receptor desensitization. To this end, we tested ethanol responsiveness of acutely isolated CA1 pyramidal cells which we have shown have elevated expression of $\alpha 4$ and δ GABAR subunits (Shen et al., 2005) to ethanol applied either with or without a 2-min preapplication period to mimic the conditions used in these two recombinant studies. Differential effects of ethanol across these two different exposures may be relevant for the well-established relationship between the speed of ethanol consumption and ethanol effect (Hiltunen et al., 2000). Further, the “inverted U” dose–response effect of ethanol in our studies may also be relevant for acute tolerance (Ponomarev and Crabbe, 2004; Quertemont et al., 2003) reported across a slower ethanol consumption period, when an initial lower concentration of ethanol produces a greater effect than later higher concentrations.

For this study, we tested effects of ethanol on CA1 hippocampal pyramidal cells after 48 h exposure of female rats to THP when we have shown that $\alpha 4$ and δ subunits are increased in CA1 hippocampus (Shen et al., 2005). This has been verified both using Western blot procedures and selective pharmacological tests on the tonic current.

Methods

Animals

Adult, female Long Evans rats (120–140 g) were used for all studies. Animals were housed in groups of three under-controlled conditions (14:10 light:dark cycle, 24°C) with free access to food and water. All conditions were in accordance with IACUC guidelines. Animals were tested on the day of diestrus 1, a low-hormone state, to minimize variability in endogenous hormone levels. Estrous cycle stage was determined by vaginal lavage and vaginal impedance measurements as described by Gulinello and Smith (2003). Animals were injected intraperitoneally (i.p.) with 10 mg/kg $3\alpha,5\beta$ -THP (3α -OH- 5β -pregnan-20-one or preganolone, in oil) or vehicle, once daily for 3 days across a 48 h period, and tested 1–2 h after the last injection. In some cases, animals were injected i.p. with 0.5 g/kg ethanol or saline (3 $\times$ every 40 min) for the final 2 h of the steroid administration protocol (Smith and Gong, 2004).

Patch clamp electrophysiology

Pyramidal neurons were acutely dissociated from CA1 hippocampus and GABA-gated Cl^- currents analyzed using whole-cell patch-clamp procedures as previously described by Smith et al. (1998). Briefly, tissue was digested at 32°C for 50–60 min under 100% O_2 in 1,4 piperazinediethane-sulfonic acid (PIPES)-buffered saline containing (in mM) NaCl 120, KCl 5, CaCl_2 1, MgCl_2 1, D-glucose 25, PIPES 20 and trypsin (type XI, sigma) 0.8 mg/ml, and pH 7.0. Following a 1 h enzyme-free incubation at room temperature, tissue was dissociated by trituration in 1 ml of 20 mM N-(2-hydroxyethyl)piperazine-N'-2-ethane sulfonic acid (HEPES)-buffered Dulbecco's Modified Eagle's Medium (DMEM) which was replaced by recording medium following transfer to the recording chamber. GABA-gated current was recorded at room temperature (20–25°C) at a holding potential of –50 mV in a bath containing (in mM) NaCl 120, CsCl 5, CaCl_2 2, MgCl_2 1, tetraethylammonium-cl (TEA Cl) 15, 4-aminopyridine 5, HEPES 10 and glucose 25, pH 7.4, and 320 mOsm/kg H_2O . The pipet solution contained (in mM) N-methyl-D-glucamine chloride 120, 1,2-bis(2-aminophenoxy)ethane-N,N,N',N'-tetraacetate (Cs_4 BAPTA) 5, and Mg-ATP 5. The ATP regeneration system Tris phosphocreatine (20 mM) and creatine kinase were added to minimize GABA rundown.

Drug delivery was accomplished with a superfusion system positioned within 50 μ M of the cell, to achieve 400 ms drug exposure times. Ethanol (0.1–30 mM) was applied in increasing concentrations either preapplied (2 min) or concomitant with GABA ($EC_{20} = 10 \mu$ M). Peak currents were calculated for all drug concentrations as % GABA potentiation relative to the current gated by 10 μ M GABA. Currents were recorded using an Axopatch 1D amplifier (Axon Instruments) filtered at 2 kHz (four-pole Bessel filter) and detected at 10 kHz using pClamp 5.1.

Drugs and steroids

Most chemicals were purchased from Sigma Chemical Company, except for 3 α ,5 β -THP which was purchased from Steraloids (Maine), and BAPTA which was purchased from Calbiochem.

Statistical analysis

Differences between two groups were analyzed using the Student's *t*-test. When concentration–response curves were analyzed, a one-way ANOVA was used to compare differences between groups, followed by the Student–Newman–Keuls post hoc analysis. Significant differences between groups were determined when $P < .05$.

Results

Ethanol effects on GABA-gated current

To test the modulatory effects of ethanol on GABA-gated current following 48 h THP administration, two different drug application protocols were implemented. When preapplied (2 min) prior to the GABA pulse, ethanol effects on GABA-gated current were maximal at lower concentrations of the drug (Fig. 1A, B). Following 48 h THP treatment, ethanol potentiated GABA-gated current by $70 \pm 1.2\%$ at an optimal concentration of 1 mM. Increasing concentrations were not as effective, with 30 mM ineffective in significantly altering GABA-gated current above predrug levels. In contrast, when ethanol was applied concomitant with GABA, without ethanol preapplication, a different concentration–response curve was obtained (Fig. 2), where increasing concentrations produced greater effects on the current following 48 h THP treatment. In this case, a concentration of 10 mM ethanol was the threshold dose for significant ethanol effects on GABA-induced current. In contrast, none of the concentrations tested produced significant effects on GABA-gated current recorded from control neurons.

In vivo ethanol preadministration

Because our previous study (Smith and Gong, 2004) suggests that in vivo administration of a low dose of ethanol (0.5 g/kg, 3 $\times$, across a 2 h period) prevents the increase in $\alpha 4$ expression after 48 h THP treatment, we implemented this in vivo ethanol preadministration paradigm prior to cell isolation and electrophysiological tests. Under these conditions, in vivo ethanol preadministration prevented the increase in ethanol sensitivity observed following 48 h THP administration (Fig. 3), when ethanol was unable to significantly potentiate GABA-gated current at any dose tested, similar to control. In vivo administration of vehicle, however, did not prevent the increase in ethanol sensitivity following 48 h THP treatment.

Discussion

The results from the present study demonstrate an increased responsiveness to ethanol following short-term administration of a neurosteroid to female rats. Further, the effective concentration of ethanol on acutely isolated CA1 pyramidal cells was dependent upon the time course and sequence of ethanol exposure; preapplication of sequentially increasing concentrations of ethanol resulted in a robust response to low (1 mM) concentrations of ethanol,

which increased GABA-gated current, but decreased responses to higher concentrations of the drug. In contrast, higher concentrations were highly effective when applied to isolated pyramidal neurons without preapplication of lower concentrations. Thus, the effective ethanol concentration for enhancing GABA-gated current was dependent upon preexposure of neurons to low concentrations of the drug.

The increase in ethanol sensitivity following short-term THP treatment is most likely due to increased hippocampal expression of $\alpha 4\beta\delta$ GABAR. Our previous work demonstrated that 48 h THP administration paradigm increases expression of both the $\alpha 4$ and δ GABAR subunits in CA1 hippocampus (Shen et al., 2005). Coexpression of $\alpha 4$ and δ subunits was suggested (Shen et al., 2005) by pharmacological findings demonstrating an increased response to the GABA agonist gaboxadol and inhibitory effects of the trivalent cation, La^{3+} on CA1 pyramidal cells, acutely isolated or recorded in the slice. These findings are characteristic of increased expression of $\alpha 4\beta\delta$ GABAR, for which gaboxadol produces a greater maximal current than GABA and which are nearly completely inhibited by La^{3+} (Brown et al., 2002). In contrast, most other receptors are either unaffected or potentiated by La^{3+} (Saxena et al., 1997; Zhu et al., 1998). The increased response of native GABAR to 1–3 mM ethanol after short-term steroid treatment in the present study is nearly identical to what we have observed in recombinant $\alpha 4\beta 2\delta$ receptors as well as following steroid withdrawal (Sundstrom-Poromaa et al., 2002), when hippocampal $\alpha 4\beta\delta$ GABAR expression is increased.

Significant potentiating effects of 1–3 mM ethanol on GABA-gated current were only seen when the drug was preapplied for 30 s to 2 min. This is consistent with our findings in recombinant GABAR (Sundstrom-Poromaa et al., 2002). The longer exposure time required for peak potentiation by 1 mM ethanol may be due to the lower open probability reported for $\alpha 4\beta\delta$ GABAR (Akk et al., 2004) or to allosteric effects which would require a longer exposure (Jung et al., 2005). Alternatively, second messenger systems, such as phosphorylation (Song and Messing, 2005), may also play a role.

Temporal factors involved in the effect of low dose ethanol have been reported previously. In fact, one recent study (Serra et al., 2006) demonstrated that low dose (25 mM) ethanol increased inhibitory current in hippocampal neurons under conditions of high $\alpha 4\beta\delta$ GABAR expression. This effect increased progressively over time, with peak level noted by 20 min after ethanol exposure. Although presynaptic effects cannot be ruled out, the results from this study imply that ethanol actions can require extended time for a maximal effect.

In addition, early studies showed low dose ethanol effects on Cl^- flux in brain homogenates containing synaptoneurosomes (Morrow et al., 1988; Suzdak et al., 1986b; Suzdak and Paul, 1987) or in cell cultures (Ticku et al., 1986). These assays showed clear effects of low dose ethanol (3–30 mM) without the addition of GABA suggesting that high affinity, presumably extrasynaptic GABAR that were activated by low, ambient levels of GABA. In these studies, as well, a longer time course was necessarily used.

However, when tested without preapplication, 30 mM ethanol produced optimal potentiation of GABA-gated current. These results are similar to findings from Wallner et al. (2003), who used a paradigm where GABA was continuously applied, while ethanol was administered in pulses, without extensive preapplication. Similarly, a single application of 30 mM ethanol has been shown to potentiate tonic current in the dentate gyrus, where $\alpha 4\beta\delta$ or $\alpha 1\beta\delta$ GABAR are expressed at high levels (Glykys et al., 2007; Wei et al., 2004). GABAR other than δ -GABAR have also been shown to respond to low dose ethanol. In one recent report (Akk et al., 2007), low dose ethanol increased the affinity of the steroid site on recombinant $\alpha 1\beta 2\gamma 2$ GABAR for 3 α -OH-5 α -pregnan-20-one (3 α ,5 α -THP). In this study, concentrations of ethanol as low as 17

mM lowered the effective steroid concentration by two orders of magnitude for increasing single channel open time.

However, the results from the present study suggest that the effect of 30 mM ethanol was dependent upon previous exposure to lower concentrations of the drug. Preapplication of increasing concentrations of ethanol, or a single prolonged exposure to 1 mM ethanol, prevented the potentiating effects of higher concentrations. The most likely explanation for this phenomenon is that the low concentration, acting through a high affinity site, accelerates desensitization in response to the higher concentration of ethanol, acting through a low affinity site. Although this would be a novel phenomenon, a recent study has also shown that low 10 mM concentrations of ethanol selectively stabilize the desensitized state of alpha-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA receptors (Moykkynen et al., 2003), effectively decreasing steady state current without altering the rate of desensitization. In addition, there may be separate desensitizing effects of low dose ethanol at the ethanol site.

The mechanism of low dose ethanol effects on receptor desensitization is currently unknown, but will be tested in future studies. Recent reports have demonstrated desensitized states of the GABAR, which precede channel opening (Burkat et al., 2001), suggesting that low levels of receptor activation can lead to slow desensitized states. Other reports demonstrating slow desensitization of GABAR by low concentrations of GABA have appeared (Chang et al., 2002; Overstreet et al., 2000). If proven to occur with low dose ethanol, this would be the first demonstration of a desensitizing effect of a GABA modulator on the receptor.

Alternatively, inhibitory actions at a second receptor subtype could also explain such an effect. Inhibitory effects of ethanol have been identified on other atypical GABA receptors, such as the homomeric rho 1 receptor (Mihic and Harris, 1996), where mutations of the TM2-TM3 region abolish the effect (Mihic et al., 1997), suggesting a structural mechanism for such an action. In contrast, this biphasic effect of ethanol may be due to effects of higher doses of ethanol (10 mM) directed at other receptors.

Thus, these studies are consistent with the idea that the $\alpha 4\beta\delta$ GABAR is a highly sensitive target for ethanol, as is the homologous $\alpha 6\beta\delta$ GABAR (Wallner et al., 2003). It would be predicted that similar experiments carried out in untreated animals using acutely isolated dentate gyrus granule cells or thalamic relay neurons, which normally express high levels of $\alpha 4\beta\delta$ GABAR (Wisden et al., 1992), would yield similar results as the present study, with GABA responses highly sensitive to low concentrations of ethanol. Low dose ethanol effects in animals not treated with steroids may in fact be due to actions at these CNS sites. In contrast, the increase in $\alpha 4$ and δ subunits after 48 h steroid treatment may produce a greater response to the lower doses of ethanol. Future studies comparing the behavioral effects of ethanol in isolated neurons of untreated animals are warranted, where results could be compared with those from $\alpha 4$ or δ knockout animals. Additional studies examining low dose ethanol effects in neuronal systems could also attempt to isolate δ -GABAR with the use of selective blockers of synaptic GABAR and $\alpha 5$ -containing GABAR.

In support of the idea that δ -containing GABAR mediate effects of low dose ethanol are the findings in δ knockout mice of reduced ethanol consumption and decreased anti-convulsant effects of ethanol (Mihalek et al., 2001). However, the discriminative stimulus effects of ethanol are also intact in the δ knockout mouse (Shannon et al., 2004), suggesting that other receptors are involved. For these studies, compensatory changes in other channels or receptors may have been produced by knocking out the δ subunit, and such changes may also play a role in the ethanol response.

In addition to wild-type δ -containing GABAR, a recent mutation found in rodent cerebellar granule cells, $\alpha 6$ (R100Q) $\beta 3\delta$ (Hanchar et al., 2005) possesses an even greater sensitivity to

ethanol, which is reflected physiologically by increased ethanol responses of the tonic current and reflected behaviorally by ethanol-induced ataxia at a low dose of the drug.

In addition to acute preapplication protocols, we also used an *in vivo* preadministration protocol. Prolonged administration of a low dose of ethanol, *in vivo*, prevented the increase in ethanol responsiveness produced by 48 h THP. This effect is likely due to downregulation of the $\alpha 4$ subunit, which we have previously shown (Smith and Gong, 2004) is reduced by this ethanol administration paradigm following 48 h THP exposure. In contrast, effects on receptor desensitization may also have been produced by the ethanol preexposure.

The time course of ethanol application in the present study may be relevant for social consumption, where ethanol intake can take place across an extended period of time. In fact, in a recent study (Nyberg et al., 2004a, 2004b), intravenous administration of a low concentration (0.2 g/kg) of ethanol to simulate social drinking resulted in a 3 mM blood ethanol concentration after a 5 min infusion, when it altered mood, a dose, and time course nearly identical to that reported here. Effects of isolated application of higher concentrations of ethanol, in contrast, may be relevant for more rapid consumption of ethanol, which leads to intoxication in response to fast elevation of blood alcohol levels. There is considerable evidence that the rate of increase in blood alcohol concentration (BAC) is an important factor in determining ethanol effects (Hiltunen, 1997), as is the exposure time.

The inverted U concentration–response relationship observed in the present study may be relevant for acute tolerance to ethanol reported following *in vivo* ethanol administration in animals (Ponomarev and Crabbe, 2004; Quertemont et al., 2003; Tampier and Quintanilla, 2002) and humans (Bennett et al., 2005; Hiltunen et al., 2000). That is, the response to a low concentration of ethanol during the initial rise in BACs can, in some cases, produce a greater effect behaviorally than higher concentrations achieved after prolonged exposure. This type of acute tolerance appears to be greater in individuals at risk for developing alcoholism (Cohen et al., 1993), suggesting a possible reinforcing effect on ethanol consumption. In fact, ethanol-preferring rats that voluntarily consume ethanol also exhibit a heightened behavioral response to low dose ethanol, where increasing doses have less of an effect (Jones et al., 2000).

The 48 h THP treatment used in the present study may produce conditions relevant for premenstrual dysphoric disorder (PMDD), when anxiety and dysphoric mood are reported early in the luteal phase (Endicott et al., 1999), shortly after the increase in circulating levels of progesterone (P) and its THP metabolite. In fact, our earlier studies have demonstrated that 48 h THP administration also increases anxiety in female rats (Gulinello et al., 2001), an effect closely correlated with increased expression of $\alpha 4$ and δ GABAR subunits (Shen et al., 2005). Interestingly, one study reported increased responsiveness to low concentrations of ethanol in women with PMDD (Sundstrom et al., 1998), although a second study (Nyberg et al., 2004a) by the same group found this increased sensitivity to be nonsignificant due to variability in response. Acute tolerance to higher concentrations of the drug has also been noted in PMDD women (I. Sundstrom-Poromaa, personal communication), suggesting an additional comparison with the findings from the present study.

In conclusion, the results from the present study suggest that under conditions of increased expression of $\alpha 4\beta\delta$ GABAR, low dose ethanol significantly enhances GABA-gated current. The effective concentration for this effect was dependent both upon the time course and sequence of administration of different doses of the drug. It is possible that ethanol-induced desensitization of $\alpha 4\beta 2\delta$ GABAR may explain the apparent discrepancy in dose-related effects of ethanol on these receptors.

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Fig. 1.

Low concentrations of ethanol potentiate γ -aminobutyric acid (GABA)-gated current following short-term neurosteroid treatment. Ethanol-potentiated GABA-gated current recorded with whole-cell recording techniques from pyramidal cells acutely isolated after 48 h THP (3 α -OH-5 β -pregnan-20-one) treatment, an effect dependent upon the mode of drug application. (A, B) When preapplied (2–5 min) before the GABA pulse, low (1 mM) concentrations of ethanol (ETOH) significantly potentiated GABA(EC_{20})-gated current, while higher concentrations were ineffective after 48 h steroid treatment. A. Representative traces and (B) averaged data. ($n = 15$ –18 cells/group; * $P < .01$ vs. GABA, control data; ** $P < .05$ vs. GABA, control data).

Fig. 2. Ethanol concentration–response relationship without preapplication of low concentrations. When concentrations of ethanol were applied independently without preapplication, increasing concentrations of ETOH produced a greater potentiation of γ -aminobutyric acid (GABA)-gated current. ($n = 10$ -15 cells/group; * $P < .05$ vs. GABA, control data).

Fig. 3.

In vivo preadministration of ethanol prevents the γ -aminobutyric acid (GABA)-modulatory effects of low concentrations of ethanol in vitro. When a low concentration of ethanol was administered intraperitoneally across a 2 h period prior to neuronal isolation, the resultant GABA-gated current was not responsive to modulation by 1 mM ETOH, following 48 h THP (3 α -OH-5 β -pregnan-20-one) treatment. ($n = 10-15$ cells/group; * $P < .01$ vs. GABA, control data; ** $P < .05$ vs. GABA, control data).