Magnitude and Ethanol Sensitivity of Tonic GABA<sub>A</sub> Receptor-Mediated Inhibition in Dentate Gyrus Changes From Adolescence to Adulthood

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INTRODUCTION

The consumption of ethanol (EtOH) by adolescents is a public health problem of immense proportions (NIAAA 2000), and occurs during a developmental period when the brain undergoes rapid changes in structure and function that make it uniquely vulnerable to negative consequences of ethanol exposure (Monti et al. 2005). Memory impairment is one of many behavioral effects of ethanol, and several lines of research suggest that adolescents are highly sensitive to this effect (White and Swartzwelder 2004). Adolescent rats show greater ethanol-induced learning impairment than adults (Land and Spear 2004; Markwiese et al. 1998), and humans in their early 20s are more sensitive to ethanol-induced memory impairment than those in their late 20s (Acheson et al. 1998). Despite the striking implications of such studies, little is known about the mechanisms that underlie these developmental differences in ethanol sensitivity. One plausible hypothesis is that memory-mediating mechanisms within the hippocampal formation are more powerfully suppressed by ethanol during adolescence, and there is growing support for this view (Li et al. 2006; Pyapali et al. 1999; Swartzwelder et al. 1995).

Ethanol has powerful effects on GABAergic neurotransmission, and some of these effects may be mediated by ethanol interactions with type-A GABA receptors (GABA<sub>A</sub>Rs). Although traditionally associated primarily with ethanol-induced sedation and anxiolysis, it is now clear that GABA<sub>A</sub>Rs are also likely to be involved in regulating memory mechanisms and information processing in the hippocampal formation (Paulsen and Moser 1998). Tonic GABA<sub>A</sub>R subtypes are relatively insensitive to physiologically relevant concentrations of ethanol (Criswell et al. 2003; Sapp and Yeh 1998; Whitten et al. 1996). Therefore it is possible that extrasynaptic GABA<sub>A</sub>R subtypes containing the δ subunit mediate the effects of low to moderate ethanol concentrations that are known to affect learning and other cognitive functions (Eckardt et al. 1998; Wallner et al. 2003). Because low to moderate concentrations of ethanol have been shown to impair learning (Markwiese et al. 1998) and learning-related neurophysiological activity (Pyapali et al. 1999; Swartzwelder et al. 1995) more potently in adolescent animals than in adults, the present study was designed to test the hypothesis that ethanol promotes extrasynaptic GABA<sub>A</sub>R function more potently in hippocampal neurons from adolescent, compared with adult rats.

METHODS

Animals and brain slice preparation

Hippocampal brain slices were prepared from adolescent (30- to 40-day old) or adult (70- to 80-day old) male Sprague-Dawley rats (Charles River). The age group used for each preparation was randomized. Rats were anesthetized with halothane vapor and decapitated. The brain was removed, and 350-μm sagittal slices containing the hippocampus were cut using a Vibratome Series 1000. The ice-cold cutting solution consisted of (in mM) 120 NaCl, 3.3 KCl, 25 NaHCO<sub>3</sub>, 1.23 Na<sub>2</sub>HPO<sub>4</sub>, 15 d-glucose, 3 myo-inositol, 2 Na pyruvate, 0.4 Na ascorbate, 0.1 CaCl<sub>2</sub>, and 12 MgCl<sub>2</sub> equilibrated with a gas mixture of 95% O<sub>2</sub>-5% CO<sub>2</sub>. Slices were incubated at room temperature (RT: 21–23°C) for a minimum of 1 h in a holding chamber containing artificial cerebral spinal fluid (ACSF: in mM, 120 NaCl, 3.3 KCl, 25 NaHCO<sub>3</sub>, 1.23 Na<sub>2</sub>HPO<sub>4</sub>, 15 d-glucose, 3 myo-inositol, 2 Na pyruvate, 0.4 Na ascorbate, 2.0 CaCl<sub>2</sub>, and 1.3 MgCl<sub>2</sub>) bubbled with 95% O<sub>2</sub>-5% CO<sub>2</sub>.

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Drug perfusion

Drugs were diluted in ACSF and applied under pressure (2–3 psi) using a manually controlled four-channel valve manifold system and a Millimaniifold (ALA Scientific Instruments) with a tip inner diameter of 200 μm. This system allows for rapid replacement of the solution surrounding the target neuron. Under a ×5 objective, Fast Green dissolved in ACSF was used to visualize the drug flow, and the center of the perfused area was positioned on the soma of the recorded neuron. To reduce turbulence that could result in dilution of drug by mixing with bath ACSF, the tip angle and height was carefully adjusted to produce laminar flow of the drug solution over the slice. However, it is possible that in some cases concentration of drug at the site of the neuron may be lower than that stated.

Electrophysiology

Individual dentate gyrus granule cells (DGCs) were visually identified (Zeiss Axioskop, IR-DIC videomicroscopy, ×40 water-immersion objective). Slices were perfused with RT 95% O2-5% CO2-bubbled ACSF at a rate of 2–4 ml/min. Microelectrodes with a tip resistance of 5–10 MΩ when filled were pulled from thin-walled borosilicate glass capillaries (World Precision Instruments) using a Sutter Instrument Co. P-2000 puller. The electrode solution consisted of (in mM) 130 CsCl, 10 HEPEs, 4 NaCl, 0.2 EGTA, 10 Na2CreatinePO4, 4 MgATP, 0.3 TrisGTP, 6 QX-314, pH 7.2, 290 osM. Whole cell voltage-clamp recordings were performed using an Axopatch 200B amplifier (Axon Instruments). Signals were low-pass filtered at 2 kHz and digitized at 10 kHz using a National Instruments PCI-6251 DAQ board and WinWCP (V3.2.9, University of Strathclyde). Series and input resistance were continuously monitored using a 200-ms, −10-mV pulse applied every 12.6 s.

Data analysis and statistics

All data analysis was performed on data records that had been stripped of age group information. Tonic current measurement was performed using an in-house function written for MATLAB (V7.1, The Mathworks). Transients produced by voltage steps were removed from the data. For each condition, all-point histograms were generated for 57.5 s of data and the function \( f(x) = A \exp[-(x - \mu)^2/2\sigma^2] \) was fitted to each histogram. The center of this distribution (\( \mu \)) represented the mean holding current, while \( \sigma \) represented the root-mean-square (RMS) noise over the 57.5-s interval. Spontaneous inhibitory postsynaptic potentials (sIPSCs) were detected using the Mini Analysis program by Synaptosoft (Ver. 6.0.3). For each condition, IPSC frequency and average peak amplitude were calculated. Rise time and decay time constant (\( \tau_d \)) of a single-exponential fit were calculated from a “typical” IPSC produced by scaling and averaging all individual events. Statistical analysis within age groups was performed using paired \( t \)-test to compare predrug baseline recordings to recordings made under drug conditions. Comparisons across groups were made using independent samples \( t \)-test. The criterion for significance was set at \( P < 0.05 \), all data are presented as means ± SE.

RESULTS

For these experiments, we used an adaptation of methods described previously by (Nusser and Mody 2002). In rats, a developmental period analogous to human adolescence begins at postnatal day 30, whereas rats older than 60 days are considered adult (Spear 2000). Therefore we measured tonic inhibitory currents in two groups of rats, 30–40 days old and 70–80 days old. Inhibitory currents were isolated by including 2 mM kynurenic acid in the bath perfusate. Granule cells were voltage clamped at −80 mV and the holding current was recorded during three 3-min periods: a no-drug baseline, during application of 30 mM ethanol, and during application of 200 μM picrotoxin. Figure 1 shows current traces recorded from DGCs from two adolescent (C) and two adult rats (D). All-point histograms and fitted distributions for the same cells are shown in A and B. Average baseline holding current (\( \mu \)) and RMS noise (\( \sigma \)) did not differ between the two age groups (\( \mu \) in pA: −61.8 ± 9.0 adolescents, −72.5 ± 9.5 adults; \( \sigma \) in pA: 3.4 ± 0.2 adolescents, 4.2 ± 0.3 adults).

Developmental changes in tonic inhibition

Tonic current was measured as the difference between the centers of the fitted distributions obtained for the baseline and picrotoxin conditions (\( \mu_{\text{picrotoxin}} - \mu_{\text{baseline}} \)), and tonic noise was measured as the difference in RMS noise between these two conditions (\( \sigma_{\text{baseline}} - \sigma_{\text{picrotoxin}} \)). GABA_A-mediated tonic current was detected in cells from both adolescent and adult rats. As shown in Fig. 2A, in the absence of ethanol, tonic current was significantly greater in neurons from adult rats (17.3 ± 1.9 pA, \( n = 8 \) cells, 7 rats) compared with those from adolescents (8.2 ± 0.7 pA \( n = 8 \) cells, 7 rats). Average tonic noise was also larger in adults compared with adolescents (2.1 ± 0.2 pA for adolescents and 1.0 ± 0.2 pA for adults, Fig. 2B). These findings are consistent with greater activation of extrasynaptic GABA_ARs in DGCs from adults compared with adolescents.

Effect of ethanol on tonic inhibition in adolescents and adults

Ethanol increased the size of the holding current in both adolescent and adult rats (\( \mu_{\text{EtOH}} \) (pA): adolescent −70.0 ± 9.7, adult −80.5 ± 10.1) but had little effect on RMS noise (\( \mu_{\text{EtOH}} \) (pA): adolescent 4.0 ± 0.4, adult 4.6 ± 0.4)). Within-group comparisons of the holding currents in the absence and presence of ethanol indicated that ethanol increased the currents in slices from both adolescent (\( P < 0.001 \)) and adult (\( P = 0.004 \)) rats. For each cell, ethanol enhancement of tonic current was calculated as the increase in holding current during ethanol exposure as percentage of the tonic current in the absence of ethanol: (\( \mu_{\text{baseline}} - \mu_{\text{EtOH}} \))/(\( \mu_{\text{picrotoxin}} - \mu_{\text{baseline}} \))×100. Ethanol enhancement of tonic current was significantly greater in adolescent rats (107.8 ± 18.4%) than in adults (46.7 ± 9.7%, Fig. 3).

Effect of ethanol on synaptic inhibition

We also investigated whether ethanol altered the frequency, amplitude, or kinetics of sIPSCs in DGCs from adolescent and adult rats. As shown in Table 1, in the baseline condition, sIPSCs occurred with similar frequency in cells from adolescents and adults, and sIPSC peak amplitude, rise time, and decay time constant (\( \tau_d \)) also did not differ between the two age groups. Ethanol did not significantly alter frequency, rise time, or \( \tau_d \) in either adolescents or adults. However, in adolescent rats, ethanol increased sIPSC amplitude, whereas in adult rats, ethanol did not increase sIPSC amplitude. These findings are generally consistent with those previously reported by Wei et al. (2004), who found that 30 mM ethanol selectively enhanced tonic, but not synaptic, inhibition in DCGs from adult mice.
DISCUSSION

We have found that the size of the tonic current measured in DGCs is larger in adult rats than in adolescents and that the enhancement of tonic current by ethanol is significantly greater in DGCs from adolescent animals than in those from adults. These findings add to a growing literature on the developmental regulation of GABAA receptor function and are of mechanistic significance in understanding the developmental sensitivity of GABAAR-mediated synaptic function, and behavioral learning, to ethanol.

Tonic inhibitory current mediated by extrasynaptic GABAARs has been described in several types of neurons including granule cells from DG (Nusser and Mody 2002) and cerebellum (Kaneda et al. 1995) and interneurons in CA1 (Semyanov et al. 2003). Extrasynaptic GABAARs are expressed diffusely on the dendrites and cell body of neurons where they are activated by the low ambient GABA levels present in the extracellular space. Specifically, in granule cells of the cerebellum and dentate gyrus, tonic current is mediated by GABAARs that contain the δ subunit (Stell et al. 2003). These δ-type GABAARs are more sensitive to ethanol than any other subtypes (Wallner et al. 2003). In fact, it has been suggested that this subtype may be the primary target for direct effects of ethanol on GABAAR function and may play a role in mediating ethanol’s amnestic and motor-incoordinating effects (Hanchar et al. 2005; Wei et al. 2004).

Ethanol and tonic current in DGCs

Although several labs have reported that intoxicating concentrations of ethanol potentiate δ-type GABAARs both in expression systems and in neurons (Hanchar et al. 2005; Liang et al. 2006; Wallner et al. 2003; Wei et al. 2004), recent work has failed to replicate this effect (Borghese et al. 2006; Yamashita et al. 2006). In particular, Borghese et al. (2006) found that 30 mM ethanol did not alter tonic currents in mouse DGCs, and Yamashita et al. (2006) found that 30 mM ethanol had either no effect or a small inhibitory effect on responses to exogenously applied GABA in cultured cerebellar granule cells. These inconsistent findings suggest that ethanol effects on these receptors may be highly sensitive to experimental variation among laboratories. Our results, indicating that 30
mM ethanol increased tonic current in DGCs from both adolescent and adult animals, provide additional confirmation that intoxicating concentrations of ethanol can enhance inhibitory tonic currents. Although this finding is consistent with others’ claims that ethanol acts directly at δ-type GABA<sub>A</sub>Rs, it should be noted that our experiments do not conclusively demonstrate this. In cerebellar granule cells, some evidence suggests that increases in tonic current during ethanol exposure may occur indirectly as a result of increased spillover from sIPSC events (Carta et al. 2004), but this finding is also controversial. Hanchar et al. (2005) have reported ethanol-induced increases in tonic current in cerebellar granule cells in the absence of increases in GABA release. Furthermore, they have shown that this effect is altered by mutation of extrasynaptic GABA<sub>A</sub>Rs on granule cells, which also suggests a “postsynaptic” mechanism (Hanchar et al. 2005). We did not find increases in sIPSC frequency during ethanol exposure, suggesting that increased spillover does not contribute to increases in tonic current in this preparation. However, it is possible that other mechanisms that modulate extracellular GABA concentration could have contributed to our results; see Semyanov et al. (2004) for review.

**Extrasynaptic GABA<sub>A</sub>Rs and learning**

Activation of extrasynaptic GABA<sub>A</sub>Rs produces shunting inhibition that decreases the excitability of a neuron and alters its membrane time constant. These changes in membrane properties alter both the time window for summation of inputs and the threshold for action potential firing and could thereby influence memory mechanisms such as long-term potentiation (LTP) (Semyanov et al. 2004). In the hippocampus, extrasynaptic receptors have been shown to play a role in learning and memory. In CA1 pyramidal cells, GABA<sub>A</sub>R α<sub>5</sub> subunits are expressed extrasynaptically (Fritschy and Brunig 2003) and in mice, reduction or loss of the α<sub>5</sub> subunit improves performance in trace fear conditioning and water maze tasks (Collinson et al. 2002; Crestani et al. 2002). Similarly, α<sub>5</sub> selective inverse agonists also improve performance in the water maze (Chambers et al. 2003). In DGCs, ethanol sensitive δ-type GABA<sub>A</sub>Rs mediate a tonic current that is about four times larger than the average total current carried by spontaneous synaptic activity (Nusser and Mody 2002), suggesting that these receptors are powerful regulators of DGC function. Knockout of the δ subunit enhances trace fear conditioning in female mice (Wiltgen et al. 2005), providing further evidence that tonic current mediated by extrasynaptic GABA<sub>A</sub>Rs suppresses hippocampal-dependent learning.

**GABA<sub>A</sub>R tonic inhibition in the dentate gyrus increases during development**

We have found that the size of tonic currents measured in DGCs in the absence of ethanol increases as rats mature from adolescence to adulthood. Developmental changes in tonic inhibition have been reported in other neurons (Brickley et al.

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**TABLE 1. Effects of ethanol on phasic inhibition in rat dentate gyrus granule cells**

<table>
<thead>
<tr>
<th></th>
<th>Adolescent (30-40 Days)</th>
<th>Adult (70-80 Days)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Frequency, Hz</td>
<td>2.0 ± 0.4</td>
<td>1.8 ± 0.4</td>
</tr>
<tr>
<td>Amplitude, pA</td>
<td>45.6 ± 5.1</td>
<td>55.0 ± 6.9</td>
</tr>
<tr>
<td>RT&lt;sub&gt;10-90%, ms&lt;/sub&gt;</td>
<td>0.98 ± 0.09</td>
<td>1.00 ± 0.12</td>
</tr>
<tr>
<td>τ&lt;sub&gt;E&lt;/sub&gt;, ms</td>
<td>18.0 ± 1.0</td>
<td>21.7 ± 1.6</td>
</tr>
<tr>
<td>Number of rats</td>
<td>8</td>
<td>7</td>
</tr>
<tr>
<td>Number of cells</td>
<td>8</td>
<td>8</td>
</tr>
</tbody>
</table>

Spontaneous inhibitory postsynaptic current (IPSC) frequency, amplitude, and kinetic parameters are shown for cells from adolescent and adult rats during the baseline condition and during application of 30 mM ethanol. Ethanol significantly increased spontaneous IPSC (sIPSC) amplitude in cells from adolescents, but not adults. Values are means ± SE. †, P = 0.02 by paired t-test.
1996; Semyanov et al. 2004), but this is the first time such studies have been conducted in the dentate. In the hippocampus, levels of mRNA for the δ subunit increase during development with the highest levels of expression occurring in adult animals (Laurie et al. 1992). The neurosteroid sensitivity of GABA_A Rs on DGCs also increases during postnatal development (Mchedlishvili et al. 2003). Because δ-type GABA_A Rs are highly sensitive to neurosteroids (Brown et al. 2002), this provides additional evidence that the number of δ-type GABA_A Rs increases in DGCs as animals mature. Thus greater expression of extrasynaptic receptors could contribute to the larger tonic currents that we measured in adult animals.

However, if δ-type GABA_A Rs are the most ethanol sensitive of the GABA_A R subtypes and if extrasynaptic expression of these receptors by DGCs increases between adolescence and adulthood, one would predict greater ethanol sensitivity of tonic currents from adult animals. In fact our data show the reverse, that ethanol enhancement of tonic current is greater in adolescent animals. This finding suggests that developmental regulation of ethanol’s effect on tonic inhibition is more complex than a simple switch in GABA_A R subunits. Several other potential mechanisms are also plausible. First, the amount of ambient GABA present under the baseline condition could change during brain maturation, altering the degree of saturation of extrasynaptic receptors and changing their apparent ethanol sensitivity, even if the mechanism of ethanol action were directly at extrasynaptic GABA_A Rs. Second, developmental changes in intracellular signaling in DGCs could reduce ethanol sensitivity of δ-type GABA_A Rs in adult animals. GABA_A R function is constitutively regulated by phosphorylation, and GABA_A R sensitivity to ethanol is regulated by intracellular signaling mechanisms in both the cerebellum (Freud and Palmer 1997) and hippocampus (Weiner et al. 1997). Furthermore, differences in phosphorylation have been invoked to explain the greater ethanol sensitivity of GABA_A Rs when expressed in Xenopus oocytes rather than mammalian cells (Yamashita et al. 2006). More work is needed to determine whether either of these mechanisms may underlie our results.

Increased levels of tonic current in adults relative to adolescents are consistent with continued maturation of the inhibitory network in the DG during the adolescent period. Recently it has been shown that hippocampal expression of several GABA_A R subunits changes throughout adolescence and early adulthood (Yu et al. 2006). Furthermore, maturation of inhibition in the hippocampus is thought to be important in determining the rules for the induction of LTP, although this work has been done in CA1 rather than in the dentate. In slices prepared from juvenile hippocampus, pairing a presynaptic stimulus with a single postsynaptic action potential can produce spike-timing dependent LTP of excitatory synapses. However, in slices from adult animals a single postsynaptic action potential is insufficient and presynaptic stimulation must be paired with a burst of action potentials to produce potentiation. Blocking all GABA_A Rs in adult slices restores the ability of a single postsynaptic action potential to produce LTP (Meredith et al. 2003). Although this study did not discriminate between the synaptic and extrasynaptic GABA_A R populations, the powerful effects that extrasynaptic GABA_A Rs have on cell excitability suggest that they may have a role in mediating this effect. Thus our finding that tonic inhibition on DGCs increases as rats age from adolescent to adult suggests that the inhibitory mechanisms that modulate learning in this brain region are still maturing and may help explain differences in memory performance between adolescents and adults.

**Developmental differences in ethanol enhancement of tonic inhibition**

Adolescents are highly sensitive to ethanol’s effects on memory (White and Swartzwelder 2004). In rats, ethanol produces greater impairment in the Morris water maze task in adolescents than in adults (Markwiese et al. 1998). Furthermore, ethanol interferes with memory of a discrimination task in adolescents at doses that do not affect adults (Land and Spear 2004). It is likely that ethanol’s effects on memory are mediated by alterations in neurotransmission and plasticity in the hippocampus (White et al. 2000), and in fact, in vitro experiments have shown that ethanol inhibits LTP more powerfully in hippocampal slices from adolescent animals (Pyapali et al. 1999). Although some of this is likely due to developmental differences in ethanol sensitivity of NMDA receptors (Swartzwelder et al. 1995), a growing body of evidence suggests that in the hippocampus, increased activation of extrasynaptic GABA_A Rs impairs learning and memory and that extrasynaptic GABA_A Rs play a role in regulating LTP. Therefore, it seems likely that the increased ethanol enhancement of inhibitory tonic current that we report in DGCs from adolescent rats contributes to the greater vulnerability of adolescents to EtOH-induced memory impairment. This study provides the first evidence to link differences in the effect of ethanol on GABA_A R-mediated tonic inhibition in the hippocampus to differences in sensitivity to ethanol’s effects on memory.

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