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GABA Transport Modulates the Ethanol Sensitivity of Tonic Inhibition in the Rat Dentate Gyrus

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Abstract

In recent years, the effect of ethanol on tonic inhibition mediated by extrasynaptic GABA_A receptors (GABA_ARs) has become a topic of intensive investigation and some controversy. The high ethanol sensitivity of extrasynaptic GABA_A receptors containing the δ subunit combined with the role of tonic inhibition in maintaining the background inhibitory ‘tone’ in hippocampal circuits has suggested that they may play a key role mediating certain behavioral effects of ethanol, including those related to learning and memory. We have found that ethanol disrupts learning and learning-related hippocampal function more potently in adolescent animals than in adults and that ethanol promotes extrasynaptic receptor-mediated GABAergic tonic currents more potently in adolescents than in adults. However, there have been no studies of potential mechanisms that may underlie the enhanced ethanol sensitivity of the tonic current in adolescents. In this study, we recorded GABA_A receptor-mediated tonic currents in dentate gyrus granule cells in hippocampal slices from adolescent and adult rats. As previously reported, we found that ethanol potentiated the currents more efficaciously in cells from adolescents than in those from adults. We also found that the GAT-1 blocker, NO-711, eliminated this developmental difference in ethanol sensitivity. These findings suggest that regulation of ambient GABA by GABA transporters may contribute to the difference in ethanol sensitivity between adolescents and adults.

Keywords

ethanol; GABA_A receptor; tonic inhibition; extrasynaptic receptors; hippocampus; dentate gyrus; adolescence; development

Introduction

The effects of ethanol on extrasynaptic GABA_A receptors (GABA_ARs) is a rapidly growing area of research that may hold great promise for increasing our understanding of a wide range of ethanol effects on behavior and cognition. Extrasynaptic GABA_ARs are activated

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by ambient GABA in the brain and differ from synaptic subtypes in their location on the neuron, pharmacology, and functional properties (Fritschy and Brunig, 2003; Nusser and Mody, 2002; Rossi et al., 2003; Yeung et al., 2003). For many years, the effects of drugs that modulate the GABA system were attributed to their effects on synaptic GABA_AR function. However, in recent years, evidence has emerged that suggests that many of these effects may actually be mediated by extrasynaptic receptors (Liang et al., 2009). In particular, extrasynaptic GABA_AR subtypes that contain the δ subunit (δ GABA_ARs) can be potentiated by ethanol concentrations as low as 3 mM; humans report feeling intoxicated at blood alcohol concentrations of 2 to 7 mM (Eckardt et al., 1998; Sundstrom-Poromaa et al., 2002; Wallner et al., 2003). Therefore, it is now thought that extrasynaptic δ GABA_ARs may play a crucial role in mediating the behavioral effects of ethanol (Hanchar et al., 2004).

Activation of extrasynaptic GABA_ARs produces shunting inhibition that regulates both the threshold for action potential firing and the time window for summation of inputs, and thereby influences memory mechanisms such as long-term potentiation (LTP) (Semyanov et al., 2004). In the hippocampus, extrasynaptic GABA_ARs, including δ GABA_ARs, have been shown to regulate learning and memory. Trace fear conditioning is enhanced in female δ knockout mice, and knockout of the α_4 subunit, which is predominantly extrasynaptic and often pairs with the δ subunit, enhances fear conditioning in mice of both sexes (Moore et al., 2010; Wiltgen et al., 2005). Furthermore, increased pubertal expression of $\alpha_4\beta\delta$ GABA_ARs impairs both LTP induction and acquisition of a hippocampus-dependent spatial learning task in mice (Shen et al., 2010). Extrasynaptic α_5 GABA_ARs on CA1 pyramidal neurons control the threshold for LTP, and antagonism or knockout of these receptors enhances fear conditioning and improves spatial memory (Chambers et al., 2003; Collinson et al., 2002; Crestani et al., 2002; Martin et al., 2010). These results strongly suggest that ethanol's amnesic effects may be mediated, at least in part, by enhancement of extrasynaptic GABA_AR-mediated tonic inhibition.

In the U.S., most people begin to drink ethanol during adolescence, a developmental period when the brain is undergoing structural and functional changes that may make it uniquely vulnerable to ethanol exposure (Monti et al., 2005; SAaMHSA, 2009). Among other differences, adolescents are more sensitive than adults to ethanol's effects on memory (White and Swartzwelder, 2004). We have previously shown that tonic inhibitory current mediated by extrasynaptic GABA_ARs is larger in dentate gyrus granule cells (DGCs) from adults than from adolescents; however, enhancement of this tonic inhibition by 30 mM ethanol was approximately twice as great in DGCs from adolescent animals (Fleming et al., 2007). While tonic inhibition in DGCs is likely mediated by ethanol-sensitive δ GABA_ARs, other mechanisms, such as changes in extracellular GABA concentration due to changes in the rates of GABA spillover or uptake, may also contribute to these developmental changes. We did not find differences in the baseline rate of spontaneous inhibitory postsynaptic currents (sIPSCs) between the two age groups, and ethanol did not increase sIPSC frequency in DGCs from either age group. Therefore, it is unlikely that differences in GABA spillover contributed to the effects that we reported. However, changes in the rate of GABA uptake by GABA transporters, such as GAT-1, could also contribute to the age differences in the size of the tonic current or its sensitivity to ethanol enhancement. In this study, we blocked GABA uptake by GAT-1 to determine if GABA transport plays a mechanistic role in those differences.

Materials and 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 or isoflurane vapor and decapitated. The brain was removed and 350 μ m 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 NaHCO₃, 1.23 NaH₂PO₄, 15 D-Glucose, 3 Myo-Inositol, 2 Na Pyruvate, 0.4 Na Ascorbate, 0.1 CaCl₂, 12 MgCl equilibrated with a gas mixture of 95 / 5% O₂ / CO₂. Slices were incubated at room temperature (RT: 21–23°C) for a minimum of one hour in a holding chamber containing artificial cerebral spinal fluid (ACSF: in mM 120 NaCl, 3.3 KCl, 25 NaHCO₃, 1.23 NaH₂PO₄, 15 D-Glucose, 3 Myo-Inositol, 2 Na Pyruvate, 0.4 Na Ascorbate, 2.0 CaCl₂, 1.3 MgCl) bubbled with 95%O₂ / 5%CO₂. Drugs were diluted in ACSF and applied in the bath.

Electrophysiology

Individual DGCs were visually identified using a Zeiss Axioskop equipped with IR-DIC videomicroscopy and a 40X water immersion objective. While in the recording chamber, the slices were perfused with RT 95 / 5% O₂ / CO₂-bubbled ACSF at a rate of 2–4 ml/min. Microelectrodes with a tip resistance of 5–10 M Ω when filled were pulled from thin-walled borosilicate glass capillaries (World Precision Instruments, Inc.) 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, osm 290. Whole-cell voltage-clamp recordings were performed using an Axopatch 200B amplifier (Molecular Devices). 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) or an Axon Instruments Digidata 1440A and Clampex 10.2 (Molecular Devices).

Tonic inhibitory currents were measured, as previously described, in two age groups of rats: adolescents, which were 30–40 days old, and adults, which were 70–80 days old (Fleming et al., 2007; Spear, 2000). Granule cells were voltage clamped at –80 mV. For control cells, in which GAT-1 was not blocked, the holding current was recorded during 3 periods: (1) baseline, ACSF only; (2) during bath application of 30 mM ethanol; and (3) during application of 200 μ M picrotoxin. For cells in which GAT-1 was blocked, 4 periods were recorded: (1) ACSF only (pre-baseline); (2) the baseline period with 2.5 μ M NO-711 in the bath; (3) during coapplication of NO-711 and 30 mM ethanol; and (4) coapplication of NO-711 and 200 μ M picrotoxin. To avoid repeat exposures to ethanol, cells were assigned to either the control group or the NO-711 group and were not tested under both conditions.

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 (The Mathworks). Transients produced by voltage steps were removed from the data. For each condition, all-point histograms were generated for 2 min of data and the Gaussian function $f(x) = A \cdot \exp(-(x-\mu)^2/2\sigma^2)$ was fitted to each histogram, as shown in Figure 1. The center of this distribution (μ) represents the mean holding current, while σ represents the root-mean-square (RMS) noise over the 2 min interval. *Statistical analysis* within age groups was performed using paired t-tests to compare pre-drug baseline recordings to recordings made under drug conditions. Comparisons across groups were made using 2-way ANOVA or t-test, as appropriate. The criterion for significance was set at $p < 0.05$; all data are presented as mean $\pm$ standard error.

Results

To determine the role that GABA uptake plays in the effects of ethanol on tonic inhibitory current, GABA uptake by GAT-1 was blocked using 2.5 μ M NO-711. NO-711 significantly increased the holding current in DGCs from both adolescent [paired $t(6) = 4.34$, $p = 0.005$] and adult rats [paired $t(4) = 6.13$, $p = 0.004$]. However, there was no significant difference in the size of the increase in holding current ($\mu_{\text{ACSF}} - \mu_{\text{NO-711}}$) between adolescents and adults (55 ± 13 pA and 30.4 ± 5.0 pA, respectively). As shown in Figure 2A, NO-711 also increased the size of the tonic current ($\mu_{\text{picrotoxin}} - \mu_{\text{baseline}}$) in both adolescents and adults. In adolescent slices, tonic current was 7.7 ± 3.3 pA in control neurons and 59 ± 17 pA in NO-711 neurons. In slices from adults, tonic current was 13.9 ± 3.0 pA in control neurons and 30.0 ± 5.5 pA in NO-711 neurons. Two-way ANOVA revealed a significant effect of GAT-1 block [control vs. NO-711, $F(1,16) = 9.54$, $p = 0.007$], but no effect of age and no age by condition interaction.

We also determined if GAT-1 block by NO-711 altered ethanol's effects on GABAergic tonic current. Within-group comparisons of the holding current before and during bath application of 30 mM ethanol indicated that ethanol significantly increased the holding current under control conditions and in the presence of NO-711 in DGCs from both age groups: adolescent control $t(3) = 4.84$, $p = 0.017$; adolescent NO-711 $t(6) = 3.35$, $p = 0.016$; adult control $t(3) = 3.39$, $p = 0.043$; adult NO-711 $t(3) = 5.56$, $p = 0.011$ (also see Figure 1). In adolescent slices, the ethanol-induced increase in the holding current ($\mu_{\text{baseline}} - \mu_{\text{EtOH}}$) was 6.3 ± 1.0 pA in control neurons and 29.7 ± 9.3 pA in NO-711 neurons. In slices from adults, this increase was 2.8 ± 0.8 pA in control neurons and 10.4 ± 1.9 pA in NO-711 neurons. Two-way ANOVA revealed a significant effect of GAT-1 block [control vs. NO-711, $F(1,14) = 5.44$, $p = 0.035$], but not of age. There was no age by condition interaction.

To control for the variability in the size of the tonic current among the groups, ethanol enhancement of the tonic current was calculated for each cell as the increase in holding current during ethanol exposure as a percentage of the tonic current in the baseline condition: $100 \cdot (\mu_{\text{baseline}} - \mu_{\text{ethanol}}) / (\mu_{\text{picrotoxin}} - \mu_{\text{baseline}})$. In adolescent DGCs, the ethanol enhancement of the holding current was $105 \pm 32\%$ in control neurons and $52 \pm 11\%$ in NO-711 neurons; in adult rats, ethanol enhancement was $22.9 \pm 6.3\%$ in control neurons and $42 \pm 16\%$ in NO-711 neurons (Figure 2B). As we previously reported, under control conditions, DGCs from adolescent rats showed significantly greater ethanol enhancement of tonic current than DGCs from adults (Fleming et al., 2007). Two-way ANOVA revealed a significant effect of age [adolescent vs. adult, $F(1,14) = 8.41$, $p = 0.012$] but no overall effect of GAT-1 block (control vs. NO-711). However, there was a significant age by condition interaction [$F(1,14) = 5.13$, $p = 0.04$]. This interaction indicates that in the presence of NO-711, the difference between adolescent and adult DGCs disappeared. Therefore, it appears that GAT-1 activity is necessary to maintain the lower baseline levels of tonic inhibition that are required for the expression of developmental differences in ethanol enhancement of tonic inhibition.

Discussion

The principal finding of this study is that in DGCs, GAT-1 activity is necessary for the expression of developmental differences in the sensitivity of extrasynaptic GABA_AR-mediated tonic current to ethanol. As we have previously shown, when GAT-1 is functional, tonic current is enhanced more by ethanol in adolescent DGCs than in those from adults (Fleming et al., 2007). This difference in ethanol sensitivity was eliminated by blocking GABA uptake by GAT-1, suggesting that during adolescence, the low levels of tonic current

that are maintained by GABA uptake are essential for the expression of high sensitivity to ethanol. Because GAT-1 is the main GABA transporter in the hippocampal formation and clears diffusely distributed GABA from the hippocampal extracellular space (Frahm et al., 2001), these results suggest that regulation of ambient GABA by GABA transporters modulates the ethanol sensitivity of tonic inhibitory current and may shed new light on the differences in adolescent and adult ethanol sensitivity that continue to emerge in the literature.

It is noteworthy that the trend we observed toward baseline tonic current being larger in adults than in adolescents was not statistically significant. This is inconsistent with our previous observations (Fleming et al., 2007). The tonic current levels we recorded in adolescents were similar in the two studies: 7.7 ± 3.3 pA in this study, compared to 8.2 ± 0.7 reported previously (Fleming et al., 2007). However, for the adult group, the baseline tonic current was lower in this study (13.9 ± 3.0 pA) compared to the previous one (17.3 ± 1.9 pA). The variability in the present study is also larger, which in itself may account for the loss of statistical significance in this comparison.

Tonic inhibitory current mediated by extrasynaptic GABA_ARs is present in many neurons in the brain, including granule cells from the dentate gyrus and cerebellum (Kaneda et al., 1995; Nusser and Mody, 2002), CA1 interneurons and pyramidal cells (Martin et al., 2010; Semyanov et al., 2003; Shen et al., 2010), and neurons in the nucleus accumbens (Nie et al., 2011), thalamus (Jia et al., 2008), and suprachiasmatic nucleus (McElroy et al., 2009). In DGCs, tonic current is mediated by δ GABA_ARs, which may be more sensitive to ethanol than other receptor subtypes (Stell et al., 2003; Wallner et al., 2003). In mice, the extrasynaptic receptors on DGCs are of the $\alpha_4\beta_2\delta$ or $\alpha_4\beta_{(\text{not } 2)}\gamma_2$ types (Herd et al., 2008). While some studies have shown that the β_3 subunit is required for high ethanol sensitivity (Wallner et al., 2003), other studies have shown that $\alpha_4\beta_2\delta$ receptors in oocytes and hippocampal neurons can also be potentiated by low doses of ethanol (Sundstrom-Poromaa et al., 2002). Therefore, it is likely that the ethanol enhancement of tonic current in our slices is due in part to direct ethanol actions at extrasynaptic GABA_ARs.

Developmental changes in tonic inhibition have been reported in other neurons, and the hippocampal expression of several GABA_AR subunits changes during adolescence and early adulthood (Brickley et al., 1996; Semyanov et al., 2004; Yu et al., 2006). In the hippocampus, the highest levels of mRNA for the δ subunit occur during adulthood, and the neurosteroid sensitivity of DGC GABA_ARs increases during postnatal development, which also suggests that δ subunits are more highly expressed in mature animals (Brown et al., 2002; Laurie et al., 1992; Mtchedlishvili et al., 2003). This developmental increase in δ expression is inconsistent with the lower ethanol sensitivity of adult DGCs that we have previously reported, and which we reproduce here, strongly suggesting that factors other than subunit composition also influence the ethanol sensitivity of tonic current; see Fleming et al. (2007) for a more complete discussion of this discrepancy.

Several factors may have contributed to the reduction in ethanol sensitivity of tonic current in the presence of GAT-1 block. First, the increase in ambient GABA presumably alters the degree of saturation of the extrasynaptic receptors, thus changing their apparent ethanol sensitivity. However, the size of the ethanol increase was greater under the NO-711 condition for both the adolescent and adult groups, suggesting that overall saturation of the receptors was not a limiting factor under the NO-711 condition. Second, the increase in ambient GABA level may have activated another population of extrasynaptic receptors, such as the $\alpha_4\beta\gamma_2$ receptors reported by Herd et al. (2008), which are less sensitive to ethanol. Therefore, developmental differences in the expression of both δ and γ extrasynaptic

GABA_ARs could contribute to the differences in ethanol enhancement between adolescents and adults, both in the normal condition and under GAT-1 block.

Some studies have suggested that δ GABA_ARs may be the primary target for the direct actions of physiologically-relevant intoxicating concentrations of ethanol (Hanchar et al., 2005; Sundstrom-Poromaa et al., 2002; Wei et al., 2004). However, other studies have not found direct actions of low ethanol concentrations on δ GABA_ARs (Borghese and Harris, 2007; Borghese et al., 2006; Yamashita et al., 2006). Global knockouts of α_4 and α_6 , the two most common binding partners for the δ subunit, do not alter several behavioral tests of ethanol sensitivity in mice (Chandra et al., 2008; Homanics et al., 1998), and knockout of the δ subunit does not decrease ethanol-induced sedation (Linden et al., 2011). Furthermore, in α_4 knockouts, ethanol enhancement of tonic inhibition in DGCs is reduced despite the apparent lack of effects on behavioral ethanol sensitivity (Chandra et al., 2008; Liang et al., 2008); however, the behavioral tests employed by Chandra et al. (2008) may not have adequately assessed hippocampus-dependent learning.

In spite of the difficulty with finding altered ethanol phenotypes in transgenic mice, other studies in rats have provided evidence supporting a role for δ GABA_ARs in mediating some behavioral effects of ethanol. In rats, a mutation in the α_6 subunit increases the ethanol enhancement of tonic inhibition in cerebellar granule cells, which is mediated by $\alpha_6\beta_3\delta$ GABA_ARs, and increases sensitivity to ethanol-induced motor incoordination (Hanchar et al., 2005). Progesterone withdrawal increases the expression of $\alpha_4\beta\delta$ GABA_ARs in the hippocampus, increases the effect of low doses of ethanol on GABAergic currents in CA1 pyramidal neurons, and increases ethanol-induced anxiolysis (Sundstrom-Poromaa et al., 2002). RNAi knockdown of the δ subunit in the medial shell region of the nucleus accumbens reduces alcohol intake (Nie et al., 2011).

Our data suggests that ambient GABA concentration modulates the effects of ethanol on tonic inhibitory current. Many, but not all, of the electrophysiological studies in mice that show effects of ethanol on tonic current add GABA (up to 5 μ M) to the bath (Choi et al., 2008; Glykys et al., 2007; Wei et al., 2004), while similar studies that have not shown ethanol effects on tonic current did not add GABA to the bath (Borghese et al., 2006). For studies that did find ethanol effects on tonic current in mice but did not add GABA see Liang et al. (2008) and Glykys and Mody (2007). Electrophysiological studies in rats typically do not add GABA (Fleming et al., 2007; Hanchar et al., 2005; Liang et al., 2006). These discrepancies suggest that different levels of ambient GABA, either in the slice or *in vivo*, possibly due to different levels of GAT activity, may contribute to differences in ethanol sensitivity between rats and mice.

Clearly, the role of δ GABA_ARs in mediating ethanol intoxication remains controversial. The discrepancies in the literature suggest that the regulation of ethanol's effect on tonic inhibition and the role of this effect in mediating behaviors are not determined exclusively by GABA_AR subunit composition. Regulation of ambient GABA levels by changes in GABA uptake may be an important mechanism that modulates the ethanol sensitivity of extrasynaptic GABA_ARs, and receptors with high affinity for GABA, such as δ GABA_ARs, may be particularly sensitive to this modulation. Thus, changes in GAT-1 activity and ambient GABA concentration may contribute not only to developmental differences in ethanol sensitivity, as shown here, but to other differences, such as differences between *in vivo* and *in vitro* systems and differences between mice and rats.

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Figure 1. Ethanol increases GABA_AR-mediated tonic current in the presence of the GABA transporter-1 blocker NO-711

Left column: Representative voltage-clamp data traces from DGCs from an adolescent (A) and adult (C) rat. **Right column:** For the same cells shown at left, all-point histograms of 120 s of whole-cell current data recorded in the presence of 2.5 μ M NO-711 in the bath (N) and during co-application of NO-711 and 30 mM ethanol (e) or 200 μ M picrotoxin (p). In order to facilitate comparison between cells, the histogram data is plotted relative to the center of the distribution for the picrotoxin condition, and the height of each histogram is normalized to the peak of the picrotoxin condition. Each histogram is shown overlaid with the Gaussian function (line) fit to the binned data.

Figure 2. GABA transporter-1 block alters tonic inhibitory current and attenuates the developmental difference in its sensitivity to ethanol

A: GABAergic inhibitory tonic current in the absence (light bars) and presence (dark bars) of 2.5 μ M NO-711. NO-711 increased tonic current in neurons from both age groups. Tonic current was measured as the difference between the centers of the holding current distributions (μ) for the baseline, either with or without NO-711 in the bath, and picrotoxin conditions; control and NO-711 measurements were made in different cells. Two-way ANOVA revealed a significant effect of drug condition [$F(1,16) = 9.54$, $p = 0.007$] but not of age; there was no significant age by condition interaction. * $p < 0.05$. **B:** Enhancement of tonic current by 30 mM ethanol was calculated as the increase in holding current during ethanol exposure as a percentage of the tonic current for each cell. Under control conditions, granule cells from adolescent rats showed significantly greater ethanol enhancement compared to adults, as we have previously reported (Fleming et al., 2007); * $t(6) = 3.01$, $p = 0.024$. However, in the presence of 2.5 μ M NO-711, this difference disappears. Two-way ANOVA revealed a significant effect of age [$F(1,14) = 8.41$, $p = 0.012$], no effect of condition, and a significant age by condition interaction [$F(1,14) = 5.13$, $p = 0.04$].