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Loss of δ -GABA_A receptor-mediated tonic currents in the adult prelimbic cortex following adolescent alcohol exposure

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Abstract

Delayed maturation of the adolescent prefrontal cortex (PFC) may render it particularly vulnerable to insults, including those associated with drugs of abuse. Using a rat model of binge alcohol exposure, the present study examined the effect of adolescent intermittent ethanol (AIE) exposure during post-natal days (PD) 28-42 on GABAergic neurotransmission in the prelimbic cortex (PrL-C). In control rats, patch-clamp electrophysiology in acute slices obtained at different postnatal ages revealed a developmental increase in the GABA_A receptor-mediated tonic current in layer V pyramidal neurons but no change in layers II/III when measured in the adult. In slices from AIE exposed rats, the amplitude of the tonic current was significantly reduced compared to controls when tested at PD 45, 60 and 90-120. This AIE-induced reduction in tonic current was found to reflect attenuation of currents mediated by δ -subunit containing receptors. Consistent with this, facilitation of the tonic current by bath application of either ethanol or allopregnanolone was attenuated in slices from AIE exposed adult rats compared to control rats. However, expression of this facilitation as a percent of the amplitude of the total current mediated by δ -GABA_A receptors revealed that AIE did not alter their sensitivity to either agonist. Lastly, immunohistochemistry and Western blot analysis revealed no change in the expression of δ -GABA_A subunits or their surface expression. Taken together, these studies reveal that AIE exposure results in persistent deficits in δ -GABA_A tonic currents in the adult PrL-C that may contribute to deficits in decision-making and behavioral control in adulthood.

Keywords

adolescence; alcohol; GABA_A receptors; prefrontal cortex; pyramidal; tonic current

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Introduction

Inhibitory neurotransmission critically regulates neuronal function and behavioral control. In the brain, the majority of inhibition is mediated by activation of the A-type of γ -aminobutyric acid receptors (GABA_AR). Stimulation of GABA_ARs generates two forms of neurotransmission: a rapidly desensitizing phasic current that is principally associated with activation of synaptic receptors, and a non-desensitizing tonic current that is associated with activation of extrasynaptic receptors [for review see (Farrant and Nusser, 2005)].

Accumulating evidence indicates that the tonic current is especially important in modulating neurodevelopment and plasticity (Hines et al., 2012). Consistent with this, alterations in tonic GABA_AR currents have been implicated in diverse neuropathological conditions such as autism, epilepsy, addiction, and various psychiatric disorders (Akbarian et al., 1995; Ben-Ari et al., 2007; Cramer et al., 2010; Hashimoto et al., 2008; LeBlanc and Fagioli, 2011; Tabuchi et al., 2007; Wang and Kriegstein, 2009).

Adolescence is a unique period of neurodevelopment that is characterized by changes in both brain structure and function. It is also a period of increased risk-taking and impulsive decision-making that is thought to relate, at least in part, to the delayed maturation of the executive function of the prefrontal cortex (PFC) (Casey and Jones, 2010). It is widely believed that the enhanced ability of the adolescent PFC to undergo experience-dependent changes renders it particularly vulnerable to adverse environmental insults such as that associated with binge consumption of alcohol. Alcohol consumption during adolescence typically occurs in excessive binge-like patterns that often result in dangerous levels of intoxication that may adversely affect brain development and function. Consistent with this, there is increasing evidence that adolescent alcohol abuse results in deficits in cognitive function mediated by the PFC that persist well into adulthood. Given the high prevalence of adolescent alcohol abuse worldwide, gaining greater insight into how adolescent alcohol abuse impacts on the adult behavior and decision-making is of high clinical significance.

A number of studies in both humans and animals have reported that adolescent alcohol exposure results in long-lasting deficits in the executive function of the PFC (De Bellis et al., 2005; Gass et al., 2014; Spear, 2000; Squeglia et al., 2014; Squeglia et al., 2009). However, the cellular mechanisms that underlie these cognitive deficits have been have received little attention and are poorly understood. In light of the critical role that tonic GABA_A currents play in neurodevelopment and neuroplasticity and the high sensitivity of the developing PFC to environmental insults, the present study investigated the impact of binge-like adolescent alcohol exposure on GABAergic neurotransmission in the PFC.

Materials and Methods

Animals and Adolescent Ethanol Exposure

Long-Evans rats were obtained from our in-house breeding colony (breeder stocks obtained from Charles River). All experimental procedures were conducted with the approval of the Institutional Animal Care and Use Committee at the Medical University of South Carolina, and were within guidelines set forth by the National Research Council's Guideline for the Care and Use of Mammals in Neuroscience and Behavioral Research (2003).

The adolescent intermittent ethanol (AIE) exposure model involved intermittent binge-like exposure to alcohol by vapor inhalation as previously described (Gass et al., 2014). Unless otherwise noted, all studies were conducted in male rats and a litter size consisted of at least 4 males in order to be included in the study. Upon weaning at PD 21, the pups were divided into control and experimental groups and pair-housed as littermates in standard polycarbonate cages for the duration of the study. The animal colony room was maintained on a 12:12 reverse light-dark cycle with lights extinguished at 08:00. AIE exposure occurred during early to middle adolescence (PD28-42) and involved 4 cycles of 2 consecutive episodes of alcohol vapor inhalation, with each episode consisting of 14 hrs in the vapor chambers (18:00-08:00) followed by 10 hrs out of the chambers (08:00-18:00). Rats were exposed to alcohol vapor or air (control) on PD28 & 29 (cycle 1), PD32 & 33 (cycle 2), PD36 & 37 (cycle 3), and PD40 & 41 (cycle 4). Intoxication levels were determined at the end of each 14 hr period of exposure using a 5-point behavioral intoxication rating scale (Supplemental Figure S1A). Tail vein blood was collected from the alcohol exposed but not Air exposed rats at the end of each 2-day cycle and used for determination of blood ethanol concentration (Supplemental Figure S1B).

Electrophysiological Recordings

Recordings were performed using acute brain slices (300 μ m thickness) containing the prelimbic cortex (PrL-C) obtained at different ages as previously described (Gass et al., 2014; Trantham-Davidson et al., 2014). Unless otherwise specified, all recordings were obtained from pyramidal neurons in layer V that were identified visually with infrared-differential interference contrast optics and video-microscopy as previously described. For all experiments, dl-2-amino-5-phosphonovalerate (dl-APV, 50 μ M) and 6-cyano-2,3-dihydroxy-7-nitro-quinoxaline (CNQX, 10 μ M) were added to the recording artificial cerebral spinal fluid (ACSF) to block *N*-methyl-D-aspartic acid (NMDA) and α -Amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) currents, respectively. IPSCs were evoked using a tungsten bipolar stimulating electrode placed in layer V within 200 μ m of the soma of the pyramidal neuron being recorded. Tonic GABA_AR currents were isolated as previously described (Fleming et al., 2007) using standard pharmacological procedures to manipulate the holding current. Detailed information about electrophysiological recording methods is presented in the Supplemental Methods and Material.

Western Blotting and Immunohistochemistry

The expression of GABA_AR subunits in the PrL-C was examined using standard Western blotting and immunohistochemical procedures as previously described (Trantham-Davidson et al., 2014). Analysis of the surface expression of GABA_AR containing the δ -subunit was also assayed as previously described (Grosshans et al., 2002). Detailed information about the blotting and immunohistochemistry methods is presented in the Supplemental Methods and Material.

Results

Expression of GABA_A Tonic Current in Layer V of the PrL-C is Developmentally Regulated

The first set of studies examined the developmental changes in GABAergic neurotransmission in layer V pyramidal neurons of the PrL-C using acute slice patch-clamp electrophysiology. A typical voltage clamp (-70 mV clamp) recording (Figure 1A) consisted of a 15 min baseline period followed by 20 minutes of bath application of the GABA_AR antagonist picrotoxin (100 μ M) in order to determine the amount of tonic current. A cell was considered to have a tonic current if the change in holding current (I_{hold}) in response to picrotoxin was greater than 5 pA. As shown in Figure 1B, both GABA_AR sIPSCs and GABA_AR-mediated tonic currents were of similar amplitude when measured in slices obtained from rats across development and adulthood (age blocks = PD16-30, PD31-45, PD46-60, PD61-75, PD75-90, and >PD90). There was, however, a difference in the number of pyramidal neurons that exhibited a tonic current across development. As shown in Figure 1C, the percentage of neurons that exhibited a change in holding current in response to picrotoxin was very low early in development (~20% at PD16-45). This percentage progressively increased during the transition from adolescence to adulthood such that virtually all cells recorded in slices obtained from fully mature adults exhibited a picrotoxin sensitive tonic current ($\chi^2_{(5)} = 19.6$, $p = 0.002$). In contrast to changes in the tonic current, all cells recorded from across all age groups exhibited similar sIPSC amplitude and frequency and thus, unlike the tonic current, there were no developmental changes in synaptic GABA_AR currents.

Developmental emergence of the GABA_AR Tonic Current is Absent Following Adolescent Alcohol Exposure

The next set of studies examined the effect of AIE exposure on both phasic and tonic GABAergic neurotransmission. As shown in Figure 2A-B, the frequency of the sIPSCs in control and AIE exposed adult rats was not significantly different ($t_{(30)} = 0.06$, $p = 0.948$), suggesting that AIE did not alter synaptic GABA release. Consistent with this, neither the paired-pulse ratio ($t_{(16)} = 0.96$, $p = 0.352$; Figure 2C-D), nor the frequency of miniature IPSCs measured in the presence of tetrodotoxin (TTX, 1 μ M) were affected by AIE exposure ($t_{(15)} = 0.33$, $p = 0.744$; Figure 2E-F). In contrast to the lack of an effect of AIE on presynaptic GABAergic neurotransmission, there was a significant reduction in the amplitude of the sIPSC ($t_{(39)} = 3.1$, $p = 0.004$) in slices from AIE exposed adult rats compared to controls (Figure 2A-B), which could potentially indicate an AIE-induced reduction in the postsynaptic response to the action potential generated synaptic release of GABA. However, this reduction in amplitude of sIPSCs was not reflected by a similar reduction in the amplitude of miniature IPSCs ($t_{(15)} = 1.05$, $p = 0.310$; Figure 2E-F). As supported by additional data shown in Supplemental Figure S2, this effect most likely reflects reduced activation of perisynaptic GABA_ARs during spillover of action potential dependent synaptic release of GABA.

To determine whether AIE altered the tonic GABA_AR current, we next examined changes in holding current in response to bath application of picrotoxin in slices from rats of different developmental ages. A Gaussian distribution was fit to all-point histograms for each cell to

determine the tonic current by subtracting the mean baseline I_{hold} from the mean I_{hold} after 100 μM picrotoxin (Figure 3A,B). Consistent with the data presented in Figure 1, there were no significant differences in the amplitude of the tonic current as a function of age ($F_{(3,22)} = 0.74$, $p = 0.538$; Figure 3C). However, as shown in Figure 3A-C, there was a significant difference in tonic current amplitude between control and AIE exposed rats ($F_{(1,37)} = 32.2$, $p < 0.0001$) at all three ages tested (PD45 $\pm$ 3 days, $t_{(9)} = 2.7$, $p = 0.025$; PD60 $\pm$ 3 days, $t_{(5.415)} = 3.2$, $p = 0.021$; PD90 $\pm$ 6 days, $t_{(8.421)} = 3.2$, $p = 0.012$). Furthermore, AIE exposure also significantly attenuated tonic GABA currents in female rats ($t_{(13)} = 4.9$, $p = 0.0003$; Figure 3D), suggesting that the observed AIE-induced reduction in tonic GABA currents is not sex specific. These data demonstrate that AIE exposure results in the blunting of GABA_AR-mediated tonic current in layer V PrL-C pyramidal neurons that persists well into adulthood.

One potential explanation for the observed AIE-induced reduction in tonic GABA current is a reduction in basal levels of extracellular GABA that could result in reduced tone on extrasynaptic GABA_ARs. To examine this possibility, we measured GABA_AR currents following bath application of the GABA reuptake inhibitor nipecotic acid (500 μM). As shown in Figure 4A,B, this resulted in a very large hyperpolarizing current that presumably reflected activation of all GABA_ARs (i.e. synaptic and extrasynaptic). To determine the contribution of the GABA_A component of the nipecotic acid-induced current, picrotoxin was applied in combination with nipecotic acid. Once again, recordings taken from AIE exposed rats exhibited a significant reduction ($t_{(13)} = 2.3$, $p = 0.041$) in the amplitude of the GABA_AR tonic current suggesting that AIE induced attenuation of the tonic GABA_AR current was not the result of reduced levels of ambient GABA, and instead most likely reflects altered function and/or availability of extrasynaptic GABA_AR.

Delta-subunit containing GABA_AR (δ -GABA_AR) are located almost exclusively in the extrasynaptic membrane, and together with $\alpha 5$ -containing receptors, are thought to mediate the majority of the tonic current in layer V neurons (Drasbek and Jensen, 2006; Gill and Grace, 2014; Moss and Smart, 2001; Zhelezнова et al., 2009). To determine whether the AIE-induced attenuation of tonic current reflected a reduction in δ -GABA_AR currents, we used the δ -GABA_AR agonist 4,5,6,7-tetrahydroisoxazolo[5,4-*c*] pyridin-3-ol hydrochloride (THIP, 1 μM) to induce tonic currents in slices from control and AIE exposed rat. Gaussian distributions fit to all-point histograms for each cell were used to determine THIP-mediated current by subtracting the mean baseline I_{hold} from the mean I_{hold} after 1 μM THIP (Figure 4A,B). In slices obtained from control rats, there was a progressive increase in the amplitude of THIP-mediated currents from PD28 to PD90 ($F_{(3,22)} = 4.4$, $p = 0.015$). This is in contrast to the studies shown in Figure 3C in which the amplitude of the GABA_AR (picrotoxin sensitive) tonic current did not change across development. Examination of the effect of AIE on the developmental time-course of δ -GABA_AR currents revealed a significant effect of age in the control but not AIE treated rats. No between group differences were observed in the amplitude of δ -GABA_AR tonic current in slices obtained shortly after the last cycle of ethanol exposure (PD43-PD47; $t_{(8)} = 0.28$, $p = 0.783$) or at the onset of adulthood (PD58-PD62; $t_{(12)} = 1.3$, $p = 0.203$; Figure 5C). In contrast, in fully mature adult AIE exposed rats, the amplitude of the δ -GABA_AR current was attenuated compared to controls (PD90-PD100; $t_{(17)} = 2.2$, $p = 0.041$). In a separate set of experiments, bath application of THIP had

no effect on sIPSC (amplitude ($F_{(1,8)} = 1.68$, $p = 0.2310$), frequency ($F_{(1,8)} = 0.26$, $p = 0.625$), rise ($F_{(1,8)} = 2.1$, $p = 0.809$) and decay ($F_{(1,8)} = 4.3$, $p = 0.073$) times, or area ($F_{(1,8)} = 0.88$, $p = 0.375$), confirming the selectivity of THIP for extrasynaptic δ -GABA_A receptors (Supplemental Figure S3). These data indicate that early in development, the GABA tonic current is mediated by GABA_ARs that do not contain the δ -subunit, whereas later in development and into adulthood a shift occurs toward increasing involvement of δ -GABA_ARs. Importantly, this developmental emergence of the δ -GABA_AR-mediated tonic current is not observed following AIE exposure. Because the expression of GABA_A receptors, specifically those that mediate the tonic current, have been shown to be hormonally regulated and can fluctuate throughout the estrous cycle (Sundstrom-Poromaa et al., 2002), we tested whether or not the effect of AIE on δ -GABA_A receptor-mediated tonic currents was sex specific. Thus, THIP-mediated currents were measured in slices from control and AIE exposed adult female rats. As shown in Figure 5D, AIE significantly attenuated THIP-mediated currents in AIE exposed adult female rats ($t_{(13)} = 4.0$, $p = 0.002$), indicating that the effect of AIE on the tonic GABA_A current is not sex specific.

Recent studies have shown that not only are there developmental changes in GABA_AR subunit expression within the PFC but also that these changes are layer specific, with different cortical layers exhibiting unique developmental GABA_AR subunit profiles (Datta et al., 2014). We therefore examined the layer-specific effects of AIE on tonic GABA_AR currents by recording from layer II/III pyramidal neurons in slices obtained from control and AIE exposed adult rats. As shown in Figure 5A & B, slices obtained from both groups exhibited similar tonic current amplitudes ($t_{(8)} = 0.5$, $p = 0.655$). In addition, in experiments using THIP to activate δ -GABA_AR, AIE exposure had no effect on the amplitude of the tonic current ($t_{(9)} = 0.6$, $p = 0.571$; Figure 6B). Taken together, these data indicate that the AIE-induced reduction of the δ -GABA_AR tonic current is specific to layer V PrL-C pyramidal neurons.

Efficacy of Facilitation of the GABA_AR Tonic Current by Positive Allosteric Modulation is Reduced Following Adolescent Alcohol Exposure

Acute ethanol enhances extrasynaptic δ -GABA_AR tonic currents, and evidence suggests that this sensitivity to ethanol is imparted by the presence of the δ -subunit (Breese et al., 2006; Wallner et al., 2003). To determine whether the AIE-induced reduction in δ -subunit containing GABA_AR-mediated tonic current was associated with a change in the acute sensitivity to ethanol, we next examined the effect of bath application of ethanol on the GABA_AR tonic current in layer V PrL-C slices obtained from control and AIE exposed adult rats. As expected, bath application of ethanol (10-100 mM) produced a dose-dependent facilitation of I_{hold} amplitude in slices from both control ($F_{(1.8,10.7)} = 33.1$, $p < 0.0001$; Figure 7A,B) and AIE exposed rats ($F_{(1.5,10.5)} = 21.51$, $p = 0.0004$). Post-hoc analysis revealed a significant reduction in I_{hold} in slices from AIE exposed rats at 50 mM ethanol ($t_{(14)} = 2.5$, $p = 0.026$) and 100 mM ethanol ($t_{(13)} = 3.3$, $p = 0.005$), but not 10 mM ethanol. The effect of ethanol was specific to the tonic GABA current, as ethanol had no effect on sIPSC kinetics (amplitude ($F_{(3,36)} = 0.57$, $p = 0.637$), frequency ($F_{(3,36)} = 0.39$, $p = 0.395$), rise ($F_{(3,36)} = 0.48$, $p = 0.698$) and decay ($F_{(3,36)} = 0.47$, $p = 0.706$) times, or area ($F_{(3,36)} = 0.70$, $p = 0.560$)) at any dose (Supplemental Figure S4). As shown in Figure 7C, when the

amplitude of the ethanol-facilitated current was expressed as a percentage of the amplitude of the average THIP-induced current (data from Figure 5), the percentage of the ethanol-facilitated increase was identical between control and AIE slices (Figure 7C).

Since GABA_AR tonic currents are also sensitive to neurosteroids, which like alcohol also appear to act as positive allosteric modulators of δ -GABA_AR, we examined the effects of bath application of allopregnanolone (0.1 and 1.0 μ M) on the tonic current. As expected, allopregnanolone enhanced I_{hold} in slices from control ($F_{(1,2,11,08)} = 36.5$, $p < 0.0001$) and AIE exposed ($F_{(1,3,9,3)} = 10.7$, $p < 0.007$) adult rats (Figure 7D,E). Similar to the effect of AIE on ethanol-facilitated tonic current, AIE also significantly attenuated the allopregnanolone-facilitated tonic current at both low and high concentrations (0.1 μ M $t_{(13,6)} = 2.2$, $p = 0.048$; 1.0 μ M $t_{(16)} = 2.25$, $p = 0.039$). Again, when expressed as a percentage of the average amplitude of THIP-activated current, no significant between group differences were observed (Figure 7F). While the above observations further demonstrate that AIE exposure results in reduced δ -GABA_AR receptor-mediated tonic currents (observed as a reduced efficacy of both ethanol and allopregnanolone to facilitate channel conductance), the sensitivity of δ -GABA_ARs to the facilitatory effect of either compound does not appear to be affected.

Altered Expression of δ -GABA_AR Subunits does not Underlie Attenuation of the Tonic Current

One potential explanation for the AIE-induced attenuation of the tonic current is a reduction in the expression of δ -GABA_ARs. To investigate this possibility, we obtained tissue punches of PrL-C from control and AIE-treated rats. Immunoblot analysis revealed no significant between group differences in the expression $\alpha 1$, $\alpha 4$, $\alpha 5$, δ or $\gamma 2$ GABA_AR subunits (Figure 8A). Since the reduction in the δ -mediated tonic current may have resulted from a reduction in their surface expression that was offset by an increase in their internal expression, we also used the BS³ crosslinking procedure to specifically measure surface protein expression. Immunoblot analysis of the total and intracellular (non cross-linked) levels of the δ -subunit again showed no significant difference between control and AIE exposed rats (Figure 8B). Because our slice electrophysiology studies revealed layer-specific effects of AIE on δ -GABA_AR-mediated tonic currents, it is possible that our immunoblot procedures were unable to detect any changes in GABA_AR subunit expression because the analysis was performed using tissue punches that contained all cortical layers within the PrL-C. We therefore performed a follow-up experiment using immunohistochemistry to examine layer-specific expression of the δ -subunit in the PrL-C of both control and AIE exposed adult rats. Consistent with previous reports regarding δ -subunit distribution (Pirker et al., 2000), immunohistochemistry revealed high levels of expression in the granule cell layer of the cerebellum, the dentate gyrus of the hippocampus and the anterodorsal and anteroventral nuclei of the thalamus (not shown), with lower levels of cortical expression (Figure 8C,D). Also in agreement with previous reports (Datta et al., 2014), we observed marked differences in δ -subunit expression across the cortical layers, with superficial layers (I and II/III) exhibiting significantly higher levels of δ -subunit expression compared to the deep layers (layer V/VI) ($F_{(1,8)} = 182.1$, $p < 0.0001$). However, consistent with our immunoblotting experiments, we did not observe a significant difference between control

and AIE exposed rats in the staining intensity of the δ -subunit in either the superficial or deep layers of the PrL-C.

Discussion

The present study demonstrates that layer V pyramidal neurons in the PrL-C of the rat undergo a striking increase in tonic GABA_AR-mediated inhibition during adolescence. The developmental emergence of this current was observed as a change in the number of pyramidal neurons that expressed a measurable tonic GABA_AR current and a development-dependent increase in current mediated by δ -subunit containing GABA_ARs. Importantly, intermittent exposure to alcohol during the early to middle period of adolescence (PD28-42) resulted in a reduction in the amplitude of the tonic current. This reduction was observed shortly after the last period of AIE exposure (i.e., PD43-47) and persisted well into adulthood (i.e., >PD90). This effect was not sex specific as AIE exposure attenuated the tonic current to a similar extent in both male and female adult rats. In contrast to these observations in layer V pyramidal neurons, AIE exposure had no effect on the tonic GABA_AR current in layers II/III. Taken together, these observations demonstrate that AIE exposure results in a remarkably protracted and layer-specific deficit in tonic GABA_AR currents in PrL-C. The reduced inhibitory tone of layer V projection neurons may contribute to recent demonstrations of AIE-induced deficits in decision-making and behavioral control in adulthood (Boutros et al., 2014; Clark et al., 2012; Gass et al., 2014; Mejia-Toiber et al., 2014; Schindler et al., 2014).

GABA neurotransmission can operate in two modes. Phasic GABA currents produce IPSCs through classic synaptic processes that induce short bursts of inhibition (Farrant and Nusser, 2005). This mode of transmission provides spatial and temporal precision. In contrast, tonic GABA currents principally arise from ambient GABA in the extrasynaptic space that activates high affinity peri-/extrasynaptic GABA_ARs. The persistent activation of these receptors induces a strong inhibitory action on the cell by shunting the electrical signal. This mode of GABA neurotransmission is increasingly recognized as playing a critical role in the pathophysiology of various CNS and neurodevelopmental disorders (Egawa and Fukuda, 2013; Whissell et al., 2015). While tonic GABA currents have been observed in a number of brain regions, several studies have reported that acute slices from the mPFC lack a tonic GABA current (Drasbek and Jensen, 2006; Weitlauf and Woodward, 2008). It is important to note, however, that those observations were obtained in slices prepared from young animals. In the present study, we also observed that in slices from young rats (PD16-45), only a small percentage of neurons exhibited a measureable tonic GABA_AR current. However, as development progressed, the tonic current emerged such that in the fully mature adult (i.e., >PD90), virtually all layer V pyramidal neurons recorded from exhibited a robust GABA_AR tonic current. This observation is consistent with recent findings in layer V of the dorsolateral PFC of the rhesus monkey that demonstrated developmental changes in GABA_AR subunits that are thought to contribute to the tonic current (i.e., δ , $\gamma 2$, and $\alpha 5$) (Datta et al., 2014). We also found that exposure of rats to intermittent ethanol during adolescence prevented subsequent developmental increases in the expression of the tonic current. Importantly, this AIE-induced reduction in tonic current was still observed in adulthood (e.g. >PD90) several months after the last period of exposure to alcohol.

Therefore, AIE did not simply shift the time-course of the developmental trajectory of the tonic current and instead produced a long-lasting, potentially permanent, reduction in tonic GABAergic inhibition of layer V PrL-C pyramidal neurons.

A particularly interesting observation in the present study was that the AIE-induced reduction in tonic inhibition was layer specific, with attenuation of the tonic current in layer V but no change in layers II/III. Thus, protracted reduction in tonic inhibition associated with AIE exposure may be selective for pyramidal neurons of the PrL-C that provide output to subcortical structures. We, and others, have recently demonstrated that AIE exposure results in deficits in behavioral control in the adult that are consistent with PFC dysfunction (Gass et al., 2014; Spear, 2000; Squeglia et al., 2012). Although it is likely that alterations in the activity of other neurotransmitter and signaling systems contribute to these cognitive deficits, it is reasonable to suggest that the reduction in tonic inhibition we observed may be of particular importance to alterations in how the PFC processes information and directs behavior. Although the role that the tonic GABA_AR current plays in modulating network organization in the PFC is not clear, alterations in the level of tonic inhibition would be expected to significantly alter the input-output relationship and impact information processing within the mPFC.

It has been previously reported that adult chronic alcohol exposure results in alterations in the expression of the δ -subunit in the hippocampus (Cagetti et al., 2003; Liang et al., 2007; Liang et al., 2006), nucleus accumbens (Liang et al., 2014), and basolateral amygdala (Lindemeyer et al., 2014). Similarly, we recently reported that AIE exposure results in reduced expression of the δ -subunit in the adult hippocampus (Centanni et al., 2014), a finding that was consistent with a reduction of tonic currents in the AIE exposed adult hippocampus (Fleming et al., 2012; Fleming et al., 2013). Thus, it was unexpected that we did not observe any significant effect of AIE on either total or surface expression of the δ -subunit in the mPFC in the present study. Since AIE-induced attenuation of the tonic current was observed only for layer V pyramidal neurons with no alteration of tonic currents in layers II/III, it is possible that an AIE-induced reduction in δ -subunit expression specifically in layer V could have been obscured by subunit expression in other layers as a result of the tissue punch procedure that included all cortical layers. However, follow-up immunohistochemistry studies revealed that this did not appear to be the case. An additional caveat to these observations is that since neither the immunoblot nor immunohistochemistry procedures differentiated surface from non-surface receptors, the possibility remained that a reduction in the expression of surface GABA_ARs could have been offset by an increase in their intracellular expression. Once again, follow-up cross-linking studies showed that AIE exposure did not alter the surface expression of δ -GABA_ARs. Taken together, these data strongly suggest that the AIE-induced reduction in tonic current in the mPFC is not the result of reduced δ -GABA_AR expression but instead may be related to a change in receptor efficacy. Of particular interest are observations that PKA mediated phosphorylation of the $\beta 1$ and $\beta 3$ subunits of GABA_AR reduce and increase, respectively, channel conductance without altering surface expression (Brandon et al., 2002). Although speculative, the AIE-induced reduction of tonic current amplitude observed in the present study may relate to either an increase in the basal level of PKA-mediated phosphorylation of $\beta 1$ and/or a decrease in phosphorylation of $\beta 3$ subunits of extrasynaptic GABA_ARs.

There is significant evidence that both ethanol and neurosteroids act as high affinity positive allosteric modulators of δ -GABA_ARs. Our observations of an AIE-induced reduction in the amplitude of the tonic current led us to hypothesize that PrL-C neurons in AIE exposed adult rats would exhibit reduced facilitation in response to acute exposure of either ethanol or neurosteroids. As expected, bath application of ethanol and allopregnanolone resulted in a dose-dependent facilitation of the tonic current. In slices from AIE exposed rats, the amplitude of facilitation of the tonic current by either ethanol or allopregnanolone was significantly reduced. However, expression of these data as a percentage of the amplitude of the THIP-activated tonic current obtained in the respective control and AIE conditions revealed no difference in facilitation by either ethanol or allopregnanolone. These observations indicate that AIE exposure results in reduced efficacy but not affinity of the GABA_AR tonic current to facilitation by either ethanol or allopregnanolone. At least in the case of the acute effects of alcohol, these observations are consistent with behavioral studies showing that adolescents are less sensitive to the physiological effects of alcohol when similar doses are administered (Hollstedt et al., 1980a; Hollstedt et al., 1980b; Ramirez and Spear, 2010). In addition, exposure to alcohol during adolescence alters the efficacy of acute ethanol in the adult dentate gyrus of the hippocampus (Fleming et al., 2007). It has been suggested that adolescent alcohol abuse can result in a “lock-in” of certain cellular and behavioral aspects of the adolescent phenotype in the adult (Spear and Swartzwelder, 2014). The results of the present study are in agreement with this construct and further suggest that this lock-in may include reduced GABAergic activity of the adolescent PFC that persists into adulthood.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1. Expression of the GABA_AR tonic current is developmentally regulated in layer V pyramidal neurons of the PrL-C

A) Representative trace showing the measurement of tonic current as a change in holding current (I_{hold}) after bath application of the GABA_AR antagonist picrotoxin (100 μM , gray dashed line). **B)** Phasic (black) and tonic (white) GABA_AR currents measured at specific developmental time-points (PD: postnatal day) revealed that the amplitude of both tonic and phasic GABA currents remained stable throughout development. **C)** Although there was no developmental change in tonic current amplitude, the percentage of cells expressing a tonic current exhibited a dramatic developmental increase ($p = 0.002$).

Figure 2. Effect of adolescent ethanol exposure on phasic GABA_AR currents in layer V pyramidal neurons of the adult PrL-C

A) Representative traces of IPSCs from adult PrL-C slice obtained from control and AIE exposed rats. Cells were voltage-clamped at -70mV. **B)** While the frequency was similar in slices from both control and AIE treated rats, spontaneous IPSC (sIPSC) amplitude was significantly reduced in slices from AIE exposed rats ($n = 20-21$ cells/group; $**p = 0.004$). **C)** Representative paired-pulse traces of evoked IPSC (eIPSC) currents from adult PrL-C slices obtained from control and AIE treated rats. **D)** The paired-pulse ratio (PPR) was not altered by AIE exposure ($n = 8-9$ cells/group). **E)** Representative traces of miniature IPSCs (mIPSC) from adult PrL-C slices obtained from control and AIE exposure rats. **F)** AIE did not alter either the amplitude or frequency of mIPSCs ($n = 8-9$ cells/group).

Figure 3. Adolescent ethanol exposure results in a reduction in the amplitude of the GABA_AR tonic current

A) Representative trace showing tonic GABA_AR current, measured as the magnitude of change in holding current after bath application of the GABA_AR antagonist picrotoxin (100 μ M), in PrL-C slices obtained from control and AIE exposed rats. **B)** Representative all-point histograms and fitted Gaussian functions for the whole-cell current data showing the peak I_{hold} before and after picrotoxin in slices from control (left panel) and AIE (middle panel) rats. **C)** Slices obtained from control rats revealed that the amplitude of the tonic GABA_AR current remained stable throughout development and AIE exposure significantly attenuated tonic GABA current at all time-points examined, including shortly after exposure (PD 45 $\pm$ 3 days; n = 5-6 cells/group; $*p < 0.05$), in early adulthood (PD 60 $\pm$ 3 days; n = 6-8 cells/group; $**p < 0.01$), and in the fully mature adult (PD90 $\pm$ 6 days; n = 8-10 cells/group; $**p < 0.01$). **D)** AIE exposure significantly attenuated tonic GABA current in layer V pyramidal neurons from adult female rats (n = 7-8 cells/group, $***p < 0.001$).

Figure 4. Blocking GABA reuptake does not reverse AIE-associated reduction in tonic GABA_AR currents

A) Representative trace showing changes in holding current that occur following bath application of nipecotic acid in slices from the adult PrL-C. After establishing a stable baseline holding current, nipecotic acid (500 μ M) was bath applied for 5 minutes. Next, picrotoxin (100 μ M) was also added to the bath. The tonic current was measured as the change in I_{hold} from baseline (gray line) to that observed after picrotoxin. **B)** AIE exposure resulted in a significant decrease in the amplitude of the GABA_AR tonic current amplitude compared to slices from control rats ($n = 6-7$ cells/group; * $p < 0.05$).

Figure 5. The decrease in the tonic GABA_AR current following adolescent ethanol exposure reflects attenuation of current mediated by δ -subunit containing receptors

A) Representative trace showing the effect of bath application of the selective δ -GABA_AR agonist THIP. **B)** Representative all-point histogram and Gaussian function that were used to measure the effect of THIP as a change in the I_{hold} for each individual cell. **C)** THIP-mediated change in the I_{hold} in PrL-C slices obtained from control and AIE exposed rats at different developmental ages. In slices obtained prior to initiation of AIE exposure (PD28, $n = 5$ cells/group), shortly after the last cycle of AIE exposure (PD45, $n = 5$ cells/group), and in early adulthood (PD60, $n = 7$ cells/group) there were no differences in THIP modulation of holding current. However, in slices from adult rats, there was a significant reduction in the THIP mediated current ($n = 9-10$ cells/group, $*p = 0.04$). **D)** AIE exposure significantly attenuated THIP-mediated current in layer V pyramidal neurons from adult female rats ($n = 6-9$ cells/group $**p < 0.01$).

Figure 6. Adolescent ethanol exposure has no effect on the tonic GABA_AR current of pyramidal neurons in layer II/III of the adult PrL-C

A) Representative traces showing the effect of picrotoxin (100 μ M) on GABA_AR tonic currents in layer II/III pyramidal neurons. **B)** AIE had no effect on picrotoxin-sensitive GABA_AR tonic current in pyramidal neurons of layer II/III ($n = 4-6$ cells/group). **C)** Representative traces showing current mediated by δ -subunit containing GABA_AR receptors in layer II/III pyramidal neurons measured using the δ -GABA_AR agonist THIP (1 μ M). **D)** THIP-mediated changes in the holding current revealed that AIE had no effect on δ -GABA_AR current amplitude in layer II/III of PrL-C ($n = 5-6$ cells/group).

Figure 7. Adolescent ethanol exposure significantly decreases ethanol and allopregnanolone-induced facilitation of the tonic $GABA_A$ R current in adult PrL-C

A) Representative traces showing the change in I_{hold} in layer V pyramidal neurons in response to bath application of ethanol (10 mM, 50 mM and 100 mM) in PrL-C slices obtained from control and AIE exposed rats. **B)** Bath application of ethanol resulted in a dose-dependent facilitation of the tonic current, especially at higher doses ($n = 15$ cells/dose, $p < 0.0001$). The enhancement of tonic current that occurred with bath application of 50 and 100 mM ethanol was significantly attenuated in slices obtained from AIE exposed rats

compared to slices from control rats ($n = 6-10$ cells/group for each dose; $*p < 0.05$, $***p < 0.001$). **C)** Comparison of ethanol facilitation of the tonic current to the average THIP current in control and AIE exposed conditions indicates that AIE did not alter the affinity of the δ -GABA_AR to acute alcohol and instead reduced the efficacy of facilitation of tonic currents. **D)** Representative traces showing the change in I_{hold} in layer V pyramidal neurons in response to bath application of a low ($0.1 \mu\text{M}$) and high ($1.0 \mu\text{M}$) dose of the allopregnanolone in PrL-C slices obtained from control and AIE exposed adult rats **E)** Allopregnanolone dose-dependently facilitated the tonic current ($n = 18$ cells/dose, $p = 0.0009$), and this enhancement was significantly attenuated in slices obtained from AIE exposed rats compared to slices from control rats ($n = 8-9$ cells/group for each dose, $*p < 0.05$). **F)** Comparison of allopregnanolone facilitation of the tonic current to the average THIP current in control and AIE exposed conditions indicates that AIE did not alter the affinity of the δ -GABA_AR tonic current to acute allopregnanolone and instead reduced the efficacy of facilitation.

Figure 8. Adolescent ethanol exposure has no effect on expression of GABA_AR subunits that contribute to the tonic current

A) Protein expression of GABA_AR subunit in the PrL-C of AIE exposed adult rats measured in total tissue extracts (n = 8-9 rats/group). **B)** Surface expression of δ -GABA_ARs using BS³ cross-linking of surface membrane proteins in acute slices. Shown on the left is loss of the δ -subunit and total protein upon detergent permeabilization of the slice in the presence and absence of the cross-linking reagent BS³. Shown on the right is quantification of δ -subunit surface expression expressed as the difference between the non-cross-linked intracellular and the total subunit expression (n = 13-14 rats/group). **C)** Representative image from an Air exposed control rat of immunohistochemical labeling for the δ -subunit showing differential expression in superficial and deep layers of the cortex. **D)** Quantification of the boxed area in C revealed significantly greater δ -subunit expression in superficial versus deep layers of the PrL-C ($p < 0.001$) but a significant effect of AIE exposure was not observed.