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Sensitivity of GABAergic tonic currents to acute ethanol in cerebellar granule neurons is not age- or δ subunit-dependent in developing rats

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Abstract

Background—The age of first exposure to ethanol (EtOH), as well as reduced sensitivity to its motor-impairing effects, are associated with a future predisposition to abuse EtOH. In adolescence, acute EtOH potentiates GABA transmission, including tonic inhibition mediated by δ -containing extrasynaptic GABA_A receptors (GABA_ARs) in cerebellar granule neurons (CGNs), an effect that likely contributes to EtOH-induced motor impairment. Prenatal EtOH exposure is strikingly prevalent and is associated with increased EtOH abuse later in life; however, the acute effects of EtOH on GABA transmission in developing CGNs are unknown.

Methods—Using whole-cell patch-clamp electrophysiological techniques in acute brain slices, we examined the acute effects of EtOH on GABA transmission and functionally assessed the role of δ -containing GABA_ARs in CGNs of pre-weanling (postnatal day (P) 12–14) and post-weanling (P28–30) male Sprague-Dawley rats.

Results—The magnitude of basal tonic currents were similar at both ages. However, 4,5,6,7-Tetrahydroisoxazolo[5,4-c]pyridin-3-ol hydrochloride (THIP), an agonist with preferential affinity for δ -containing GABA_ARs, significantly potentiated tonic currents to a larger magnitude in CGNs from post-weanlings compared to pre-weanlings. Conversely, acute application of EtOH (80 mM) significantly increased tonic currents and the frequency of spontaneous inhibitory postsynaptic currents to a similar extent in CGNs from pre- and post-weanlings.

Conclusions—These findings highlight the sensitivity of the developing cerebellum to EtOH. Furthermore, this study demonstrates age-dependent functional changes in a well characterized circuitry that may contribute to the short- and long-term effects of prenatal exposure to ethanol.

Keywords

Ethanol; cerebellum; tonic GABA; development; prenatal

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Introduction

Ethanol (EtOH) is known to alter multiple systems in numerous brain regions, resulting in a combination of behavioral alterations. Some EtOH-induced behavioral alterations, such as reduced sensitivity to EtOH and motor ataxia (Schuckit, 1994), are related to age of exposure (Spear, 2015, DeWit et al., 2000, Masten et al., 2009). Importantly, prenatal exposure to EtOH is also associated with future manifestations of neurobehavioral dysfunction and abuse propensity (Valenzuela et al., 2012, Baer et al., 1998, Baer et al., 2003, Barbier et al., 2009); yet, the neurobiological effects of EtOH during development are largely unknown.

Cerebellar granule neurons (CGNs), which function to filter information into the cerebellar cortex, are well-known targets of EtOH, and have been shown to be highly sensitive to the acute and chronic effects of EtOH throughout development (Luo, 2015, Luo, 2012). Acute exposure to EtOH, at concentrations equal to or greater than the legal U.S. intoxication limit (0.08 g/dl or 17 mM) robustly impairs motor performance in rats at the end of their brain growth spurt (Silveri and Spear, 2001, Van Skike et al., 2010), equivalent to the 3rd trimester of human pregnancy (Biran et al., 2012). Similarly, acute EtOH exposure impairs motor performance in adolescent rats, and this is, in part, related to the actions of EtOH on CGNs (Hanchar et al., 2005). It is well established that GABAergic transmission is a primary target of acute EtOH (Weiner and Valenzuela, 2006), particularly in CGNs during adolescence (Valenzuela and Jotty, 2015). Despite these known interactions between age and EtOH, the acute effects of EtOH on GABA transmission in immature CGNs have not been studied.

GABA_A receptor (GABA_AR) tonic inhibition has been shown to be particularly sensitive to EtOH at doses relevant for motor ataxia (Diaz and Morton, 2014). Tonic inhibition is mediated via activation of extrasynaptic GABA_ARs primarily by ambient GABA in the extracellular space (Farrant and Nusser, 2005). While the source of ambient GABA remains unclear (Diaz et al., 2011, Lee et al., 2010, Yoon et al., 2014), we and others have demonstrated that GABA release from Golgi cells significantly contributes to tonic inhibition of CGNs (Bright et al., 2011, Diaz et al., 2013, Rossi et al., 2003). Since their discovery, the majority of extrasynaptic GABA_ARs throughout the central nervous system have been found to express the δ -subunit which significantly contributes to their functional activity [reviewed by (Farrant and Nusser, 2005)]. In mature CGNs, extrasynaptic GABA_ARs are comprised of $\alpha_6\beta\gamma\delta$ and heavily contribute to the inhibitory tone in these neurons via tonic inhibition (Brickley and Mody, 2012, Farrant and Nusser, 2005). In older animals, deletion of the δ -subunit has been shown to abolish tonic currents in mature CGNs (Stell et al., 2003), consistent with the notion that the δ -subunit is essential for expression of a tonic current later in development. In the mature cerebellum, we and others have shown that acute EtOH can increase Golgi cell firing *in vivo* (Huang et al., 2012) and *in vitro* (Botta et al., 2010, Botta et al., 2011, Kaplan et al., 2013) via various mechanisms, ultimately resulting in potentiation of tonic currents in CGNs (Carta et al., 2004, Diaz et al., 2013).

One potential contribution to the long lasting effects of EtOH following developmental EtOH exposure is the age-dependent maturation of GABA transmission in CGNs (Laurie et

al., 1992). In contrast to the $\gamma 2$ subunit, which is expressed prenatally and remains stable through adulthood in rodents, δ subunit mRNA is not apparent until postnatal day (P) 12 (Laurie et al., 1992) and δ protein levels are not appreciable until ~P21, with a further increase at P28 (Diaz et al., 2014). Given that the expression of tonic currents in CGNs is relatively stable as early as P12 (Brickley et al., 1996, Diaz et al., 2014), there is an apparent developmental mismatch between the expression of tonic currents and the δ subunit. Recent studies, however, have shown that functional extrasynaptic receptors also express the γ -subunit (Brickley and Mody, 2012), indicating region-specific expression of receptors with unique subunit composition. Given that immature CGNs express tonic currents before expressing the δ subunit (Diaz et al., 2014) and the effects of acute EtOH on GABA transmission in immature CGNs are unknown, the objective of this study was to 1) determine the functional contribution of the GABA_AR δ -subunit in developing CGNs and 2) characterize the acute effects of EtOH on tonic currents in developing CGNs.

Methods

Drugs and chemicals

All drugs and chemicals were from Sigma-Aldrich (St. Louis, MO) unless indicated.

Animals

All experiments were approved by the University of New Mexico Health Sciences Center and Binghamton University Institutional Animal Care and Use Committee and conformed to the National Institutes of Health guidelines. Timed-pregnant Sprague-Dawley rats (Harlan Laboratories) were shipped to either institute. Litters were weaned at P21, group-housed, and received food and water *ad libitum*.

Brain Slice Preparation

Using methods previously described (Diaz et al., 2014), pre-weanling (P12–14) or post-weanling (P28–30) male rats were sacrificed by rapid decapitation under deep anesthesia with ketamine (250 mg/kg i.p.). Brains were removed and placed for 2 min in cold sucrose artificial cerebrospinal fluid (aCSF) that contained in mM: 220 sucrose, 2 KCl, 1.25 NaH₂PO₄, 26 NaHCO₃, 12 MgSO₄, 10 glucose, 0.2 CaCl₂ and 0.43 ketamine, pre-equilibrated with 95% O₂/5% CO₂. Cerebellar vermis parasagittal slices (300 μ m thick in pre-weanling and 200 μ m thick in post-weanling) were prepared in sucrose aCSF and transferred to normal aCSF for 40 min at 35–36°C; normal aCSF contained (in mM): 126 NaCl, 2 KCl, 1.25 NaH₂PO₄, 26 NaHCO₃, 10 glucose, 1 MgSO₄, 2 CaCl₂, and 0.4 ascorbic acid that was continuously bubbled with 95% O₂/5% CO₂. Slices were then stored in normal aCSF at 25°C.

Cerebellar Slice Electrophysiology

Whole-cell patch clamp recordings of CGNs were performed as previously described (Diaz et al., 2014). Briefly, identification of CGNs was based on their location in the internal granule cell layer and their size (capacitance = 2–10 pF). To record GABA_AR-mediated tonic currents and spontaneous inhibitory postsynaptic currents (sIPSCs) from CGNs, patch pipettes (tip resistance = 3–5 M Ω) were filled with an internal solution containing (in mM):

135 KCl, 10 HEPES, 2 MgCl₂, 0.5 EGTA, 5 Mg-ATP, 1 Na-GTP, and 1 QX314-Br (Tocris, Minneapolis, MN), pH 7.25, osmolarity 280–290 mOsm. GABAergic currents were pharmacologically isolated with the ionotropic glutamate receptor blockers kynurenic acid (1mM) and D, L-APV (50 μ M, Tocris). These glutamate antagonists were applied for ~5 min prior to starting a recording. The holding potential was kept at –70 mV. Data were acquired in gap-free mode at 10 kHz and filtered at 2 kHz.

Electrophysiology data were analyzed with Clampfit-10 (Molecular Devices, Sunnyvale, CA) or MiniAnalysis (Synaptosoft, Decatur, GA). As previously described (Diaz et al., 2014), tonic currents were calculated by fitting a Gaussian distribution to an all-point histogram for every 30 seconds or every minute of the recording and constraining the fit to eliminate the contribution of sIPSCs. The tonic current amplitude was calculated as the mean current in the absence minus the mean current in the presence of picrotoxin (50 μ M, PTX).

Statistics

Data were statistically analyzed with Prism 5 (Graphpad, San Diego, CA). Initially, data were analyzed with the Pearson omnibus normality test. Parametric tests were used if data followed a normal distribution; otherwise, non-parametric tests were used. Data are presented as mean $\pm$ SEM. A $p < 0.05$ was considered statistically significant. For all statistical analyses, the experimental unit is a cell.

Results

Basal GABA transmission in CGNs does not differ between pre- and post-weanlings

Consistent with previous developmental studies (Brickley et al., 1996, Diaz et al., 2014), the magnitude of the basal tonic current was similar between pre-weanlings (Fig. 1A; $n = 15$) and post-weanlings (Fig. 1B; $n = 13$) and there was no significant difference between the two ages (Fig. 1C: $t = 1.197$, $df = 26$; $p > 0.05$ by unpaired t-test). There was also no significant age-dependent difference in sIPSC frequency (Fig. 1D and F; $n = 13–15$; $t = 1.43$, $df = 26$; $p = 0.16$ by unpaired t-test). While there was a trend toward smaller sIPSC amplitude in post-weanlings, it was not statistically different (Fig. 1E and G; $n = 13–15$; $t = 1.74$, $df = 25$; $p = 0.09$ by unpaired t-test). Interestingly, specific components of sIPSC kinetics varied between the two ages (Fig. 1H). Calculating sIPSC decay time using 2-exponentials, post-weanlings exhibited a significantly faster sIPSC tau 1 compared to pre-weanlings (Fig. 1I; $n = 13–15$; Mann-Whitney $U = 41$, sum of ranks = 246,132, $p < 0.05$ by Mann-Whitney test); but, there was no significant difference in tau 2 (pre-weanling: 63.21 ± 29.10 ms, $n = 13$; post-weanling: 43.52 ± 13.40 ms, $n = 13$; Mann-Whitney $U = 78$, sum of ranks = 182,169, $p > 0.05$ by Mann-Whitney test).

THIP-modulation of tonic GABA currents is age-dependent

Although the magnitude of basal tonic currents was similar in CGNs of pre- and post-weanlings, we previously found that expression of the δ subunit in the internal granule cell layer of the cerebellar cortex was lower in pre- vs. post-weanling animals (Diaz et al., 2014). Therefore, to pharmacologically assess the contribution of δ -containing GABA_AR function,

we acutely applied THIP, an agonist with preferential selectivity for δ subunit-containing GABA_ARs at nanomolar concentrations (Meera et al., 2011), onto cerebellar slices of pre- (Fig. 2A) and post- (Fig. 2B) weanling animals. A 2-way ANOVA showed a significant effect of age ($F(1,24) = 5.033$, $p < 0.05$), THIP ($F(1,24) = 19.889$, $p < 0.001$), and an age $\times$ THIP interaction ($F(1,24) = 6.156$, $p < 0.05$). Post-hoc analysis showed THIP (500 nM) only significantly increased tonic currents in CGNs of post-weanlings ($t = 4.49$, $df = 24$, $p < 0.05$ - Bonferroni post-hoc test). Importantly, the effect of THIP on tonic currents was significantly greater in post-weanling compared to pre-weanling animals (Fig. 2D; $n = 6-8$; $t = 2.59$, $df = 12$; $p < 0.05$ by un-paired t-test) consistent with the notion that tonic currents are mediated by δ subunit-containing GABA_ARs in the post-weanlings but not in pre-weanlings.

Tonic current sensitivity to acute ethanol is not age-dependent

Acute EtOH has been shown to potentiate phasic and tonic GABA transmission in CGNs of adolescent and adult animals (Bright et al., 2011, Carta et al., 2004, Diaz et al., 2013, Hancher et al., 2005), but the sensitivity of the GABA system to acute EtOH in immature CGNs is unknown. We found a significant effect of acute EtOH (80 mM) on tonic currents regardless of age (Fig. 3A–C; age ($F(1,22) = 49.42$, $p < 0.0001$), with no significant effect of age ($F(1,22) = 0.098$, $p > 0.05$) or an age $\times$ drug interaction ($F(1,22) = 0.459$, $p > 0.05$). Importantly, this effect was not significantly different between the two ages (Fig. 3D; Mann-Whitney $U = 12$, sum of ranks = 48,43, $p > 0.05$, Mann Whitney test). Likewise, 80 mM EtOH significantly increased sIPSC frequency at both ages (pre-weanling: $51.92 \pm 13.00\%$, $n = 8$, $t = 3.99$, $df = 7$, $p < 0.001$, one-sample t-test vs. 100%; post-weanling: $134.10 \pm 44.60\%$, $n = 5$, $t = 3.00$, $df = 4$, $p < 0.05$ by one-sample t-test vs. 100%), and this was also not significantly different between the two ages (Mann-Whitney $U = 7$, sum of ranks = 43,48, $p > 0.05$ by Mann Whitney test). There was also no significant effect of 80 mM EtOH on sIPSC amplitude at either age (pre-weanling: $7.62 \pm 4.69\%$, $n = 8$, $t = 1.62$, $df = 7$, $p > 0.05$ by one-sample t-test; post-weanling: $5.24 \pm 9.76\%$, $n = 5$, $t = 0.54$, $df = 4$, $p > 0.05$ by one-sample t-test).

Ethanol-induced potentiation of GABA transmission is action potential-dependent in pre-weanlings

We have previously demonstrated that tetrodotoxin (TTX), an action potential blocker, decreases sIPSC frequency and blocks the component of the tonic current that is dependent on spillover of GABA release in a spontaneous action potential-dependent manner (Carta et al., 2004, Diaz et al., 2013). In addition, TTX also completely blocks the acute effect of EtOH in mature CGNs, suggesting that the actions of EtOH are action potential-dependent in older rats (Carta et al., 2004, Diaz et al., 2013). Here, we tested whether this is also the case in pre-weanlings. Consistent with our previous study in CGNs from P13 rats (Diaz et al., 2014), TTX alone significantly reduced tonic currents (Fig. 4A and C; $t = 2.91$, $df = 4$, $n = 5$, $p < 0.05$ by one-sample t-test) and sIPSC frequency (Fig. 4B and C; $t = 11.16$, $df = 4$, $n = 5$, $p < 0.001$ by one-sample t-test) in CGNs of pre-weanlings, with no significant change in sIPSC amplitude (Fig. 4B; % change from baseline = $-12.68 \pm 25.23\%$, $t = 0.50$, $df = 4$, $n = 5$, $p > 0.05$ by one-sample t-test). Importantly, while 80 mM EtOH significantly increased tonic currents in pre-weanlings (Fig. 4D; $t = 3.89$, $df = 7$, $n = 8$, $p < 0.001$ by one-sample t-

test), EtOH did not significantly change tonic currents in the presence of TTX (Fig. 4D; $t = 0.015$, $df = 4$, $n = 5$, $p > 0.05$ by one-sample t-test). Likewise, the 80 mM EtOH-induced significant increase of sIPSC frequency (Fig. 4E; $t = 3.10$, $df = 7$, $n = 8$, $p < 0.05$ by one-sample t-test), was not observed in the presence of TTX (Fig. 4E; $t = 0.97$, $df = 4$, $n = 5$, $p > 0.05$).

Sensitivity to acute EtOH is dose-dependent in CGNs from pre-weanlings

We next determined if the effect of acute EtOH was dose-dependent in pre-weanlings as we have previously shown in adolescent animals (Carta et al., 2004). We found a significant dose-dependent effect of EtOH on tonic currents (Fig. 5A; $F(2,19) = 5.576$, $n = 6-8$, $p < 0.05$ by one-way ANOVA). Post-hoc analysis revealed that the effect of 80 mM EtOH was significantly different than 20 mM (Bonferroni's multiple comparisons). Conversely, while all concentrations of EtOH did significantly increase sIPSC frequency (20 mM: $t = 4.25$, $df = 7$, $p < 0.01$ by one-sample t-test; 40 mM: sum of signed ranks (W) = 21, $n = 6$, $p < 0.05$ by Wilcoxon Signed Rank Test; 80 mM: $t = 3.10$, $df = 7$, $n = 8$, $p < 0.05$ by one-sample t-test) the effect was not dose-dependent (Fig. 5B; $F(2,19) = 0.96$, $n = 6-8$, $p > 0.05$ by one-way ANOVA). None of the EtOH concentrations tested significantly altered sIPSC amplitude (20 mM: -0.48 ± 3.88 pA, sum of signed ranks (W) = -2.0 , $n = 4$, $p > 0.05$ by Wilcoxon Signed Rank Test; 40 mM: 2.29 ± 1.79 pA, $t = 1.27$, $df = 5$, $n = 6$, $p > 0.05$ by one-sample t-test; 80 mM: -3.08 ± 2.68 pA, $t = 1.15$, $df = 7$, $n = 8$, $p > 0.05$ by one-sample t-test).

Discussion

The GABA system is a primary target of EtOH throughout development, therefore, the current study examined the effects of acute EtOH on GABA transmission in immature CGNs and investigated the functional contribution of the GABA_AR δ -subunit in CGNs as a function of age. Despite robust neuronal maturation, migration, and re-organization of the cerebellar cortex during the first 2–3 postnatal weeks in rodents, a period equivalent to the 3rd trimester of human pregnancy (Biran et al., 2012), we found that the magnitude of basal tonic currents was similar in pre-weanlings (~P12) and post-weanlings (~P28). Consistent with previous characterization of δ -subunit protein expression in CGNs (Diaz et al., 2014), we found a significant and large age-dependent increase in THIP-induced tonic currents. However, tonic GABA currents were similarly potentiated by acute EtOH at both ages. Additionally, we found that the effect of acute EtOH on GABA transmission was action potential-dependent and dose-dependent on tonic currents in pre-weanlings, similar to studies in post-weanlings. Overall, this study reveals that δ -containing GABA_ARs do not functionally contribute to tonic currents in immature CGNs and that the effects of acute EtOH on GABA transmission in CGNs is relatively consistent across development.

GABA transmission in CGNs has been shown to be developmentally regulated, particularly during the first 2 postnatal weeks (Brickley et al., 1996, Diaz et al., 2014). However, we found that from ~P12 and onward, tonic currents remain relatively stable, consistent with our previous study (Diaz et al., 2014). Interestingly, while the prevailing view has been that tonic currents in CGNs are mediated by δ -containing extrasynaptic GABA_ARs (Farrant and Nusser, 2005, Stell et al., 2003), we and others have shown that δ -subunit protein (Diaz et

al., 2014) and mRNA (Laurie et al., 1992, Wisden et al., 1992) expression is lower in early postnatal development. Furthermore, THIP binding in the cerebellar granule cell layer has been shown to be significantly lower in the first 10 postnatal days compared to P25 (Friemel et al., 2007). Consistent with these biochemical analyses, we found that tonic currents in pre-weanlings were insensitive to THIP, contrasting the large THIP-induced potentiation of tonic currents in post-weanlings. One possible explanation for the presence of tonic currents in the absence of δ -containing extrasynaptic GABA_ARs is that extrasynaptic GABA_ARs with a different subunit composition may mediate tonic currents in early postnatal development of CGNs. Particularly, γ -containing extrasynaptic GABA_ARs may mediate tonic currents in developing CGNs as $\alpha 6\beta\gamma 2$ GABA_ARs have been shown to replace δ -containing receptors in the cerebellar cortex of adult δ knockout animals (Tretter et al., 2001).

There are also several reports of tonic currents mediated by γ -containing extrasynaptic GABA_ARs in pyramidal neurons of hippocampal CA1 and CA3 (Wei et al., 2004, Caraiscos et al., 2004, Glykys and Mody, 2006, Glykys and Mody, 2007, Pavlov et al., 2009, Prenosil et al., 2006, Semyanov et al., 2004), neocortex (Yamada et al., 2007), and central amygdala (Herman et al., 2013). Furthermore, there is strong and stable γ -subunit mRNA expression in the internal granule cell layer beginning in early development (Laurie et al., 1992), suggesting that γ -containing extrasynaptic receptors may be a likely candidate. Reports of high affinity $\alpha\beta$ GABA_ARs have also been shown to replace δ -containing GABA_ARs in cerebellum (Tretter et al., 2001), which can mediate tonic conductances in the hippocampus (Mortensen and Smart, 2006), even in the absence of agonists (Hadley and Amin, 2007), particularly in immature neurons (Birnir et al., 2000). Another explanation is that ambient GABA levels may be much higher in pre-weanlings to compensate for the low expression of δ -containing GABA_ARs in order to maintain a sufficient tonic current throughout development. As we had previously shown (Diaz et al., 2014), we found that the Golgi cell-dependent component of tonic currents, as measured by the effect of TTX, was similar between pre-weanlings (current study) and previous reports in older animals (Carta et al., 2004, Diaz et al., 2013, Bright et al., 2011). Since Golgi cells significantly contribute to tonic currents in CGNs (Carta et al., 2004, Diaz et al., 2013, Crowley et al., 2009), our data suggest that the synaptically released-dependent pool of ambient GABA cannot account for the similar magnitude of tonic currents between the two ages. However, this does not rule out fluctuations in ambient GABA levels from other sources, including glial cells (Yoon et al., 2014). Future studies should examine expression of other GABA_AR subunits and levels of ambient GABA to better understand the mechanisms underlying the mismatch between basal tonic currents and δ -subunit expression in early postnatal development.

We also found that the fast decay component of sIPSCs (τ_1) was significantly longer in pre- compared to post-weanlings indicative of slower synaptic GABA_AR function in immature CGNs. Similar findings have been reported in cultured CGNs across days *in vitro* (Ortinski et al., 2004). These observations suggest that a developmental switch in synaptic GABA_AR subunit composition also occurs in CGNs. $\alpha 1$ -containing GABA_ARs exhibit faster deactivation than $\alpha 4$ - (Lagrange et al., 2007) and $\alpha 2$ -containing GABA_ARs (Dixon et al., 2014). Modest $\alpha 2$ and $\alpha 4$ mRNA expression in the cerebellum has been shown through

P12 but not in adults (Laurie et al., 1992), while $\alpha 1$ expression gradually increases and peaks at P12. Therefore, it is possible that a switch from $\alpha 2/\alpha 4$ - to $\alpha 1$ -containing GABA_ARs explains the change in decay kinetics.

While it is well established that acute EtOH can potentiate tonic currents in adolescent and adult CGNs (Carta et al., 2004, Diaz et al., 2013, Hanchar et al., 2005, Kaplan et al., 2013), the sensitivity of tonic currents to acute EtOH in CGNs of pre-weanlings, a developmental period equivalent to the 3rd trimester of human pregnancy (Biran et al., 2012), was previously unknown. Importantly, our data shows that even in early development, EtOH can significantly potentiate tonic currents in CGNs similar to adolescents, suggesting that these two developmental ages may have similar EtOH-induced motor impairment. Consistent with this, it was previously shown that rats of similar ages to those in the current study did not differ in either EtOH-induced swim impairment (Silveri and Spear, 2001) or in the areal righting reflex (Van Skike et al., 2010) with blood EtOH concentrations within the range used in the current study. Given that pre-weanlings and post-weanlings exhibit similar EtOH-induced motor impairment (Silveri and Spear, 2001, Van Skike et al., 2010), our data provide a neurophysiological mechanism that likely contributes to the ataxic effects of EtOH in these early developmental periods.

The cerebellum has been suggested to be one of the brain structures most vulnerable to the teratogenic effects of EtOH during development, particularly during the 3rd trimester-equivalent (Luo, 2015, Luo, 2012). Numerous reports indicate significant CGN loss following binge-like EtOH exposure [reviewed in (Luo, 2012)], with blood ethanol concentrations < 150 mg/dl (~35 mM EtOH) reducing CGN levels by 20% (Maier and West, 2001). Importantly, these doses are well in the range of EtOH concentrations that produced significant effects on GABA transmission in the current study. EtOH-induced cell death of CGNs has been investigated extensively and has revealed that many factors can contribute to death of CGNs, including activation of NMDA receptors, imbalance of neurotrophic factors and retinoic acid processes, oxidative stress, thiamine deficiency, and alterations in potassium channel function [reviewed by (Luo, 2012)]. Given that in early development GABA transmission is involved in depolarization of neuronal membrane potential, rather than hyperpolarization (Ben-Ari, 2014), it is possible that significant EtOH-induced potentiation of tonic currents in CGNs may be another contributor to excitotoxicity and eventual death of CGNs. Based on the current findings, it is possible that developmental exposure to agonists for GABA tonic currents would mimic EtOH teratogenicity in CGNs. Therefore, studies examining the role of EtOH-induced potentiation of tonic currents in developing CGNs and cell death are warranted.

We and others have shown that in adolescent and adult rodents, acute EtOH can increase tonic currents in CGNs by increasing Golgi cell firing via various mechanisms (Botta et al., 2010, Botta et al., 2011, Kaplan et al., 2013); however, this has been debated as others have shown that tonic currents in CGNs can be potentiated as a result of direct activation of the δ subunit (Hanchar et al., 2005, Santhakumar et al., 2013). Importantly, we found that in pre-weanlings that have little to no δ expression, acute EtOH potentiated tonic currents to a similar extent as in post-weanling animals that exhibit greater δ expression. We also found a significant increase in sIPSC frequency without any changes in sIPSC amplitudes at both

ages, indicative of and consistent with a presynaptic effect. Additionally, the effects of acute EtOH were attenuated when inhibiting spontaneous action potentials in Golgi cells with TTX, as has previously been shown in adolescent and adult animals (Carta et al., 2004, Diaz et al., 2013, Kaplan et al., 2013). Therefore, these data are consistent with the notion that in CGNs, δ subunit-containing GABA_ARs are not a primary target of EtOH, and that regardless of age, EtOH can increase tonic inhibition via presynaptic mechanisms.

Taken together, the current study addresses a number of important questions that had not previously been explored. Our advances in the understanding of the dynamic development of CGNs, particularly the expression and function of the δ subunit, demonstrate our little understanding of the development of the nervous system. Furthermore, the robust sensitivity of GABA transmission to acute EtOH in immature CGNs convincingly demonstrates the vulnerability of this system to EtOH in early development that likely contributes to the long-lasting effects of prenatal exposure to EtOH.

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Figure 1. Developmental characterization of tonic and phasic currents

Exemplar traces of tonic currents from (A) pre-weanlings and (B) post-weanlings. (C) Basal tonic current amplitudes are not different between pre- and post-weanlings. (D) Exemplar traces from pre- and post-weanlings of sIPSC frequency; (E) sIPSC frequency was not different. (F) Expanded averaged traces of sIPSC amplitudes from pre- (green) and post-weanlings (red); (G) sIPSC amplitude was not different. (H) Scaled averaged traces of sIPSC emphasize decay kinetics; (I) sIPSC tau 1 was significantly smaller in post-weanlings (* - $p < 0.05$). PTX – picrotoxin.

Figure 2. THIP-induced potentiation of tonic currents across development

Exemplar traces of the effect of THIP (500 nM) on tonic currents in (A) pre-weanlings and (B) post-weanlings. (C) THIP significantly increased tonic currents only in post-weanlings (* - $p < 0.05$). (D) The effect of THIP was significantly larger in post-weanlings relative to pre-weanlings (# - $p < 0.05$ compared to pre-weanling).

Figure 3. Acute EtOH-induced potentiation of tonic currents across development

Exemplar traces of the effect of EtOH (80 mM) on tonic currents in (A) pre-weanlings and (B) post-weanlings. (C) EtOH significantly increased tonic currents at both ages (* - $p < 0.05$ compared to pre-weanling baseline, \$ - $p < 0.05$ compared to post-weanling baseline). (D) There was no difference in the effect of 80 mM EtOH potentiation of tonic currents between pre- and post-weanlings.

Figure 4. EtOH-induced potentiation of tonic currents is TTX-sensitive in pre-weanlings
 Exemplar traces of the effect of TTX on (A) tonic currents and (B) sIPSC frequency and amplitude. (C) TTX significantly decreased tonic current amplitude (* - $p < 0.05$ compared to 0) and sIPSC frequency in pre-weanlings (** - $p < 0.001$). (D) The significant EtOH (80 mM)-induced potentiation of tonic currents was abolished in the presence of TTX (* - $p < 0.05$ compared to 0). (E) The significant EtOH (80 mM)-induced increase of sIPSC frequency was abolished in the presence of TTX (* - $p < 0.05$ compared to 0). TTX – tetrodotoxin.

Figure 5. Acute EtOH effect on GABA transmission is dose-dependent in pre-weanlings
(A) Application of 20 mM EtOH did not change tonic currents, while 40 and 80 mM EtOH significantly increased tonic currents in pre-weanlings (* - $p < 0.05$ compared to 0). The effect of 80 mM was significantly different than 20 mM (# - $p < 0.05$). (B) 20, 40 and 80 mM EtOH significantly increased sIPSC frequency in pre-weanlings (* - $p < 0.05$ compared to 0).