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Repeated intermittent alcohol exposure during the third trimester-equivalent increases expression of the GABA_A receptor δ subunit in cerebellar granule neurons and delays motor development in rats

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Abstract

Exposure to ethanol (EtOH) during fetal development can lead to long-lasting alterations, including deficits in fine motor skills and motor learning. Studies suggest that these are, in part, a consequence of cerebellar damage. Cerebellar granule neurons (CGNs) are the gateway of information into the cerebellar cortex. Functionally, CGNs are heavily regulated by phasic and tonic GABAergic inhibition from Golgi cell interneurons; however, the effect of EtOH exposure on the development of GABAergic transmission in immature CGNs has not been investigated. To model EtOH exposure during the 3rd trimester-equivalent of human pregnancy, neonatal pups were exposed intermittently to high levels of vaporized EtOH from postnatal day (P) 2 to P12. This exposure gradually increased pup serum EtOH concentrations (SECs) to ~60 mM (~0.28 g/dl) during the 4 hours of exposure. EtOH levels gradually decreased to baseline 8 hrs after the end of exposure. Surprisingly, basal tonic and phasic GABAergic currents in CGNs were not significantly affected by postnatal alcohol exposure (PAE). However, PAE increased the expression of δ subunit expression at P28 as detected by immunohistochemical and western blot analyses. Also, electrophysiological studies with an agonist that is highly selective for δ -containing GABA_A receptors, 4,5,6,7-tetrahydroisoxazolo[4,5-c]pyridine-3-ol (THIP), showed an increase in THIP-induced tonic current. Behavioral studies of PAE rats did not reveal any deficits in motor coordination, except for a delay in the acquisition of the mid-air righting reflex that was apparent at P15 to P18. These findings demonstrate that repeated intermittent exposure to high levels of EtOH during the equivalent of the last trimester of human pregnancy has significant but relatively subtle effects on motor coordination and GABAergic transmission in CGNs in rats.

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Keywords

Fetal; cerebellum; GABA; tonic current; ethanol; extrasynaptic

Introduction

The cerebellum is best known for its role in motor coordination and motor learning; however, it also critically regulates numerous cognitive processes including attention, the speed of information processing, visuo-spatial functions, and language (O'Halloran et al., 2012). Alterations in cerebellar functions have been demonstrated in neurodevelopmental disorders such as autism, Down's syndrome, and fetal alcohol spectrum disorders (FASDs) [reviewed in (O'Halloran et al., 2012; Steinlin, 2008)]. FASDs are among the most common neurodevelopmental disorders, affecting up to 5% of the population in the United States and Western Europe (May et al., 2009). Individuals with FASDs often exhibit impairments in balance, delays in gross and fine motor skills (Jaatinen and Rintala, 2008; Mattson and Riley, 1998; Nguyen et al., 2012), and deficits in motor learning (i.e., cerebellar-mediated eyeblink conditioning (Jacobson et al., 2011; Jacobson et al., 2008)), and additional cognitive deficits that could also be related to cerebellar damage, such as: impaired math skills (Lebel et al., 2010) and dyslexia (Coffin et al., 2005). A reduction in cerebellar volume and white matter tracts have been detected in patients with FASDs (Autti-Ramo et al., 2002; O'Hare et al., 2005; Roebuck et al., 1998; Sowell et al., 1996; Wozniak et al., 2006; Wozniak and Muetzel, 2011). However, the relationship between the pattern of alcohol exposure and the extent of the cerebellar alterations is not fully understood. Furthermore, the precise molecular mechanisms underlying the cerebellar deficits associated with FASDs have yet to be elucidated.

Cerebellar granule neurons (CGNs) are considered to be the gateway of information to the cerebellar cortex. CGNs receive afferent input from the brain stem and spinal cord via glutamatergic mossy fibers (MF). The glutamatergic axons from CGNs relay MF input to Purkinje cells (PCs), which is the sole output of the cerebellar cortex. PC activity is also regulated by glutamatergic climbing fibers, GABAergic molecular layer interneurons, and collateral inputs from neighboring PCs (Watt et al., 2009). Numerous animal models of FASDs have shown that binge-like ethanol (EtOH) exposure during development induces apoptosis of PCs [reviewed in (Green, 2004; Luo, 2010)]. Functionally, prenatal EtOH exposure during the equivalent to the 1st and 2nd trimester of human pregnancy altered spontaneous firing, synaptic transmission, and plasticity in PCs in mice (Servais et al., 2007), whereas exposure during the equivalent of the 3rd trimester decreased the proportion of PCs that exhibit complex spikes in anesthetized rats (Backman et al., 1998). CGNs have also been shown to be vulnerable to developmental EtOH exposure. Binge-like exposure to low-to-high doses of EtOH during the 1st, 2nd, or 3rd trimester-equivalents disrupts the migration of CGNs and triggers apoptosis of these neurons in mice [reviewed in (Luo, 2010)]. However, the effects of EtOH exposure during the equivalent of the human 3rd trimester on synaptic transmission at CGNs have not been characterized using animal models of FASDs. It is critically important to determine the effects of EtOH during the 3rd trimester-equivalent because one of the most common patterns of alcohol abuse in pregnant women is abstinence during the first two trimesters of pregnancy followed by consumption during the last trimester (Ethen et al., 2009).

CGNs receive two distinct forms of GABAergic transmission: phasic and tonic. Phasic inhibition is mediated by conventional vesicular GABA release from specialized interneurons, referred to as Golgi cells (GoCs), which activate synaptic GABA_A receptors (GABA_ARs). Tonic inhibition is mediated by extrasynaptic GABA_ARs that are activated by

ambient levels of GABA whose source remains controversial (Brickley and Mody, 2012; Diaz et al., 2011; Lee et al., 2010). While GABAergic transmission at CGNs has been shown to be an important target for acute EtOH in the mature cerebellar cortex (Botta et al., 2010; Botta et al., 2007; Botta et al., 2011; Carta et al., 2004; Hancher et al., 2005), the impact of EtOH exposure during development has not been explored. Importantly, the development of GABAergic transmission in other brain regions has been shown to be impaired in various models of 3rd trimester-equivalent EtOH exposure (Everett et al., 2012; Hsiao et al., 1998; Zucca and Valenzuela, 2010). In this study, we tested the hypothesis that repeated intermittent exposure to EtOH during the 3rd trimester-equivalent also impairs GABAergic transmission at CGNs.

Methods

Animals

All animal procedures were approved by the UNM-Health Sciences Center Institutional Animal Care and Use Committee and conformed to National Institutes of Health Guidelines. Timed-pregnant Sprague-Dawley rats from Harlan (Indianapolis, IN) were received at the UNM-Health Science Center at embryonic day (E) 12–16. Dams were individually housed, received food and water *ad libitum*, and were housed in cages with a plastic hut to reduce stress (lights on at 6 am and off at 6 pm). Animals were observed daily for signs of labor, with minimal handling of the housing cages to accurately determine the date of postnatal day (P) 0. From P2 to P13 all pups within a litter were counted and weighed together every day and average pup weights calculated. At P21 and 28, remaining offspring were individually weighed and the weights were averaged for the litter. Litters were culled at ~P4 to 8–12 pups (# of pups were matched for paired litters).

Ethanol Vapor Chamber Exposure

We used vapor chambers purchased from La Jolla Alcohol Research Inc (La Jolla, CA) (Fig. 1A). Briefly, 95 % EtOH was dripped via a peristaltic pump into a heated round-bottom glass flask to allow the EtOH to vaporize. In-house air flowed through the flask, mixed with the EtOH vapor, and was delivered into a sealed rat cage. The flow of EtOH vapor/air mixture was regulated by flow meters. The cages had a port that was connected to the in-house vacuum line to maintain constant pressure inside the cage. During the equivalent of the 3rd trimester, rats were subject to postnatal alcohol exposure (PAE) from P2 to P12 and litters were exposed from 10 am to 2 pm each day. The concentration of EtOH vapor samples was analyzed using a breathalyzer (Intoximeters, St. Louis, MO) every hour to ensure constant EtOH levels throughout the exposure. Paired control (CON) litters underwent the same exposure in forced gas chambers; however, the vaporized EtOH was excluded. After the exposure, cages were returned to the animal research housing facilities. Bedding was changed 1 time during the exposure at P6 or P7. For all experiments performed at P6, animals were taken 20 hrs after the end of EtOH vapor exposure.

To determine the average serum EtOH concentrations (SECs), pups were taken immediately after the exposure on P6 and P12 (Fig. 1B). To determine a time course of SECs on a single day of exposure, litters were exposed from P2 to P6. At P6, a single pup from each litter was taken every hour throughout the exposure (1, 2, 3, and 4 hrs) and every two hrs post exposure (6, 8, 10, and 12 hrs). For comparison, we also collected samples from P12 pups at the end of the 4 hr exposure. SECs were averaged from pups from different litters. Dam SECs were taken after weaning following a 4 hr EtOH vapor exposure. Animals were anesthetized with ketamine (250 mg/kg i.p.), decapitated, and trunk blood (50 μ L) was collected and mixed with 6.6 % perchloric acid (450 μ L). SECs were measured using a

standard alcohol dehydrogenase-based assay, as previously described (Galindo and Valenzuela, 2006).

Maternal Care Assessment

Maternal care during the exposure to EtOH was assessed as previously reported (Ponce et al., 2011; Smith et al., 2004). Immediately before the exposure on P6–7, dams were briefly removed from the home cage and pups were scattered in the cage. Dams were returned and vapor exposure was started. Litters were video-recorded for the first 1.5–2 hrs of exposure for later analysis of: 1) time for the dam to reunite with all the pups; 2) percent of time the dam groomed pups; 3) percent of time the dam nursed at least 1 pup; and 4) percent of time the dam spent with at least 1 pup. Video analysis was performed in a blind fashion to avoid experimenter bias.

Cerebellar slice electrophysiology

The animals that were used for behavioral experiments were not used for electrophysiology experiments (Fig. 1B). For experiments at P6, we did not differentiate between males and females in order to conserve animals for subsequent experiments. Although some sex differences in the functional properties of immature hippocampal neurons and Purkinje neurons grown in culture have been reported (Dean et al., 2012; Nunez and McCarthy, 2009; Wittmann and McLennan, 2011), we are unaware of any reports of sex-dependent differences in the properties of cerebellar neurons in neonatal rats studied using the slice preparation and therefore do not anticipate problems by the inclusion of both sexes at this developmental time point. For experiments performed at P13, we utilized only male pups. Using slicing methods that have been previously described (Diaz et al., 2011), animals were sacrificed by rapid decapitation under deep anesthesia with ketamine (250 mg/kg i.p.). Brains were quickly removed and submerged for 2 minutes 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. The vermis of the cerebellum was sliced parasagittally in cold sucrose aCSF at 300 μ m (200 μ m at P6 and 13) using a vibrating tissue slicer (Leica Microsystems, Bannockburn, IL). Slices were immediately transferred to a chamber containing normal aCSF and allowed to recover for 40 minutes 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 and was continuously bubbled with 95 % O₂/5 % CO₂. Slices were then stored in normal aCSF at 21–22°C.

Whole-cell patch clamp recordings were performed in a chamber perfused with aCSF at a rate of 2–3 ml/min and maintained at 32–33°C. Neurons were visualized using infrared-differential interference contrast microscopy and recordings were performed with Axopatch 200B amplifiers (Molecular Devices, Sunnyvale, CA). CGNs were identified on the basis of their location in the granule cell layer and their size (capacitance = 5–10 pF). To record CGN GABA_AR-mediated tonic currents (CGN-I_{tonic}) and GABA_AR-dependent spontaneous postsynaptic currents (GABA_A-sPSCs), 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 N-(2,6-Dimethylphenyl)carbamoylmethyltriethylammonium bromide (QX314-Br), pH 7.25, osmolarity 280–290 mOsm. GABAergic transmission was pharmacologically isolated by blocking AMPA and NMDA receptors using kynurenic acid (1 mM) and D,L-APV (50 μ M), respectively. The holding potential was –70 mV. During application of glutamate antagonists, neurons were allowed to equilibrate (~5 min) prior to beginning an experiment. Data were acquired in gap-free mode at 10 kHz and filtered at 2 kHz.

Electrophysiology recordings were analyzed with Clampfit-10 (Molecular Devices) or MiniAnalysis (Synaptosoft, Decatur, GA). $CGN-I_{tonic}$ was calculated by fitting a Gaussian distribution to an all-point histogram for every minute of the recording, constraining the fit to eliminate any contribution from GABA_A-sPSCs. $CGN-I_{tonic}$ amplitude was defined as the mean current recorded under control conditions minus that recorded in the presence of 50 μ M picrotoxin (PTX).

Immunohistochemistry

Rats were euthanized by decapitation under deep anesthesia with ketamine (250 mg/kg). Brains were quickly extracted and placed on ice, and then rinsed briefly in ice-cold phosphate-buffered saline (PBS). The cerebellar vermis was then isolated and fixed in 4% paraformaldehyde in PBS for 24 – 48 hrs at 4°C, followed by cryoprotection in 30% sucrose in PBS for an additional 24 hrs at 4°C. For δ -antibody validation, C57BL/6 wildtype (WT) and δ -subunit knockout (δ -KO) mice were euthanized by transcardial perfusion with paraformaldehyde (Dr. Orser's Lab at U. of Toronto). Mouse brains were fixed in 4% paraformaldehyde and shipped to the University of New Mexico. The cerebellar vermis was then blocked and cryo-protected in 30% sucrose in PBS. Following cryoprotection, all procedures were exactly the same in rat and mouse tissues. The vermis was embedded in optimal cutting temperature compound (Sakura Finetek, Torrance, CA, USA) and flash-frozen by submersion in isopentane cooled in a dry ice/ethanol bath. Embedded vermis was stored at -80°C until they were cryo-sectioned (16 μ m) in the sagittal plane. Sections were placed on SuperFrost Plus microscope slides (VWR international, West Chester, PA, USA; Electron Microscopy Sciences, Hatfield, PA, USA). Sections were rinsed in PBS then incubated with blocking solution containing 1% bovine serum albumin (BSA), 5% normal donkey serum (Jackson ImmunoResearch Laboratories, Inc., PA, USA), and 0.2% Triton X-100. This was followed by overnight incubation at 4°C with anti- δ GABA_AR subunit antibody (1:100; PhosphoSolutions, Aurora, CO, USA) plus Alexa 488-conjugated FluoroPan Neuronal Marker (1:1000; Millipore, Billerica, MA, USA) diluted in blocking solution. Sections were rinsed and incubated for 2 hrs with Alexa Fluor 555 donkey anti-rabbit secondary antibody (1:1000; Invitrogen, Grand Island, NY, USA) and nuclear counterstain diaminidino-2-phenylindole (DAPI; 1:1000; Invitrogen) diluted in blocking solution. Sections were rinsed and mounted with Vectashield (Vector Laboratories, Burlingame, CA, USA). For δ -antibody validation and high magnification analysis of δ -antibody staining in rats, sections were imaged using an LSM510 META confocal microscope (Carl Zeiss, Oberkochen, Germany) at 20 and 63 $\times$ magnification with a 0.5 N.A. objective (confocal microscopy facility at the UNM Cancer Center). For assessment of δ -subunit expression, sections were imaged at 20 $\times$. Stained sections were imaged using an LSM510 META confocal microscope (Carl Zeiss, Oberkochen, Germany) at 10 and 20 $\times$ magnification with a 0.5 N.A. objective. Microscope parameters were maintained for all images taken from all litters. Staining intensity of GABA_AR δ subunit in the internal granule cell layer (IGCL) was quantified using SlideBook 5 software (Intelligent Imaging Innovations, Inc., Denver, CO, USA). Briefly, five square masks were randomly applied throughout the IGCL of images taken at 20 $\times$ magnification and total intensity within these masks was normalized to the total area of the IGCL. Intensity was normalized to blank sections where primary antibody was omitted. Intensity measurements were averaged over three images for each animal.

Western Blotting

For validation of the anti-GABA_AR δ subunit antibody, 3 month-old WT and δ -KO were studied. The δ -KO mice were obtained from a breeding colony maintained by Dr. Orser at the University of Toronto (Mihalek et al., 1999). Whole cerebella from these mice were homogenized in lysis buffer containing 20 mM HEPES (pH 7.4), 150 mM NaCl, 2 mM

EDTA, 1% (v/v) Triton X-100, 0.1% sodium dodecyl sulfate (SDS), plus protease inhibitors (Roche, catalog # 11836170001). Tissues were triturated by passing them 10 times through 1 ml pipette tips and 20 times through 200 μ l pipette tips followed by sonication. Samples were incubated for 30 min at 4 °C under constant rotation. The homogenate was centrifuged at 18000 g for 20 min at 4 °C to remove insoluble material and the supernatant was saved for subsequent assays. Protein concentration was determined using the bicinchoninic acid assay (Thermo Scientific, Rockford, IL) using BSA as standard. Samples (10 μ g/lane) were separated by SDS polyacrylamide gel electrophoresis using 12% gels (stacking layer: 125 mM Tris (pH 6.8), 4% polyacrylamide, 0.1% SDS, 0.05% ammonium persulfate and 0.001% TEMED; separating layer: 375mM Tris (pH 8.8), 12% polyacrylamide, 0.1% SDS, 0.05% ammonium persulfate, 0.0005% TEMED). Gels were run at 100 V for 90 min and electro-blotted onto nitrocellulose membranes (0.2 μ m pore size; 0.12A overnight at 4° C). Non-specific binding was blocked by incubating for 2 hrs at room temperature with 5% milk in Tris buffered saline containing 0.1% Tween-20 (TBST). Membranes were rinsed once with TBST and incubated overnight at 4° C with rabbit polyclonal anti-GABA_AR δ subunit antibody (1:1000 dilution; PhosphoSolutions) in TBST containing 3% BSA. Membranes were washed 3 $\times$ 15 min in TBST and incubated with anti-rabbit secondary antibodies for 1.5 hrs at room temperature (1:1000, Cell Signaling, Danvers, MA) in TBST containing 3% BSA. Membranes were washed 3 $\times$ 15 min in TBST. Detection of blots was performed using enhanced chemiluminescence (Thermo Scientific, Rockford, IL) and the Chemidoc XRS+ Imager (Bio-Rad, Hercules, CA). Membranes were stripped with Restore Western Blot Stripping Buffer (Thermo Scientific, Rockford, IL), washed 5 times with TBST, and reprobed overnight at 4° C with rabbit anti- β -actin monoclonal antibody (1:5000 dilution, Millipore).

For the western blot experiments with tissue from the CON and PAE groups, rats were anesthetized with ketamine as described above, the cerebellar vermis was collected, flash frozen in liquid nitrogen, and stored at -80° C. Tissue was processed and assayed as described above with the exception that samples were loaded at a concentration of 15 μ g per lane and separated in 7.5% Tris-HCl precast gels (BioRad) at 150 V for 60 min. Control experiments demonstrated that this concentration of protein was within the linear dynamic range for the western blot assay (not shown). Proteins were electroblotted onto polyvinylidene fluoride (0.45 μ m pore size) at 100 V for 70 min. Non-specific binding was blocked with Odyssey Blocking Buffer (Li-Cor, Lincoln, NE) for 2 hrs at room temperature. Membranes were incubated overnight at 4° C with rabbit anti-GABA_AR δ subunit polyclonal antibody (1:1000 in PBS plus 0.1% Tween-20; PhosphoSolutions) and mouse anti- β -actin monoclonal antibody (1:50000, Sigma-Aldrich). Membranes were simultaneously incubated with goat anti-rabbit IR-Dye 800CW (Li-Cor # 926-32211) and goat anti-mouse IR-Dye 680 (Li-Cor # 926-32220) secondary antibodies for 2 hrs at room temperature (1:1000 dilution in PBS plus 0.1% Tween-20). Detection and quantification of results were performed with an ODYSSEY fluorescent imager (Li-Cor). Membranes were then incubated for 20 sec in 0.025% (w/v) Coomassie Blue R-250, 40% methanol, and 7% acetic acid and washed overnight with a de-staining solution containing 50% methanol and 10% acetic acid. Membranes were scanned and Coomassie staining quantified using Quantity One Analysis Software (Bio-Rad). An average of the intensity of randomly selected bands from the Coomassie stain was used to control for differences in lane loading. GABA_AR δ subunit levels were normalized to intensity of the average of these Coomassie-stained bands within each respective lane.

Behavioral Assessment

We utilized a battery of behavioral tests to assess effects of 3rd trimester EtOH exposure on developmental milestones and motor coordination. For the air-righting reflex, balance beam,

rotarod and gait studies, we used different animals for each assay to avoid any confounds or interactions between different behavioral tests (Fig. 1C). Moreover, tissue from these animals was not used for any of the *in vitro* assays mentioned above (Fig 1B). For developmental milestones, we examined age of eye opening and righting reflex (Mesquita et al., 2007; Slamberova et al., 2006) prior to the start of the exposure. For age of eye opening, animals were monitored every day beginning on P2 and the number of pups per litter with eyes opened was documented, until 100% of animals in each litter had opened their eyes (approximately P16). For measurement of the righting reflex, animals were placed on their back and the time it took them to stand on all four legs was recorded. This was repeated on P2, P7, and P12 at which time all the animals within a litter could right themselves (100% passed). For measurement of the air-righting reflex, animals were held 10 cm above a cushioned surface with their backs facing down and were dropped (Dommett and Rostron, 2011). The number of animals/litter that were able to right themselves – or “pass the test” – on a given day was documented. This was started on P13 and was repeated until all animals within a litter could right themselves before landing (100% passed). When an animal “passed” the air righting reflex test, it was marked and not re-tested.

To examine motor coordination, we assessed gait and performance on a balance beam and a rotarod (Carter et al., 2001). For all behavioral experiments, animals were transported to the testing room ~1 hr before testing to allow acclimation to ambient noise and lighting. Equipment was wiped down with 70% EtOH and water and allowed to dry between animals. Animals were tested for 3 days (P21–23), 4 trials/animal/day. To measure gait, paws were dipped in non-toxic paint (front-red, hind-blue) and animals were placed in a closed runway, 100 cm long and 10 cm wide with 20 cm sides (bottom lined with white paper) and allowed to walk down the runway to a black goal box (a plastic hiding tube from the housing cage was used to encourage animals to walk to the box). Animals were trained for 3 consecutive days, and measurements from the 4th day were analyzed for various parameters as described below. For balance beam experiments, 100 cm long square beams of varying diameters (14, 20, and 40 mm) were placed 60 cm above a cushioned surface. There was an illuminated starting platform at one end of the beam. On the other end of the beam was a black goal box used to encourage animals to traverse the beam and seek shelter. Animals were placed on the starting platform and allowed to traverse the beam. If an animal did not move, then the animal was nudged gently to encourage movement to the goal box. Animals were trained for 3 days using the 40 mm beam, and on the 4th day animals were sequentially tested on the 40, 20, and 14 mm beams. Training and tests were video-recorded for later analysis. Video analysis was performed in a blind fashion to avoid experimenter bias. For rotarod experiments, animals were placed on a moving rotarod (Ugo Basile, Italy) rotating at 16 rotations per minute. The latency to fall was documented for each animal, with a maximum time of 60 s.

Statistics

Data were statistically analyzed with Prism 5 (Graphpad, San Diego, CA). Data were initially analyzed with the Pearson omnibus normality test. If data followed a normal distribution, these were analyzed using parametric tests. If this was not the case, then non-parametric tests were used. A $p < 0.05$ was considered to be statistically significant. For all statistical analyses, the experimental unit is a litter. For slice electrophysiological experiments, results obtained with all the slices from a single animal were averaged and results from animals from a single litter were averaged to yield a unit of determination. For histological experiments, results obtained from a single tissue section from a single animal (each from a different litter) were averaged to yield a unit of determination. For western blot experiments, results from a single animal from a single litter yielded a unit of determination.

For behavioral experiments, results from animals from a single litter were averaged to yield a unit of determination.

Drugs and Chemicals

D,L-APV; PTX; 4,5,6,7-tetrahydroisoxazolo[4,5-c]pyridine-3-ol (THIP) and QX-314 (Br⁻) were purchased from Tocris (Ellisville, MO). Tetrodotoxin (TTX) was from Calbiochem (San Diego, CA). All other drugs and chemicals were from Sigma-Aldrich (St. Louis, MO).

Results

Characterization of a 3rd trimester equivalent repeated intermittent EtOH vapor exposure model

In rodents, the equivalent developmental time period to the human 3rd trimester occurs during the first two postnatal weeks in rodents. To model exposure of the human fetus that mimics heavy drinking during the 3rd trimester of pregnancy (Burd et al., 2012), postnatal pups housed with their respective dams were repeatedly exposed to EtOH via vapor inhalation. Analysis of the time course of SECs during the 4th day of exposure on P6 (Fig. 2A) demonstrated that peak SECs of ~60 mM (U.S.A. legal limit = 17.4 mM or 0.08 g/dL) were achieved after 4 hrs of EtOH exposure and these levels gradually returned to baseline 12 hrs after the start of the EtOH exposure. SECs measured in P12 pups (64.70 ± 11.45 mM; $n = 7$) and were not significantly different from those concentrations measured in P6 pups (Mann-Whitney $U = 9$, sum of ranks = 15,40; $p > 0.05$ by Mann-Whitney test) indicating that metabolic tolerance to EtOH did not develop as the duration of exposure increased. Dams had peak SECs under the legal limit (~13 mM at 4 hrs; Table 1). We chose this level of neonatal EtOH exposure because: 1) studies suggest that higher concentrations of ethanol are required to produce developmental neurotoxicity in rodents compared to humans (Gohlke et al., 2008); 2) neonatal rats are less sensitive than older rats to the intoxicating effects of EtOH (Hollstedt et al., 1980); and 3) several studies have demonstrated that binge-like EtOH exposure during the 3rd trimester-equivalent generally causes significant cerebellar damage at concentrations of approximately 40 mM (Goodlett et al., 1998; Green, 2004). Further, we wished to compare our results to those from the studies cited above.

As shown in Table 1, the initial number of pups per litter, number of pups left after culling each litter, and the number of dead pups per litter were not significantly different between treatment groups. An analysis of pup weight gain (Fig. 2B) revealed a main effect of age [$F(12,707) = 917.5$, $p < 0.0001$], exposure [$F(1,707) = 261.1$, $p < 0.0001$], and an exposure $\times$ age interaction [$F(12,707) = 11.71$, $p < 0.0001$] as evidenced by a 2-way ANOVA test. A Bonferroni multiple comparisons post-hoc test showed that PAE pups weighed significantly less than CON pups beginning at P6 and this difference persisted through P28 when pups weighed ~9% less than CON rats (Fig. 2B, P6, $p < 0.05$; P7, $p < 0.001$; P8–21, $p < 0.0001$; P28, $p < 0.001$). Assessment of maternal care showed that time for all pups to reunite with the dam, the nursing time (% of total time), and the time spent with at least 1 pup (% of total time) were not significantly different between CON and PAE at P6–7 (Table 1; Mann-Whitney test performed for each measure, $p > 0.05$). However, maternal grooming time (% of total time) was significantly reduced in PAE litters compared to CON (Table 1; Mann-Whitney $U = 2$, sum of ranks = 43,12; $p = 0.038$ by Mann-Whitney test).

EtOH exposure neither affects the membrane properties nor the I_{tonic} in developing CGNs

We next examined the effect of PAE on the functional maturation of the membrane properties of CGNs. The capacitance values of CGNs were significantly larger at P6 compared to older ages in both CON and PAE groups (Table 2; age [$F(3,56) = 20.17$, $p < 0.0001$]), with no differences found between CON and PAE (exposure [$F(1,56) = 0.14$, $p >$

0.05], interaction of age x exposure [$F(3,56) = 0.23$, $p > 0.05$]). Analysis of the membrane resistance showed a main effect of age ($[F(3,56) = 4.91$, $p = 0.004]$) and a Bonferroni post-hoc analysis revealed that the membrane resistance was significantly lower at P28 with respect to P13 in the PAE group; however, there was no difference between the CON and PAE groups (exposure [$F(1,56) = 0.25$, $p > 0.05$], interaction of age x exposure [$F(3,56) = 1.54$, $p > 0.05$]). Fig 3 shows that CGN- I_{tonic} was absent at P6 and could be detected starting at P13. A 2-way ANOVA test confirmed an effect of age on the development of the CGN- I_{tonic} (Fig. 3A–B; age [$F(3,56) = 10.53$, $p < 0.0001$]), but did not an effect of exposure ($[F(1,56) = 0.69$, $p > 0.05]$) or an interaction of age x exposure [$F(3,56) = 1.20$, $p > 0.05$]). CGN- I_{tonic} in unhandled P28 rats that were born in our animal facility had a similar amplitude to those of CON and PAE P28 rats (Fig 3B; [$F(2,19) = 0.85$, $p > 0.05$] by one-way ANOVA).

Several studies have reported that the release of GABA from GoCs is driven by spontaneous firing of these neurons and this GABA contributes to the pool of transmitter that generates the CGN- I_{tonic} (Botta et al., 2010; Carta et al., 2004; Diaz et al., 2011; Diaz et al., 2013; Hamann et al., 2002; Rossi et al., 2003; Wall and Usowicz, 1997). Therefore, we examined the impact of PAE on the amplitude of the GoC-dependent component of CGN- I_{tonic} , which is sensitive to TTX. As mentioned above, CGN- I_{tonic} was undetectable at P6 and TTX did not change the holding current in neurons from CON or PAE rats (data not shown). At later time points of development, a 2-way ANOVA did not show an effect of age [$F(2, 42) = 0.44$, $p > 0.05$], exposure [$F(1,42) = 1.03$, $p > 0.05$] or an interaction of age x exposure [$F(2,42) = 0.65$, $p > 0.05$] on the TTX-induced change in CGN- I_{tonic} (Fig. 3C). The effect of the TTX on CGN- I_{tonic} at P28 in CON and PAE was not significantly different from that of unhandled rats at P28 (Fig. 3C; [$F(2,19) = 0.47$, $p > 0.05$] by one-way ANOVA).

Effect of PAE on GABA_A-R δ subunit levels

We next characterized the levels of protein expression of the GABA_A-R δ subunit, which is present in the receptors that mediate the CGN- I_{tonic} and has been shown to be developmentally regulated (Laurie et al., 1992; Takayama and Inoue, 2004). The specificity of the anti- GABA_A-R δ subunit antibody was first assessed by immunohistochemistry and western blot analysis using cerebellar tissue from adult WT mice and δ KO mice (Mihalek et al., 1999). As shown in Fig 4, the anti- GABA_A-R δ subunit antibody strongly labeled the granule cell layer in sections from WT mice. This antibody labeling was absent in sections from δ -KO mice (Fig. 4A and B; similar results were obtained with sections from two separate WT and δ -KO mice). Weak staining was observed in the Purkinje cell layer and the molecular layer, but was also observed in sections from δ -KO mice, suggesting that it corresponds to binding to a non-specific protein (Fig. 4C and D). In agreement with these results, western blot assays revealed that the anti-GABA_A-R δ subunit antibody strongly labeled two bands of molecular weights near ~53 kDa in cerebellar homogenates from WT mice and these bands were not detected in homogenates from δ -KO mice (Fig. 4E). These closely spaced bands probably correspond to differentially phosphorylated forms of the GABA_A-R δ subunit (Fig 4E). A non-specific band was detected near 80 kDa in homogenates from both WT and knockout mice (Fig 4E).

We next evaluated the impact of PAE on expression of the δ subunit in the cerebellum using immunohistochemical techniques. In agreement with studies of the mRNA expression of this subunit (Laurie et al., 1992), immunohistochemical experiments revealed that GABA_A-R δ subunit expression did not peak until P21 in either CON or PAE in the IGCL (Fig. 5). A 2-way ANOVA detected a main effect of age [$F(3,18) = 50.75$, $p < 0.0001$] and a main interaction of age x exposure [$F(3,18) = 3.40$, $p = 0.04$], but no significant effect of exposure [$F(1,18) = 0.92$, $p > 0.05$]. A Bonferroni multiple comparisons post-hoc analysis revealed a

significant increase in GABA_AR δ subunit levels at P28 in PAE compared to CON ($p < 0.05$, $n = 4$).

To more quantitatively assess the impact of PAE on δ subunit expression, we performed western blotting assays of cerebellar vermis homogenates. These assays showed that the expression of the δ subunit increases with age, and peaks at P28 (Fig. 6A and B). β -actin levels decrease with age, consistent with previous reports (Fig. 6A) (Schmitt et al., 1977). Therefore, we did not use this widely used house-keeping protein to control for differences in lane loading; instead, we used an average of Coomassie-stained bands in each lane. A 2-way ANOVA showed a significant effect of age on δ subunit expression ($[F(3,46) = 629.9, p < 0.0001]$). We also found a significant effect of exposure [$F(1,46) = 9.44, p = 0.0036$], and a significant interaction of age x exposure [$F(9,46) = 22.26, p < 0.0001$]. A Bonferroni's multiple comparisons post-hoc analysis revealed a significant increase of GABA_AR δ subunit levels at P28 in PAE ($n = 7$) compared to CON ($n = 5, p < 0.05$) (Fig 6B).

To pharmacologically assess levels of the functional δ subunit-containing GABA_ARs we tested the effect of THIP, an agonist that potentiates receptors containing this subunit. We focused on P28 rats because this is when we found that GABA_AR δ subunit levels were significantly different between the CON and PAE groups. Consistent with the increase in δ -subunit expression, we found that the THIP (0.5 μ M)-induced potentiation of CGN-I_{tonic} was significantly enhanced in PAE ($n = 7$) compared to CON ($n = 6$; Fig. 7; $t = 2.61, df = 11, p < 0.05$ by unpaired t -test).

PAE does not alter phasic GABAergic transmission

Similar to CGN-I_{tonic}, phasic GABAergic transmission dramatically changes in CGNs during the first days of life. Consistent with previous findings (Brickley et al., 1996), we found an age-dependent decrease in GABA_A-sPSC amplitude in CON slices (Fig. 8A and C; age [$F(3,56) = 11.91, p < 0.0001$]) but no effect of PAE on amplitude (exposure [$F(1,56) = 1.60, p > 0.05$]; interaction of age x exposure [$F(3,56) = 1.37, p > 0.05$] by 2-way ANOVA, $n = 8$). We also found a main effect of age on spontaneous GABA_A-sPSC frequency in CON slices (Fig. 8B and D; [$F(3,56) = 4.22, p = 0.0093$]) and this parameter was also unaffected by PAE (exposure [$F(1,56) = 1.74, p > 0.05$]; interaction of age x exposure [$F(3,56) = 0.10, p > 0.05$] by 2-way ANOVA, $n = 8$).

To further characterize phasic GABAergic transmission, we examined action potential-independent GABA_AR-mediated miniature postsynaptic currents (GABA_A-mPSCs) in the presence of TTX. Age influenced the effect of TTX on the amplitude of GABA_A-sPSCs in slices from both CON and PAE rats (expressed as TTX-induced % change from baseline; CON: P6 = $-40.16 \pm 12.30\%$, P13 = $-32.35 \pm 13.53\%$, P21 = $13.48 \pm 23.56\%$, P28 = $5.75 \pm 10.08\%$; PAE: P6 = $-52.57 \pm 9.48\%$, P13 = $-28.61 \pm 12.19\%$, P21 = $6.66 \pm 11.51\%$, P28 = $-2.12 \pm 22.99\%$; age [$F(3,56) = 6.07, p = 0.0012$]; exposure [$F(1,56) = 0.26, p > 0.05$]; interaction of age x exposure [$F(3,56) = 0.09, p > 0.05$] by 2-way ANOVA; $n = 8$). A Bonferroni's posthoc test did not detect any significant differences among groups. Similarly, a 2-way ANOVA of the effect of TTX on GABA_A-sPSC frequency between slices from CON and PAE rats only showed a main effect of age (expressed as TTX-induced % change from baseline; CON: P6 = $-54.97 \pm 10.05\%$, P13 = $-93.69 \pm 2.33\%$, P21 = $-89.88 \pm 4.99\%$, P28 = $-64.74 \pm 15.40\%$; PAE: P6 = $-64.66 \pm 10.43\%$, P13 = $-91.16 \pm 6.39\%$, P21 = $-90.84 \pm 4.56\%$, P28 = $-72.60 \pm 11.56\%$; age [$F(3,56) = 6.17, p = 0.0011$]; exposure [$F(1,56) = 0.38, p > 0.05$]; interaction of age x exposure [$F(3,56) = 0.19, p > 0.05$]; $n = 8$). A Bonferroni's posthoc test did not detect any significant differences among groups.

As with sPSCs, analysis of GABA_A-mPSCs showed a main effect of age on GABA_A-mPSC amplitude (Fig. 8C; [$F(3,56) = 4.98, p = 0.0039$]) with no effect of PAE (exposure [$F(1,56)$])

= 1.15, $p > 0.05$]; interaction of age x exposure [$F(3,56) = 0.14$, $p > 0.05$] by 2-way ANOVA, $n = 8$). A Bonferroni's posthoc test did not detect any significant differences among groups. GABA_A-mPSC frequency was not affected by age (Fig. 8D; [$F(3,56) = 2.75$, $p > 0.05$]), or PAE (exposure [$F(1,56) = 0.007$, $p > 0.05$]; interaction of age x exposure [$F(3,56) = 0.82$, $p > 0.05$]).

Effects of PAE on developmental behaviors and motor coordination

We compared the onset of eye opening, righting reflex, and air-righting reflex between CON and PAE groups. The day of eye opening was not significantly altered by PAE (Fig. 9A; exposure [$F(1,24) = 1.3$, $p > 0.05$]; interaction of age x exposure [$F(3,24) = 0.42$, $p > 0.05$] by 2-way ANOVA, $n = 4$; Bonferroni multiple comparison, $p > 0.05$). Also, the acquisition of the righting reflex was not different between the treatment groups at any of the ages tested (Fig. 9B; exposure [$F(1, 18) = 0.4777$, $p > 0.05$]; interaction of age x exposure [$F(2, 18) = 0.1492$, $p > 0.05$] by 2-way ANOVA, $n = 4$; Bonferroni multiple comparison, $p > 0.05$). Interestingly, EtOH impaired the air-righting reflex (Fig. 9C), as a 2-way ANOVA with repeated measures revealed a main effect of exposure [$F(1,48) = 54.35$, $p < 0.0001$] and interaction of age x exposure [$F(7,48) = 5.11$, $p = 0.0002$]. A Bonferroni's multiple comparison post-hoc analysis showed that the percent of PAE animals that passed the air-righting reflex was significantly lower than CON animals from P15 to 18 (Fig. 9C; $n = 4$, # - $p < 0.001$, * - $p < 0.01$). The majority of pups in both groups passed the air-righting reflex by P19 consistent with a delay in the acquisition of the air-righting reflex as a result of PAE.

We next assessed the effect of PAE on various cerebellar-dependent motor behaviors at P22 to P24. Investigation of motor performance on a balance beam test showed that PAE ($n = 4$) animals trained equally well to CON ($n = 5$) animals (Fig. 9D; exposure [$F(1,21) = 1.26$, $p > 0.05$], interaction of age x exposure [$F(2,21) = 0.01$, $p > 0.05$]) and also tested equally well to CON animals (exposure [$F(1,21) = 0.244$, $p > 0.05$], interaction of age x exposure [$F(2,21) = 1.15$, $p > 0.05$]). Likewise, gait was not affected by PAE (Fig. 9E; $n = 4$, $p > 0.05$; Mann-Whitney test for each measure). Finally, while both CON and PAE animals showed a significant day-dependent improvement in rotarod performance (Fig. 9F; day [$F(2,14) = 20.54$, $p < 0.001$]), PAE animals showed no difference in performance on the rotarod compared to CON rats (exposure [$F(1,42) = 2.24$, $p > 0.05$], interaction of days x exposure [$F(2,42) = 1.31$, $p > 0.05$] by repeated measures 2-way ANOVA, $p > 0.05$, $n = 8$).

Discussion

Summary of findings

This study characterized the impact of EtOH exposure during the 3rd trimester-equivalent on the developing cerebellum. The major findings of this study can be summarized as follows. Repeated intermittent exposure to a high dose of EtOH during P2 to P12, using a paradigm where EtOH levels gradually increase and decrease, caused mild growth delay without any significant changes in the indices of maternal care, except for a decrease in maternal grooming time. PAE caused growth retardation that was clearly evident even at P28. In CGNs, PAE did not affect either basal tonic or phasic currents; however, PAE increased the expression of the GABA_A δ -subunit and the THIP-induced tonic current at P28. PAE delayed the acquisition of the air-righting reflex without affecting performance in other behavioral assays of motor coordination. Thus, this exposure paradigm had significant, but relatively subtle effects on motor coordination and tonic GABAergic currents recorded in developing CGNs.

PAE produced growth retardation

It is well established that prenatal EtOH exposure causes persistent growth deficits in humans that persist into adulthood (Calhoun and Warren, 2007; Day et al., 1999) and our PAE animal model reproduced this effect. The decrease in pup weight is comparable to that observed in studies that utilized an artificial rearing paradigm to model 3rd trimester-equivalent EtOH exposure (Stanton and Goodlett, 1998). However, other studies have found minimal changes in body weight in neonatal rats exposed to similar levels to those used in our study (Heaton et al., 1999). The lower weight gain in our PAE rats may be indirectly related to a decrease in maternal grooming behavior. Although studies with neonatal rats have shown that exposure to high doses of EtOH can impair sucking behavior, leading to a decrease in body weight gain (Cheslock et al., 2000), we did not detect significant alterations in nursing time, suggesting that alternative mechanisms are responsible for the growth deficits observed in our rats. Studies suggest that EtOH may stunt postnatal growth by interfering with the actions of insulin and insulin-like growth factor (Aros et al., 2011; Ting and Lutt, 2006). Future studies could determine whether these factors contribute to the growth deficits produced by our exposure paradigm.

Interestingly, down-regulation of receptors for insulin and insulin-like growth factor during the neonatal period was recently shown to produce cerebellar alterations similar to those produced by EtOH exposure (de la Monte et al., 2011), raising the possibility that a common factor participates in the growth and brain alterations associated with FASDs. It should be noted that timing of exposure during the 3rd trimester-equivalent, rather than alterations in body weight, is a better predictor of severity of the cerebellar insult induced by EtOH exposure (e.g., greater loss of PCs occurs with P4 to P5 exposure) (Hamre and West, 1993; Heaton et al., 1999). Nevertheless, an uncertainty that must be kept in mind when interpreting our findings is that nutritional and/or global growth deficits may have contributed, in part, to the results described below (Keen et al., 2010).

Basal CGN- I_{tonic} is not affected but δ subunit expression is increased by PAE

We observed that tonic GABAergic currents in CGNs are gradually acquired over the course of the first month of life in rats, as shown previously (Brickley et al., 1996). We found no significant effect of PAE on basal CGN- I_{tonic} or the TTX-sensitive component of CGN- I_{tonic} . Surprisingly, immunohistochemical and western blot analysis of the GABA_AR δ -subunit, performed using an antibody whose specificity was validated with tissue from knockout mice, revealed a significant increase in the expression of this subunit in PAE rats at P28. This finding was confirmed pharmacologically using the δ subunit-selective agonist THIP. The mechanism responsible for this effect is presently unknown. Expression of the GABA_AR δ -subunit in developing CGNs has been shown to be induced and maintained by membrane potential depolarization, elevation of intracellular Ca^{2+} levels, and activation of Ca^{2+} /calmodulin-dependent kinase (Gault and Siegel, 1997), possibly driven by mossy fiber inputs (Dave and Bordey, 2009; Engblom et al., 2003; Gault and Siegel, 1998; Payne et al., 2008; Takayama and Inoue, 2004). PAE could increase expression levels of the δ subunit by enhancing any of these mechanisms. Alternatively, an EtOH-induced increase of GABAergic transmission could have increased excitability of CGNs via depolarizing actions of GABA_A-Rs, which are thought to exist in CGNs during the neonatal period in rodents, and could also contribute to the programming and regulation of GABA_AR δ -subunit expression in these cells (Dave and Bordey, 2009; Takayama and Inoue, 2006).

The PAE-induced increase of GABA_AR δ -subunit expression would have been expected to result in greater CGN- I_{tonic} . However, we found no significant differences in CGN- I_{tonic} between CON and PAE rats. The lack of an effect of PAE on CGN- I_{tonic} could be a consequence of a compensatory decrease in ambient GABA levels or a decrease in

sensitivity to GABA of extrasynaptic receptors that ultimately result in normal CGN- I_{tonic} even under conditions of higher GABA_AR δ -subunit levels in extrasynaptic GABA_ARs. It is worth noting that although the experiments with THIP demonstrating enhanced potentiation of CGN- I_{tonic} suggest that PAE induced an increase in δ -subunit expression at extrasynaptic receptors, we cannot eliminate the possibility that PAE induced an increase in the expression of δ -subunits in synaptic or peri-synaptic receptors. In fact, we were unable to assess the effect of THIP on GABA_A-sPSCs because these phasic events could not be clearly identified due to the dramatic increase in CGN- I_{tonic} noise induced by this agent. Regarding the potential physiological impact of an increase in δ subunit-containing extrasynaptic GABA_ARs in juvenile PAE rats, it should be kept in mind that endogenous neurosteroids, such as 3 α ,21-dihydroxy-5 α -pregnan-20-one (allotetrahydrodeoxycorticosterone, THDOC) are known to potentiate these receptors at concentrations that may occur *in vivo* [see review (Farrant and Nusser, 2005)]. Therefore, future experiments should examine if PAE causes dysregulation of the modulatory actions of these agents at CGNs.

Unexpectedly, there is a temporal mismatch between GABA_AR δ subunit expression and the appearance of CGN- I_{tonic} . Specifically, the CGN- I_{tonic} reached maximum levels at P13, while δ expression did not peak until P21 by IHC and P28 by western blot, consistent with mRNA expression patterns (Laurie et al., 1992; Pirker et al., 2000; Takayama and Inoue, 2004). This mismatch raises questions about the subtype of GABA_ARs that mediates the CGN- I_{tonic} at P13. $\alpha 6\gamma$ - and $\alpha 1\alpha 6\gamma$ -containing GABA_ARs are known to exist in the IGCL of the cerebellar cortex (Nusser et al., 1998; Poltl et al., 2003), and are up-regulated in δ -knockout mice (Tretter et al., 2001). It is possible that GABA_ARs containing these subunits contribute to the generation of CGN- I_{tonic} in immature CGNs. We were unable to assess the effect of PAE on $\alpha 1$ or $\alpha 6$ expression due to an inability to rigorously validate the specificity of antibodies against these subunits. Nevertheless, both the developmental expression of these subunits and the potential impact of PAE on this process require further investigation.

Phasic GABAergic transmission at CGNs was not affected by PAE

Phasic GABAergic currents also changed during the 3rd trimester-equivalent period. GABA_A-sPSC amplitude gradually decreased between P6 and P28 whereas GABA_A-sPSC frequency transiently increased at P13. GABA_A-mPSCs underwent similar developmental changes in amplitude, although this effect was much more subtle. The age-dependent decrease in GABA_A-sPSC amplitude can be a result of a decrease in action potential duration in GoCs as this can significantly impact the amplitude of evoked postsynaptic potentials (Hochner et al., 1986; Wheeler et al., 1996) and sPSCs (Sabatini and Regehr, 1997) without affecting frequency. The transient increase in GABA_A-sPSC frequency at P13 could be due to a transient increase in spontaneous action potential firing of GoCs (Crowley et al., 2009). Surprisingly, we found no effect of PAE on phasic currents. Consistent with the lack of change in sPSCs at any of the ages studied, no deficits in the righting reflex at P6, or balance beam, gait, and rotarod were observed between P21 and P23.

As with the lack of effect on the CGN- I_{tonic} , these data were striking as we have observed transient increases in GABAergic transmission at rat hippocampal neurons as a result of a similar EtOH vapor exposure during the 3rd trimester-equivalent. Specifically, we detected an increase in GABA_A-mPSC amplitude in CA3 hippocampal pyramidal neurons from P5–8 and P17–21 rats; PAE also delayed the increase in GABA_A-mPSC frequency that normally occurs in these neurons (Everett et al., 2012). Additionally, the frequency of GABA_A-sPSCs was increased with no change in amplitude in CA3 pyramidal hippocampal neurons from rats exposed between P2–6 (Zucca and Valenzuela, 2010). However, animals used for these experiments were euthanized within 30 minutes after the end of EtOH exposure (rather than

20 hr into withdrawal on P6 and P13 as in our current study), suggesting that these changes may, in part, be related to the effects of acute withdrawal from EtOH. Other studies have also shown that 3rd trimester-equivalent EtOH exposure alters the normal developmental up-regulation of GABA_AR function, specifically in cultured medial septum/diagonal band neurons and PCs (DuBois et al., 2004; DuBois et al., 2006; Hsiao et al., 1999), though these studies used binge-like exposures. Therefore, it is apparent that development of GABAergic transmission is an important target of EtOH; however, the spectrum of effects is dependent not only on the brain region, but also the time post exposure.

PAE delayed the acquisition of the air-righting reflex

The air-righting reflex was acquired 4–5 days later in PAE rats than CON rats. In general agreement with our results, a study using a mouse model of drinking during the 1st and 2nd trimester-equivalent found a deficit in the acquisition of the air-righting reflex in EtOH-exposed offspring, although the delay was more subtle (Kleiber et al., 2011). This suggests that EtOH exposure during the 3rd trimester-equivalent has a greater impact on the acquisition of the air-righting reflex than EtOH exposure during earlier stages of development. Although the neuronal circuits that mediate this reflex have not been fully characterized, they may involve vestibular and visual neuronal networks (Pellis et al., 1989; Pellis et al., 1991), the superior colliculus (Yan et al., 2010), the striatum (Masuda and Yamaguchi, 2000), and the cerebellum (Bouet et al., 2004). Particularly, the cerebellar vermis contributes to this reflex, where PCs inhibit neurons in the fastigial nucleus, one of the deep cerebellar nuclei involved in vestibular integration (Brooks and Cullen, 2009).

Our study identified no functional deficits in GABAergic transmission in vermal CGNs, suggesting that PAE may impact the mid-air reflex by altering other intra- and/or extra-cerebellar neuronal circuits. Others showed that rats conceived, born, and reared in high gravity until P10 exhibited a delayed development of the air-righting reflex (Bouet et al., 2004), and this deficit correlated with a decrease in calbindin-D28k and inositol-3-phosphate labeling in PCs specifically in lobule IX (Bouet et al., 2005). A binge-like EtOH exposure via artificial rearing from P4 to P9 decreases PCs in lobules IX and X of the vermis (Marcussen et al., 1994). Interestingly, lobule IX of the cerebellar vermis, also known as the uvula, plays a role in integrating otolithic vestibular reflexes (Walker et al., 2010) and PCs of lobule IXB respond to vestibular stimulation in decerebrate rabbits (Bradley et al., 1990). It should be noted that deficits in the striatum, another brain region involved in this reflex, have also been reported following prenatal EtOH exposure (Zhou et al., 2012). Future experiments should explore whether alterations in other intra- and extra-cerebellar regions play a role in the mechanism by which PAE delays the air-righting reflex. Particularly, a detailed analysis of the effect of EtOH during the 3rd trimester-equivalent on the vestibulo-cerebellar system should be undertaken in the future.

Conclusion

This study illustrates that repeated intermittent exposure to high levels of EtOH during the 3rd trimester-equivalent does alter the development of phasic and tonic GABAergic transmission in immature CGNs. However, this EtOH exposure increases the expression of the GABA_AR δ subunit in mature CGNs, suggesting that EtOH exposure alters the programming of these neurons. Furthermore, this EtOH exposure delayed the acquisition of the air-righting reflex. In contrast to our findings, other studies have found more dramatic alterations in the cerebellar cortex using intragastric gavage models of binge-like exposure to high doses of EtOH during the 3rd trimester-equivalent where blood alcohol levels may increase more rapidly than in our vapor chamber exposure paradigm (Green, 2004). It is possible that the time it takes to reach peak blood EtOH concentrations is an important

factor that determines the magnitude of the effects of EtOH on the cerebellum (West et al., 1989).

In summary, our study suggests that repeated intermittent drinking that results in a gradual increase in blood EtOH concentrations causes relatively subtle, but persistent alterations in cerebellar function. Given that human studies suggest that binge drinking is particularly damaging to the cerebellum (Jacobson et al., 2008), it will be interesting to investigate whether brief, binge-like EtOH exposure has more dramatic effects on the development of GABAergic transmission in the different cellular components of the cerebellar cortex.

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Highlights

- Impact of 3rd trimester-equivalent ethanol exposure on the cerebellum was studied
- Rats were exposed to ethanol vapor for 4hr/day between postnatal days 2–12
- Basal GABA transmission in developing granule cells was unaffected by ethanol
- Ethanol exposure increased GABA_A receptor δ -subunit expression at postnatal day 28
- Behaviorally, ethanol exposure delayed the acquisition of the air righting reflex

Figure 1. Ethanol (EtOH) vapor chamber setup and experimental time-line

(A) Basic setup of EtOH vapor chambers. **(B)** Time line illustrating that animals from a set of litters were used for maternal care assessment, serum ethanol concentration (SEC), electrophysiology (E-phys), immunohistochemistry (IHC), and western blots (WB). Blue color indicates EtOH exposure days. These animals were handled daily from P2–P12 for weights only. **(C)** Time line showing that animals from another set of litters were used for all behavioral experiments (blue color indicates EtOH exposure days). Each behavioral test was done on separate sets of animals. These animals were handled daily from P2–12 for weights and on testing days.

Figure 2. Serum ethanol concentrations (SECs) and pup weights

(A) Time course for SECs during and following exposure to ethanol (EtOH) at P6. SECs peak at the end of the exposure (time = 4 hrs) and remain elevated for an additional 8 hrs following exposure (time = 12 hrs); each point represents the average of 3–4 litters. (B) Pup weights (averaged by litter) for control (CON; white squares) and postnatal alcohol exposure (PAE; black squares); $n = 32$ litters for all conditions; * - $p < 0.05$, *** - $p < 0.001$, ## - $p < 0.0001$ by 2-way ANOVA followed by Bonferroni's multiple comparisons post-hoc test.

Figure 3. Effect of postnatal ethanol exposure on the acquisition of tonic GABAergic currents in cerebellar granule neurons (CGN-I_{tonic})
 Exemplar traces of CGN-I_{tonic} through development in (A1) for the control (CON) and (A2) postnatal alcohol exposure (PAE) treatment groups. The effect of tetrodotoxin (TTX; 0.5 μ M) and picrotoxin (PTX; 50 μ M) is also shown on these panels. (B) Pooled data illustrating the developmental time course of PTX-sensitive CGN-I_{tonic} in the CON and PAE groups. CGN-I_{tonic} was greater at P13, P21 and P28 compared to P6 in both treatment groups ($n = 8$ litters for all conditions; $p < 0.05$ by 2-way ANOVA followed by Bonferroni's post hoc test). Also shown is the PTX-sensitive CGN-I_{tonic} from unhandled animals at P28 ($n = 6$ animals) (C) Summary graph of the TTX-induced change in CGN-I_{tonic} in the CON and PAE groups ($n = 8$ litters for all conditions). Also shown is the TTX-induced change in CGN-I_{tonic} from unhandled animals at P28 ($n = 6$ animals).

Figure 4. Validation of anti-GABA_A δ subunit antibody

Confocal microscopy images of 16 μm cerebellar vermis parasagittal sections from wildtype C57BL/6 (WT) and δ-subunit knockout (δ-KO) adult mice magnified at 20× (**A and B**) and 63× (**C and D**). Each panel shows the GABA_A δ subunit (red), Pan Neuronal marker (green), nuclear counterstaining diaminidino-2-phenylindole (DAPI; blue), and merge of the three markers. Cerebellar layers are indicated for each merged picture (molecular layer = ML; Purkinje cell layer = PCL; granule cell layer = GCL). Scale bar = 10 μm. Images from WT show intense staining for δ subunit, specifically in the GCL. δ-KO mice show no staining for δ subunit in the GCL. Same results obtained with sections from another pair of WT and δ-KO mice (not shown). (**E**) Results of western blot studies with anti- GABA_A δ subunit antibodies, which recognized closely spaced bands at ~53 kDa in homogenates from two different WT mice but not δ-KO adult mice. A non-specific band of ~80 kDa was recognized in tissue from WT and δ-KO mice. The lower panel shows β-actin expression levels. Each lane corresponds to a sample from a different mouse.

Figure 5. Immunohistochemical characterization of GABA_A-δ subunit expression

(A) Magnified (20×) confocal microscopy images of cerebellar cortex parasagittal sections from control (CON) and postnatal alcohol exposed (PAE) animals that were collected at the indicated ages. Left panel for each picture demonstrates staining with GABA_A-δ antibodies (red). Right panel for each picture demonstrates merge of GABA_A δ subunit, nuclear counterstaining diamidino-2-phenylindole (DAPI; blue), and Pan Neuronal marker (green). Scale bar = 100 μm. (B) Summary graph illustrates developmental time course of GABA_A δ subunit expression in section from CON and PAE rats ($n = 4$ litters for all conditions; * - $p < 0.05$ by 2-way ANOVA, Bonferroni's multiple comparisons post-hoc test). GABA_A δ subunit antibody fluorescence was quantified only in the internal granule cell layer (IGCL).

Figure 6. Characterization of GABA_A- δ subunit expression by western immunoblotting
(A) Exemplar western blots illustrating that GABA_AR δ subunit levels increase with age in samples from air controls (CON) and postnatal alcohol exposed (PAE) rats (upper panel). Conversely, β -actin levels decreased with age (middle panel). An average of randomly selected Coomassie-stained bands was used to control for differences in protein loading (lower panel). **(B)** Summary of results ($n = 5-7$ litters). PAE significantly increased GABA_AR δ subunit levels at P28 (* - $p < 0.05$ by 2-way ANOVA, Bonferroni's multiple comparisons post-hoc).

Figure 7. Sensitivity of tonic GABAergic currents in cerebellar granule neurons (CGN- I_{tonic}) to THIP is enhanced by postnatal alcohol exposure

(A) Exemplar traces of the effect of THIP (0.5 μM) on CGN- I_{tonic} in control (CON) and postnatal alcohol exposed (PAE) rats at P28. Blue arrows demonstrate the difference in the effect of THIP between CON and PAE rats. (B) The effect of THIP on CGN- I_{tonic} was significantly larger in slices from PAE rats ($n = 7$ litters) compared to CON rat ($n = 6$ litters; * - $p < 0.05$ by unpaired t -test).

Figure 8. Age-dependent changes in GABA_A receptor-mediated spontaneous and miniature postsynaptic currents (GABA_A sPSCs and mPSCs) at cerebellar granule neurons
(A) Exemplar expanded scale traces of averaged GABA_A-sPSCs for control (CON) and prenatal alcohol exposed (PAE) rats at the indicated ages. **(B)** Exemplar compressed traces of GABA_A-sPSC recordings obtained at the indicated ages in slices from CON and PAE rats. Summary graphs for GABA_A-sPSC and -mPSC **(C)** amplitude and **(D)** frequency for CON and PAE at the indicated ages ($n = 8$ litters for all conditions).

Figure 9. Effect of postnatal ethanol exposure on developmental milestones and motor coordination

(A) The percentage of animals/litter that opened their eyes from P13–16 did not differ between the control (CON; white squares) and postnatal alcohol exposure (PAE; black squares) groups ($n = 4$ litters for both conditions). (B) Assessment of righting reflex time showed no difference between CON (white squares) and PAE (black squares) at any age ($n = 4$ litters for all conditions). (C) The percentage of animals/litter that passed the air-righting reflex was significantly lower in PAE compared to CON from P15–18 ($n = 4$ litters for all conditions; # - $p < 0.001$, * - $p < 0.01$ repeated measures 2-way ANOVA followed by Bonferroni's multiple comparisons post-hoc test). CON and PAE did not differ in (D) time to cross balance beam (CON $n = 5$ litters and PAE $n = 4$ litters) or (E) gait ($n = 4$ litters for all conditions; FS = forelimb stride, HS = hindlimb stride, F-H = fore-hindpaw overlap, H-B = hind-base width, F-B = front-base width). (F) Latency to fall from the rotarod during three days testing was not different between CON and PAE rats ($n = 6$ litters for all conditions).

Table 1

Characterization of Postnatal EtOH Vapor Exposure Paradigm

	Control	PAE
^a Number of pups/litter	11.56 ± 0.58, <i>n</i> = 18	13.05 ± 0.47, <i>n</i> = 20
^a Number of pups after culling/litter	9.37 ± 0.36, <i>n</i> = 18	9.44 ± 0.40, <i>n</i> = 20
^b Number of dead pups/litter	0.58 ± 0.29, <i>n</i> = 18	0.84 ± 0.28, <i>n</i> = 20
Average ethanol vapor level	N/A	3.26 ± 0.06 g/dL, <i>n</i> = 24
Mother Serum EtOH Concentration	N/A	12.99 ± 2.62 mM, <i>n</i> = 6
^c Time away from all pups	1,942 ± 1,019 seconds	1,398 ± 1,208 seconds
^c Maternal grooming time (%)	4.92 ± 0.82 %	2.32 ± 0.79 % *
^c Nursing time (%)	78.69 ± 4.25 %	62.54 ± 9.98 %
^c Total time with at least 1 pup (%)	84.10 ± 5.34 %	71.05 ± 10.92 %

^a Student *t*-test^b Mann-Whitney test^c Maternal care assessment, *n* = 4 for all parameters; Mann-Whitney test,* *p* < 0.05

Table 2

Developmental electrophysiological characteristics of cerebellar granule neurons

Age (P)	Capacitance (pF)		Membrane Resistance (GΩ)	
	CON	PAE	CON	PAE
6	7.63 ± 0.58	7.31 ± 0.85	1.28 ± 0.14	1.43 ± 0.14
13	3.76 ± 0.32 *	3.81 ± 0.09 #	1.70 ± 0.33	2.44 ± 0.46
21	3.76 ± 0.40 *	4.10 ± 0.64 #	2.17 ± 0.35	1.74 ± 0.26
28	4.42 ± 0.54 *	4.93 ± 0.55 #	1.23 ± 0.14	1.17 ± 0.15 ^a

n = 8 for all groups* *p* < 0.05 compared to P6 CON;# *p* < 0.05 compared to P6 PAE;^a *p* < 0.05 compared to P13 PAE