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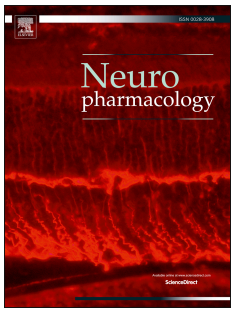

Rescue of ethanol-induced FASD-like phenotypes via prenatal co-administration of choline

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ABSTRACT

Maternal consumption of alcohol during pregnancy can generate a multitude of deficits in the offspring. Fetal Alcohol Spectrum Disorders, or FASD, describe a palette of potentially life-long phenotypes that result from exposure to ethanol during human gestation. There is no cure for FASD and cognitive-behavioral therapies typically have low success rates, especially in severe cases. The neocortex, responsible for complex cognitive and behavioral function, is altered by prenatal ethanol exposure (PrEE). Supplementation with choline, an essential nutrient, during the prenatal ethanol insult has been associated with a reduction of negative outcomes associated with PrEE. However, choline's ability to prevent deficits within the developing neocortex, as well as the underlying mechanisms, remain unclear. Here, we exposed pregnant mice to 25% ethanol in addition to a 642 mg/L choline chloride supplement throughout gestation to determine the impact of choline supplementation on neocortical and behavioral development in ethanol-exposed offspring. We found that concurrent choline supplementation prevented gross developmental abnormalities associated with PrEE including reduced body weight, brain weight, and cortical length as well as partially ameliorated PrEE-induced abnormalities in intraneocortical circuitry. Additionally, choline supplementation prevented altered expression of *RZRβ* and *Id2*, two genes implicated in postmitotic patterning of neocortex, and global DNA hypomethylation within developing neocortex. Lastly, choline supplementation prevented sensorimotor behavioral dysfunction and partially ameliorated increased anxiety-like behavior observed in PrEE mice, as assessed by the Suok and Ledge tests. Our results suggest

that choline supplementation may represent a potent preventative measure for the adverse outcomes associated with PrEE.

KEYWORDS

prenatal, ethanol, choline, neocortex, behavior

1. INTRODUCTION

Fetal Alcohol Spectrum Disorders, or FASD, refer to a wide range of life-long physical, neurological, and behavioral deficits that result from prenatal ethanol, or alcohol exposure. The more commonly known designation, Fetal Alcohol Syndrome, or FAS, represents the most severe cases on the spectrum. Prenatal ethanol exposure, or PrEE, is the leading cause of *preventable* intellectual disability in the Western world, and the prevalence of FASD has been recently estimated to be as high as 5% in the United States (US) (May et al., 2018), carrying an estimated financial burden of approximately $23,000 per year per diagnosed individual (Greenmyer et al., 2018). Alarmingly, the absence of diagnosis or misidentification of FASD has been reported to occur frequently (Chasnoff et al., 2015). Thus, the prevalence of FASD and the corresponding economic load are likely to be greatly underestimated. Despite a plethora of preclinical and clinical research studies, as well as government health agency recommendations identifying the dangers of PrEE to the developing baby, 11.5% of all

pregnant women in the US admit to drinking alcohol during pregnancy and some estimates for older women are greater than 18% (Denny et al., 2019). Thus, it is critical to look at preventative and therapeutic approaches beyond abstinence to address this serious health issue.

One such approach that has been studied is the use of choline. Choline, a vitamin-like essential nutrient (IOM, 1998) important for proper brain development, is essential for cell membrane formation, synthesis of the neurotransmitter acetylcholine, and is a source of the methyl groups needed to form the primary methyl donor *S*-adenosylmethionine (SAM) (reviewed in Zeisel, 2006). Additionally, alcohol consumption reduces brain choline levels (Biller et al., 2009) and the availability of related metabolites in liver (Barak et al., 1987), functionally increasing demand for choline.

Preclinical research has explored the ability of choline to prevent or ameliorate systemic PrEE-induced alterations in brain and behavior using rodent models for nearly twenty years (Thomas et al., 2000). Specifically, promising results from these studies have shown that prenatal or early postnatal choline supplementation can prevent altered behavior due to PrEE such as hyperactivity (Thomas et al., 2004b; 2007), spatial learning and memory deficits (Ryan et al., 2008; Thomas et al., 2010; Waddell and Mooney, 2017), and fear conditioning (Wagner and Hunt, 2006). In addition, choline supplementation has been shown to prevent or reduce PrEE-induced abnormalities within the brain such as altered DNA methylation in the hippocampus and prefrontal cortex (Otero et al., 2012), atypical gene expression and epigenetic regulation in the hypothalamus (Bekdash et al., 2013), as well as hippocampal microRNA (miRNA)

92 expression variance (Balaraman et al., 2017). Supporting results have been also
93 demonstrated in human cohorts, which exhibited reduced behavioral deficits following
94 choline supplementation in patients afflicted with FASD (Wozniak, et al., 2015;
95 Jacobson et al., 2018). Thus, the use of choline may have potent therapeutic promise in
96 humans with FASD.

97 Despite the number of studies supporting choline's use a preventative measure
98 for FASD-like phenotypes, one gap in the existing literature which has yet to be fully
99 investigated is the impact of choline on neocortical development in the context of PrEE.
100 The neocortex is responsible for processing several modalities of sensory information,
101 coordinating motor output, and mediating higher order cognitive function. The highly
102 ordered and complex circuitry that is characteristic of this structure has been postulated
103 as a focal point of ethanol's impact due to the observed phenotypes in patients with
104 FASD. Indeed, a mass of both preclinical (Ikonomidou et al., 2000; Cuzon et al., 2008;
105 Hashimoto-Torii et al., 2011; Delatour et al., 2018) and clinical evidence (Infante et al.,
106 2015; Kodali et al, 2017; Zhou et al., 2018) have confirmed the adverse influence of
107 PrEE on the development of the neocortex.

108 Additionally, our laboratory has published a series of studies demonstrating the
109 consistent, simultaneous disruption of neocortical connectivity and expression of genes
110 thought to govern the development of these connections (such as *RZRβ* and *Id2*) in
111 newborn mice. These phenotypes may underlie PrEE's ability to alter several aspects of
112 behavior in pre-pubescent mice, such as sensorimotor integration, coordination, and
113 anxiety-like behaviors (El Shawa et al., 2013; Abbott et al., 2016; Abbott et al., 2018).
114 Taken together, these results indicate the need for a thorough examination of choline's

ability to prevent both abnormal neocortical development and subsequent behavioral deficiencies induced by PrEE, which has yet to be evaluated in any previous study.

In this study, we employed the same FASD murine model to assess the ability of concurrent choline supplementation to fully or partially ameliorate atypical neocortical and behavioral development following PrEE. Specifically, we examined gross physical and morphological development, patterns of neuronal connectivity, gene expression, and epigenetic regulation within the cortex at postnatal day (P)0 following PrEE with or without choline supplementation. We then examined adolescent behavior using a battery of assays at P20. We hypothesized that choline, when given at the time of the PrEE insult, would partially or fully prevent aberrant development of cortex at P0 and subsequent altered behavior at P20, and that the underlying mechanisms would be epigenetically-mediated. Results from this study will provide both key neocortical and mechanistic information on this potential human preventative treatment, as well as on the general underlying mechanisms of FASD.

2. MATERIALS AND METHODS

2.1. Mouse colony

All breeding and experimental studies were conducted in accordance with protocol guidelines approved by the Institutional Animal Care and Use Committee at the University of California, Riverside. CD-1 background mice were originally purchased from Charles River Laboratories and the breeding colony was maintained at UCR. Mice were housed in an environmentally controlled vivarium and were kept on a 12-hour

light/dark cycle. Standard lab chow and water were provided *ad libitum* (except in gestational liquid treatment period).

2.2. FASD Mouse model, choline supplementation, and dam measures

2 to 3-month-old mice were paired overnight for breeding. Upon confirmation of vaginal plug, the gestational day was recorded as 0.5. Immediately after, dams were randomly assigned to one of four treatment groups for the entirety of gestation: group 1-Water (Control), group 2-25% EtOH in water (EtOH), group 3-25% EtOH in water with 642 mg/L choline chloride (Sigma; St. Louis, MO, USA) (CE), and group 4-642 mg/L choline chloride in water (CW). For all groups, 8 litters were treated for offspring generation. These treatments were available *ad libitum* to dams and were the only source of liquid provided. Choline dosage was based off a previous report which found choline supplementation ameliorated PrEE effects in rodent brain (Bekdash et al., 2013). Daily liquid and food intakes of all dams were measured daily at 0900h using a standard scale and graduated drinking bottle. Separate subsets of dams were sacrificed at either GD9 and 19 for blood ethanol content (BEC) and plasma osmolality (POSM) measurements during gestation (n=10, all groups). BEC was assayed in duplicate using whole blood samples. Briefly, whole blood samples were processed to obtain 5 µL of serum which was then mixed with 1 mL of alcohol reagent (Pointe Scientific; Canton, MI, USA) and assayed immediately using a Nanodrop 2000 spectrophotometer at 340 nm wavelength. POSM was assayed using whole blood samples taken from dams at GD 19. Blood samples were added to ice-cold tubes and centrifuged at 4°C/1100 g for

10 min, obtaining a clear supernatant. Blood plasma osmolality was then measured using a Vapro 5520 vapor pressure osmometer (Wescor; Logan, UT, USA).

2.3. Pup measurement and selection criteria

For consistent staging of pups, the day of birth was designated as P0. Total litter sizes (# of pups) were recorded on P0. For experimental analyses in brain, 1 ± 1 pups were selected pseudo-randomly from each litter. Pups were then weighed via Fisher Scientific scale and sacrificed at P0 via 1 of 2 methods dependent on experimental endpoint (details below). Only pups used at P0 endpoints were weighed at P0 to avoid excess disturbance of both pups and dams to ensure successful viability. Psuedo-randomly chosen subsets of P0 brains from each group were weighed and imaged for cortical length measurements accomplished via digital micrometer (ImageJ, NIH; Bethesda, MD). Although subsequent behavioral analyses were sex-specific, no P0 endpoints were conducted in a sex-specific manner, due to the difficulty of accurately sexing P0 pups non-genetically. Absence of distinguishing sexual characteristics at P0, relative inaccuracy of using conventional methods, such as anogenital distance (Wolterink-Donselaar et al., 2009), as well as the inability to use newly validated methods (Deeney et al., 2016) due to use of an albino mouse strain prevented P0 sex-separated analysis. For behavioral analysis, subsets of each litter in all groups were cross-fostered to alcohol-naïve mothers until P20 when behavioral testing of 3 ± 1 pups per litter took place. All rearing and housing conditions were consistent among cross-fostered groups and all litters were pseudo-randomly culled to 6 pups prior to cross fostering to prevent any off-target effects of varied rearing experience. Experimental use

of pups had strict upper limits of 2 pups/per litter/per P0 endpoint and 4 pups/per litter/per P20 endpoint to reduce the potential confounder of litter effects. A larger per-litter subset was used as the upper limit for P20 behavioral analyses due to the inclusion of both male and female offspring in near-equal ratios, as well as to control for the potential of non-participants in behavioral tests. For global methylation and gene expression assays, only a single pup per litter was included in analysis to prevent confounding litter effects.

2.4. Dye tracing and ISH tissue preparation

P0 pups used in dye tracing and *in situ* hybridization (ISH) studies were sacrificed via hypothermia, and transcardially perfused with 4% paraformaldehyde (PFA) in 0.1M phosphate buffer, pH 7.4. Brains were quickly removed, weighed, and dorsal whole brain photos taken via a Zeiss Axio HRm (Carl Zeiss; Oberkochen, Germany) camera attached to a dissecting microscope. Cortical length was measured using NIH ImageJ's measure function by drawing a diagonal line from the most anterior pole of the cortex to the furthest posterior pole. Following post-fixation in 4% PFA, brains were hemisected, and hemispheres were designated for either dye tracing or ISH assays to ensure even sampling of each litter for each assay. Previous studies from our laboratory (El Shawa et al., 2013; Abbott et al., 2018) have examined the potential asymmetric impact of PrEE on the early postnatal brain and have found no significant lateralization effects. Thus, random sampling of both right and left hemispheres was used in all P0 brain-based experiments to produce a homogenous population of data.

205 *2.5. Global methylation tissue preparation*

206 A separate subset from litters of each group were sacrificed for global methylation
207 assays at P0 as follows. Pups were briefly placed on ice until a deep plane of
208 anesthesia was reached via hypothermia which was confirmed via toe pinch. Once this
209 was achieved, pups were sacrificed via decapitation and the brain was quickly removed
210 from the skull and underlying membranes. The brain was then hemisected and the
211 whole neocortex was dissected from all subcortical structures. Once isolated, whole
212 hemisected neocortical samples were measured using a grid system and partitioned
213 into even rostral and caudal samples. Whole rostral and caudal divided portions of
214 neocortex from each animal were then placed into Buffer RLT (Qiagen; Venlo,
215 Netherlands) for further processing and DNA extraction via Qiagen AllPrep DNA/RNA
216 extraction kit.

217

218 *2.6. Anatomical tracing*

219 To determine the development of ipsilateral, intraneocortical connections (INCs) in P0
220 mice 1,1-Dioctadecyl-3,3,3,3-tetramethylindocarbocyanine (DiI; Invitrogen; Carlsbad,
221 CA, USA) dye crystals were placed in putative primary visual cortex (VCx), as described
222 previously (El Shawa et al. 2013). A coordinate grid was used for reliability of dye
223 placement locations (DPLs) across cases. Following placement of crystals,
224 hemispheres were placed in 4% PFA in the dark at room temperature for 4-6 weeks to
225 allow for dye transport. Upon confirmation of retrograde thalamic nuclei labeling, brains
226 were embedded in 5% low melt agarose, and sectioned at 100µm via vibratome in 1X
227 phosphate buffered saline (PBS). Sections were then counterstained with 4′, 6-

diamidino-2-phenylindole dihydrochloride (DAPI; Roche; Basel, Switzerland), mounted onto glass slides, and coverslipped using FluoroMount (Sigma; St. Louis, MO, USA).

2.7. Dye tracing analyses

A Zeiss Axio Upright Imager microscope equipped with a Zeiss Axio high resolution (HRm) camera and two filters was used to visualize and capture images of dye labelled sections via PC running Axiovision software. The two filters used are detailed in the following: red for DiI and blue for DAPI counterstain labeling (Excitation wavelengths-red: Cyanine 3,550 nm; blue: DAPI, 359 nm. Emission wavelengths-red: Cyanine 3, 570 nm; blue: DAPI, 461 nm). Images from both filters per section were merged for subsequent analysis. Matched sections from all groups' brains using DAPI counter-stained landmarks were used to compare the development and trajectory of INCs among treatment and control groups. Retrogradely-labeled cell bodies were plotted and aligned using subcortical and blood vessel anatomy as guidelines to generate 2D reconstructions of "flattened" neocortex, illustrating the position of INCs labelled from DPLs. Section data was further quantified by projection zone and cell counting analyses. Projection zone analysis was accomplished by using combined 2D reconstruction and individual section data, measuring the furthest rostral and caudal cell labeling originating from a distinct cortical DPL. This parameter was measured in relationship to the center of the DPL and quantified as a percentage of total cortical length to control for possible neocortical reduction in experimental groups. DPL spreads were also measured in all cases to ensure consistent dye crystal size and uptake and are presented as percentage of total cortical length. This measurement technique

controls for inherent cortical differences between brains and allows for statistical analysis of INC development between the 4 groups. Frontal cortex cell counts took place in an electronically placed region of interest (ROI) (ImageJ) in anatomically matched sections across cases, as confirmed via the Paxinos developing mouse atlas (Paxinos et al., 2007), and retrogradely-labeled cells were counted by a trained researcher blind to condition. ROIs remained static in size and location for all cases and groups.

2.8. In Situ Hybridization (ISH) and transcript density analyses

Standard protocols for free-floating non-radioactive ISH were used to visualize neocortical *Id2* and *RZRβ* gene expression of P0 brains, as previously described in detail elsewhere (Dye, et al., 2011a). Briefly, hemispheres were first embedded in gelatin-albumin, and sectioned at 100μm via vibratome. After hybridization to digoxigenin-labeled probes for *Id2* and *RZRβ* (gifts from John Rubenstein, UCSF), sections were developed in NBT/BCIP (Roche; Basel, Switzerland), mounted in a 50% glycerol solution onto glass slides, coverslipped, and imaged using a Zeiss Axio HRm camera attached to a dissecting microscope. Relative levels of *Id2* and *RZRβ* were measured via semi-quantitative mRNA transcript density analyses (Dye et al., 2012; El Shawa et al., 2013). Briefly, raw ISH data images among all groups were strictly matched anatomically and confirmed via atlas and subcortical landmarks, converted to binary via ImageJ and matched to a set threshold. ROIs were chosen based on qualitative observations of potential phenotypic variation due to condition. ROIs were electronically placed onto matching locations within sections and the level of pixel

density within ROIs was measured as a proxy for transcript signal density. Transcript densities are reported as area fraction of total ROIs. ROIs remained static in location for all cases and groups for each gene analyzed, however, due to the consistent reduction in brain size in EtOH-exposed pups, ROIs for the EtOH group were also reduced to control for this difference. Briefly, section area was calculated for all cases to produce an average percent reduction in section size from controls, which was determined to be 12%. Thus, ROIs in EtOH cases were reduced in area by 12% to account for overall brain size differences. Because no other groups displayed a brain size reduction, ROI size remained static within those analyses.

2.9. DNA extraction and global DNA methylation assay

Following homogenization, genomic DNA was isolated from rapidly dissected rostral and caudal cortical samples at P0 via the Qiagen AllPrep DNA/RNA Mini kit. Extracted DNA was then assessed for quality and quantity via NanoDrop 2000 spectrophotometer (ThermoScientific; Waltham, MA, USA). 5-methylcytosine (5-mC) levels were then measured in DNA isolated from rostral and caudal cortical samples using the MethylFlash Global DNA Methylation (5-mC) ELISA easy kit (Epigentek; Brooklyn, NY, USA). Briefly, 200 ng of DNA was bound to plates treated to have high affinity for DNA. Methylated fractions of DNA (i.e. 5-mC) in samples were then assessed using detection antibodies and absorbance was measured at 450 nm on a Victor 2 plate reader. The level of 5-mC in cortical tissue samples were quantified from absorbance measurements according to manufacturer's instructions and is reported as % of 5-mC relative to the total genomic DNA content.

297

298 *2.10. Behavioral analyses*

299 Prior to all behavioral testing, P20 mice from all 4 experimental groups were acclimated
300 to the dimly-lit experiment room. All behavioral scoring was conducted by trained
301 researchers blind to condition. Each mouse tested underwent a single trial of the Suok
302 test, followed by a single trial in the Ledge test after a period of rest. The Suok test was
303 used to simultaneously assess potential anxiety-like behaviors and motor function, as
304 previously described (Kalueff et al., 2008). In the Suok test, mice traverse an elevated
305 cylindrical bar (2 m length, 3 cm diameter, 20 cm off ground) for a single 5-minute trial
306 and are scored for several parameters. Anxiety-like behavioral parameters measured in
307 the Suok assay include latency to leave the center of the bar (start position), directed
308 exploration events, and rearing/grooming events. Potential sensorimotor integration and
309 motor coordination are also assessed in the Suok test via number of missteps and falls
310 from the rod. Additionally, the Ledge test was used to measure the animal's ability to
311 integrate sensory inputs and motor outputs, as previously described (Wang et al.,
312 2002). Here, mice were scored for their ability to maintain balance on a vertically-
313 stabilized Plexiglass sheet, which was 50 cm long, 30 cm tall, and 0.7 cm in width for a
314 maximum score of 60 seconds (s). If the mouse was able to successfully traverse the
315 length of ledge and returned to the starting position, a maximum score of 60 s was
316 assigned. The ledge test is used to directly measure coordination in mice (Guyenet et
317 al., 2010). As such, if a mouse displays adequate coordination to successfully traverse
318 the very thin (0.7 cm) ledge twice, it can be considered to display no significant
319 coordination impairments, and thus is awarded a maximum score (60 seconds). This

established scoring methodology was used as previously published for the Ledge test (Schaefer et al., 2000; Wang et al., 2002; El Shawa et al., 2013). Following each trial in both Suok and Ledge tests, the behavioral apparatuses were thoroughly cleaned to remove any olfactory cues.

2.11. Statistical analyses

All statistical analyses took place using GraphPad Prism 6 software (La Jolla, CA, USA). All data was initially analyzed for Gaussian distribution using the Shapiro-Wilk normality test. For between group comparisons that displayed normal distributions, two-way analysis of variance (ANOVA; factors: ethanol and choline) followed by Tukey's multiple comparisons test was used. For the 9 measures (Dam BEC, P0 body weight, frontal cortex labeled cell counts, ledge test scores, and latency to leave center, directed exploration events, rearing/grooming events, and falls on the Suok test) that did not display normal distributions, the Kruskal-Wallis test followed by Dunn's multiple comparison test was used. For all behavioral measures at P20, individual animals from each group were separated by sex and additionally analyzed using tests described above. For all measures, statistical significance was set as $p < 0.05$. Data are presented as mean ± SEM. All group means and SEMs for quantified data are presented in supplemental materials (Supplementary Table 1).

3. RESULTS

3.1. Dam measures

In order to assess possible nutritional confounds in dams, we measured gestational food intake, liquid intake, blood ethanol content (BEC), plasma osmolality (POSM), weight gain, and litter size (**Fig. 1**). There was a significant effect of ethanol on gestational food intake [$F_{1,28} = 5.488$; $p = 0.027$], however post hoc tests revealed no significant differences between any groups (**Fig. 1A**). No significant effects or interaction were present in dam liquid intake measurements (**Fig. 1B**). A significant effect due to treatment was found in BEC levels at GD 9 and 19 [$H = 61.22$; $p < 0.0001$]. As expected, BEC levels (**Fig. 1C**) were only present in EtOH (106.5 ± 1.693 mg/dL, *n*= 10) and CE (106.9 ± 1.040 mg/dL, *n*= 10) dams at GD 9, and rose to 136.9 ± 2.794 mg/dL (EtOH, *n*= 10) and 136.3 ± 2.894 mg/dL (CE, *n*= 10) at GD 19. Post hoc comparisons revealed no significant differences between EtOH and CE dams BEC levels at either time point (GD 9: $p > 0.999$; GD 19: $p > 0.999$), suggesting similar levels of ethanol intoxication. Additionally, no statistically significant differences were present between GD 9 and GD 19 BEC measures within EtOH (*p = 0.303)* or CE dams (*p = 0.667)*. Dam POSM, which serves as a measure of hydration, displayed no significant main effects and post hoc tests revealed no significant differences among groups (**Fig. 1D**) at GD 19. No main effects were found for gestational weight gain, however a significant ethanol X choline interaction was present [$F_{1,28} = 6.375$; $p = 0.018$] and post hoc tests revealed that EtOH dams displayed a reduction in weight gain (**Fig. 1E**) compared to controls (*$p = 0.022$). Furthermore, a significant main effect of ethanol [$F_{1, 28} = 4.233$, $p= 0.049$] and an ethanol X choline interaction [$F_{1, 28} = 5.635$, $p= 0.025$] was found for litter size. Post hoc analysis revealed that EtOH dams also displayed a concomitant reduction in litter size (**Fig. 1F**) compared to both control (*$p = 0.020$) and

CE dams (*p = 0.043), suggesting choline can prevent adverse pregnancy outcomes due to PrEE. As an additional control, we investigated potential group differences in gestational length and found no significant changes due to treatment [$F_{3, 28}$ = 2.333, p = 0.096; Control: 19.88 ± 0.227 d, CW: 20.25 ± 0.164 d, EtOH: 20.50 ± 0.189 d, CE: 20.50 ± 0.189 d].

3.2. Physical development of pups

Body weights and brain weights were measured in offspring at P0 to assess physical development (**Fig. 2**). A significant effect of treatment on P0 body weight was detected [H = 19.49, p= 0.0002], with post hoc analyses revealed a reduction in EtOH pups compared to control (**p = 0.024), CW (***p = 0.029), and CE pups (**p = (**Fig. 2A**). In addition, 2-way ANOVA revealed significant main effects of ethanol [$F_{1, 51}$ = 8.281, p= 0.005] and choline [$F_{1, 51}$ = 6.954, p= 0.011] on P0 brain weight, as well as an ethanol X choline interaction [$F_{1, 51}$ = 8.281, p= 0.005]. Post hoc analyses showed that EtOH pups have reduced brain weights at P0 (**Fig. 2B**) compared to control (***p = 0.0002), CW (**p = 0.001), and CE pups (**p = 0.003). No main effects or interactions were present for brain/body weight ratios (**Fig. 2C**), which were not significantly altered among groups, likely reflecting the association of *both* reduced brain and body weights at P0 in EtOH pups.

Cortical length was measured at P0 (**Fig. 3**) in order to analyze gross neocortical developmental abnormalities associated with PrEE and the ability of choline to prevent such deficits. Black arrows in **Fig. 3A-D** indicate the cortical length measurements as assessed electronically in ImageJ. A significant main effect of ethanol [$F_{1, 36}$ = 12.07, p=

388 0.001] and an ethanol X choline interaction [$F_{1, 36}$ = 4.433, *p*= 0.042] was found for P0
389 cortical length, and post hoc analyses revealed that EtOH cortical lengths were
390 significantly reduced (**Fig. 3E**) compared to control (***p* = 0.002), CW (***p* = 0.004), and
391 CE brains (**p* = 0.049) at P0. Overall, these results suggest concurrent choline
392 supplementation can prevent adverse PrEE-induced physical and gross brain
393 developmental deficits in very early life.
394
395 *3.3. Development of ipsilateral INCs*
396 Intraneocortical connections (INCs) are a defining characteristic of distinct cortical areas
397 and are crucial for integration of sensory inputs and coordinating outputs of the
398 neocortex (Kaas, 1982). Previously, our lab has shown PrEE significantly disrupts INC
399 development of putative sensory areas in offspring at P0, specifically resulting in a
400 severely disorganized pattern of neocortical connectivity (El Shawa et al., 2013; Abbott
401 et al, 2018). Here, we analyze INC patterns of putative visual cortex (VCx) using
402 lipophilic dye tracing in order to assess the ability of choline supplementation to mitigate
403 abnormal neocortical connectivity due to PrEE.
404 Analysis of ipsilateral INCs at P0 confirmed the ability of PrEE to perturb
405 development of patterns of neocortical connections (**Fig. 4**). EtOH brains displayed
406 abnormal patterns of labeled cells stemming from putative primary visual cortex DPLs
407 (*V, **Fig. 4D1-4**). Specifically, EtOH brains contained labeled cells that extended into
408 cortical areas within very far rostral sections (arrows, **Fig. 4A3, B3**) not present in either
409 control (**Fig. 4A1, B1**) nor CW brains (**Fig. 4A2, B2**) at P0. In contrast, CE brains did
410 not contain labeled cells in very rostral sections that were present in the EtOH group at

P0 (compare **Fig., 4A3 to A4**). However, CE brains still displayed an extended pattern of INCs from VCx injections (arrow, **Fig. 4B4**) which were not present in either control group (**Fig. 4B1-2**), suggesting concurrent choline administration can only partially ameliorate PrEE-induced disruption of sensory cortical area INC patterns. Importantly, the altered patterns of INCs were present without gross alteration of thalamocortical afferent connectivity, as labeled cells were present within the dorsal lateral geniculate nucleus in all groups (arrows, **Fig. 4D1-4**).

These overall patterns of INCs are more easily visualized via flattened lateral view reconstructions of the cortex in **Fig. 4F1-4**. Whereas control (**Fig. 4F1**) and CW (**Fig. 4F2**) brains displayed a tight pattern of labeled cells that reside in caudal cortical areas, EtOH (**Fig. 4F3**) and CE (**Fig. 4F4**) both show patterns of INCs that extended into rostral sections not present at any age in control animals, (Dye et al., 2011a; 2011b), suggesting an abnormally defined border of putative visual cortex. However, the partial reduction of this extended pattern by choline was apparent when EtOH and CE brains were compared, where labeled cells did not extend as far rostrally in the CE group.

Analysis of DPL spread (to assess size of injections) revealed no significant main effects or an interaction (**Fig. 5A**), suggesting size of dye injections is consistent, and does not confound the observed results. In contrast, significant effects of ethanol [$F_{1, 18}$ = 173.8, $p < 0.0001$] and choline [$F_{1, 18}$ = 7.991, $p = 0.011$], as well as an ethanol X choline interaction [$F_{1, 18}$ = 9.367, $p = 0.007$] was observed for VCx projection zones. Post hoc analyses revealed that both EtOH and CE brains have significantly increased projection zones compared to control (EtOH: ****$p < 0.0001$*; CE:* ****$p < 0.0001$) and

CW groups (EtOH: ****$p < 0.0001$*; CE:* ****$p < 0.0001$) (**Fig. 5B**). However, post hoc analysis also revealed a significant reduction in projection zone in CE brains compared to the EtOH group (**$p = 0.002$), suggesting choline has the ability to partially prevent abnormal INC patterns due to PrEE. This partial reduction by choline is further demonstrated by the count of retrogradely-labeled cell bodies within the region of interest (ROI) at the anatomical level illustrated in **Fig. 5C**. The static ROI spans the putative prelimbic (PrL), cingulate (Cg), secondary motor (M2), and primary motor (M1) cortical areas. Cell counts within this ROI (**Fig. 5D**) yielded no results for any treatment group except for the EtOH treated animals, which contained 25.33 ± 3.43 cells at this anatomical level [H= 20.33, $p < 0.0001$]. Post hoc analyses expectedly revealed significant increases in EtOH brains compared to control (**$p = 0.002$), CW (**$p = 0.002$), and CE brains (**$p = 0.001$). Overall, results from these dye tracing experiments suggest that concurrent choline supplementation can partially prevent the disruption of INC pattern development of an early sensory cortical area that results due to PrEE.

3.4. Cortical gene expression

Age-dependent patterns of expression of a subset of genes within the neocortex have been experimentally implicated in guiding both the development of distinct cortical areas and the characteristic patterns of INCs in these areas (Huffman et al., 2004; Dye et al., 2011a; 2011b). Expression of two particular genes within this subset, *RZRβ* and *Id2*, have been consistently shown to be altered in the neocortex of newborn mice due to PrEE (El Shawa et al., 2013; Abbott et al., 2018). In order to assess choline's ability to

ameliorate PrEE-induced abnormal gene expression of *RZRβ* and *Id2*, we employed *in situ* hybridization in coronal hemisphere sections at P0 in all 4 groups (**Fig. 6**).

As demarcated by the arrow in **Fig. 6A1**, P0 control brains displayed a distinct boundary of *RZRβ* expression in layer IV of rostral parietal cortex, which distinguishes the gradient of low expression in medial cortex and gradual high expression in lateral cortex, which was also present in the CW group (arrow, **Fig. 6B1**). In comparison, EtOH mice displayed a dramatic medial shift in this boundary (arrow, **Fig. 6C1**), and, thus, *RZRβ* expression extended into the most medial regions of cortex, confirming the results of our previous reports (El Shawa et al., 2013; Abbott et al., 2018). However, in CE mice, this abnormal medial shift in *RZRβ* expression was not present (arrow, **Fig. 6D1**) and expression patterns were qualitatively indistinguishable from either control groups, suggesting choline can prevent altered cortical gene expression due to PrEE at P0.

Id2 expression patterns in early postnatal control mice contain laminar specific expression gradients that vary depending on the area of the neocortex (Rubenstein et al., 1999). At the level of the rostral parietal cortex of controls in our study, Id2 was expressed in the subplate, deep layers V and VI, as well as in superficial layers I and II/III, where a boundary denoted a gradient of high medial expression and lower lateral expression (arrow, **Fig. 6A2**), which was also present in CW mice (arrow, **Fig. 6B2**). However, in EtOH mice this boundary was shifted medially (arrow, **Fig. 6C2**), and thus low expression of *Id2* was similarly shifted medially. In contrast, CE mice did not display this aberrant shift in *Id2* expression (arrow, **Fig. D2**), further suggesting that choline can mitigate gene expression deficits induced by PrEE in the neocortex at P0.

Semiquantitative analyses of these gene expression patterns were conducted using binary-converted raw images and transcript density counts (**Fig. 7**). Transcript densities were measured in static ROIs indicated on the illustrations in **Fig. 7A1** (*RZRβ*) and **Fig. 7B1** (*Id2*) and were reported as area fractions (%) of black pixel containment within the ROIs. Significant main effects of ethanol [$F_{1, 18} = 44.32$, $p < 0.0001$] and choline [$F_{1, 18} = 50.69$, $p < 0.0001$], as well as an ethanol X choline interaction [$F_{1, 18} = 30.63$, $p < 0.0001$], were present for *RZRβ* transcript density (**Fig. 7A2)**. Post hoc analyses revealed a significant increase of *RZRβ* transcript density in the ROI of EtOH mice compared to control (****$p < 0.0001$), CW (****$p < 0.0001$), and CE groups (****$p < 0.0001$). Additionally, 2-way ANOVA revealed significant main effects of ethanol [$F_{1, 13} = 14.72$, $p = 0.016$] and choline [$F_{1, 13} = 28.65$, $p = 0.002$], as well as an ethanol X choline interaction [$F_{1, 13} = 24.15$, $p = 0.004$], on *Id2* transcript density (**Fig. 7B2)**. Post hoc analyses reveal a significant decrease in *Id2* area fractions within the ROI of EtOH compared to control (**$p = 0.002$), CW (**$p = 0.002$), and CE mice (**$p = 0.001$). Together, these results confirm the qualitative analyses and suggest choline supplementation can prevent PrEE-induced abnormal expression patterns of two genes known to participate in the guidance of development of INCs at P0.

3.5. Global DNA methylation

In order to investigate choline's function in DNA methylation and whether its supplementation can prevent PrEE-induced epigenetic dysregulation, we examined global DNA 5-methylcytosine (5mC) levels in rostral and caudal cortex at P0 in all 4 groups (**Fig. 8**). Previously, our laboratory has shown significant hypomethylation in

both rostral and caudal cortical samples in PrEE mice compared to controls at P0 (Abbott et al., 2018). Using the same ELISA-like colorimetric assay, a two-way ANOVA revealed significant main effects due to ethanol [$F_{1, 13} = 8.696$, $p = 0.011$] and choline [$F_{1, 13} = 14.90$, $p = 0.002$] on global DNA %5mC in rostral cortex. For caudal cortex, significant main effects due to ethanol [$F_{1, 13} = 21.08$, $p = 0.001$] and choline [$F_{1, 13} = 6.099$, $p = 0.028$] were also present. No ethanol X choline interactions were present for either region. Post hoc analyses detected a significant DNA hypomethylation in EtOH mice in both rostral and caudal cortex compared to controls (rostral: *$p = 0.049$; caudal: *$p = 0.030$). In contrast, CE 5mC DNA levels were not statistically significant from control animals in either rostral ($p = 0.924$) or caudal cortex ($p = 0.498$) and were significantly increased when compared to EtOH mice in rostral cortex (*$p = 0.050$), suggesting that choline supplementation can prevent PrEE-induced hypomethylation in newborn cortex. Choline supplemented control animals (CW) seemed to display a trend in increased DNA methylation, which was supported by the post hoc comparisons which revealed a stronger difference when compared to EtOH (rostral: **$p = 0.001$; caudal: ***$p = 0.001$) than what was seen in control vs. EtOH comparisons (rostral: *$p = 0.049$; caudal: *$p = 0.030$). However, CW 5mC DNA levels were not statistically different from the control group in rostral ($p = 0.105$) or caudal cortex ($p = 0.180$), suggesting that our choline dose alone does not significantly impact DNA methylation in the cortex of normally developing mice at P0. Overall, these results suggest that choline supplementation, when administered at the time of PrEE insult, can prevent PrEE-associated hypomethylation in the cortex at P0.

525 *3.6. Behavioral analyses*

526 At P20, two separate behavioral assays were used to test the hypothesis that
527 concurrent prenatal choline supplementation can prevent the adverse behavioral
528 outcomes associated with PrEE (**Fig. 9**). Previously, our laboratory has shown motor
529 deficits (which may be rooted in sensorimotor integration) and increased incidence of
530 behaviors that may be indicative of anxiety-like states in PrEE mice at P20 (El Shawa et
531 al., 2013; Abbott et al., 2018) using these same behavioral assays.

532 Potential anxiety-like behavioral measures scored included latency to leave
533 center of the bar (**Fig. 9A**), directed exploration events (**Fig. 9B**), and rearing/grooming
534 events (**Fig. 9C**). A significant effect of treatment was found on all anxiety-like
535 measures: [Latency to leave center: H = 8.196, p = 0.042; Directed exploration: H =
536 16.61, p = 0.001; Rearing/grooming: H = 15.33, p= 0.002]. Post hoc analyses revealed
537 a significant increase in latency to leave center in EtOH animals (**Fig. 9A**) compared to
538 control (*p = 0.043) mice. Post hoc analyses also revealed a significant decrease in
539 directed exploration events in EtOH mice (**Fig. 9B**) compared to control (*p = 0.026),
540 CW (**p = 0.002), and CE (**p = 0.001) mice. Lastly, both EtOH and CE mice displayed
541 decreased rearing and grooming events (**Fig. 9C**) compared to controls (EtOH: *p =
542 0.010; CE: *p = 0.021) at P20. Overall, these results suggest that prenatal choline has
543 the ability to partially ameliorate potential anxiety-like behaviors induced by PrEE at
544 P20.

545 Sensorimotor integration and motor function were also assessed by missteps
546 and falls within the Suok test, as well as by the overall score of the Ledge test.
547 Significant main effects of ethanol [$F_{1,61}$ = 23.79, p < 0.0001], choline [$F_{1,61}$ = 10.31, p =

548 0.0010] and an ethanol X choline interaction [$F_{1, 61}$ = 13.33, p = 0.0002] were found on
549 missteps. A significant effect of treatment was also found on falls [H= 23.54, p < 0.0001]
550 in the Suok test. Post hoc test revealed that EtOH mice showed significantly increased
551 missteps (**Fig. 9D**) compared to control (****p < 0.0001), CW (****p < 0.0001), and CE
552 (****p < 0.0001) groups. Additionally, EtOH mice displayed significantly increased falls
553 (**Fig. 9E**) compared to control (****p < 0.0001), CW (**p = 0.004), and CE (*p = 0.036)
554 groups. Finally, we report a significant effect of treatment on Ledge test score [H =
555 9.864, p = 0.020], and post hoc analyses revealed a decrease in the EtOH groups
556 scores (**Fig. 9F**) compared to control (**p = 0.033). In contrast, CE mice did not display
557 scores that were statistically significant from either control group. Overall, these data
558 suggest that choline supplementation, when given at the time of PrEE insult, can
559 partially prevent potential anxiety-like behaviors and fully prevent deficits that may be
560 rooted in motor function or sensorimotor integration in offspring at P20.
561
562 *3.7. Sex-specific behavioral analyses*
563 Both EtOH and choline supplementation have been reported to perturb and prevent
564 PrEE-induced behavioral deficits, respectively, in a sex-specific manner (Thomas et al.,
565 2007; Bearer et al., 2015; Schneider and Thomas, 2016). Thus, we also analyzed
566 behavioral data separated by sex, as detailed below, to determine if any sex-specific
567 effects are present within our model.
568
569 *3.7.1. Behavioral analyses- Females*

For the Suok test, no differences were present in latency to leave center in females [H = 4.553; p = 0.208], however main effects of treatment were found on both directed exploration [H = 13.35; p = 0.004], and rearing/grooming [H = 13.16; p = 0.004]. Post hoc tests revealed female EtOH mice displayed significantly decreased directed exploration events compared to control (*p = 0.011), CW (*p = 0.026), and CE (*p = 0.046) females (**Fig. 10B**). Both female EtOH and CE mice display decreased rearing/grooming events compared to controls (EtOH: *p = 0.030; CE: **p = 0.006) (**Fig. 10C**).

Significant main effects of both ethanol [$F_{1,27}$ = 5.043, p =0.033] and choline [$F_{1,27}$ = 7.588, p = 0.010] were found for missteps in females. Post hoc tests showed EtOH females had increased missteps compared to control (*p = 0.036), CW (*p = 0.011), and CE (*p = 0.028) females (**Fig. 10D**). Additionally, a significant effect of treatment was found on falls [H = 12.24, p = 0.007], as EtOH females had increased falls compared to control females (**p = 0.006) (**Fig. 10E**). Lastly, no effect of treatment was found on female Ledge test scores.

3.7.2. Behavioral analysis- Males

For the Suok test, no effects of treatment were present in latency to leave center in males [H = 1.990; p = 0.575], directed exploration [H = 5.654; p = 0.130], or rearing/grooming [H = 3.314; p = 0.346].

A significant main effect of ethanol [$F_{1,27}$ = 6.298, p =0.014] was found on missteps for males. Post hoc tests showed EtOH males have increased missteps compared to control (*p = 0.018), CW (*p = 0.033) males (**Fig. 11D**). Additionally, a

593 significant effect of treatment was found on falls [H = 12.15, p = 0.007], as EtOH males
594 increased falls compared to control (**p = 0.007) and CW males (*p = 0.036) (**Fig. 11E**).
595 Lastly, no effect of treatment was found on male Ledge test scores.
596
597 **4. DISCUSSION**
598 We report that prenatal choline supplementation can attenuate some physical
599 and behavioral dysfunction associated with PrEE. Specifically, gestational choline
600 supplementation was able to rescue gross developmental deficits, as well as
601 dysregulated intraneocortical connectivity and gene expression gradients. Choline also
602 mitigated performance deficits in most behavioral tasks which may model anxiety-like
603 behavior and sensory-motor integration in PrEE mice. Choline, a primary methyl group
604 donor, also prevented global DNA hypomethylation that results from PrEE, providing a
605 window of insight into a possible epigenetic mechanism of ethanol's detrimental
606 developmental effects. Together, these results broaden our understanding of choline's
607 potential as a prophylactic agent for FASD and provide key, novel insight on how it
608 affects patterns of neuronal connectivity and cortical development. As behavioral testing
609 was conducted 20 days following cessation of choline supplementation, it is likely the
610 beneficial effects of choline were not related to acute action, but rather to its inhibition of
611 long-lasting organizational changes in brain development normally generated by PrEE.
612
613 *4.1. Choline prevents PrEE-induced developmental alterations in brain*
614 PrEE causes gross developmental abnormalities in rodent offspring, including
615 decreased perinatal body weight (Datta et al., 2008) and total brain weight (Komada et

al., 2017). In the present study, we report significant reductions in body weight, brain weight, and cortical length in newborn EtOH-exposed pups. In contrast, concurrent choline supplementation prevented these effects in CE pups. These results support those observed in rats who received concurrent choline supplementation over the course of a timed gestational day (GD) 5-20 EtOH exposure (Thomas et al., 2009; Thomas et al., 2010). Despite promising results when co-administered with EtOH at early developmental timepoints, choline is unable to prevent body weight reductions when administered after EtOH exposure in a rodent model of third-trimester PrEE (Ryan et al., 2008; Thomas and Tran, 2012), likely due to its absence at the time of PrEE insult. Together, these results suggest that choline can mitigate EtOH-induced gross developmental abnormalities within certain critical periods.

Development of a functional neocortex results from a series of highly-regulated processes, which are consistently disrupted following PrEE (Ikonomidou, et al., 2000; Cuzon et al., 2008; Pascual et al., 2017). These effects can manifest as reductions in cortical length, volume and area in PrEE offspring (El Shawa et al., 2013; Smiley et al., 2015). Despite the ability of preventative treatments such as choline supplementation to ameliorate these effects, there remains a paucity of research examining the mode, and extent of impact on PrEE-impaired cortical development. In the present report, we characterize genetic and epigenetic aspects of early mammalian CNS development, and associate these changes with juvenile behavior.

Choline's prophylactic action is likely achieved through the positive regulation of multiple developmental pathways, including direct influence of cortical neurogenesis and migration (Wang et al., 2016; Trujillo-Gonzales, et al., 2019), as well as the

prevention of underlying aberrant gene expression. Indeed, by mapping INC patterns of putative V1 we found that CE pups, while still displaying an altered areal boundary compared to control mice, exhibit a marked reduction in visual cortex projection zones compared to EtOH animals. This recovery may also be related to the observed ability of choline supplementation to prevent altered patterns of *RZRβ* and *Id2* expression in newborn neocortex due to PrEE. These findings expand on previous work which showed that choline can mitigate PrEE-induced alterations to gene expression in hypothalamus (Bekdash et al., 2013) and miRNA expression in hippocampus (Balaraman et al., 2017), suggesting that choline may be sufficient for prevention of widespread gene expression changes induced by PrEE.

The two genes analyzed within the current study*, RZRβ* and *Id2,* play key roles in cortical development. *Id2,* a transcription factor, and *RZRβ* (also known as *RORβ*) are known to influence several key factors of neurodevelopment including neurogenesis (Toma et al., 2000), apoptosis (Gleichmann et al., 2002) and layer formation (Oishi et al., 2016). However, in late embryonic and early postnatal ages in mice, both genes are instrumental in proper patterning of postmitotic neurons into functionally distinct areas, a phenomenon known as arealization (Rubenstein et al., 1999). More specifically, *Id2* affects axonal/neurite outgrowth (Lasorella et al., 2006; Ko et al., 2016; Huang et al., 2019) and *RZRβ* is involved in clustering of neurons to generate proper connectivity patterns (Jabaudon et al., 2012). Thus, by preventing ectopic cortical expression of *RZRβ* and *Id2,* which is hypothesized to underlie aberrant INC patterning in both genetically manipulated (Huffman et al., 2004) and PrEE cortex (Abbott et al., 2018), choline provides a potential novel mechanistic framework for the partial prevention of

662 altered INC patterns and areal size perturbations in CE neocortex. Furthermore,
663 abnormal areal boundaries within the neocortex have been shown to directly impact
664 mouse behavior (Leingärtner et al., 2007; Scearce-Levie et al., 2008), a facet of
665 development heavily affected by PrEE.

666 One attractive, alternative hypothesis to purely aberrant expression of genes and
667 INC patterns is that PrEE produces a developmental delay, an idea based largely on
668 behavioral observations in humans with FASD (Davies et al., 2011). A review of
669 previous work done in our laboratory in wild-type CD1 mice demonstrates the life-long
670 natural progression of developmental changes in cortical gene expression and
671 connections (Dye et al., 2011a; 2011b). From these data, we can conclude that neither
672 the observed ectopic expression of *RZRβ* and *Id2* or the aberrant INC patterns reported
673 here exist at any time within the normally developing mouse cortex, suggesting a
674 developmental delay likely does not describe and/or underlie the phenotypes reported
675 within the current study.

676 It is important to note that P0 endpoints, where neocortical aspects of
677 neurodevelopment were analyzed, were not examined in a sex-specific manner.
678 Although the late embryonic period is considered a critical time for the effects of
679 hormones on sexual dimorphic development (McCarthy, 2016), a recent study has
680 found that anatomical changes in dimorphic brain structures typically emerge in later
681 postnatal life (e.g. around P10) (Qiu et al., 2018). Thus, we do not expect sexually
682 dimorphic features to be present in the infant mouse brain. However, future studies
683 must take into account the potential sex differences within brain at all ages due to the

684 differential effect of both EtOH and choline on offspring behavior reported here and
685 elsewhere (Thomas et al., 2007; Schneider and Thomas et al., 2016).
686
687 *4.2. Choline prevents PrEE-induced developmental alterations in behavior*
688 Several previous studies have examined choline's ability to mitigate PrEE-induced
689 abnormal behavior, with a majority focusing on learning and memory behaviors
690 (Thomas et al., 2007; Ryan et al., 2008; Schneider and Thomas, 2016). Here, we
691 demonstrate that concurrent choline supplementation fully or partially prevents the
692 development of motor deficits and increased potential anxiety-like behaviors present in
693 peripubescent EtOH-exposed mice. Notably, the interpreted, specific behavioral
694 alterations that choline prevented within the current study are among the myriad of
695 dysfunctions consistently observed in rodent models (Thomas et al., 1996; Akers et al.,
696 2011; Baculis et al., 2015), and humans with FASD (Barr et al., 2006; Connor et al.,
697 2006; Carr et al., 2010). This is in contrast to previous studies which have reported that
698 concurrent choline supplementation during a P4-9 EtOH exposure does not prevent
699 motor deficits (Thomas et al., 2004a). However, EtOH-induced motor developmental
700 delays in offspring were also prevented by concurrent choline during a GD 5-20
701 exposure (Thomas et al., 2009). Taken together with our findings, this suggests that
702 choline may have a critical period for mitigating sensorimotor deficits, likely restricted to
703 specific prenatal ages.
704 The behavioral parameters assessed via the Suok test and Ledge tests have
705 largely been interpreted as anxiety-like behaviors and derivatives of sensorimotor
706 function. The anxiety-like interpretations are rooted in the classical approach-avoidance

707 theory (Montgomery, 1955; Montgomery and Monkman, 1955) and are supported by the
708 observed results of mice in the Suok test who have undertaken pharmacological
709 anxiogenics or anxiolytics or have undergone predator stress (Kalueff et al., 2007;
710 2008). The interpretations of sensorimotor function modalities assayed in the test are,
711 more simply, based on the parameters of the test that seemingly require both proper
712 sensory processing of the apparatus environment and the ultimate motor function
713 required to traverse this challenging apparatus.

714 However, it should be noted that like most behavioral assays, alternate
715 interpretations of the parameters scored are also apparent. For example, missteps or
716 falls may reflect pure motor or balance deficits rather than integration of sensorimotor
717 information, supported by the likely cerebellar dysfunction reported that results due to
718 PrEE (Maier et al., 1999; Thomas et al., 2010) which was not directly examined within
719 the current study. Additionally, the anxiety-like measures reported here (i.e. latency to
720 leave center, directed exploration) may also reflect motor deficits due to the necessity of
721 proper motor function to execute these phenomena. Similarly, rearing/grooming
722 behaviors have also been interpreted as either increased or decreased in anxiety-like
723 behavior. However, it is believed that low-stress grooming (indicative of low anxiety-like
724 behavior) typically takes place in a highly-stereotyped cephalocaudal order, whereas
725 stress-evoked grooming does not (Smolinksy et al., 2009). Within the current study, only
726 stereotyped-cephalocaudal grooming was quantified in the Suok test and thus was
727 interpreted as decreased anxiety-like behavior. Taken together, more in-depth testing is
728 required in the future to fully parse out the behavioral modalities both disrupted by PrEE
729 and potentially prevented by choline.

Lastly, we have included additional sex-separated behavioral analysis to determine the presence of sex-dependent variable effects of both PrEE and the ameliorative ability of choline. Largely, the behavioral trends observed in the sex-specific analyses closely mirror those seen in the combined dataset. However, it does appear that female mice may be both more sensitive to the potential increased anxiety-like behavior induced by PrEE, as well as to the ability of choline to prevent these behavioral abnormalities. This notion is supported by the finding that females display a significant decrease in directed exploration events and that choline supplementation prevents this phenomenon, which is not seen in the male-separated dataset, suggesting the observed effect in the combined pool may be largely driven by females. Interestingly, these female-driven observations support previous findings in preclinical choline FASD models examining other behavioral modalities (Thomas et al., 2007; Schneider and Thomas et al., 2016). However, others have found a greater ameliorative effect of choline on EtOH-disrupted motor function and balance in males (Bearer et al., 2015), suggesting that the sex-specific effects of PrEE and choline may be a highly complex process that relies on many different factors, including timing and dose of both choline supplementation and EtOH insult, as well as sex of offspring. Further studies need to address this concept in depth in order to develop a more effective intervention or treatment for the harmful consequences of PrEE. In summary, our findings extend the established ability of choline to mitigate PrEE-induced behavioral deficits to novel modalities that are relevant to clinical FASD.

4.3. Potential mechanisms

753 Choline influences neurodevelopment in a multitude of ways including acting as a
754 methyl-group donor in one-carbon metabolism (OCM), the cellular pathway that
755 generates methyl groups used for epigenetic modifications to DNA and histones
756 (reviewed in Zeisel, 2006). Here, we report global DNA hypomethylation in rostral and
757 caudal cortices of newborn EtOH mice, similar to previous reports (Özturk et al., 2017).
758 However, this effect was mitigated by choline supplementation, suggesting choline
759 supplementation may prevent epigenetic dysregulation due to PrEE as previously
760 reported (Otero et al., 2012; Bekdash et al., 2013). Since choline availability has been
761 shown to directly influence DNA and histone methylation levels and, thus, gene
762 expression within the developing brain (Mellot et al., 2007; Mehedint et al., 2010), the
763 observed amelioration in cortical DNA methylation could potentially underlie the
764 prevention of gene expression changes in PrEE neocortex observed here, however no
765 direct testing of this hypothesis took place in the current study. Together, prevention of
766 global epigenetic and subsequent genetic perturbations in PrEE neocortex by choline
767 constitutes an appealing hypothesis for how choline may prevent brain and behavioral
768 abnormalities associated with PrEE.

769 Despite the strong influence of choline availability on epigenetic regulation,
770 choline also functions as a precursor to both membrane lipids and the neurotransmitter
771 acetylcholine. Indeed, previous reports have shown supplementation can prevent PrEE-
772 induced dysregulation in both of these systems (Monk et al., 2012; Tang et al., 2014),
773 suggesting choline may exert its beneficial effects against PrEE insult via multiple
774 mechanistic pathways. Additionally, it must be made clear that choline supplementation
775 in the current report and in other studies (Thomas et al., 2004a; Hunt et al., 2014) does

not reverse all adverse PrEE effects to control levels. Furthermore, although prenatal choline availability by itself has been reported to affect DNA methylation (Kovacheva et al., 2007) and gene expression (Mehedint et al., 2010) within the brain, we do not report similar differences here. Even though our significant levels for DNA methylation and gene expression results are robust with low measured variability, we recognize that small samples size can limit the strength of conclusions drawn from the data. Thus, further work must be done to determine the precise mechanisms by which choline has its effects to develop the most effective preventative and/or therapeutic treatment for FASD.

5. CONCLUSIONS

In summary, prenatal choline supplementation, when administered at the time of prenatal ethanol exposure, ameliorates abnormal brain and behavioral development. Specifically, the benefits of co-administration of choline during ethanol exposure are recovery of abnormal brain and body size and partial prevention of aberrant INC connectivity at birth, as well as the rescue of atypical *RZRβ* and *Id2* gene expression patterns and deficits in sensorimotor and anxiety-like behaviors at P20. The prevention of PrEE-induced global cortical DNA hypomethylation, suggests choline may be acting through canonical epigenetic pathways to achieve these protective effects. These findings suggest that choline supplementation offers significant protection from PrEE associated fetal growth abnormalities, and that optimal management of methyl group donors during pregnancy may be an effective way to reduce the extent of PrEE-based toxicity in high-risk individuals.

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FIGURE LEGENDS

Figure 1. *Dam measures*. No differences in food (**A**) or liquid (**B**) intake are present among groups (n = 8, all groups). **C,** EtOH (n = 10) and CE dams (n = 10) show elevated blood ethanol content (BEC; mg/dL) at gestational day (GD) 9 and 19 compared to CW (n = 7) and Controls (n = 7) (****p < 0.0001). **D,** No differences are present among groups in plasma osmolality (mosm/kg) (n = 10, all groups). **E, F,** Dam weight gain and litter size are significantly decreased in EtOH dams compared to

Controls (*$p < 0.05$, $n = 8$, all groups), and EtOH litter size is significantly different from CE dam's average litter size (*$p < 0.05$).

Figure 2. *Pup measures at P0.* **A,** EtOH pups also display decreased body weights at P0 ($n = 24$) compared to controls ($n = 26$; **$p < 0.01$), CW ($n = 25$; **$p < 0.01$), and CE pups ($n = 23$; **$p < 0.01$). **B,** EtOH pups display decreased brain weights at P0 ($n = 15$) compared to controls (n = 17; **$p < 0.01$), CW (n = 13; **$p<0.01$), and CE pups (n = 10; **$p<0.01$). **C,** No differences are present in brain/body weight ratios among any groups.

Figure 3. *Cortical lengths at P0.* **A-D,** Representative dorsal views of whole brains of control (**A**), CW (**B**), EtOH (**C**), and CE (**D**) pups at P0. **E,** EtOH pups display decreased cortical lengths at P0 ($n= 10$) compared to controls ($n = 10$; **$p<0.01$), CW ($n = 10$; **$p<0.01$), and CE pups ($n = 10$; *$p<0.05$). Scale bar, 2 mm.

Figure 4. *Analysis of intraneocortical connections (INCs) at P0.* Rostral to caudal series of P0 100m coronal sections of control (**A1-E1**), CW (**A2-E2**), EtOH (**A3-E3**), and CE (**A4-E4**) brains that have been injected with DiI in putative primary visual cortex (VCx, *V in **D1-4**). EtOH brains display altered patterns of connections stemming from VCx injections, specifically red-labeled cells are present in far rostral sections (**A3, B3**, arrows) not present in control or CW brains. CE brains show labeled cells not present in control or CW brains (**B4**, arrow), but do not in far rostral sections as occurs in EtOH brains (**A4** vs. **A3**). Altered connectivity in EtOH and CE brains is present in absence of altered thalamocortical connectivity i.e. appropriate dorsal lateral geniculate nucleus

labeling is present in all groups (**D1-D4**, arrows). These overall patterns are clearly visualized in 2D flattened reconstructions of the neocortex in F1-4, where CE brains (**F4**) display altered INC connectivity compared to control and CW brains, but do not show the magnitude of cortical disorganization seen in EtOH brains (**F3**). Coronal hemispheres: Scale bar: 500μm (white). Images oriented dorsal (D) up and lateral (L) right. Reconstructions: red dots: labeled cells, red fills: injection locations; scale bar: 1mm (black). Images oriented dorsal (D) up and caudal (C) right.

Figure 5. *Quantitative analysis of visual INC development at P0.* **A,** Putative visual cortex (VCx) dye placement location (DPL) spread as a function of total cortical length. No differences are present among groups. **B,** VCx projection zones as a function of total cortical length. EtOH brains display increased projection zones (n = 6) compared to Control (n = 5, ****p < 0.0001) and CW (n = 5, ****p < 0.0001). CE brains also display altered projection zones (n = 6) compared to Control and CW brains, but are significantly reduced when compared to the EtOH group (**p < 0.01). **C,** Representative section of anatomical level of cell count in graph **D.** Region of interest (ROI), box denotes where cells were counted amongst sections/groups. Dorsal (D) up, lateral (L) left; Scale bar 500m. **D,** EtOH brains (n = 6) contain 25.33± 3.43 labeled cells from VCx injections in the ROI. No other groups contain labeled cells in any region at this level (**p < 0.01; Con, n = 5; CW, n = 5; CE, n = 6).

Figure 6. *Cortical expression of RZR and Id2 at P0. In situ* hybridizations in P0 100m coronal sections of hemispheres at the level of rostral parietal cortex. **A1-D1,** EtOH

brains display an abnormal medial shift in expression in *RZR* (**C1**, arrow), compared to Control (**A1**), CW (**B1**), and CE (**D1**) brains. **A2-D2,** EtOH brains show a medial shift of expression (**C2**, arrow) in the most superficial layer of *Id2* expression, which normally extend laterally in Controls, CW, and CE brains (**A2, B2, D2**, arrows). In both genes analyzed, Choline supplementation (CE) ameliorates altered expression to levels indistinguishable from controls. Images oriented dorsal (D) up, medial (M) left. Scale bar, 500 μm.

Figure 7. *Transcript density analysis of RZRβ and Id2 at P0.* **A1, B1,** Line drawings of the anatomical level in which static electronically-drawn regions of interest (ROIs) were placed on sections of binary-converted ISH experiments to quantify levels of mRNA expression. **A2,** EtOH brains display an increase in *RZRβ* transcript densities (n = 6) compared to Control (n = 6; ****p<0.0001), CW (n = 4; ****p<0.0001), and CE (n = 6; ****p<0.0001) in the ROI defined in A1. **B2,** EtOH brains display a significant decrease in *Id2* transcript densities (n = 5) compared to Control (n = 4; **p<0.01), CW (n = 4; **p<0.01), and CE (n = 4; ***p<0.005) in the ROI defined in B1. Scale bar, 500 μm.

Figure 8. *Global DNA methylation in rostral and caudal cortex at P0*. EtOH brains at P0 display significantly reduced %5-mC DNA (n = *5*) compared to controls (n = *5;* *p<0.05), and CW mice (n = *4;* **p < 0.01, ***p < 0.005) in both rostral and caudal cortex. CE brains show significantly increased %5-mC DNA (n = *3*) compared to EtOH (*p < 0.05, rostral cortex), and are not different from controls in rostral or caudal cortex.

Figure 9. *Behavioral measures at P20 in the Suok (**A-E**) and Ledge (**F**) tests*. EtOH animals (*n* = 15) display increased anxiety-like behavior as measured by latency to leave center (**A**), number of directed exploration events (**B**), and rearing/grooming events (**C**) compared to control mice (*n* = 18; **p* < 0.05). CE mice (*n* = 16) show decreased number of rearing/grooming events compared to controls but show significantly different directed exploration events compared to EtOH mice (***p* < 0.01) indicating partial amelioration of PrEE-induced anxiety-like behaviors. EtOH mice also display sensorimotor deficits as indicated by increased missteps (**D**) and falls (**E**) on the Suok test, and decreased time on the Ledge test (**F**) compared to controls (***p* < 0.01, ****p < 0.0001), CW (*n* = 14; ***p* < 0.01, *****p* < 0.0001) and CE (**p* < 0.05, *****p* < 0.0001) mice. In contrast, CE mice do not show any significant sensorimotor or motor deficits in any aforementioned measure compared to control or CW mice at P20.

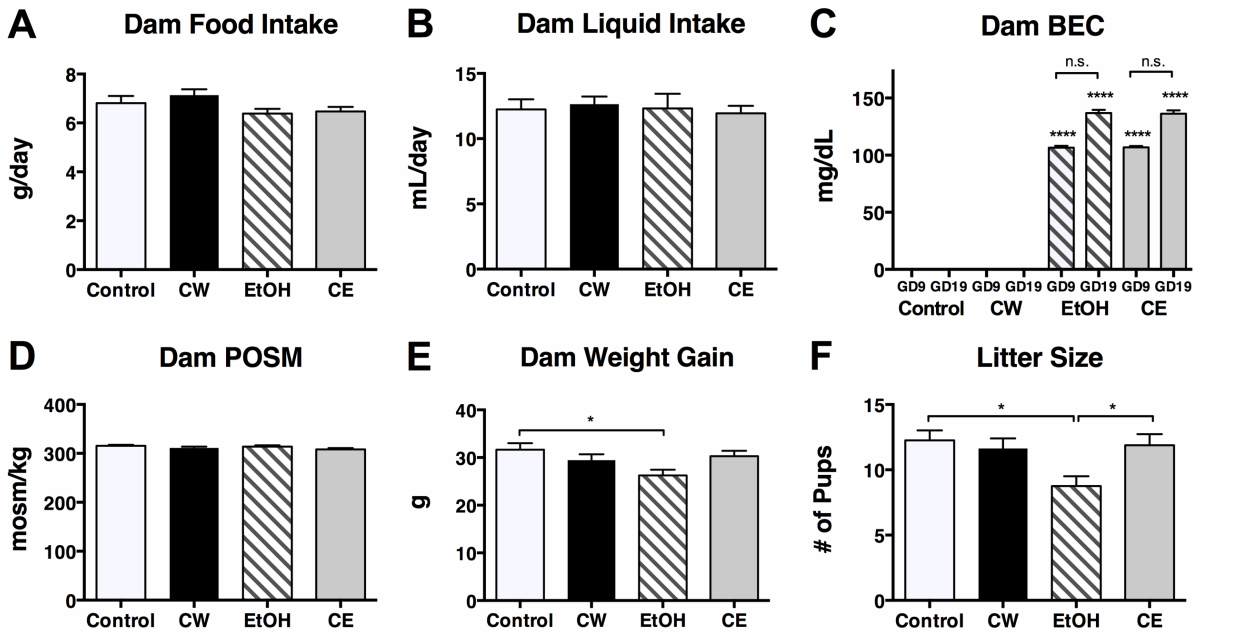
Figure 10. *Behavioral measures at P20- Females*. No significant differences present between groups in latency to leave center (**A**). EtOH female mice (*n* = 10) display significantly decreased directed exploration events (**B**) compared to control (*n* = 10; **p* < 0.05) CW (*n* = 7; **p* < 0.05) and CE (*n* = 7; **p* < 0.05) female mice. Rearing and grooming events are significantly decreased in both EtOH and CE females (**C**). EtOH females also display increased missteps (**D**) and falls (**E**) compared to control (**p* < 0.05, ***p* < 0.01). No differences are present on the Ledge test (**F**) for females only. CE females do not display any differences compared to controls in any 3 motor tests.

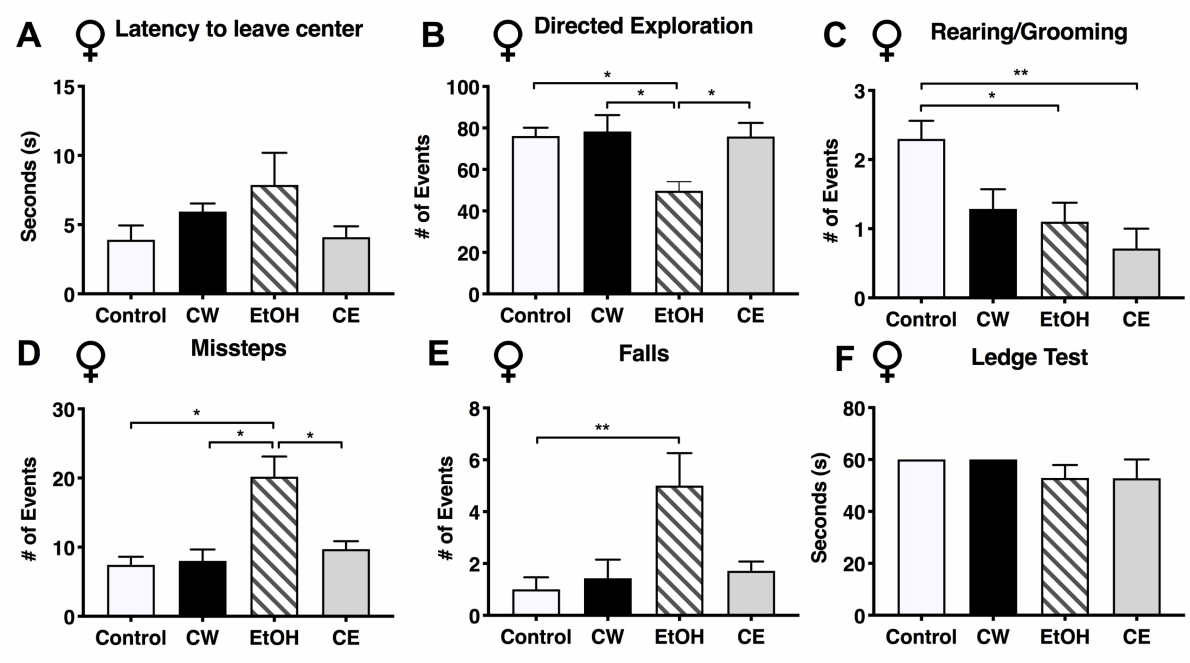
Figure 11. *Behavioral measures at P20- Males*. No significant differences are present in latency to leave center (**A**), directed exploration (**B**) or rearing/grooming events (**C**) among any group of males. EtOH males (n = 7) display increased missteps (**D**) and falls (**E**) compared to control (n = 8;*p < 0.05, **p < 0.01) and CW males (n = 7;*p < 0.05). No differences are present among any groups in Ledge test (**F**). CE males (n = 9) do not display any difference compared to controls in any measure tested.

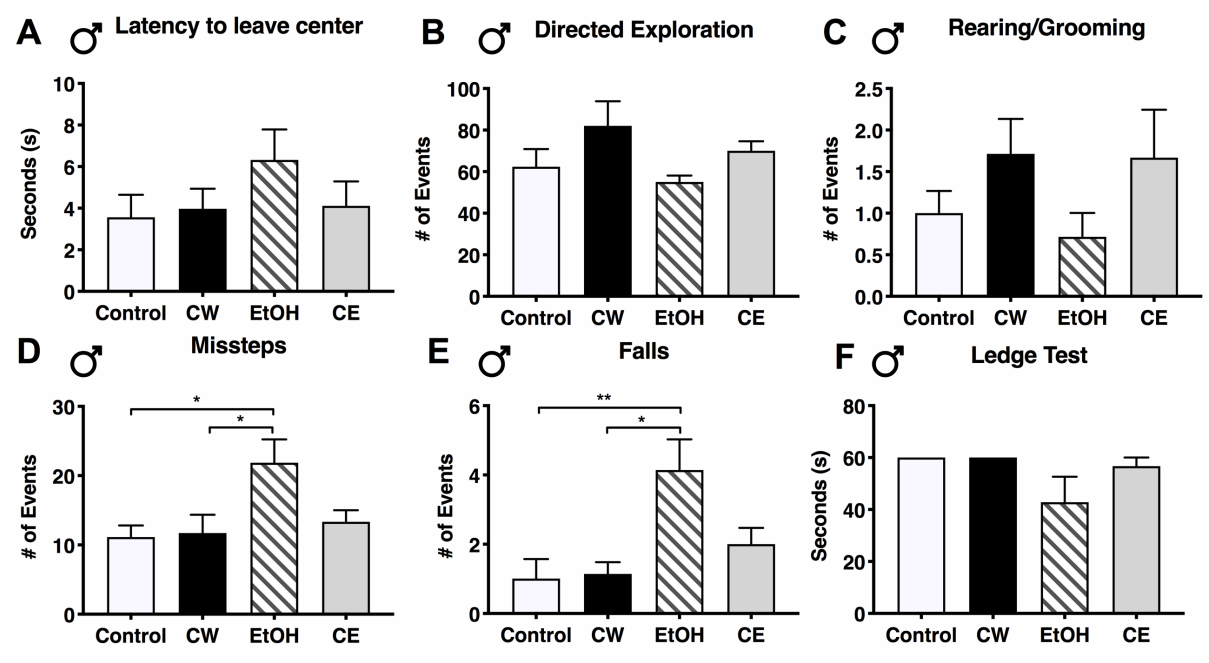
Measure (units)	**Control, *n***	**CW, *n***	**EtOH, *n***	**CE, *n***
Dam food intake (g/day)	6.812 ± 0.293, *8*	7.133 ± 0.242, *8*	6.381 ± 0.198, *8*	6.469 ± 0.188, *8*
Dam liquid intake (mL/day)	12.24 ± 0.776, *8*	12.64 ± 0.594, *8*	12.31 ± 1.133, *8*	11.94 ± 0.583, *8*
Dam GD9 BEC (mg/dL)	0.0 ± 0.0, *7*	0.0 ± 0.0, *5*	106.5 ± 1.693, *10*	106.9 ± 1.040, *10*
Dam GD19 BEC (mg/dL)	0.0 ± 0.0, *7*	0.0 ± 0.0, *5*	136.9 ± 2.794, *10*	136.3 ± 2.894, *10*
Dam POSM (mosm/kg)	315.4 ± 2.001, *10*	311.0 ± 2.813, *10*	313.9 ± 2.614, *10*	308.0 ± 2.801, *10*
Dam weight gain (g/day)	31.65 ± 1.362, *8*	29.46 ± 1.216, *8*	26.25 ± 1.212, *8*	30.29 ± 1.134, *8*
Litter size (# of pups)	12.25 ± 0.773, *8*	11.63 ± 0.778, *8*	8.750 ± 0.750, *8*	11.88 ± 0.854, *8*
P0 body weight (g)	1.615 ± 0.023, 26	1.628 ± 0.031, *25*	1.454 ± 0.024, *24*	1.543 ± 0.023, *23*
P0 brain weight (g)	0.09877 ± 0.002, 17	0.09838 ± 0.002, *13*	0.0894 ± 0.001, *15*	0.09834 ± 0.001, *10*
P0 brain/body weight ratio	0.05868 ± 0.001, 17	0.05730 ± 0.001, *13*	0.06090 ± 0.002, *15*	0.05925 ± 0.002, *10*
P0 cortical length (mm)	4.623 ± 0.020, *10*	4.612 ± 0.017, *10*	4.464 ± 0.037, *10*	4.573 ± 0.034, *10*
DPL spread (% total cortical length)	13.33 ± 0.960, *5*	12.82 ± 0.811, *5*	13.25 ± 0.428, *6*	13.33 ± 0.983, *6*
Dye projection zone (% total cortical length)	49.18 ± 1.461, *5*	49.61 ± 1.572, *5*	79.06 ± 1.920, *6*	68.23 ± 2.078, *6*
Frontal cortex cell count (# of cells)	0.0 ± 0.0, *5*	0.0 ± 0.0, *5*	25.33 ± 3.43, *6*	0.0 ± 0.0, *6*
RZRβ transcript density (% area fraction)	4.345 ± 0.851, *6*	2.629 ± 0.315, *4*	17.54 ± 1.548, *6*	3.844 ± 0.768, *6*
Id2 transcript density (% area fraction)	23.67 ± 3.634, *4*	24.59 ± 3.556, *4*	5.322 ± 1.628, *5*	26.85 ± 2.810, *4*
Global DNA methylation- rostral cortex (% 5-mC DNA)	0.761 ± 0.007, *5*	0.951 ± 0.094, *4*	0.570 ± 0.051, *5*	0.812 ± 0.033, *3*

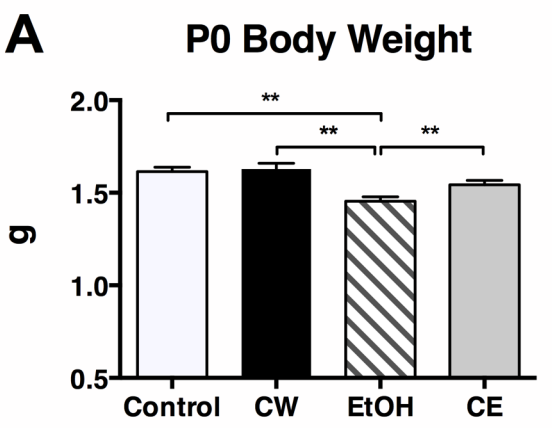
Global DNA methylation- caudal cortex (% 5-mC DNA)	0.734 ± 0.016, *5*	0.891 ± 0.073, *4*	0.515 ± 0.049, *5*	0.621 ± 0.074, *3*
Latency to leave center (s)	3.750 ± 0.732, *18*	4.899 ± 0.610, *14*	7.824 ± 1.366, *17*	4.103 ± 0.702, *16*
Directed exploration (# of events)	70.00 ± 4.567, *18*	80.14 ± 6.874, *14*	51.94 ± 2.895, *17*	72.56 ± 3.824, *16*
Rearing/grooming (# of events)	1.944 ± 0.189, *18*	1.929 ± 0.245, *14*	0.9412 ± 0.201, *17*	1.125 ± 0.221, *16*
Missteps (# of events)	9.176 ± 1.075, *18*	9.857 ± 1.586, *14*	22.35 ± 1.819, *17*	11.75 ± 1.135, *16*
Falls (# of events)	1.000 ± 0.352, *18*	1.533 ± 0.376, *14*	4.706 ± 0.803, *17*	1.875 ± 0.301, *16*
Ledge test score (s)	60.00 ± 0.000, *18*	60.00 ± 0.000, *14*	49.18 ± 4.829, *17*	54.99 ± 3.556, *16*

Supplementary Table 1 (above). Group means and number of replicates for all statistical measures. Data presented as mean ± SEM.





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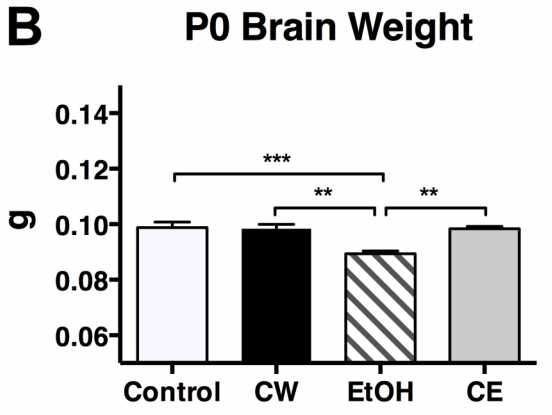
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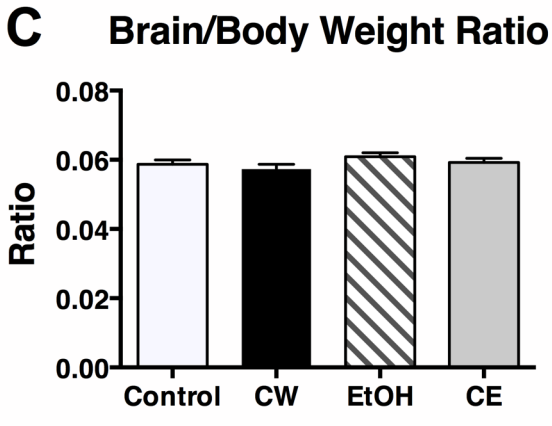
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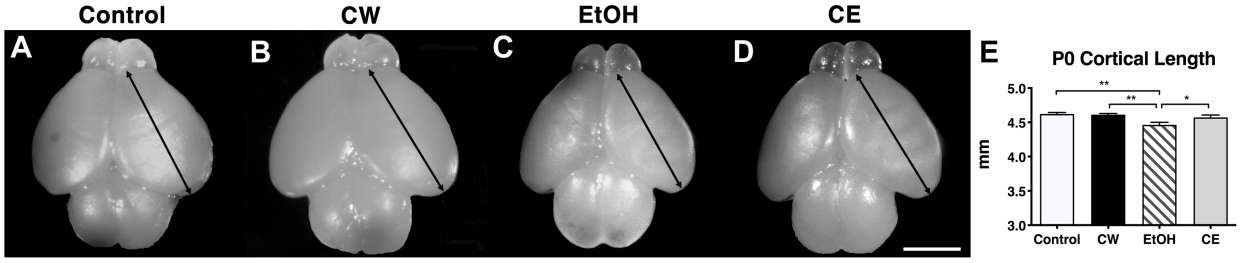

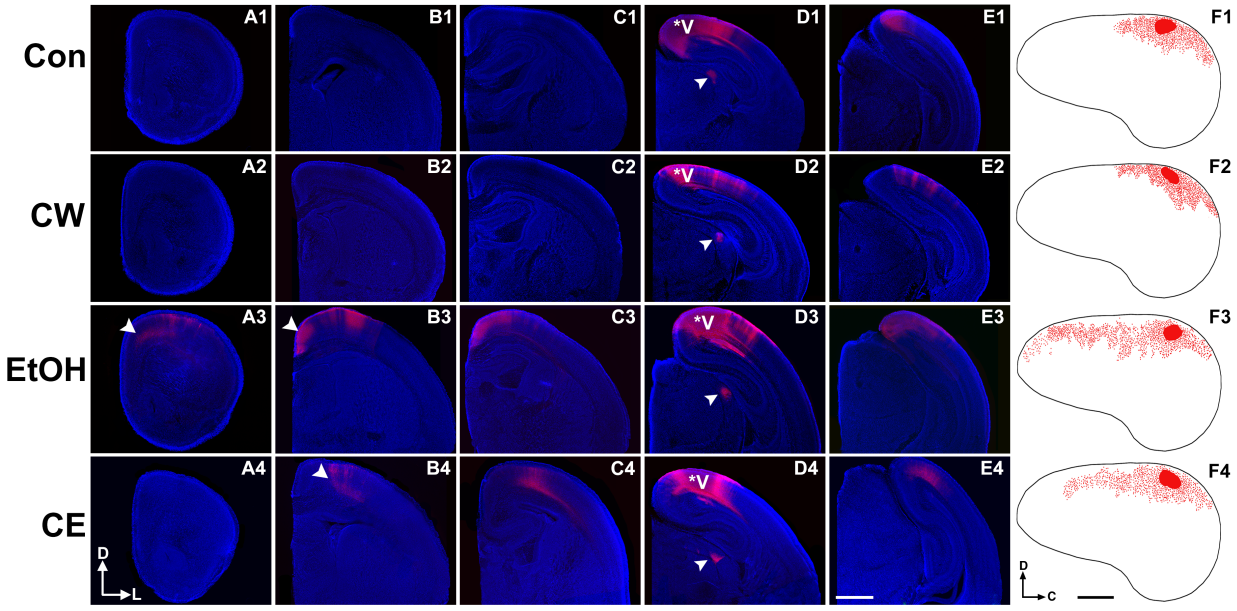

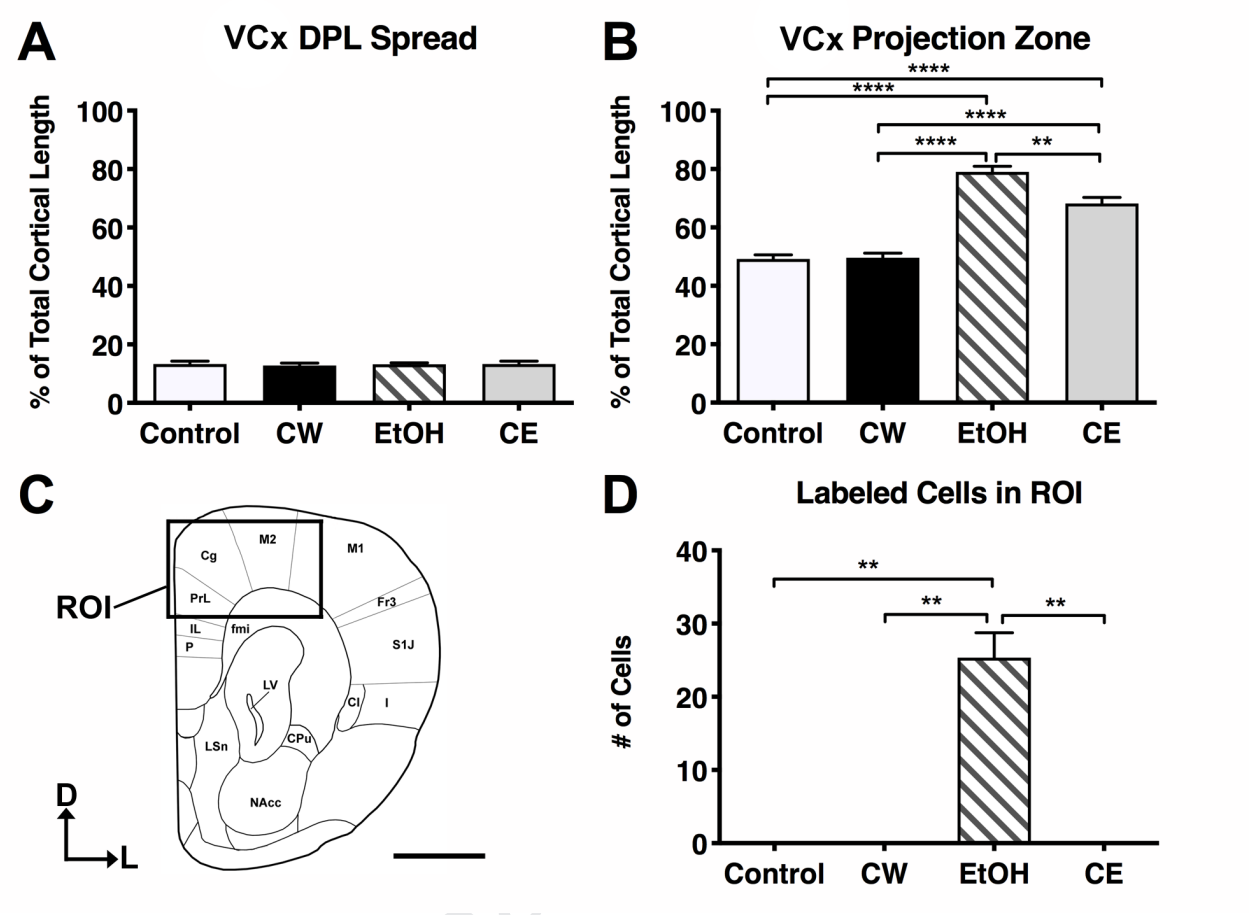
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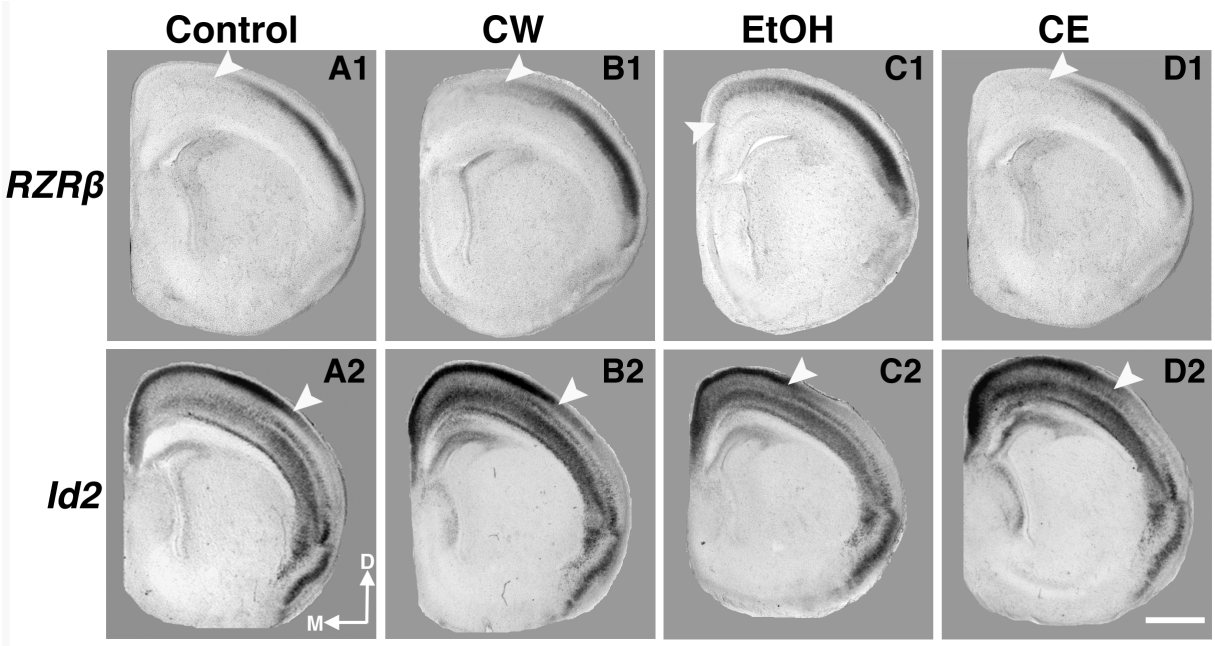

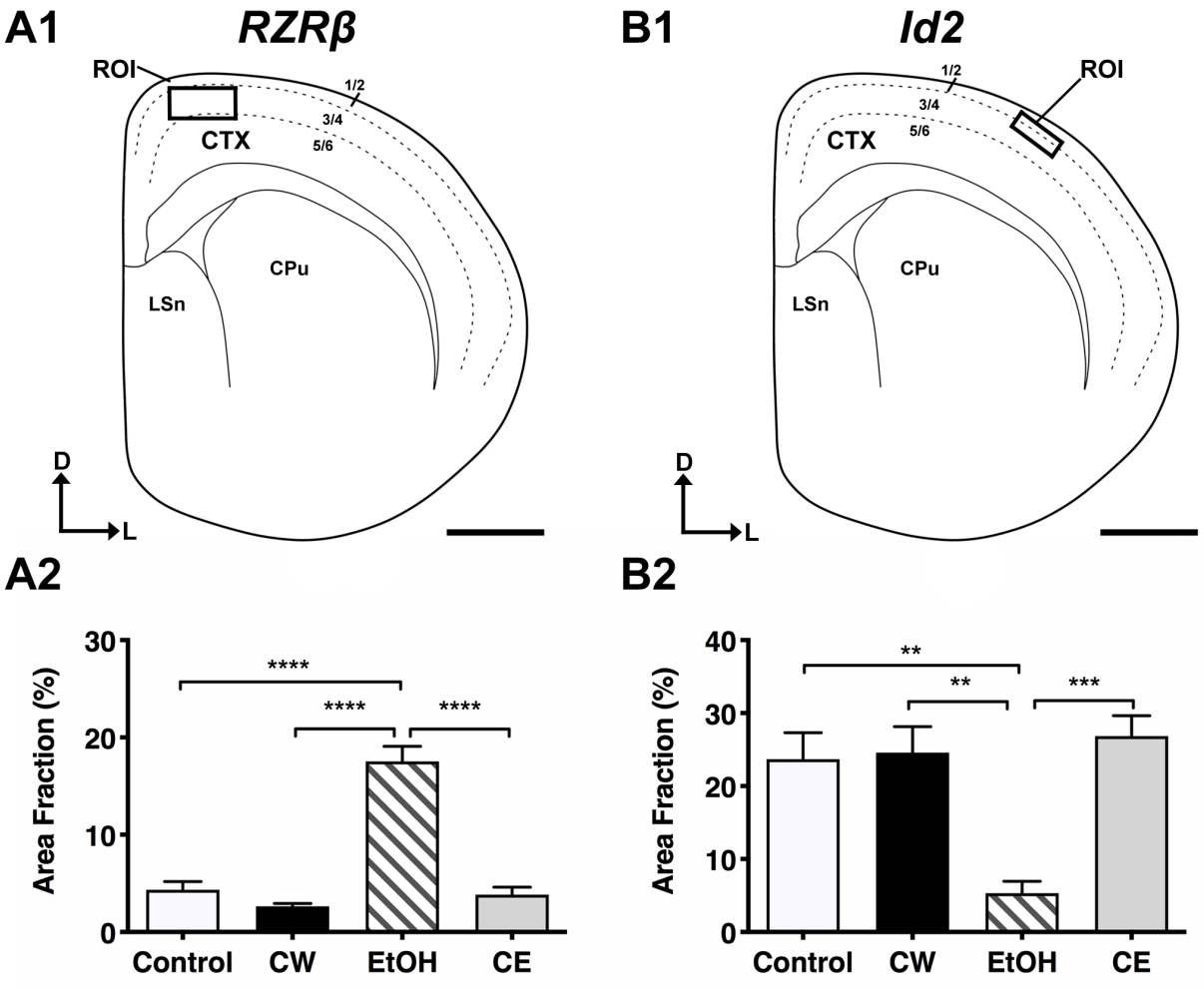
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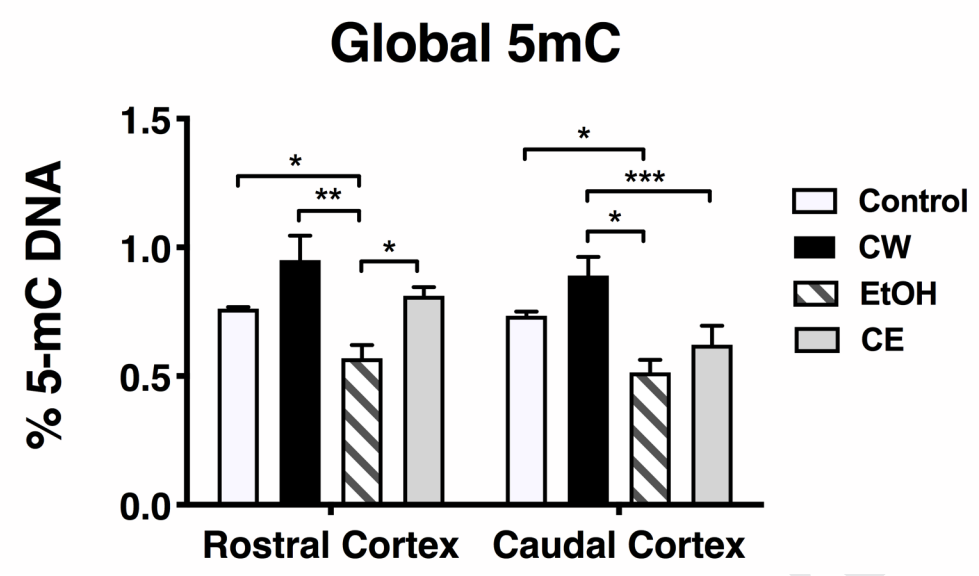

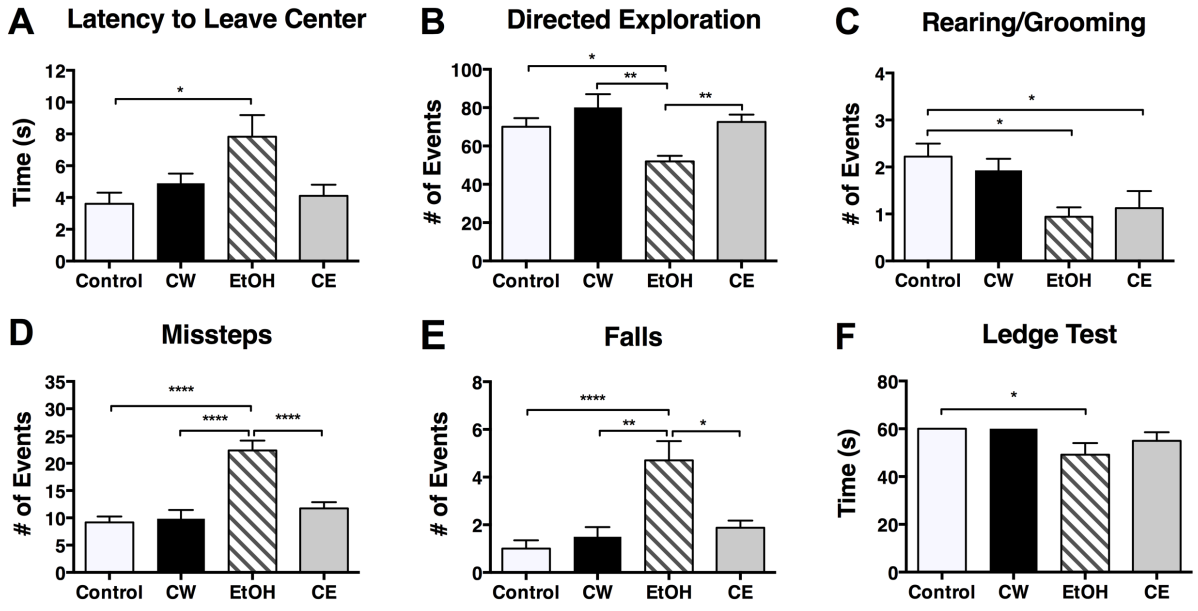

HIGHLIGHTS

- Choline supplementation protects against effects of prenatal ethanol exposure.
- Choline administered with ethanol in pregnancy may protect offspring from FASD.
- Choline ameliorates PrEE-related abnormal development of cortical connections.
- Choline inhibits PrEE-related development of sensorimotor deficits in mice.
- Choline prevents PrEE-related ectopic gene expression in murine neocortex.

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Conflict of Interest Statement.

All authors have read and approved the manuscript for submission to *Neuropharmacology*; have made a substantial contribution to the conception, design, gathering, analysis and/or interpretation of data and a contribution to the writing and intellectual content of the article; and acknowledge that they have exercised due care in ensuring the integrity of the work. None of the original material contained in the manuscript has been submitted for consideration nor will any of it be published elsewhere except in abstract form in connection with scientific meetings.

All authors are free of any conflict of interests.

Sincerely,

Kelly Huffman, Ph.D.