Published in final edited form as:

Alcohol Clin Exp Res. 2016 January ; 40(1): 122–133. doi:10.1111/acer.12936.

The impact of prenatal ethanol exposure on neuroanatomical and behavioral development in mice

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Abstract

Background—*In utero* alcohol, or ethanol, exposure produces developmental abnormalities in the brain of the fetus, which can result in lifelong behavioral abnormalities. Fetal alcohol spectrum disorders (FASD) is a term used to describe a range of adverse developmental conditions caused by ethanol exposure during gestation. Children diagnosed with FASD potentially exhibit a host of phenotypes including growth retardation, facial dysmorphology, central nervous system anomalies, abnormal behavior and cognitive deficits. Previous research suggests that abnormal gene expression and circuitry in the neocortex may underlie reported disabilities of learning, memory, and behavior resulting from early exposure to alcohol (El Shawa et al., 2013).

Methods—Here, we utilize a mouse model of FASD to examine effects of prenatal ethanol exposure, or PrEE, on brain anatomy in newborn (P0), weanling (P20) and early adult (P50) mice. We correlate abnormal cortical and subcortical anatomy with atypical behavior in adult P50 PrEE mice. In this model, experimental dams self-administered a 25% ethanol solution throughout gestation (gestational day, GD, 0 to 19, day of birth), generating the exposure to the offspring.

Results—Results from these experiments reveal long-term alterations to cortical anatomy, including atypical developmental cortical thinning, and abnormal subcortical development as a result of *in utero* ethanol exposure. Furthermore, offspring exposed to ethanol during the prenatal period performed poorly on behavioral tasks measuring sensorimotor integration and anxiety.

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Conclusions—Insight from this study will help provide new information on developmental trajectories of prenatal ethanol exposure and the biological etiologies of abnormal behavior in people diagnosed with FASD.

Keywords

prenatal alcohol exposure; brain development; neocortex; FASD; anatomy

Introduction

Fetal alcohol spectrum disorders (FASD) describe a continuum of deleterious effects on offspring from *in utero* ethanol exposure. Individuals with FASD often exhibit neurological, craniofacial, skeletal, cardiovascular and cognitive-behavioral deficits (Clarren et al., 1978; Murawski et al., 2015). Fetal Alcohol Syndrome, or FAS, represents the most severe form of FASD where the child is physically, cognitively and/or behaviorally impaired from prenatal ethanol exposure (PrEE). FAS epidemiological data show an incidence rate of 0.5–2.0 cases per 1,000 births (May and Gossage, 2001). However, rates of the *spectrum*, where all levels of exposure-related deficits are included, are believed to be much higher, as 18.6% of U.S. pregnant women age 35–44 reported drinking during pregnancy (Tan et al., 2015). According to the World Health Organization, PrEE is the leading cause of *preventable* mental retardation in the Western world. Despite this, the biological mechanisms that lead to altered brain development in offspring prenatally exposed to ethanol are not fully understood. Identification of brain regions susceptible to the effects of *in utero* ethanol exposure in humans and animal models provide critical insight that can be applied to future diagnoses and treatments in people with FASD.

The Collaborative Initiative on Fetal Alcohol Spectrum Disorders (CIFASD) has provided support for links between prenatal ethanol exposure and anatomical irregularities in the human brain. For example, PrEE-related reductions in brain volume have been identified using human brain imaging (Archibald et al., 2001; Norman et al., 2009; Lebel et al., 2011). MRI studies in humans have revealed abnormalities in corpus callosum, with high variability in structural form persisting into adulthood (Bookstein et al., 2002; 2007), reduced basal ganglia (Mattson et al., 1996) and left-right hippocampal asymmetry (Riikonen et al., 1999). The neocortex is differentially affected by PrEE (Riikonen et al., 1999; Archibald et al., 2001; Sowell et al., 2002) with cortical thinning reported in middle-frontal, inferior-occipital and paracingulate areas (Zhou et al., 2011), and thickening reported in inferior-parietal, anterior-frontal and orbital-frontal cortex of subjects exposed ethanol during gestation (Sowell, et al. 2002a; Sowell et al., 2002b; Yang et al., 2011). Abnormal hippocampal and cortical information processing may largely underlie reported disabilities of learning, memory, and behavior as result of early exposure to alcohol (Streissguth et al., 1994; Granato et. al., 2006, El Shawa et al., 2013).

Many experimental studies in animal models have also demonstrated the risk of maternal ethanol consumption during pregnancy. Drinking while pregnant significantly impacts the offspring's brain development, with the cerebral cortex being one of the key structures damaged by ethanol's teratogenicity. PrEE leads to varying levels of cortical dysfunction,

including defects in neuronal migration, changes in apoptosis or cell death, alterations in cortical thickness, callosal connectivity and selective reduction of GABA neurons (Miller 1993; Sampson et al., 1994; Ikonomidou et al., 2000; O'leary-Moore et al., 2011; Cao et al., 2014; Smiley et al., 2015). Furthermore, in our CD-1 mouse model of FASD, PrEE generated ectopic neocortical gene expression and disrupted targeting of intraneocortical connections (El Shawa et al., 2013). These atypical features of brain development were correlated with behavioral deficits around weaning. Other studies have identified non-cortical damage to the developing nervous system from PrEE. For example, West and colleagues described abnormal development in the hippocampus, cerebellum and corpus callosum following prenatal ethanol exposure in a rat model of FASD (Maier and West, 2001; Livy et al., 2003; Livy and Elberger, 2008).

In this study, we measure cortical thickness, cortical and subcortical structures in PrEE and control mice at three developmental timepoints in a novel mouse model of FASD: postnatal day (P)0 (newborn), P20 (weanling) and P50 (young adult). We predicted that moderate PrEE in the developing CD-1 mouse would result in abnormal region-specific neocortical thickness as well as altered cortical and sub-cortical structural development. Furthermore, we speculated that these PrEE-induced brain defects would persist across development, from birth to early adulthood. By assessing multiple locations throughout the brain at different stages of development we were able to map transient and persistent changes in the neuroanatomy of mice exposed to ethanol *in utero*.

Methods

Animal care

All experimental procedures conducted were approved by the University of California, Riverside Institutional Animal Care and Use Committee. Ten-week-old Female CD-1 mice were bred with non-sibling males, and housed alone during the entire gestational period following confirmation of a vaginal plug (gestational day, GD, 0.5). Dams were weight-matched, separated into ethanol-treated and control groups, and provided *ad libitum* access to standard mouse chow. A series of dam measures were taken to validate the exposure model; detailed methods for the measures are located in Supplemental Information. Upon birth, post-natal day (P) 0 male mice were weight-matched, euthanized (described in Supplemental Information) or cross-fostered with ethanol-naïve dams until age P20. P20 male weanlings were weight-matched, euthanized or weaned and raised to P50, when they were euthanized after behavioral testing.

Ethanol Administration

Ethanol administration in the CD-1 model was carried out as described previously (El Shawa et al., 2013). Dams received either 25% EtOH in water (experimental), or an isocaloric maltodextrin solution (control). Both solutions were available *ad libitum* alongside standard mouse chow for the entirety of gestation.

Tissue Processing

After euthanasia and perfusion, brains were collected and weighed at three ages (P0, P20, after behavioral experiments at P50) and cryoprotected in a sucrose solution for 1–3 days. Tissue was cryosectioned at 30 μ M in the coronal plane, mounted onto glass slides, stained for Nissl and imaged using a Zeiss Microscope and Zeiss Axiocamera.

Anatomical Measures

Gross dorsal views of P0, P20 and P50 control and PrEE brains were imaged using Zeiss microscopy. Regions of interest (ROI) in individual tissue sections were measured across all cases using an electronic micrometer in ImageJ by a trained researcher blind to the treatment. Cortical thickness measures in sections stained for Nissl substance were taken along a line perpendicular to the cortical sheet extending between the most superficial portion of layer I to the deepest portion of layer VI. These included prelimbic cortex (PL), frontal cortex (FC), primary auditory cortex (A1), primary somatosensory cortex (S1) and primary visual cortex (V1). ROIs were identified with the Paxinos atlas using specific cortical and subcortical landmarks such as the genu of the corpus callosum, the anterior commissure, the fimbria of hippocampus and the superior colliculus. Corpus callosum (CC) thickness was measured from the midline region. Measures of hippocampal subfield thickness were taken in CA3. Measures of basal ganglia (BG) were limited to caudate putamen, globus pallidus, and ventral palladium. Boundaries for dorsal lateral geniculate nucleus (dLGN) measures were taken using specific anatomical landmarks surrounding the dLGN, including: the intergeniculate leaf (IGL) for the ventral lateral border, the external medullary lamina (EM) for the ventral medial border, the lateral posterior nucleus (LP) for the dorsal border, and the brachium of the superior colliculus (BSC) for the dorsal lateral border of the dLGN. Basal Ganglia and dLGN volumes were calculated by drawing borders around the BG and dLGN in serial sections at a fixed magnification using ImageJ. Cell packing density within these ROIs was measured; these methods are located in Supplemental Information. Cortical thickness measures were as follows: P0 PrEE n = 15 and P0 Control n = 15; P20 PrEE n = 12 and P20 Control n = 12; P50 PrEE n = 12 and P50 Control n = 15. Subcortical measures were as follows: P0 PrEE n = 8 and P0 Control n = 8; P20 PrEE n = 8 and P20 Control n = 8; P50 PrEE n = 8 and Control n = 8). See Table 1 for a list of abbreviations.

Behavioral assays

The effects of PrEE on sensorimotor integration, balance and anxiety-like behaviors were measured at P50 using two behavioral assays: the Suok test and the platform test. These tests have been used previously in this PrEE mouse model (El Shawa et al., 2013); methods for these behavioral assays are located in Supplemental Information.

Statistical Analyses

A 2 $\times$ 3 ANOVA with Holm-Šídák post hoc analysis was used to establish significant differences between body and brain weight, cortical thickness and volume differences of PrEE and control mice. To achieve homogenous variation data were log (base 10) transformed before analysis. Student's t-test was used to analyze dam measures and

offspring behavior. For data displayed as percent change, mean baseline corrected control was set as 100%, with experimental measures expressed as percentage variation from that mean.

Results

Dam measures

Multiple dam measures were recorded to verify effects generated by our ethanol exposure paradigm (Figure 1). To ensure our exposure paradigm did not cause malnutrition or dehydration measures of blood plasma osmolality, food intake, liquid intake and dam weight were taken. No significant variation was detected in daily food consumption (Figure 1A; control 5.19 ± 0.17 g/day; EtOH-exposed 4.78 ± 0.20 g/day; $p = 0.16$), though a significant reduction in weight gain over the entirety of gestation was observed (Figure 1B; control 27.63 ± 0.96 g; EtOH-exposed 24.18 ± 1.17 g; $p < 0.05$). The reduced weight gain correlated with reduced litter size in EtOH-exposed dams (Figure 1C; control 11.25 ± 0.47 pups; EtOH-exposed 9.20 ± 0.60 pups; $p < 0.05$). Liquid intake (Figure 1D; control 6.48 ± 0.25 ml/day; EtOH-exposed 6.55 ± 0.20 ml/day; $p = 0.81$) and measures of blood plasma osmolality revealed no significant variance in hydration between treatments (Figure 1E; control 308.2 ± 1.77 mosm/kg; EtOH-exposed 309.2 ± 1.65 mosm/kg; $p = 0.68$).

Blood ethanol content was measured at two gestational timepoints: GD9 and GD19. EtOH-exposed dams had elevated BEC levels at both GD9 (Figure 1F; control 0.00 ± 0.0 mg EtOH/dl; EtOH-exposed 104.4 ± 1.21 mg EtOH/dl; $p < 0.001$) and GD19 (Figure 1F; control 0.00 ± 0.0 mg EtOH/dl; EtOH-exposed 135.2 ± 4.12 mg EtOH/dl; $p < 0.0001$) when compared to controls.

Body and brain weights of PrEE and control mice

Body weights were significantly reduced across both treatment [$F(1, 101) = 53.32$, $p < 0.0001$] and age [$F(2, 101) = 3230$, $p < 0.0001$]. Post hoc analysis indicated significant decreases in body weight across development in PrEE mice (Figure 2A: P0, control 1.794 ± 0.041 and PrEE 1.515 ± 0.029 , $p < 0.001$; P20, control 9.882 ± 0.428 and PrEE 7.953 ± 0.375 , $p < 0.0001$; P50, control 32.27 ± 1.446 and PrEE 25.04 ± 0.990 , $p < 0.0001$). Average daily growth rate from P0 to P20 was similar in control (9.35% per day) and PrEE animals (9.12% per day), while average growth rate from P20 to P50 was slightly accelerated in PrEE animals (4.01% per day) compared to controls (3.3% per day). Brain weight measurements of PrEE and control brains revealed significant effect across both treatment [$F(1, 101) = 94.57$, $p < 0.0001$] and age [$F(2, 101) = 3583$, $p < 0.0001$]. Post hoc analysis indicated that significant variation between control and PrEE mice was present at P0 (Figure 2B; control 0.104 ± 0.004 and PrEE 0.089 ± 0.001 ; $p < 0.0001$), P20 (Figure 2B; control 0.438 ± 0.018 and PrEE 0.377 ± 0.024 ; $p < 0.01$) and P50 (Figure 2B; control 0.590 ± 0.010 and PrEE 0.457 ± 0.014 ; $p < 0.0001$). Average daily growth rate of whole brain from P0 to P20 and P20 to P50 was consistent in control and PrEE animals (P0-20: control = 7.7% per day, PrEE = 8.4% per day; P20-P50: control = 1.3% per day, PrEE = 0.07% per day). These changes occur without altering brain/body weight ratios at any age (Figure 2C: P0, control 0.057 ± 0.002 and PrEE 0.060 ± 0.002 , $p = 0.73$; P20, control 0.033 ± 0.003 and PrEE 0.034

± 0.003 , $p = 0.99$; P50, control 0.019 ± 0.001 and PrEE 0.018 ± 0.001 , $p = 0.99$). Consistent with body and brain weight reductions, visual assessment of brain size across conditions and ages revealed that PrEE mice are born with smaller brains than age-matched controls, which persists into early adulthood (dorsal view images of brains shown in Figure 3).

Cortical thickness in PrEE and control mice

Frontal cortex—Significant thickening of frontal cortex was observed in PrEE mice across development [$F(2, 78) = 288.4$, $p < 0.0001$]. Post hoc analysis at P0 (Figure 4A1-A3, control $100 \pm 2.231\%$, PrEE $117.1 \pm 4.023\%$, $p < 0.001$), P20 (Figure 5A1-A3, control $100 \pm 3.305\%$, PrEE $116.7 \pm 2.254\%$, $p < 0.01$) and P50 (Figure 6A1-A3, control $100 \pm 3.869\%$, PrEE $108.8 \pm 3.134\%$, $p < 0.05$) revealed significant variance between control and PrEE animals.

Prelimbic cortex—Experimental animals exhibited an inverted U-shape developmental trajectory in prefrontal cortex that varied significantly across age [$F(2, 78) = 1575$, $p < 0.0001$]. Post hoc analysis revealed that PrEE induced significant thinning in PL at birth (Figure 4B1-B3, control $100 \pm 2.711\%$, PrEE $87.46 \pm 1.424\%$, $p < 0.05$). By P20 significant thickening in the PL was found in PrEE mice (Figure 5B1-B3, control $100 \pm 4.460\%$, PrEE $115.9 \pm 1.301\%$, $p < 0.05$), with the PL reverting to substantial thinning in experimental groups by P50 (Figure 6B1-B3, control $100 \pm 0.8254\%$, PrEE $87.80 \pm 1.094\%$, $p < 0.001$).

Somatosensory cortex—PrEE inhibited early developmental cortical thinning in somatosensory cortex at both P0 (Figure 4C1-C3, control $100 \pm 1.412\%$; PrEE $109.9 \pm 1.310\%$, $p < 0.05$), and P20 (Figure 5C1-C3, control $100 \pm 4.091\%$, PrEE $117.8 \pm 3.692\%$, $p < 0.01$). The absence of developmental thinning of S1 early on was reversed by early adulthood with PrEE generating significant thinning at P50 (Figure 6C1-C3, control $100 \pm 0.4849\%$, PrEE $95.29 \pm 2.144\%$, $p < 0.05$). An effect of PrEE was also detected across development [$F(2, 78) = 902.1$, $p < 0.0001$].

Auditory cortex—Significant variance in auditory cortex was detected across treatment [$F(1, 78) = 6.834$, $p < 0.05$] and age [$F(2, 78) = 614.0$, $p < 0.0001$]. Post hoc analysis indicated significant thinning of auditory cortex in P0 PrEE brains, compared to P0 control (Figure 4D1-D3, control $100 \pm 1.433\%$, PrEE $84.74 \pm 1.277\%$, $p < 0.0001$). At P20, however, PrEE induced significant thickening of auditory cortex (Figure 5D1-D3, control $100 \pm 3.910\%$, PrEE $121.4 \pm 5.567\%$, $p < 0.05$). Significant changes persisted into early adulthood, with PrEE brains having significantly thinner A1 (Figure 6D1-D3, control $100 \pm 1.260\%$, PrEE $91.18 \pm 2.018\%$, $p < 0.05$).

Visual cortex—Significant variance in visual cortex was detected across treatment [$F(1, 78) = 36.20$, $p < 0.05$] and age [$F(2, 78) = 3259$, $p < 0.0001$]. Visual cortex thickness was significantly increased in P0 PrEE brains (Figure 4E1-E3, control $100 \pm 0.8511\%$, PrEE $120.0 \pm 1.305\%$, $p < 0.0001$), when compared to controls. No significant variance persisted into later developmental time points (Figure 5E1-E3, $p = 0.21$; Figure 6E1-E3, $p = 0.21$).

Dorsal lateral geniculate nucleus

Significant variance in dLGN was detected across treatment [$F(1, 78) = 4.938, p < 0.05$] and age [$F(2, 78) = 13.08, p < 0.0001$]. Post hoc analysis of dLGN size revealed no significant change in P0 PrEE mice (Figure 7A1-A3, control $100 \pm 1.767\%$, PrEE $92.99 \pm 0.7395\%$; $p = 0.65$). A reduction in size of dLGN was present at P20 in PrEE mice (Figure 8A1-A3, control $100 \pm 3.567\%$, PrEE $84.99 \pm 1.673\%$; $p < 0.05$). Analysis of P50 PrEE and control dLGN revealed no differences in nuclear size (Figure 9A1-A3; $p = 0.70$).

Basal ganglia

The basal ganglia exhibited significant variance across treatment [$F(1, 78) = 10.38, p < 0.01$] and age [$F(2, 78) = 26.49, p < 0.0001$]. Post hoc analysis of newborn PrEE mice revealed reduced BG size (Figure 7B1-B3, control $100 \pm 1.894\%$, PrEE $88.38 \pm 1.501\%$; $p < 0.05$). Further analyses of BG in weanling and early adult mice revealed no persistent variance between treatments (Figure 8B1-B3; $p = 0.65$; Figure 9B1-B3, $p = 0.77$).

Hippocampus

The CA3 region of the hippocampus varied significantly across development [$F(2, 78) = 44.33, p < 0.0001$]. Post hoc analysis revealed significant thinning in P0 PrEE (Figure 7C1-C3, control $100 \pm 5.353\%$, PrEE $84.38 \pm 3.917\%$; $p < 0.05$) and P20 PrEE mice (Figure 8C1-C3, $p < 0.05$). By early adulthood a significant thickening of CA3 was observed in PrEE brains, compared to controls (Figure 9C1-C3, control $100 \pm 1.963\%$, PrEE $108.1 \pm 0.826\%$; $p < 0.05$).

Corpus Callosum

Significant differences in midline callosal thickness were observed between treatments [$F(1, 78) = 16.32, p < 0.001$] across early development [$F(2, 78) = 1135, p < 0.0001$]. Post hoc analysis indicated PrEE mice had a significantly thinner CC at P0 (Figure 7D1-D3, control $100 \pm 1.844\%$, PrEE $91.85 \pm 1.430\%$; $p < 0.0001$), and P20 (Figure 8D1-D3, control $100 \pm 2.347\%$, PrEE $92.20 \pm 1.049\%$; $p < 0.01$). However, CC thinning in P0 and P20 PrEE mice was no longer evident in young adult brains (P50). Instead, a statistically significant, dramatic increase in thickness was observed at P50 in PrEE mice as compared to controls (Figure 9D1-D3, $p < 0.05$).

Cell packing density

To determine if altered cortical thickness observed in PrEE mice was generated by varied total cell number or degree of compaction, cell nuclei packing density was measured at P0, P20 and P50 (table 2). Regional cell packing density in cortex did not significantly differ at any of the developmental timepoints measured. Thus, the varied region-specific volumetric changes in cortical thickness are not simply caused by a change in cell packing density.

Behavioral analyses

Performance on the Suok and platform apparatus was recorded at P50, revealing increased anxiety and reduced balance and sensorimotor integration in PrEE mice compared to age-matched controls. Balance, as defined by performance on the platform test, was significantly

different across treatments (Fig 10A, control 45.07 ± 5.056 s, PrEE 29.18 ± 5.538 s; $p < 0.05$). Falls per segment crossed, a measure of sensorimotor integration, revealed a pronounced deficit in PrEE mice when compared to controls (Figure 10B, control 0.015 ± 0.005 , PrEE 0.04 ± 0.007 ; $p < 0.01$). Rearing and stereotyped cephalo-caudal grooming was reduced significantly in PrEE animals indicating increased anxiety (Figure 10C, control 0.9375 ± 0.309 , PrEE 0.1250 ± 0.085 ; $p < 0.05$). Additional anxiety-like behaviors were detected in measures on the Suok apparatus including an increased latency to leave the central portion of the apparatus (Figure 10D, control 6.817 ± 1.471 s, PrEE 25.03 ± 7.114 s; $p < 0.05$), increased time spent immobile (Figure 10E, control 23.07 ± 4.947 s, PrEE 55.73 ± 11.04 s; $p < 0.05$) and reduced directed exploration (Fig 10F, control 100.1 ± 4.352 , PrEE 80.53 ± 6.187 ; $p < 0.05$).

Discussion

In this report we describe structural abnormalities in PrEE mice at postnatal days 0, 20 and 50, with behavioral analyses performed at P50. By detailing developmental changes in the neuroanatomy of PrEE mice, we gain a more complete understanding of ethanol's impact on the trajectory of cortical thinning, nuclear size and structural features during development. Furthermore, measuring behavioral dysfunction in the same animals at P50 suggests that these changes may underlie some commonly observed cognitive and behavioral impairments observed in children with FASD.

Effects of PrEE on offspring body and brain weights

In this study, we found that newborn PrEE mice had decreased body and brain weights when compared to controls, although brain to body weight ratio remained unchanged following prenatal ethanol exposure, suggesting that we are non-selectively inhibiting CNS development. Neuronal loss in the cortex, coupled with reports of specific loss of cerebellar and hippocampal neurons, and decreased white matter in the brain as a result of ethanol exposure during development, could account for the overall loss of brain mass observed in children diagnosed with FASD (Bauer-Moffett and Altman, 1977; Goodlett et al., 1990; Ikonomidou et al., 2000). In addition to our PrEE mice displaying lower brain weights throughout development when compared to their control counterparts, ethanol exposed mice also had decreased body weights at birth, an effect that was maintained through early adulthood (P50). Decreased body weight across development in our mouse model is consistent with outcomes observed in humans and rodents exposed to ethanol via maternal consumption during pregnancy (Margret et al., 2005; Chappell et al., 2007; May et al., 2014). Interestingly, however, despite this effect average growth rates were marginally accelerated in weanling (P20) and young adult (P50) PrEE animals when compared to controls. This time period is considered a sensitive developmental time-period, as the body and brain undergo considerable maturation. Although a growth spurt may represent a form of recovery from P20-P50 in PrEE mice, weight increases outside of normal programmed growth rates may have long-term adverse consequences. Perhaps the offspring are compensating for low body weights by increasing caloric intake. This increased rate of weight gain when compared to controls may negatively impact programmed developmental mechanisms and cause unwanted consequences related to obesity later in life.

PrEE and cortical thickness

Prenatal ethanol exposure disrupts the normal cortical developmental trajectory, beginning with a significant thickening of FC, S1 and V1 and thinning of PL and A1 at P0. In control P20 mice, developmental cortical thinning is usually observed across cortical areas; however, in the PrEE mice, all cortical areas except V1 are thicker than controls, suggesting a delay in area maturation. By P50, PrEE PL, S1 and A1 exhibit distinct thinning, while FC shows thickening and V1 show no observable changes in thickness compared to controls. The observation of reduced developmental cortical thinning during a time related to ‘middle childhood’ in children and a maintained perturbation during early adulthood in our model is reminiscent of variation seen in humans with FASD. For example, these changes mark an alteration to an essential developmental process thought to be broadly driven by arealization, and in particular, the refinement of neuronal networks, white matter myelination and synaptic pruning (Sowell et al., 2004; Treit et al., 2014). The process of cortical thinning, beginning in early development and carrying through to adulthood, proceeds at varying rates dependent upon brain region, with frontal and prefrontal regions thinning at lower rates in humans (Tamnes et al., 2010). The persistence of thickened FC in PrEE mice throughout early development is stereotaxically consistent with dysregulated intraneocortical connections previously reported by our laboratory (El Shawa et al., 2013). The failure to prune these aberrant connections may be involved in delayed cortical thinning, with these alterations potentially underlying the impaired coordination and increased anxiety we observed in P50 PrEE mice. Further evidence demonstrating the dysfunctional impact of inhibited FC thinning can be found in human studies which have demonstrated that FC thickness during development is increased in individuals with both attention-deficit/hyperactivity disorder and FASD when compared to those suffering from FASD alone, a finding they attribute to immature brain development (Fernández-Jaén et al., 2011).

Prenatal ethanol exposure alters the development of sensory areas in the neocortex and thus impacts sensory processing. One area altered by PrEE is the V1 cortical retinotopic map, which is the recipient of projections from the dLGN. Studies show a lack of connectional and electrophysiological refinement in response to prenatal ethanol exposure in V1 (Lantz et al., 2014). Somatosensory and auditory information show similar multi-leveled processing deficits ranging from thalamic defects to altered cortical pyramidal cell morphology and function (Mooney et al., 2010; Church et al., 2012; De Giorgio and Granato, 2015). Taken together, these findings suggest that impaired higher-order cortical processing and integration of auditory, somatosensory and visual information may contribute to balance and motor control impairments in our mouse model of FASD.

Cortical thickness is influenced by a number of factors including cytoarchitecture, organization and overall cell number (Dunty et al., 2001; Miller, 1988). Analysis of cell packing density in the present study revealed no significant change amongst treatments. This suggests that the maintenance of cortical thickness in PrEE animals occurs independently of cell packing density of specific cortical populations. Instead, it is probable that these changes occur at least partially through a comprehensive change in cell number, possibly generated via up/down regulation of migration or proliferation pathways, which maintain cortical cell packing density despite altered cortical thickness.

Extra-Neocortical brain structures altered by PrEE

Commensurate with PrEE induced changes in brain size and cortical thickness, widespread extra-neocortical abnormalities have been detected in both mice exposed to ethanol during gestation and children with FASD, with neuroimaging studies revealing the largest variation residing in BG, CA3 regions of the hippocampus and the CC (Derauf et al., 2009; Norman et al., 2009; Godin et al., 2011). As such, these regions, along with the dLGN, were investigated from birth to early adulthood.

dLGN—Our data demonstrate a reduction in the size of dLGN in P20 PrEE mice when compared to controls; however, this effect is not present at either P0 or P50. We believe the observed change is two-fold: reduction of the dLGN in the PrEE animals may result from altered expression of *ephrin A5* in the nucleus, as its development is linked to the expression (Dye et al., 2012). We also suggest that PrEE, in addition to disrupting other developmental programs, induces a developmental delay.

Basal Ganglia—We initially observed decreased volume of the PrEE BG at P0, with recovery by P20, which persisted into early adulthood. While measures of BG volume typically reveal an overall reduction, these changes are commonly measured only during early postnatal and adolescent periods (Mattson et al., 1996; Godin et al., 2011). Our data demonstrates an increase in both anxiety-like behavior and deficits in motor function in PrEE mice at P50, behaviors associated with BG function, without persistent anatomical variation of BG size across PrEE and control mice at this age. Perturbations in early development may be sufficient for the generation of long-lasting behavioral consequences, even in the absence of persistent anatomical change.

Hippocampus—Clinical investigations have established a distinct association between gestational ethanol consumption and learning and memory deficits in exposed offspring (Willford et al., 2004). Owing to this vulnerability even moderate levels of ethanol exposure during development are sufficient to reduce hippocampal CA1 and CA3 pyramidal cell number, and alter dendritic morphology. Furthermore, timing of exposure has been demonstrated to play a role in both the persistence, and magnitude of effect (Maier and West, 2001; Livy et al., 2003). In the present report, measurement of the CA3 region of hippocampus revealed an initial reduction in CA3 thickness at birth in PrEE mice. However, by weaning and persisting to early adulthood, a significant thickening was observed in PrEE mice compared to controls. It is likely that the early reduction detected at P0 results from a combination of factors including reduced neurogenesis, synaptogenesis and decreased cell and spine density (Tarelo-Acuna et al., 2000; Livy et al., 2003; Cullen et al., 2014). Later recovery, and thickening observed in CA3 suggests the possibility of a preliminary period of a reversible neurodegenerative process, such as cell swelling due to the acute toxic effect of ethanol, which could become exacerbated following cessation of ethanol administration to the dams (Paula-Barbosa et al., 1993).

Corpus Callosum—Midline callosal hypoplasia was detected in both P0 and P20 PrEE mice, consistent with previous studies in both human and mouse that demonstrate an association between prenatal ethanol exposure and shape abnormalities with volumetric

changes in the CC (Livy and Elberger, 2008; Gautam et al., 2014). Our results show an initial thinning of the CC at birth, lasting through P20, due to PrEE. However, by P50, we observe a recovery of the phenotype that led to increased thickness of the CC in early adulthood. These results have been observed in humans with FASD, and may be associated with deficits in executive function (Bookstein et al., 2002).

PrEE impacts behavior in Early Adulthood

Sensory/motor cortical and extra-neocortical development is disrupted by PrEE with effects on behavior lasting into early adulthood. These results corroborate similar findings reported previously in P20 PrEE mice (El Shawa et al., 2013). Furthermore, our results are consistent with cognitive and behavioral deficits described in children with FASD (Vernescu et al., 2012; Jirikowic et al., 2013).

Some anatomical measures show recovery of PrEE-related phenotypes by adulthood at P50 in our model (for example, the BG, dLGN, V1) without rescue of behavioral effects. We suggest that ectopic connectivity, as shown in our previous report (El Shawa et al., 2013), persists into adulthood. Despite a recovery in volume of some structures, a persistence of abnormal connectivity could account for the maintenance of the behavioral phenotype.

In summary, we report alterations to developmental cortical thinning across functionally diverse regions of cortex, and altered development of extra-neocortical structures, with correlative behavioral deficits in early adulthood. The observed group of PrEE-induced alterations is consistent with documented human patterns of birth defects present at early developmental stages. By extending our findings into childhood and early adulthood, we identify possible markers, which if investigated using non-invasive methods, such as MRI, could prove informative for clinical diagnosis of individuals with FASD.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

The authors would like to thank Hani El Shawa for his role in the early development of the mouse model.

Support: NIAAA 1R03AA021545-01 to K.J.H.

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Figure 1. Measurements in ethanol (EtOH)-exposed and control dams

A, No significant differences were detected in average dam food intake. (EtOH-exposed, N=24; control, N=26). **B**, EtOH-exposed dams (N=12) gained significantly less weight during gestation when compared with controls (N=14; * $p < 0.05$). **C**, Gestational exposure to EtOH resulted in significantly reduced litter size in experimental cases (N=11) when compared with controls (N=10; * $p < 0.05$). **D**, No significant difference was detected in average dam liquid intake (EtOH-exposed, N=24; control, N=26). **E**, No significant difference was detected in average dam plasma osmolality (EtOH-exposed, N=15; control, N=15). **F**, EtOH-exposed dams (GD9 N=11, GD19 N=12) had elevated BEC levels compared to untreated controls (GD9 N=5, GD19 N=7; * ** $p < 0.001$, * *** $p < 0.0001$).

Figure 2. Body and brain weight measures

A, Average body weight was significantly reduced in PrEE animals at all timepoints (P0, Control: n=20, PrEE: n=20, $p<0.001$; P20, Control: n=17, PrEE: n=17 $p<0.0001$; P50, Control: n=17, PrEE: n=17, $p<0.0001$). **B**, A significant reduction in average brain weight was observed in PrEE mice at all timepoints (P0, Control: n=20, PrEE: n=20, $p<0.0001$; P20, Control: n=17, PrEE: n=17, $p<0.01$; P50, Control: n=17, PrEE: n=17, $p<0.0001$). **C**, Brain/body weight ratios were not significantly altered between treatments at any age.

Figure 3. Brain size at P0, P20 and P50

Dorsal images of control and PrEE mice brains. PrEE brains are reduced in size when compared to age-matched controls. Scale bar = 2 mm.

Figure 4. Cortical thickness measures in P0 PrEE and control mice

Coronal sections in control (top row) and PrEE mice (middle row). Arrows indicate area of measure. A significant reduction was detected in prelimbic (B3; $p < 0.05$) and auditory cortices (D3; $p < 0.0001$). PrEE mice exhibited significantly thicker dorsal frontal (A3; $p < 0.001$), somatosensory (C3; $p < 0.05$) and visual cortices (E; $p < 0.0001$). Data is expressed as mean percent of control $\pm$ S.E.M. Dorsal (D) up, lateral (L) to the right. Scale bars = 500 μ M. Control: $n=15$, PrEE: $n=15$.

Figure 5. Cortical thickness measures in P20 PrEE and control mice

Coronal sections of representative control (top row) and PrEE (middle row) tissue. Arrows indicate area of measure. PrEE mice exhibited significantly thicker frontal (A3; $p < 0.01$), prelimbic (B3; $p < 0.05$), somatosensory (C3; $p < 0.01$) and auditory cortices (D3; $p < 0.05$). No significant variation was detected in visual cortex of PrEE animals (E3; $P = 0.19$). Data is expressed as mean percent of control $\pm$ S.E.M. Dorsal (D) up, lateral (L) to the right. Scale bar = 500 μ M. Control: $n=12$, PrEE: $n=12$.

Figure 6. Cortical thickness measures in P50 PrEE and control mice

Coronal view of representative sections in P50 control (top row) and PrEE mice (middle row). Arrows indicate area of measure. Significant thinning of PrEE cortical tissue was found in prelimbic (B3; $p < 0.001$), somatosensory (C3; $p < 0.05$) and auditory cortices (D3; $p < 0.05$). Significant variation was found in frontal (A3; $p < 0.05$), but not visual cortices (E3; $p = 0.13$) of PrEE animals. Data is expressed as mean percent of baseline corrected control $\pm$ S.E.M. Dorsal (D) up, lateral (L) to the right. Scale bar = 500 μ M. Control: $n=12$, PrEE: $n=12$

Figure 8. Anatomical volume and thickness measures in P20 PrEE and control mice

Representative coronal sections in control (top row) and PrEE mice (middle row). Outlines and arrows indicate area of measure. Significant reductions were detected in dLGN (A3; $p < 0.05$) and corpus callosum (D3; $p < 0.01$). CA3 pyramidal layer thickness was significantly increased in PrEE animals (C3; $p < 0.05$). PrEE basal ganglia (B3; $p = 0.11$) did not differ significantly from that of controls. Data is expressed as mean percent of baseline corrected control $\pm$ S.E.M. Dorsal (D) up, lateral (L) to the right. Scale bar = 500 μ M. Control: $n=8$, PrEE: $n=8$.

Figure 9. Anatomical volume and thickness measures in P50 PrEE and control mice

Representative coronal sections in P50 control (top row) and PrEE mice (middle row).

Outlines and arrows indicate area of measure. No significant variance was detected in dLGN (A3; $p = 0.09$) or basal ganglia (B3; $p = 0.54$). Pyramidal layer thickness increased within CA3 of PrEE animals (C3; $p < 0.05$). A significant increase of callosal thickness was detected at the midline of PrEE corpus callosum (D3; $p < 0.05$). Data is expressed as mean percent control $\pm$ S.E.M. Dorsal (D) up, lateral (L) to the right. Scale bar = 500 μ M. Control: $n=8$, PrEE: $n=8$.

Figure 10. Behavioral measures in young adult mice

In the platform test (A) PrEE mice exhibited a significant reduction in balance compared to control ($p < 0.05$). In the Suok test PrEE animals fell significantly more for each segment crossed, a measure of sensorimotor integration (B; $p < 0.01$). Anxiety-like behaviors including rearing/cephalo-caudal grooming (C), latency to leave central zone (D), time spent immobile (E) and directed exploration all indicated increased anxiety in PrEE mice ($p < 0.05$). Control: $n = 16$, PrEE: $n = 17$.

Table 1

Abbreviation	Structure
A1	Primary auditory cortex
BG	Basal ganglia
CA3	Cornu ammonis region 3, hippocampus
CC	Corpus callosum
dLGN	Dorsal lateral geniculate nucleus
FAS	Fetal alcohol syndrome
FASD	Fetal alcohol spectrum disorders
FC	Frontal cortex
P	Postnatal day
PL	Prelimbic cortex
PrEE	Prenatal ethanol exposure
ROI	Region of Interest
S1	Primary somatosensory cortex
V1	Primary visual cortex

Table 2

Cortical cell packing density in PrEE and control mice expressed in cells $\times 10^3/\text{mm}^2$ as mean $\pm$ s.e.m.

	PL		dFC		S1		A1		V1	
	Con	PrEE	Con	PrEE	Con	PrEE	Con	PrEE	Con	PrEE
P0	101.4 $\pm$ 3.1	100.5 $\pm$ 1.9	102.6 $\pm$ 3.8	100.6 $\pm$ 2.7	115.5 $\pm$ 1.1	111.7 $\pm$ 1.4	104.7 $\pm$ 2.4	103.1 $\pm$ 2.0	121.0 $\pm$ 1.8	119.6 $\pm$ 2.3
P20	87.8 $\pm$ 3.5	89.5 $\pm$ 0.9	89.8 $\pm$ 1.8	89.9 $\pm$ 2.7	94.0 $\pm$ 1.5	98.9 $\pm$ 3.2	91.1 $\pm$ 0.9	92.1 $\pm$ 3.0	95.3 $\pm$ 1.5	98.1 $\pm$ 3.8
P50	89.0 $\pm$ 0.2	86.5 $\pm$ 2.2	83.5 $\pm$ 0.8	88.1 $\pm$ 2.2	86.0 $\pm$ 0.4	86.6 $\pm$ 0.2	86.3 $\pm$ 1.2	87.7 $\pm$ 0.6	90.4 $\pm$ 1.6	93.0 $\pm$ 1.5

Note: PL, prelimbic cortex; dFC, dorsal frontal cortex; S1, primary somatosensory cortex; A1, primary auditory cortex; V1, primary visual cortex.