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The Enduring Impact of Neurulation Stage Alcohol Exposure: A Combined Behavioral and Structural Neuroimaging Study in Adult Male and Female C57BL/6J Mice

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Abstract

Prenatal alcohol exposure (PAE) can cause behavioral and brain alterations over the lifespan. In animal models, these effects can occur following PAE confined to critical developmental periods, equivalent to the third and fourth weeks of human gestation, before pregnancy is usually recognized. The current study focuses on PAE during early neurulation and examines the behavioral and brain structural consequences that appear in adulthood. On gestational day 8 C57BL/6J dams received two alcohol (2.8 g/kg, i.p), or vehicle, administrations, four hours apart. Male and female offspring were reared to adulthood and examined for performance on the elevated plus maze, rotarod, open field, Morris water maze, acoustic startle, social preference (i.e. three-chambered social approach test), and the hot plate. A subset of these mice was later evaluated using magnetic resonance imaging to detect changes in regional brain volumes and shapes. In males, PAE increased exploratory behaviors on the elevated plus maze and in the open field; these changes were associated with increased fractional anisotropy in the anterior commissure. In females, PAE reduced social preference and the startle response, and decreased cerebral cortex and brain stem volumes. Vehicle-treated females had larger pituitaries than did vehicle-treated males, but PAE attenuated this sex difference. In males, pituitary size correlated with open field activity, while in females, pituitary size correlated with social activity. These findings indicate that early neurulation PAE causes sex specific behavioral and brain changes in adulthood. Changes in the pituitary suggest that this structure is especially vulnerable to neurulation stage PAE.

Keywords

Fetal Alcohol Spectrum Disorder; Behavior; Mouse; Brain; Ethanol

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1. Introduction

When a fetus is exposed to alcohol, critical developmental events can be altered, leading to physical malformations and neurobehavioral symptoms identified as fetal alcohol spectrum disorder (FASD), which includes both fetal alcohol syndrome (FAS) and alcohol-related neurodevelopmental disorder (ARND). The expression of the consequences of fetal alcohol exposure depends, in part, on which developmental events are occurring when the fetus is exposed. Neurulation is the developmental period when the neural tube folds along its rostrocaudal axis, laying the foundation for continued growth of the central nervous system. The events of neurulation occur during the late third to fourth week of human gestation, before most pregnancies are recognized (Floyd et al., 1999). Alcohol exposures that occur during neurulation may have significant developmental consequences.

The behavioral symptoms of FASD are among the most pervasive symptoms, though there is considerable variability in their expression. Cognitive and learning impairments, hyperactivity, lack of behavioral control, and social difficulties are frequent among children prenatally exposed to alcohol (Streissguth et al., 1991, Mattson and Riley, 1998). For some of the FASD symptoms, exposure during early pregnancy is reported to be the most damaging, but the timing of exposure is often difficult to precisely identify in clinical studies. In this regard, the greater experimental control of animal models of PAE makes them essential to determining what kinds of PAE at which developmental stages are important for particular behavioral outcomes. However, no single laboratory animal model can completely mirror the complexity of FAS and FASD, as each model approximates only a fraction of the patterns of human alcohol exposure (Patten et al., 2014). The current model is designed to approximate binge-like alcohol exposure that occurs during neurulation (i.e. before pregnancy is recognized). Binge alcohol drinking is increasingly common, especially in young adults, and though most alcohol drinking stops once pregnancy is recognized, about 50% of pregnancies in North America are unplanned and therefore less likely to be identified early in gestation (Sedgh et al., 2014, Finer and Zolna, 2016).

In laboratory rodent models, neurulation takes place between gestational days eight and eleven (GD 8-10 in the mouse; GD 9-11 in the rat). Previous studies of neurulation alcohol-exposed C57BL/6J mice have revealed significant changes in the brains and craniofacial structures of near-term fetus following a single high dose binge-like exposure, or even when alcohol is consumed as part of a liquid diet. Alcohol drinking during neurulation increases the incidence of eye defects (Parnell et al., 2006), which may reflect concurrent brain abnormalities, as the brain and eyes are derived from the same progenitor cell populations. When measured directly, overall fetal brain size is reduced, but the relative sizes of some ventral midline structures, such as the hypothalamus/diencephalon, pituitary, cerebral ventricles, and the septal region are expanded (Parnell et al., 2009, O'leary-Moore et al., 2010, Parnell et al., 2013). Moreover, shape changes are noted in the cortex, hippocampus and striatum (Parnell et al., 2014, Parnell et al., 2013). Interestingly, by the time of late adolescence, the changes in brain volume become attenuated, though there remain significant shape changes in the cerebellum, corpus callosum, striatum, and anterior hypothalamus (Fish et al., 2016). These findings imply that developmental compensation occurs to allow neurulation alcohol-exposed brains to “catch-up” to their normal size, and

that some structures may persist to be structurally abnormal. The presence of behavioral alterations on tasks of motor coordination and exploration, social behavior, and water maze performance during adolescence further supports long-lasting CNS consequences of neurulation alcohol exposure (Fish et al., 2016). Other studies have also found behavioral differences in adolescent or adult rodents after acute neurulation stage PAE (Endres et al., 2005, Incerti et al., 2010, Minetti et al., 1996, Summers et al., 2008), depending on the task (Sadrian et al., 2014) and effects have been shown following repeated exposures throughout gastrulation and neurulation (Sanchez Vega et al., 2013, Mooney and Varlinskaya, 2011, Kelly and Tran, 1997). Similarly, exposure to valproic acid during neurulation have also been shown to produce behavioral changes later in life (Roullet et al., 2013). Together, these findings indicate that even transient disruptions in the events of neurulation can have lasting repercussions on behavior.

The current experiments are designed to test whether the brain and behavioral consequences of acute neurulation PAE observed during the previous study on adolescent mice (Fish et al. 2016) also extend into adulthood. Prior studies have demonstrated that the age of behavioral testing is an important variable in the expression of PAE (Wozniak et al., 2004, Mooney and Varlinskaya, 2011, Varlinskaya and Mooney, 2014, Wagner et al., 2014, Schambra et al., 2016), and it was hypothesized that the adult mice would have a different profile of behaviors affected by PAE than did the adolescent mice. Since male and female adolescents expressed different behavioral effects of neurulation PAE, male and female adult mice were also tested to determine if there were similar sex differences. Finally, magnetic resonance imaging (MRI) and diffusion tensor imaging (DTI) of a subset of brains were performed following behavioral testing to examine brain structure. Based on a prior study of adult behavior following gastrulation alcohol exposure, and the neurulation stage exposure adolescent study (Wieczorek et al., 2015, Fish et al., 2016), it was hypothesized that behavioral consequences of neurulation PAE would be observed on tests related to exploration and reactivity to novelty, but that performance of the water maze task would be relatively unaffected. Changes in brain volume were not predicted, based on the adolescent brain volumes, but if observed would likely be the result of continued adaptation and compensation to the original damage induced by neurulation PAE. However, it was hypothesized that changes in the shape of regions affected by neurulation PAE, the cerebellum, hypothalamus, striatum, and corpus callosum, would persist into adulthood.

2. Materials and Methods

2.1. Mice

Female C57BL/6J mice (n=14 treated dams) arrived from The Jackson Laboratory (Bar Harbor, ME) weighing about 20g, and were group-housed in standard polycarbonate cages. The cages were lined with cob bedding, contained cotton nesting material, and provided free access to rodent chow (Isopro RMH 3000; Purina, St. Louis, MO) and tap water. The vivarium was maintained at $21\pm1^{\circ}\text{C}$, 30-40% humidity, and kept on a 12:12 light:dark cycle with lights on at 07:00. Three hours into the light cycle, females were time-mated with male C57BL/6J mice for one to two hours; gestational day 0, 0 hr (GD 0) was the beginning of the breeding period when a copulatory plug was detected. At GD8, dams received two

intraperitoneal (i.p.) injections, 4 hrs apart, of 23.7% (v/v) ethyl alcohol (Pharmaco-Aaper, Brookfield, CT) in a lactated Ringer's solution at a dose of 2.8 g/kg, or the equivalent volume of the vehicle alone. Blood alcohol levels following this procedure peak at approximately 381 mg/dl, 30 min after the second alcohol injection (Parnell et al., 2009). While there are many alcohol exposure procedures that model different aspects of FASDs (e.g. Cudd, 2005, Kelly et al., 2009, Patten et al., 2014), exposure during critical early gestational periods is sufficient to cause many of the defects associated with FASD. Importantly, GD8 alcohol exposure mimics exposure that occurs during the third and fourth weeks of human pregnancy, before pregnancy is typically recognized. After alcohol treatment, the dams were disturbed only for cage changes. The date of birth was considered postnatal day 0 (PD0) and alcohol- and control- treated litters were left housed with their dams.

On PD3, pups were culled to a maximum of 8/litter, and were not handled until PD28, when two male and two female mice were weaned and housed in same sex pairs with a littermate. A total of 12 male (6 litters) and 14 female (7 litters) prenatal alcohol-exposed mice and 12 male (6 litters) and 12 female (6 litters) vehicle-exposed mice were tested for behavioral experiments beginning at PD70. All experiments were conducted following the guidelines of the National Institutes of Health using methods approved by the Institutional Animal Care and Use Committee of the University of North Carolina at Chapel Hill.

2.2. Procedures

All behavioral experiments were conducted in the UNC Behavioral Phenotyping Core during the light portion of the 12:12hr light:dark schedule and occurred every other weekday, except for the daily testing required during the Morris water maze. The mice were evaluated on the tests described below, in the order in which they appear, by an experimenter who was blinded to the treatment groups.

2.2.1. Elevated Plus Maze—Mice were placed in the center of a metal maze elevated 50 cm above the floor that contained two open arms (30 cm length, 220 lux) and two closed arms (20 cm high walls, 120 lux). The number of entries and time spent in each of the arms during the 5-min test were used to calculate the percent of open arm time $[(\text{open arm time} / \text{total arm time}) \times 100]$, the total arm entries, and the percent of open arm entries $[(\text{open arm entries} / \text{total arm entries}) \times 100]$.

2.2.2. Open Field—Mice were placed in the corner of an illuminated open field (41×41×30 cm, 120 lux at the edge, 155 lux in the center) that was housed within a sound-attenuated chamber and equipped with upper and lower grids of photobeams for the detection of both horizontal and vertical activity as well as the position of the mouse in the chamber (Versamax System: Accuscan Instruments, Columbus, OH). Activity was calculated every 5 min over the 60-min test. Distance travelled in the entire chamber and in the center of the chamber were analyzed as the primary dependent measures.

2.2.3. Rotarod—Mice were placed on a rotating barrel (3 cm) of a rotarod apparatus (Ugo-Basile, Stoelting Co., Wood Dale, IL) which progressively accelerated from 3 rpm to 30 rpm

during the maximum of a 5-min test. The rotarod was illuminated at 140 lux. The latency to fall off or rotate around the top of the barrel was recorded during three repeated trials on the first day of testing and two repeated trials on the second day of testing. Each trial was separated by about 45 s.

2.2.4. Social Preference Testing—Mice were placed in the center of a three-chambered Plexiglass box ($60 \times 42 \times 20$ cm, 60 lux) fitted with retractable doorways in the dividing walls. During an initial 10-min habituation phase, the doors were removed and the mice could freely explore the chambers. In the 10-min sociability phase, an unfamiliar same sex adult C57BL/6J mouse was placed in a Plexiglass protective cage ($17 \times 11 \times 18$ cm) in one of the side chambers while an empty protective cage was placed in the other side. In the final 10-min social novelty phase, a second unfamiliar conspecific was placed in the empty protective cage. Time spent in the immediate proximity (within 5 cm) of the stimulus cage and entries into each side chamber were recorded using image tracking (Ethovision, Noldus Information Technology, Wageningen, the Netherlands).

2.2.5. Acoustic Startle/Prepulse Inhibition—Mice were tested with the San Diego Instruments SR-Lab system consisting of a Plexiglass cylinder atop of a piezoelectric transducer housed in a sound attenuating chamber containing a houselight (80 lux), fan, and loudspeaker for the presentation of acoustic stimuli (background 70 dB). The transducer quantified vibrations caused by the mouse's movements in the cylinder. Following a 5-min habituation period, the test session had 42 trials (six repetitions of seven different types, randomly presently with an average inter-trial interval of 15 s ranging from 10-20 s): no-stimulus baseline trials; acoustic startle alone (40ms; 120dB); and prepulse trials (20ms; 74, 78, 82, 86, or 90dB) beginning 100ms before the startle stimulus. The startle amplitude for each trial was the peak response during a 65-ms sampling window from the onset of the startle stimulus.

2.2.6. Morris Water Maze—Mice were placed in one of four quadrants of a large circular pool (122 cm diameter, 125 lux at the edges, 155 lux in the center), filled with opaque water ($24\text{--}26^\circ\text{C}$), inside in a room with distinct visual cues. The pool contained an escape platform (12 cm diameter) in a fixed location submerged just beneath the surface of the water. Each mouse had 60 s to find the platform and received 4 trials per day for 5 days to learn the location of the platform. If the platform was not found in 60 s, the mouse was placed on the platform for 10 s and then given the next trial. Each trial was separated by about 30 s and began in a different quadrant in a randomized order. Twenty-four hours following acquisition testing, a 60-s probe trial was conducted with the platform removed. Movements of the mice were measured using video tracking (Ethovision, Noldus Information Technology, Wageningen, the Netherlands), calculating the latency to find the platform, swimming speed, and the durations of time spent near the wall or in the center of the pool. Latency to find the platform on each day of acquisition and the percent of time spent in each quadrant of the probe trial were the primary dependent variables.

2.2.7. Hot Plate—Mice were placed on a 55°C hot plate illuminated at 440 lux (IITC Life Science, Woodland Hills, CA) for a maximum of 30 s, while an observer recorded the latency for the mouse to lift or lick one of its hind paws from the surface.

2.2.8. Image Processing—On PD100, after the completion of all behavioral tests, six male and six females from each treatment group were randomly selected (n=1/litter), deeply anesthetized with tribromo-ethanol (250 mg/kg, i.p.) and perfused with heparinized physiological saline followed by 10% formalin containing the MR contrast agent ProHance (10:1 formalin:ProHance). The brains were post-fixed in formalin overnight at 4°C and rehydrated in 0.1M phosphate buffered saline with ProHance (100:1 PBS:ProHance) at 4°C until they were scanned on a 9.4T magnet at the Duke University Center for *In Vivo* Microscopy. The field of view was 22 mm × 11 mm × 11 mm, the TR was 100 ms, and the TE was 11.82 ms.

The data were processed using an in-house pipeline consisting of unbiased, atlas based, regional segmentation (Budin et al., 2013). The images were rigidly aligned (translation and rotation) (Johnson et al., 2007) to an external template for the C57BL/6 mouse (The C57 Brookhaven Atlas, Ma et al., 2005) and then skull-stripped using an atlas-based tissue classification method (Oguz et al., 2011). After skull-stripping, the images were deformably co-registered in a group-wise manner to compute an unbiased population average with diffeomorphic non-rigid registration using Advanced Normalization Tools (ANTs) (Avants et al., 2009). The C57 Brookhaven atlas (Ma et al., 2005) segmentation was then registered to the population average and then propagated to the individual subjects for region based analysis of DTI metrics and volume. Each subject's final segmentation was carefully checked by an anatomical expert for quality control.

2.3. Statistical Analysis

The percentage of open arm time, percentage of open arm entries, and total arm entries on the elevated plus maze as well as the hindpaw response latency on the hot plate tests were analyzed with two-way (sex by prenatal exposure) between-subjects analyses of variance (ANOVA). For all other measures three-way, mixed ANOVAs were performed with prenatal exposure and sex as the between- and time or trial as the within-subjects factors. The data from the rotarod trials 1-3 were analyzed separately from trials 4-5.

Region-based statistics were calculated for each subject and included volumes, means, and standard deviations of the intensity in the segmented regions or over the whole mask. Regions of interest (ROIs) used for this study include: cortex, cerebellum, brain stem, olfactory bulbs, thalamus, hippocampus, striatum, external capsule, midbrain, basal forebrain, amygdala, hypothalamus, superior colliculus, inferior colliculus, globus pallidus, central gray, fimbria, ventricles, pituitary, internal capsule, corpus callosum, and the anterior commissure. Brain volumes were analyzed using three-way, mixed (sex by prenatal exposure by brain region) ANOVAs. Significant F-tests were further analyzed with post-hoc Bonferroni comparisons with alpha levels set at $p < 0.05$. Pearson product-moment correlations evaluated the relationship between behaviors and brain regions affected by PAE,

using unadjusted p-values set at <0.05 . To minimize the number of possible correlation tests, we analyzed only those behaviors and brain regions that were most affected by PAE.

Surface-based shape analysis was performed using dense surface models (DSM) (Hammond et al., 2004, Hutton et al., 2003) on 3D extractions of the segmented regions. A series of sparse anatomical landmark points were manually annotated on each region by a single individual (MS), and used to align surfaces for the generation of a dense correspondence of points. DSM models were used to visualize mean regional shape morphology in PAE mice relative to the vehicle group, producing heat maps of normalized differences. Male and female mice were combined for each group given the small numbers available.

3. Results

3.1. Gross Morphology

There was no effect of PAE on post-natal body weights in either sex. The average body weights across test days were: 26.0 ± 0.5 and 25.9 ± 0.4 for vehicle- and alcohol-exposed males, respectively; and 20.5 ± 0.3 and 20.4 ± 0.1 for vehicle- and alcohol-exposed females, respectively. One prenatal alcohol-exposed male mouse was anophthalmic on the right side but showed no marked behavioral differences from the other prenatal alcohol-exposed mice.

3.2. Elevated Plus Maze and Open Field

There were significant interactions between sex and prenatal exposure on the % open arm time ($F_{1,46}=4.8$; $p=0.03$; Fig 1A and 1B), % of open arm entries ($F_{1,46}=4.2$; $p=0.047$; Fig 1A and 1B), and total entries ($F_{1,46}=6.9$; $p=0.01$; Fig 1A and 1B). Post-hoc analyses revealed that, as compared to vehicle-treated males, vehicle-treated females spent a greater percent of time on the open arms, made more total arm entries and made a greater proportion of these entries onto the open arms ($p<0.009$ for all comparisons). PAE increased the percent of time spent on the open arms in male mice ($p=0.03$) and normalized the sex differences on the total arm entries and the percent of open arm entries (Fig 1A and 1B). In the open field, there were significant sex by prenatal exposure by time bin interactions on total distance ($F_{11,506}=2.2$; $p=0.01$ Fig 1C and 1D) and center distance in the open field ($F_{11,506}=2.4$; $p=0.008$; Fig 1C and 1D). Post-hoc analysis revealed that in the male mice only, PAE increased total activity in the open field during the first 5 minutes of the test ($p=0.03$). Vehicle-treated female mice, as compared to vehicle-treated male mice, were more active in the center of the open field during the 1st and 4th 5-min time bins ($p<0.02$). PAE reversed this sex difference by significantly increasing the activity of the male mice in the center of the open field during the 1st, 2nd, and 4th 5-min time bins ($p<0.02$). PAE did not significantly affect female mice on the elevated plus maze (Fig 1B) or open field (Fig 1D).

3.3. Rotarod

The latency to fall off the rotarod apparatus increased over successive trials for all groups of mice (Table 1), but neither sex nor PAE significantly affected rotarod performance.

3.4. Social Preference

In the sociability test, there was a significant main effect of social stimulus ($F_{1,45}=52.7$; $p<0.001$; Fig 2A and Fig 2B) but no significant effects of sex or PAE. All groups of mice spent more time next to the social partner than they did the non-social stimulus. In the social novelty phase, there was a significant main effect of social side ($F_{1,45}=94.7$; $p<0.001$; Fig 2C and Fig 2D) such that all groups of mice spent more time with the novel mouse than they did with the familiar mouse. Furthermore, there was a significant interaction between sex and prenatal exposure on the time spent near either the familiar or novel mouse ($F_{1,45}=10.0$; $p=0.003$; Fig 2C and 2D). Subsequent post-hoc analysis revealed that, as compared to vehicle-treated male mice, vehicle-treated female mice were significantly more social. They spent more time with either social stimulus than did the males ($p=0.02$). PAE reduced the sociability of the female mice, as these females spent less time in proximity to either the novel partner or the familiar partner ($p=0.001$) than did the vehicle-treated females. Instead, PAE females spent significantly more time in the non-social, center chamber ($t_{23}=3.0$; $p=0.007$; $86.3\pm5.7s$ vs. $109.9\pm5.4s$ for vehicle and prenatal alcohol exposed females, respectively). There were no significant effects of PAE on social behavior in the male mice.

3.5. Acoustic Startle/Prepulse Inhibition

All mice reacted to the acoustic startle stimulus and demonstrated inhibition of startle responses with presentation of a prepulse, dependent on the decibel level (Fig 3). There was no significant sex by prenatal exposure by startle stimulus interaction. However, there was a significant prenatal exposure by startle stimulus interaction ($F_{6,270}=2.5$; $p=0.02$; Fig 3A and 3B), such that PAE reduced the amplitude of the startle response ($p=0.005$), as well as the startle that was preceded by a 74 dB prepulse ($p=0.02$). Since the effect of PAE on the startle response appeared to be caused by a large PAE effect in the female, but not the male mice, we further analyzed the startle response in males and females separately. Indeed, this analysis confirmed that, in the female, but not the male mice, there was a significant interaction between PAE and the startle stimulus ($F_{6,138}=2.9$; $p=0.011$; Fig 3B), such that PAE reduced the amplitude of the startle response ($p=0.006$), as well as the startle that was preceded by a 74 dB prepulse ($p=0.02$). PAE did not significantly affect prepulse inhibition.

3.6. Morris Water Maze

During the acquisition phase, all groups of mice found the location of the hidden platform (quadrant I) faster on the 5th day of testing than they did on the 1st day and there were no significant differences between males and females or between prenatal alcohol-exposed and vehicle-exposed mice (Table 2). During the probe test, all groups of mice spent more time in quadrant I than they did in at least one other quadrant and there were no significant effects of PAE (Table 3).

3.7. Hot Plate

There was a significant main effect of sex on the latency to lift or lick the hindpaw during the hot plate test ($F_{1,48}=16.3$; $p<0.001$), such that female mice had longer response latencies than did male mice, regardless of prenatal treatment. PAE did not significantly affect response latencies. The mean response latencies were: 23.6 ± 1.1 and 23.4 ± 0.5 for vehicle

and PAE male mice, respectively; and 28.3 ± 0.6 and 26.1 ± 1.1 for vehicle and PAE female mice, respectively.

3.8. Brain Regional Volumes, DTI, and Shape Measurements

PAE did not significantly affect the total brain volume in either male or female mice (Sup Fig 1). There was a significant sex by prenatal exposure by brain region interaction for volume measurements ($F_{21,420}=5.0$; $p<0.001$; Fig 4A and 4B; Sup. Fig 1). Post-hoc analysis revealed that regardless of PAE treatment, females had a larger cerebral cortex ($p=0.008$) and striatum ($p=0.04$) than did males. Furthermore, PAE reduced the volume of the brain stem in females, but not males ($p<0.001$). Importantly, vehicle-treated females had significantly larger pituitary volumes than did vehicle-treated males ($p<0.001$), but this sex difference was absent in the PAE mice, as PAE significantly decreased the pituitary size in females ($p=0.008$) and tended to increase pituitary size in the males ($p=0.09$).

On measures of fractional anisotropy (FA) in the anterior commissure, corpus callosum, and internal capsule, there was a significant interaction between sex and prenatal exposure ($F_{1,20}=5.8$; $p=0.026$; Fig 4C and Fig 4D). Vehicle-treated females tended to have higher overall FA than did vehicle-treated males ($p=0.052$), but PAE significantly increased FA in males ($p=0.01$), and did not affect FA in females. Although there were regional differences in axial and medial diffusivity, there were no significant effects of PAE on these measures.

In males, PAE-induced increases in pituitary size were significantly correlated with PAE-induced increases in the distance travelled in the center of the open field ($p=0.004$; Fig 5), but not with the % of time on the open arms of the elevated plus maze ($p=0.32$). PAE-induced increases in anterior commissure FA were significantly correlated with PAE-induced measures in the % of time on the open arms of the elevated plus maze ($p=0.004$; Fig 5) and distance travelled in the center of the open field during the first 15 minutes ($p=0.049$; Fig 5). In females, PAE-induced decreases in pituitary size were significantly correlated with PAE-induced decreases in social behavior during the social novelty test ($p=0.002$; Fig 5), but not the startle magnitude. However, PAE-induced reductions in the brain stem size were not significantly correlated with PAE-induced changes in social behavior or the startle response, although a trend toward a correlation was observed between the brain stem and social behavior during the social novelty test (Fig 5). Given the relatively small number of observations, these correlations should be viewed cautiously, as preliminary findings which are specific to the current dataset.

Shape analysis of the cerebellum and hippocampus revealed clusters of significant differences between alcohol- and vehicle-treated mice in the antero-medial, and postero-medial cerebellum, as well as the postero-lateral cerebellum (Fig 6). Significant differences were also found in the antero- and postero-dorsal hippocampus (Fig 6). In both structures, PAE reduced the size of the anterior portions, but increased the size of the posterior portions.

4. Discussion

Acute, binge-like alcohol exposure to a mouse embryo at the beginning of neurulation (GD 8, equivalent to the late third gestational week in the human), can produce behavioral and

brain structural consequences that are identifiable in adulthood. In general, this finding replicates those from other alcohol exposure procedures and test species, and highlights the fact that even a single, high dose, alcohol exposure can be sufficient to induce long-lasting consequences. When compared to the previous study in adolescent mice, some behavioral alterations intensified into adulthood (i.e. elevated plus maze, open field, and acoustic startle), while others (i.e. rotarod, water maze, hot plate) were attenuated, indicating an interaction between PAE and age of testing. Interestingly, brain structural consequences were dynamic as well; while no volumetric differences were observed during adolescence, adult prenatal alcohol-exposed females had smaller brain stems, as compared to vehicle exposed females, and prenatal alcohol-exposed adult males developed higher FA in the major fiber tracts, particularly the anterior commissure. The effects of PAE on certain behavioral and brain measures interacted with sex effects in adulthood, further confirming that an individual's sex is a strong modifier of the long-term effects of PAE. The pituitary gland may be especially relevant to sex differences, as changes in pituitary volume were correlated with changes in exploratory and social behavior, albeit in a small sample size. Taken together, the current results suggest that the trajectory of neurulation stage PAE on the brain and behavior depends to a large extent on both the age and sex of the affected individual. Aberrant brain and behavioral outcomes may thus evolve out of disruptions in typical sex-dependent development.

Sex differences following various PAEs have been observed repeatedly in laboratory animals, as well as clinical populations (e.g. McGivern et al., 1984, Mattson et al., 2011, Weinberg et al., 2008, Hellemans et al., 2010, Mooney and Varlinskaya, 2011, Wiczorek et al., 2015). The timing of the current GD8 exposure is before the major events of sexual differentiation (development of the genital ridge; expression of Sry; prenatal androgen exposure), so it is unlikely that alcohol directly interferes with sexual differentiation. Accordingly, no sex differences have been observed in the fetal MRI studies, which are measured at GD17 (Parnell et al., 2009, Parnell et al., 2013, Parnell et al., 2014, O'leary-Moore et al., 2010, Godin et al., 2010) before the peak of fetal androgen exposure (Ward and Weisz, 1980). However, in the GD17 fetus, there are identifiable PAE effects on hypothalamic and pituitary structure (Parnell et al., 2009, Parnell et al., 2013), suggesting that these regions could respond differently to subsequent androgen exposure. Pituitary volumes are sensitive to sex hormone levels, and alcohol exposure throughout gestation has been shown to influence the sensitivity to androgens, as well as a variety of pituitary functions (Redei et al., 1993, Weinberg et al., 2008, Lan et al., 2015). Therefore, it is possible that the early PAE-induced changes in pituitary and/or hypothalamic structure contribute to the behavioral and brain sex differences eventually observed in adulthood. Further studies on the effects of neurulation PAE on pituitary sensitivity would help address the mechanism for the changes in pituitary volume and its correlated behavioral outcomes.

PAE effects on the adult pituitary were sex dependent; in males, PAE increased pituitary size, while in females, PAE decreased pituitary size, effectively attenuating the inherent sex difference in the mouse pituitary. Similar effects of PAE on pituitary volumes have been observed in adolescent humans (Moore et al., 2016), and the current results suggest that early alcohol exposure may have been sufficient for those structural changes. In the small sample size available from the current dataset, pituitary volume changes were correlated

with distinct behaviors in males and females, distance travelled in the center of the open field and time with the familiar social stimulus during the novelty phase, respectively. As indicated above, both gastrulation and neurulation PAE affect fetal pituitary morphology, so it is likely that the pituitary differences observed in adulthood have been present throughout the lifespan, although the effects of post-natal life experiences, such as the stress of weaning, pair-housing, and the behavioral testing itself, on pituitary volumes cannot be ruled out. Although a causative mechanism is not indicated by the volume changes, it is possible that the control of stress reactivity by the pituitary may be altered by neurulation PAE, as has been shown for gastrulation and full-term gestational alcohol exposure (Weinberg, 1988, Glavas et al., 2006, Wieczorek et al., 2015), in a way that prevents typical behavioral responses to environmental stimuli. Nonetheless, the pituitary-behavior correlations further strengthen the biological relevance of statistically significant differences between prenatal alcohol-exposed and control adults for exploratory behaviors.

In addition to changing the pituitary, PAE increased FA in the major fiber tracts of male mice. The effects were largest in the anterior commissure which, like the corpus callosum, contains fibers connecting the hemispheres, especially the temporal lobes, but also the amygdala and olfactory regions (Fox et al., 1948, Brodal, 1948). This is the first study to find significant effects on FA in a mouse model of FASD, but a previous study in neonatal rats found increased FA in the cerebral cortex, an effect that was interpreted as representing a decrease in morphological complexity (Leigland et al., 2013). Many studies in human FASDs have shown decreases in FA, but some have found increased FA in certain regions (see Wozniak and Muetzel, 2011, for review). Recently, Uban et al. (2017) reported that PAE decreased FA in adolescent females, but increased FA in adolescent males, a similar pattern to those observed in the present study. FA, which is derived from the orientation of water molecules diffusing in the brain tissue, may indicate the degree that a structure is integrated and organized. Whether the higher FA values reflect denser fibers, more highly crossed fibers, altered synaptic pruning, or increased connectivity is not clear, but these differences are interpreted as evidence for structural pathology following neurulation PAE.

PAE-induced increases in FA were positively correlated with increases in both center distance and time on the open arms of the elevated plus maze, in the male mice only. Similar increases in elevated plus maze behavior have been reported elsewhere (Osborn et al., 1998, Carneiro et al., 2005, Diaz et al., 2016, Burton et al., 2017) and may reflect behavioral disinhibition, a feature of FASD and other developmental disorders (Kumar et al., 2014, Mattson and Riley, 2000, Lange et al., 2017). Baseline differences between males and females were observed on both center distance and elevated plus maze activity, as has been previously reported (Zimmerberg and Kajunski, 2004, Johnston and File, 1991), and similar to its effects on the sociability, PAE normalized these baseline sex differences. However, many animal studies also report decreased exploratory behaviors after repeated PAE and interpret this as increased anxiety-like behavior (Dursun et al., 2006, Cullen et al., 2013, Baculis et al., 2015, Abbott et al., 2016). While species and procedural variables, such as prior testing experience, may contribute to the different results, the largest influence is likely to be the timing of the alcohol exposure. Exposure restricted to neurulation will affect the fetus differently than will repeated exposures and the systems modulating increased anxiety-like behavior may develop at later time points. Thus, the current results reiterate the

importance of identifying when a fetus was exposed to alcohol and the major developmental events taking place at the time of exposure. A complete understanding of the effects of exposure timing on neurobehavioral outcomes may help make neurobehavioral outcomes useful predictors in establishing the patterns of alcohol exposure a fetus may have experienced. Alternatively, knowledge of when a fetus was exposed may help predict the neurobehavioral symptoms expected later in life.

The brain stem of female mice following PAE was significantly smaller than in control mice. Along with slight reductions in other regions, these differences contributed to a trend toward an overall reduction in brain size, an effect that has been observed in humans and in animal models of PAE (Donald et al., 2015). Reduced brain size has been reported in the fetal mouse after acute gastrulation or neurulation exposure (Parnell et al., 2009, O'leary-Moore et al., 2010, Lipinski et al., 2012) however, by adolescence there was no difference between prenatal alcohol exposed and control brains (Fish et al., 2016), suggesting a compensatory neural growth after birth. The fact that a volumetric difference, particularly in the brain stem, re-emerges during adulthood, suggests there may have been increased post-adolescent pruning, or an accelerated age-related neuronal loss or decreased neurogenesis. This pattern of volumetric differences in females is similar to that frequently observed in clinical samples of autism spectrum disorder (ASD), where an early overgrowth is followed by a period of growth arrest and subsequent decline (Courchesne et al., 2001, Courchesne et al., 2011, Sussman et al., 2015). Interestingly, the PAE-induced decrease in brain stem volume tended to be correlated with altered behavior during the social novelty test ($p=0.075$), a test commonly used in studies on ASD-related behavioral phenotypes. During this test, PAE reduced sociability in females to the level of the less social male mice, providing another example of how in the current study PAE normalized baseline behavioral sex differences. Longitudinal structural MRI studies of human FASD have also revealed dynamic age-related anatomical changes (Treit et al., 2013, Lebel et al., 2012), but most studies have focused primarily on childhood and early adolescent ages (Donald et al., 2015). Thus, it is not clearly known how PAE affects the continued trajectory of the human brain into adulthood. The current results, in a mouse model, suggest that a discrete alcohol exposure is sufficient to induce dynamic neuroanatomical changes into adulthood, though these may still be the result of potential interactions between PAE and post-natal environmental experiences.

Further evidence for PAE-induced changes in neuroanatomical structure comes from the findings of significant shape changes in the cerebellum and hippocampus, even though there were no differences in overall volume. It should be noted that these shape changes were measured by combining males and females to provide sufficient power for the analysis. Therefore, the changes in shape represent an effect of PAE that is consistent between the sexes. PAE reduced the size of the antero-dorsal cerebellum, while it increased the size of the postero-dorsal cerebellum. Significant reductions were also observed in the postero-lateral cerebellum. Similarly, the antero-dorsal hippocampus was reduced in size, whereas the postero-dorsal hippocampus was increased in size, relative to the vehicle controls. These findings of a smaller anterior cerebellum and a larger posterior cerebellum are consistent with those observed in adolescent mice, indicating some stability of shape between adolescence and adulthood. However, hippocampal shape differences were not observed during adolescence, while extensive shape changes were noted in the corpus callosum, indicating

that PAE continues to interact with ongoing developmental events to impact brain growth. The meaning of shape changes and how they may impact function is unclear, but regional shape changes are a consistent feature of the brains of prenatal alcohol exposed humans and laboratory animals (Donald et al., 2015).

One of the limitations of the current study is the use of a single, inbred mouse strain. Although the C57BL/6J mouse is among the most actively studied mouse strains in alcohol research, several features reduce the generalizability of studies with this strain. First, the C57BL/6J strain appears to be more sensitive than other strains, including the closely related C57BL/6N substrain, to the dysmorphic effects of gastrulation and neurulation stage PAE (Boehm et al., 1997, Downing et al., 2012, Downing et al., 2009, Dou et al., 2013, Gilliam, 2014). The enhanced sensitivity may be related to strain differences in developmental timing, alcohol metabolizing enzymes, ERK1/2 signaling, glucose sensitivity, or oxidative stress, among other physiological processes (Toye et al., 2005, Rydstrom, 2006, Lee et al., 2010, Kumar et al., 2013, Simon et al., 2013, Fergusson et al., 2014, Eberhart and Parnell, 2016). Additionally, differences in behavioral performance between mouse strains are common (e.g. Crawley et al., 1997, Bryant et al., 2008, Simon et al., 2013) and likely impact how the behavioral consequences of PAE are expressed in each strain. Further investigation of strain differences, which are also apparent in other species (e.g. rats, chicken, fish) as well as specific gene by drug exposure interactions may help reveal the genes that underlie susceptibility to PAE (Eberhart and Parnell, 2016).

Conclusions

Overall, the current results support the hypothesis that a single neurulation stage alcohol exposure is sufficient to change adult behavior and brain structure. Affective behaviors, rather than learning and memory, appear to be the most perturbed by neurulation stage alcohol, and these behavioral changes are associated with changes in FA, pituitary and brain stem volumes, as well as shape changes in the hippocampus and cerebellum. These results reiterate the importance of early gestational stages to the development of species-typical male and female behavior as well as the importance of avoiding fetal alcohol exposure. Although the mechanisms by which neurulation stage alcohol exposure affect the embryo are only now being clarified, it is evident that the consequences of these mechanisms are pervasive and long-lasting.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

- Acute, neurulation stage (GD 8) alcohol exposure altered the behavior of adult male and female mice.
- Sex differences were observed in the effects of neurulation stage alcohol on exploratory behavior, social preference, and acoustic startle reactivity.
- Neurulation stage alcohol reduced the volume of the brain stem in females and increased fractional anisotropy in the males.
- Neurulation stage alcohol attenuated the sex difference in the volume of the pituitary, an effect that correlated with behavioral changes.

Figure 1.

The effects of GD8 alcohol exposure on elevated plus maze and open field behavior. A and B. In each panel the *left pair of bars* represents the percentage of time on the open arms and corresponds to the *left y-axis*. The *center pair of bars* corresponds to the *left y-axis* and represents the percent of total arm entries that were made onto the open arms. The total arm entries are represented by the *right pair of bars*, corresponding to the *right y-axis*. C and D. In each panel, the *left symbols* correspond to the *left y-axis* and represent the distance travelled in the open field in each 5-min time bin. The *right symbols* correspond to the *right y-axis* and represent the distance (cm) that was travelled in the center of open field in each 5-min time bin. *Filled gray bars and symbols* represent PAE mice, while *open bars and symbols* represent vehicle-treated mice. All data are means $\pm$ 1 SEM. *Asterisks* denote within sex significance (Alc vs. Veh); *daggers* denote between sex significance within Veh treatment (Males vs. Females), $p < 0.05$.

Figure 2.

The effects of GD8 alcohol exposure on social behaviors in the three-chambered test. A and B. In each panel, the *left and right pairs of bars* represent the time spent on the side that contained the non-social, or the social stimulus, respectively. C and D. In each panel, the *left and right pairs of bars* represent the time spent on the side that contained the familiar, or novel, social stimulus, respectively. *Filled gray bars* represent PAE mice, while *open bars* represent vehicle-treated mice. All data are means $\pm$ 1 SEM. *Carets* denote significance vs. Non-Social or Familiar; *asterisks* denote within sex significance (Alc vs. Veh); *daggers* denote between sex significance within Veh treatment (Males vs. Females), $p < 0.05$.

Figure 3.

The effects of GD8 alcohol exposure on acoustic startle responses. A and B. In each left panel, the *left and right pair of bars* represents the response amplitude after no-stimulus trials (70 dB background stimulus alone) or a 120 dB startle stimulus, respectively. In each right panel, the *open and filled gray symbols* represent the response amplitude following the presentation of a 74 – 90 dB prepulse, before the startle stimulus. *Filled gray symbols* represent PAE mice, while *open symbols* represent vehicle-treated mice. All data are means $\pm$ 1 SEM. *Asterisks* denote within sex significance (Alc vs. Veh), $p < 0.05$.

Figure 4.

The effects of GD8 alcohol exposure on brain regional volumes and fractional anisotropy. A and B. In each panel, the *open and filled gray bars* represent the volume of the brain stem and pituitary as measured by MRI. C and D. In each panel, the *open and filled gray bars* represent the fractional anisotropy of the internal capsule, anterior commissure, and corpus callosum, as measured by DTI. *Filled gray bars* represent PAE mice, while *open bars* represent vehicle-treated mice. All data are means $\pm$ 1 SEM. *Asterisks* denote within sex significance (Alc vs. Veh); *daggers* denote between sex significance within Veh treatment (Males vs. Females), $p < 0.05$.

Figure 5.

Correlations between the brain and behavioral effects of GD8 alcohol exposure. The *top and bottom panels* portray data from male and female mice, respectively. *Filled gray symbols* represent individual PAE mice, while *open symbols* represent individual vehicle-treated mice. A and B. Correlations between anterior commissure fractional anisotropy (A. *x-axis*) or pituitary volume (B. *x-axis*), and the % of time on the open arms of the elevated plus maze (*y-axis, left panels*) or the distance traveled in the center of the open field (*y-axis, right panels*). C and D. Correlations between brain stem (C. *x-axis*) or pituitary volume (D. *x-axis*), and the time spent near the social stimulus (*y-axis, left panels*) or the amplitude of the startle response (*y-axis, right panels*).

Figure 6.

The effects of GD8 alcohol exposure on regional brain shapes. Areas portrayed in green are statistically unchanged from vehicle, while areas in other colors are different between alcohol and vehicle treated mice. Red indicates areas that are significantly smaller in PAE mice, while blue indicates areas that are significantly larger PAE mice. a and b) portray the anterior and posterior views of the hippocampus. c, d and e) portray the anterior, posterior, and left views of the cerebellum.

Table 1
Effects of GD 8 Alcohol Exposure on the Latency (s) to Fall off the Rotarod in Male and Female Mice

Rotarod Trial	1.	2.	3.	4.	5.
<u>Males</u>					
Veh	126±18	155±17	141±22	218±20	215±18
Alc	112±12	109±20	153±23	195±23	229±19
<u>Females</u>					
Veh	140±20	170±24	189±22	205±18	223±21
Alc	102±13	150±21	157±21	203±20	219±18

All data are expressed as mean (+ SEM). Trials 1-3 were conducted on Day 1, and trials 4-5, on Day 2 of testing.

Table 2
Effects of GD 8 Alcohol Exposure on the Latency (s) to Find the Hidden Platform of the Morris Water Maze in Male and Female Mice

Test Day	1.	2.	3.	4.	5.
<u>Males</u>					
Veh	45.7±4.0	33.4±4.0	33.6±4.6	25.3±5.4	19.9±4.0*
Alc	38.8±4.8	31.9±2.0	22.1±2.3	20.5±2.8	18.4±2.5*
<u>Females</u>					
Veh	40.3±4.6	28.4±5.1	25.1±3.7	22.6±4.4	18.2±3.4*
Alc	40.5±3.4	28.0±4.0	20.0±3.7	20.1±3.1	20.9±1.8*

All data are expressed as mean (+ SEM) of four trials per day across five days of testing.

Asterisks denote significance vs. day 1, $p < 0.05$.

Table 3

Effects of GD 8 Alcohol Exposure on the Percent of Time Spent in Each Quadrant of the Morris Water Maze during the Probe Trial in Male and Female Mice.

Water Maze Quadrant	I.	II.	III.	IV.
<u>Males</u>				
Veh	37.7±3.6*	13.8±2.3	21.9±3.2	26.6±3.9
Alc	33.5±4.7*	19.2±3.4	22.1±3.5	25.1±3.1
<u>Females</u>				
Veh	39.7±4.1*	18.7±1.1	18.5±2.4	23.1±2.5
Alc	33.2±4.8*	19.8±3.7	20.2±3.2	26.8±3.5

The hidden platform, which was located in Quadrant I during the acquisition phase, was removed for the probe trial. All data are expressed as mean (± SEM).

Asterisks denote significant effect of quadrant following within-treatment repeated measures ANOVA, $p < 0.05$.