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Neurodevelopmental Timing of Ethanol Exposure May Contribute to Observed Heterogeneity of Behavioral Deficits in a Mouse Model of Fetal Alcohol Spectrum Disorder (FASD)

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ABSTRACT

Maternal drinking during pregnancy can result in a wide spectrum of cognitive and behavioral abnormalities termed fetal alcohol spectrum disorders (FASD). The heterogeneity observed in FASD-related phenotypes can be attributed to a number of environmental and genetic factors; however, ethanol dose and timing of exposure may have significant influences. Here, we report the behavioral effects of acute, binge-like ethanol exposure at three neurodevelopmental times corresponding to the first, second, and third trimester of human development in C57BL/6J mice. Results show that developmental ethanol exposure consistently delays the development of basic motor skill reflexes and coordination as well as impairs spatial learning and memory. Observed changes in activity and anxiety-related behaviors, however, appear to be dependent on timing of alcohol exposure. The variability in behaviors between different treatment models suggests that these may be useful in evaluating the mechanisms disrupted by ethanol at specific neurodevelopmental times. The results provide further evidence that, regardless of developmental stage, the developing brain is acutely sensitive to alcohol exposure.

Keywords: Fetal Alcohol Spectrum Disorder (FASD); Ethanol; Behavior; Neurodevelopment; Mouse Model; C57BL/6J

1. Introduction

Maternal alcohol consumption during pregnancy can result in morphological, behavioral and neurological abnormalities, collectively termed Fetal Alcohol Spectrum Disorders (FASD) [1,2]. The prevalence of FASD is estimated to be approximately 1 in 100 live births in North America, and the occurrence and severity of FASD phenotypes, including variable behavioral effects, have been attributed to the timing and dosage of alcohol [3-6]. The nature of the heterogeneity associated with variability of manifestation is poorly understood, as is the mechanism that causes these phenotypes to persist throughout the lifetime of an individual. These phenotypes often include delayed early-life development of motor control and coordination, hyperactivity, increased risk for anxiety-related psychopathologies, impulsivity, inattentiveness and intellectual impairment [7-13]. Further, many children with FASD show deficits in cognition and learning, executive functioning, memory and social adaptation [13-17].

A number of animal models have been used to explore the relationship between ethanol exposure and specific FASD-related phenotypes [11,18-21]. Specifically, the generation of well-established behavioral battery protocols has led to a better characterization of behaviors and cognition in animals that have been prenatally exposure to alcohols. In particular, the C57BL/6J mouse has been shown to be acutely sensitive to both the physiological and the behavioral effects of neurodevelopmental ethanol exposure. This strain of mouse has been successfully used to replicate a number of FASD-relevant phenotypes including impairments in cognitive function, activity levels, novel-environment anxiety, and depression-related behaviors [22-27]. Such results are considered representative of humans for a variety of reasons, including the fact that rodent and human neurodevelopmental timelines are comparable [28-30], which allows for experimentation on timing-of-exposure [31-33]. Most previous work, however, differs substantially in experimental factors such as ethanol dosage, timing of exposure, time of testing, be-

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havioral phenotype evaluated, and testing protocol, making comparisons across studies difficult. Thisstudy is novel as it assesses the effects of exposure at specific neurodevelopmental times using a consistent dosage regiment. The results will provide a realistic framework for future studies.

Specifically, we have used the C57BL/6J (B6) mouse strain to model the behavioral effects of acute, binge-like alcohol exposure at three neurodevelopmental time points corresponding to human trimesters one (T1), two (T2) and three (T3). There is evidence that binge alcohol exposures are prevalent in pregnant women of certain high-risk groups, and can lead to FASD-related clinical diagnoses in resulting children [34,35]. We evaluated the resulting offspring across a battery of behavioral tests relevant to FASD-associated phenotypes from early neonatal development to adulthood. The results offer a perspective on the range of neurological and cognitive functions affected by alcohol that is dependent on timing of exposure.

2. Materials and Methods

2.1. Animals and Breeding

Male and female C57BL/6J mice were originally obtained from Jackson Laboratories (Bar Harbor, ME, USE) and subsequently bred at the Health Sciences Animal Care Facility at the University of Western Ontario. All procedures involving the use of mice met the ethical standards outlined by the Canadian Council on Animal Care and were approved by the Animal Use Subcommittee of the University. Mice were housed in standard same-sex colony cages with a temperature range of 21˚C - 24˚C and at 14-h light/10-h dark schedule, with free access to food and water. For breeding, females of approximately eight weeks of age were housed in individual cages and time-mated overnight with 8 - 12 weeks old males. At the end of the mating period, females were examined for presence of a vaginal plug, indicating gestational day 0 (G0), and males were removed from cages.

2.2. Ethanol Injections

Pregnant females were randomly assigned to two groups in each of the three trimesters: control dams injected subcutaneously (abdomen) with 0.15 M saline solution and ethanol-treated dams injected with 20% ethanol in the saline solution at the same site. An injection of 2.5 g/kg was given twice, spaced two hours apart to model a heavy, binge-like exposure (blood alcohol concentration or BAC remains over 200 mg/dl for at least 4 hours) [29]. Subcutaneous injections were given on G8 and G11 to model the first trimester [36,37], and on G14 and G16 to model the second trimester equivalent [31,38]. In order

to model for the third trimester equivalent, pups at postnatal day 4 (P4) and P7 were subcutaneously injected using the same dosage. This stage parallels the human equivalent of third trimester alcohol exposure [28,29,39]. It is not possible to perfectly match such stages across species. Consequently, these timings should be viewed as the best approximation for the human equivalent. Dams were randomly assigned to control and ethanol-treated groups. Third trimester pups from each litter were matched where possible to control for litter effects. The number of litters used for T1 include 4 ethanol and 3 control litters, and 6 ethanol and 4 control litters for T2. For T3 we were able to assign pups from the same litter to two treatments from a total of 6 litters. Dams and pups were monitored following ethanol injections until full recovery, and pups were weaned on P21 into colony cages of two to four same-sex littermates. All pups were run through the same battery of tests and treated as statistically independent observations.

2.3. Early Postnatal Development

From P2 to P21, pups were assessed for the ability to reach critical developmental milestones evaluating the appearance of age-appropriate motor skills such as balance, motor coordination, strength and reflexes, following Hill and colleagues [40]. Tests were performed at the same time each day until the pup was able to perform the task in the prescribed amount of time for two consecutive days [41]. The tests used included surface righting, negative geotaxis, cliff aversion, forelimb grasp, auditory startle, ear twitch, open field traversal, air righting and eye opening. Pups from T3 (P4 and P7) underwent developmental milestone testing beginning at 9:30 am, followed by injections at 12:00 pm.

2.4. Open-Field Locomotor Activity to Test Activity and Anxiety

At P25 pups were assessed for activity in a novel open-field environment using the infrared Actimeter system and measured using Acti-Track software (Panlab, Barcelona, Spain) [41]. We chose mice at young adolescence following previous reports that this testing during this period results in measurable differences between ethanol-treated and control mice [41]. Also, this timing corresponds to the prepubescent period of development in mice [42]. The prepubescent period is the time markedly used in human studies assessing disorders involving hyperactive behaviors [43,44]. The open field arena consisted of a 45 cm (W) × 45 cm (L) surface constructed of black plexiglass enclosed by four 35 cm-high clear acrylic walls, as well as an infrared frame that produced a 16 × 16 grid of intersecting beams used to track the movement of each mouse. Infrared beam-break data were used

to calculate locomotor activity. Movement data were also analyzed by dividing the arena into an outer periphery zone and a central zone to allow for the evaluation of thigmotaxis. Testing was conducted during the light phase between 1000 h and 1300 h, and the lighting of the arena was 100 lx. Each mouse was placed in the same corner of the arena when beginning the trial, and was allowed to freely explore for 15 min. At the end of the testing, the mouse was removed and returned to its home cage. Between trials, the arena was cleaned with 30% isopropanol.

2.5. Home Cage Activity Testing

Activity in a familiar environment was measured using the infrared Actimeter system (Panlab, Barcelona, Spain). At P35, mice were placed individually into 38 cm (*L*) × 24 cm (*W*) × 14 cm (*H*) transparent plastic cages (Innovive, San Diego, CA, USA) with standard woodchip bedding and free access to food and water. Following a 24 h acclimation period, cages were placed in the Actimeter frame and testing was conducted overnight from 1900 h to 0600 h, spanning the dark phase of the light/dark cycle and 1 h of light at the beginning. Recordings were taken for two consecutive nights and averaged. Mice were re-housed in their original cages at the end of activity testing.

2.6. Light/Dark Box for Testing Anxiety

The light/dark box was used as a measure of exploratory behavior and anxiety in a novel, illuminated environment to further characterize the thigmotaxis behavior observed during the open-field test. The apparatus was constructed following Crawley and colleagues [45] and included two compartments consisting of a 27 cm (*L*) × 27 cm (*W*) × 27 cm (*H*) light arena and a 18 cm (*L*) × 27 cm (*W*) × 27 cm (*H*) dark arena, with a 7.5 cm × 7.5 cm opening between the light and dark regions. At age P40, each mouse was placed in the light arena facing the opening, and allowed to freely explore both light and dark areas for 5 min. The overhead light in the room was 200 lx and all trials were recorded by a ceiling-mounted camera. Any-Maze digital tracking software (Stoelting, Wood Dale, IL, USA) was used to analyze movements and track the amount of time spent in the light arena versus the dark arena. Following testing, the mouse was returned to its home cage.

2.7. Barnes Maze to Test Spatial Learning and Memory

A modified version of Barnes maze for mice was constructed following Sunyer and colleagues [46] and was conducted as previously described [41]. Briefly, the test consisted of four 3-minute training trials per day, spaced 15 minutes apart, over four consecutive days beginning at P50 (acquisition days). For each trial, the mouse was placed in a start chamber in the centre of the platform, and after 10 seconds, an overhead 220 lx light and 85 dB computer-generated white noise were turned on, and the mouse was released to explore the platform. The trial ended once the mouse successfully located the target and entered the escape box. The light and white noise were terminated immediately after entry. If the 3 minutes had elapsed without the mouse entering the escape box, the mouse was guided into the box by the experimenter and left for 1 minute undisturbed. Between trials, the platform was cleaned with 30% isopropanol. Trials were also qualitatively scored based on whether the mouse used a direct strategy (mouse moves directly to the escape hole or an adjacent hole), a serial strategy (the first visit to the escape box was preceded by visiting at least two adjacent holes in a serial manner, in a clockwise or counter-clockwise direction), or a mixed strategy (hole explorations were separated by crossing through the centre of the maze or unorganized search).

The day following the last acquisition day (day 5 of testing), the escape box was covered and the mouse was allowed to explore the maze for 1 minute before being returned to its home cage (probe trials). The number of errors and explorations to the target hole were recorded. The same probe trial protocol was also performed seven days following the first probe trial (day 12 of testing) without further training between these days. Trials were recorded using a ceiling-mounted camera and analyzed using AnyMaze digital tracking software (Stoelting, Wood Dale, IL, USA) for latency to reach the escape box. Explorations and search strategy was scored manually by an independent observer unaware of the treatment group.

2.8. Statistical Analysis

Data were analyzed using appropriate analysis of variance methods depending of the number of independent variables (sex, treatment, repeated day of testing). Where data were analyzed across days, repeated-measures analysis of variance (ANOVA) with treatment as the between-subjects factor and day as the within-subjects factor was used. For overnight activity, we performed hypothesis-based, step-down analyses and corrected for multiple testing. All data are reported as mean ± standard error of the mean. For the Barnes maze acquisition days, data were $\log_3$-transformed to account for the differences in variance between latencies to reach the target across days. Data analysis was stratified by sex if sexually dimorphic effects were observed. Effects of litter were analyzed using "litter" as a covariate in the ANOVA, however, no litter effects were observed for any of the beha-

vioral measures. All statistical analyses were performed using SPSS v.16 (SPSS Inc, Chicago, IL, USA).

3. Results

3.1. Postnatal Developmental Milestones

All pups were evaluated from P2 to P21 for the abilities to reach critical neurodevelopmental milestones. The average day each specific milestone was achieved by each treatment group is shown in **Table 1**. The ethanol exposure during T1 significantly delayed the ability of pups to surface right themselves when placed on their backs ($F_{1,46} = 29.90$, $p < 0.001$), grasp a rod with their forelimbs ($F_{1,46} = 15.50$, $p < 0.001$), extinguish pivoting behavior and transverse out of a 15 cm-diameter circle ($F_{1,46} = 15.10$, $p < 0.001$), and right themselves in the air when dropped from upside down from 5 cm ($F_{1,46} = 23.71$, $p < 0.001$). Interestingly, a significant interaction between sex and treatment was observed for the ability of the pup to right itself in the air when dropped ($F_{1,46} = 7.60$, $p = 0.008$), with control males taking longer to right themselves than control females but exposed females taking longer to right themselves than exposed males. T2 ethanol-treated pups were significantly delayed in time it took to surface right ($F_{1,54} = 4.93$, $p = 0.03$), turn 180° upward when placed downward on a screen set at 45° ($F_{1,54} = 34.88$, $p < 0.001$), crawl away from the edge of a cliff ($F_{1,54} = 25.10$, $p < 0.001$), grasp the rod ($F_{1,54} = 6.65$, $p = 0.01$), transverse out of the circle ($F_{1,54} = 17.02$, $p < 0.001$), right themselves in the air ($F_{1,54} = 10.06$, $p = 0.003$) and open their eyes for the first time ($F_{1,54} = 6.21$, $p = 0.02$). Finally, T3 ethanol-treated pups had significant delays in surface righting ($F_{1,39} = 97.15$, $p < 0.001$), grasping the rod ($F_{1,39} = 10.96$, $p = 0.002$), reacting to a handclap at a distance of 10 cm ($F_{1,39} = 9.52$, $p = 0.004$), flattening their ear in response to an applicator ($F_{1,39} = 6.34$, $p = 0.02$), traversing outside of the circle ($F_{1,39} = 27.67$, $p < 0.001$), opening their eyes ($F_{1,39} = 12.72$, $p < 0.001$), and righting in the air ($F_{1,39} = 63.24$, $p < 0.001$).

3.2. Locomotor Activity and Anxiety-Related Traits

Spontaneous activity of juvenile (P25) offspring from control and ethanol-treated mice for each trimester was assessed over a 15 minute period in a novel open-field environment. Two-way ANOVA did not result in a significant effect of sex or an interaction between sex and treatment, therefore the groups were collapsed and a one-way ANOVA with treatment as the main factor was applied. It showed a significant effect of treatment in T1 ($F_{1,46} = 6.48$, $p = 0.01$) and T2 ($F_{1,55} = 69.89$, $p < 0.001$), where the number of beam breaks was increased in ethanol-treated mice versus control mice (**Figures 1(a)** and

(b)). No significant effect of treatment was observed for T3 mice (**Figure 1(c)**).

To evaluate the effects of novelty induced anxiety-related traits in the open field task, we also examined differences in thigmotaxis during open-field testing. Although no statistically significant effect of treatment was observed for T1 mice (**Figure 2(a)**), a significant main effect of treatment was observed between T2 exposed and control mice ($F_{1,55} = 59.65$, $p < 0.001$), with ethanol-treated offspring spending significantly more time in the centre zone than control mice (**Figure 2(b)**). Interestingly, mice treated with ethanol during the T3 equivalent spent significantly less time than control mice in the centre
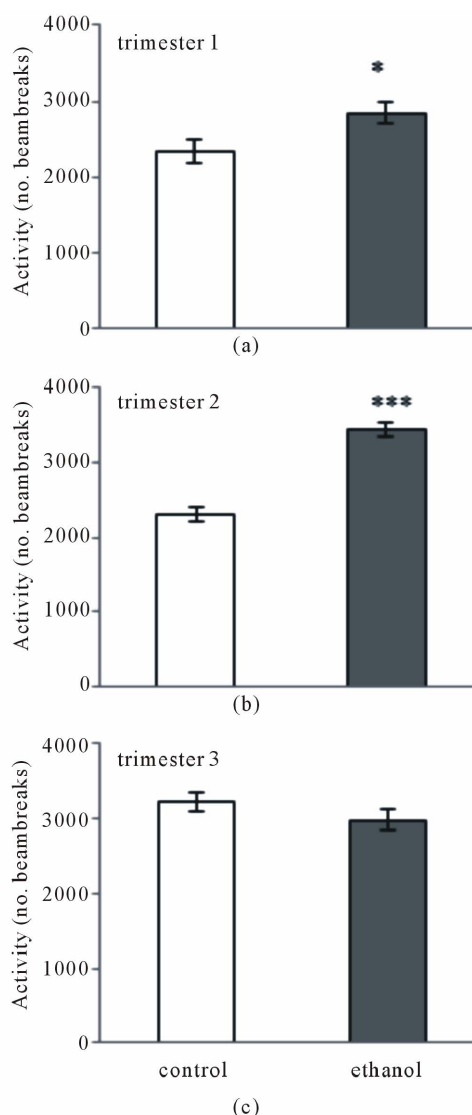

Figure 1. Locomotor activity in a novel open-field environment. Mean (±SEM) infrared beam breaks of ethanol-treated and control mice from trimester 1 (a), trimester 2 (b) and trimester 3 (c) over a 15 min period (n = 21 - 31 mice per group). $^*p < 0.05$; $^{***}p < 0.001$.
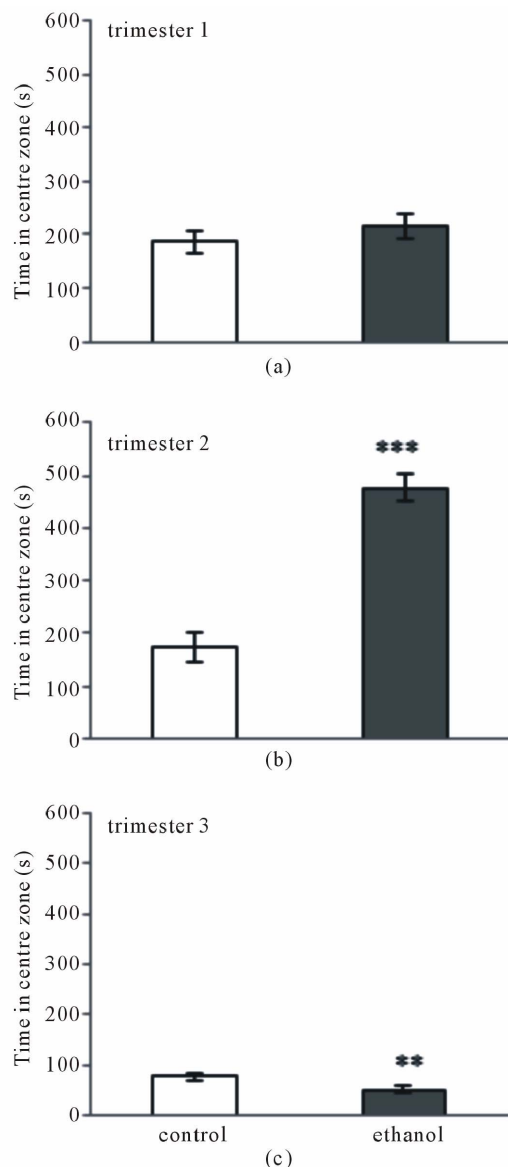
Figure 2. Time spent in the centre of a novel open-field environment to assess anxiety-related behaviours. Mean (±SEM) time in seconds spent in the centre of the apparatus of ethanol-treated and control mice from trimester 1 (a), trimester 2 (b) and trimester 3 (c) over a 15 min period (*n* = 21 - 31 mice per group). ***p* < 0.01; ****p* < 0.001.

zone of the open-field arena ($F_{1,164}$ = 10.40, *p* = 0.002) (**Figure 2(c)**).

Home-cage (familiar environment) locomotor activity was also assessed in ethanol-exposed and control adolescent mice between P30-40. Repeated-measures two-way ANOVA detected significant main effects of hour and treatment, but no significant effects of hour and treatment or sex and treatment. A significant main effect of treatment in T1 was found ($F_{1,46}$ = 12.09, *p* = 0.001) with ethanol-treated mice demonstrating increased activity throughout the nocturnal phase versus control mice (**Figures 3(a)**

and (**b**)). A significant main effect of treatment was found in T3 with ethanol-exposed mice demonstrating increased activity versus control mice.

Finally, we used a light/dark box apparatus to further evaluate the anxiety-related phenotypes we had observed in (novel) open field activity. Here, T2 ethanol-treated mice spent significantly ($F_{1,55}$ = 7.08, *p* = 0.01) more time (average: 152.46 ± 4.65 s) in the light area of the box than control mice (average: 134.50 ± 4.89 s). Mice from T1 were not significantly different ($F_{1,46}$ = 1.82, *p* = 0.18) between control mice (160.90 ± 7.92 s) and ethanol-treated offspring (146.12 ± 7.58 s). Analysis of T3 data also did not result in a significant difference ($F_{1,24}$ = 3.67, *p* = 0.067) between ethanol-treated offspring (141.06 ± 6.80 s) and controls (121.86 ± 7.35 s).

3.3. Barnes Maze Task for Spatial Learning and Memory

Mixed-model ANOVA showed a significant interaction of treatment by acquisition day on latency to reach the escape box for all trimester treatments. There was no significant effect of sex or an interaction between sex and treatment observed for any treatment time. Mice treated with ethanol in T1 displayed increased latency to reach the escape box than control mice on acquisition days 3 ($F_{1,46}$ = 12.54, *p* = 0.001) and 4 ($F_{1,46}$ = 9.86, *p* = 0.003) (**Figure 4(b)**). Mice treated with ethanol in T2 had significantly increased latency to reach the escape box on day 1 ($F_{1,55}$ = 10.60, *p* = 0.002) versus control mice but performed similarly to control mice on days 2 to 4 (**Figure 4(c)**). Ethanol-exposed mice from T3 had increased latency to reach the escape box on days 2 ($F_{1,39}$ = 12.78, *p* = 0.001), 3 ($F_{1,39}$ = 42.77, *p* < 0.001) and 4 ($F_{1,39}$ = 35.99, *p* < 0.001) in comparison to control mice (**Figure 4(d)**). The efficiency of learning can be measured by the strategy a mouse uses to locate a target, with an organized (direct) search providing evidence of spatial memory retention [47,48] We qualitatively scored search strategies used by each mouse (data not shown). Mice from T1 were not observably different in the search strategies used over trial days. Control T2 mice showed a general trend of increased direct search strategies and a decreased percentage of mixed strategies, while their ethanol treated counterparts used mixed strategies in a large proportion of trials across all learning days. T3 control mice showed a pronounced trend of increased direct search strategies and decreased serial strategies across every learning day as compared to ethanol-treated mice that more often used mixed strategies.

Short-term and long-term retention of memory in the Barnes maze was assessed on days 5 and 12, respectively, by calculating the number of explorations to the target hole location. On probe day 5, T1 ethanol-treated mice
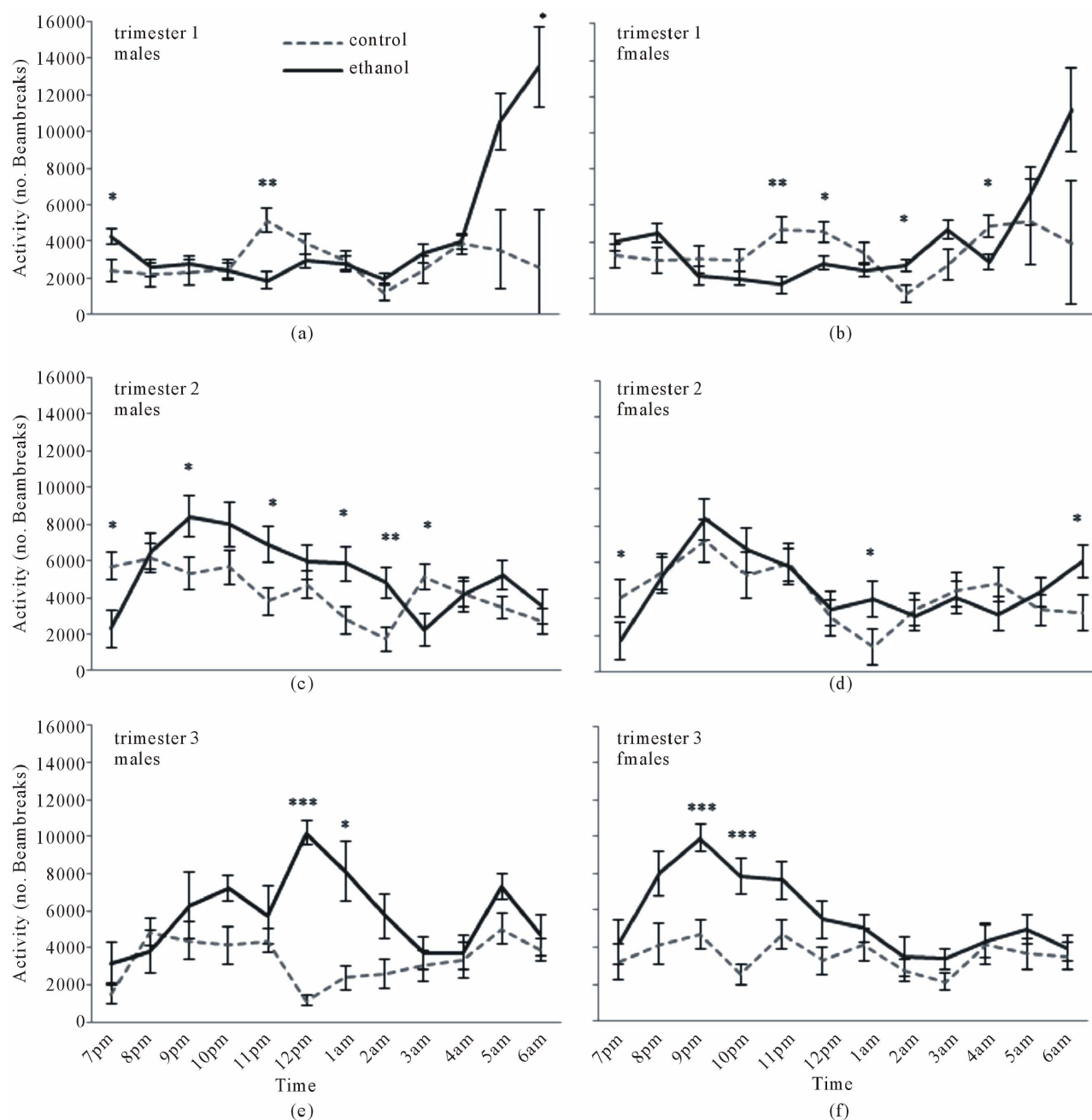
Figure 3. Locomotor activity in a familiar home-cage environment stratified by sex. Mean (±SEM) infrared beam breaks by ethanol-exposed and control mice from trimester 1 males (a) and females (b), trimester 2 males (c) and females (d), and trimester 3 males (e) and females (f) over an 11-h period (n = 9 - 20 mice per group). $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$.

were not significantly different from controls in the number of explorations to the escape box (**Figure 4(a)**). Generally, ethanol-treated mice displayed increased explorations to the opposite end of the target than control mice (**Figures 5(a)** and **(b)**). On probe day 12, we found a main effect of sex ($F_{1,46}$ = 4.22, p = 0.046), with females exhibiting more explorations to the target (average female: 2.64 ± 0.15 explorations) than males (average male: 2.21 ± 0.15 explorations).

No significant effects of sex or an interaction between sex and treatment was observed for the explorations of ethanol-treated and control T2 mice on probe day 5. Significant effects of treatment were observed for the explorations to the escape hole location ($F_{1,55}$ = 5.21, p = 0.03) and hole position −1 ($F_{1,55}$ = 14.27, $p < 0.001$), with control mice displaying increased exploration around the target than exposed mice (**Figure 5(c)**). Ethanol-treated mice exhibited more explorations on the opposite site of
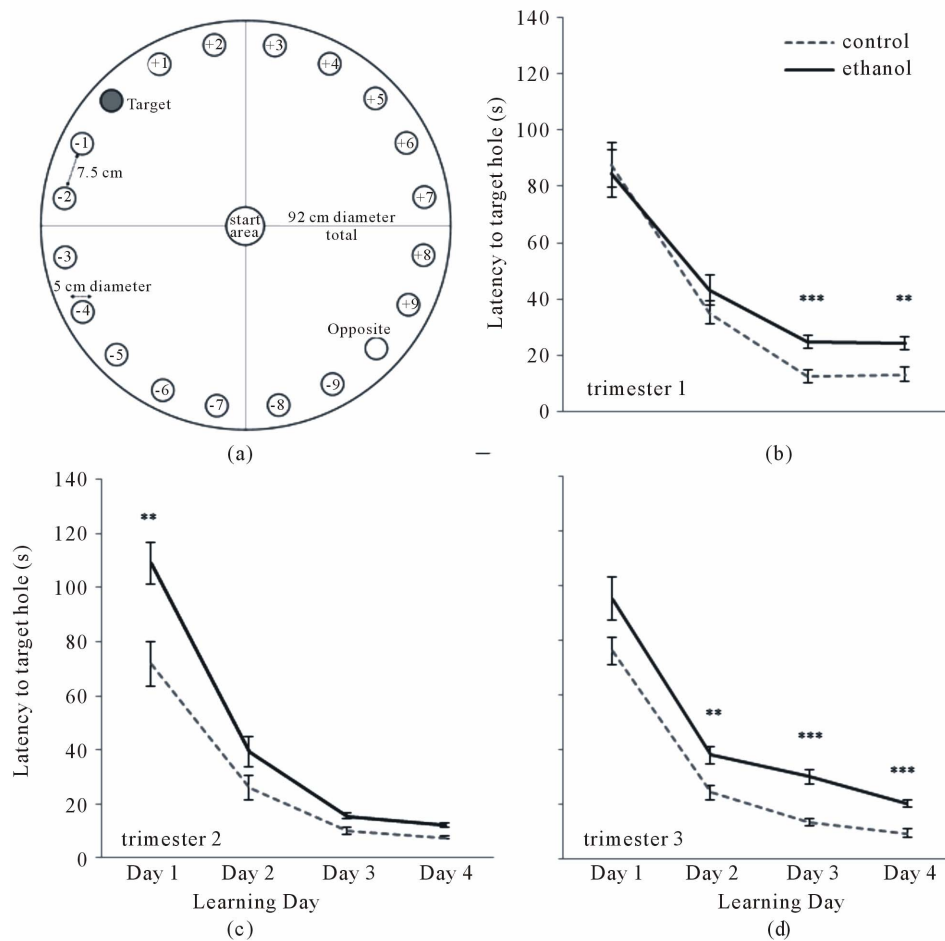
Figure 4. Latency to reach the target in the Barnes maze (a) used for spatial learning and memory. Average latencies to the target represent mean (±SEM) of four trials per day across four acquisition days for ethanol-treated and control mice from trimester 1 (b), trimester 2 (c) and trimester 3 (d). Data shown are collapsed across sex (n = 21 - 31 mice per group). $^{**}p < 0.01$; $^{***}p < 0.001$.

the target zone than control mice (**Figure 5(c)**). Significant effects of treatment were observed for the explorations to the escape hole ($F_{1,55}$ = 12.29, p = 0.001), with control mice spending more time within the target area than ethanol-treated mice (**Figure 5(d)**).

For T3 probe days 5 and 12 explorations, we did not observe any significant effects of sex or an interaction between sex and treatment. A significant effect of treatment was observed on probe day 5 for explorations to the target ($F_{1,39}$ = 14.22, p < 0.001) and positions near the target, with control mice displaying significantly more explorations around the target than ethanol-treated mice (**Figure 5(e)**). We also found a significant effect of treatment on probe day 12 for the target hole ($F_{1,39}$ = 7.80, p = 0.008), with control mice spending more time in the target area than ethanol-treated mice **Figure 5(f)**).

4. Discussion

This study evaluates the effects of heavy binge-like etha-

nol exposure at neurodevelopmental times approximating human trimesters one, two and three across a battery of behavioral assays. The phenotypes assessed fall into three categories: motor skill development, locomotor activity including behaviors relevant to anxiety, and spatial learning and memory. The specific assays are well-established and have been used extensively in the literature to model human behaviors that are most relevant to FASD [40,46, 49-53]. Our results show that ethanol exposure causes changes in a number of behaviors examined and that the extent of alterations may be dependent upon the gestational timing of ethanol treatment.

4.1. Motor Skill Development

The results show that ethanol exposure at any time during gestation may cause delays in motor skill and reflex development. Specifically, our results show delays in surface righting, open-field traversal and air righting (**Table 1**), which follows the literature [54,55]. Such phenotypes

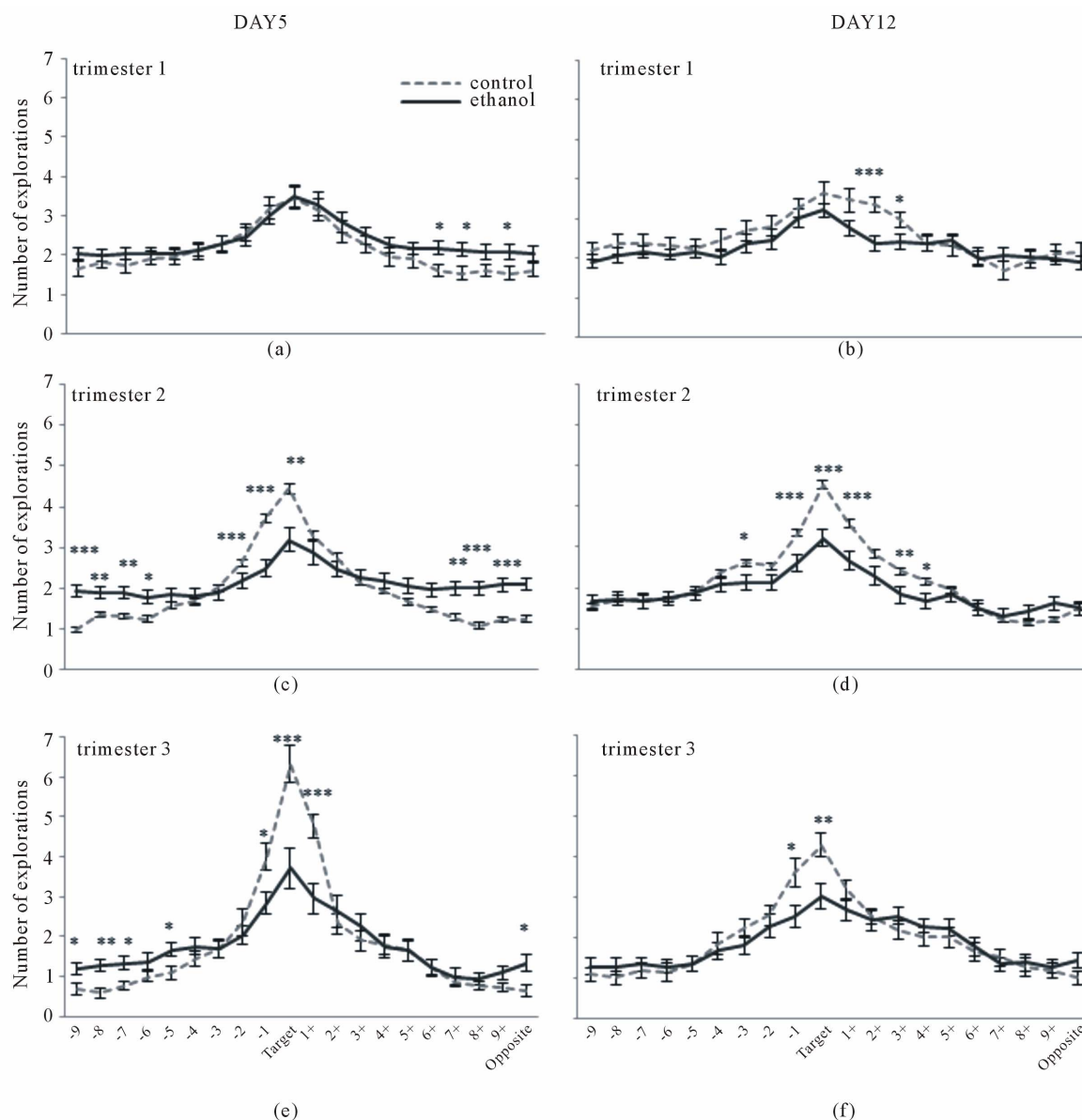DAY5   DAY12

Figure 5. Number of explorations for control and ethanol-treated mice during Barnes maze on days 5 and 12 to represent short-term and long-term memory retention, respectively. Mean (±SEM) number of explorations to each hole of the Barnes maze for ethanol-treated and control mice on day 5 for trimester 1 (a), trimester 2 (c) and trimester 3 (e), and day 12 for trimester 1 (b), trimester 2 (d) and trimester 3 (f). The position of numbered holes (*x*-axis) of Barnes maze are shown in Figure 3(a). Data shown here are collapsed across sex (*n* = 21 - 31 mice per group). ** $p < 0.05$; **** $p < 0.01$; ****** $p < 0.001$.

are early indicators of the rostro-caudal gradient of limb coordination maturation [56]. Ethanol exposure during the trimester three equivalent appears to produce delays in most measures, followed closely by trimester two, while ethanol exposure at the first trimester produced subtle effects, with less than half of the milestones significantly altered. This pattern may suggest that the brain regions responsible for the development of these neuro-motor skills may be less sensitive to ethanol at early stages of neurodevelopment, such as neurulation and cell proliferation [32]. Also, fetal ethanol exposure can cause cell

death and neuronal reduction [57-62], that is known to lead to motor skill deficits [63]. Further, the impairments caused by late gestation alcohol exposure may be attributed to specific brain region development including synapse formation in the cerebellum and prefrontal cortex [33,61,63-65]. Such results are relevant to FASD as young children with FASD also show delayed motor development and fine-motor dysfunction including weak grasp, poor hand-eye coordination and poor balance [66-70], which is thought to result from damage to the cerebellum given its involvement in motor function control

Table 1. Achievement of developmental milestones of postnatal day ethanol-exposed and control offspring.

	Control Male T1 (n = 11)	T2 (n = 18)	T3 (n = 10)	Female T1 (n = 12)	T2 (n = 10)	T3 (n = 12)	Ethanol Male T1 (n = 13)	T2 (n = 11)	T3 (n = 9)	Female T1 (n = 14)	T2 (n = 20)	T3 (n = 12)
SR	6.5 ± 0.4	8.7 ± 0.3	7.3 ± 0.2	6.4 ± 0.4	8.4 ± 0.3	7.5 ± 0.3	***9.1 ± 0.4	*8.9 ± 0.3	***9.6 ± 0.3	***8.7 ± 0.5	*9.7 ± 0.3	***9.8 ± 0.2
NG	7.0 ± 0.4	6.2 ± 0.4	5.4 ± 0.2	7.5 ± 0.4	7.4 ± 0.3	5.8 ± 0.2	6.6 ± 0.5	***9.5 ± 0.7	6.0 ± 0.2	6.6 ± 0.4	***9.4 ± 0.3	5.9 ± 0.2
CA	9.0 ± 0.4	7.9 ± 0.5	5.1 ± 0.3	9.7 ± 0.4	6.4 ± 0.9	5.5 ± 0.2	9.1 ± 0.5	***9.7 ± 0.4	5.4 ± 0.2	8.7 ± 0.3	***10.4 ± 0.4	5.8 ± 0.2
FG	9.6 ± 0.6	10.0 ± 0.7	10.8 ± 0.3	9.3 ± 0.4	9.4 ± 0.8	11.1 ± 0.3	***11.5 ± 0.7	11.1 ± 0.5	**12.0 ± 0.3	***11.9 ± 0.5	11.6 ± 0.3	**11.8 ± 0.3
AS	14.7 ± 0.2	13.9 ± 0.4	10.3 ± 0.3	13.8 ± 0.5	14.0 ± 0.3	10.3 ± 0.3	13.1 ± 0.5	13.4 ± 0.8	**11.1 ± 0.3	13.8 ± 0.6	14.2 ± 0.4	**11.2 ± 0.2
ET	9.8 ± 0.3	9.3 ± 0.3	9.6 ± 0.2	9.5 ± 0.3	10.4 ± 0.5	9.7 ± 0.2	9.3 ± 0.4	10.4 ± 0.3	*10.3 ± 0.2	9.9 ± 0.5	10.4 ± 0.3	*10.2 ± 0.3
OFT	10.6 ± 0.6	10.9 ± 0.4	11.7 ± 0.1	11.4 ± 0.4	10.0 ± 0.4	11.8 ± 0.2	***12.5 ± 0.3	***12.4 ± 0.6	***13.0 ± 0.3	***12.8 ± 0.4	***12.5 ± 0.4	***13.3 ± 0.4
EO	14.7 ± 0.1	13.8 ± 0.2	14.6 ± 0.2	14.3 ± 0.1	14.2 ± 0.3	14.9 ± 0.2	14.5 ± 0.1	*14.6 ± 0.2	***15.4 ± 0.2	14.6 ± 0.2	*14.4 ± 0.2	***15.5 ± 0.2
AR	12.9 ± 0.7	12.1 ± 0.6	10.4 ± 0.3	11.0 ± 0.6	12.4 ± 0.5	10.8 ± 0.9	***14.1 ± 0.5	**14.0 ± 0.7	***14.0 ± 0.6	***15.2 ± 0.4	**14.2 ± 0.4	***13.6 ± 0.5

Table 1. Milestones include surface righting (SR), negative geotaxis (NG), cliff aversion (CA), forelimb grasp (FG), auditory startle (AS), ear twitch (ET), open field traversal (OFT), eye opening (EO) and air righting (AR). Mean (±SEM) number of days to complete each task is compared across males and females from trimester one (T1), trimester two (T2) and trimester 3 (T3), ethanol-exposed and control pups. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$.

[63,71]. The cerebellum is also known to be quite sensitive to the apoptotic effects of ethanol, particularly during later neurodevelopmental stages [72]. Our results support these findings, and suggest that ethanol exposure later in gestation may be more detrimental to the development of motor skills, reflexes and coordination.

4.2. Locomotor Activity and Anxiety-Related Behaviors

The literature is divided on the impact of prenatal alcohol exposure on activity levels [49,73,74]. Results included in this report suggest that this apparent discrepancy may be due, at least in part, to the timing of exposure during neurodevelopment (**Figure 1**). Our findings are consistent with other research that has observed increased activity in animal models following ethanol administration during earlier stages of fetal development [32,49,74,75], but the same pattern was not observed for mice treated during the trimester three equivalent. However, given that increased activity levels as well as anxiety-related traits are commonly found both in children and animal models of FASD [10,24,49,76], we sought to differentiate the effects of alcohol on activity versus novelty-induced stress by measuring nocturnal activity levels of the mouse (**Figure 3**). In a familiar, home-cage setting, the ethanol-treated mice, independent of timing of exposure, are significantly more active than control mice at certain peaks throughout the night, which agrees with the literature [7, 20,25,41,49]. Although the mechanism by which ethanol exposure leads to increased activity levels is unknown, however children with FASD often develop Attention Deficit Hyperactivity Disorder (ADHD) comorbidity [77-79]. This phenotype may be associated with neuronal

reduction within the basal ganglia, cerebellum, and the prefrontal cortex [80,81] or abnormal cortical thickness [82]. These abnormalities have been observed in both FASD and ADHD, and are implicated in executive functioning and motor activity [83,84].

Interestingly, our results indicate that anxiety-related traits may confound analysis of locomotor behaviors in FASD models, particularly in novel environments. In this study, we utilized two independent measures of both anxiety and activity, with the light/dark box test to confirm the thigmotaxis observations of the open-field assay and the home cage activity test to validate our open-field locomotor observations. The thigmotaxis we observed in the open-field locomotor tests was dependent upon the timing of ethanol treatment (**Figure 2**), although the observation of a decrease in anxiety-related behaviors in T2 does not follow previous reports in the literature. It is possible that trimester two exposure may not reduce anxiety, per se, but increases the risk of other FASD-related phenotypes, such as impulsivity [85], and/or an anxiolytic effect [23]. Conversely, increased anxiety-related behaviors, such as those in our T3 data, have been commonly reported in FASD-related literature [11,13,14, 86]. This may be due to ethanol's ability to affect the development and function of the hypothalamic-pituitary-adrenal (HPA) axis [87,88], leading to increased vulnerability to anxiety-like phenotypes during adolescence and adulthood [89].

4.3. Spatial Learning and Memory

Our results (**Figure 4**) provide further support for deficits in learning and memory caused by prenatal alcohol exposure [15,16]. Results from T1 and T3 (**Figures 4(b)**

and (**d**)) support other studies that have observed mice treated with ethanol either early in prenatal development or early neonatal development show significant impairment on spatial learning tasks [41,90-92]. This may be due to ethanol-induced neurodegeneration at these developmental times in specific brain regions, such as the hippocampus and prefrontal cortex [93-95] that are associated with learning and memory deficits in young adult mice [18,21,90,96].

Interestingly, mice exposed to ethanol during mid-gestation (T2) took longer than control mice to learn the location of the target on the first acquisition day, but were able to improve over subsequent training days (**Figure 4(c)**). This pattern is consistent with the suggestion that, with repetition, ethanol-exposed mice are able to perform as well as control mice by the end of the acquisition days [41]. These results are consistent with reinforcement learning in some children with FASD [9]. Search strategies used by T2 and T3 ethanol-treated mice provide further evidence that ethanol-treated mice have difficulty in remembering the location of the target in this task, which may be attributed to the direct or indirect effect of ethanol during neurodevelopment. This follows previous reports in children with FASD who show altered developmental transitioning from visual to verbal memory strategies [97], and who also improve on learning and memory tasks when given an implicit learning strategy [98].

4.4. Sources of Error

We recognize that our study may include some still unrecognized effects that are not easily eliminated. These include potential litter and circadian effects. Also, we have relied on BAC data from previous studies from rodents, including C57BL/6 mice [29,94,99,100], rather than directly measuring BAC in our experimental animals. This has allowed us to predict blood alcohol levels to be above a critical threshold of neurodegeneration (200 mg/dl) [29]. In addition, the results from direct alcohol treatment of pups in trimester three, compared to the indirect treatment of fetuses in trimesters one and two, must be interpreted with caution. The dose used in our experiment accommodates the weight of the animals; however, our experimental paradigm does not match the direct exposure of pups in trimester three with the other treatments. Therefore, the pups' sensitivity to alcohol may contribute to the severity of behavioral results.

4.5. Conclusion

We have examined the effects of alcohol exposure during specific neurodevelopmental times approximating the three human trimesters using a consistent dosage. We show that acute ethanol exposure during neurodevelopment consistently leads to delays in achievement of mo-tor skill coordination and spatial learning, regardless of timing of exposure. However, activity and anxiety phenotypes may be more sensitive to the timing of alcohol exposure and potentially confounded with one another, as well as other FASD-associated endophenotypes. This study provides a framework for a comparison of phenotypic outcomes using a consistent treatment paradigm across major neurodevelopmental stages, which may be useful in examining the underlying biological mechanisms. The results add to the growing body of evidence that the brain is sensitive to alcohol throughout neurodevelopment, and that the timing of exposure may offer an explanation for the extensive heterogeneity associated with developmental spectrum disorders including FASD.

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