NEONATAL ETHANOL EXPOSURE IMPAIRS OBJECT-IN-PLACE LEARNING AND TRACE FEAR CONDITIONING IN JUVENILE RATS

by

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ABSTRACT

The purpose of this study was to extend our previous work on neonatal alcohol exposure and object recognition performance (Jablonski et al., 2013) to the object-in-place (OiP) task. This task requires memory of both object identity and object location, and therefore serves as a combination of the standard object-recognition (OR) and object-location (OL) tasks (Barker et al., 2007; Barker & Warburton, 2011; Jablonski et al., 2013). The present study utilized 4-Object and 2-Object variants of the OiP task that were modeled after other studies (Barker et al., 2007; Ainge & Langston, 2012, respectively). Rats preferentially explore novelty—in the OiP task, an object is novel if it changed locations with another object (4-Obj) or replaced another object (2-Obj) between sample and test phases. In the 4-Object variant, 4 different objects are presented during the sample phase; the locations of the 2 left or right objects are interchanged for the test phase. The 2-Object variant consists of 2 different objects during the sample phase; one of these objects is replaced with an identical copy of the remaining object for the test phase. Lesion studies have implicated roles of prefrontal cortex (PFC), hippocampus (HPC), and perirhinal cortex (PRH) in this task (Barker studies, cited above), while trace fear conditioning (TFC) is believed to engage both PFC (Gilmartin & Helmstetter, 2010) and HPC (McEchron et al., 1998). Additionally, neonatal alcohol disrupts maturation of PFC (Whitcher & Klintsova, 2008) and HPC (Marino et al., 2004). Therefore, we predicted that neonatal alcohol would disrupt OiP learning, as well as visual TFC as a "positive control" task (Schreiber et al., 2012). We report that normative PD26 rats can perform the 4-Object,

but not 2-Object, variant of the OiP task, which may imply reliance of these tasks on different neural mechanisms. Neonatal ethanol exposure during postnatal days (PD) 7-9 disrupted both the 4-Object variant of OiP in PD26 rats, as well as TFC—but not background contextual conditioning during TFC—in PD30-31 rats. These findings underscore the previously supported claim that OR, OL, and OiP tasks rely on different neural regions and/or systems; furthermore, they are interesting in the context of our recent report that PD7-9 ethanol exposure does not impair OR and OL tasks (Jablonski et al., 2013). Future studies could better inform understanding of the relationship between ethanol exposure window, brain targeting, and behavioral deficits.

Chapter 1

INTRODUCTION

Disorders resulting from human prenatal alcohol exposure have introduced challenges in societal, medical, and economic domains. Research citing specific values of incidence rate is limited due to difficulties related to diagnostic criteria as well as measurement. A study performed in 1991 estimated the incidence rate of FAS alone at approximately 0.33 cases per 1,000 live births in the "western world." Depending on the socioeconomic status of a given region, this estimate may range from 0-1.9 cases out of 1,000 live births (Abel & Sokol, 1991). Because of diagnostic ambiguities, these values do not include non-FAS diagnoses, such as alcohol-related neurodevelopmental disorder (ARND). A second, longitudinal study in the western United States estimated the combined incidence of FAS and ARND to be much greater—1 in 100 live births (Sampson et al., 1997). Therefore, a comparison of these values leads to the conclusion that FAS individuals compose only a small fraction of total diagnoses related to prenatal alcohol exposure, all of which have been labeled under the umbrella term "fetal alcohol spectrum disorders" (FASDs; for review, Kodituwakku & Kodituwakku, 2013). More recent research suggests that FASDs are significantly more common than previously thought, estimating the incidence in school children of the United States and certain European countries to be 2-5% (May et al., 2009). The diagnosis of fetal alcohol-type disorders is made on the basis of three categories: growth retardation, facial dysmorphology, and central nervous system (CNS) dysfunction. Moreover, detrimental effects on intelligence and various

neurological functions, such as attention, learning and memory, language, motor skills, and spatial reasoning, have been observed (for review, Mattson & Riley, 1998). General morphological brain abnormalities, such as thinning of cortical regions, have been reported in individuals with FASD as well (Zhou et al., 2011).

In a study of adolescent children, those diagnosed with FAS suffered from deficits related to spatial and delayed object recall tasks, as well as visuospatial drawing tasks (Uecker & Nadel, 1996). In a virtual Morris water maze task, human adolescent males diagnosed with FAS were impaired in locating a hidden platform without a cue, which adds further support to the claim that alcohol exposure disrupts spatial navigation (Hamilton, Kodituwakku, Sutherland, & Savage, 2003). This finding parallels research that examined Morris water maze (MWM) learning in rats, where neonatal ethanol exposure significantly impaired performance (Goodlett & Johnson, 1997; Marino, Aksenov, & Kelly, 2004).

Disorders related to early-life ethanol exposure are commonly studied using a rodent model; this approach offers investigative opportunities that would be unethical or inefficient in humans. For one, rodent studies allow researchers to target developmental periods in a highly controlled manner. In rats, the brain growth spurt occurs postnatally and peaks just before 10 days of age (Dobbing & Sands, 1979). This window has been chosen by many investigators to model the human brain growth spurt, which occurs prenatally during the third trimester of gestation (Dobbing & Sands, 1973). Rodent model studies have revealed detrimental outcomes in a range of domains. Many studies have reported reduced cell counts within specific brain regions following ethanol exposure. Following exposure during the third trimester equivalent, studies reported decreased cell counts in hippocampal regions CA1 (Marino et al.,

2004), CA3, and the dentate gyrus (Livy, Miller, Maier, & West, 2003). Bonthius & West (1991) found long-term loss of mitral cells within the olfactory bulb. Somatic growth reduction, in combination with reduction of cerebellar Purkinje cells, has also been associated with such exposure (Goodlett, Pearlman, and Lundahl, 1998; Goodlett, Marcussen, & West, 1990). Some evidence suggests that the prefrontal cortex (PFC) may also be targeted by neonatal ethanol exposure (Whitcher & Klintsova, 2008). Finally, various reports have rescued ethanol-induced deficits via employment of pharmacological (Wagner & Hunt, 2006; Dokovna, Jablonski, & Stanton, 2013) and environmental (Hamilton et al., 2014) interventions.

In the behavioral realm, tasks engaging the hippocampus, sometimes in conjunction with other brain regions, are often chosen to measure ethanol-related deficits. The present study utilized two paradigms involving the hippocampus: object-in-place learning (OiP) and trace fear conditioning (TFC). The OiP task, like other object and object-location recognition paradigms, capitalizes on the rat's innate preference for novelty—rats typically explore new objects and locations instead of familiar ones (Berlyne, 1950). In the present study, the OiP task was first given in two different variants. In both variants, subjects received a sample phase and a test phase, which were separated by a delay period; during the delay, the sample phase presentation was altered to involve novelty. Trace fear conditioning involved the presentation of a conditioned stimulus (CS), followed by a stimulus-free trace interval, and then an unconditioned stimulus (US). Here, a flashing light served as the CS, while the US was a brief foot shock. Background contextual conditioning during TFC was also measured.

Both OiP (Barker, Bird, Alexander, & Warburton, 2007; Barker & Warburton, 2011) and TFC (Kronforst-Collins & Disterhoft, 1998; Quinn, Oommen, Morrison, & Fanselow, 2002; Gilmartin & Helmstetter, 2010; Schreiber, Brennan, Robinson-Drummer, & Stanton, submitted) require intact functioning of both the hippocampus and prefrontal cortex. Because previous research has reported detrimental effects on both regions following neonatal ethanol exposure, we hypothesized that both OiP and TFC may be disrupted following ethanol exposure in the present study (Whitcher & Klintsova, 1998; Marino et al., 2004).

Overall, the purpose of this investigation was to examine the effects of early-life alcohol exposure using a rodent model, during a specific time frame analogous to a highly sensitive period of human brain development. Experiment 1 examined performance of normally developing animals on two different variants of the object-in-place task. Experiment 2 added the ethanol-exposure manipulation and featured a within subjects design, such that rats were first tested on OiP performance and then on TFC, as well as background contextual conditioning during TFC. We predicted that neonatal alcohol would disrupt the OiP task, in contrast with the lack of deficit on object-recognition (OR) and object-location (OL) tasks, as discussed in our recent report (Jablonski, Schreiber, Westbrook, Brennan, & Stanton, 2013). Additionally, we expected that alcohol exposure may also impair TFC; results could be compared to another of our recent studies investigating the effect of hippocampal NMDA receptor blockade on TFC (Schreiber et al., submitted).

Chapter 2

EXPERIMENT 1: NORMATIVE OBJECT-IN-PLACE PERFORMANCE

Introduction

The purpose of Experiment 1 was to explore two variants of the object-in-place task using 2 objects (as described in Ainge & Langston, 2012) or 4 objects (described in Barker et al., 2007) with juvenile rats aged PD26. This paradigm had not been previously performed in our lab, and, although the 2-object task has been used in developing rats (Ainge & Langston, 2012), the literature lacks data on developing rats using the 4-object task. Our lab has previously determined that PD26 rats can perform both novel object recognition and novel object-location tasks (Jablonski et al., 2013). The OiP task is of special interest because it combines characteristics of both the novel object recognition and novel object-location tasks, which we have studied recently.

Materials and Methods

Subjects

Subjects were 24 Long-Evans rats (14 males and 10 females) derived from a total of 6 litters. There were two instances in which same-sex littermates assigned to the same condition (Task, Sex) contributed to the experimental data (4-Object Task, M, n=1; 2-Object Task, M, n=1). Within each pair, data were averaged together, and each pair was therefore counted as a single observation. Maintenance of the animal colony was the same as described in my previous papers (Jablonski et al., 2013;

Schreiber et al., submitted). Animals were bred at the University of Delaware, Office of Laboratory Animal Medicine (OLAM), where females were time-mated and housed overnight with breeder males. Dams and their pups were housed in clear, polypropylene cages (45 x 24 x 21 cm) with standard bedding and *ad libitum* access to water and rat chow. Cages were checked for births during the light cycle, and the date of birth was designated as postnatal day (PD) 0. Litters were transferred on PD2 from the breeding facility to the laboratory colony rooms, where they were maintained on a 12:12 hour light/dark cycle with lights on at 8:00 am. On the following day (PD3), litters were weighed and culled to 8 pups (typically 4 males and 4 females) and were paw-marked with subcutaneous injections of non-toxic black ink for identification. Pups were weaned from their dams on PD21 and housed with same-sex litter mates in clear, polypropylene cages (45 x 24 x 17 cm) with *ad libitum* access to water and rat chow. Animals remained in these cages until they were individually housed in small, white polypropylene cages (24 x 18 x 13 cm) on PD23, two days prior to experimentation.

Apparatus and Stimuli

With minor exceptions noted below, the apparatus used has been described previously (see Jablonski et al., 2013). Two or four identical circular arenas, measuring 78.7 cm in diameter and 48.9 cm in wall height, were used for the study. The walls and floor of the arena were constructed of white polyester resin panels, and the arena was raised 26.7 cm from the floor of the room. Each chamber featured two spatial cues, placed at the top of the wall. A black "X" made with electrical tape (10.5 x 9 in) was placed at the north position, and a paper circle striped with two colors (diameter of 8.5 in) was placed at the west position. These cues were placed at a

sufficient height to prevent the rats from interacting with them. Some distal cues (within the room, external to the chamber) were also observable. A tripod was placed outside of the chamber at the south position to allow video recording of the sessions for later analysis.

Four types of objects were used in this study (Figure 1). Three of them (objects A-C) were discussed in Jablonski et al. (2013), and object D was not. Object A was a fake green apple. Object B was a white plastic hook with a flat bottom. Object C was a small glass jar with a handle; it was filled with blue aquarium gravel. Object D was a small soda can (8 fl oz) filled with gravel for added weight. In the 2-Object task, objects A and C were used exclusively. All objects were chosen for their similarity in size but variance in color, texture, and shape. They were made of nonporous materials, which permitted easy cleaning. Velcro was used to secure the objects in place on the chamber floor. A rectangular section of the loop component of the Velcro (Velcro USA Inc., Manchester, NH) was attached to the bottom of each object; rectangular sections of the hook component of Velcro were placed on the chamber floor.

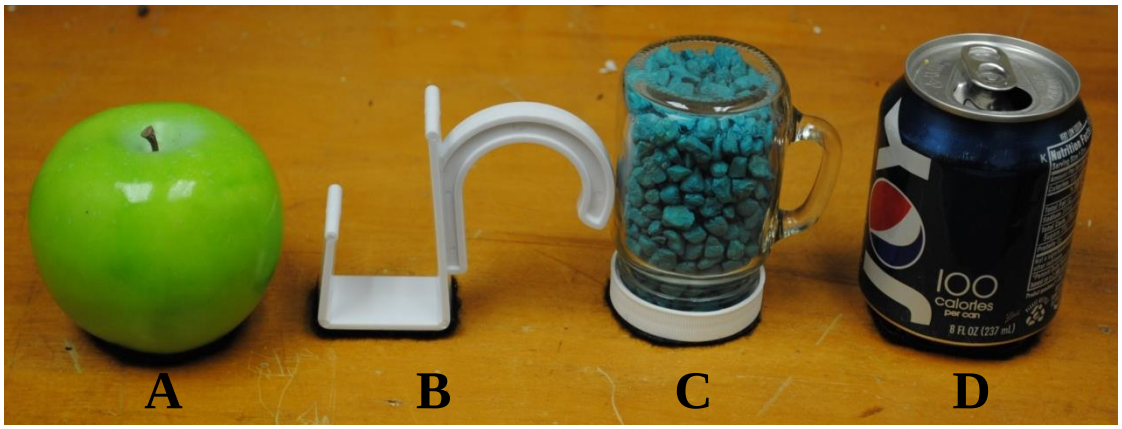

Figure 1 The four objects (A-D) used in the OiP tasks. Objects B and C were positioned so the hook or handle was faced eastward. Object D was positioned so the opening of the can was faced south. All four objects were used in the 4-Object variant of the task, while only objects A and C were used in the 2-Object variant.

Procedure

The general procedure used was similar to that which was previously reported (Jablonski et al., 2013). The 4-Object task was derived from Barker et al. (2007), while the 2-Object version was similar to that described in Ainge and Langston (2012).

Habituation

All rats received three habituation sessions before testing. At the beginning of a session, all rats were handled in the colony room for 3 minutes each. Rats were then weighed and carted two or four at once in their home cages to the behavioral testing room. The cart remained within a hallway outside of the chamber rooms while the chambers were cleaned with 70% ethanol. Each rat was then placed into a chamber, facing north, and the 10-minute exposure period began. There were no objects within the chambers during habituation. After 10 minutes, each rat was removed from the

chamber, returned to its home cage, and carted back to the colony room where it was replaced on the shelf of its "home" rack. The first and second habituation sessions began during the morning (8:00 am – 12:00 pm) and afternoon (1:00 pm – 6:00 pm), respectively, of the first day of experimentation, while the third session occurred during the morning of the second and final day. All sessions were separated by 5 hours ± 30 minutes, from "start" time to "start" time. Between days, sessions occurred at the time of the previous day's session ± 1 hour. Each habituation session was video recorded.

Testing

Testing occurred during the final session of the task, in the afternoon of the second day of experimentation. Rats were weighed and carted to the behavioral testing room in their home cages. They waited in the hallway while the chambers and objects were cleaned with 70% ethanol and while the objects were arranged in the proper configuration, with one object in each of four chamber quadrants (Figure 2). Rats were placed in the chamber in the north direction and allowed to explore for a 5-minute sample phase. After 5 minutes, rats were returned to their cages and replaced on the cart for a 5-minute delay period while the chambers and objects were cleaned and the objects were rearranged into the novel configuration. Rats were then replaced into the same chambers, featuring the novel object-place configuration, and allowed to explore for a 3-minute test phase. Following testing, rats were returned to their home cages and carted back to the colony room. The testing session occurred 5 hours ± 30 minutes following the beginning of the morning habituation session and ± 1 hour from the previous day's afternoon habituation session.

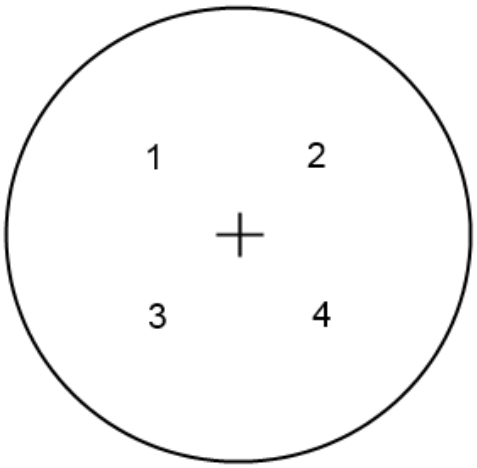

Figure 2 Diagram of object locations within the chamber. The "+" marks the center of the arena where each rat was placed at the beginning of each session (rat facing north direction).

4-Object Variant

Four different objects, one of each A-D, were placed in the arena locations 1-4 for the sample phase (Figures 2 and 3). During the delay period, either the two left objects (1 and 3) or the two right objects (2 and 4) exchanged locations. Objects A (apple) and C (jar) were always the novel objects; the lab has acquired a significant body of data to suggest that these objects are effective in both object recognition and object location tasks. Object location and side of switch (left or right) were counterbalanced across different rats.

2-Object Variant

Objects A and C were placed in the arena at locations 1 and 4, or 2 and 3, for the sample phase (Figures 2 and 3). During the delay period, one of the two objects

was removed and was replaced with an identical copy of the remaining object. Objects B and D were not used in this variant of the task.

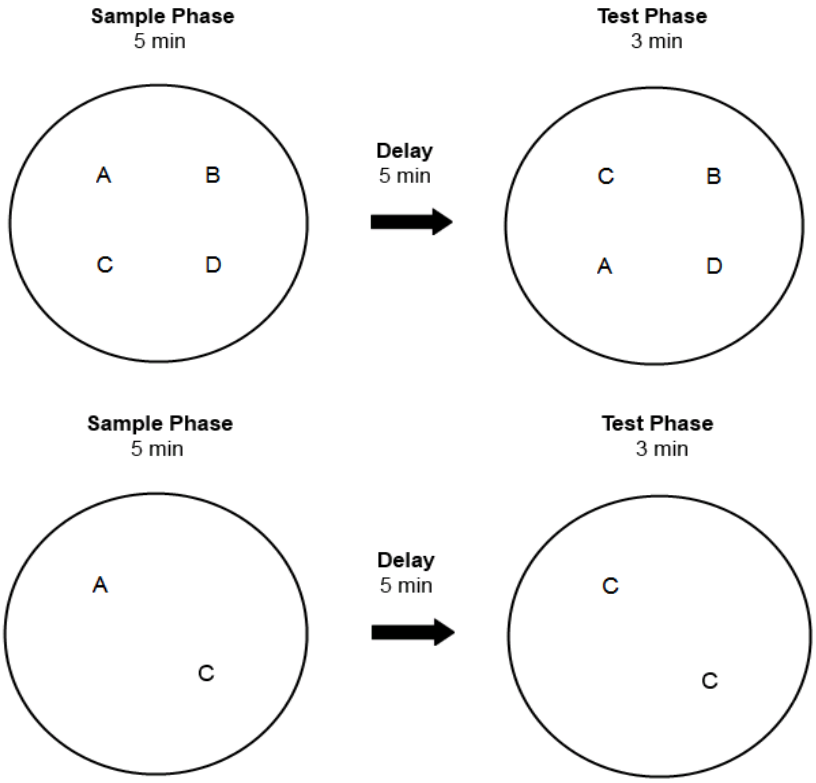

Figure 3 **(Top)** Schematic of the 4-Object variant of OiP showing object identity and orientation in sample and test phases. Four objects (A-D) were placed in the chamber for the sample phase. During the 5-min delay period, the experimenter exchanged the locations of the two left objects (A and C, shown) or two right objects for the test phase. **(Bottom)** Schematic of the 2-Object variant. Two different objects (A and C) were placed in the chamber for the sample phase. During the delay, one of the two objects was removed (A) and replaced with an identical version of the remaining object (C).

Data and Statistical Analysis

All 4 sessions of OiP were recorded using video camcorders (Panasonic USA, Models SDR-H80 and SDR-H85P). Video files were stored on a laboratory PC for analysis by observers blind to the experimental conditions. The sample and test phases of the final session were manually analyzed using a timer application (NILA Lab, Temple University) featuring "start time" and "stop time" buttons corresponding with left and right objects. This software allowed for the comparison of the rat's total time spent exploring the objects. Both the sample and test phases were scored in minute-long blocks, and overall exploration time (across the 3-minute test phase), as well as individual minute exploration times, were computed. Two second observers, blind to experimental conditions, scored a subset of the data to allow for verification of inter-observer reliability. Independent samples *t*-tests were run between observers for Total ($p>0.70$; $t=-0.39$), Min 1 ($p>0.49$, $t=-0.71$), Min 2 ($p>0.84$, $t=-0.21$), and Min 3 ($p>0.99$, $t=0.01$). Results demonstrate that observers were not different.

Total exploration times of left and right objects during sample and test phases were analyzed using factorial ANOVAs with Sex and Task as between subject factors.

Exploration ratios for novel versus familiar objects were computed for the overall test, as well as for individual minutes, to measure task performance. The following equation takes as a ratio the time spent exploring the novel objects out of the entire time spent exploring any object: $t_{novel} / (t_{novel} + t_{familiar})$. These ratios were compared to the ratio of 0.5, or chance performance. This value would result if the rat showed no preference, i.e., explored all objects equally: 1 min / (1 min + 1 min) = 0.5. Matched-pair *t*-tests were used to compare experimental exploration ratios to the chance performance exploration ratio of 0.5. For each measurement interval in the test phase (Total, Minute 1, Minute 2, Minute 3), outliers were defined as any novelty ratio

that exceeded ± 2 standard deviations from the group mean. Outliers were removed separately within each measurement interval (i.e., if a subject was an outlier for Total novelty score, its values for individual minutes were not necessarily removed). Additionally, animals that did not explore any object within a given minute were removed from the analyses for that minute. This minimized the number of scores that were removed from the analyses to a median of 1.5 scores per group.

Other statistical analyses were completed with STATISTICA 12 software. Factorial analyses of variance (ANOVA) were calculated with Task (4-Object or 2-Object) and Sex as between subject factors. Four factorial ANOVAs were run on each of Total, Minute 1, Minute 2, and Minute 3 novelty ratios.

Results and Discussion

Results

Sample Phase

Exploration Time

A factorial ANOVA was computed to examine effects of Sex or Task on exploration time during the sample phase. There was a significant effect of Task [$F(1, 18)=16.60$, $p<0.001$], such that the 4-Object group (84.22 ± 8.51 s) explored significantly more than the 2-Object group (43.19 ± 4.51 s), probably reflecting the greater number of objects to explore in the 4-Object task. There was no main effect of Sex, nor was there a Sex x Task interaction (all ps>0.69).

Test Phase

Exploration Time

A factorial ANOVA revealed a significant effect of Task [$F(1, 18)=9.790$, $p<0.01$], where the 4-Object group (33.82 ± 4.23 s) explored for significantly more time than the 2-Object group (17.22 ± 3.49 s). The effect of Sex was marginally significant ($p>0.06$), where males (31.16 ± 4.80 s) explored more than females (20.83 ± 4.05 s). There was no Sex by Task interaction ($p>0.72$).

Novelty Preference

The results from Experiment 1 are displayed in Figure 4. Across the total and individual minute novelty ratios, 8 scores were removed as outliers [4-Object Task: Min 1 (1 F), Min 2 (1 M), Min 3 (2 M); 2-Object Task: Total (1 M), Min 1 (2 M, 1F)]. Five animals did not explore the objects within a given minute and were removed from the analyses for that minute [2-Object Task: Min 2 (2 M), Min 3 (2 M, 1 F)]. After outliers and animals that did not explore were removed, the remaining group sizes were as follows: [4-Object Task: Total (n=11; 6 M, 5 F), Min 1 (n=10; 6 M, 4 F), Min 2 (n=10; 5 M, 5 F), Min 3 (n=9; 4 M, 5 F); 2-Object Task: Total (n=10; 5 M, 5 F), Min 1 (n=8; 4 M, 4 F), Min 2 (n=9; 4 M, 5 F), Min 3 (n=8; 4 M, 4 F)].

Effects of Sex and Task on the novelty ratios were examined using four factorial ANOVAs—one for each of Total, Min 1, Min 2, and Min 3 novelty scores. There were no main effects or interactions within any measurement interval (all Fs<3.125; all ps>0.09). Matched-pair t-tests were conducted against chance performance (0.5) and revealed strong preference for the novel objects in the 4-Object task, but not in the 2-Object task (Figure 4). Subjects in the 4-Object group failed to exhibit a significant total novelty ratio ($p>0.22$) but demonstrated strong novelty

preference within Minutes 1 (p<0.01) and 2 (p<0.05). In Minute 3, the 4-Object group displayed significant preference for the familiar objects (p<0.01). The 2-Object group failed to exhibit significant novelty preference within any minutes of the test (all ps>0.23).

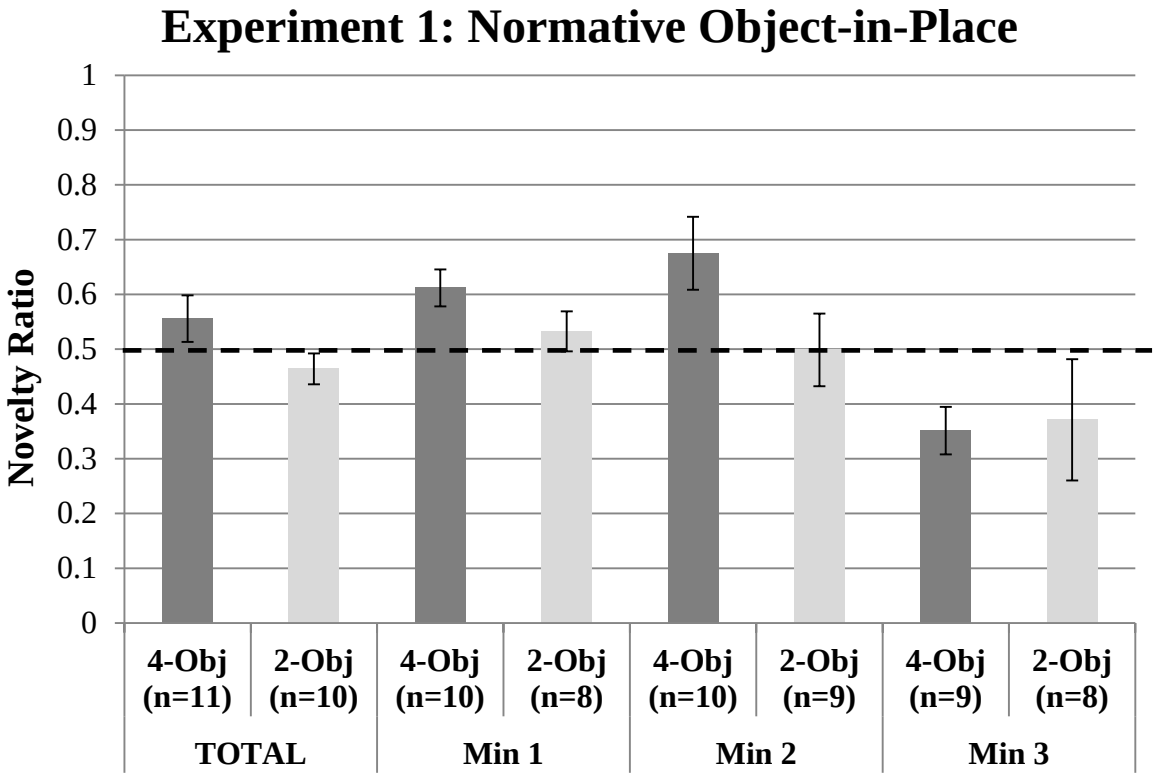

Figure 4 Results of Experiment 1 displayed with total and individual minute novelty ratios for the 4-Object and 2-Object tasks. Novelty ratio is calculated using the following equation: $(t_{novel}) / (t_{novel} + t_{familiar})$. Mean novelty ratios are depicted with error bars (SEM). The dotted line represents chance performance (0.5). The 4-Object group demonstrated significant novelty preference during Minutes 1 (p<0.01) and 2 (p<0.05) of the 3-min test, while the 2-Object group failed to exhibit novelty preference in any minute of the test.

Summary and Discussion

The 4-Object group demonstrated significant novelty preference in Minutes 1 and 2. In Minute 3, this group displayed significant familiarity preference, which drove the lack of total significance across the entire test. The failure to achieve significance for the Total exploration ratio could be attributed to an insufficiently large sample size; this possibility is discussed in greater detail within the General Discussion. Overall, these findings extend the current knowledge of the 4-Obj OiP task, which has never been studied in developing rats.

Chapter 3

EXPERIMENT 2: ETHANOL EFFECTS ON OBJECT-IN-PLACE PERFORMANCE AND TRACE FEAR CONDITIONING

Introduction

The results of Experiment 1 showed that normally developing PD26 rats exhibit novelty preference during the 1st and 2nd minutes of the 4-Object OiP task. Experiment 2 sought to examine the effects of neonatal ethanol exposure on PD7-9 on the OiP task and TFC. Animals were tested on OiP first and then on TFC. Previous research has suggested a role of the hippocampus and prefrontal cortex in both tasks (Barker & Warburton, 2011; Quinn et al., 2002; Gilmartin & Helmstetter, 2010), and alcohol exposure is believed to impair hippocampal (Marino et al., 2004) and prefrontal (Whitcher & Klintsova, 2008) function. Therefore, we predicted that ethanol may disrupt performance on both tasks.

Materials and Methods

Subjects

Maintenance of the animal colony, weaning, and housing were the same as described in Experiment 1. As in Experiment 1, animals remained in weaning cages until they were individually housed in small, white polypropylene cages (24 x 18 x 13 cm) on PD23, two days prior to the start of the OiP task. Additionally, one animal was removed from the analyses because its BAC value was a statistical outlier (BAC=180.7 mg/dl).

Object-in-Place

Subjects were 39 Long-Evans rats (20 males and 19 females) derived from a total of 11 litters. There was one instance of same-litter sampling (Sham, F, n=1).

Trace Fear Conditioning

Subjects were 32 Long-Evans rats (16 males and 16 females) derived from a total of 9 litters. There was one instance of same-litter sampling (Sham, F, n=1). The total number of subjects differs between object-in-place and trace fear conditioning because one cohort of OiP animals was not trained on TFC.

Ethanol Dosing

The ethanol dosing procedure was similar to that previously described (Murawski & Stanton, 2011). Pups from 9 litters were randomly assigned to one of two groups (ethanol or sham-intubated) for PD7-9 dosing sessions. For 3 consecutive days, the ethanol (EtOH) group received 1 dose per day of 5.25 g/kg ethanol-milk formula solution administered at 23.94% v/v and 0.02778 ml/g body weight. Rat milk was custom-made (see Kelly & Lawrence, 2008 for details). Fluids were delivered via intragastric intubation: Thin PE10 tubing, lubricated with corn oil, was passed through the animal's esophagus into its stomach where the liquid was infused. On the first day of the procedure, subjects in the EtOH group received a morning dose (beginning between 8:00 am – 10:00 am) of ethanol followed by two doses of rat milk formula (without ethanol) at 2 h ± 5 min intervals from the first dose; the second and third days included one morning dose of ethanol followed by one milk-only dose 2 h ± 5 min later. Milk-only doses were administered as nutritional supplementation to maintain body weight, as rat pups do not suckle while intoxicated. The sham intubation (SI) group received intubation persisting approximately 10 s (without any infusion of

liquid) at the same 2 h ± 5 min intervals as the EtOH group. Weights for all animals were recorded on each day prior to the first dosing session. Individual dosing sessions were completed within approximately 20-30 min per litter. During this period, pups were separated from their dam and kept in plastic containers on a heating pad.

All subjects were tail-clipped for blood sampling on the first day of the procedure, during the second dosing session of that day. Approximately 20 μL of blood was collected from each pup in a heparinized capillary tube. Samples from SI animals were discarded, and samples from EtOH animals were stored for later analysis.

Blood Alcohol Concentration (BAC) Analysis

Blood alcohol content (BAC) analysis was performed as previously described (Murawski & Stanton, 2011). On the day of collection, blood samples taken during the dosing procedure were centrifuged; the plasma was then collected and stored at -20 °C. An Analox GL5 Analyzer (Analox Instruments, Lunenburg, MA) was used for sample analysis. BAC values represent the rate of oxidation of alcohol in the samples. BACs were calculated based on comparisons to values acquired with an alcohol standard solution.

Apparatus and Stimuli

Object-in-Place

The apparatus and stimuli used for this task were the same as described in Experiment 1, except pink (instead of blue) aquarium gravel was sometimes used for Object C (the jar).

Trace Fear Conditioning

Training

The apparatus used for trace fear conditioning has been previously described (Schreiber et al., submitted). Training occurred in a Med Associates (Med Associates Inc., Georgia, VT) ENV-008 Standard Modular Chamber with an interior measurement of 30.5 x 24.1 x 21.0 cm. An ENV-005A shock grid served as the chamber flooring, and a modified ENV-227 house light (contained within a red cap) provided low-level illumination. Each chamber was housed within an ENV-022MD medium-density fiberboard cubicle (22 x 15 x 16 in). Each cubicle was fitted with a small fan that was allowed to run during training for background noise. The conditioned stimulus (CS) was provided by a white light bulb (25-W; A19 Frost), which was mounted on the cubicle interior at a height matching the top of the training chamber's back wall. The unconditioned stimulus (US) was a 1-s, 2 mA foot shock that was delivered via the shock grid (i.e., chamber floor). Between subjects, the paper towel underneath the grid bars was replaced, and chambers were washed with deionized water and then dried. This apparatus was controlled using a PC computer equipped with MED-PC (Med Associates Inc., St. Albans, VT) software.

Testing

The testing context was the same as previously described (Schreiber et al., submitted). Testing chambers were located on the opposite wall of the room that housed the training chambers, and they were constructed of Plexiglass and wire mesh (23.5 x 23.2 x 29 cm). A white piece of standard printer paper (8.5 x 11 in) was attached to the outside of the back wall of each chamber. Each chamber was housed within an open animal chamber outfitted with acoustic foam (BRS/LVE, Laurel, MD).

The CS light bulb was identical to that used during training and was mounted on a wood block exterior to the right chamber wall. Low-level illumination was provided by two small, red light bulbs (5-W)—one located at the center of the chamber ceiling (exterior to the chamber) and the other installed adjacent to the CS bulb. Between subjects, the paper towel underneath the grid bars was replaced, and chambers were washed with deionized water and then dried. Testing sessions were videotaped with video cameras (Panasonic USA, Models SDR-H80 and SDR-H85P).

Procedure

Experiment 2 compared performance on the OiP and TFC tasks in the same subjects. Animals were trained on the object-in-place task (PD25) prior to trace fear conditioning (PD29-30).

Object-in-Place

The OiP task in Experiment 2 was essentially identical to the 4-Object task used in Experiment 1. Rats were individually housed on PD23; from PD25-26, they were handled, habituated to chambers, and tested.

Trace Fear Conditioning

Training

Animals were trained on PD29-30 and presented with 10 CS-US trials over a roughly 50-min period. Subjects were given a 5-min adaptation period prior to the onset of the first trial. Each trial consisted of a 10-s CS presentation, followed by a 10-s stimulus-free trace interval, followed immediately by a 1-s US. The CS flashed two

times per second. The inter-trial interval (ITI) was 250 ± 50 s. Following training, animals were returned to their home cages and replaced in the colony room.

Testing

Cued and contexts tests were administered 24 h ± 2 h and 48 h ± 2 h, respectively, following training. The cued testing session occurred in a novel context and consisted of a 1-min baseline period followed by a constant 1-min CS-alone presentation. (The floor of the novel context consisted of wire mesh and so shocks could not be administered during testing.) After a total of 10 min, subjects were placed back into their cages and returned to the colony room. The context test, administered two days following training, consisted of a 5-min, stimulus-free period in the training chambers. All conditions were the same as in training, except neither the CS nor the US was presented. Both the cued and context tests were videotaped for later analysis.

Data and Statistical Analysis

Blood Alcohol Concentration (BAC) and Body Weights

An independent samples *t*-test was used to compute any effect of Sex on BAC. Factorial ANOVAs were run on body weights with Sex and Condition as between subject factors: Effects were measured for the three days of dosing, PD7-9, as well as for the first days of OiP (PD25) and TFC (PD29 or 30). Additionally, a repeated measures ANOVA was run across PD7-9 to measure the effects of Day (PD7, 8, and 9), Sex, and Condition on body weight.

Object-in-Place

Analysis of OiP data was the same as described in Experiment 1, except the comparison between tasks (4-Object vs. 2-Object) was replaced with the comparison between condition (Ethanol vs. Sham). Once outliers and "zero exploration" scores were removed, the median number of scores removed per group was 2. As in Experiment 1, two second observers who were blind to experimental conditions scored a subset of the data to allow for verification of inter-observer reliability. Independent samples *t*-tests revealed that there were no differences between observers for Total ($p>0.82$, $t=-0.22$), Min 1 ($p>0.79$, $t=-0.27$), Min 2 ($p>0.82$, $t=-0.23$), or Min 3 ($p>0.90$, $t=-0.12$) novelty ratios.

Trace Fear Conditioning

Trace fear conditioning was analyzed by manually measuring freezing behavior, in a manner identical to the analysis method previously described (Schreiber et al., submitted). Freezing has been defined as the lack of any movement, save for that associated with breathing (Fanselow, 1980). A time-sampling procedure was used for analysis of both cued and context testing. For cued testing analysis, 5 observations were made during the 1-min pre-CS baseline period, and 5 observations were made during the 1-min CS presentation. Observations were separated by 12-s intervals. A judgment of whether or not the animal was freezing was made based on behavior observed over the first 10 frames of the 1-s observation period. Context testing analysis consisted of 25 observations, occurring at 12-s intervals over the 5-min testing period. Again, judgments regarding freezing behavior were made by observing the first 10 frames of the second. The amount of freezing behavior was quantified as a % change value, which was acquired by subtracting pre-CS (baseline) freezing from

CS-elicited freezing (% change in freezing = CS freezing – pre-CS freezing). All video files were analyzed by the primary experimenter, who was blind to the experimental conditions. Additionally, approximately 25% of the video files were scored by a second, blind observer for inter-rater reliability analysis. For cued analysis, inter-rater agreement was 96.7%, and for context analysis, it was 94.2%.

Results and Discussion

Results

BAC and Body Weight Analysis

Ethanol group BACs, as well as body weights of Ethanol and Sham group animals, are displayed in Table 1. An independent samples *t*-test revealed no effect of Sex on BAC ($p>0.56$).

A repeated measures ANOVA for body weights across PD7-9 revealed a significant effect of Day [$F(2, 66)=229.3$, $p<0.001$], such that average body weight increased with each day. Additionally, there was a significant Day by Condition interaction [$F(2, 66)=147.3$, $p<0.001$], where Sham animals had greater weight gain than Ethanol animals across PD7-9. Growth retardation in Ethanol animals has been discussed previously within the literature (Dokovna et al., 2013; Jablonski et al., 2013). Finally, there was a significant Day by Sex interaction [$F(2, 66)=3.588$, $p<0.05$], such that males gained more weight than females with each day.

A factorial ANOVA compared effects of Sex and Condition on body weight measured on PD25, the first day of the first experiment (OiP). There were significant effects of both Sex [$F(1, 33)=8.722$, $p<0.01$] and Condition [$F(1, 33)=8.860$, $p<0.01$]. Ethanol group animals (59.44 ± 1.35 g) weighed less than Sham group animals (65.12

± 1.35 g), and females (59.62 ± 1.44 g) weighed less than males (65.25 ± 1.30 g). There was no interaction between Sex and Condition (p>0.95). Finally, a factorial ANOVA computed effects of Sex and Condition on PD29-30 body weight, which was measured on the first day of the second experiment (TFC). There was a significant effect of Sex [F(1, 33)=8.039, p<0.01], such that females (83.85 ± 2.30 g) weighed less than males (93.15 ± 2.12 g). There were no other main effects or interactions related to PD29-30 body weight (all ps>0.08). Consistent with previous reports, EtOH produced a transient decrease in body-weight gain that recovered by PD29-30.

Table 1 Body weights and BACs are displayed for Ethanol and Sham groups. Weights are in grams.

Condition	*n*=	PD7 Weight	PD8 Weight	PD9 Weight	PD25 (M) Weight	PD25 (F) Weight	PD29-30 (M)Weight	PD29-30 (F)Weight	BAC (mg/dl; PD7)
EtOH (5.25 g/kg/d)	**16**	**15.09** ± 0.273	**15.19** ± 0.335	**15.59** ± 0.382	**62.13** ± 1.32	**56.75** ± 2.01	**89.38** ± 2.05	**81.38** ± 3.59	**376.1** ± 17.38
SI	**21**	**15.17** ± 0.374	**17.67** ± 0.490	**19.59** ± 0.490	**67.33** ± 1.77	**62.17** ± 1.71	**95.67** ± 3.12	**86.06** ± 2.93	**N/A**

Object-in-Place

Sample Phase

Exploration Time

Exploration times during the sample phase were analyzed via a Sex and Ethanol condition factorial ANOVA. There was a marginally significant Sex by Condition interaction (p=0.063), such that in the Ethanol group, males (56.57 ± 9.99 s) explored more than females (43.70 ± 7.02 s). In the Sham group, females (62.96 ± 6.41 s) explored more than males (46.25 ± 7.20 s). Neither Sex nor Condition main effects were significant (all ps>0.56). The marginal exploration time effects did not influence novelty preference (see below).

Test Phase

Exploration Time

A factorial ANOVA measuring effects of Sex and Condition on test phase exploration revealed a marginally significant effect of Condition (p=0.065), where Ethanol animals (25.05 ± 3.84 s) explored more than Sham animals (17.29 ± 1.88 s). There were no other significant effects (ps>0.21). This effect may reflect poorer memory in ethanol animals.

Novelty Preference

The results from Experiment 2 OiP are displayed in Figure 5. Across the total and individual minute novelty ratios, 12 scores were removed as outliers: [EtOH: Total (1 M, 1F), Min 1 (1 M), Min 2 (1 F), Min 3 (1 M); SI: Total (1 M, 2 F), Min 1 (1

M, 1 F), Min 3 (2 F)]. Five animals did not explore the objects within a given minute and were removed from the analyses for that minute: [EtOH: Min 3 (2 F); SI: Min 2 (1 F), Min 3 (1 M, 1 F)]. After outliers and animals that did not explore were removed, the remaining group sizes were as follows: [EtOH: Total (n=14; 7 M, 7 F), Min 1 (n=15; 7 M, 8 F), Min 2 (n=15; 8 M, 7 F), Min 3 (n=13; 7 M, 6 F); SI: Total (n=18; 10 M, 8 F), Min 1 (n=19; 10 M, 9 F), Min 2 (n=20; 11 M, 9 F), Min 3 (n=17, 10M, 7 F)].

Novelty ratios were analyzed across the entire 3-minute test phase, as well as within the individual minutes of the test (Figure 6). Matched-pair *t*-tests were conducted against chance performance (0.5) and revealed significant novelty preference in the Sham group but not in the Ethanol group (Figure 6). The Sham group demonstrated significant novelty preference overall ($p<0.01$), which was driven by a significant novelty preference within Minute 3 of the test phase ($p<0.05$). Sham performance during Minute 1 ($p>0.27$) and Minute 2 ($p>0.35$) was non-significant when compared to chance performance. The Ethanol group failed to display significant novelty preference overall ($p>0.25$), or during Minute 1 ($p>0.72$), Minute 2 ($p>0.15$), or Minute 3 ($p>0.16$).

Four factorial ANOVAs were used to determine effects of Sex or Condition on novelty ratios. For the total novelty score, there was a main effect of Condition [$F(1, 28)=12.47$, $p<0.01$], such that the Sham novelty ratio (0.585 ± 0.022) was significantly higher than the Ethanol novelty ratio (0.472 ± 0.024). There were no other main effects or interactions for Total novelty score (all *p*s>0.25) or for Minute 1 (all *p*s>0.24). For Minute 2, there was a marginally significant effect of Condition ($p=0.069$), but there were no other significant main effects or interactions (all *p*s>0.16). There was a main effect of Condition in Min 3 [$F(1, 26)=7.751$, $p<0.01$],

such that the Sham group novelty ratio was greater than the Ethanol group novelty ratio. There were no other significant effects within Min 3 (all *p*s>0.33).

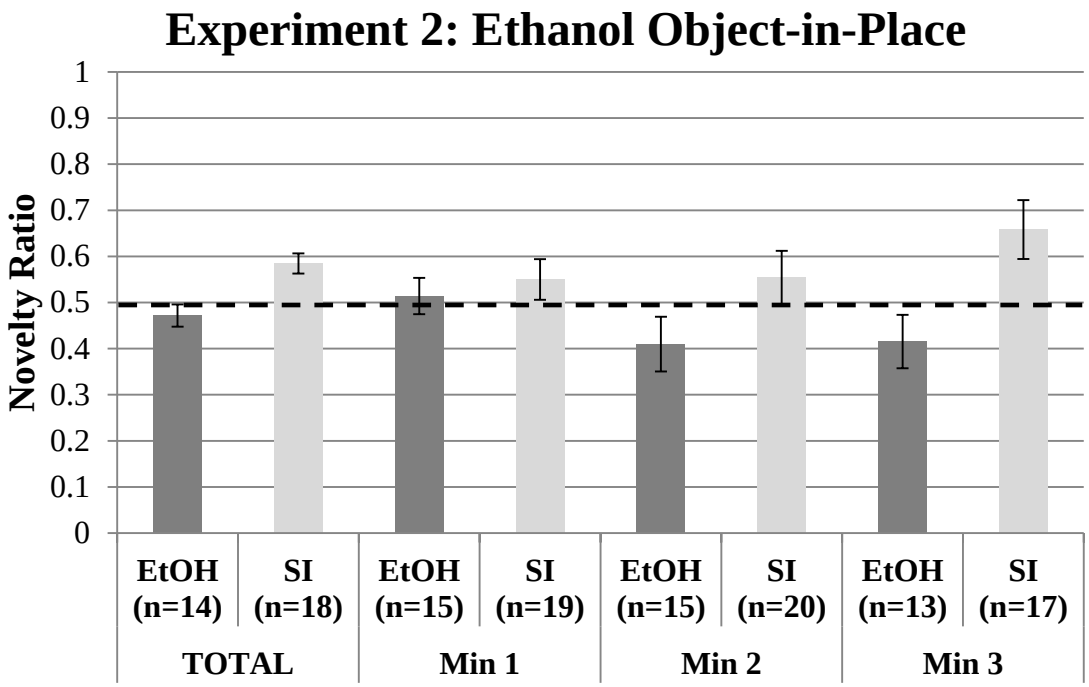

Figure 5 Results of Ethanol OiP displayed with total and individual-minute novelty ratios for the Ethanol and Sham groups. Novelty ratio is calculated using the following equation: $(t_{novel})/(t_{novel} + t_{familiar})$. Mean novelty ratios are depicted with error bars (SEM). The dotted line represents chance performance (0.5). The Sham group exhibited significant novelty preference overall ($p<0.01$), as well as within Minute 3 ($p<0.05$). The Ethanol group failed to demonstrate novelty preference during any minute of the 3-min test phase. Ethanol and Sham groups were significantly different from each other for both Total and Minute 3 scores (both *p*s<0.01).

Trace Fear Conditioning

Cued Fear Conditioning

The results for percent change in freezing from baseline are shown in Figure 7. Percentage freezing during the 1-minute baseline period was subtracted from freezing during the 1-min conditioned stimulus (CS)-presentation period. A total of 3 animals (EtOH, F, n=2; SI, F, n=1) met the criteria for statistical outlier and were removed.

Factorial ANOVAs were computed to analyze effects of Sex and Condition (EtOH vs. Sham) on baseline freezing (before CS onset), CS-elicited freezing, and the difference between CS-elicited and baseline freezing. There were no significant main effects or interactions for baseline or CS-elicited freezing (all *p*s>0.19). There was a significant effect of Condition [$F(1, 23)=10.81$, $p<0.01$] on the CS-baseline difference score, such that the Sham group (31.25 ± 4.07 %) froze significantly more than the Ethanol group (7.273 ± 5.47 %). There were no other significant main effects or interactions (all *p*s>0.37).

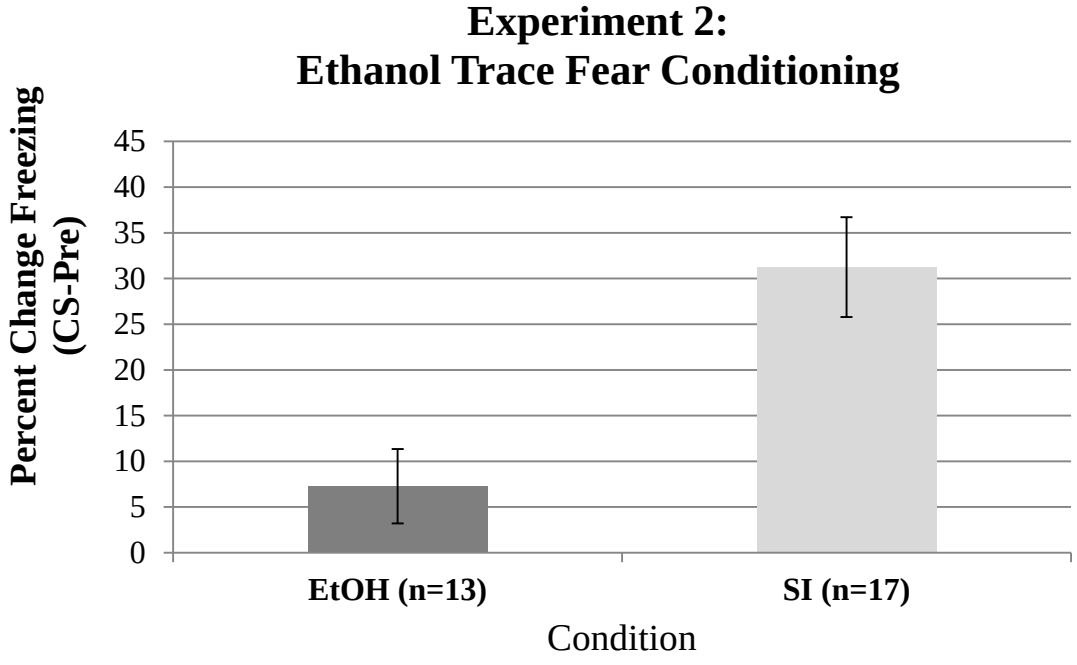

Figure 6 The mean percent changes in freezing scores (CS-Pre) are shown for Ethanol and Sham groups. The error bars represent SEM. These data reveal a statistically significant ethanol deficit on trace fear conditioning ($p<0.01$).

Contextual Fear Conditioning

A factorial ANOVA was used to examine effects of Sex and Condition on context testing. Two outliers were removed for the context test analysis using the same criterion for other measures (EtOH, F, n=1; SI, M, n=1). There were no significant effects related to Sex or Condition on context conditioning (all Fs<2.02; all ps>0.16; EtOH: 42.67 ± 5.83 %; SI: 54.13 ± 4.51 %). Therefore, alcohol treatment did not impair contextual fear conditioning.

Summary and Discussion

Object-in-Place

When ethanol and sham-intubated groups were compared, it was evident that PD7-9 ethanol exposure significantly impaired performance on both tasks. The OiP findings are especially interesting in the context of our recent research showing no ethanol deficit on OR and OL tasks following PD7-9 ethanol exposure (Jablonski et al., 2013). Differences between normative (Experiment 1) and Sham (Experiment 2) groups are discussed in the General Discussion.

Trace Fear Conditioning

A significant ethanol deficit was observed in the trace fear conditioning task, which suggests that neonatal ethanol exposure during PD7-9 is sufficient to disrupt acquisition of the CS-US association across the trace interval. However, contextual fear conditioning was not impaired. Further discussion is to follow.

Chapter 4

GENERAL DISCUSSION

Overall, this study reveals converging evidence for learning and/or memory deficits associated with neonatal exposure to alcohol. Experiment 1 examined learning of the object-in-place task in normative animals aged PD26; subjects in the 4-Object task demonstrated significant novelty preference in the 1st and 2nd minutes of the 3-minute test, while subjects in the 2-Object task failed to exhibit novelty preference in any minute of the test. The aim of Experiment 2 was to investigate the effects of neonatal alcohol exposure (PD7-9) on two different tasks—4-Object OiP and trace fear conditioning—that were expected to be sensitive to alcohol exposure. The results of this experiment demonstrated that neonatal ethanol exposure impaired performance in both the object-in-place and trace fear conditioning tasks. The implications of these results are discussed in relation to (1) other studies utilizing the same or similar tasks and treatment, as well as (2) the neurobiological mechanisms associated with alcohol-induced deficits in performance on these tasks.

Normative Object-in-Place

The current study adds to knowledge regarding the ontogenetic profile of the object-in-place (OiP) task. Generally, this paradigm combines object-recognition (OR) and object-location (OL) tasks, which each engage two separate cognitive demands. The OR task requires subjects to learn only the objects' identities, whereas the OL task involves memory of the objects' spatial locations (but not their identities). In the OiP

task, subjects must integrate information regarding both object identity and location to develop a conjunctive representation of a given object and location. Few comparisons can be made between the present 2-Object task results and results from other studies, as there are only two published articles that feature this task. This research is discussed below.

Ainge and Langston (2012) tested rats aged PD24 and PD30 on the 2-Object variant of the OiP task and found that PD24 rats failed to discriminate between novel and familiar objects; however, PD30 rats demonstrated significant novelty preference. In the present study, PD26 rats did not perform the task. This suggests that the ability of rats to perform this task may emerge sometime between PD26 and PD30. Therefore, the present findings have potentially narrowed the window of emergence of 2-Object OiP performance ability. (However, I have been unable to confirm 2-Object OiP performance in P31 rats—unpublished preliminary observations—suggesting other differences across studies may be important.) In comparison to the 2-Object variant, the 4-Object variant has been utilized in substantially more studies. However, the present study, to my knowledge, is the first study to examine performance of this task in juvenile rats. Additionally, the current results suggest that there may be differences between the two task variants. Whether performance on 4-Obj OiP is present at even younger ages, and why the 4-Object but not the 2-Object variants can be performed during the juvenile period, are questions for future research.

Because the OiP task features an object-location component, it seems reasonable to assume that it may be sensitive to hippocampal injury, which is a known consequence of PD7-9 ethanol exposure (Marino et al., 2004). Barker and Warburton (2011) determined that hippocampal lesions eliminate performance of the 4-Object

variant of the task, while Langston and Wood (2010) determined that hippocampal lesions did not disrupt adult rats' performance on the 2-Object variant of this task. For this reason, the findings of Langston and Wood (2010) are intriguing and potentially imply true differences between the 2- and 4-Objects variants of the object-in-place task. Perhaps, the 2-Object variant of the task requires a brain area or memory system interaction that emerges later in development than the hippocampus, thus explaining the inability of PD26 rats to perform this task in the current report.

Other lesion studies have elucidated the roles of brain regions underlying object-in-place learning (using the 4-Object procedure). The perirhinal cortex is necessary for object recognition, which includes the ability to discriminate between novel and familiar objects (Murray and Richmond, 2001; Buckley, 2005; Bussey, Saksida, & Murray, 2005). Therefore, it is expected that perirhinal lesions would disrupt object-recognition tasks (requires recognition of object identity only), as well as the object-in-place task (requires integrated object-identity and object-location information). This is precisely what Barker et al. (2007) observed in their disconnection analysis, which involved bilateral lesions of the medial prefrontal cortex (mPFC) or perirhinal cortex (PRH), as well as a simultaneous contralateral lesions of these regions. Another supporting finding is derived from the Barker and Warburton (2008) study, which determined involvement of medial prefrontal cortex NMDA receptors, as well as kainate receptors of the perirhinal cortex, in short-term (5 min delay) memory ability for the OiP task.

Additionally, the role of the hippocampus in spatial memory is well-documented; Barker and Warburton (2011) found that contralateral hippocampal-perirhinal or hippocampal-prefrontal lesion disrupted performance of the 4-Object-in-

place task. Together, the Barker (2007, 2008, 2011) studies suggest that a neural system comprised of these three regions (PRH, mPFC, HPC) underlies object-in-place task ability. Such an integrative relationship is supported by the known anatomical connectivity between the prefrontal cortex, hippocampus, and perirhinal cortex (Jay & Witter, 1991; Burwell, Witter, & Amaral, 1995). Yet another study utilized a disconnection analysis design with mediodorsal thalamic nuclei and the prefrontal cortex and also observed significant impairment on the OiP task (Cross, Brown, Aggleton, & Warburton, 2013). Wilton, Baird, Muir, Honey, and Aggleton (2001) found that excitotoxic lesions of the anterior and lateral dorsal thalamic nuclei disrupted performance of this task, which provides further support for a role of the thalamus. In sum, these studies suggest not only that these regions are required for OiP, but also that they function as an integrated system in a manner that is presently not well understood; because of this uncertainty regarding the precise function of these regions as a cooperative system, further investigation would be highly valuable.

Overall, the present study suggests that the 4-Object task is more robust than the 2-Object task, which may explain the greater prevalence of the 4-Object variant in the literature. The lack of consistency in results between studies (in conjunction with the overall lack of research utilizing the 2-Object tasks) necessitates further investigation of this paradigm. Effects of ethanol exposure on 4-Object OiP task performance, as well as differences between the normative and ethanol OiP experiments, are discussed later.

General Ethanol Effects

Neonatal ethanol exposure has been shown to produce a variety of impairments, which have been observed on both behavioral and cellular levels. Some

studies have associated learning and memory impairments with neuronal cell loss (Wozniak et al., 2004). Murawski, Klintsova, and Stanton (2012) reported ethanol-related decreases in hippocampal CA1 pyramidal cells, CA1 c-Fos^{+} cells, as well as freezing behavior during behavioral testing. Reduction in CA1 hippocampal cells is supported by other studies as well (Livy et al., 2003; Marino et al., 2004). Using the same ethanol exposure window as the Murawski et al. (2012) study, Hamilton et al. (2011) reported similar behavioral deficits, as well as reduced cell survival in the adult dentate gyrus. Other research has identified the cholinergic system as a target. Physostigmine, an acetylcholinesterase inhibitor, reversed the ethanol deficit when administered acutely during testing of the CPFE (Dokovna et al., 2013). Additionally, choline supplementation via subcutaneous injection has been shown to mitigate negative behavioral effects associated with neonatal (Wagner & Hunt, 2006) as well as post-training (Hunt, Levillain, Spector, & Kostelnik, 2009b) ethanol exposure. *N*-methyl D-aspartate (NMDA) receptors in the hippocampus have been associated with neural plasticity and learning (see Shapiro, 2001 for review); within our own lab, antagonism of these receptors via MK-801 in juvenile rats impairs a spatial task (object-location – Jablonski et al., 2013), a context task (CPFE – Schiffino, Murawski, Rosen, & Stanton, 2011), and trace fear conditioning (Schreiber et al., submitted). There is significant overlap between the underlying brain mechanisms of learning and memory on object-in-place and trace fear conditioning tasks—both tasks require the prefrontal cortex as well as the hippocampus. If these tasks share common learning/memory mechanisms that are also sensitive to alcohol, it may be predicted that ethanol-exposed animals will exhibit deficits in performance of both tasks.

Ethanol Object-in-Place

The novelty ratios reported in the current study are comparable to at least one other study using the same task (Dix & Aggleton, 1999), though the minute-by-minute trends vary. When compared to novel object-recognition (OR) and object-location (OL) tasks (Jablonski et al., 2013), the present novelty scores are lower, but this may be expected due to differences task complexity. When Sham animals from the present study are compared against sham lesion animals from other research using the same task (Barker et al., 2007; Barker & Warburton, 2008), it is again evident that subjects in the present study exhibited relatively lower levels of novelty preference. However, the other studies tested adult rats, while the present study tested juveniles. It is possible that the task is more "difficult" when performed close to the functional emergence of the brain regions or processes necessary to perform the task (i.e., during the juvenile period).

The disparity observed between the Sham (Experiment 2; significant total novelty ratio; n=21) and Normative (Experiment 1; non-significant novelty ratio; n=11) groups of the present study may be related to differences in sample size. The normative group displayed significant preference for familiar objects during the 3rd minute of the test, which drove the non-significance of the total novelty score. This result could be related to habituation effects (the "novel" objects may lose their salience with the passage of two-thirds of the total test time) or perhaps more likely, to recency effects (the "familiar" objects become relatively novel following extended exploration of the true "novel" objects); both of these effects serve to increase "noise" within the data. The Sham group (Experiment 2) trend from the present study differs from this pattern and from the OL findings in the Jablonski et al. (2013) study: in the present study, the Sham group average novelty score was not significantly different

from "no preference" performance until the 3rd minute of the test. This finding could potentially be attributed to early-life stress-induced impairment (i.e., recognition of the novel objects may require more time in comparison to normative animals). Alternatively, the 4-Object task Sham subjects may have required more time to orient themselves among 4 objects within the arena, as compared with only 2 objects used in 2-Object OiP, as well as in the OR and OL studies (Jablonski et al., 2013); this may explain the delay of significant novelty preference until the 3rd minute. Finally, if the test were extended beyond 3 minutes, we may expect to observe an eventual "drop-off" in novelty exploration by the Sham group, as was seen in the normative group.

Other data from our lab have shown that PD7-9 ethanol exposure does not disrupt the object-location task (Jablonski et al., 2013). This finding is interesting for two reasons. First, the Jablonski et al. (2013) study can be closely compared with the present study, as both were performed in the same lab, using nearly identical protocols, except for task—OL versus OiP. Second, the lack of ethanol deficit in the present study suggests several alternative hypotheses. First, the difference in results of the Jablonski et al. (2013) study versus the present study could potentially be explained by task difficulty; the demands of the object-in-place task are arguably more complex than the object-location task, primarily due to the required integration of object identity and location information. The object-location task is believed to require information on object location but not object identity because perirhinal lesions do not impair performance on OL (Barker et al., 2007). Second, it is possible that the OiP task engages hippocampus to a greater degree than OL and is therefore impaired by partial hippocampal injury to a greater extent than OL. Finally, the OiP task may require at least one process or brain region that is additional to the region(s) necessary

for OL. Here, the prefrontal cortex is a primary candidate, and sensitivity of this region to alcohol exposure is supported in the literature (Whitcher & Klintsova, 2008).

The ethanol OiP data reported in the present study offer the novel finding that the 4-Object OiP task is sensitive to neonatal (PD7-9) ethanol exposure. Furthermore, the data reported here strengthen evidence arguing for the necessity of the HPC for this task, though the role of disruption of additional brain regions or systems could also be an important factor. These findings are significant considering the lack of research investigating ethanol disruption of this task.

Trace Fear Conditioning

The freezing scores for cued fear conditioning in the current study are generally similar to those reported in other studies that determined impairments following PD4-9 exposure. To our knowledge, there are no published studies reporting significant deficits on TFC following PD7-9 exposure. Freezing of ethanol-dosed animals in the present study was very similar to experimental group (PD4-9 ethanol or MK-801) freezing in two studies (Hunt, Jacobson, & Torok, 2009a; Schreiber et al., submitted), and the effect sizes for both the ethanol and sham groups within the present study closely resembled those in another ethanol TFC study (Wagner & Hunt, 2006). Hunt et al. (2009a), as well as Schreiber & Hunt (2013a), reported increased freezing (average freezing score of ~ 50-60%) for sham-group animals of the same age, relative to the current study (avg. score of ~ 30%). The case was similar with the Schreiber et al. (submitted) study (intracranial infusion of MK-801), where sham-surgery animals exhibited comparatively higher levels of freezing (avg. score of ~ 50%). The source of these differences is unclear, but they could be related to parametric factors. The positive PD7-9 exposure findings reported here are supported

by studies utilizing other hippocampal-dependent tasks; Goodlett & Johnson (1997) observed significant impairment on the Morris water maze task in animals exposed to ethanol during PD7-9 and PD4-9. Additionally, our own lab has shown that PD7-9 exposure disrupts an alternative contextual fear paradigm, named the context pre-exposure facilitation effect (CPFE; Murawski & Stanton, 2011; Dokovna et al., 2013; Jablonski & Stanton, 2014).

Ethanol exposure did not significantly impair contextual fear conditioning in the present study, but ethanol animals did exhibit a slightly decreased percent freezing score in comparison to sham animals (Ethanol – 42.67 ± 5.83%; Sham – 54.13 ± 4.51%). This finding is interesting in juxtaposition with other studies that have determined significant disruption of contextual conditioning in animals with impaired hippocampal function. Schreiber et al. (submitted) found that intra-hippocampal infusion of MK-801 (NMDA receptor antagonist) prior to training produced significant deficits in contextual fear conditioning two days later (collapsed across task: MK-801 – 19.65% ± 4.17; PBS vehicle control – 36.89% ± 4.79). This disparity in findings could perhaps be attributed to differences between neonatal alcohol exposure and transient pharmacological inactivation of the hippocampus: Ethanol is associated with widely-distributed neural effects, while MK-801 specifically targeted NMDA receptors in HPC in the Schreiber et al. (submitted) study discussed above. Research on the medial prefrontal cortex has supported its role in TFC (Gilmartin & Helmstetter, 2010) and as previously mentioned, there is evidence to support alcohol-targeting of this region (Whitcher & Klintsova, 2008). Recent research within our lab found that PD7-9 ethanol exposure disrupted retention of the context-shock association in the CPFE paradigm; this effect was associated with impaired mPFC

function (Jablonski, under review). This finding is consistent with the Runyan, Moore, and Dash (2004) study, which determined PFC to be necessary for retention, but not encoding, of TFC. Other research from our own lab (Westbrook, 2014) showed that PD7-9 exposure does not disrupt the object-location task, while PD4-9 exposure prevents performance of this task. Therefore, it seems possible that PD7-9 ethanol exposure more specifically targets the prefrontal cortex, as opposed to the hippocampus. Perhaps, the window of neonatal alcohol exposure that targets the hippocampus occurs earlier than the window that targets prefrontal cortex. The PD4-9 exposure window may target the prefrontal cortex as well as the hippocampus, while the PD7-9 window may target mostly prefrontal cortex with only moderate effects on the hippocampus. In summary, prefrontal targeting by PD7-9 alcohol may explain why a deficit on trace fear conditioning, but not background contextual fear conditioning, was observed in the present study.

The findings of the trace fear conditioning task of Experiment 2 contradict at least one previous report. Hunt et al. (2009a) reported significant ethanol deficits on this task when PD4-9 exposed animals were trained on PD30; however, performance of their PD7-9 exposed group was no different from sham-intubated controls. Of the parametric differences existing between the Hunt et al. study (2009a) and the present study, it appears that only one could differentially influence the results. In their study, each day's ethanol dose was administered in two half-doses, instead of in a single dose as in the present study. Some research (Bonthius, Goodlett, & West, 1988) has suggested that the severity of ethanol-induced damage depends on the concentration of the dose (i.e., height of BAC peak), which could explain the different outcomes between the Hunt et al. (2009a) study versus the present study. Ikonomidou et al.

(2000) examined the relationship between total ethanol dose (and the number of sub-doses over which total dose was administered) and neuronal apoptosis; they found that the most critical factor was the duration for which BAC was maintained above the toxic threshold of 200 mg/dl. Their "5 g/kg over 2 doses" group exhibited the most severe neuronal loss, but they did not compare against a "5 g/kg over 1 dose" group, which would have closely modeled the present study's protocol. At least part of the neural damage resulting from ethanol exposure is related to the post-exposure withdrawal period. During this time, the inhibitory effects of alcohol induce NMDA receptor up-regulation, which provides the mechanism for the excitotoxic effects of alcohol withdrawal (Grant, Valverius, Hudspith, & Tabakoff, 1990; Gulya, Grant, Valverius, Hoffman, & Tabakoff, 1991). It is possible that 5.25 g/kg ethanol administered in a single dose (present study), as compared to 2 doses, is sufficient to not only maintain BACs above the toxic threshold, but also induce a higher peak BAC, which could be associated with a more severe withdrawal period. In sum, there exists some disparity within reported findings on the effects of PD7-9 ethanol exposure on trace fear conditioning. Further investigation of this issue, as well as the differences between administration of 1 versus 2 doses of a total ethanol dose, could be valuable.

Overall, the present findings suggest that trace fear conditioning, but not context fear conditioning, requires the function of brain regions that are impaired by neonatal ethanol exposure from PD7-9. The differences in findings between the current report and Hunt et al. (2009a) warrant further investigation on the effect of PD7-9 exposure on trace fear conditioning. Finally, further work could develop a

clearer understanding of the relationship between ethanol exposure window and targeting of various brain regions.

Summary and Conclusion

Taken together, the findings of the present study suggest that neonatal ethanol exposure from postnatal days 7-9 significantly impairs learning and/or memory on both an incidental learning task (object-in-place) and on a reinforcement-based task (trace fear conditioning). To my knowledge, the ethanol deficit observed with the object-in-place task is a novel finding and may serve as the foundation for additional work in this area. Further investigation of TFC may focus on the outcomes of ethanol exposure during a different developmental window (i.e., PD4-9) and/or may involve transient pharmacological manipulation. Future studies could help identify major brain structures or systems underlying these tasks, especially with regard to specific task demands, including CS-US association (TFC), as well as memory consolidation and recall.

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