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Moderate Prenatal Alcohol Exposure Impairs Performance in an Object-Place-Paired-Associate Task

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Abstract

Memory impairments, including spatial and object processing, are often observed in individuals with Fetal Alcohol Spectrum Disorder. Much attention has been directed towards the hippocampus, which displays significant alterations after moderate prenatal alcohol exposure (PAE). In the present study, we tested a moderate PAE rat model in an object-place-paired-associate (OPPA) task, previously shown to require hippocampal processing. The OPPA task was composed of training rats to discriminate between an identical pair of objects presented in 180° opposite arms of a radial arm maze. Animals were given a total of 10 trials per day over 14 consecutive days of training and were rewarded with a piece of cereal after the correct selection of an object. We observed that PAE rats (n = 8) made significantly more errors than saccharin (SACC) control (n = 8) rats during acquisition of the OPPA task. In Experiment 2, rats performed an object-discrimination task in which rats were trained in a single arm to accurately select a rewarded object from a pair of objects. Moderate PAE and SACC control rats exhibited comparable performance. In Experiment 3, rats performed a Morris water task experiment in

which rats were trained to find a hidden platform. The moderate PAE rats by the probe test performed at levels largely similar to the SACC rats. All three experiments combined we found that moderate PAE rats can learn to discriminate objects, places but they are impaired when given a more complex task of combining the two, in an OPPA task.

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Introduction

Consumption of alcohol while pregnant can lead to developmental disability including profound altered physical features, behavior, and neurological function (Berman & Hannigan, 2000a; Hamilton, Kodituwakku, Sutherland, & Savage, 2003; Kodituwakku, 2007; Mattson, Goodman, Caine, Delis, & Riley, 1999; Mattson et al., 1999). Fetal Alcohol Spectrum Disorder, which includes Fetal Alcohol Syndrome (confirmed alcohol exposure), partial FAS and Alcohol related Neurodevelopmental disorder are a major public health concern in the United States impacting approximately 2-5% of children especially since it is an avoidable public health concern (Chasnoff, Wells, Telford, Schmidt, & Messer, 2010; Rodriguez et al., 2016). While a great deal of research has been done to understand the effects of high dose Prenatal Alcohol Exposure (PAE), there is increasing evidence that moderate PAE is much more common and can also have a long-lasting impact on cognition and behavior (Marquardt & Brigman, 2016). One of the most striking behavioral abnormalities after PAE are deficits in learning and memory which can have serious repercussions for scholastic performance. Learning impairments are particularly noticeable once children begin to engage in complex math and reading comprehension assignments (Willford, Richardson, Leech, & Day, 2004). Since moderate PAE is usually diagnosed later in childhood, there is a considerable need to establish behavioral assessments for early detection and subsequent intervention.

Although there is currently no consensus regarding the precise neuroanatomical locus for impairments in learning and memory after PAE, considerable attention has been directed towards

the hippocampus, which displays significant synaptic and structural alterations in animal models of PAE (Berman & Hannigan, 2000b; Berman, Hannigan, Sperry, & Zajac, 1996). Theoretical and experimental work have linked the hippocampus to episodic memory, i.e., the recollection of the components of a personal experience, such as the spatial location of the event, the items, people, and objects encountered (Eichenbaum, 2014; Hasselmo et al., 2010; Knierim et al., 2006; Kesner & Hunsaker, 2010). Studies investigating the relationship between objects and places have utilized behavioral tasks which pair specific items and places (Gilbert & Kesner, 2002; Lee & Solivan, 2008; Sanchez et al., 2016). For example, subjects are rewarded when selecting object A only when it appears in location 1, but not in location 2. Object B is rewarded only when it is encountered in location 2, but not in location 1. The subject is therefore required to select a specific object on the basis of where it is encountered in the environment, often referred to as an object-place-paired associate. A large body of research has shown that damage to the hippocampus impairs the acquisition of object-place-paired associations (Crane & Milner, 2005; Gilbert & Kesner, 2002; 2003; 2004; Lee & Solivan, 2008).

It is currently unclear whether exposure to PAE results in deficits in object-place-paired associate learning. However, studies have investigated object learning in PAE animals (Kim et al., 1997a; Popovic, Caballerobleda, & Guerri, 2006a; Terasaki & Schwarz, 2016), with some reporting intact object discrimination, and others showing mild deficits in acquisition. A study by Kim et al., found no differences between PAE and control animals in the delayed non-matching to sample task for object recognition, in which animals were required to select a novel object after a delay. Although, impaired performance was detected in a variant of the delayed non-matching sample task in which during the delay rats were rewarded for distractor objects then the

matching sample to place task was resumed. The distractors were used to place greater load on the memory of objects (Kim et al., 1997b). In addition, Popovic et al., found mild learning impairments by PAE animals in a visual discrimination object recognition task (Popovic, Caballerobleda, & Guerri, 2006b). Further, Terasaki et al., reported learning impairments in a similar object recognition task in PAE animals, but only after animals received injections of a lipopolysaccharide, which induces neuroinflammation (Terasaki & Schwarz, 2016). It is important to note, however, that the studies above used rat models that were exposed to high doses of ethanol. The impact of moderate levels of PAE on these behaviors is unknown.

Several studies have investigated the impact of high dose and moderate PAE on spatial learning and memory. With respect to moderate PAE, several studies have reported intact place learning—the ability of animals to use cues to navigate to a location in an environment (Hamilton et al., 2010, 2014a; Sutherland, McDonald, & Savage, 2000). In the Morris water task, in which animals are trained to swim to a fixed hidden platform location, Hamilton et al., found intact acquisition in the Morris water task, and only detected impairments after moving the platform to a novel location (Hamilton et al., 2010, 2014a). In the moving platform manipulation, PAE animals largely swam at the platforms previous location and perseverated at this location during testing suggesting that PAE exposure reduces spatial flexibility. Sutherland et al. observed a similar pattern of impairments with normal acquisition of the Morris water task by PAE rats but reported longer swim latencies and impairments in the moving platform task. Sutherland et al also tested PAE rats in a version of the Morris water task where the proximal cues and the distal cues were placed in conflict due to the movement of the platform to the diagonal quadrant of the pool in which the animals had already been trained to go to. This tested

the relative use of distal cues versus the proximal cue (the platform) in this task. In this case the PAE rats showed proximal cue usage, going straight to the moved platform. In contrast, the control animals used the distal cues going to the previous platform location, thereby exhibiting place learning and suggesting that this latter form of learning might be impaired after PAE (Sutherland et al., 2000).

To summarize, few studies have been conducted to address the impact of PAE on object-place paired associate learning while some studies have investigated object discrimination. Of the studies on object discrimination, most have utilized rat models aimed at determining the effects of high dose PAE on behavior. Given these considerations, the aims of the present study were twofold: first, in Experiment 1 we evaluated the impact of moderate prenatal alcohol exposure on object-place paired associate learning using an established procedure in our laboratory (Fig. 1A; Sanchez et al., 2016). As described above, the task requires that rats learn to discriminate between two objects in two different locations in a radial arm maze. Second, in Experiment 2, we evaluated performance by moderately exposed PAE rats in an object-discrimination task in which rats had to accurately distinguish between two objects based on their unique features (Fig. 1B). Lastly, in Experiment 3, we tested moderate PAE and control rats in a hidden platform variant of the Morris water task, which required that animals learn to navigate to a particular place in the pool. The same animals were tested in each experiment and a well-established rat model of PAE was used. Briefly, animals were exposed to moderate levels of alcohol (BEC levels of ~ .80mg/dl). Pregnant rat dams voluntarily drank moderate amounts of ethanol during their pregnancy (Savage et al., 2010). Since the object-place task is more complex and perhaps closer to the requirements of demanding scholastic performance such as reading

comprehension and math, we hypothesized that moderate PAE rats will be impaired at establishing an association between sensory information and place in the maze environment in Experiment 1. Further, we hypothesized that moderate PAE rats will display intact acquisition of object discrimination and the Morris water task procedures in Experiment 2 and 3. The results are discussed in relation to the notion that object-place-paired associate learning could be utilized in test batteries assessing cognitive performance in animal models of PAE, for investigating the neurobiological basis of impaired learning in PAE, and the possibility that this task could be expanded to cognitive assessments of PAE in humans.

Materials and Methods

Subjects

Subjects included 16 male Long Evans rats which were obtained from the University of New Mexico Health Sciences Animal Resource Facility (breeding protocol can be seen below). All rats used were produced in the same breeding round. Following weaning all animals were pair housed with animals given the same prenatal treatment (either alcohol or saccharine exposed) in standard plastic cages on a reverse 12-hour light: dark cycle at a room temperature of 22 ^{o}C with food and water provided ad libitum. At approximately 120 days of age (4 months) during pre-training and behavioral experiments rats were placed on a food restricted diet of 90% of their Ad libitum diet weight and given access to water ad libitum. Food restriction continued through Experiments 1 and 2, but animals were provided food *ad lib* during Experiment 3. The

Institutional Animal Care and Use Committee (IACUC) at the University of New Mexico central campus and/or Health Sciences Center approved all procedures for the studies reported here.

Breeding and Voluntary Ethanol Consumption During Gestation

Breeding procedures were conducted at the University of New Mexico Health Sciences Animal Resource Facility (ARF). Three to four-month-old rat breeders (Harlan Industries, Indianapolis, IN) were single housed in standard plastic cages and placed on a 12-hour reverse light: dark cycle (lights on from 2100-0900 hours) and kept at 22 ^{o}C with ad libitum food and water. Following a brief one-week acclimation period in the animal facility, the breeders were exposed to a voluntary ethanol drinking paradigm. Female rats were provided 0.066% (w/v) saccharin (sac) in tap water from 10:00 to 14:00 hours (4 hours) each day. On days 1-2, the saccharin water contained 0% ethanol, days 3-4, saccharin water contained 2.5% ethanol (v/v). On Day 5 and subsequently, saccharin water contained 5% ethanol (v/v). The daily four-hour consumption of ethanol was monitored for at least two weeks and mean daily ethanol consumption was determined for each female breeder. After two weeks of daily ethanol consumption, females that drank at levels more/less than one standard deviation of the entire group mean (~12-15%) were removed from the study. The remaining females were then assigned to either a saccharin control or 5% ethanol drinking group. These breeding females were matched such that the mean pre-pregnancy ethanol consumption by each group corresponded.

Female rats were matched with a male breeder rat until pregnancy was verified. There was no ethanol consumption during breeding. On day one of gestation, for four hours a day,

from 10:00 to 14:00 hours the rat dams were given access to saccharine water containing either 0% (v/v) or 5% (v/v) ethanol. The volume of the 0% ethanol saccharine water provided to the control group was matched to the mean volume of the 5% ethanol saccharine water consumed by the ethanol group. During gestation and including the four hour ethanol/saccharine drinking period, rats were provided with ad libitum water and rat chow. The volume of ethanol consumed was recorded daily for each pregnant rat dam, and in the present study, the volume ranged from 1.87g/kg to 2.92g/kg. After birth, daily ethanol consumption ended and the litters were weighed and culled to 10 pups. At 24 days of age, rats were weaned and transferred to the Department of Psychology Animal Research Facility (ARF) from HSC-ARF. To minimize potential litter effects, only 1-2 animals were used from each breeding pair litter.

Experiment 1: Object-Place-Paired-Associate Task

Objects and Environment. The maze was composed of two arms (each 40.1cm × 9.30cm) of an 8-arm radial-arm maze (Sanchez, Thompson, & Clark, 2016). The two arms were separated by 180° and were fixed to a center stage (25cm in diameter). The end of each arm was a rectangular platform (20cm × 30cm), each containing three recessed food wells separated by transparent vertical Plexiglas dividers (each 5.1cm × 5.1cm). Transparent Plexiglas doors (20cm × 9.5cm) were present at the entrance of each arm from the center stage. A set of toy objects were presented at the end of the two opposite arms and above two of the recessed food wells. The maze was placed in the center of a testing room that contained many extra-maze cues, including a sink, desk, chair, shelves, and wall posters (Harvey et al., 2017; Sanchez et al.,

2016). Session recordings were conducted using a camera above the maze and under normal lighting conditions. Digital videos were obtained for off-line analysis.

Pre-training. Rats were handled for 5 days for approximately 5 minutes per day. To help increase motivation to forage, rats were placed on a food restricted diet to a weight of 90% of ad libitum feeding diet. After rats displayed signs of comfort with the experimenter (i.e. no defecation/urination), shape training began. On the first day of shape training, 5 Fruit Loop pieces were placed along each of the two arms and animals were allowed to freely explore and consume the food. In subsequent training session, food was restricted to the recessed cups located at the end of each arm. Once rats retrieved 20 Fruit Loop pieces from the recessed food wells (10 pieces per arm), metal washers (2.5cm in diameter) were placed above the recessed food wells and rats had to successfully push the metal washer to retrieve the Fruit Loop. Shape training continued for 6 days. Rats were then trained for 20 trials per day. A trial consisted of the rat being placed in the center of the maze within the closed doors of the maze. One of the two arm doors were pseudo-randomly opened. The rat was then allowed to locomote to the end of the arm to the choice platform where it displaced the metal washer, retrieved the half Fruit Loop, and was carefully guided back to the center to consume the food with the arm door closed. After a few days of washer displacement, the rats, naturally, with little experimenter guidance ran back to the center for Fruit Loop consumption (Whishaw & Tomie, 1997). Once rats could displace the washer 20 times within a 20-minute time interval, they had completed pre-training and moved on to object-place-paired-associate training.

Object-Place-Paired-Associate Training. Animals were trained in an object-place paired-associate task similar to Sanchez, Thompson, & Clark, (2016), (Hernandez et al., 2015; Jo &

Lee, 2010). Washers were replaced with two distinctive objects (i.e. a toy superman and a Ping-Pong ball). The toys were placed above the left and right recessed food wells on the choice platform and rats were required to displace one of the objects to uncover the reward. If rats displaced the incorrect object, they were not allowed to change their choice but were gently guided back to the center of the maze. The positions of objects on the choice platform was pseudo-randomly selected across trials to prevent animals from learning a specific egocentric response to obtain the food reward. This required the animals to learn a rule which was associated with a particular object (superman or ball), and a particular place (1 of the 2 arms) in the maze. This rule is typically referred to as an object–place paired association. The sequence of arm visits was pseudo-randomized with two different sequences that were alternated between days. Rats were trained to acquire the task for a total of 14 days. Each day consisted of a total of 10 trials with 5 trials on each arm. The maximum time allotted for each rat per day was 20 min. Based upon our previous findings, to prevent use of proximal cues from day to day (Sanchez et al., 2016), the maze was cleaned between animals and rotated each day.

Experiment 2: Object-Discrimination Task

Rats were trained to discriminate between two novel objects (i.e., black conical tube and red box) to receive a reward. One arm of the maze was used for the experiment. As in the tasks above, rats were placed in the center of the maze, the experimenter opened the maze arm, and the rats were each (Sanchez et al., 2016) allowed to push one of the two objects to uncover a half Fruit Loop. The same object was rewarded throughout training. The left-right position of the object was pseudo-randomly selected to prevent acquisition of an egocentric response (e.g., a left

turn to obtain a reward). As in previous training, if the incorrect object was chosen, the rat was not allowed to correct the choice but was guided back to the center without reward. Each day consisted of a total of 10 trials administered over 8 days. We chose this particular type of object discrimination in following what has previously been done by Lee(Lee & Solivan, 2008).

Experiment 3: Morris Water Task

After testing in the object-discrimination task, animals were trained in the hidden platform variant of the Morris Water task (Williams, 1998; C. Gianoulakis et al.1990, Hamilton et al., 2014; Vorhees & Williams, 2006). A large circular pool was used (1.5 m in diameter; height of 48 cm). The pool was filled with opaque water (~22-23^{o}C) to a depth of 26 cm. The escape platform (15 cm × 15 cm) was submerged ~1 cm below the surface of the water. The testing room used was different than that used in object-place and object-discrimination training, but had a variety of posters, chairs, shelves, and distinct visual cues. Trials were conducted in blocks of four starting from four different pseudo random release points around the perimeter of the pool (N, S, E, W). A trial ended when the animal either reached the platform or after 60 seconds had passed. If 60 seconds passed, and the rat had not reached the platform, the experimenter gently guided the animal to the platform where it remained for 10 seconds. The rat was then removed from the platform, dried off and placed in a holding cage between trials. The intertrial interval was ~ 5 minutes. Testing was performed during the dark cycle. Animals were trained for five days and a probe test was performed on the fifth day after the final trial. During the probe test, the platform was removed from the pool and all animals were released from a randomly selected release location. The probe test continued for 30 seconds. Digital copies of

behavior were recorded using an overhead camera and the videos were transferred to a computer for tracking and analysis. The analysis was done using a circular location with a diameter of 25cm, around the actual platform area. The videos were then analyzed using custom scripts in Matlab (Hamilton et al., 2007).

Data Analysis:

Prior to learning the object-place rule we observed that the rats used a response bias for a particular side on the choice platform or particular object (Jo & Lee, 2010; Hernandez et al., 2015; Sanchez et al., 2016). To create these response bias indices, we calculated the absolute value of object 1 choice minus the total number of object 2 choices divided by the total number of trials. The same calculations were used for side bias on the choice platform were very similar. The absolute value of the total number of left choices minus the total number of right choices divided by the total number of trials. For the object place association task, the performance of each animal was calculated based upon the average percent correct per day (correct choices minus incorrect choices divided by total choices multiplied by 100. Then group (PAE and SACC rats) means were analyzed using mixed model repeated measures analysis of variance (ANOVA) with between subject's factors of treatment. Mean comparisons were conducted each day using an independent samples t-test. Performance in the Morris water task was assessed by measuring escape latency to the platform and an ANOVA was conducted to determine group differences. We used an independent sample t-tests (two tailed) for group comparisons on measures for the water task probe test (no platform).

Results

Experiment 1: Object-Place Paired-Associate Task

Animals were first tested on an object-place-paired-associate task in which they were required to discriminate between a set of objects based upon their spatial position in the environment. Figure 3A plots the percentage of correct choices from PAE and SACC animals over 14 days of training. In general, animals in both groups improved their performance over days ($F(13,182) = 27.88$, $p < 0.001$, $\eta_p^2 = 0.637$). However, improvement in performance was greater for the SACC group. In contrast, PAE animals improved across training but not comparably to SACC animals. These observations were confirmed by an mixed model repeated measure ANOVA conducted on the percent correct across training day which indicated a significant group by day interaction effect ($F(13,182) = 1.89$, $p = 0.034$, $\eta_p^2 = 0.043$), and a significant group effect and treatment effect ($F(1,14) = 5.46$, $p = 0.035$, $\eta_p^2 = 0.281$). We also conducted mean comparison of groups each day using independent samples t-tests. We found significantly different or trending towards significant percent correct between groups on days 9,11,12,13, and 14 ($p = 0.024$, $p = 0.080$, $p = 0.008$, $p = 0.048$, $p = 0.094$, respectively).

To try to understand the specifics of the acquisition impairment, we investigated whether the PAE animals improved their performance within a day's 10 trial training session. We hypothesized that if rats were impaired earlier in the training session, and improved by the final trials, then this might suggest that the animals had a memory impairment rather than a general learning deficit. When we compared the percent correct for the first 5 trials versus the final 5 trials for each PAE animal, we found no evidence of a significant difference indicating

equivalently poor performance across the 10 daily trials (Figure 3B). We confirmed this finding with a significant ANOVA for the day effect, both PAE and SACC animals improved performance over 14 days ($F(13, 182) = 7.65$, $p < 0.001$, $\eta_p^2 = 0.353$), but a nonsignificant time-bin effect (i.e., trials 1-5 versus 6-10; $F(13, 182) = 0.865$, $p = 0.591$, $\eta_p^2 = 0.058$).

Figure 3C plots the side bias index for each group across training in the object-place task. Animals in both groups typically began acquisition training turning to one side regardless of the spatial position of the maze arm and reward; an observation consistent with previous work (Sanchez et al., 2016). Both SACC and PAE animals showed similar use of this rule early in training, but the bias index decreased in SACC animals as a function of testing day (Figure 3C). Notably, by day 14, the side bias for the SACC group was lower compared to PAE animals. Supporting these general observations, an ANOVA indicated that this group difference was trending towards significance ($F(1,14) = 3.155$, $p = 0.097$, $\eta_p^2 = 0.184$). We also looked at a possible object bias, meaning that animals had a preference for a particular object independent of the location of the object in the maze. We found no object bias (Figure 3D) amongst the SACC or PAE animals. Although, interestingly we observed that PAE rats made similar use of the object bias throughout the training, while SACC animals had almost zero object bias by the end of training, while the object bias was greater in the PAE group.

Experiment 2: Object-Discrimination Task

With the exception of one rat that died after Experiment 1, animals were tested in an object-discrimination task in which they were required to discriminate between two distinct

objects in a single maze arm. Animals were no longer required to discriminate between the two objects based on spatial position. Figure 4 plots the percentage of correct choices by PAE and SACC groups across the eight days of testing. On average, rats in each group showed improvement in selecting the reinforced object across training. This observation was confirmed by a mixed model repeated measures ANOVA indicating a significant day effect (F(df greenhouse geisser adjusted: 3.41, 44.31) = 40.23, $p < 0.001$, $\eta_p^2 = 0.756$). By day 8, animals showed significant object discrimination well above chance performance (PAE: 94.29 $\pm$ 7.87%; SACC: 96.25 $\pm$ 5.18%; t(14) = 27.44, $p < 0.001$, $\eta_p^2 = 0.294$). Importantly, animals in both groups demonstrated similar performance across training as revealed by the absence of a significant group (F(1,13) = 0.001, $p = 0.981$, $\eta_p^2 = 0.000$), or group by day effect (F(df greenhouse geisser adjusted: 3.41, 44.31) = 1.00, $p = 0.407$, $\eta_p^2 = 0.072$). In sum, PAE animals displayed intact performance in the object -discrimination task.

Experiment 3: Morris Water Task

We then chose to test animals in the Morris water task to test animals place learning because PAE animals have previously been characterized in this task. Animals were trained and tested in a hidden platform variant of the Morris water task. Animals were trained to discriminate between spatial quadrants of a pool to find a hidden platform. We measured swim latencies to reach the platform, which are plotted in Figure 5A. We observed no differences in latencies to the platform across training days for SACC and PAE animals. All subject spent less time finding the platform over the training day (F(4,52) = 59.08, $p < 0.001$, $\eta_p^2 = 0.940$), and there was no significant interaction in their performance (F(4,52) = 0.755, $p < 0.559$, $\eta_p^2 = 0.055$). Although there was an effect of group (F(1,13) = 6.91, $p = 0.021$, $\eta_p^2 = 0.347$). This latter difference might

be driven by mean differences on day 4. However, by day 5, there were no group differences in the mean swim latencies.

To evaluate spatial memory, we conducted a probe test (last day of training) in which the platform was removed from the pool and rats were permitted to swim for 30 seconds (Fig. 5B). The probe test was performed immediately after the last training trial of day 5. Measures of swim latency to the platform ($t(13) = 0.751$, $p = 0.233$, $\eta_p^2 = 0.400$), path length ($t(13) = 1.25$, $p = 0.117$, $\eta_p^2 = 0.667$), and time in the target zone ($t(13) = -0.292$, $p = 0.388$, $\eta_p^2 = 0.151$) were all found to be non-significant (Fig. 5C). Overall, in the Morris water task we found that spatial learning and memory by PAE animals were largely intact.

Discussion

We hypothesized that moderate PAE rats would have impaired learning in an object place paired associate learning task because of the complexity of the task. In Experiment 1, animals were trained to discriminate between two objects in two different locations. In moderate PAE rats, we observed slower learning compared to SACC rats. In addition, by the end of training, PAE animals failed to obtain similar performance on the task and appeared to adopt a simple rule of either entering a particular side of the reward platform or selecting a specific object. Thus, PAE animals may have difficulties in inhibiting the use of these simple strategies and to make way for the more complex associations (i.e., the object-place paired-associate). In Experiment 2, animals were trained to discriminate between two objects. We found that both moderate PAE and SACC control rats were able to learn the object discrimination at similar competence. In

Experiment 3, rats were trained in a fixed hidden platform Morris water task in which animals learned to navigate to a place. Both PAE and SACC rats learned to navigate to the location of the platform by the final day, although there was some evidence of slower learning in the PAE group. These results indicate that moderate PAE rats can learn to discriminate objects, display similar learning of places, but they are impaired when they are given a more complex task of associating the two.

The results of the present study point to three main conclusions. First, moderate PAE impairs the information processing required for acquisition of an object-place paired associate task. To our knowledge this is the first study to look at the object place paired associate learning in moderate PAE animals. A number of studies have indicated that neurobiological mechanisms underlying the object-place paired-associate learning may involve the hippocampus, and it is well known that PAE can damage the hippocampus. For instance, there is evidence that PAE can produce alterations in LTP transmission along the perforant pathway from the entorhinal cortex to the dentate gyrus (Galindo et al., 2004; Savage et al., 2010).

Consistent with the observations above, selective damage to the hippocampus produces similar deficits in object-place paired-associate learning. In a hippocampal lesion study, Solivan and Lee found clear impairments in object-place associations (Lee & Solivan, 2008). Rats with hippocampal lesions performed at only 60 percent correct while control animals performed at 80 percent correct or greater throughout acquisition trials. Solivan and Lee also found a side bias in lesioned animals and not in control animals. There is also data that indicates that specific damage to CA3 of the hippocampus might be the true place of issue with object-place-paired-associate type tasks. Gilbert et al. performed lesions of specific regions of the hippocampus, CA1, CA3

and DG. When each of these hippocampal sub regions were lesioned, it was found that only after CA3 damage that impairments were expressed in an object-place-paired-associate task. Neither lesions of the CA1 or DG had outcomes that showed impairments in object-place-paired-associate tasks. The CA3 region of the hippocampus may have a special role in paired-associate learning due to the fact that the region contains recurrent collaterals potentially responsible for mediating an auto-associative network. These recurrent collaterals are thought to play an important role in pattern completion or generalization; the notion that a small fragment of a memory (e.g., a spatial location) can trigger the full representation of a previous event (e.g., move a specific object to obtain a food reward).

While most alcohol exposure studies have focused attention on the impact on hippocampal processing, there is evidence that damage to areas outside the hippocampus might also contribute to the PAE behavioral deficits described here. The reasoning is that if areas outside the hippocampus are lesioned, and experimenters observe deficits in object place paired associate information processing, we could conclude that these areas also contribute to the mechanism of PAE damage. Jo and Lee induced axon sparing lesions of the perirhinal cortex with NMDA and then trained rats on a very similar object place paired associate learning task (Jo & Lee, 2010). They found very similar behavioral deficits as was found in our study. Damage to the perirhinal cortex rats were not able to learn to the same level as control rats and they exhibited similar side bias responses rather than object place paired associate responses (Gilbert & Kesner, 2003). This might mean that moderate PAE animals have some damage to the perirhinal cortex resulting in similar deficits.

A second conclusion drawn from the present study is that moderate PAE animals are not impaired at a simple object-discrimination task. While some studies have reported impairments in object-discrimination tasks, others have not. One possible explanation is that the previous studies involved "spontaneous" exploration behaviors, which could be influenced by differences in locomotor behavior rather than discrimination impairments per se. Second, previous studies have mostly utilized high doses of PAE, whereas the present study was the first to explore the impact of moderate PAE on object discrimination. It is important to note that the discrimination in the present study did not use objects with overlapping features. Thus, perhaps deficits might be observed if the discrimination was made more difficult by systematically varying the degree of stimulus overlap.

The findings are also consistent with the interpretation that deficits in hippocampal and CA3 processing might mediate the impairments described in the present study. Notably, while lesions of the hippocampus and the perirhinal cortex impair object-place learning, lesions to these areas do not result in impairments in object discrimination learning (Jo & Lee, 2010; Lee & Solivan, 2008). Theoretical work has pointed to the possibility that the medial and lateral entorhinal cortices have differential roles in object and object-place learning with the later region more involved in object processing and the medial having a more specialized role in spatial and object-spatial processing. Whether the medial entorhinal cortex is disrupted in moderate PAE is currently unknown.

One caveat to the interpretations presented above is that the relatively poor performance by PAE animal in the object-place task reflects the greater complexity of the task demands. As a consequence, PAE animals may have had difficulties attending to the appropriate object-place-

paired associate. Comparatively, the object-discrimination task involved locomoting along only a single arm, perhaps reducing complexity of the environment in turn reducing the possibility for exposing impairments in the PAE group. Indeed, a recent study found greater impairments by animals with hippocampal lesions in a complex context relative to a simple context (Moses, Winocur, Ryan, & Moscovitch, 2007). Nonetheless, this interpretation is not mutually exclusive with our proposal described in the paragraphs above as PAE animals could at the same time express impairments in associating the appropriate object-place pair.

The final conclusion of the present study is that spatial learning is subtly impaired in the moderate PAE animals based upon our Morris water task results. A study of high dose PAE showed moderate learning impairments, specifically, these animals were able to perform the Morris water task but it took them longer and they swam longer distances to get to the hidden platform (Gianoulakis, 1990). An et al. also found similar impairments from high PAE but also found behavioral flexibility deficiency which corresponded with reduced fEPSP slopes suggesting that long term potentiation (LTP) was also impaired based upon in vivo LTP and depotentiation recordings (An, Yang, & Zhang, 2013). Conversely, in varying dosages PAE, others found no impairments in acquisition in the Morris water task (Sutherland et al., 2000; Thomas, Idrus, Monk, & Dominguez, 2010; Hamilton et al., 2014b; Savage, Becher, de la Torre, & Sutherland, 2002). Although, Savage et al. did mention that in a moving platform variant of the Morris water task, the low (3%) and moderate (5%) PAE animals did not improve their escape latency times for first and second trials, although thought to be a transient deficit. This showed that there may be issues once again with flexibility as well. Together, these findings suggest that even low and moderate PAE can produce lasting deficits in the hidden platform

Morris water task but these impairments appear to be mild in the stable platform variant. These findings could show that there is a challenge in behavioral flexibility (Savage et al., 2002).

To understand further the neurobiological basis of navigation in the Morris water task, lesion studies again are useful. From the present findings and previous lesion studies, one area of interest is the CA3 sub region of hippocampus as well as the medial entorhinal cortex (MEC). Several studies have damaged the MEC and then had rats perform the Morris water task, many of these studies have found slight impairments. In particular the studies determined that the MEC damaged rats swam longer distances in spite of swimming at a faster speed than the controls (Ferbinteanu, Holsinger, & McDonald, 1999; Van Cauter et al., 2013). Others have found that the MEC cortex damage did not affect performance in a similar hidden platform task (Burwell, Saddoris, Bucci, & Wiig, 2004). The fact there is faster swim speed can be a little hard to reconcile but it may be due in part to the speculation that the MEC is involved in temporal organization of behavior (Schlesiger et al., 2015). However, the deficits might be accounted by a motivational change resulting in faster movements.

Moderate Prenatal Alcohol exposure is of great importance because of the elevated likelihood that women of childbearing age drink alcohol. Moderate alcohol exposure can sometimes result from a woman drinking alcohol before knowing that she is pregnant. Considering that moderate alcohol exposure is often diagnosed later in childhood and has more subtle disabilities it often goes undiagnosed or misdiagnosed. The work that we have done here will help us to better understand the impairment in humans especially regarding associative, and hippocampal-dependent, memory in children. This understanding of the impairments of

moderate prenatal exposure will lead us to a discovery of sooner/better diagnosis in children and possible treatment options.

Figures

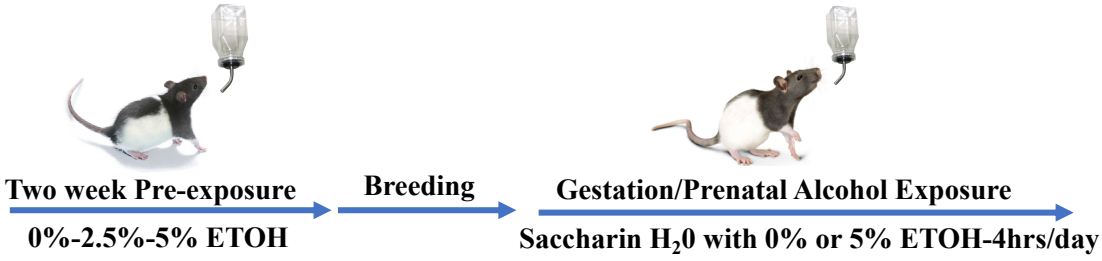

Figure 1. Exposure timeline for moderate Prenatal Alcohol Exposure (PAE). Over two weeks rats are exposed to 0, 2.5, and 5% ethanol in sucrose water. Female rat breeders that drank either above or below one standard deviation from the mean of the group were removed from the study. Rats are then matched with a male breeder until pregnancy is verified. There was no ethanol consumption during breeding. Once pregnant female rats were given access to 4 hours daily of 5% ethanol in sucrose or just sucrose water corresponding to their group assignment (either SACC or PAE).

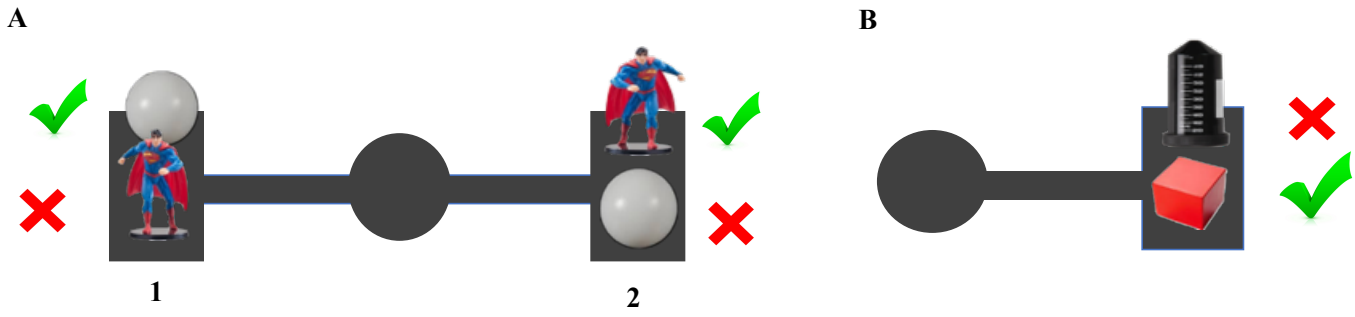

Figure 2A. Schematics of the maze. Rats running to side 1 must push the ball to receive the half fruit loop reward irrespective of which side the toy is placed upon on the arm. Rats running to side 2 must push the superman in order to receive the half fruit loop reward, irrespective of which side the superman is placed upon. B. Schematics of object discrimination. Red box object was always correct regardless of the location of the box.

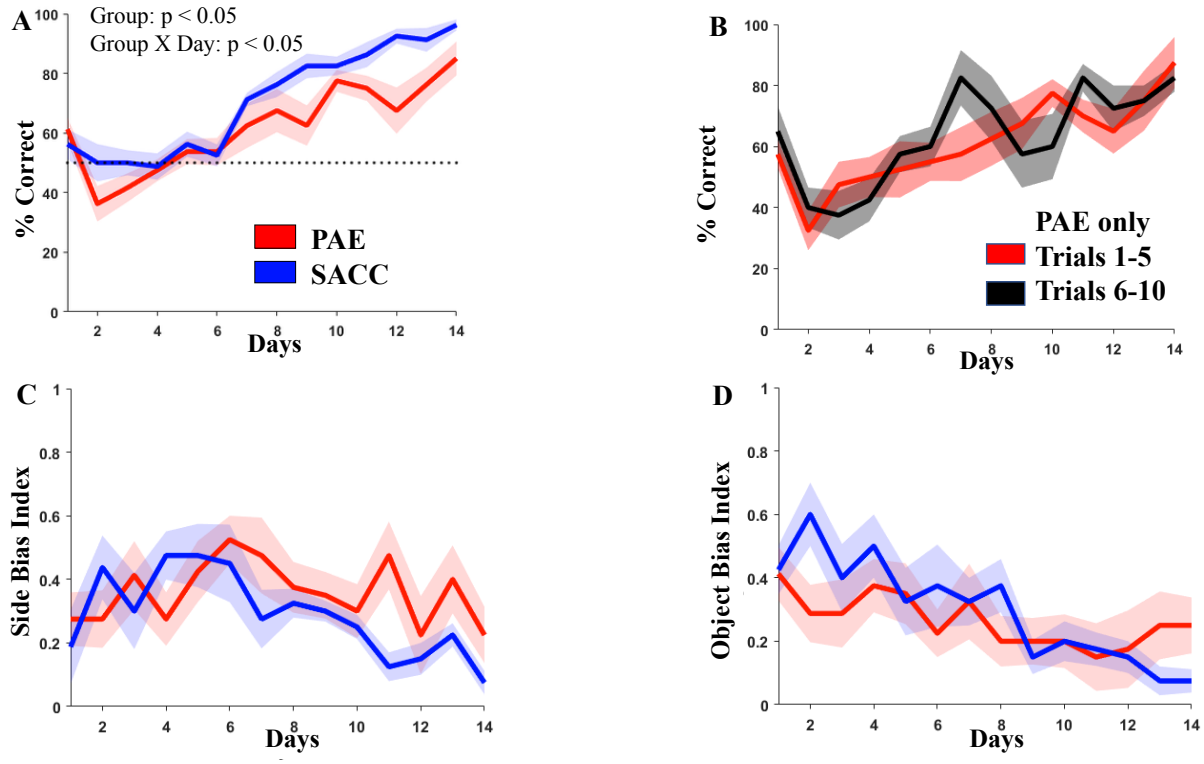

Figure 3A. Object place paired associate acquisition curve showing mean and SEM for the percentage correct object association as a function of 14 training days. Dashed line represents chance performance. Both PAE and SACC learn the task but PAE never learn it to the level that SACC learn the task. B. PAE trials 1-5 vs. 6-10 mean percent correct per day. This was done to try to identify if there was a difference between learning at the beginning of the training day and at the end of the training day. No significant differences were found between the beginning trials and the last trials suggesting PAE were equally poor across the entire day. C. Side bias index, |left side - right/ total trials| as a function of days. Both PAE and SACC animals showed side bias early in training while PAE continued to use a side bias in later days of training as well. D. Object bias index, |object1-object2/total trials|, as a function of days. PAE and SACC have an

object bias, days 1-8, while SACC drop off but PAE continue along the same trend of object bias.

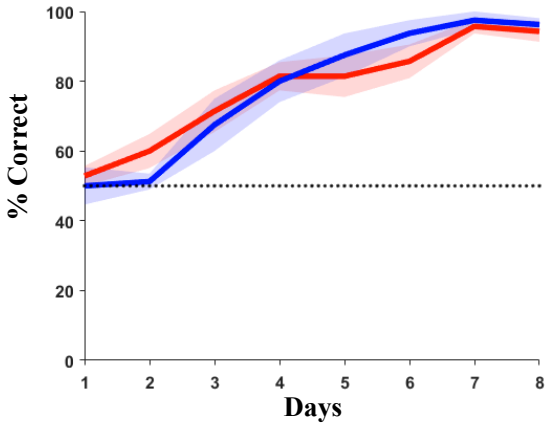

Figure 4. Object discrimination. PAE and SACC performed equally as well discriminating between objects.

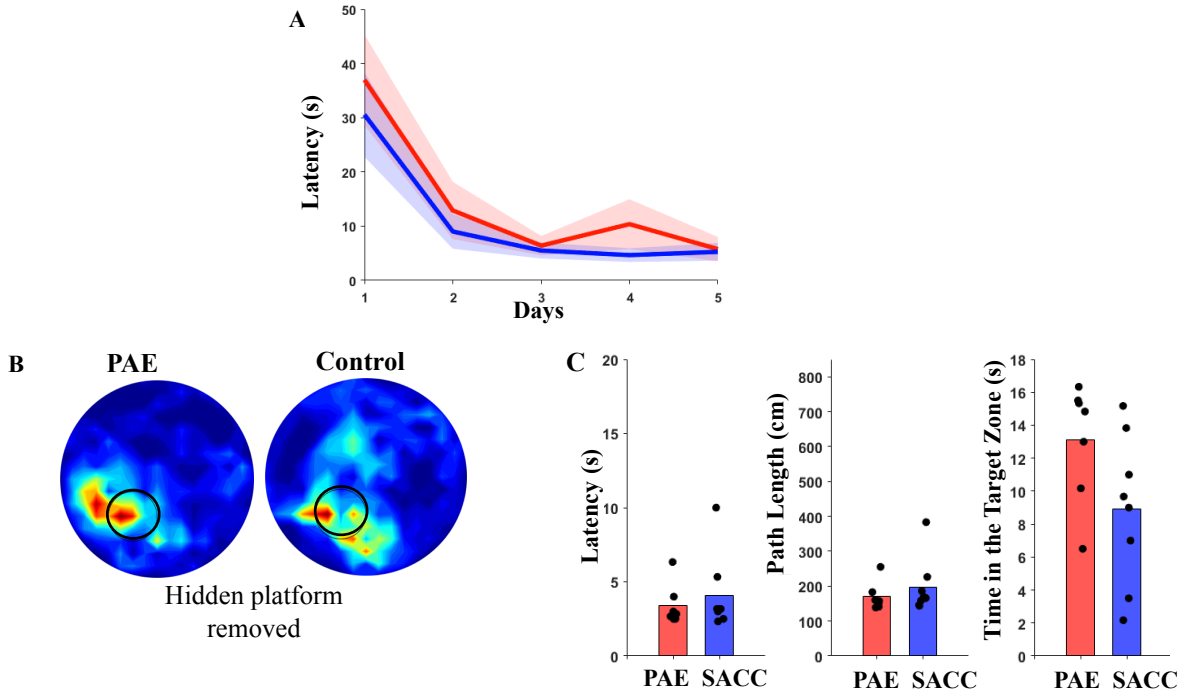

Figure 5A. Morris water task acquisition curve showing mean and SEM as a function of days. A. Latency to the platform in seconds as a function of training days. B. Heat maps for PAE and SACC during Probe test. D. Probe test mean latencies, path length and time in the target zone as a function of PAE or SACC.

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