Spring 2-27-2024

The Effects of Moderate Prenatal Alcohol Exposure on Navigation in a Delayed Non- Match-To-Place Spatial Alternation Task by Adult Male and Female Rats

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THESIS
Submitted in Partial Fulfillment of the
Requirements for the Degree of

Master of Science
Psychology

The University of New Mexico
Albuquerque, New Mexico

May, 2024

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ABSTRACT

Prenatal alcohol exposure has been found to alter brain regions involved in spatial memory. Previous studies have shown that moderate PAE (mPAE; ~30-120 mg/dL) impairs spatial memory in male rats and damages the limbic-thalamus and hippocampus. Recent work has shown that visual discrimination memory is impaired after mPAE in a sex-specific manner such that female mice exhibit greater deficits after 15sec delay. It is unclear whether similar-sex-specific deficits would be observed in a spatial memory task or in a rat model of mPAE. Thus, the present study tests the hypothesis that mPAE would produce sex-specific deficits in a delayed non-match-to-place variant of an alternation task. Saccharine and mPAE adult Long-Evans rats were trained to alternate between outbound arms of an M-maze after a delay. Here we report that female rats required a significantly greater number of training days; mPAE does not have a considerable effect on delayed non-match spatial alternation behavior.

TABLE OF CONTENTS

LIST OF FIGURES

List of Tables

INTRODUCTION

Fetal Alcohol Spectrum Disorders

Prenatal alcohol exposure remains a leading and persistent global concern, as it is reported that roughly 10% of women engage in alcohol consumption duing their pregnancy (Popova et al., 2017). Exposure to teratogens such as ethanol during the gestational period can lead to the onset of Fetal Alcohol Spectrum Disorders (FASD). FASD are a set of neurodevelopmental deficits that follow prenatal alcohol exposure (PAE) and are marked by cognitive and anatomical dysfunction in offspring. The consequences of PAE are profound and can have lifelong effects on neurodevelopment. Assessing the repercussions of prenatal ethanol exposure presents a multifaceted challenge. Consequently, rodent models have become an essential tool for investigating the ramifications of PAE. Factors including the methods of ethanol administration, dose, and exposure period play a pivotal role in shaping the magnitude of cognitive and behavioral impairments (reviewed in Harvey et al., 2019; Marquardt & Brigman, 2016; Valenzuela et al., 2011). While apoptosis constitutes a physiological mechanism innate to the regulation of neurodevelopment during synaptogenesis, substantial neuronal attrition caused by toxins like ethanol profoundly impacts the morphological attributes of specific brain regions and cell populations. PAE has been found to result in apoptotic neurodegeneration, altering the development of regions in the brain critical to learning and memory (Harvey et al., 2019; Valenzuela et al., 2011), including the thalamus (Ikonomidou et al., 2000) and hippocampus (Wozniak et al., 2004). Understanding these effects at the cellular and anatomical levels is critical for developing effective interventions and support for individuals affected by FASD

Fetal Alcohol Spectrum Disorders and Spatial Navigation

Spatial cognition, the ability to acquire spatial relationships about one's location and goals and use this information for navigation (Gallistel, 1990; O'Keefe & Nadel, 1978), represents one of the most common behavioral deficits that follow PAE (Harvey et al., 2019; Marquardt & Brigman, 2016). It is notable that previous reports have shown a consistent pattern of impairments in spatial processing in human subjects with FASD (Uecker & Nadel, 1996), even demonstrating impairments in variants of tasks frequently used in animal studies, e.g., a virtual Morris water task (Hamilton et al., 2003; Mattson et al., 2010; Woods et al., 2018).

In rodents, numerous studies have employed behavioral paradigms to assess spatial impairments associated with PAE in animal models. The Morris Water Maze (MWM) has been extensively utilized for comprehensive assessment of both place learning and progression of escape latency (Morris, 1981). This task entails animals locating a precisely positioned platform submerged beneath opaque water. Typically, discernible deficits manifest following a substantial dose of alcohol following PAE (Harvey et al., 2019; Marquardt & Brigman, 2016). Although spatial navigation impairments across both acquisition and retention in the water task are consistently reported after high dose developmental exposure to alcohol, water task deficits have also been reported after more milder forms of alcohol exposure including moderate PAE (mPAE), which typically ranges between 30-120 mg/dL (Harvey et al., 2019; Marquardt & Brigman, 2016). In the water task, spatial deficits after mPAE become apparent when introducing a considerable delay to the task and evaluating cognitive flexibility. Cognitive flexibility is evaluated by administrating a probe test where a platform is relocated from its initial position. Animals are then required to

adapt and acquire the change in placement. For instance, Savage and colleagues have reported that adult male rats show spatial memory and spatial reversal learning deficits in the hidden platform variant of the Morris water task after mPAE (Rodriguez et al., 2016; Savage et al., 2010). More recently, an object-place association paradigm has detected a notable disruption in paired association following mPAE. For instance, Sanchez et al (2019) reported that adult male rats that had been exposed to mPAE were impaired at discriminating between objects on the bases of spatial location (object-place paired associate learning).

Spatial working memory, the online processing of spatial information, has also been assessed in tests where animals are required to alternate between spatial locations with a delay between the choice of location. Working memory, a concept initially introduced by Miller et al. (1960), describes the cognitive system responsible for temporarily holding and manipulating information required for various tasks. While Miller's original description emphasized short-term online storage often tied to prefrontal cortical function, the concept of working memory has also been described in terms of the memory for recent spatial experience (i.e., spatial working memory) which is often linked to hippocampal function (Olton et al., 1979). In this thesis, the term spatial working memory will be used consistent with this latter definition which is the capacity to remember information that was obtained in a single experience and to retain this information after a delay period exceeding the traditional definition of working memory (Eichenbaum, 2012). Spatial working memory can be contrasted with spatial reference memory which relies on long-term memory of spatial layout cues to guide behaviors within an environment. Unlike spatial working memory which involves acquiring spatial information during a single experience, spatial reference memory involves the retention and retrieval of spatial information that is consistently presented across

several experiences. Using a radial arm procedure, Jarrard (1993) reported spatial working and reference memory deficits after hippocampal damage.

Previous studies have used an M-shaped maze environment (also termed the ‘W-maze”) with an alternating task to test the hypothesis that the hippocampus contributes to working and reference components of spatial memory. In brief, the task uses both spatial reference memory (long-term memory), when animals are required to return to the starting area and spatial working memory when recalling recently visited outbound arms (short-term memory). Animals with extensive lesions to the hippocampus exhibited impairment during the acquisition of a continuous alternating task in the M-maze, and were found to make errors on both spatial working and spatial reference memory components (Kim & Frank, 2009). In addition, a recent study using optogenetic inhibition of the anterior thalamus and terminal projections from the anterior thalamus to parahippocampal cortex impaired accurate performance on a delayed spatial alternation variant of the M-maze task (Roy et al., 2022). Thus, many studies have used this particular maze and task to demonstrate that hippocampal dynamics and thalamo-parahippocampal circuitry are critical to spatial navigation and the online processing of spatial information (Fernández-Ruiz et al., 2019; Frank et al., 2000; Jadhav et al., 2012; Singer et al., 2010; 2013; Zhang et al., 2021).

The use of spatial working memory assessments has received limited attention in mPAE, however one study (Brady et al., 2012; also see **Table 1** below) reported that impairments could be observed in male mPAE mice after a short delay and when the spatial locations had considerable location overlap (i.e., the discrimination was made between adjacent maze arms). In addition, a preliminary study from our laboratory has also reported that mPAE produces spatial working memory deficits in a radial arm maze spatial task (Sanchez et al.,

unpublished observations). Thus, integrating the M-maze with alternation and spatial working memory components may provide insight into mPAE effects on spatial cognition and provide insight on neurodevelopmental processes. In summary, mPAE, which produces more subtle changes in hippocampal morphology and synaptic plasticity (Brady et al., 2013; Madden et al., 2020; Savage et al., 1998; Sutherland et al., 1998; Varaschin et al., 2010; 2014), can lead to a range of cognitive and behavioral impairments, including deficits in spatial learning and memory in adult male rats, characterized by difficulties acquiring and retaining the spatial locations of objects, items, and destinations to goal locations.

Sex-Specific Effects of Moderate Prenatal Alcohol Exposure on Spatial Navigation

Although the studies summarized above have shown that adult male rats are impaired after mPAE (Sanchez et al., 2019; Savage et al., 2010), whether similar spatial impairments can be observed in adult female rats has received less attention (see **Table 1** below). However, a recent study in adult mice has shown that mPAE can lead to sex specific deficits in visual discrimination memory (Kenton et al, 2020). In this study, animals were tasked with selecting an illuminated square during the sample phase, followed by a variable delay. In the subsequent test phase, the sample and new stimulus were presented, and the mice were required to select the novel option (non-match). Initially, mice did not demonstrate significant impairments within the context of a basic trial-unique delayed non-matching to location (TUNL) paradigm. However, upon subjecting the animals to a notably more challenging iteration of the TUNL task, characterized by varying delay intervals and minimal interstice between sample and choice trials, it became evident that female PAE mice exhibited a deficit in performance. Interestingly, similar deficits were not observed in male

mice, suggesting that non-match visual-spatial discrimination learning is impaired in a sex-specific manner following mPAE.

Moderate Prenatal Alcohol Exposure and Neural Circuits of Spatial Navigation

While mPAE may yield subtle influences on spatial behavior and cognition, empirical findings indicate the occurrence of morphological and structural alterations that impact neural circuitry crucial for spatial navigation. For instance, mPAE can disrupt NMDA subunit expression in the dentate gyrus (Brady et al., 2013), disrupt synaptic (long term potentiation) at these same perforant path synapses (Savage et al., 2002; 2010), and alter the expression of interneurons in the hippocampus in both male and female rats (Madden et al., 2020). Recent research has also shown that neural activity within the hippocampus can be affected by mPAE. The hippocampus contains neurons called place cells that fire as a function of an animal's spatial location in an environment (O'Keefe & Nadel, 1978). Place cells are thought to have an important role in generating "maps" of environmental space and may have an important role in spatial navigation. In a previous study (Harvey et al., 2020), it was reported that hippocampal place cells recording in mPAE male rats were less likely to fire distinctly (i.e., discriminate) between different contextual experiences such as when the animal was travelling in opposite directions of travel. Place cells in mPAE male rats also expressed reductions in spatial tuning and location instability across recording sessions. Place cells in mPAE male rats were also less likely to coordinate their firing with the local theta rhythm (i.e., phase precession). Thus, mPAE can alter many features of hippocampal structure and activity relevant to spatial learning and memory. However, while some of these

features have been extended to female mPAE animal models (Madden et al., 2020), most previous studies have been restricted to male animal subject.

Key:
Behavioral Task
MWM = Morris Water Maze
RAM= Radial Arm Maze
OPPA= Object-Place Paired-Association
DNMP= Delayed non-match
TUNL= Trial- Unique Non-matching to Location

Ethanol Exposure:
Table consists of moderate Prenatal Alcohol exposure between 1st and 2nd trimester and blood alcohol concentration (BAC) ranging from 30 - 120mg/dl.

Reference	Testing Age	Task	Impaired	Sex Trained	Effects
Savage et at. 2002	P180-P220	MWM	No	Male	Decrease in learning the moving platform task with 30 or 83 mg/dL.
Clausing et al. 1995	P68-P80	Complex Maze	Yes	Male	Increase in errors.
Cullen et al. 2014	P270, 540	Y-Maze MWM	No	Both Female and Male	No effect.
Sanchez et al. 2019	P120-P202	OPPA	Yes	Male	Increase in errors.
Hamilton et al. 2014	P135-P315	MWM	No	Male	Slight preservation in males.
Rodriguez et al. 2016	P135-P315	MWM	No	Both Female and Male	Slight preservation in males.
Savage et al. 2010	P130-160	MWM	Yes	Male	Impaired retention, rescued by histamine H3 receptor antagonist.
Brady et al. 2012	P90-150	DNMP	Yes	Male	Impaired on difficult spatial discrimination variant.
Sutherland et al. 2000	P100-200	MWM	No	Male	Most likely to use cue over place navigation. Impaired on moving platform task.
Matthews et al. 1998	P86-P95	MWM	No	Male	Impairment following delay period.
Kenton et al. 2020		TUNL	Yes	Both Female and Male	Sex-specific Impairment in visual spatial discrimination in female PAE treatment group

Table 1 compiles studies involving moderate prenatal alcohol exposure (PAE) occurring between the 1st and 2nd trimesters, with corresponding blood alcohol concentrations (BAC) ranging from 30 to 120 mg/dl. The table provides details on spatial tasks employed, the gender subjected to testing, and the resultant effects observed for each task.

SPECIFIC AIM & HYPOTHESIS

It is currently unknown whether the sex-specific deficits in visual discrimination learning in mPAE mice reported by Kenton et al (2020) would extend to tests of spatial discrimination in a rat model of mPAE. While both species share many similarities in neurobiological changes and spatial navigation impairments after mPAE (summarized in Harvey et al., 2019; Marquardt & Brigman, 2016), sex-specificity in deficits of spatial discrimination behavior has received limited attention in rat models of mPAE, especially in tasks where animals are required to alternate between spatial locations (i.e., a variant of non-match-to-location procedure used by Kenton et al. 2020). The previous observations that spatial navigation deficits are common in adult male rats (Rodriguez et al., 2016; Sanchez et al., 2019; Savage et al., 2010), coupled with the findings by Kenton et al (2020), motivate the aim of this thesis proposal to test the hypothesis that mPAE produces impairments in spatial discrimination behavior in both male and female rats. To test this hypothesis, we used a delayed non-match-to-place variant of a spatial alternation task, a well-established paradigm for assessing spatial working memory. In this task, rats were required to alternate between two locations (arms) of an ‘m-maze’ environment (**Fig. 1**; Jadhav et al., 2012; Kim et al., 2009; Singer et al., 2013). During the sample phase, rats navigated from a rest area located in the central stem to one of the maze arms to receive a reward. Following this, rats were held in the central stem for a 15 second delay, assessing their working memory, as they must remember the location they just

visited. Recent research has shown that thalamic nuclei are selective in encoding and consolidation of spatial information critical for working memory in an alternating task (Roy et al., 2022). After the delay phase of the task, rats are required to navigate to the opposite (non-match) maze arm for reward, called the choice phase. The choice phase evaluates reference memory, as rats must recall the previously learned spatial information about the maze to make the correct choice. The task is repeated for 10 trials daily until rats reach a criterion of two consecutive days of greater than 90% accuracy (9 out of 10 trials). Following task acquisition, rats received two consecutive days of probe trials in which the delay interval varies between 15sec and 30sec. The task therefore allowed assessment of both acquisition and retention of spatial information.

Aim: To determine whether mPAE produces sex-specific deficits *in a delayed non-match-to-place variant of a spatial alternation task*

Hypothesis: mPAE will impair non-match spatial alternation in male and female rats following mPAE

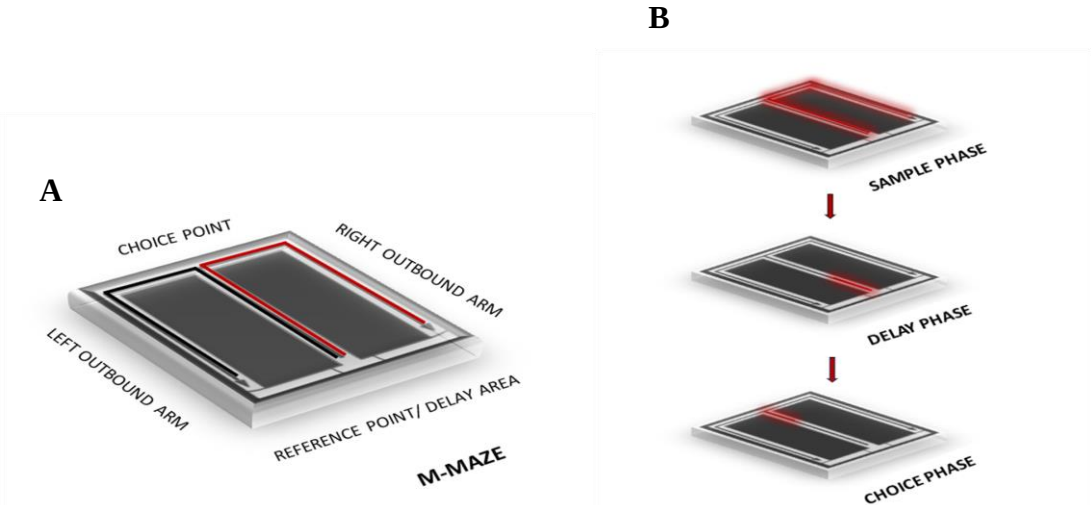

Figure 1. A) Illustrates the apparatus for the non-match spatial alternation task, with labeled components including the reference point, outbound arms, and choice point. The reference point marks the task's starting and returning point, while the outbound arms are referenced during decision-making at the choice point. Panel **B)** provides a detailed breakdown of each task phase. The sample phase initiates with a barrier obstructing an outbound arm, guiding animals in a predetermined direction. Subsequently, the delay phase confines animals at the reference point for 15 seconds. Finally, the choice phase occurs at the choice point, where animals select their subsequent direction.

EXPECTED & ALTERNATIVE OUTCOMES

Following the observations by Kenton et al (2020), we predicted that female mPAE rats will require a greater number of trials to reach the learning criteria, and will express lower performance accuracy in probe tests. Based on previous work in rat models of mPAE (e.g., Rodriguez et al., 2016; Sanchez et al., 2019; Savage et al., 2010), it is likely that deficits in spatial acquisition and retention will also be observed in male rats. Thus, while Kenton et al., (2020) produced sex-specific deficits in performance, we hypothesized that impairments will be expressed in both male and female rats. However, it is important to note that few studies have investigated sex differences in delayed non-match spatial alternation tasks (see **Table 1** above). It is therefore possible that non-match task procedures, with a variable delay as in Kenton et al, are particularly sensitive to sex-specific impairments. Specifically, Kenton et al (2020) reported impairments in female mPAE rats after delay intervals of 15sec. In the delayed non-match spatial alternation task, we will therefore use 15sec delay intervals during both acquisition and probe testing. Thus, we predicted to observe deficits in female mPAE rats during acquisition and probe tests. The aims of this study are designed to evaluate these outcomes.

METHOD

Subjects.

Subjects included 40 female (n=20) and male (n=20) Long Evans rats. Rats will be obtained from the New Mexico Alcohol Research Center located at the University of New Mexico Health Sciences Animal Resource Facility (Dr. Dan Savage's colony; see breeding protocol and **Table 2** below). Following weaning, all rats are pair-housed in standard plastic

cages on a reverse 12-hour light:dark cycle at a room temperature of 22° C with food and water provided ad libitum. Pair-housed rat offspring are given the same prenatal treatment of either moderate PAE or saccharin (SACC). SACC rats (n=20; counterbalanced for sex) and PAE rats (n=20; counterbalanced for sex) will begin testing at three months of age. The University of New Mexico central campus and Health Sciences Center Institutional Animal Care and Use Committee (IACUC) approved all procedures for the current study.

Table 2. The effects of daily 4-h consumption of 5% ethanol on female rat dams and their offspring.

	Saccharin Control	5% Ethanol Group
Daily 4 hour 5% ethanol consumption	NA	2.33 ± 0.07[a] (13)
Maternal Weight Gain during pregnancy	129 ± 8[b] (10)	117 ± 4 (13)
Litter Size	11.8 ± 0.4[c] (10)	10.9 ± 0.8 (13)
Pup birth weight	8.10 ± 0.33[d] (10)	8.33 ± 0.22 (13)

a- Mean ± S.E.M. grams ethanol consumed/kg body weight/day
b- Mean ± S.E.M. grams increase in body weight from GD 1 through GD 21
c- Mean ± S.E.M. number of live births/litter
d- Mean ± S.E.M. grams pup birth weight
NA-not applicable
(n)-Group sample size

Breeding and Voluntary Ethanol Consumption During Gestation

The University of New Mexico Health Sciences Animal Resource Facility (ARF) personnel conducted the breeding procedures using previously described procedures (Savage, Rosenberg et al. 2010, Davies, Ballesteros-Merino et al. 2019, Sanchez, Goss et al. 2019, Davies et al., 2023)). Briefly, rat breeders approximately three to four months old (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° C with ad libitum food and water. After a brief one-week acclimation period in the animal facility, the female breeders were exposed to a voluntary ethanol drinking paradigm. Female rats are

provided 0.066% (w/v) saccharin in tap water from 10:00 to 14:00 hours (four hours) each day. On days one and two, saccharin water contains 0% ethanol, and on days three and four, saccharin water contains 2.5% ethanol (v/v). On day five and subsequent days, saccharin water contained 5% ethanol (v/v). The consumption of ethanol was monitored daily during the four-hour consumption period for at least two weeks, and mean daily ethanol consumption were determined for each female breeder. Following two weeks of daily ethanol consumption, females that drink at levels less than one standard deviation below of the entire group mean (~12-15% of breeders) were removed from the study. The remaining female breeders were then assigned to either a saccharin control or 5% ethanol drinking group. The breeding females were matched based on their mean pre-pregnancy ethanol consumption. Thus, dams from both SACC and PAE groups experienced equivalent pre-conceptual exposure to ethanol.

The female breeder rats were then housed with the male breeders until conception is achieved which was assessed by the presence of the vaginal plug. Ethanol was reintroduced post-breeding during gestation. On day one of gestation for four hours a day (10:00-14:00), the 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 all of gestation, rats were provided with ad libitum water and rat chow (5LOD-Laboratory Diet, PicoLab), in addition to the four-hour ethanol/saccharine drinking period. The volume of ethanol consumed was recorded daily for each pregnant rat dam and ranged from 1.38 to 3.01 g/kg in the present study. Previous studies have shown that this level of exposure produces a mean peak maternal serum ethanol concentration of 46.0 + 3.2

mg/dL (Davies et al., 2023). After birth, daily ethanol consumption is discontinued, and the litters are weighed and culled to 10 pups. At 24 days of age, rats were weaned and transferred to the Department of Psychology Animal Research Facility. To minimize potential litter effects, one to two female or male rats were used from each breeding pair.

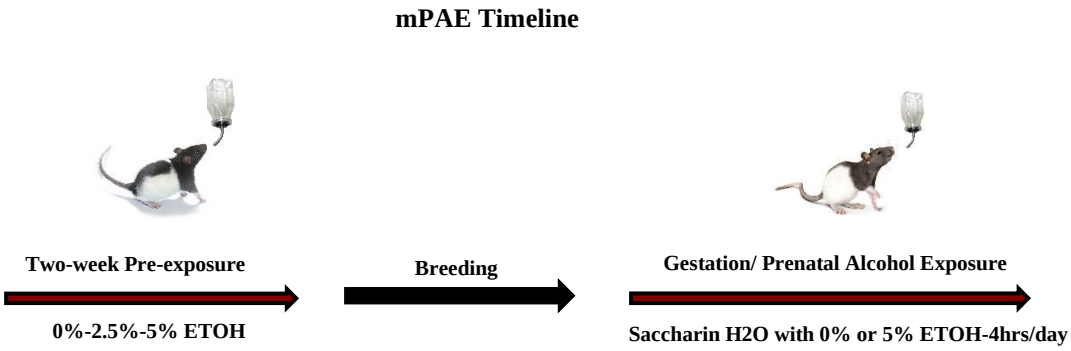

Figure 2. illustrates the temporal progression of the moderate prenatal alcohol exposure (PAE) paradigm, presenting the daily and hourly ethanol consumption of the mother, as well as the corresponding ethanol concentration.

M-Maze Apparatus

To investigate delayed non-match spatial alternation behavior, we used an M-maze configuration. Behavioral testing was conducted within a custom M-maze configuration, comprised of three vertical arms measuring 140cm in length, and two horizontal tracks measuring 110cm in width, positioned at the ends of the arms. Together the apparatus formed a figure eight manipulated by removable and adjustable dividers. Each arm and track had a width of 10.2cm, and cemented walls measuring 4.4cm in height. The entire apparatus was elevated 13.9cm above a blue-gray floor, providing a suitable point for observation. The maze was located at the center of a well-lit testing room containing visible objects such as a

white noise generator, computers, computer benches, a sink, a cabinet, shelves, and an overhead camera. An overhead camera connected to Mac computer was used to create digital recordings of each test session.

Delayed Spatial Alternation Training Procedures

Prior to training, rats were carefully handled in the testing room and subsequently exposed to the maze. Before placing the animals on the apparatus, treats were strategically distributed in a specific configuration over a four-day period. During training, rewards were allocated at the end of each arm of the apparatus. The animals were trained to alternate between the outer arms of the maze. Initially, the animals will make their way from the center arm toward either the left or right outbound arm, where they will collect a reward, called the *Sample* phase of the task. Subsequently, they will return to the center stem reference point, where they will collect a treat. Animals will then be held at the reference point for 15sec called the *Delay* phase of the task. Following the delay at the reference point, the *Choice* phase begins in which rats are released and are required to navigate to the opposite arm. Thus, rats are required to recall what direction they came from initially (e.g., left arm) during the *Sample* phase and produce the correct trajectory for reward (e.g., proceed to the right arm) during the *Choice* phase. Failing to navigate to the opposite arm during the *Choice* phase of the task is considered an error and resulted in the animal not receiving a reward. The spatial alternation task allows assessment of memory encoding (*Sample*) and consolidation (*Delay*). Animals were trained until they achieved 90% accuracy in 10 trials for two consecutive days (i.e., 9 correct trials per day). The left and right arms were counterbalanced during the *Sample* phase. After reaching criteria, animals were tested in the probe test described below.

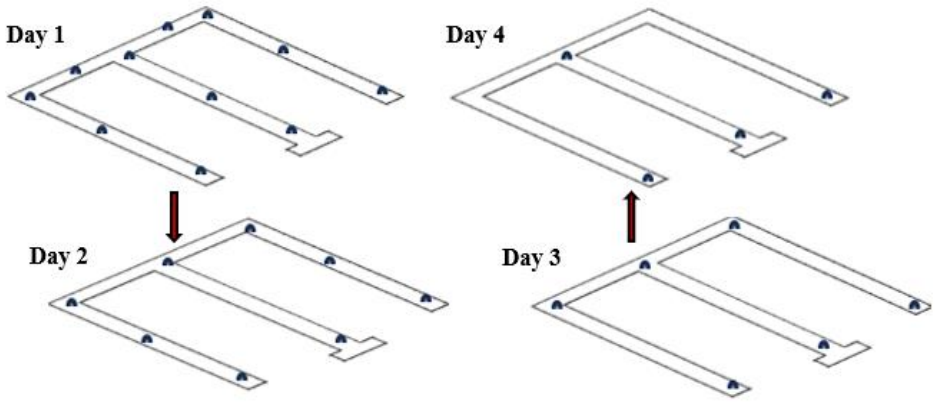

Figure 3. Illustration of the maze habituation procedure which shows the progressive configuration of treat placement in preparation for task training. Treats were progressively reduced each day in order acclimate animals to daily training treat distribution and enhancing motivation.

M-Maze Probe Test

Once the rats reached a criterion of 90% accuracy for two consecutive days, two probe tests will be administered on two separate (consecutive) days. Implementing varying delay periods ensured that the rats spatial working memory and memory retention were challenged. Each session consisted of ten trials. During the probe tests, subjects proceeded to alternate between outbound arms and produce a trajectory to the opposite arm after varying delays, as previously described for task acquisition (see above). Delays used were 15 and 30 seconds, which were evenly counterbalanced during each 10 trial probe test.

Behavior Analysis

Spatial alternation behavior was classified into three categories: correct, incorrect, or a non-responsive error. A trial was deemed correct if the rat successfully navigated down the alternate arm during the choice phase, deviating from the initial (Sample) trajectory. Conversely, a trial was classified as incorrect if rats, during the choice phase, continued

along the arm they explored during the sample phase. Furthermore, if an animal failed to exhibit a response (i.e., absence of an entry into an outbound arm during the choice phase), it was categorized as a non-responsive error.

Statistics

ANOVAs and linear mixed model approaches were used to evaluate the main effects of treatment group and sex and their interactions, with an alpha set at 0.05. For ANOVAs, a Greenhouse-Geisser correction was used in analyses where Mauchly's test indicates significant departure from the assumption of sphericity. Partial eta squared (η^2p) values was reported for each main effect and interaction as a measure of effect size. The Tukey post-hoc method was used to compare statistically significant differences. For a more comprehensive examination of the data, a linear mixed model was implemented. This approach allowed us to further examine both the main effect and interaction of probe trials, litters, and cohorts. Statistical analyses was conducted using R. An estimate marginal mean (EMMs) analysis was used to further assess the effects of the Delay variable in the probe tests. This analysis also accounted for the imbalance in sample size.

RESULTS

Delayed Spatial Alternation is Acquired More Slowly in Female Rats but Similarly Across mPAE and Control Groups.

Homoscedasticity was assessed to determine whether the assumption of equal variances across groups was met. Levene's test yielded a p- value of 0.750, indicating that the variances among groups were not significantly different. As a result, no corrections for

unequal variances were necessary in subsequent analysis. The ANOVA resulted in a significant difference in days to criteria based on the sex variable ($F(1,36) = 6.92$, $p = 0.012$, $\eta_p^2 = 0.161$). This finding indicates that sex has an effect on the time it took to meet the criteria, with females requiring more testing days to reach criteria compared to male rats.

Although 3 rats within the mPAE treatment group, comprising of two females and one male, did not meet criterion over the course of an 18-day training period, the ANOVA did not indicate significant differences for the type of treatment administered on days to criteria ($F(1,36) = 0.043$, $p = 0.837$, $\eta_p^2 = 0.001$). Thus, the results indicate that the type of treatment administered did not appear to have any significant impact on the time it took animals to meet the criteria.

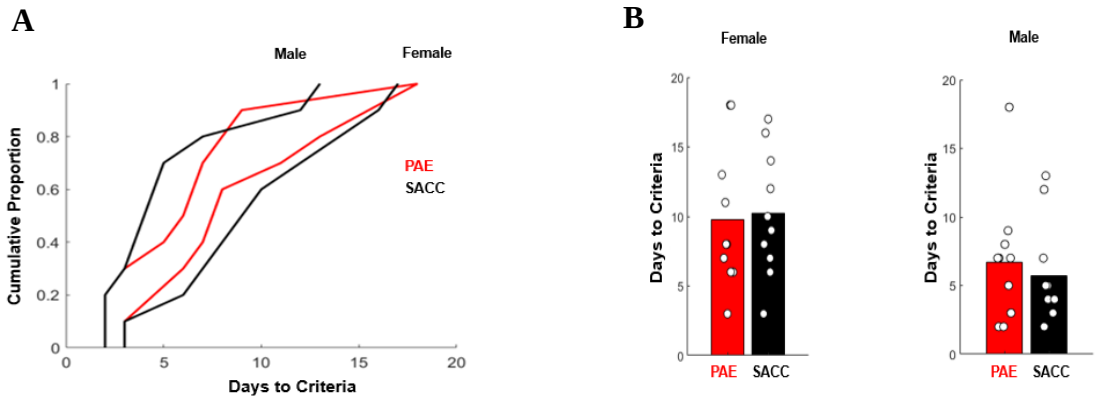

Figure 4. Days to criteria. A.) The cumulative proportion graph depicts the proportion of animals that met criteria as a function of days to criteria. There was a greater proportion of male rats that met criteria in a shorter period compared to female rats; however, there were no significant treatment differences. B.) Alternatively the bar graphs represent the days to criteria as a function of treatment group. Female rats were slower to meet criteria compared to male rats.

Varying the Delay Interval Between Sample and Choice Did Not Disrupt Performance by mPAE and Control Rats.

A linear mixed model was implemented to examine both the main effect and interaction of probe trials, litters, and cohorts. The analysis indicated that the prenatal treatment administered to rats had no significant effect on performance accuracy ($\beta_1 = 0.16$, p= 0.117), indicating that mPAE animals performed, on average, 0.16 units higher than the animals in the control group. In addition, the effect of sex ($\beta_2 = 0.17$, p= 0.113) was not statistically significant, suggesting that sex did not influence performance accuracy during probe testing. Additionally, delay intervals ($\beta_3 = 0$, p= 0.519) were also not significant, suggesting that delay intervals do not impact animals' performance in the retention delayed testing in the task. At a 15-second delay, the mean score was 4.28 (SE = 0.0679, 95% CI [4.15, 4.42]), and at a 30-second delay, the mean score was 4.23 (SE = 0.0679, 95% CI [4.09, 4.36]). Although a slight decrease in mean score was observed with a longer delay, these differences were not statistically significant.

The two-way interactions between treatment and delay ($\beta = 0.06459$, SE = 0.18004, t(DF) = 0.359, p = 0.720), and between delay and sex ($\beta = -0.05074$, SE = 0.17951, t(DF) = -0.283, p = 0.778) were also found to be non-significant. Moreover, the analysis indicated a non-significant three-way interaction among treatment, sex, and delay ($\beta = -0.27889$, SE = 0.36094, t(DF) = -0.773, p = 0.441). This suggests that the relationship between the outcome variable and the predictors is not significantly influenced by the simultaneous presence and interaction of prenatal treatment, sex, and delay. Overall, the delay did not have a significant effect on the animals' performance during the probe test.

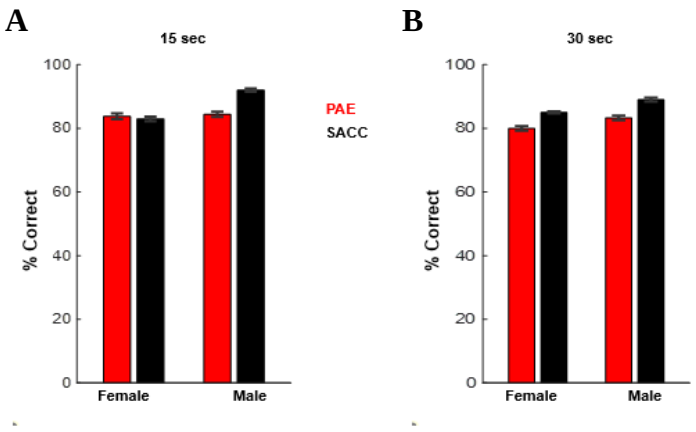

Figure 5. Probe test performance accuracy. A.) Performance accuracy in percentage at 15 second delay for each sex and treatment group. There were no significant differences in performance between sex and treatment B.) Performance accuracy in percentage at 30 second delay for each sex and treatment group. There were no significant differences in performance between sex and treatment.

DISCUSSION

The present study was aimed at testing the hypothesis that male and female mPAE rats would express impairments in the acquisition and retention of a delayed non-match to place variant of a spatial alternation tasks. Here we report that female rats required a significantly greater number of training days to reach criteria in the spatial task. However, there were no significant differences between treatment groups in acquiring the task. After reaching criteria, all rats were administered probe tests in which the delay between sample and choice was doubled in time. In sum, the probe test did not reveal significant treatment or sex differences. The results suggest that mPAE does not have a considerable effect on delayed non-match spatial alternation behavior.

The primary conclusion of the present study is that mPAE does not produce deficits in the acquisition or retention of delayed non-match spatial alternation behavior. This finding

stands in contrast with studies demonstrating an impact of mPAE on adult male rats performing novel platform learning in the MWT (behavioral flexibility), discrimination of objects conditionally associated with places (Sanchez et al., 2019), and discriminating between spatial locations in a radial maze (Brady et al., 2012; Sanchez et al., unpublished observations) (see **Table 1**).

It is possible that the discrepancy between the present study and previous studies relates to task design. For instance, Brady et al (2012) reported deficits only when spatial locations had considerable feature overlap (were adjacent arms on a radial maze). In the present study, we used an M-maze where the maze arms, while having considerable length (to elicit substantial place cell expression in hippocampus), were on opposite sides of the maze and did not carry featural (spatial cue) overlap (as in Brady et al., 2012). In other words, the two outbound segments were located on opposite sides of the test room and were likely associated with distinct distal (extra-maze) landmarks.

A second consideration is that the task in the present study was highly structured with distinct task phases (sample, delay, choice) and did not allow for expression of spontaneous investigation (re-entries) of maze arms during the choice phase. In a previous study, mPAE rats were reported to display "perseverative" spatial behaviors in which they repeatedly return to recently visited locations during trials (Sanchez et al., unpublished observation). This pattern of spatial working memory impairments has also been reported after a hidden platform has been displaced to another location in the MWT (Hamilton et al., 2014; Rodriguez et al., 2016; Sutherland et al., 2000), suggesting behavioral flexibility may be an important feature of impairment after mPAE. Future work could use an alternative hippocampal-dependent variant in which alternation between M-maze locations is continuous

for long durations. Such a design may place a greater load on spatial working memory and as a result may be more sensitive to observing online errors during navigation. This type of design would also allow comparisons between spatial working memory and spatial reference working memory (i.e., returns to the reference central maze arm).

An additional consideration is that the delay interval may need to be greater than 30sec to observe an impairment in retention. The design and selection of 15 and 30 sec interval used in the present study was based on a pilot experiment in which we observed poor performance when 30 sec was used as the delay. This pilot study used Long Evans male rats as control subjects. It is unclear why the pilot subjects showed greater difficulty with the 30sec delay interval, however, it is noteworthy that a previous study showed deficits in mPAE male mice in a visual-spatial discrimination using only a 15sec interval. Future studies could explore lengthening the delay interval in this task design.

A final conclusion of the present study is the observation that female rats (pooled across both control and mPAE groups) required a greater number of training days to reach criteria. It is unclear why sex-specific learning deficits would be observed in the present study. However, previous studies have revealed conflicting sex differences in spatial alternating tasks. A study assessing spatial learning in rats with PAE utilizing a water T-maze indicated that male subject from the PAE treatment group generally performed more poorly, with deficits persisting into adulthood (Zimmerberg et al., 1991). Both female and male animals from the PAE treatment groups made more reference errors on the task compared control animals; however, male PAE subjects also exhibited a higher frequency of working memory errors. This study suggests that sex-specific differences can be detected in some task designs, wherein females performed better compared to male animals regardless of treatment

condition. Consistent with the Zimmerberg et al. (1991) study, Gué et al. (2004) identified sex-specific differences in a delayed alternation variant among control animals in a prenatal stress (PS) experimental design. Control females spent more time in the novel arm relative to control males; however, this sex difference diminished when PS offspring were introduced. Conversely, Ulloa et al. (2004) reported that control female subjects consistently preserved their initial arm choice in a spontaneous alternation task, while male animals alternated contingent to their initial choice. These sex differences can be explained by morphological changes caused by hormones produced in the endocrine system during hippocampal formation neurodevelopment (Ulloa et al., 2004; Gould et al., 1991). Discrepancies in sex-difference could be attributed to variation in experimental parameters across studies.

In conclusion, the results suggest that mPAE does not have a considerable effect on delayed non-match spatial alternation behaviors in the M-maze. Future experiments should consider test designs in which maze arms have overlapping (perceptually similar) spatial cues, and perhaps longer delay intervals. Future designs may also consider continuous delayed alternation designs where animals are allowed to return to recently visited locations, as has been demonstrated to be impaired after hippocampal damage and mPAE in previous studies using the water maze or radial arm maze task.

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