SELECTIVE DAMAGE OF THALAMIC NUCLEUS REUNIENS IN A RAT MODEL OF FETAL ALCOHOL SPECTRUM DISORDERS: ALTERATIONS TO HIPPOCAMPUS- AND PREFRONTAL CORTEX-DEPENDENT BEHAVIORS

by

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ABSTRACT

Individuals diagnosed with fetal alcohol spectrum disorders (FASD) often display behavioral impairments in executive functioning. Mechanistic studies have implicated coordination between prefrontal cortex and hippocampus (through thalamic nucleus reuniens) as critical for such executive functions. The current study characterized the neuroanatomical alterations to midline thalamus and alterations in executive function in a rodent model of fetal alcohol spectrum disorders. Alcohol diluted in milk formula was administered to Long Evans rat pups on postnatal days (PD) 4-9 (5.25 g/kg/day of ethanol, 11.9% v/v, intragastric intubation). Control animals were intubated without administration of liquid. In adulthood (PD72), brains of females were analyzed for total cell number and neuron number in three midline thalamic nuclei. Males from the same litters were run on a battery of behavioral assays in adulthood (postnatal day >55). This battery consisted of novel object recognition, object in place, spontaneous alternations, and behavioral flexibility tasks. Nucleus-specific damage was found in midline thalamus, resulting in long term loss of neurons as well as volume specifically in reuniens of alcohol-exposed females. Males demonstrated behavioral impairments in short-term spatial memory and rule-switching, but not object recognition or levels of activity, suggesting impairments in executive functioning. This constitutes the first investigation into the circuit-specific consequence of third trimester alcohol exposure on the midline thalamus. These specific neuroanatomical and behavioral alterations suggest that prenatal alcohol exposure results in damage to prefrontal-thalamo-hippocampal circuitry.

Chapter 1

INTRODUCTION

1.1 Fetal Alcohol Spectrum Disorders

Alcohol consumption is prevalent among women of childbearing age in the United States (Tan, Denny, Cheal, Sniezek, & Kanny, 2015), with many of them continuing to consume alcohol between conception, recognition of pregnancy and advanced pregnancy (McCormack et al., 2017). Prenatal alcohol exposure often results in diagnosis of a fetal alcohol spectrum disorder (FASD) (Hoyme et al., 2016). The prevalence of FASD has a heterogeneous distribution through out the world, but is estimated between 1-10% for most countries (Roozen et al., 2016). FASD-afflicted individuals display impairments in a subset of cognitive tasks, which can vary by age, but are collectively referred to as "executive function" (Connor, Sampson, Bookstein, Barr, & Streissguth, 2000; Rasmussen, 2005; Rasmussen & Bisanz, 2009).

1.2 Modeling Third Trimester Fetal Alcohol Exposure using Rodents

A number of animal models have been developed to assess the impact of alcohol exposure at certain developmental time points, and in different doses and methods of administration (reviewed in Gursky & Klintsova, 2017; Kelly & Lawrence, 2008). One particular model, administering ethanol in a milk substitute to rats during the first two postnatal weeks of life via intragastric intubation (e.g., Goodlett, Pearlman, & Lundahl, 1998; Helfer et al., 2009), has gained popularity for its ability to precisely control the dose of ethanol administered to the pup. The first

two postnatal weeks of life in rat also model a period in nervous system development referred to as the "brain growth spurt" (Dobbing & Sands, 1979), which occurs in the third trimester of humans, making it ideal to study the impact of late gestational alcohol exposure.

The brain growth spurt model of developmental alcohol exposure has consistently produced damage to structures critical for learning and memory, such as the hippocampus (HPC) (Livy, Miller, Maier, & West, 2003; Murawski, Klintsova, & Stanton, 2012; Otero, Thomas, Saski, Xia, & Kelly, 2012) and medial prefrontal cortex (mPFC) (G. F. Hamilton, Hernandez, Krebs, Bucko, & Rhodes, 2017; G. F. Hamilton, Whitcher, & Klintsova, 2010; Lawrence, Otero, & Kelly, 2012; Otero et al., 2012). These two structures form a functional circuit along with the thalamus, and have the strongest reciprocal connectivity with nucleus reuniens (Re) of the ventral midline thalamus. Despite its role in the mPFC-HPC circuit, the Re has not yet been studied in the context of alcohol exposure during the brain growth spurt.

1.3 Role of mPFC-Re-HPC Circuit

Hippocampus has long been implicated in memory processes (Fanselow & Dong, 2010). More recently, coordination between mPFC and HPC has been implicated in memory as well. mPFC appears to be involved in context learning, especially in conjunction with HPC (Heroux et al., 2018; Heroux, Robinson-Drummer, Sanders, Rosen, & Stanton, 2017) or in compensation for HPC impairment (Zelikowsky et al., 2013). Interactions between mPFC and HPC during working memory are facilitated through the Re (Hallock, Wang, & Griffin, 2016), a structure with recurrent connectivity with both mPFC and HPC (Varela, Kumar, Yang, & Wilson, 2014). Spatial memory testing activates immediate early genes expression in

the neuronal populations within the Re (Vann, Brown, & Aggleton, 2000), supporting the notion that neurons in this region increase activity during some forms of memory or executive function. The "cognitive" behaviors that ventral midline thalamic nuclei (e.g., Re) underlie (Cassel et al., 2013) bear resemblance to the deficits in "executive functioning" observed in humans diagnosed with FASD (Khoury, Milligan, & Girard, 2015).

1.4 Thalamic Damage in Fetal Alcohol Spectrum Disorders

To our knowledge, no published data exists, to date, characterizing nucleus-specific damage to the ventral midline thalamus in either human studies or rodent models of FASD. Previous work has examined the impact of developmental alcohol exposure on gestational days 6-21 (modeling first and second trimester human alcohol exposure) on dorsal thalamus of rat did not find alterations in rates of cell death (Mooney & Miller, 2001), but the dorsal and ventral thalamus are distinct in the timing of their development and each possess a large number of distinct nuclei (Altman & Bayer, 1979). The ventrolateral nucleus of thalamus also seems to be largely unaffected by developmental alcohol exposure unless it is exclusively targeted at 2-day period of neurogenesis within the nucleus (on gestational days 14-15) (Livy, Maier, Se, & West, 2001).

1.5 Rationale for Current Research

The current study was conducted to test the hypothesis that mPFC-Re-HPC circuit is damaged comprehensively in FASD. To test this, we assess the impact of early postnatal alcohol exposure on Re of midline thalamus – an understudied but critical substrate for mPFC-HPC interactions. We additionally analyze neighboring

thalamic nuclei (both ventral and dorsal) to determine the specificity of alcohol's influence on thalamic development, and test whether behaviors known to be dependent on mPFC-Re-HPC circuitry are altered by this alcohol exposure paradigm.

Chapter 2

MATERIALS AND METHODS

2.1 Experimental Subjects

All animal procedures were performed in accordance with the University of Delaware animal care committee's regulations. A total of 41 Long Evans rats were used in the current study: 28 males (for behavioral testing) and 13 females (for neuroanatomical analysis). All animals were obtained from in-house breeding colony at the University of Delaware and housed with a 12-hour light-dark cycle (lights on at 9 AM). Food and water were administered *ad libitum*. On postnatal day (PD) 3, litters were culled to 8 pups (4 males and 4 females, where possible). Pups were cross-fostered on PD 3 when necessary to establish consistent litter size and sex distribution. Pups were also administered subcutaneous injections of small amount of black India ink in the pad of the paw to provide a unique identifier for each animal. Pups within a litter were randomly placed into one of two experimental groups: alcohol exposed (AE) or sham-intubated (SI). Each litter consisted of 4 AE and 4 SI animals, counterbalanced by sex.

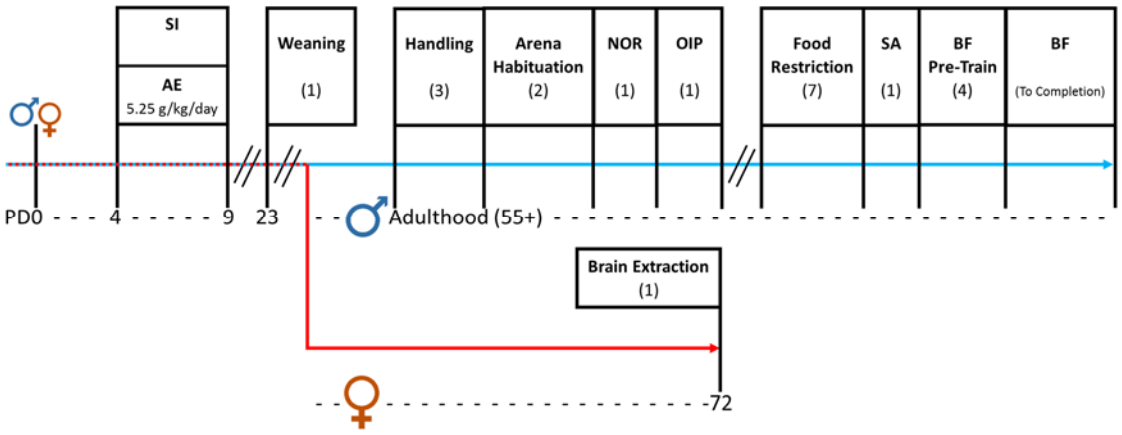


Figure 1: Experimental timeline for all manipulations. All behavioral assays were performed on male Long Evans rats while all neuroanatomical analyses were performed on female Long Evans rats. The number in parentheses below each task on the timeline indicates the number of days over which the task occurred.

2.2 Rodent Model of Human Third-trimester Alcohol Exposure

On PD 4-9, all animals were weighed daily at 9AM. AE animals were administered 5.25 g/kg/day of 11.9% v/v ethanol in a milk substitute via intragastric intubation over 2 doses, 2 hours apart. AE animals also received 1 daily supplemental dose of milk substitute (2 supplemental doses on PD 4) 2 hours after the second ethanol administration to prevent confounding weight differences from insufficient feeding during intoxication. Blood samples were collected from the tail vein on PD 4 at 90 minutes following the second ethanol administration, when blood alcohol content (BAC) is at peak (Kelly, Bonthius, & West, 1987). Collected blood samples were centrifuged at 15,000 x g for 25 minutes at 4 °C; plasma was removed from supernatant and stored at -20°C until analysis. BAC was analyzed using an Analox GL5 Alcohol Analyzer (Analox Instruments, Boston, MA).

SI animals underwent identical procedures on PD 4-9 (intubation, blood collection) but did not receive any milk substitute (due to potential weight confounds (Goodlett & Johnson, 1997)) or ethanol. All animals were earpunched on PD 9 following the final milk dose, for identification purposes.

On PD 23, all animals were weaned and subsequently housed in social cages of 2-3 same-sex animals, as is common in studies of developmental alcohol exposure (G. F. Hamilton et al., 2011; Thomas, Sather, & Whinery, 2008).

2.3 Behavioral Testing

2.3.1 Behavioral Apparati

Two distinct behavioral apparati were utilized to assess hippocampus- and prefrontal cortex-dependent behaviors in adulthood: an open field arena and a plus maze. The open field arena (used in novel object recognition and object in place tasks) is identical to the apparatus used by Hall and Savage (2016), and measures 90 cm x 90 cm x 60 cm. The plus maze (used in spontaneous alternation and behavioral flexibility tasks) consisted of 40 cm x 15 cm painted black PVC arms with 20 cm tall Plexiglas walls and was similar to the apparatus used in Young and Shapiro (2009).

2.3.2 Habituation

Once male animals reached adulthood (PD 52-72; at least 300g), each animal was handled for 5 min per day for 3 consecutive days. After 3 days of handling by experimenters, animals were habituated to the open field arena (to be used for the novel object recognition and object in place tasks) for 2 days, 10 min on each day. During habituation, animals could freely explore the empty arena with no experimenters present in the room. At the end of 10 min, the animal would be returned

to its home cage, and the arena would be cleaned. This process would be repeated for every animal within the cage before returning the cage to the colony room.

2.3.3 Novel Object Recognition

Animals were first assayed on a novel object recognition task to confirm that all animals were capable of retaining object identity over a 5 min delay period and show preference for novel objects (Antunes & Biala, 2012). The day following the second habituation day, animals were subject to novel object recognition testing as in G. R. Barker and Warburton (2008). NOR testing consisted of a 5 min habituation phase, a 5 min sample phase, and a 5 min testing phase. Each phase would be separated by a 5 min inter-phase interval when animal was returned to home cage and the arena and objects would be cleaned using diluted Mr. Clean cleaning solution or Quadricide disinfectant, respectively. During the habituation phase, the animal would be allowed to freely explore the empty arena. During the sample phase, the animal would be placed in the arena with two similar objects and video-recorded using a Sony Handycam Camcorder (Sony Corporation; DCR-HC28). During the testing phase, the animal would be placed in the arena with one familiar and one novel object and video-recorded. The location of the novel object was counterbalanced within neonatal treatment.

Exploratory behavior was coded by several experienced research assistants blind to postnatal treatment using the criteria in G. R. Barker and Warburton (2008) and values for each measure were averaged across research assistants to generate a single value of each measure for each animal. A discrimination index was generated for each animal which was defined as the difference between the amount of time spent exploring the novel object and the familiar object divided by the total amount of time

exploring both objects (+1 being exclusive novel object exploration, 0 being equal amounts of novel and familiar object exploration, and -1 being exclusive familiar object preference).

2.3.4 Object in Place

The day following NOR testing, animals were subject to object in place testing (OIP), similar to the procedures used by G. R. Barker and Warburton (2008). The OIP task was chosen due to its dependence on mPFC and HPC and robustness following NMDA alterations to perirhinal cortex (G. R. Barker & Warburton, 2015). OIP testing consisted of a 5 min habituation phase, a 5 min sample phase, and a 5 min testing phase with each phase separated by a 5 min inter-phase interval (see NOR testing). During the habituation phase, the animal was allowed to freely explore the empty arena. At the start of the sample phase, each animal was placed in the arena with four distinct objects and video-recorded. Similairly, in the testing phase, the animal was placed in the arena with two of the four objects having swapped locations and video-recorded. The location and identity of the moved objects were counterbalanced within neonatal treatment.

Exploratory behavior was coded by research assistants blind to postnatal treatment using the criteria in G. R. Barker and Warburton (2008) (see above, NOR). A discrimination index was generated for each animal which is defined as the difference between the amount of time spent exploring the moved objects and the unmoved objects divided by the total amount of time exploring all objects (with +1 being exclusive moved object exploration, 0 being equal amounts of moved and unmoved object exploration, and -1 being exclusive unmoved object preference).

2.3.5 Food Restriction

Following completion of NOR and OIP testing, animals were individually housed and fed a reduced diet of standard lab chow. Animals were weighed daily and food consumption was individually monitored such that each individually housed animal's weight was restricted to 85-90 % of *ad lib.* weight over 7 days. Animals remained on food restricted diets to maintain 85-90 % *ad lib.* weight throughout spontaneous alternation, behavioral flexibility pre-training, and behavioral flexibility tasks.

2.3.6 Spontaneous Alternation

After 7 days of food restriction and appropriate weight loss, animals were tested for spontaneous alternation behaviors as in Bobal and Savage (2015). SA was chosen to probe hippocampal function as it is robustly hippocampal-dependent across many manifestations of the task (Lalonde, 2002). In brief, animals were allowed to freely explore a plus maze for 18 minutes while an experimenter recorded behavior. An arm entry was defined as all 4 paws of an animal passing onto the PVC plank that constitutes a given arm. Each arm entry was recorded by the experimenter and the total number of entries were quantified and an alternation score was determined for each animal as in Bobal and Savage (2015).

2.3.7 Pre-training

The two days immediately following SA testing, animals were habituated to foraging for food reward (Dustless Precision Sucrose Pellets; F06233, Bio-Serv, Flemington, NJ) on the plus maze apparatus. Animals were placed in the center of the plus maze and required to consume a food reward placed in the cup of all 4 arms

within 2.5 min over 2 consecutive trials. Following 2 consecutive trials of successful foraging, the animal was returned to its home cage and returned to the colony room.

Starting on the day following foraging training, animals had 2 consecutive days of pre-training. The plus maze apparatus was converted into a T-maze by placing a weighted divider into one of two opposing arms. Animals were placed in the arm opposite the weighted divider ("start arm") and required to forage both open arms ("goal arms") for 4 consecutive trials per day, over 2 consecutive days. The direction and location of the animal's first goal arm entry was recorded to determine whether the animal had any location or action preferences. No animals displayed marked location- or first turn-preference.

2.3.8 Behavioral Flexibility

Following pre-training, animals began training and testing on the behavioral flexibility (BF) task, as outlined in Young and Shapiro (2009). The BF task is designed to assess functionality of prefrontal cortex, with different phases of the task probing functionality of distinct prefrontal subregions (Bissonette, Powell, & Roesch, 2013). Animals were tested during approximately 6 of 7 days in a given week. The BF task consists of 5 phases, in order: initial discrimination learning ("Disc"), a first reversal ("Rev1"), a second reversal ("Rev2"), a rule switch ("RS"), and a third reversal ("Rev3"). The rules for the BF task were determined by either the action that the animal would have to perform at the center of the maze ("choice point") to enter the goal arm containing the reward (Bio-Serv Dustless Precision Pellets®, 45 mg, Sucrose, Product # F0023), or the goal arm (location) in which the reward was placed. Each rule (action or location) had 2 contingencies (action: right or left; location: north or south). Each animal was randomly assigned to an initial discrimination rule (action

or location) and contingency (left or right; north or south). A schematic representation of reversals and rule switches is provided in Figure 2.

Animals were not allowed to self-correct following an unsuccessful trial. In a given phase, an animal underwent "training days" during which the animal was given 40 trials to reach a criterion of 6 consecutive correct trials. Every 2 consecutive successful trials, the start arm would switch to the opposing (previously blocked) arm. If an animal failed to reach criterion in 40 trials, the subsequent day would be an additional training day. If the animal reached criterion within 40 trials, the animal would undergo a "retention day" the following day. On a retention day, the animal would perform 24 trials with the start arm randomly determined by a random number generating algorithm. If the animal performed at an 85 % or greater success rate on a retention day, it would progress to a training day for the next phase of the task on the following day; if the animal performed below an 85 % success rate, it would return to a training day for the same phase on the subsequent day. Animals that could not acquire an initial discrimination within 100 total training day trials were no longer run on the BF task (6 total animals [4 SI, 2 AE] excluded for this reason). One SI animal did not complete Rev3 due to mechanical error in the testing room during behavioral testing. Due to this attrition in our original cohort, an additional cohort was generated using identical methods to appropriately power analyses for the behavioral flexibility task. The total number of trials to criterion (TTC), successes to criterion (STC), errors to criterion (ETC), and training days to criterion (DTC) were recorded for each animal on each phase of the task.

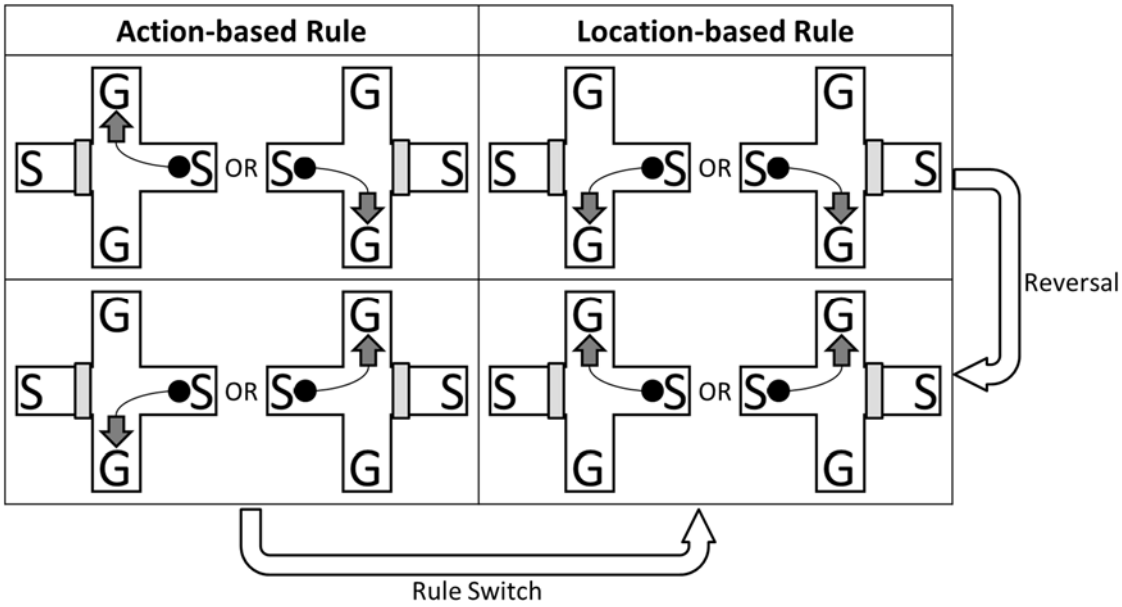


Figure 2: Schematic Diagram illustrating Behavioral Flexibility task progression. Start arms are labeled with an "S". Goal arms are labeled with a "G". The rewarded goal arm is indicated with a shaded arrow, with the curved path illustrating the path from start point (black circle) to the reward.

2.4 Tissue Preparation

On PD 72, female animals were lightly anesthetized with gas isoflurane then deeply anesthetized with a ketamine-xylazine mixture via intraperitoneal injection. Animals were then perfused with heparinized 0.1 M phosphate buffered saline (PBS) followed by 4 % paraformaldehyde in 0.1 M PBS, pH 7.2. Brains were postfixed in 4% paraformaldehyde in 0.1M PBS for 24 hrs, then equilibrated with 30 % sucrose in fixative solution. Brains were sectioned coronally at 40 μm using a cryostat (CM3050S; Leica Biosystems, Wetzlar, Germany) and serial sections were collected in a cryoprotectant buffer solution for histological and immunofluorescent processing.

2.5 Histology and Immunohistochemistry

2.5.1 Cresyl Violet

Tissue was first stained histologically to generate total cell counts within multiple thalamic nuclei (reuniens: Re, rhomboid: Rh, and mediodorsal: Md). For histological processing, every 6th serial section was mounted on slides maintaining the order and air dried. Re, Rh, and Md were exhaustively sectioned, stained, and slide-mounted, resulting in 8-12 sections for analysis per animal. Sections were then rehydrated in dH_2O followed by staining with 0.1 % Cresyl "Fast" Violet (RT12780; Electron Microscopy Science, Hatfield, PA) in acetic acid buffer (pH=4.45) for 30 min. Following staining, sections were dehydrated in increasing concentration ethanol rinses (70%, 95%, and 100% ethanol), and cleared in SafeClear II (044-192; Fisher Scientific, Hampton, NH) prior to cover-slipping with DPX mounting medium (RT13512; Electron Microscopy Science, Hatfield, PA).

2.5.2 NeuN Immunohistochemistry and Hoechst Staining

An additional set of every 6th serial section was used for free-floating immunohistochemical processing. Immunofluorescent staining with Hoechst33342 provides an alternative measure to verify the reliability of histological measures and allows for co-localization with NeuN, a robust neuronal indicator (Lind, Franken, Kappler, Jankowski, & Schilling, 2005) to determine potential loss of neurons in Re and Rh due to PD 4-9 alcohol exposure. Again, Re and Rh were exhaustively mounted resulting in 8-12 sections for analysis per animal. Tissue was first rinsed in 0.1 M tris-buffered saline (TBS; pH=7.40; BP153; Fisher Scientific, Hampton, NH) and blocked in a solution of 3 % donkey serum (S30; Merck Millipore, Burlington, MA) and 0.5% Triton X-100 (BP-151; Fisher Scientific, Hampton, NH) in 0.1M TBS for 1 hr. Then, tissue was incubated in primary anti-NeuN antibody (Millipore Cat# MAB377,

RRID:AB_2298772) in TBS/goat serum/Triton-X blocking solution at 1:500 dilution for 48 hrs at 4 °C. Next, tissue was washed in 0.1M TBS then incubated in a Cy3-conjugated secondary antibody (Jackson ImmunoResearch Labs Cat# 715-165-151, RRID:AB_2315777) in blocking solution at 1:250 dilution for 2 hrs. Tissue was washed once more in 0.1M TBS prior to slide mounting in dH_2O. After air drying for 1 hr, tissue was rehydrated with 0.1 M PBS and stained with Hoechst33342 (H3570; ThermoFisher Scientific, Waltham, MA) in 0.1M PBS (1:1000) for 5 min. Tissue then washed with 0.1 M PBS prior to cover-slipping with Vectashield Anti-fade Mounting Medium (H-1000; Vector Laboratories, Burlingame, CA). After allowing mounting medium to set for 12 hrs, slides were sealed with clear coat nail polish (Insta-Dri 106; Sally Hansen) and stored at 4 °C.

2.6 Unbiased Stereological Quantification

Cell number and volume within each thalamic nucleus was quantified using the optical fractionator (West, Slomianka, & Gundersen, 1991) workflow in Stereo Investigator software (Stereo Investigator, RRID:SCR_002526). Tissue was imaged using an Axioskop2Plus outfitted with a 5x Plan-Neofluar objective for contouring the regions of interest and either a 40x Plan-Neofluar objective for brightfield histological quantification or a 63x oil-immersion Plan-Neofluar for immunofluorescent quantification (Carl Zeiss Inc., Thornwood, NY). The regions of interest were outlined according to (Paxinos & Watson, 2013). Tissue thickness measurements for all tissue was determined at every 5^{th} counting site. The following stereological parameters were utilized for brightfield histological quantification: 50 µm x 50 µm counting frame, 200 µm x 200 µm grid size, 12.00 µm disector height with 1.00 µm guard zones, and 1/6 section counting fraction. The following stereological parameters were utilized for

immunofluorescent quantification: 30 µm x 30 µm counting frame, 210 µm x 210 µm grid size, 25.00 µm disector height with 1.00 µm guard zones, and 1/6 section counting fraction. To assure unbiasedness in the event of damaged or missing tissue, missing sections were accounted for using the "account for missing sections" function in Stero Investigator, resulting in an adjusted missing sections fraction to be applied to the final stereological estimate. Co-localization of NeuN and Hoechst is demonstrated in *Figure 4C*. Means for Gundersen coefficient of error (m=1) for total cell number estimates were below 0.10 for all structures and postnatal treatments, and can be seen in *Table 1*. Confocal images were prepared using Fiji image analysis software (Schindelin et al., 2012).

Postnatal Treatment	Thalamic Nucleus	Methodology	(n)	Coefficient of Error (mean ± SEM)
SI	Re	Cresyl Violet	(8)	0.051 ± 0.014
		Immunofluorescence	(8)	0.055 ± 0.002
	Rh	Cresyl Violet	(8)	0.090 ± 0.027
		Immunofluorescence	(8)	0.073 ± 0.004
	Md	Cresyl Violet	(8)	0.034 ± 0.007
AE	Re	Cresyl Violet	(5)	0.042 ± 0.002
		Immunofluorescence	(5)	0.064 ± 0.005
	Rh	Cresyl Violet	(5)	0.054 ± 0.002
		Immunofluorescence	(5)	0.082 ± 0.005
	Md	Cresyl Violet	(5)	0.030 ± 0.004

Table 1: Average Gundersen coefficient of error (m=1) for all stereological estimates of total cell number distributed by postnatal treatment, thalamic nucleus, and methodology.

2.7 Statistical Analyses

All statistical analyses were performed using RStudio (RStudio, RRID:SCR_000432) with R version 3.4.3 (R Core Team, 2018) running the "tidyverse" (Wickham, 2017), "pwr" (Champely, 2018), "effsize" (Torchiano, 2017), and "powerAnalysis " (Fan, 2017) packages. To appropriately analyse all response variables while accounting for potential influence of extreme values (*i.e.*, outliers), the structure of data were comprehensively characterized prior to statistical inference testing. Prior to any between-group analyses or correlational analyses, measures were analyzed for violations of normality using a Shapiro-Wilks test ($\alpha = 0.050$). Response variables that violated assumptions of normality ($p \leq .05$ on Shapiro-Wilks test) were analyzed using nonparametic analyses (Wilcoxon rank sum test for between-groups

comparisons using the unpaired version of the “wilcox.test” function, Spearman rank correlation for correlational analyses using the “spearman” method of the “cor.test” function). Data that did not demonstrate violations of normality ($p > 0.05$ on Shapiro-Wilks test) were analyzed using either a t-test for equal variances (Base R var.test function, $p > 0.05$) or a Welch t-test for unequal variances (Base R var.test function, $p \leq 0.05$). Correlations that did not violate assumptions of normality were performed as bivariate Pearson’s product-moment correlations. Power analyses for 2-group comparisons, 1-group comparisons, and correlations were performed using the pwr.t2n.test, pwr.t.test, and pwr.r.test functions (pwr package), respectively. Effect sizes (Cohen’s d) to be used in power analyses were calculated using the cohen.d function (effsize package) for 2-sample t-tests and ES.t.one (powerAnalysis package) for one-sample t-tests. Statistics are reported with test statistic with subscript degrees of freedom (N-2 for 2-group analyses and bivariate correlation; N-1 for one-group analyses), p-values, effect size (Cohen’s D), and achieved power.

Chapter 3

RESULTS

3.1 Weights and Blood Alcohol Concentrations

Average weights of pups in SI and AE groups did not significantly differ on PD4 prior to treatment (t_{39}=-1.47, p=0.149, Cohen's d=-0.46, power=0.30).

Postnatal Treatment	Sex	(n)	Weight (g) on PD4 (mean ± SEM)	Weight (g) on PD9 (mean ± SEM)	Weight (g) in Adulthood (Males: Arena Habituation; Females: Perfusion; mean ± SEM)
SI	Total	(21)	10.60 ± 0.17	19.11 ± 0.45	295.62 ± 13.67
	F	(8)	10.25 ± 0.32	19.20 ± 0.62	231.63 ± 7.24
	M	(13)	10.82 ± 0.18	19.06 ± 0.63	335.00 ± 12.03
AE	Total	(20)	10.93 ± 0.13	16.45 ± 0.45	308.90 ± 10.75
	F	(5)	11.24 ± 0.24	17.24 ± 0.48	255.20 ± 22.19
	M	(15)	10.82 ± 0.15	16.19 ± 0.57	326.80 ± 8.40

Table 2: Weights and numbers of all experimental animals distributed by postnatal treatment and sex.

BAC at 90 minutes following the second administration of ethanol on PD4 did not differ between sexes of AE pups (W_{18}=32.00, p=0.672, Cohen's d=0.13, power=0.06). AE pups weighed significantly less than SI pups on PD9, the final day of treatment (t_{39}=-4.22, p<0.001, Cohen's d=1.32, power=0.98). Average weight of SI and AE adult animals (males at time of first arena habituation; females at time of perfusion) did not significantly differ at in adulthood (t_{39}=0.76, p=0.452, Cohen's d=-0.24, power=0.11).

Sex	(n)	BAC (mg/dL) on PD4 (mean ± SEM)
Total	(20)	365.06 ± 30.64
F	(5)	378.60 ± 37.64
M	(15)	360.54 ± 39.47

Table 3: BAC of AE animals at 90 minutes following second alcohol administration on PD4.

3.2 Neuroanatomy

3.2.1 Midline Thalamic Cell Number and Volume Estimates (Figure 3)

Re of AE female rats displayed significant reductions in total cell number (t_{11}=2.31, p=0.041, Cohen's d=1.32, power=0.56) and volume (t_{11}=2.66, p=0.022, Cohen's d=1.52, power=0.68), relative to SI controls. Md of AE females rats showed a significant reduction in volume (W_{11}=36.00, p=0.019, Cohen's d=1.11, power=0.43). but not total cell number (t_{11}=1.17, p =0.266, Cohen's d=0.67, power=0.19). In contrast, neither total cell number nor volume of Rh significantly differed between AE and SI females (p's≥0.379). BAC following final alcohol administration on PD4 did not significantly correlate with any measure (p's≥0.480).

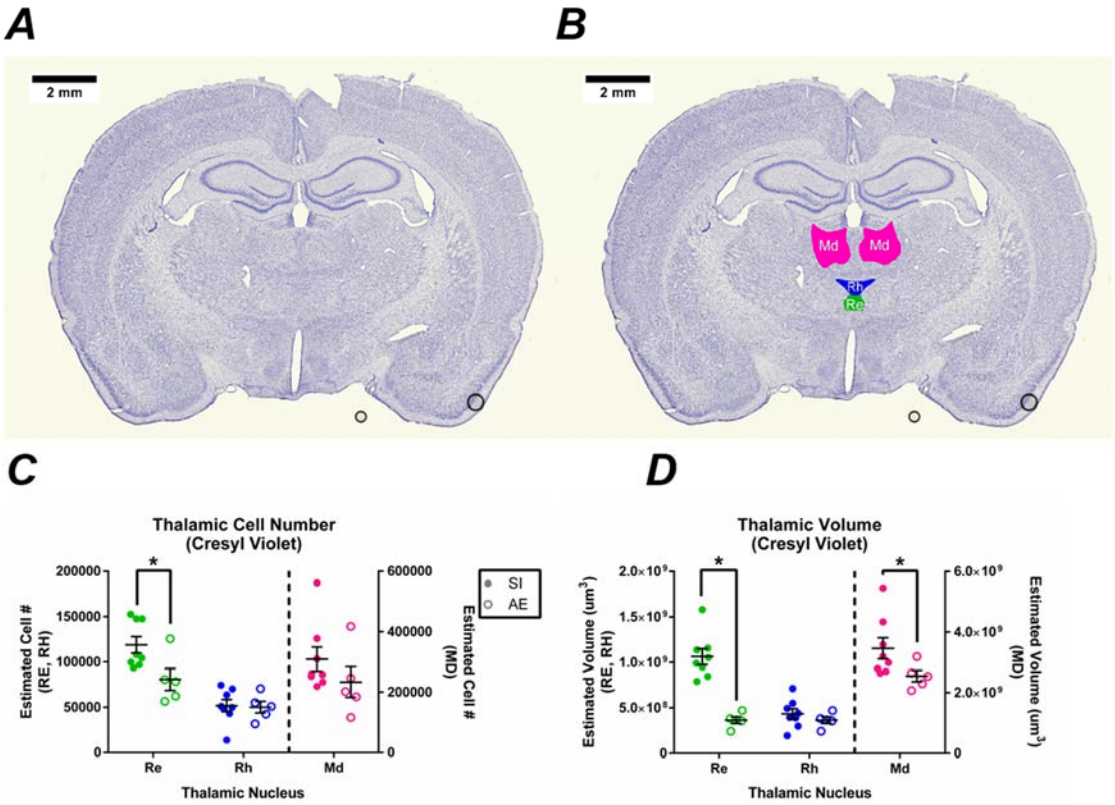


Figure 3: Thalamic nucleus reuniens selectively loses cells within midline thalamus of adult female rats following PD4-9 alcohol exposure. *3A)* An acquired photomicrograph of a representative coronal section located approximately 2.40mm caudal to bregma in the rat brain. Image was acquired and compiled simultaneously using the "Virtual Tissue" feature on StereoInvestigator software (MBF Bioscience, Williston, VT) with a 20x Plan-Neofluar objective on a Zeiss Axioskop 2Plus (Carl Zeiss Inc., Thornwood, NY). Scale bar: 2mm. *3B)* Photomicrograph from 3A overlaid with representative contours and labels for thalamic nucleus reuniens (Re, green), rhomboid nucleus of thalamus (Rh, blue), and mediodorsal thalamus (Md, magenta). Contours were superimposed over the micrograph using Fiji software (Schindelin et al., 2012). Scale bar: 2mm. *3C)* Only Re displays significant cell loss in adult AE females relative to SI controls. *3D)* Both Re and Md display significant reductions in volume in adult AE females relative to SI controls. Data are presented as individual data points with arithmetic mean and error bars (± SEM). SI = filled-in shapes, AE = hollow shapes, *p≤0.05

3.2.2 Ventral Midline Thalamic Neuron Number and Volume Estimates (Figure 4)

AE females displayed significant reductions in total Re neuron number (t_{11}=3.21, p=0.008, Cohen's d=1.83, power=0.83), as well as total cell number (W_{11}=35.00, p=0.030, Cohen's d=1.64, power=0.74) and volume (t_{11}=2.54, p=0.027, Cohen's d=1.45, power=0.64), relative to SI controls. In contrast, total neuron number, total cell number, and volume in Rh was not significantly different between AE and SI females (p's≥0.153). BAC following final alcohol administration on PD4 was not significantly correlated with any measure (p's≥0.134).

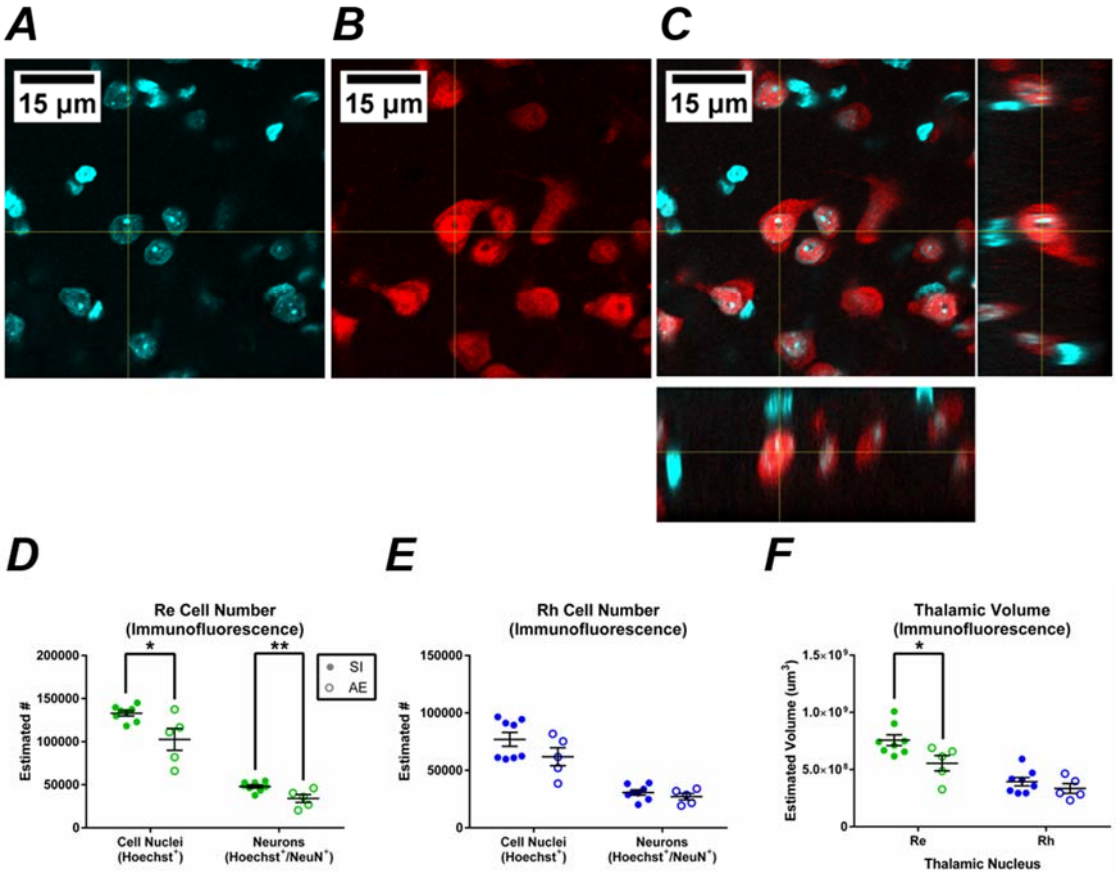


Figure 4: Neuron loss in ventral midline thalamus of adult AE females is specific to thalamic nucleus reuniens. *4A, 4B, 4C)* Representative confocal micrographs of Re illustrating Hoechst33342 *(4A)* and anti-NeuN conjugated with Cy3 *(4B)* immunofluorescence (pseudocolored cyan and red, respectively), as well as a merged image (*4C*; with x-y [center], y-z [right], and x-z [bottom] planes). Neuronal phenotype was determined by co-localization of Hoechst and NeuN immunofluorescent signal (e.g., cell in yellow crosshair). Scalebar and crosshairs were inserted using Fiji software (Schindelin et al., 2012). Scale bars: 15µm. *4D)* Both total cell number and total neuron number was significantly decreased in Re of AE females. *4E)* AE females displayed no significant differences in total cell number nor total neuron number in Rh. *4F)* AE females displayed a significant reduction in Re volume, but showed no differences in Rh volume relative to SI controls. Data are presented as individual data points with arithmetic mean and error bars (± SEM). SI = filled-in shapes, AE = hollow shapes, *p≤0.05, **p≤0.01

3.3 Behavioral Testing

3.3.1 Novel Object Recognition (Figure 5)

We found that SI (control) males displayed novel object preference (DI>0) during both 5 min of the testing phase (t_{10}=6.46, p<0.001, Cohen's d=1.95, power=0.99) and the first min of the testing phase (t_{10}=6.73, p<0.001, Cohen's d=2.03, power=0.99). Novel object preference in SI control males did not differ between the first min and full 5 min of NOR (t_{20}=-2.03, p=0.056, Cohen's d=-0.86, power=0.49). Based on the work of others that suggest that the first minute of NOR testing is a better predictor of novelty preference than whole-session preference (Jablonski, Schreiber, Westbrook, Brennan, & Stanton, 2013), we followed with comparison of only the first minute of recorded behavioral testing between groups to determine the impact of AE on NOR behaviors.

SI and AE males did not differ in the total amount of time spent exploring objects during the sample phase (t_{20}=-0.29, p=0.777, Cohen's d=-0.12, power=0.06) or 1st min of testing (t_{20}=-0.17, p=0.866, Cohen's d=-0.07, power=0.05) of the NOR task. Both SI and AE groups displayed preference for the novel object relative to the familiar object (t_{10}=6.73, p<0.001, Cohen's d=2.03, power=0.99 and t_{10}=5.53, p<0.001, Cohen's d=1.67, power=0.99, respectively). Novel object preference did not differ between AE and SI males (t_{20}=1.71, p=0.102, Cohen's d=0.73, power=0.37). However, BAC following final alcohol administration on PD4 was negatively correlated with NOR discrimination index (r_9=-0.64, p=0.034, Cohen's d=-1.66, power=0.60).

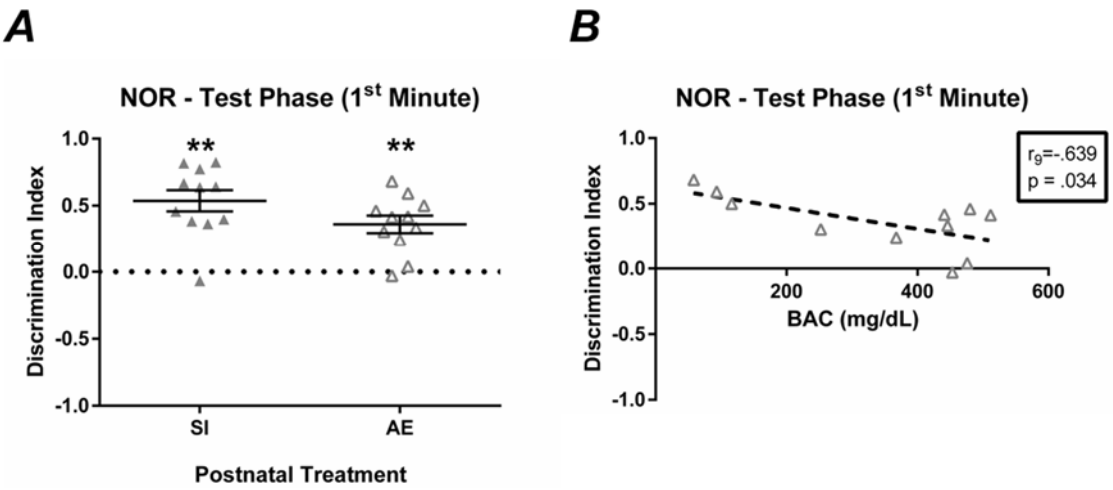


Figure 5: Males with higher BACs on PD4 show lower levels of novel object preference in males. *5A)* Both SI and AE animals did display preference for the novel object (indicated by asterisks over individual group). Discrimination index (DI) between postnatal treatments did not significantly differ. The dotted line indicates a Discrimination Index (DI) of 0 (similar amounts of time exploring both the novel and familiar object). *5B)* BAC 90 minutes following alcohol administration on PD4 showed a strong negative correlation with discrimination index during the NOR task. The dashed line indicates the line-of-best-fit for the data. Data are presented as individual data points with arithmetic mean and error bars (± SEM). SI = filled-in shapes, AE = hollow shapes, **p≤0.01 relative to chance (DI of 0.0)

3.3.2 Object in Place (Figure 6)

SI males displayed significant preference for moved objects during the first minute of the testing phase (W_9=47.00, p=0.049, Cohen's d=0.79, power=0.60), but not over 5 min of the testing phase (t_9=1.45, p=0.180, Cohen's d=0.46, power=0.26). Consistent with findings of others that spatial novelty learning is weaker than object recognition learning, and that spatial novelty learning is most robust during the first 1-2 minutes of the testing phase of the task (Jablonski et al., 2013), we therefore only

analyzed the first minute of OIP testing between groups to determine the impact of AE on NOR behaviors.

The total amount of time spent exploring objects during the sample phase did not differ between SI and AE groups for 5 min (t_{19}=-1.64, p=0.117, Cohen's d=-0.72, power=0.35), or during the 1st min of testing (t_{19}=-0.98, p=0.338, Cohen's d=-0.43, power=0.15) of the OIP task. Neither SI nor AE animals displayed biased object preference during the sample phase (t_9=-0.58, p=0.576, Cohen's d=0.18, power=0.08, and t_{10}=0.49, p=0.637, Cohen's d=0.15, power=0.07, respectively). The SI group showed significant preference for moved objects (W_9=47.00, p=0.049, Cohen's d=0.79, power=0.60) but the AE group did not (t_{10}=0.90, p=0.391, Cohen's d=0.27, power=0.13). However, despite this the SI and AE groups did not differ significantly in moved object preference (t_{19}=0.44, p=0.664, Cohen's d=0.19, power=0.07). BAC following final alcohol administration on PD4 negatively correlated with OIP discrimination index (r_9=-0.64, p=0.035, Cohen's d=-1.65, power=0.60). Within the AE group, NOR discrimination index did not significantly correlate with OIP discrimination index (r_9=0.57, p=0.066, Cohen's d=1.40, power=0.48).

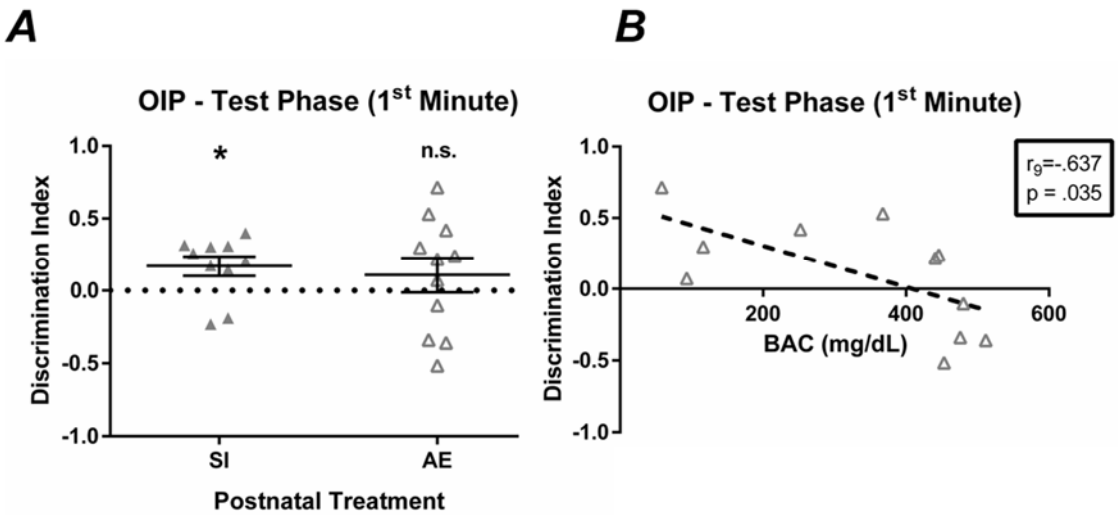


Figure 6: PD 4-9 alcohol exposure eliminates moved object preference in males. *6A)* The SI group did display preference for moved objects (indicated by asterisk over individual group) while the AE group did not. SI and AE groups did not significantly differ in their preference for moved objects. The dotted line indicates a Discrimination Index (DI) of 0 (similar amounts of time exploring both the novel and familiar object). *6B)* BAC 90 minutes following alcohol administration on PD4 showed a strong negative correlation with discrimination index during the NOR task. The dashed line indicates the line-of-best-fit for the data. Data are presented as individual data points with arithmetic mean and error bars (± SEM). SI = filled-in shapes, AE = hollow shapes, n.s. = not significant relative to chance (DI of 0.0), *p≤0.05 relative to chance (DI of 0.0)

3.3.3 Spontaneous Alternation (Figure 7)

SI and AE animals did not differ on either alternation score (t_{11}=-0.04, p=0.966, Cohen's d=-0.02, power=0.05) or total number of arm entries (W_{11}=22.00, p=0.942, Cohen's d=0.51, power=0.13). BAC following final alcohol administration on PD4 showed a strong negative correlation with alternation score (r_4=-0.87, p=0.023, Cohen's d=-3.57, power=0.70), but did not correlate with number of arm entries (r_4=0.80, p=0.055, Cohen's d=2.69, power=0.54).

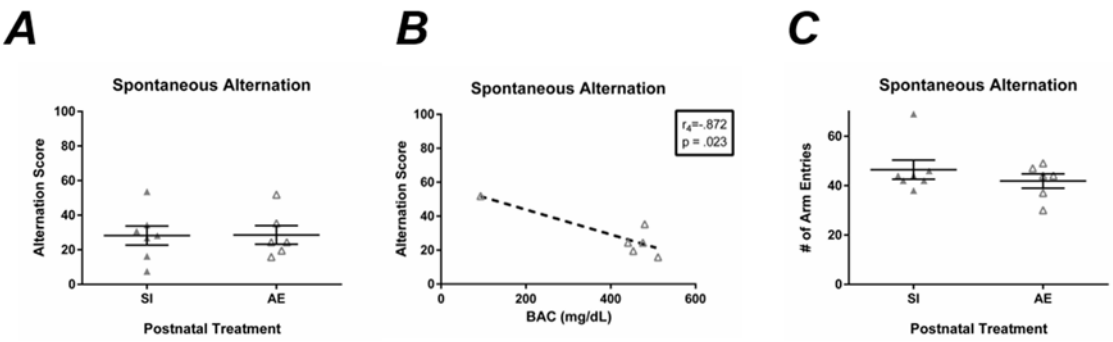
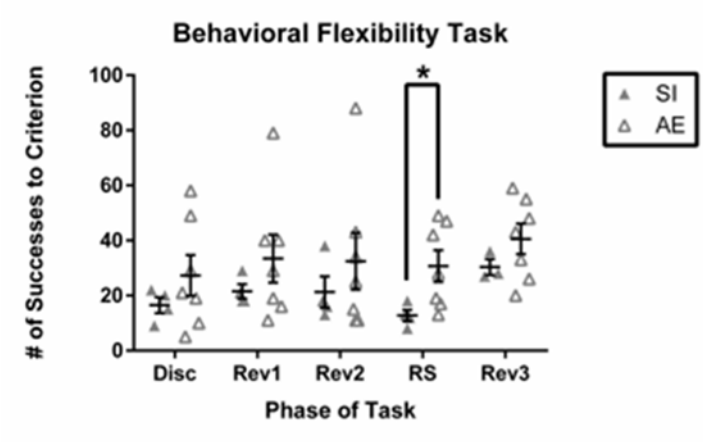


Figure 7: PD 4-9 alcohol exposure did not alter alternation or activity patterns beyond control levels in a spontaneous alternation task in males. *7A)* Alternation score did not significantly differ between AE and SI males. *7B)* BAC 90 minutes following alcohol administration on PD4 showed a strong negative correlation with alternation score. The dashed line indicates the line-of-best-fit for the data. *7C)* Total number of arm entries did not significantly differ between AE and SI males. Data are presented as individual data points with arithmetic mean and error bars (± SEM). SI = filled-in shapes, AE = hollow shapes

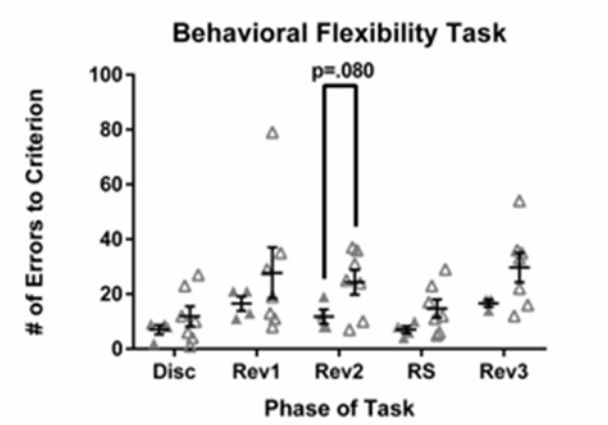
3.3.4 Behavioral Flexibility (Figure 8; Table 4)

AE males displayed increased number of successful trials to criterion during the Rule switch phase (W_9=2.50, p=0.037, Cohen's d=-1.43, power=0.53), but did not differ on number of trials, errors, or training days to criterion (p's≥0.088). AE and SI males' trials, successes, errors, and training days to criterion did not differ on any other phase of the BF task (p's≥0.071). A detailed list of statistical tests between groups for behavioral flexibility task can be found in Table 4. BAC following final alcohol administration on PD4 was not significantly correlated with any measure during the BF task (p's≥0.167).

A


Behavioral Flexibility Task
# of Successes to Criterion
100
80
60
40
20
0
*
SI
AE
Disc
Rev1
Rev2
RS
Rev3
Phase of Task

B


Behavioral Flexibility Task
# of Errors to Criterion
100
80
60
40
20
0
p=.080
Disc
Rev1
Rev2
RS
Rev3
Phase of Task

C

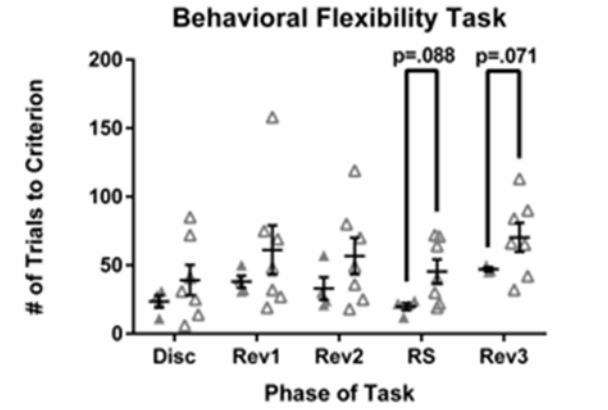

Behavioral Flexibility Task
# of Trials to Criterion
200
150
100
50
0
p=.088
p=.071
Disc
Rev1
Rev2
RS
Rev3
Phase of Task

Figure 8: AE males manifest deficits in executive functioning through impaired rule switching. *8A)* AE males took approximately 3-4x more successfully rewarded trials on average to learn a rule switch relative to SI controls. *8B)* SI and AE males did not differ on number of errors to reach criterion at any point during behavioral flexibility. *8C)* SI and AE males did not differ on number of trials to reach criterion at any point during behavioral flexibility. Data are presented as individual data points with arithmetic mean and error bars (± SEM). SI = filled-in shapes, AE = hollow shapes, *p≤0.05

Phase of Task	Measure	Test Performed	Test Statistic	Degrees of Freedom	P Value
Discrimination	Total Trials	Wilcoxon rank sum	8.500	9	0.344
	Successes	Wilcoxon rank sum	9.000	9	0.412
	Errors	T-test	-0.369	9	0.369
	Training Days	Wilcoxon rank sum	6.000	9	0.133
Reversal 1	Total Trials	Wilcoxon rank sum	11.500	9	0.705
	Successes	Wilcoxon rank sum	10.500	9	0.568
	Errors	Wilcoxon rank sum	12.000	9	0.775
	Training Days	Wilcoxon rank sum	9.000	9	0.366
Reversal 2	Total Trials	T-test	-1.238	9	0.247
	Successes	Wilcoxon rank sum	13.000	9	0.925
	Errors	T-test	-1.969	9	0.080
	Training Days	Wilcoxon rank sum	13.500	9	0.999
Rule Switch	Total Trials	Wilcoxon rank sum	4.500	9	0.088
	Successes	Wilcoxon rank sum	2.500	9	0.037
	Errors	T-test	-1.669	9	0.129
	Training Days	Wilcoxon rank sum	7.500	9	0.211
Reversal 3	Total Trials	Welch T-test	-2.176	8	0.071
	Successes	T-test	-1.142	8	0.287
	Errors	T-test	-1.519	8	0.167
	Training Days	Wilcoxon rank sum	6.000	8	0.253

Table 4: Details of the statistical analyses performed between-groups for each phase and measure within the Behavioral Flexibility task.

Chapter 4

DISCUSSION

4.1 Summary of Findings

The current study demonstrates that early postnatal alcohol exposure induces severe and persistent damage to nucleus reuniens of the ventral midline thalamus in the female rat. This damage manifests as a reduction in total neuron number, cell number, and Re volume (the latter two measures replicating when re-sampled and analyzed using two different methods of visualization). Male rats from the same cohorts displayed alcohol-induced impairment of object-in-place preference accompanied by modest impairments in rule-switching behaviors in a plus maze-based version of behavioral-flexibility, both of which implicate altered mPFC-HPC interactions.

Lack of alcohol-induced alterations in Rh, an adjacent thalamic nucleus, suggests that such damage is circuit-specific rather than exclusively region-specific. While a reduction in volume of Md was observed, total cell number was unaffected, indicating that AE may alter Md, but this damage does not appear to produce a functional deficit, as does damage to the Re, which manifests as a reduction in number of neurons, total cell number, and volume. Further, we did not observe deficits in reversal learning at any point — a behavioral pattern that emerges following lesion of Md (Chudasama, Bussey, & Muir, 2001) — suggesting that the reduction in volume that we observed in Md is unlikely to contribute behavioral impairment in this FASD model.

Additionally, tasks that require subregions of the PFC besides mPFC (i.e., reversal learning which requires orbitofrontal cortex) are not impaired, further

implicating circuit-specific alterations following early postnatal alcohol exposure. Lack of alcohol-induced alterations in control tasks indicate that AE animals were able to retain the memory of objects for the necessary delay period for OIP testing (indicated by a significant novel object preference), which did not differ from control animals, and a lack of change in motor behavior (e.g., number of arm entries in SA). Similar amounts of time exploring objects during each phase of NOR and OIP demonstrates that changes in discrimination index are driven by differences in object preference rather than changes in activity or total amounts of exploration.

4.2 Validity of Findings

Neuroanatomical findings were replicated using both histological staining (cresyl violet) evaluated by brightfield microscopy and fluorescent labeling (Hoechst33342) combined with immunofluorescence (for NeuN) and widefield epifluorescence with high-numerical aperture objective. All neuroanatomical analyses were performed using appropriate unbiased stereological procedures, maximizing the validity of the data collected and allowing for the use of re-sampling within-animal for the two methods of analysis. The rate of reduction in AE females was approximately 25% of the cell population observed in SI control females, a rate of cell loss that was consistent in either methodology used (*i.e.*, brightfield microscopy or epifluorescence).

While neuroanatomical analysis and behavioral testing occurred in different sexes, convergence of results suggests that cell loss due to early postnatal alcohol exposure maybe occurring in males, as it did in females. Previous studies examining prefrontal and hippocampal alterations following early postnatal alcohol exposure in rats have been inconsistent in finding sex-related differences. While some literature did not find significant sex differences in molecular or anatomical measures within

PFC or HPC (Livy et al., 2003; Otero et al., 2012), some behavioral studies have observed differential influence of alcohol between sexes (Goodfellow, Abdulla, & Lindquist, 2016; Goodlett & Peterson, 1995; Mooney & Varlinskaya, 2011). Therefore, the influence of sex as a biological modulator of alcohol-related brain damage still warrants further investigations within Re.

While AE animals did display reductions in weight at PD9, it is possible that observed effects are the consequence of differences in weight during early postnatal development. There were no significant differences in weight by the time of assessment (either behavioral or neuroanatomical), indicating that weight differences between treatment groups are transient and do not appear to persist across the lifespan. This supports the notion that our observed effects are not the consequence of nutritional deficits at time of assessment.

4.3 Identifying mPFC-Re-HPC Circuit Damage in Fetal Alcohol Spectrum Disorders

Taken together, these data constitute the first (to our best knowledge) report of circuit-specific damage to midline thalamus in a rodent model of prenatal alcohol exposure. This indicates that the anatomical alterations underlying this neurodevelopmental disorder may be more pervasive than previously thought, as these data demonstrate that Re, a previously unexamined portion of mPFC-Re-HPC circuitry is damaged following developmental alcohol exposure. A considerable body of work characterizes developmental alcohol exposure-related damage in HPC (Berman & Hannigan, 2000) and a small, but growing, body of literature indicates that mPFC is damaged as well (Lawrence et al., 2012; Louth, Luctkar, Heney, & Bailey, 2017; Mihalick, Crandall, Langlois, Krienke, & Dube, 2001). While non-invasive

imaging is integrating into rodent models of FASD (e.g., Rodriguez, Davies, Calhoun, Savage, & Hamilton, 2016), such techniques currently lack the spatial resolution to resolve individual thalamic nuclei, which the current study has indicated is a necessity.

Only a few studies have examined the impact of prenatal AE on corticothalamic connectivity (Granato, Santarelli, Sbriccoli, & Minciacchi, 1995; Santarelli et al., 1995), but this research looked at a markedly different time point in development: gestational days 14-19, a rodent analogue of human second trimester. Although both Granato et al. (1995) and Santarelli et al. (1995) observed reductions in corticothalamic connectivity following gestational alcohol exposure, the studies examined thalamic connectivity to somatosensory cortex and did not investigate nucleus-specific alterations to thalamus.

4.4 Role of Re in Mediating mPFC- and HPC-dependent Behaviors

Functional neuroanatomical research over the last 2 decades has implicated ventral midline thalamus in a variety of behavioral processes, all broadly encompassed under executive functioning. One critical note is that the current study assessed damage to individual nuclei within the midline thalamus, indicating that Re and Rh were differentially affected by our developmental alcohol exposure paradigm. In contrast, all reviewed studies experimentally manipulate the "ventral midline thalamus" (Re and Rh together) without distinguishing between Re and Rh (Hallock et al., 2016; Xu & Sudhof, 2013), which provide a necessary baseline for ventral midline thalamic function, but results in a lack of research on the unique contributions of Re to behavior. Regardless, ventral midline thalamus activity is necessary for mPFC-HPC synchronization, and inhibition of ventral midline thalamus via direct muscimol infusion results in reductions in mPFC-HPC coherence and impaired performance on

the (mPFC- and HPC-dependent) delay-non-match-to-place task (Hallock et al., 2016). Our observed neuron loss in Re likely impairs mPFC-Re-HPC connectivity as the major afferent projections of Re neurons are mPFC, HPC, and “”limbic” structures (Vertes, Hoover, Do Valle, Sherman, & Rodriguez, 2006). There is also a small subpopulation of Re projection neurons (<10%) that collaterally project to mPFC and HPC, which Varela et al. (2014) have hypothesized are critical for mPFC-HPC synchronization. Further research must determine whether this subpopulation of Re projection neuron are preferentially vulnerable to developmental alcohol exposure.

Re is necessary for the recall of OIP memory, as inactivation of Re 15 minutes prior to the test phase is sufficient to abolish moved object preference (G. R. I. Barker & Warburton, 2018). This suggests that damage to Re in FASD likely impairs recall processes in executive functioning, rather than the initial encoding processes. We observed deficits in rule shifting but no abnormalities in reversal learning, therefore, our data suggest that mPFC is selectively damaged within PFC, as mPFC and OFC play dissociable roles in rule switching and reversal learning, respectively (Bissonette et al., 2008; Young & Shapiro, 2009). The highest density of Re projections to PFC are located within mPFC (Vertes et al., 2006), further supporting the relationship between our observed alcohol-induced neuroanatomical and behavioral alterations.

One theory of mPFC-Re-HPC interaction is that mPFC and Re are critical in regulating the intrinsic function of HPC. mPFC inactivation impairs the ability to adapt to rule shits in a plus maze behavioral flexibility task much like the one used in the current study (Rich & Shapiro, 2007) by altering mPFC-HPC activity (Guise & Shapiro, 2017). These impairments in behavior are accompanied by reductions in prospective coding, suggesting that the mPFC is not necessary for baseline learning or

performance, but rather plays a role in reducing interference in HPC to allow prospective coding in a spatial memory task. This idea of mPFC/Re-induced modulation of HPC is extended in work by Xu and Sudhof (2013) in which activity in Re projection neurons was downregulated or upregulated using either tetanus toxin (inactivation of synaptic transmission) or neuroligin-2 knock-down (disinhibition of Re neurons), respectively. These bidirectional changes in neural activity were paralleled by bidirectional changes in context generalization in a contextual fear conditioning paradigm: Decreased Re activity resulted in generalization of contextual memory – whereas increased Re activity increased specificity of contextual memory. Increases in Re neuron activity were coupled with increased neuronal activity in both the mPFC and the CA1 region of the HPC, as indicated by quantification of immunofluorecent labeling for c-Fos. Xu and Sudhof (2013) proceed to demonstrate that bidirectionally manipulating Re activity via optogenetic photostimulation during context training is sufficient to induce similar behavioral changes, supporting the belief that Re manipulations can influence mPFC and HPC activity, whether such manipulations occur over longer timeframes (e.g., drug infusion over minutes) or shorter timeframes (e.g., optogenetic acitivty modulation over milliseconds). This supports the idea that mPFC and Re are critical in the function of HPC in tasks that are traditionally considered hippocampus-dependent, such as plus-maze behavioral flexibility tasks and contextual fear conditioning experiments. Similar reductions in levels of neuronal activation in CA1 of HPC occur following learning in late adolescence following the same alcohol exposure paradigm used in the current study (Murawski et al., 2012). Murawski et al. (2012) demonstrate that reductions in CA1 activation are strongly correlated with behavior on contextual learning. These

abovementioned studies suggest that the Re likely plays a role in generalization and memory modification, but not baseline learning and performance, through modification of mPFC and HPC activity. Therefore, we believe that alcohol-induced damage to Re impairs performance on complex HPC-dependent tasks such as OIP or rule switching by altering function of HPC and mPFC, but only on some — but not all — hippocampal tasks (i.e., OIP but not spontaneous alternation).

4.5 Conclusions

Our observation of nucleus-specific damage to midline thalamus suggests that developmental alcohol exposure damages the brain in a circuit-specific manner. Developmental alcohol exposure selectively damages neuronal populations that are undergoing circuit formation and synaptogenesis (Olney, Ishimaru, Bittigau, & Ikonomidou, 2000). Re is a nodal hub between mPFC and HPC, having high levels of recurrent connectivity with both structures (reviewed in Cassel & Pereira de Vasconcelos, 2015; Cassel et al., 2013) and undergoing neurogenesis at a similar developmental period to HPC (Altman & Bayer, 1979; Bayer, 1980), which possesses high levels of synaptogenesis over the first 2 postnatal weeks of life in rodents (Fiala, Feinberg, Popov, & Harris, 1998). The observed damage in Re converges with behavioral evidence that HPC- and mPFC-dependent behaviors are impaired, suggesting that behavioral consequences of alcohol exposure in the third trimester may be the result of damage to the mPFC-Re-HPC circuit rather than isolated damage to any of its elements.

Human studies assessing the behavioral and neuroanatomical impact of prenatal alcohol exposure suggest that function within the frontal lobe (Ware et al., 2015), as well as structure and function in the HPC (D. A. Hamilton, Kodituwakku,

Sutherland, & Savage, 2003; Joseph et al., 2014; Willoughby, Sheard, Nash, & Rovet, 2008), correlate with behavioral abnormalities in FASD-afflicted individuals. This literature also notes that functional and behavioral alterations are more pronounced on tasks with higher level of difficulty. While little work has examined changes to thalamus in humans diagnosed with FASD, some work suggests that prenatal alcohol exposure reduces whole-thalamus volume (however, individual nuclei were not discriminated in this work) (Nardelli, Lebel, Rasmussen, Andrew, & Beaulieu, 2011).

The convergence between rodent literature modeling FASD and human studies of FASD suggests that this model shows a high degree of validity for the human diagnostic condition. Further, our observed circuit-specific alterations suggest that the broader regions of interest for executive functioning (i.e., PFC, thalamus, HPC) each contain functionally distinct subregions, only a subset of which appear to be critical targets of developmental alcohol exposure. Further work needs to be done to tease apart the vulnerability of individual subregions, to assure that consequences of developmental alcohol are not overlooked due to structural heterogeneity.

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Appendix A

INSTITUTIONAL ANIMAL CARE AND USE COMMITTEE ANNUAL REVIEW

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University of Delaware
Institutional Animal Care and Use Committee
Annual Review

Title of Protocol: Therapeutic Motor Training and Fetal Alcohol Effects	
AUP Number: 1134-2018-2	← **(4 digits only)**
Principal Investigator: Anna Y Klintsova	
Common Name: Rat, Long Evans **Genus Species:** Rattus norvegicus	

Pain Category: ***(please mark one)***

USDA PAIN CATEGORY: ***(Note change of categories from previous form)***

Category	Description
☐ B	Breeding or holding where NO research is conducted
☐ C	Procedure involving momentary or no pain or distress
☒ D	Procedure where pain or distress is alleviated by appropriate means (analgesics tranquilizers, euthanasia etc.)
☐ E	Procedure where pain or distress cannot be alleviated, as this would adversely affect the procedures, results or interpretation

Official Use Only

IACUC Approval Signature: [signature] DVM

Date of Approval: 7.1.18