EFFECT OF DEVELOPMENTAL ALCOHOL EXPOSURE ON THE STRUCTURE AND FUNCTION OF THE MEDIAL PREFRONTAL CORTICAL-REUNIENS-HIPPOCAMPAL CIRCUIT IN A RODENT MODEL OF FETAL ALCOHOL SPECTRUM DISORDERS

by

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A thesis submitted to the Faculty of the University of Delaware in partial fulfillment of the requirements for the degree of Master in Science in Neuroscience

Spring 2023

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ACKNOWLEDGMENTS

I would like to thank my advisor, Dr. Anna Klintsova, for her dedicated, fierce support to push her students to self-actualize their full academic, professional, and personal potential. Thank you for taking a chance on me at the end of my freshman year, and for providing me with unimaginable opportunities since. Having the honor to work on Dr. Klintsova's research line has fostered a level of intellectual confidence that is incomparable to any success I've had in a classroom. I cannot properly articulate my gratitude for how this position has fulfilled a childhood dream, one where I get to put on my burgundy lab coat, an actual bona fide scientist, and work on what I find to be the most stimulating, fascinating thing in the universe: the complexity of the human mind. My successes would not be possible without the substantial work and dedication Dr. Klintsova has put into her passion for decades.

I would also like to thank third-year graduate student Ian Smith, who has dedicated a portion of his graduate career to serve as my mentor on this project and has guided me for the past 2.5 years. I would like to thank my professional role model, Dr. Katrina Milbocker, whose intelligence is matched equally by her professional grace, and serves as the epitome of an inspiration for women in STEM. Thank you to all members past and present of the Klintsova Lab who made this work possible, especially: SuHyeong Kim, Gillian LeBlanc, Eric Brengel, Maddie Callahan, Allison George, and the ingenious Dr. Zachary Gursky whose work inspired our current research focus. I would like to thank the dedicated undergraduates whom I've had the pleasure of mentoring and training this year Sarah Gustafson, Melissa Grogin, and

Kaitlyn Goblirsch, I wish you the very best in your pursuits. I would like to thank my thesis committee Dr. William Kenkel and Dr. Sheu Chai for their support throughout both my undergraduate and graduate theses.

It is because of the incredible staff at the Office of Laboratory Animal Medicine we are able to conduct this work, thank you to every member for their time. Additionally, I acknowledge my deep appreciation and respect for the research animals who have contributed to our scientific understanding, without whom this knowledge would not be accessible. Funding for this work has been provided by the NIH/NIAA RO1 AA027269 grant awarded to Dr. Klintsova.

I would not be here today without my friends and family who offer support far beyond requirements. Thank you to my mom Catherine Onesi, who never denies a phone call, and my stepdad Mark Teoli, the support system behind her unwavering support. I would like to thank my best friends who double as my siblings, Anthony, Elizabeth, Alex, Marissa, and Aaron. I wouldn't want to spend my life with any other group of people, SWAT team for life. Lastly, I would like to dedicate this thesis to former Carnegie Mellon professor Dr. Randy Pausch who inspired thousands to give yourself the permission to dream. At nine years old, I picked up his memoir solely because there was a rocket ship on the cover. Unknowingly, the contents inside changed my life and over a decade later I still find fragments of his advice guiding me. His words have made me who I am, all culminating to this present moment.

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LIST OF ABBREVIATIONS

Fetal Alcohol Spectrum Disorders	FASD
Alcohol Exposure	AE
Fetal Alcohol Syndrome	FAS
Partial Fetal Alcohol Syndrome	pFAS
Alcohol Related Birth Defects	ARBD
Alcohol Related Neurodevelopmental Disorder	ARND
Brain Growth Spurt	BGS
Blood Alcohol Concentration	BAC
Executive Function	EF
Hippocampus/ dorsal Hippocampus	HPC/ dHPC
Nucleus Reuniens	Re
Medial Prefrontal Cortex	mPFC
Object-in-Place	OIP
Socially Housed	SH
Tris Buffered Saline	TBS
Normal Donkey Serum	NDS
Postnatal Day	PD
Postnatal	PN
Discrimination Index	DI

Central Nervous System	CNS
Immediate Early Genes	IEGs

ABSTRACT

Fetal Alcohol Spectrum Disorders encompass an array of developmental disorders defined by physical, behavioral, and cognitive deficits as a result of prenatal exposure to alcohol. As many as 1 in 20 live births in the United States have been exposed to sufficient alcohol exposure to produce lasting, significant impairments in these areas. Among these deficits, a notable effect of AE is damage seen to executive function, a set of cognitive controls involved in goal-directed behaviors. Executive function regulation has been associated with activity in both the hippocampus and medial prefrontal cortex in the mammalian brain. However, an intermediary structure, the nucleus reuniens has been implicated in the reciprocal communication between the hippocampus and medial prefrontal cortex. Research has demonstrated that developmental AE can specifically damage the reuniens, compromising signal transmission between structures. It is therefore hypothesized that damage to the structural integrity of the medial prefrontal cortical-reuniens-hippocampal circuit is linked to executive function deficits observed in Fetal Alcohol Spectrum Disorders by compromising synchrony between the medial prefrontal cortex and hippocampus.

This study used a rodent model of third trimester binge alcohol exposure to examine the neuroanatomical alterations to the reuniens that result from neonatal alcohol exposure and linking these findings to previous work that used an associative memory behavioral task to model executive function. This study examined the effects of developmental alcohol exposure on neuron activation and loss of neurons in the nucleus reuniens. It was expected, and found, that neonatal alcohol exposure

significantly reduced the number of mature neurons, as well as the number of activated mature neurons in the reuniens. It was expected that activation of other non-neuronal cells would be negatively affected, however we did not find an effect of alcohol exposure on activation of non-neuronal cell types. This finding suggests that anatomical alterations to the reuniens may play a major role in executive dysfunction observed in Fetal Alcohol Spectrum Disorders.

Chapter 1

INTRODUCTION

1.1 Fetal Alcohol Spectrum Disorders

Fetal Alcohol Spectrum Disorders (FASD) encompass a spectrum of preventable developmental disorders that are the result of prenatal alcohol exposure (AE). Some of the earliest studies on what eventually came to be recognized as FASD started in the early 1970s after researchers identified children with confirmed cases of AE during prenatal development and compiled the shared deficits among the subjects. This was the first instance of identifying the consequential side effects of prenatal AE. In this study, they described the presence of shared deficits as "Fetal Alcohol Syndrome" (FAS) and this sparked decades of research into the devastating effects that AE has on neurodevelopment (Riley et al., 2011).

As research persisted, it became clear there are cases of AE during pregnancy that result in major deficits that do not meet FAS diagnostic criteria. It is now known that what this early study identified as FAS describes the extreme end to what is a spectrum of related developmental disorders that result from prenatal AE and falls

under the umbrella of FASD. Among others, this spectrum includes Fetal Alcohol Syndrome (FAS), Partial Fetal Alcohol Syndrome (pFAS), Alcohol Related Birth Defects (ARBD), and Alcohol Related Neurodevelopmental Disorder (ARND) (Riley et al., 2011). Where a patient falls on the FASD spectrum is variable and dependent not only on the amount of AE during gestation, but also the timing of exposure during development (Hoyme et al., 2016; Institute of Medicine, 1996; Klintsova et al., 2013), as any resulting damage is contingent on the developmental stage of a brain region during exposure. More extreme diagnoses are divided into three major symptom areas consisting of (1) prenatal and/or postnatal growth delays, (2) facial abnormalities (e.g. smooth philtrum, thin vermilion border, short palpebral fissures), and (3) central nervous system dysfunctions (e.g. cognitive and behavioral deficits) (Kingdon et al., 2016). FAS, which could result from heavy AE during the first trimester, is characterized by specific facial dysmorphologies, below average birth height and weight, deficits in motor skill developments, and underlying central nervous system dysfunction, including behavioral and cognitive changes (Hoyme et al., 2016, Kingdon et al., 2016, Klintsova et al., 2013). These extreme and distinct characteristics of FAS makes it a distinguishable and readily diagnosable disorder on the FASD spectrum. Another disorder on this spectrum, ARBD, is specified in patients with physical, congenital defects whereas ARND diagnosis is described in patients with isolated neurodevelopmental problems (Institute of Medicine, 1996). FASD diagnoses from late-term exposures are primarily based on neurological changes observed in cognitive and behavioral deficits as the teratogenic effects of AE

are presented at a time when higher-order complex processes are being developed, especially during crucial developmental milestones such as the brain growth spurt (BGS) (Hoyme et al., 2016; Klintsova et al., 2007; Kodituwakku et al., 2001). The indistinct nature of late-term exposure deficits makes them harder to detect and are easily misdiagnosed as other neurological disorders with similar symptomatology such as Attention-Deficit/Hyperactivity Disorder or Autism Spectrum Disorder leading to improperly matched treatment which may not aid in reducing deficits (Bakhireva et al., 2018). This misdiagnosis discrepancy can only be resolved by further parsing out the mechanisms and phenotypes of FASD. The need for clearer diagnostic criteria in addition to the increasingly high prevalence of FASD makes it of interest for further research. Overall, children with FASD experience an array of outcomes ranging from memory and executive functioning deficits to difficulties with socialization (reviewed in Klintsova et al., 2013), all of which have long term effects on quality of life.

Despite widespread warnings from major public health organizations about the effects of drinking during pregnancy, it is reported that as many as 5% of live births in the U.S. have been affected by prenatal AE (May et al., 2009). This makes prenatal AE a leading preventable cause of developmental disorders in the U.S. (U.S. Department of Health and Human Services, 2021) and makes FASD a major public health concern. As the prevalence of FASD persists, more research on the neural mechanisms by which alcohol structurally and functionally impairs brain development paired with potential therapeutic interventions is needed.

1.2 Rodent Model of FASD

To implement a preclinical model of FASD, there are three fundamental aspects to be considered: the timing, amount, and route of alcohol administration. Understanding the stages in the timeline of brain development allows us to target equivalent developmental time points in species with differing gestational periods. In humans, the BGS occurs during the third trimester of pregnancy and is defined by a period of rapid brain growth, neurogenesis, differentiation, migration, and synaptogenesis (Dobbins & Sands, 1979). Additionally, there are increases in axon growth, dendritic arborization, and myelination (Meyer et al., 1990), making this time period crucial for long term development. Since rats are altricial species, the BGS takes place within the first two postnatal weeks of life (Dobbins & Sands, 1979). This postnatal occurrence of the BGS in rodents allows researchers to address the effects of alcohol exposure on the developing brain by directly administering alcohol to animal subjects rather than via bloodstream or umbilical cord from the dam. There are many methods of alcohol administration used in previous studies including self-administration, intraperitoneal injections, vapor inhalation, and intragastric intubation, each with their own benefits and drawbacks. Self-administration and vapor inhalation, while effective, do not allow standardization of alcohol dose to each animal. However, the design of each does have the benefit of reducing stress during the procedure as it does not require dam and pup separation (Klintsova et al., 2013). The self-administration method is not applicable to this model as the subjects are pups in the

first days of PN life and are unable to drink yet, only suckle. Intraperitoneal injections have the benefit of monitoring exact dosage, but this procedure is stressful to pups and impractical for models of multiple alcohol exposures (Klintsova et al., 2013). Intragastric intubation is a commonly used method of administration for recreating binge-like exposures because it produces consistent, high peak of BACs (Klintsova et al., 2013) and allows precise regulation of the amount of alcohol administered to each animal. However, it does invoke stress to the dams and pups, which can present as potential confounds in experimental designs if not controlled for. This study used an established paradigm to model third trimester binge-like alcohol exposure in neonatal rat pups where a high alcohol dose is administered via intragastric intubation across two doses per day, two hours apart, during postnatal days 4-9 to specifically target the rodent BGS.

1.3 Executive Function and the Nucleus Reuniens

Executive function (EF) refers to a set of cognitive processes involved in regulation of goal-directed behavior (Kingdon et al., 2016) and consists of three core areas: inhibition, working memory, and cognitive flexibility (Diamond, 2002). Inhibition consists of two subcategories: response inhibition and interference control. Response inhibition refers to the ability to self-regulate, to have control over impulsivity or giving into temptation. Inference control refers to inhibition at a more cognitive level, mainly attentional control (Diamond, 2002). Working memory is a

type of short-term memory implicated in executing cognitive tasks and is crucial in learning as it allows us to hold information briefly and process it (Diamond, 2002). Lastly, cognitive flexibility refers to an organism's ability to quickly adjust behavior according to a changing environment and see things from a different perspective (Dajani and Uddin, 2017; Diamond, 2013; Diamond, 2014). Proper EF is crucial for maintaining an array of everyday functions that have long-term impacts on proper development, academic functioning, and mental and physical health (Anderson, 2002; Diamond, 2013). Executive dysfunction is a frequently observed deficit in FASD clinical populations, and common behavioral markers of the disorder include impulsivity, hyperactivity, and inattention (Diamond, 2013; Mattson et al., 2011; Rasmussen, 2005).

The neural underpinnings of EF have been linked to synchronous neural activity between the hippocampus (HPC) and medial prefrontal cortex (mPFC) in the mammalian brain (O'Neill et al., 2013). Previous studies have shown that while the HPC can directly communicate with the mPFC, there are no direct inputs to the HPC projecting from the mPFC (Jin & Maren, 2015). For the mPFC to send signals to the HPC, the nucleus reuniens (Re), located in the ventral midline thalamus, acts as an intermediary structure to relay neural activity and support communication (Hallock et al., 2016). Additionally, the Re also receives inputs from the HPC to project back to the mPFC, making the Re an overall critical structure for reciprocal signal transmission (Hallock et al., 2016; Jin & Maren, 2015; Layfield et al., 2015; O'Neill et al, 2013; See Figure 1). This bidirectional connectivity of the ventral midline thalamus

observed in non-human subjects has also been replicated in humans via diffusion weighted imaging (Reeders et al., 2023). Developmental AE has consistently been shown to detrimentally alter both the HPC and mPFC via reductions in region volume, cell density, and cell counts (Murawski et al., 2012; Lawrence et al., 2003; Livy et al., 2003), therefore disrupting the functional synchrony of the mPFC-Re-HPC circuit which supports EF. Consequently, it is hypothesized that the structural integrity of the mPFC-Re-HPC circuit as a whole is crucial for EF tasks and damage to its components has the potential to lead to improper EF and long-term behavioral impairments. Our lab has previously shown that in an animal model of third trimester exposure, AE damages the Re via lasting neuron loss and a reduction in structural volume (Gursky et al., 2019, Gursky et al., 2020), which may compromise the function of the circuit by disrupting the synchrony between the mPFC and HPC and lead to impairments in EF. Gursky et al. (2021) has also observed EF deficits following binge-like AE during the third-trimester equivalent. Taken together, this study sought to further elucidate the relationship between neuroanatomical alterations to the mPFC-Re-HPC circuit and EF behavioral deficits.

One component of EF is associative memory, which refers to the ability to learn and retain information between unrelated items (Diamond, 2013). To test how associative memory was implicated in our model of FASD, an Object-in-Place (OIP) associative memory task, which is known to be dependent on an intact Re, was used (Barker & Warburton, 2018; Hallock et al., 2016; Layfield et al., 2015). Proper performance on the OIP task requires associative memory formation and retention of

the original object placements after they have been altered which relies on reciprocal signal transmission between the PFC and HPC, implicating the crucial role of the Re for success in this task. It was expected that control, non-AE animals would exhibit increased exploration of the moved objects relative to unmoved objects. AE animals were expected to have reduced and undifferentiated exploration of moved objects due to impaired associative memory formation of the original object location. We found in our previous study that developmental AE did not have an effect on exploratory behavior of moved objects, indicating no observed impairments to EF in that specific study (see Supplementary Figure 2).

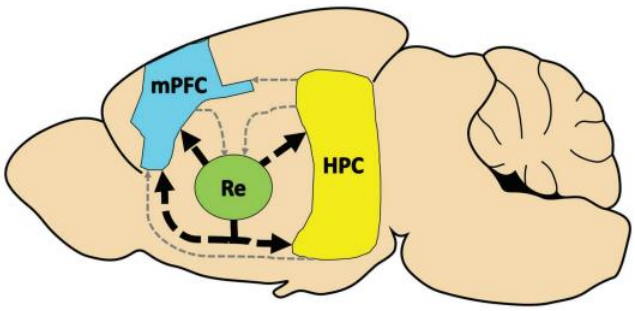


Figure 1. Schematic representing mPFC-Re-HPC reciprocal signal transmission in the rodent brain.

1.4 Neuroanatomical Changes in the Nucleus Reuniens

Previously, our lab compared the levels of neuronal activation in the Re with behavioral performance on an OIP task in AE and control animals by quantifying expression of the protein c-Fos, the product of the immediate early gene *c-fos* that is

often expressed by depolarized neurons following stimulation (Bullit, 1990). Immediate early genes (IEGs) refer to a group of transcription factors induced in the early phases of memory formation and maintenance that serve as biological markers for populations of neurons undergoing neuroplastic changes that underly formation of long-term memory (Minatohara et al., 2016). IEG expression in neurons is a rapid (within 30 min) response to synaptic activation that encodes and stores information that is required for memory formation, storage, and recall (F.F. Barbosa, 2018; Minatohara et al., 2016). While a decrease in the number of c-Fos+ cells in the Re in AE animals was expected, our study failed to discover significant change in the number of activated neurons (see Supplementary Figure 1, 3). However, this study left two considerable questions unanswered: whether the nature of the damage to the Re is rooted in a reduction of activation of the existing cells or in an AE-induced neuron loss. Secondly, c-Fos+ cell counts are only indicative of the neurons activated within 20-90 minutes post stimulation (Bullit, 1990), so it provided a measure of immediate activation and perhaps not total.

This study will build upon our recent work by implementing immunocytochemical detection of mature and activated neurons in the Re. It has previously been shown that AE damages the Re by dramatically reducing the number of mature neurons following exposure (Gursky et al., 2019, Gursky et al., 2020). In response to this, the current study examined the expression of neuronal nuclear protein (NeuN) which is expressed in nearly all neurons allowing us to visualize mature neurons separately from glial cells (Gusel'nikova and Korzhevskiy, 2015).

Additionally, this study estimated the number ΔFosB-expressing cells, another common marker for neuronal activation. ΔFosB is the result of alterations in splicing of the *FosB* gene, resulting in a shortened form that increases the stability and accumulation of expression at the promoter site (Patterson et al., 2017). Thus, it can be used to visualize the prolonged activation that may not have been represented by cFos+ cells.

It was expected that postnatal AE during the third trimester time period would result in a decrease in the number of mature neurons in the Re represented by a reduced amount of NeuN+ cells in AE animals. It was also expected that postnatal AE would reduce the activation of neurons in the Re in response to the behavioral task, represented as a decrease in the number of ΔFosB+/NeuN+ cells in AE animals. Taken together, the resulting ratio of the number of activated mature neurons to the total number of mature neurons will provide further insight into the effects of AE on Re activation during an associative memory task.

Chapter 2

METHODS

2.1 Experimental Subjects

All animal procedures were approved by the University of Delaware's Institutional Animal Care and Use Committee (AUP protocol #1134) and followed guidelines of animal care provided by the National Institutes of Health. All pups were born at the University of Delaware from timed-pregnant Long-Evans dams obtained from Charles River Laboratories (Wilmington, MA). Animals were culled to 10 pups per litter and housed on a 12 hr light-dark cycle (lights on at 7:00 AM, off at 7:00 PM) in standard opaque cages (17 x 145 x 24cm). On postnatal day (PD) 3, pups were given subcutaneous injections of black india ink in the paw pad to differentiate individual animal subjects. On PD 4 pups were randomly assigned to three experimental groups: alcohol-exposed (AE), sham-intubated (SI), or suckle-control (SC) with a total of 13 AE, 12 SI, and 10 SC animals. The final sample size used for estimated cell populations in this study was 31 animals (11 AE (6F,5M) 11 SI (6F, 5M), 9 SC (4F, 5M)). One animal was lost from the SI group due to complications from transcardial perfusions. Tissue lost from the AE group was due to experimenter error, with one being an extracted whole-brain sample accidentally thrown away, the other discounted because of a staining issue that due to time constraints will be resolved after this study and added to the sample. Lastly, one sample from the SC

group was excluded due to anatomical abnormalities that were not a result of the experimental paradigm.

2.2 Experimental Manipulations

From PD 4-9 all animals were weighed daily, and AE animals were intubated with ethanol (5.25g/kg/day) in an 11.9% milk substitute divided into two doses via intragastric intubation, separated by two hours apart, at 9:00AM and 11:00AM. AE animals received one additional milk dose to mitigate weight loss due to decreased suckling while being intoxicated after alcohol exposure. SI animals served as procedural controls and were weighed, handled, and intubated but received no liquid, accounting for any stress induced by the handling and intubation process. SC animals were weighed daily but did not undergo other experimental manipulations. All animals remained in the home cage with the dam and littermates until weaning. On PD 23, all animals were weaned and socially housed (SH) with 2-3 animals of the same sex per cage. Food and water were available *ad libitum*. Animals remained socially housed through adulthood until behavioral testing began.

2.3 Behavioral Paradigm

An object-in-place (OIP) behavioral test was used to assess associative memory formation and potential deficits induced by neonatal AE. The OIP test took place in an open field arena measuring 90 cm x 90 cm x 60 cm. Three walls of the arena were painted black and the wall opposite of the camera was painted gray to

indicate the front of the arena. When animals reached adulthood (approximately PD 60), the six-day testing process began (Figure 2). Animals were weighed daily during the behavioral paradigm. Animals were handled on days 1-3 by the same experimenter for 5 minutes each within the first two hours of the light cycle. During days 4-5, animals were placed within the open field with no objects for 5 minutes per day to habituate to the testing arena. During habituation animals could freely explore the empty arena and become familiar with the environment before being returned to their home cage. Handling and habituation serve to reduce stress induced by testing as much as possible so that it did not affect the final test phase and subsequent results. On the testing day (day 6), animals underwent a final habituation phase for 5 min immediately before starting the sample phase. In this phase, four distinct novel objects were placed in the arena for the animals to freely explore for 5 minutes before being returned to their home cage. All objects were similar in size and material and were placed equidistant from each other in the arena. In order to test for long-term memory retention there was a 3-hour intertrial interval between sample and testing phases. Exactly 3 hours after the sample phase, animals were placed back in the behavioral arena for the test phase to freely explore for 5 minutes. In this phase, the positions of two of the objects that were in a vertical line with each other switched places, while the other two remained in the same positions as during the sample phase. A visualization of the transition between sample and test phases can be found in Figure 3.

Behavioral data was quantified using a discrimination index (see Figure 4), defined as the difference between the amount of time spent exploring the moved versus unmoved objects divided by the total amount of time exploring both objects. A discrimination value of 0 would refer to an undifferentiated amount of time at the objects regardless of movement, meaning they performed at chance. A positive DI value indicates more time was spent at moved objects, and inversely a negative DI value indicates more time spent at the unmoved objects. Supplemental behavioral measures were calculated but not included in the discrimination index calculation including: the number of exploratory bouts, rearing, and locomotion (See Supplementary Figure 4). The number of exploratory bouts refers to each instance that an animal interacted with an object. Total time rearing and total locomotion calculations were done as part of an Open-Field analysis to test for anxiety-like behaviors that could have impacted behavior findings. Rearing refers to any instance in which the animal stood on their hindlegs. Total locomotion was calculated by placing a 3x3 grid over the arena in the behavior video and tracking each time the animal crossed a grid line. Exploratory behavior was coded by experimenters blind to sex and postnatal treatment.

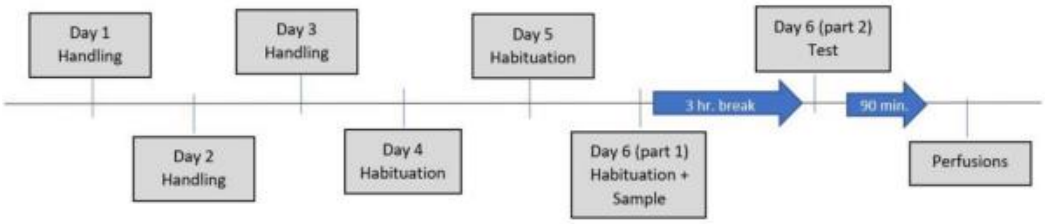


Figure 2. Behavioral paradigm timeline of six-day Object-in-Place behavior testing.

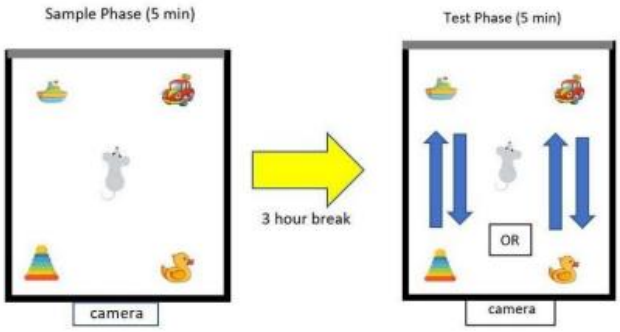


Figure 3. The figure above describes the change in the behavioral arena between the sample and test phases. To the left there are four objects in the arena representing the sample phase and to the right is an example of the test phase showing how objects move positions in a vertical line.

$$DI = \frac{(\text{time spent at moved objects (s)} - \text{time spent at unmoved objects (s)})}{\text{Total time spent at both moved and unmoved objects}}$$

Figure 4. Formula for the discrimination index (DI) used to determine behavioral outcomes of the OIP associative memory task, with *s* = seconds.

2.4 Tissue Collection

90 minutes following completion of behavioral testing, rats were heavily anesthetized with gas isoflurane and an intraperitoneal injection of a 2mL/kg ketamine/xylazine mixture followed by transcardial perfusion (heparinized 0.1M phosphate buffered solution (PBS), pH = 7.2, followed by 4% paraformaldehyde in 0.1M PBS, pH = 7.2). Brains were removed and post-fixed in 30% sucrose in the fixative solution. Brain tissue was sectioned coronally at 40 µm using a cryostat and stored, with the order maintained, in cryoprotectant at -20 ℃.

2.5 Immunohistochemistry

Every eighth section of whole-brain tissue was pulled and underwent an immunofluorescent staining procedure using primary antibodies for ΔFosB and NeuN to label both activated and mature neurons, respectively, as well as co-labeled ΔFosB/NeuN cells. Sections were first washed in distilled H_2O followed by a 30 minute 2N Hydrochloric Acid (HCl) incubation at 47℃ to improve antigen retrieval that could potentially have been altered by the fixation and processing of tissue (Krenacs et al., 2020). Tissue sections were then moved into a 0.1M Boric Acid in Tris-Buffer Saline (TBS) (pH=8.62) incubation for 10 minutes. This pre-treatment strengthens the specificity and consistency of immunostaining for target antigens (Wilson et al., 2007). Next, the tissue underwent two TBS washes, before being placed in a blocking solution (0.5% Triton X-100 + 3% Normal Donkey Serum (NDS) in

TBS) for two hours. Sections were then transferred into a primary antibody mixture of mouse anti-NeuN (Millipore Sigma, MAB377; 1:500) and rabbit anti-ΔFosB (Cell Signaling Technology, D358R; 1:1000) in TBS-NDS blocking solution at 4℃ for approximately 12-24 hours. Control sections were placed in the TBS-NDS blocking solution without primary antibodies. On the second day of the procedure, the tissue underwent three rounds of TBS washes. The lights were then turned off and sections were moved to the secondary antibody incubation of donkey anti-rabbit with Alexa Fluor488 (Jackson ImmunoResearch, 711-545-152; 1:250)and donkey anti-mouse with Alexa Fluor647 (Jackson ImmunoResearch, 715-605-151; 1:250) a in TBS-NDS blocking solution for three hours. The sections underwent three final TBS washes, were slide-mounted, and coverslipped with Gelvatol mounting media.

2.6 Unbiased Stereology

The StereoInvestigator Optical Fractionator workflow (MBF Bioscience, Williston, VT) was used for unbiased estimation of ΔFosB and NeuN labeled and co-labeled ΔFosB/NeuN cells in the nucleus reuniens. Sections were first imaged using a Zeiss AxioImager M2 microscope (Carl Zeiss AG, Oberkochen, Germany) with a high-sensitivity monochrome camera (ORCA-Flash4.0 LT + Digital CMOS Camera, Hamamatsu Corporation, Middlesex, NJ, USA), and the region-of-interest (ROI) was traced at *5x* magnification and imaged at *20x* magnification. For NeuN acquisitions, the Optical Fractionator parameters were set to dissector height of 25.00µm, guard zone of 2.00µm, grid size of 200.00µm x 200.00µm, and counting frame of 50.00µm x

50.00µm. For acquisitions of ΔFosB and co-labeled ΔFosB/NeuN population acquisitions, the Optical Fractionator parameters were set to a dissector height of 25.00µm, guard zone of 2.00µm, grid size of 200.00µm x 200.00µm, and counting frame of 200.00µm x 200.00µm. The stereological parameters were sufficient to obtain coefficient of error (CE) values of < 0.1, which has been described as a reliable estimation of stereological measures (Slomianka and West, 2005).

2.7 Statistical Analysis

Behavioral and anatomical data were analyzed using SPSS Statistics (IBM, version 28.0.0.0 (190)). The independent variables of this study were sex and postnatal treatment. Dependent variables included the performance on the OIP test measured by changes in the discrimination index as well as the estimated populations of ΔFosB+, NeuN+, and co-labeled ΔFosB/NeuN+ cells in the Re. A Two-Way ANOVA did not reveal significant sex effect on parameters measured (except weight), which prompted all further analyses to be collapsed across sex. A One-Way ANOVA was run to analyze NeuN+, ΔFosB+, and NeuN+ counts. For all analyses, Shapiro-Wilk tests confirmed normal distribution of the data and Levene's tests confirmed homogeneity of variance.

Chapter 3

RESULTS

3.1 Body Weights and Blood Alcohol Concentrations

Blood samples to measure blood alcohol concentration (BAC) were collected during intubation procedures but due to technical restraints, the data is unavailable. Despite this, the model employed has been shown to consistently produce stable BACs, in the range of 300-370 mg/dl (Gursky et al., 2019, Helfer et al., 2009, Milbocker and Klintsova, 2021). Body weights were collected each day of behavioral testing and averages were calculated for the first and last day of behavioral testing (see Table 1). A Two-Way ANOVA revealed a main effect of sex, but no significant main effect or interaction effect of postnatal treatment on average body weight.

Postnatal Treatment Group	Females - Last Day of Alcohol Administration (postnatal day 9)	Males - Last day of Alcohol Administration (postnatal day 9)	Females – First Day of Behavior Testing	Females – Last Day of Behavior Testing	Males – First Day of Behavior Testing	Males – Last Day of Behavior Testing
Alcohol-Exposed	18	19	253	255	385	460
Sham-Intubated	19	21	263	264	431	442
Suckle-Control	18	21	257	261	411	423

Table 1. Average body weight in grams (g) ± 14.93 SE separated by sex and postnatal treatment from the last day of alcohol administration, the first day of behavioral testing, and the last day of behavior testing. *n = 13 AE (8 F, 5M), 12 SI (7 F, 5M), 10 SC (4F, 6M).*

3.2 Nucleus Reuniens Regional Volume

The StereoInvestigator Optical Fractionator probe (MBF Bioscience, Williston, VT) was used to estimate the total numbers of cell populations in the Re. The Re was contoured at *5x* magnification and the software overlaid a randomly positioned grid over the contour. Within this grid, the program systematically selects counting frames to ensure accurate estimation of the total regional populations. At *20x* magnification, the focus tool was used to estimate the regional section thickness and, as a result, volume of the Re, and cells were counted. A One-Way ANOVA revealed no significant differences in the mean volume of the Re between treatment group collapsed across sex ($F_{2,28} = 2.105$, $p = 0.1407$).

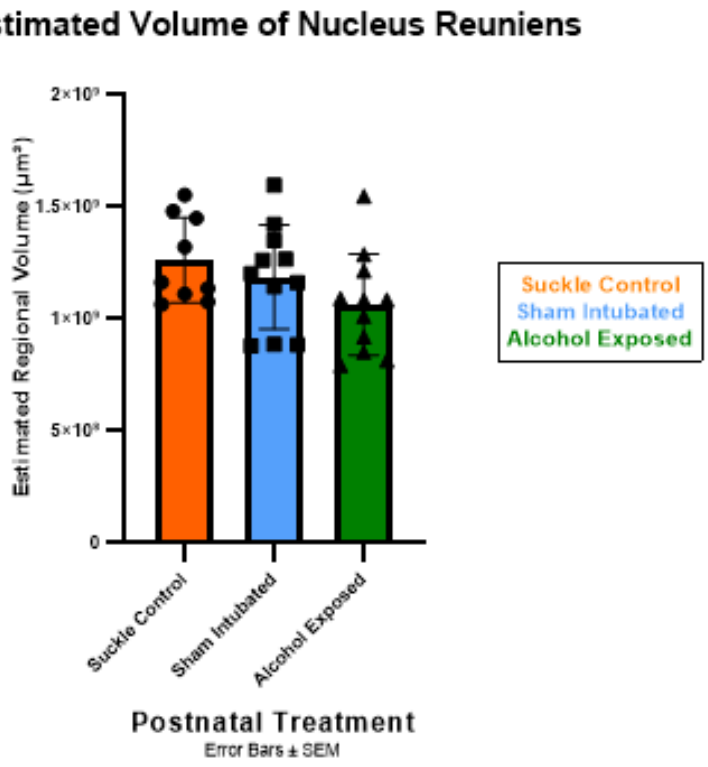


Figure 5. Estimated volume of the Re for AE, SI, and SC animals collapsed across sex. No significant main effects of postnatal treatment and sex or interaction effects were observed. *n = 11 AE, 11 SI, 9 SC;* Data presented as *mean ± 1 SEM.*

3.3 Mature Neuron Population in the Nucleus Reuniens

A One-Way ANOVA revealed a significant main effect of PN treatment on the total number of NeuN+ cells in Re ($F_{2,27} = 4.013$, $p = 0.0298$). Tukey's *post hoc* tests confirmed there was a significant decrease in NeuN+ neuron number in AE compared to SC groups ($p = 0.0235$), but no significant differences were found between SC and SI ($p = 0.2005$) or between SI and AE groups ($p = 0.235$).

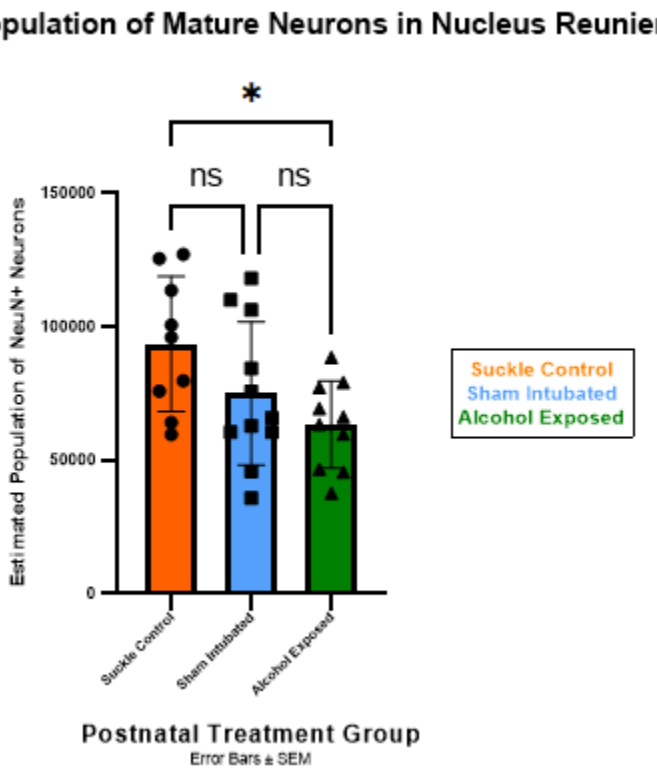


Figure 6. Estimated population of NeuN+ neurons within the Re across PN treatment groups collapsed across sex. A significant main effect of PN treatment was found,

with SC animals displaying significantly higher estimated NeuN+ cell populations than AE animals. No significant differences were found between AE and SI or SC and SI groups. *n = 11 AE, 11 SI, 9 SC;* Data presented as *mean ± SEM.*

3.4 Activated Non-Neuronal Cells in the Nucleus Reuniens

A One-Way ANOVA showed no significant main effects ($F_{2,25} = 2.299$, $p = 0.1212$) of PN treatment on global estimations of cells expressing ΔFosB. The coefficients of error averaged at 0.3, which is not in line with reliable estimation of stereological measures (Slomianka and West, 2005; see discussion for further explanation).

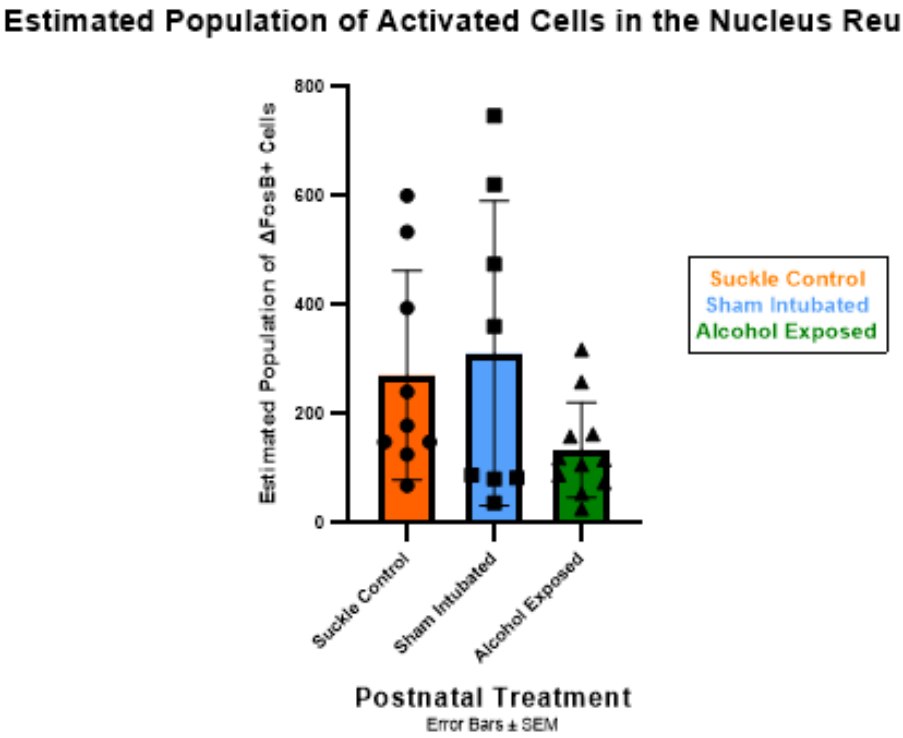


Figure 7. is representing the estimated population of total ΔFosB+ cells within the Re. No significant main effects or interaction effects were observed as a result of PN treatment conditions collapsed across sex. *n = 11 AE, 11 SI, 9 SC; mean ± SEM*

3.5 Activated Mature Neuron Population in the Nucleus Reuniens

A One-Way ANOVA revealed a significant main effect of PN treatment on estimations of activated neurons ($F_{2,27} = 5.805$, $p = 0.008$), with a 0.07 average coefficient of error. Tukey's *post hoc* tests revealed statistically significant differences between SC and AE groups ($p = 0.0083$) and SC and SI groups ($p = 0.0385$) but not between SI and AE groups ($p = 0.8131$).

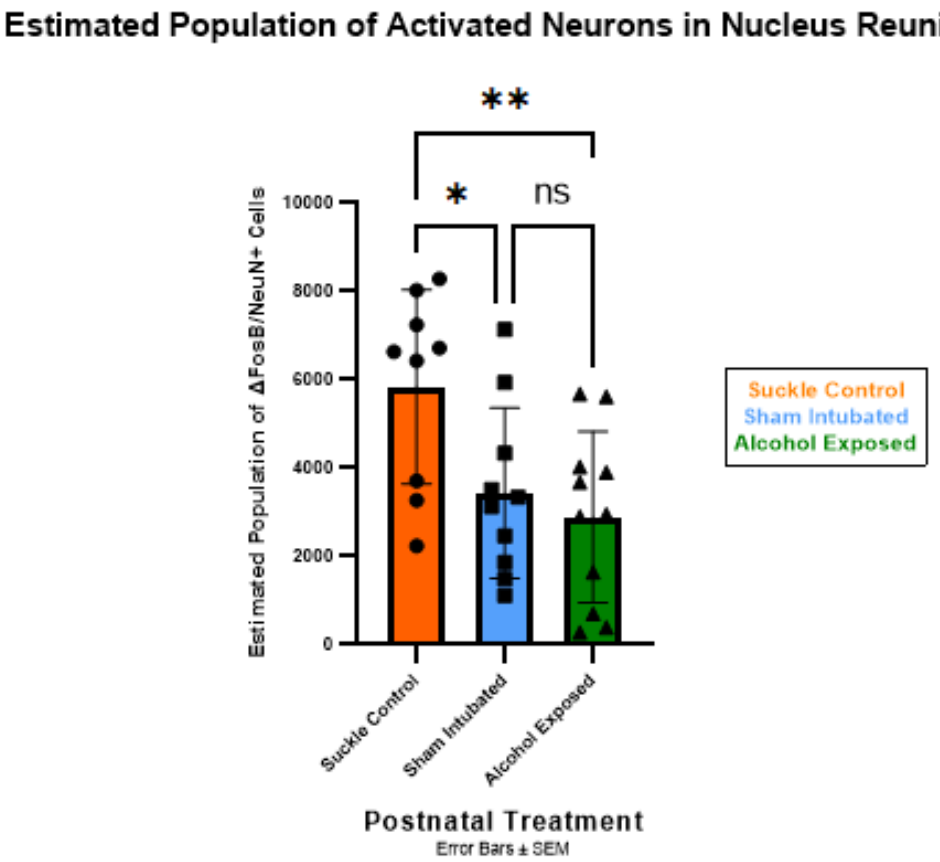


Figure 8. is representing the estimated population of NeuN+/ΔFosB+ co-labeled cells within the Re among PN treatment conditions, collapsed across sex. A significant main effect of PN treatment was found between SC and AE and SC and SI groups, with SC animals showing higher global populations of NeuN/ΔFosB+ co-labeled cells. No significant effects were found between SI and AE, with no interaction effects overall. *n = 11 AE, 11 SI, 9 SC; mean ± SEM, p <0.05*, p<0.01***

3.6 Ratio of Activated Neurons out of Total Neuron Population in the Nucleus Reuniens

A One-Way ANOVA showed a significant main effect of PN treatment on percentage of the total neuron population that was activated ($F_{2,28} = 3.411$ $p = 0.0472$). Tukey's *post hoc* analysis confirmed statistically significant differences between SC and AE ($p = 0.0373$), but not between SC and SI ($p = 0.4020$) or SI and AE groups ($p = 0.3706$).

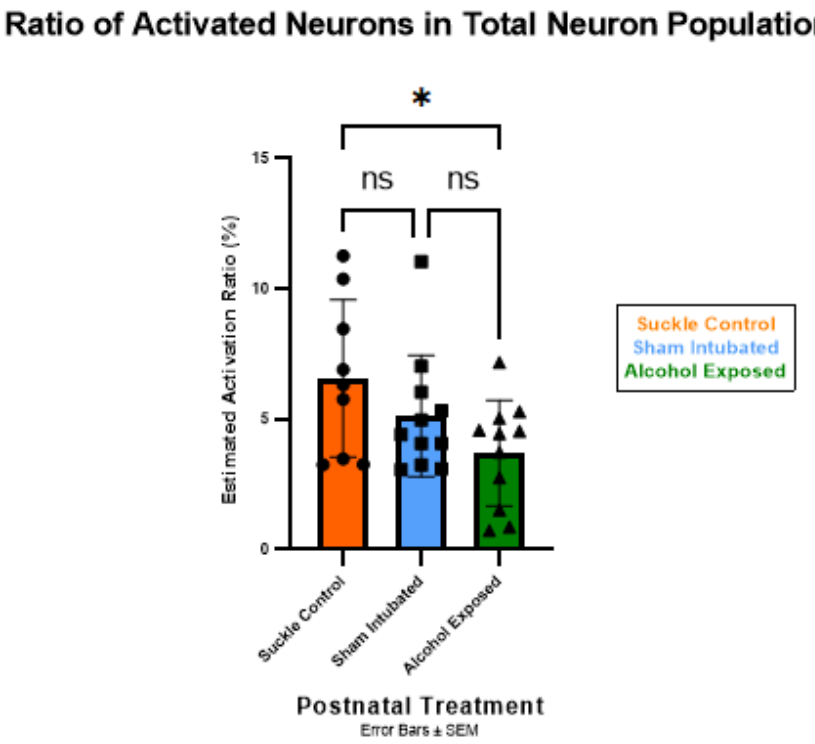
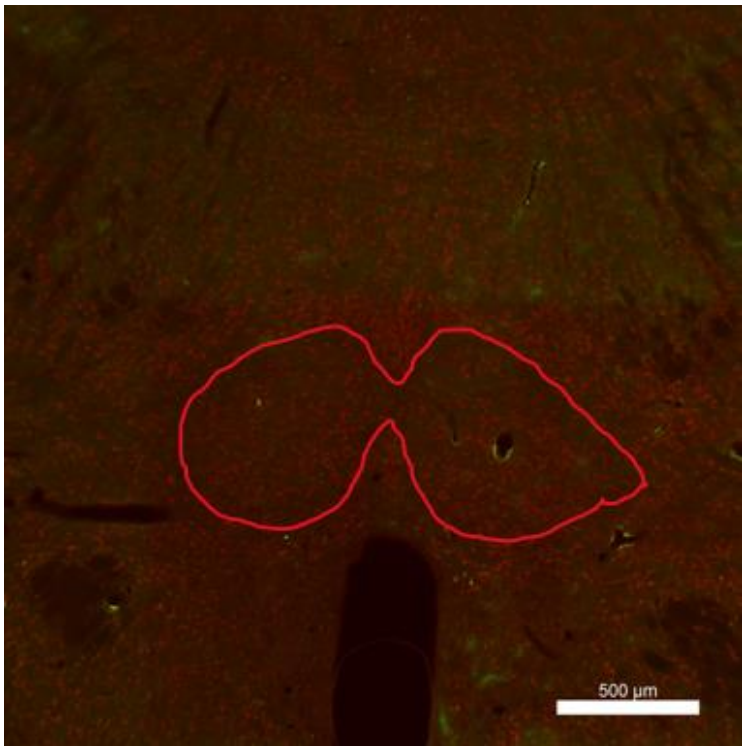


Figure 9. is representing the estimated ratio of NeuN+/ΔFosB+ co-labeled cells to total NeuN+ cell populations within the Re in PN treatment conditions, collapsed across sex. A significant main effect of PN treatment was found between SC and AE, with SC animals showing higher percentage of activation in the neuron population than AE animals, showing there is still a reduction in activation despite a loss of neurons. No significant effects were found between SC and SI nor SI and AE groups, with no interaction effects. *n = 11 AE, 11 SI, 9 SC; mean ± SEM, p <0.05*

a) 

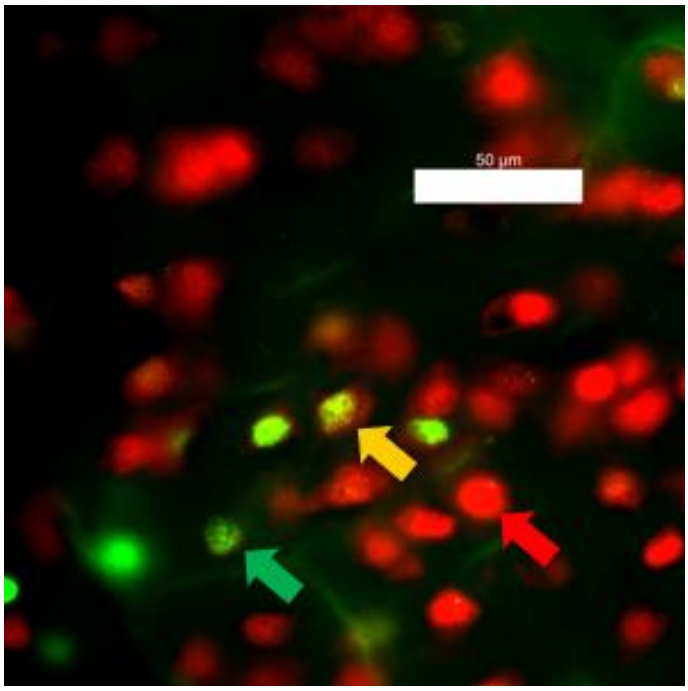
b) 

Figure 10. Illustration of fluorescent immunohistochemical detection of NeuN+/ΔFosB+ expression in the Re. 9a: whole brain image, *5x* magnification with Re outlined 9b: NeuN+/ΔFosB+ cells in Re at *65x* magnification. *NeuN+ (red), ΔFosB+ (green), NeuN+/ΔFosB+ (yellow).*

Chapter 4

DISCUSSION

4.1 Summary of Results

This study examined the effects of developmental alcohol exposure on the neuron activation and neuron loss in the nucleus reuniens in an established rat model of third trimester alcohol exposure. Data acquired from immunohistochemically stained brain tissue demonstrated that neonatal binge alcohol exposure significantly reduced the number of mature neurons in the reuniens, did not seem to affect the activation of non-neuronal cell types (meaning cells activated other than mature neurons), and significantly reduced the number of activated mature neurons in the reuniens. Overall, this study shows impairment to the medial prefrontal cortical-reuniens-hippocampal circuit via developmental exposure to alcohol. Because this triregional circuit is critical in mediating executive function, deficits to executive function could be due to a lack of neurons available in the reuniens to be recruited, compromised ability of reuniens neurons to activate, or likely a combination of both of these effects that invokes disrupted functional communication within the circuit and resultant executive dysfunction. It is notable to mention that, despite observed anatomical alterations, this was not mirrored by significantly impaired Object-in-Place behavior performance in a previous study. The overall volume of the region was not impacted by alcohol exposure.

4.2 Neonatal Alcohol Exposure Altered Mature Neuron Populations in the Nucleus Reuniens

In line with other studies conducted by the Klintsova Lab, it was expected that a marked loss of mature neurons in the Re would be observed as a result of exposure to alcohol during the rodent BGS. It was found that there was a significant difference in total Re neuron population between the AE and SC groups, but not the AE and SI groups. It is interesting that there was not a significant decrease in neuron number in AE groups compared to SI animals, however the trend of lack of statistically significant differences between AE and SI has been noted in previous publications (Hamilton et al., 2010, Helfer et al., 2009, Klintsova et al., 2007). Taking into consideration that the intubation process causes pronounced stress, this could be a contributing factor to a decrease in decreased neuron count in the SI group compared to SC, bringing the average estimated neuronal number down and closer to that of AE groups. A lack of difference between SI and AE animals could be due to the CNS depressant effects of alcohol during intoxication, which may have provided some stress relief and allow the AE animals to tolerate the stress of intubation better. High stress, especially early in life, has cascading detrimental effects and it is possible that intubation stress invoked high cortisol levels potentially eliminating newly generated neurons (Conrad, 2009; Justice, 2018) and disrupting neurogenesis supportive processes. The observed decrease in neurons due to AE is widely supported by previous studies (Gursky et al., 2019, Tateno and Saito, 2008, Vetreno and Crews, 2018) and provides support for the hypothesis that EF deficits manifest from a

decrease in the number of neurons available in the Re to support communication between the mPFC and dHPC.

4.3 Neonatal Alcohol Exposure and Activation of Cells in the Nucleus Reuniens

This study looked at activation of mature neurons and non-neuronal cells in the Re. Non-neuronal cells refer to any non-mature neuron cell types (e.g., astrocytes, precursor cells, oligodendrocytes, microglia, etc.) that have the innate ability to be activated. Activation of the Re was measured by expression of *ΔFosB*, a member of the IEGs upregulated in association with learning and memory formation. Previous studies have shown that eliminating a similar member of the *Fos* family, *c-Fos*, impairs long-term potentiation and memory retrieval in a spatial water maze (Kubik et al., 2009). *c-Fos* is upregulated in the dHPC in response to novel spatial arrangements of familiar visual cues but not novel items in general in the HPC (Kubik et al., 2009). Similarly, another study showed other IEGs including *Arc, zif268*, as well as *cFos* at elevated levels in the HPC in spatial and cued water mazes (Kubik et al., 2009), overall showing the importance of IEG involvement in spatial memory consolidation and empirical evidence using IEG expression to match hippocampal activity to behavior.

It was expected that third trimester equivalent alcohol exposure would reduce neuronal activation in the Re indicated by a decrease in the number of ΔFosB+ and/or NeuN+/ΔFosB+ cells. No significant differences in ΔFosB+ populations were found between any group, indicating that neither intubation per se nor AE affect activation of

non-neuronal cells. It should be noted the coefficient of error value for these counts averaged at 0.3, far exceeding the average accepted as a reliable estimation of stereological measures (Slomianka and West, 2005), likely due to the very limited sampling size of these types of activated cells across all PN treatment groups. To compensate for the limited sampling size of non-neuronal activated cells, the entire region of interest (ROI) was sampled, accounting for every activated non-neuronal cell in the Re. Although the coefficient of error was above 0.1, this measurement is still reliable because it took into account any and all possible samples.

Significant differences in the estimates of NeuN+/ΔFosB+ cell populations in Re were found SC and SI as well as SC and AE animals. Statistically significant reductions in the number of co-labeled cells in the SI group may be due to the slight decrease of mature neurons to begin with observed in this group, making the presence of co-labeled cells less prevalent. The present study found a major depletion of co-labeled cells in AE group regional estimates, providing support for the hypothesis that neonatal AE reduces activation of mature neurons in the Re as compared to the SC group. Further analyses of the ratio of activated neurons out of the total neuron population in each PN treatment condition was able to show a reduction in the activation percentage in neuronal populations when taking into consideration a reduction in mature neurons. This shows that AE is still reducing the activation of cells even though there are less neurons in general. This finding provides support for decreased neuronal activation as a potential cause for EF deficits, as binge-AE

compromised the ability of neurons in the Re to activate in response to a behavioral task.

4.4 Limitations

Although this study demonstrated AE-induced neuron loss as well as reduction in neuronal activation in the Re, these findings were not mirrored by deficits in performance of an EF behavioral assessment in the previous study. Because sex-by-postnatal treatment group sample sizes are still quite low, the addition of subjects to the behavioral task may provide further insight into the impact of developmental AE on EF-related behavior. Additionally, the behavioral data was scored by one individual and lacks inter-rater reliability, which should be resolved in follow-up studies with this data set. In our previously gathered behavioral data, there was a significant interaction effect between sex and phase of testing (sample vs. test phase) in which females performed better despite PN treatment condition, while males did not show signs of learning irrespective of PN treatment. This should be investigated further to determine any behavioral sex differences in the OIP task in this model.

Social isolation is known to have major implications for neurodevelopment. Though it was unlikely to cause significant stress, it should be noted that after being in social housing prior to the behavior regimen, animals were individually housed for functional purposes of the study during the six-day testing period. Due to the stress of being separated from cagemates, as well as the stress of novel behavior testing, extra

measures were put in place inside individual home cages to alleviate stress as much as possible including wooden tongue depressors for play and red plastic tubing.

Lastly, a significant decrease in regional volume of the Re was not observed, which conflicts with supporting literature showing a consistent decrease in Re volume following third trimester binge-AE. This finding could be due to a low sample size, or uncontrolled conditions that impacted the procedure.

4.5 Future Directions

This study is one of many from the Klintsova Lab to understand the functional synchronization of the mPFC-Re-HPC circuit and more specifically, how AE disrupts the synchrony between these structures and the resultant phenotype presenting as cognitive and behavioral deficits observed in FASD. One step in accomplishing this could be to extend the investigation beyond the Re and quantify the mature neuron and activated neuron cell populations, as well as regional volume changes in the dHPC and mPFC. This will provide a holistic understanding of the impact of AE on the structural and functional integrity of this circuit.

It is possible that the lack of behavioral deficits observed was due to how the behavioral data was analyzed. In supporting literature, experimenters analyzed OIP data only from the first minute of the testing session, otherwise known as the “probe phase” (Gursky et al., 2021), as it was thought this is when robust behavior would be most observed and believed to plateau for the remaining four minutes of the session. Our current data could be re-analyzed in a same or similar way, to compare the

efficacy of previous behavioral scoring. Additionally, vaginal swabs were collected from female rats before perfusion on the testing day. In the future, in order to further understand behavioral sex differences observed in the OIP task, estrus cycle day should be cross-examined against the testing day to see if hormone levels could have impacted results. There is research to suggest sex differences in memory in rodents, and notably, research has found that performance of female rodents on OIP tasks may be enhanced due to ovarian sex steroid hormone levels (Cost et al., 2012, Tuscher et al., 2015). Studies have shown that 17β-estradiol and progesterone hormones are involved in the regulation of hippocampal memory formation, which could have downstream effects on OIP performance because of the hippocampus-reliant nature of this associative memory tasks (Tuscher et al., 2015). For even further validity, it would be beneficial to test female subjects on different estrus cycle days within this model.

Lastly, there seemed to be a trend in clustering of activated mature neurons more ventrally and medially in the Re in some, but not all subjects. Due to a lack of literature breaking down the Re into specific subregions, as well as the experimenter remaining blind to PN treatment condition while counting, it is difficult to link this anecdotal evidence to a neuroanatomical underpinning. However, work might benefit from dividing Re into subregions and investigating the effect of AE on cell activation in this manner. With the current data set, it would be interesting to take image stacks with activated mature neuronal cell counts superimposed together to see if there were any differences in the density of clusters of these cells linked to PN treatment

condition. Literature showing afferent and efferent projections of the Re show different patterns of densely packed clusters of neurons in the Re (Vertes et al., 2002, Vertes et al., 2006) should be used as a guide. This data suggests there may be ventral Re specific interactions with other regions. If significant populations of cells were restricted to certain areas of the Re, this could elucidate subregions of the Re that have specific connections to either the mPFC or dHPC and which subregions are potentially more sensitive to AE-induced damage.

Overall, this study was an excellent step in elucidating the potential mechanism behind EF deficits that may arise due to developmental AE. We found an alcohol-induced neuron loss, coupled with a reduction of activated mature neurons, which can disrupt synchronous communication between the mPFC and dHPC, and result in behavioral representation of executive dysfunction.

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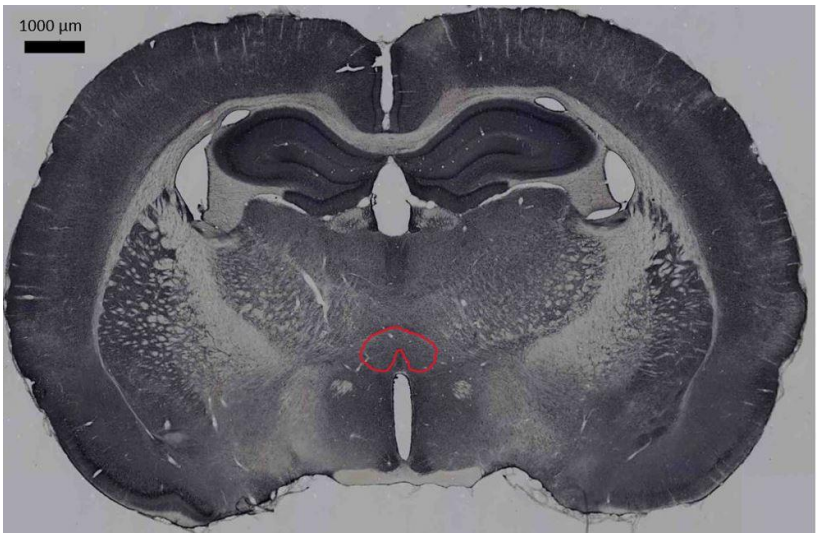
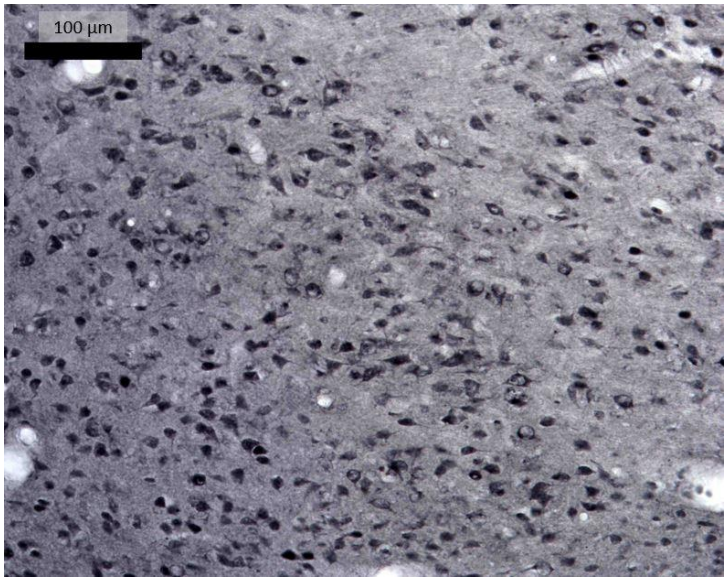
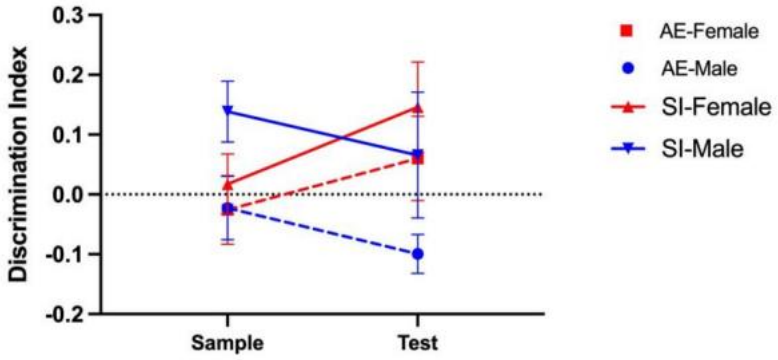
Appendix A

SUPPLEMENTARY RESULTS

a. 

b. 

Supplementary Figure A.1. Illustration of immunohistochemical detection of cFos expression with DAB visualization in brain tissue. (a): whole brain image, *5x* magnification with Re outlined. (b): cFos+ cells within the Re at *20x* magnification.

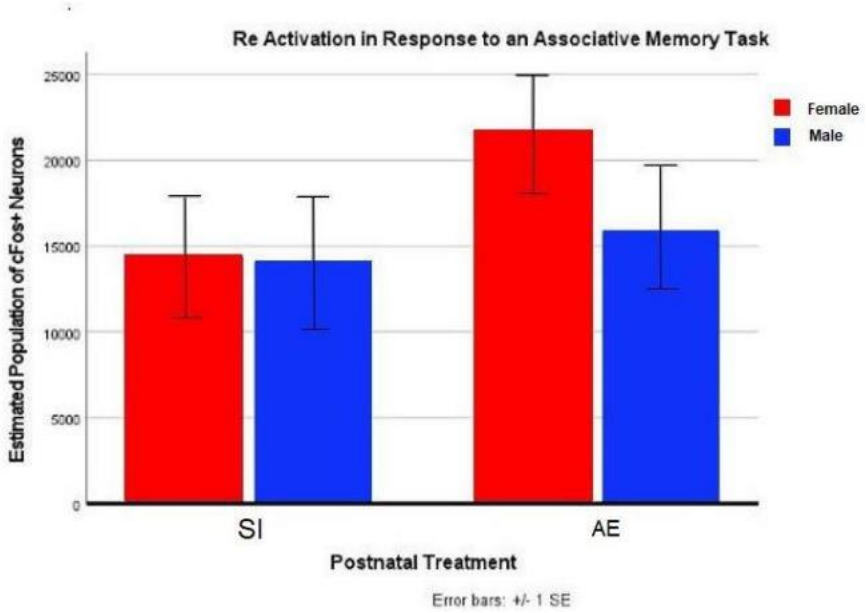


Supplementary Figure A.2. Average calculations of discrimination index of exploratory behavior in the OIP task between sample and test phases in SI vs AE subjects.

Test of Within-Subjects Effects

	Sum of Squares	df	Mean Square	F	p	n^2p
Bouts	0.00307	1	0.00307	0.1636	0.690	0.008
Bouts * PN Treatment	0.00156	1	0.00156	0.0830	0.776	0.004
Bouts * Sex	0.09579	1	0.09579	5.1048	0.035	0.203
Bouts * PN Treatment * Sex	0.00110	1	0.001100	0.0588	0.811	0.003
Residual	0.37529	20	0.01876			

Supplementary Table A.1. Mixed Repeated Measures ANOVA results within subjects. The Bouts * Sex condition seen above revealed a significant interaction between sex and phase on exploratory behavior ($p < 0.05$).



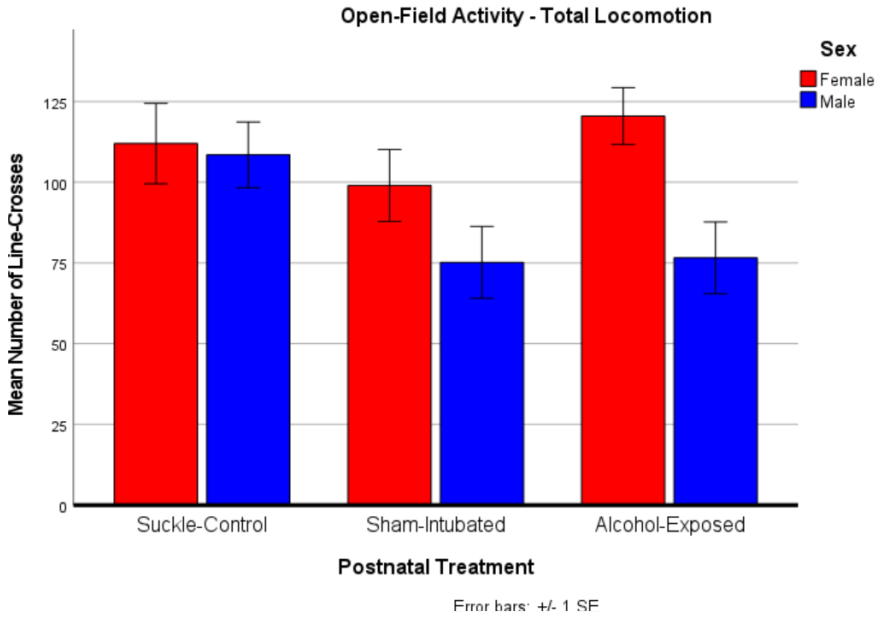
Supplementary Figure A.3. The figure above shows the estimated population count of total cFos+ neurons within the Re. No main effect was found for sex or PN treatment on global estimations.

Test of Between-Subjects Effects

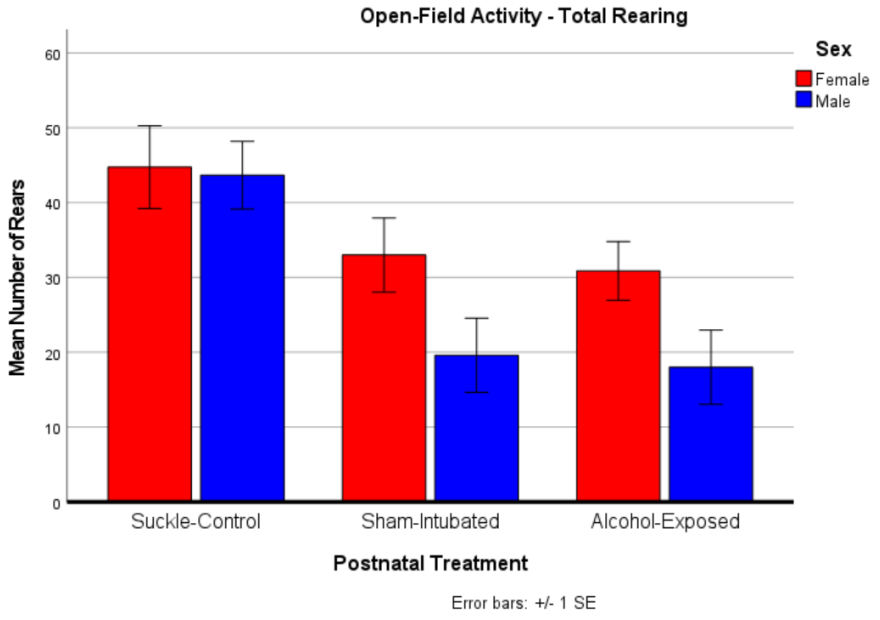
Source	Type III Sum of Squares	df	Mean Square	F	Sig.	Partial Eta Squared
Corrected Model	245785019[a]	3	81928339.74	1.087	0.379	0.146
Intercept	6117271927	1	6117271927	81.126	<0.001	0.810
Sex	53851444.12	1	53851444.12	0.714	0.409	0.036
PN Treatment	117994715.5	1	117994715.5	1.565	0.226	0.076
Sex * PN Treatment	42502035.51	1	42502035.51	0.564	0.462	0.029
Error	1432678864	19	75404150.72			
Total	8193996448	23				
Corrected Total	1678463883	22				

a. R squared = 0.146 (Adjust3ed R Squared = 0.012)

Supplementary Table A.2. Descriptive statistics of the number of estimated cFos+ neurons between subjects. No significant main effects were found for sex or PN treatment.



a)



b)

Supplementary Figure A.4. Two-Way ANOVAs show a significant main effect of sex for line crosses [Females: $F_{1,20} = 7.157$, p=0.013] (a), and significant main effects of sex and postnatal treatment on rearing behaviors [Females: $F_{1,20} = 5.335$, p=0.029], [Intubation: $F_{1,20} = 9.869$, p=<0.001] (b).

Appendix B

IACUC PROTOCOL APPROVAL

University of Delaware
Institutional Animal Care and Use Committee
Application to Use Animals in Research
(New and 3-Yr submission)

Title of Protocol: Therapeutic Motor Training and Fetal Alcohol Effects	
AUP Number: 1134-2022-0	**← (4 digits only — if new, leave blank)**
Principal Investigator: Anna Klintsova	
Common Name (Strain/Breed if Appropriate): Rat, Long Evans **Genus Species:** Rattus norvegicus	
Date of Submission: 04/17/2022	

Official Use Only

IACUC Approval Signature: Glen Tellin, DVM

Date of Approval: 7.1.2022