Investigation of Cell Type-Specific Loss in the Nucleus Reuniens of the Midline Thalamus following Single-Day Alcohol Exposure in a Rodent Model of FASD

by

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Alcohol exposure	AE
Alcohol-related birth defects	ARBD
Alcohol-related neurodevelopmental disorder	ARND
Anti-adenomatous polyposis coli clone CC1	CC1
Attention deficit/Hyperactive disorder	ADHD
Autism spectrum disorder	ASD
Brain growth spurt	BGS
Blood alcohol content	BAC
Central nervous system	CNS
Deionized water	DI
Executive function	EF
Fetal Alcohol Spectrum Disorders	FASDs
Fetal Alcohol Syndrome	FAS
High alcohol exposure	AE_H
Hippocampus	HPC
Intellectual disability	ID
Moderate alcohol exposure	AE_M
Neuronal nuclear antigen	NeuN
Nucleus reuniens of the midline thalamus	Re
Oligodendrocyte	OL
Oligodendrocyte precursor cell	OPC
Partial Fetal Alcohol Syndrome	pFAS

Phosphate buffer solution	PBS
Postnatal day	PD
Sham intubated	SI
Tris buffer solution	TBS

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ABSTRACT

Fetal Alcohol Spectrum Disorders (FASDs) is an umbrella term used to describe multiple developmental disorders stemming from the prenatal alcohol exposure that are manifested by deficits in growth development, cognitive impairments, and physical abnormalities. 2-5% of live births in the US are affected by FASD and result in lower brain volume, brain anomalies, and behavioral deficits including impaired executive function. Executive function (EF) defines a set of cognitive controls that aid in the formation of goal-directed movements and regulating self-control; EF has been shown to relate to medial prefrontal cortex (mPFC) and hippocampus (HPC) activity. An intermediary structure, the nucleus reuniens (Re) of the midline thalamus, facilitates communication between the mPFC and HPC and is known to be damaged by prenatal alcohol exposure. A damaged Re is known to produce deficits in EF due to inefficient communication in the mPFC-Re-HPC circuit.

This study employed a rodent model of third-trimester single-day binge alcohol exposure (AE) to evaluate neuroanatomical effects of neonatal alcohol exposure. Specifically, we examined moderate (3g/kg/day), and high (5/25g/kg/day) doses of ethanol compared to a sham-intubated control group. In conjunction with histological immunofluorescence staining, this study measured the numbers of specific cell populations in Re to assess levels of cell loss at 12hrs after AE. Specifically, we estimated the total number of neurons and oligodendrocytes in Re by labelling these

cells with specific antibody markers NeuN (neuronal marker) and CC1 (marker for mature oligodendrocytes).

No significant effect of postnatal treatment was found on neuron and oligodendrocyte populations in Re which indicates that cell loss might occur later than 12 hours following AE, as found in studies examining apoptotic-cell expression at 5 and 24 hours (Phanithi, et al., 2000). Additionally, no significant effect of alcohol exposure on reuniens volume was found, indicating that reuniens volume was not compromised within 12 hours of AE. This study aids in our understanding of FASD and adds to the general portrait of the effects of ethanol on the brain and Re

Chapter 1

Introduction

1.1 Fetal Alcohol Spectrum Disorders

Fetal Alcohol Spectrum Disorders (FASD) is an umbrella term used to describe symptoms and deficits that result from exposure to alcohol in utero. FASD presents us with a challenging barrier to overcome considering that alcohol is the most widely consumed psychoactive substance in the world (Choate et al., 2022). While alcohol consumption during pregnancy is entirely preventable, many individuals still drink (Umer et. al., 2020). Information regarding the negative effects of alcohol consumption while pregnant is readily accessible, however, as many as 20 to 30 percent of expecting individuals report drinking at some point during pregnancy with more than 8 percent reporting binge drinking during pregnancy (Ethen et al., 2009). As many as 2-5% of all children born in the US are estimated to have an FASD (May et al., 2014). The prevalence of FASD makes alcohol use during pregnancy the leading and most preventable cause of birth defects and disabilities in the US (Dejong et al., 2019).

The non-diagnostic term FASD covers a wide range of outcomes surrounding in-utero alcohol exposure including fetal alcohol syndrome (FAS), partial fetal alcohol syndrome (pFAS), alcohol-related neurodevelopmental disorder (ARND), and alcohol-related birth defects (ARBD), along with sudden infant death (Riley et al.,

2011). Diagnosing these disorders can prove difficult since physical signs of FASDs are often present after alcohol exposure during early gestational periods (first trimester); it is important to note that third-trimester alcohol exposures usually result in mostly neurological deficits, which can make achieving a diagnosis difficult. Even though variability is observed in FASD symptomology a diagnosis can be achieved by identifying symptoms within four categories (1) growth delays in height and weight (2) facial abnormalities including a smooth philtrum and thin upper lip (3) central nervous system (CNS) dysregulation and (4) alcohol use during pregnancy (Astley, 2011). FASD can present symptoms such as decreased brain volume, brain and facial anomalies, behavioral deficits, decreased height and weight, and impaired executive function and spatial working memory (Vorgias et al., 2021). On top of distinguishable facial abnormalities, FAS patients can present more severe neurological symptoms including seizures, deficits in fine motor function, learning and memory skills, and executive functioning, to name a few (Landgraf et al., 2013). While some symptoms can make a diagnosis easy to obtain, similarities in diagnostic criteria between other disorders such as attention-deficit/hyperactivity disorder (ADHD), intellectual disability (ID), and autism spectrum disorder (ASD) make misdiagnoses all too common (Ergun, 2021). As long as alcohol use during pregnancy continues, we will continue to observe FASDs and the deficits that come along with them, which makes this a crucial area of research in order to better understand the full impact of alcohol exposure in utero.

1.2 Rodent Model of FASD

Working with a rodent model of FASD allows us to control alcohol dose, timing of exposure, and administration methods. These key aspects allow us to target specific stages of growth to observe the developmental and anatomical changes that occur during alcohol administration. Moreover, a rodent model allows us to model AE during the human brain growth spurt (BGS). The BGS is defined as a transient period of rapid brain growth during the third trimester in humans and is marked as a time of extreme vulnerability to nutritional and metabolic deficiencies (Dobbings and Sands, 1979). It is important to note that rats are altricial rodents: newborns are naked and helpless, with poor postural or motor control, and with brain development comparable to the human brain at the end of the second trimester. The BGS occurs postnatally in rodents, which allows researchers to administer alcohol in multiple ways including, but not limited to, intragastric intubation, vapor inhalation, and intraperitoneal injections. Each administration method has its benefits and drawbacks. Vapor inhalation exposes rodents to vaporized ethanol at a specific concentration for a set period of time in order to produce an increased blood alcohol content (BAC). While this technique may prevent increasing stress levels it also lacks specificity, the amount one rodent inhales compared to another may vary and therefore produce different effects even if both received the same amount of exposure. Intraperitoneal injections deliver ethanol by administering a specific dose of ethanol into the body cavity; this route has been shown to produce rapid increases in BACs followed by a steep decline and create a stressful environment (Livy et al., 2003). Intragastric intubation is widely

used to mimic binge-like ethanol exposure due to its ability to produce high BACs, however, this is a stressful method for the dams and pups (Klintsova et al., 2013).

Our study employs a rodent model of FASD to mirror third-trimester single-day alcohol exposure on postnatal day (PD) 9 in neonatal rat pups. Pups received a moderate (3g/kg) or high (5.25 g/kg) dose of ethanol via intragastric intubation twice with two hours in between each dose. A single day of ethanol exposure was used to further examine the effects of differing amounts of alcohol exposure during a specific and isolated period during third trimester brain development.

1.3 mPFC-Re-HPC Circuit Deficits in FASD

Executive function (EF) refers to the set of cognitive abilities that allow for the planning and execution of goal-directed behavior and exerting self-control (Cheung, 2021). EF heavily relies on synchrony between the hippocampus (HPC) and medial prefrontal cortex (mPFC) which are connected by the nucleus reuniens (Re) of the ventral midline thalamus. Efficient communication within the mPFC-Re-HPC circuit relies on the circuit being intact. Reuniens damage, modeled by pharmacological inactivation, has been shown to produces EF deficits in spatial working memory (SWM) specific activity patterns (Hallock et al., 2016). Moreover, Barker and Warburton, 2018, found Re to facilitate interactions between mPFC and HPC by showing the infusion of muscimol in Re to produce delay-dependent deficits in object-in-place memory tests. Such demonstrations of Re involvement in the mPFC-Re-HPC circuit highlight the importance of this region in proper EF. Findings surrounding PD

7 AE demonstrate time-dependent levels of neuron loss that are immediate (i.e. 12 hours after AE) and persist into adulthood, which sparks the question if other neuroglia are susceptible to damage by AE (Gursky et al., 2019). Relating to this, a past Klintsova Lab study demonstrated apoptosis levels in Re to be dose-dependent. This specific study investigated time- and dose-dependent relationship between apoptotic cells in Re and postnatal treatment. Results from this experiment revealed a significant dose-dependent interaction but not a time-dependent one. For this reason, we only examine cell populations at PD 9 in the current study

Gursky et al., 2021 also found AE to increase the proportions of neurons that project to both the mPFC and HPC alongside an overall reduction in neurons; this supports the claim that AE disrupts mPFC-Re-HPC circuitry. Relating to neuron projections, one study found AE to reduce mPFC projection fibers in Re, however, this study was unable to determine if this is due to less neurons in general or if glial cell damage may have contributed (Smith et al., 2022). Decreased axonal material in this study might be attributed to a reduction in myelination or a lack of OL interaction with axon fibers. These findings lead us to investigate specific glial cells, such as oligodendrocytes (OLs) in the current study, to examine specific interactions between AE and glial cell populations.

Relating to glial cells, a more recent study revealed neuronal and non-neuronal cell loss in adulthood following a single day of moderate and high doses of ethanol, however, the number of microglia remained unchanged after postnatal treatment (Gursky et al., 2022). Oligodendrocytes are the myelinating cells of the central

nervous system and have been determined to be among some of the most vulnerable cell populations (Bradl, 2010). Furthermore, our lab has demonstrated oligodendrocyte populations undergo apoptosis after alcohol exposure, which hinders neuroimmune function and can disrupt white matter formation during adolescence (Milbocker, 2023)..

This leads to the current investigation in assessing the effects of alcohol on specific neuroglia populations to determine if certain cell populations are more vulnerable to damage by alcohol. More specifically, we are interested in assessing how alcohol exposure impacts both neuron and oligodendrocyte populations in the reuniens to assess cell-specific susceptibility to ethanol intoxication.

1.4 NeuN and CC1

To evaluate the numbers of mature neurons and oligodendrocytes, immunofluorescent staining was used to detect and localize specific antigens found in these cell populations; through the combination of primary and secondary antibodies that possess fluorophores, we are able to tag specific antigens in the cells of interest and observe their presence via fluorescent microscopy (Im, 2019). Neuronal nuclear antigen (NeuN) is a marker of neurons that belongs to nuclear proteins and has been shown to correspond to neuronal differentiation during important fetal developmental time points (Sarnat, 1998). Moreover, NeuN is especially useful for marking late stage neuron maturation with biding most present in the second and third trimesters. Our hypothesis is that AE on the PD9 would lead to a decrease in neuron number in the Re

and that the loss would be dose dependent (i.e., there will be a more pronounced loss of neurons after high-dose exposure). In addition, the monoclonal antibody anti-adenomatous polyposis coli clone CC1 (CC1) was used to label mature oligodendrocytes. This antibody identifies oligodendrocytes without labeling for myelin by binding Quaking 7, an RNA-binding protein that is demonstrated to be up regulated in mature myelinating oligodendrocytes (Bin et al, 2016). We expect high AE pups to demonstrate the greatest reduction in oligodendrocytes. Since oligodendrocytes are one of the most vulnerable cell populations (Bradl and Lassmann, 2010), we hypothesize that oligodendrocytes will exhibit the greatest reduction in cell population.

Chapter 2

Methods

2.1 Experimental Subjects

All animal procedures were approved by the University of Delaware's Institutional Animal Care and Use Committee (IACUC, protocol #1134) and followed guidelines of Animal Care provided by the National Institutes of Health. Timed pregnant Long-Evans dams were acquired from Charles River Laboratories (Wilmington, MA). All pups were maintained on a 12-hour light/dark cycle, lights on at 7:00 AM and lights off at 7:00 PM. Pups were maintained in standard opaque cages (17 X 145 X 24 cm) in a temperature-controlled colony room. On postnatal day 3, pups were given subcutaneous injections of black India ink in the paw pad to identify individual animals. On PD 4, pups were randomly assigned to 3 experimental groups: high alcohol exposure (AE_H), moderate alcohol exposure (AE_M), or sham-intubated (SI). Thirty-one animals from 7 litters were used in this study (3 female AE_H, 3 male AE_H, 5 female AE_M, 7 male AE_M, 6 female SI, and 6 male SI).

2.2 Experimental Manipulation

To target a single day within the rodent brain growth spurt that occurs postnatally, intubations were performed on PD 9. Pups within the AE_H group received 5.25 g/kg and AE_M group received 3 g/kg of ethanol in milk formula over two administrations via intragastric intubation at 9:00 AM and 11:00 AM. SI animals were handled and intubated but did not receive any liquid, which allowed to control for any intubation-related stress underwent by AE animals. Blood samples were collected

from the tail vein from all animals 90 minutes after the second dose to examine blood-alcohol concentrations. Blood samples collected from SI animals were not analyzed for BAC. Analysis of BAC plasma samples were completed on an Analox GL5 Alcohol Analyzer (Analox Instruments, Boston MA).

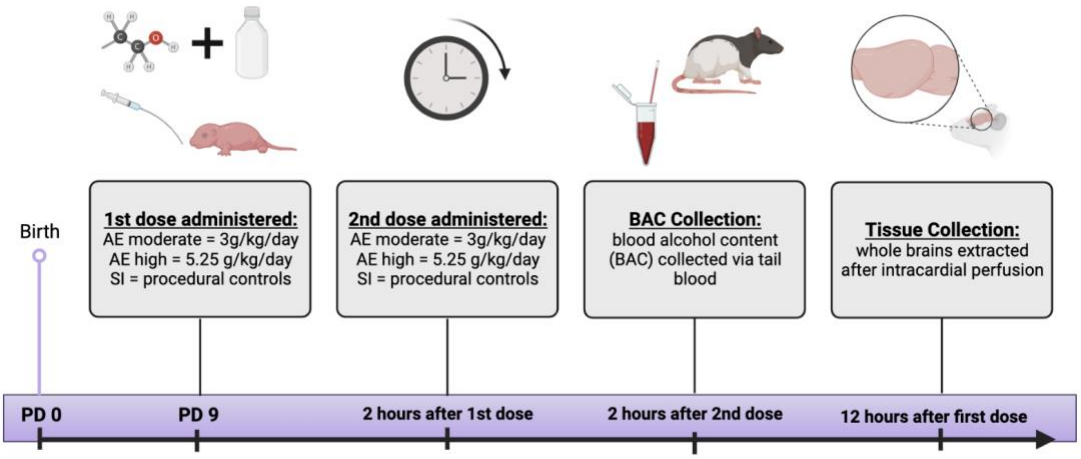


Figure 1. Single-day dosing paradigm timeline in a rodent model of FASD

2.3 Immunocytochemical Staining

12 hours following the first dose of ethanol in milk pups were heavily anesthetized with isoflurane followed by an intraperitoneal injection of 0.03ml/kg of ketamine/xylazine mixture prior to transcardial perfusion (0.1M phosphate buffered solution (PBS), pH = 7.2, followed by 4% paraformaldehyde in 0.1M PBS, pH = 7.2). Brains were then removed and post-fixed in a 30% sucrose paraformaldehyde solution. A cryostat was used to section brain tissue coronally at 40m which was then stored in cryoprotectant at -20 ℃.

Every eighth section containing the ventromedial thalamus was pulled and stained using primary antibodies against NeuN (to label mature neurons) and CC1 (to label mature oligodendrocytes). Tissue was pulled from cryoprotectant and placed into perforated bubble trays for a 5-minute deionized (DI) water wash. The tissue underwent two rounds of 5-minute 0.1M Tris Buffer Saline (1X TBS) washes. Following this, tissue was incubated for 120 minutes in a blocking solution (0.5% Triton X-100 and 3% Normal Donkey Serum (NDS) in 1X TBS). Tissue was then incubated in the primary antibody solution containing rabbit anti-NeuN antibody (Millipore Sigma, ABN78, 1:500) and mouse anti-APC/CC1 (EMD Millipore, OP-80, 1:400) in TBS-NDS blocking solution at 4℃ for 12 hours. On the second day of the procedure, tissue was placed on a shaker for 1-2 hours at room temperature. Tissue was then placed into a perforated bubble tray and underwent 3 rounds of 10-minute 1X TBS washes. A fluorescent secondary antibody solution including donkey anti-mouse antibody with Alexa Fluor647 (Jackson Immuno, 715-605-151, 1:250) and donkey anti-rabbit antibody with Alexa Fluor488 (Jackson Immuno, 711-545-152, 1:750) in TBS-NDS blocking solution was applied to the tissue for 120 minutes. It is important to note that tissue labeling became light sensitive at this point and remaining steps were completed with minimal light exposure. Once the secondary antibody incubation was complete, the tissue underwent two more 10-minute 1X TBS washes. A 5-minute 0.1M PBS wash was completed and then followed by a 5-minute 0.4g/μL Hoechst incubation in 0.1M PBS (12.3mg/mL, ThermoFisher, Catalog # 62249). Lastly, two 5-minute 0.1M PBS immersions are completed. Upon completion of all

washes and immersions, tissue was mounted onto microscope slides in dH_2O. Sections were left to dry on slides for about 60 minutes before coverslipped with gelvatol mounting media. Slides were stored away from light at 4°C.

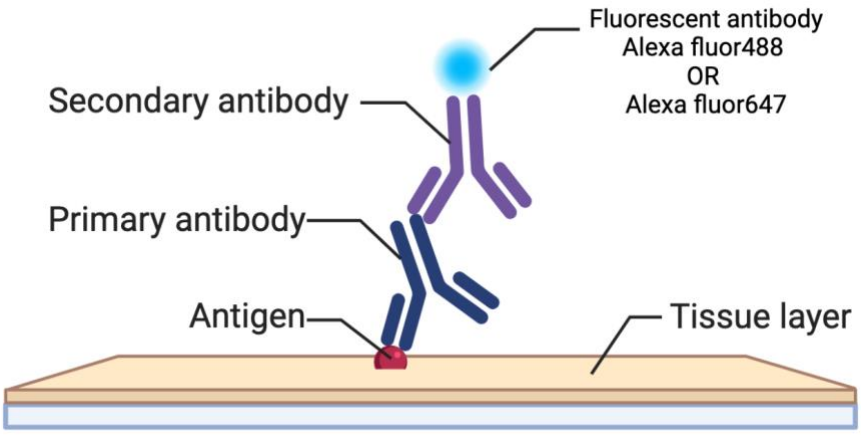


Figure 2. Use of antibodies in immunofluorescence staining to label specific cells. Binding of a fluorophore on a secondary antibody allows for fluorescence to be observed after activation using specific wavelengths of light.

2.4 Unbiased Stereology Cell Quantification

To estimate the numbers of neuron and oligodendrocyte populations, an unbiased stereological approach was used. This approach was used as it is recognized for its ability to accurately and efficiently estimate the total cell number, fiber length, and volume of the region of interest. This method uses systematic and random sampling by randomly overlaying a grid over the region of interest and systematically selecting a point within each grid to count cells of interest. We specifically used the optical fractionator probe, which is used to estimate the total number of objects in any

three-dimensional volume regardless of that region's shape. To do this, Re was outlined at 5x magnification. 3D image stacks were then acquired at 20x magnification using a Zeiss AxioImager M2 microscope with Colibri 7 LED illumination (Carl Zeiss AG, Oberkocken, Germany). For stereological estimation, counting regions within the regions of interest were randomly and systematically placed by the StereoInvestigator software (MBF Bioscience, Williston, VT). To estimate the total NeuN+ and CC1+ cell populations within Re, optical fractionator parameters were set to a dissector height of 28μm, guard zone of 28μm, sampling grid of 300μm x 300μm , and a counting frame of 50μm x 50μm.

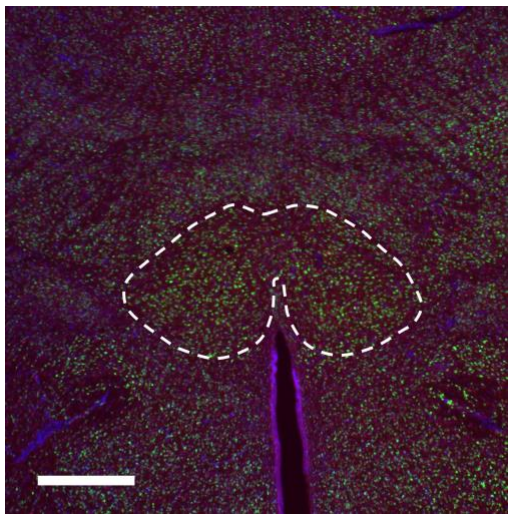
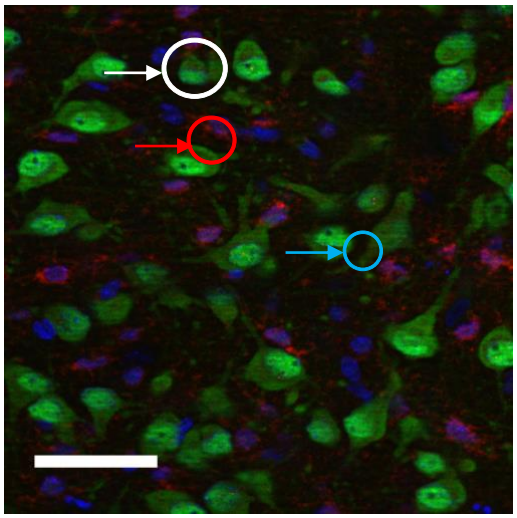
A.  B. 

Figure 3. Illustration of immunofluorescence stain used to detect neuron and oligodendrocyte populations. 3A: outlined region of interest, nucleus reuniens, at 5X magnification. Scale bar = 500μm. 3B: NeuN+ and CC1+ cells within Re at 63X magnification. White arrow indicates NeuN+ neuron, red arrow indicates CC1+ oligodendrocyte, and blue arrow represents cell nuclei labelled with Hoechst. Scale bar = 50μm.

2.5 Statistical Analysis

The data were analyzed using SPSS statistics (IBM, version 28.0.0.0 (190)). The independent variables of this study were sex and postnatal treatment. The dependent variables were the number of NeuN+ and CC1+ cells in the nucleus reuniens of the midline thalamus (Re) and reuniens volume.. A factorial 2 x 2 ANOVA was initially run to analyze NeuN+ and CC1+ counts.

Chapter 3

Results

3.1 Blood Alcohol Contents (BACs)

The mean BAC for AE pups was consistent with prior BAC's obtained from our lab using the same model of third trimester binge alcohol exposure (Smith et al., 2022, Milbocker et al., 2023).

3.2 NeuN and CC1 Expression in the Nucleus Reuniens (Re)

The optical fractionator probe (Stereo Investigator, MBF Bioscience, Williston, VT, USA, version 2021) on a Zeiss Axioscope 2 Plus (Carl Zeiss Inc., Thornwood, NY, USA) was used to quantify the number of NeuN+ and CC1+ cells in Re. The coefficients of error averaged to 0.088 for NeuN and 0.14 for CC1; both of these values have been described as reliable estimations of stereological measures (Solomianka and West, 2005). We found no effect of sex on any of the parameters of the study and therefore collapsed data across sex. A One-Way ANOVA to examine the number of NeuN+ and CC1+ cells in Re revealed no significant effect of postnatal treatment on the number of either NeuN+ ($F(2, 25) – 2.717$, $P = 0.0856$) or CC1+ ($F(2, 27) = 0.05811$, $P = 0.9437$) labeled cells in Re as seen in Figures 4 and 5. One-Way ANOVA of the volume of the Re revealed no significant difference ($F(2, 27) = 0.1746$, $P = 0.8408$) between postnatal treatment groups as seen in Figure 6.

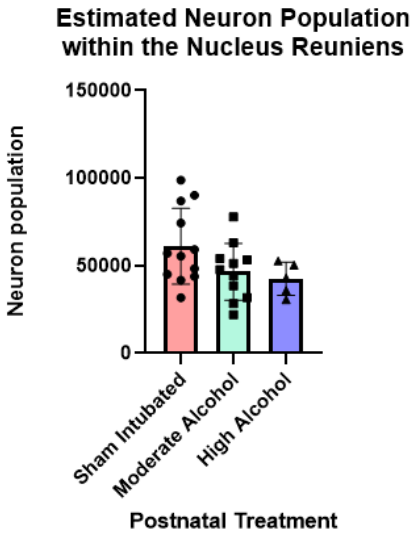


Figure 4. Estimated neuron population based on postnatal treatment. No significant effect of one-day AE on mature neuron population in Re was found. F(2, 25) = 2.717, P = 0.0856.

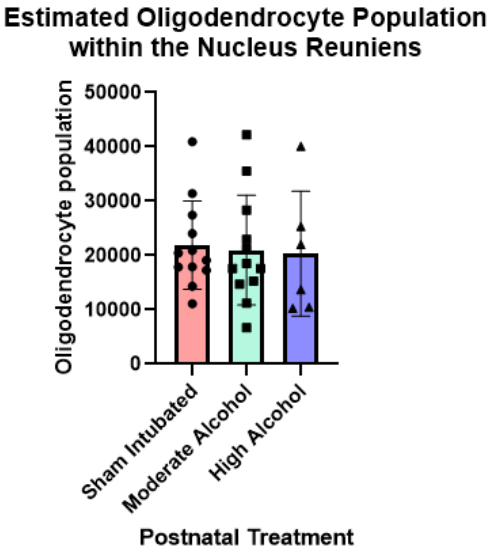


Figure 5. Estimated neuron population based on postnatal treatment. No significant effect of one-day AE on mature neuron population in Re was found. F(2, 25) = 2.717, P = 0.0856.

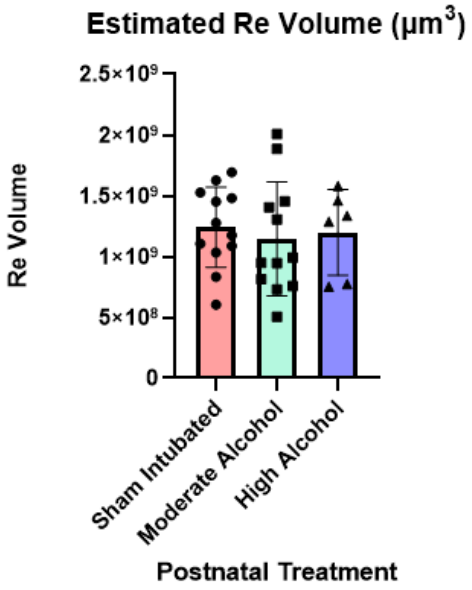


Figure 6. Estimated Reuniens volume based on postnatal treatment. No significant difference in reuniens volume based on postnatal treatment was demonstrated. F(2, 27) = 0.1746, P = 0.8408.

Sex	Postnatal Treatment	CE for Estimated Neuron Population in Re (Mean CE ± SEM)	CE for Estimated Oligodendrocyte Population in Re (Mean CE ± SEM)	CE for Estimated Reuniens Volume (Mean CE ± SEM)
Female	SI (*N* = 12)	0.09 ± 0.0141	0.14 ± 0.0319	0.0385 ± 0.0061
	Moderate AE (N = 12)	0.1 ± 0.0283 (1 outlier, *N* = 11)	0.16 ± 0.0513	0.0384 ± 0.0055
	High AE (N = 6)	0.08 ± 0.0058 (1 outlier, *N* = 5)	0.13 ± 0.0361	0.014 ± 0.00033
Male	SI	0.07 ± 0.0089	0.12 ±. 0.0103	0.0196 ± 0.0054
	Moderate AE	0.1 ± 0.0199	0.14 ± 0.0302	0.0327 ± 0.0048
	High AE	0.1 ± 0.0200	0.15 ± 0.0361	0.025 ± 0.012

Table 1. Coefficient of Error (CE) values for neuron and oligodendrocyte populations. CE values resemble proper cell estimation counts and rule out errors of over- or under-sampling. For estimated neuron population one outlier for the AE_M group was found and made *N* = 11; another outlier was found in the AE_H group for estimated neuron population and made *N* = 5.

Chapter 4

Discussion

4.1 Summary of Results

This study examined cell type-specific numbers in the nucleus reuniens of the midline thalamus in a rodent model of third trimester single-day (PD9) alcohol exposure. This brain area and thalamic nucleus was chosen because damage to Re had been demonstrated to underlie deficits in executive function; EF deficits are well characterized in Fetal Alcohol Spectrum Disorders (FASDs) (Pei et al., 2011). No significant effect of sex on any measure was found, thus the data were collapse across sex. Data obtained from this study showed that neuron and oligodendrocyte populations were not decreased significantly in comparison with sham-intubated controls, possibly due to the relatively early time point after alcohol teratogenic insult. While we did not see a significant difference between postnatal treatments, we observed a slight decrease in mean neuron populations based on treatment. Obtaining a larger sample size may reveal results of greater significance relating to mean cell populations in Re. Previous findings have revealed postnatal alcohol exposure to result in apoptotic cell death in Re and even demonstrated single-day AE to cause a permanent loss of neurons in Re in adulthood (Gurksy. et al., 2020). This study employed a third-trimester single-day alcohol exposure model of FASD on PD 7. While the current study quantified cell populations only 12 hours following PD 9 alcohol exposure, Gurksy et al., 2020 assessed Re vulnerability on PD 7, PD 11, and PD 72, which revealed significant levels of apoptosis at all three time points. Not only

did this study administer alcohol at an earlier time point and observe immediate apoptosis, but they also examined the effects of single-day exposure well into adulthood. While our study did not find significant decreases in neurons and oligodendrocytes based on postnatal treatment, we may find significant differences by administering AE at an earlier time point and collecting tissue closer to 48 hours following ethanol exposure, a peak time point for neuron loss (Phanithi, et al., 2000). Another study revealed neuronal and non-neuronal cell loss in adulthood following a single day of moderate and high doses of ethanol, however, the number of microglia remained unchanged after postnatal treatment (Gursky et al., 2022). If neuronal and non-neuronal cell loss occurs following AE, this poses the question, why do microglia not demonstrate the same loss in population? Microglia have been found to incorrectly phagocytize stressed, but viable, neurons through an event termed phagoptosis; this event occurs in times of brain development, inflammation, ischemia, and neurodegeneration (Brown and Neher, 2014, Yanuck, 2019). Phagoptosis may be one reason why reduction in neurons but not microglia were observed in Gursky et al., 2021. In addition, a previous project from our lab using a single-day binge model of FASD at PD 7 and PD 9, using the same moderate and high doses as the current study, revealed a significant increase in apoptotic cells in Re among high AE groups but not moderate AE groups. While not significant, this study showed the high dose PD 9 group to have a greater number of apoptotic cells than the high dose PD 7 group, a crucial factor in our decision to examine PD 9 AE in the current study. These findings, in conjunction with Gursky et al., 2020 indicate PD 9 to be a period of marked

vulnerability to AE and aided in our decision to examine neuron and oligodendrocyte populations at this time point. Findings from these various studies support decades of research that show alcohol exposure during synaptogenesis induces an apoptotic neurodegenerative reaction that produces deletions of many neurons in several major areas of the developing brain (Ikonomidous et al., 2000). To progress with our current findings, we may benefit from examining earlier and more vulnerable time points of the brain growth spurt to assess neuron and oligodendrocyte loss.

4.2 Neonatal Single Day Alcohol Exposure's Effect on Neuron and Oligodendrocyte Populations within the Re

The Re is known to be especially vulnerable to alcohol-related cell damage based on previous research within our lab; these findings led us to expect decreased neuron and oligodendrocyte populations within Re following alcohol exposure. More specifically, we anticipated AE_H groups to demonstrate the greatest reduction in cell populations. The anatomical analysis revealed no significant difference in the estimated neuron population in Re between AE_H, AE_M, and SI groups. Analysis of oligodendrocyte population in Re also demonstrates no significance between groups. This finding implies that AE did not significantly reduce the population of neurons and oligodendrocytes, although based on increases in apoptosis found in other studies this may be due to not enough time having elapsed between AE and time of perfusions.

4.3 Future Directions

While this study did not indicate that AE reduced cell-specific populations in Re, the overall effect of alcohol exposure on Re cell loss should be further studied. Future work still needs to be done to add subjects across postnatal treatment groups. Expanding the sample size will provide us with a more representative population and may reveal significant differences. Future work would also benefit from counting cell nuclei labeled with Hoechst to calculate a ratio of NeuN and CC1 populations to the estimated total cell population. This could allow for a more comprehensive understanding regarding total cell loss and not just neurons and oligodendrocytes. Furthermore, exploration of the vulnerability of the mPFC-Re-HPC circuit to alcohol exposure can aid in our understanding of how circuit activity correlates to executive function. Looking at cell populations in the medial prefrontal cortex (mPFC) and hippocampus (HPC) can help us understand how each of these areas work in unison. Future studies may benefit from continuing to use immunofluorescence staining to label cells, however, it may be beneficial to investigate cell activation and how levels of activation differ between AE and SI groups. We are also particularly interested in examining neuron and oligodendrocyte populations 48 hours after AE; based on our findings regarding neuron loss at 12 hours in the current study, examining this variable 48 hours after AE may reveal dynamic changes that are not evident at the peak of apoptosis. Since we did see small reductions in neuron populations in Re at 12 hours, we can hypothesize that a greater, and possibly significant, reduction would be observed at 48 hours. Keeping with this idea, we would also benefit from examining

neuron and oligodendrocyte Re populations in adulthood to assess persistent levels of cell-specific loss.

4.4 Limitations

Even though we did not see reduced neuron and oligodendrocyte populations in Re there are some limitations that may influence the validity of our findings. Most notably, our sample size did not have the same number of animals in each group. Moving forward, it should be the top priority to generate a substantial sample size to properly represent each group. The current study had a maximum of seven animals in a group, future studies may benefit from increasing the number of animals in each group. A second limitation of this study is that there was no suckle-control group; lacking this group prevents comparisons to be made to assess the stress of intubation within the sham-intubated group. From unpublished findings in our lab, we know sham intubated pups suffer cell loss due to the stress of intragastric intubation. However, it should be noted that previous findings have shown no difference in Re damage between suckle control and sham intubated animals. Addition of a suckle-control group would elucidate differences between sham intubated and alcohol exposed groups by providing us with a true baseline measure.

Stress plays a large role in not only influencing behavior but also anatomical development. The administration of alcohol during this early time point is a stressful experience not only for the pups but also for the dams. Increased levels of stress in the dam may hinder their ability to properly care for their pups and produce greater levels

of cell loss (Heun-Johnson and Levitt, 2016); increased cell loss due to stress and not AE could impact results and not allow us to draw clear conclusions regarding the effects of AE on cell-specific loss. In Re. One approach to resolving this issue may be to implement a handling period where the dams and pups become accustomed to encounter researchers.

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