EFFECT OF VOLUNTARY ALCOHOL DRINKING ON MICROGLIA ACTIVATION

An Honors Thesis

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ABSTRACT

Abstract:

Addiction is common within the U.S. Alcohol use disorder is one type of substance abuse. Dependency on alcohol may begin as early as adolescence, which is a crucial time for development. During adolescence, the brain undergoes maturational processes that are necessary for cognitive function later in life. Rodent studies have shown that medial prefrontal cortex (mPFC) and the hippocampus, are susceptible to damage from alcohol. We have previously found a greater loss of myelin of axons in the mPFC in adolescent males (Tavares et al., 2019). In this study, we test the hypothesis that voluntary alcohol drinking during adolescence leads to a greater inflammatory response in males compared to females. Adolescent male and female Wistar rats self-administered sweetened alcohol or sweetened water. Brains were collected and immunolabeled for ionized calcium-binding adapter molecule 1 (Iba-1). Microglial cell morphology was then analyzed using high content microscopy. There was no effect of sex on cumulative alcohol intake. Microglia density in the mPFC did not change for males or females. In the mPFC, there was a decrease in branching points per microglia cell distribution in both males and females. For the hippocampus, there were differences found in microglia density in males, and the microglia population shifted to a state of having fewer ramifications per cell in the alcohol group in the CA1 and DG of females, and in the CA2/3 of both males and females. No differences were found in the circularity of microglia in the hippocampus. The lack of consistent findings regarding a greater extent of microglia activation in males do not support our hypothesis that differential microglia activation is responsible for the sex differences in the myelination of axons.

LITERATURE REVIEW

Adolescence is a period of time where the brain is developing and it appears to have heightened vulnerability to the damaging effects of toxins, including alcohol . During this time period of maturation of the brain, there is a great deal of remodeling of grey and white matter (Lenroot & Giedd, 2006). Additionally, during maturation, there is a remodeling of the reward circuit (Tarazi and Baldessarini, 2000, Teicher et al., 1995). During this time of adolescence and brain maturation, individuals may engage in risky behaviors, such as binge drinking. This is thought to occur because the frontal lobes are still developing, which are the regions of the brain responsible for decision making, stress response, and attention (Wilson et al., 2010, Kesner & Churchwell, 2011). Alcohol use has been found to result in brain abnormalities, weakened memory capacity, and worsened academic performance, which has been supported by findings of negatively impacted memory and learning in experimental animals when exposed to alcohol (Crews et al., 2000, Pascual et al., 2007).

Two mechanisms have been proposed for the neurotoxic effects of alcohol usage. The first is that repeated, intermittent exposure to alcohol eventually results in short periods of withdrawal after local alcohol levels decrease, and this state induces excitotoxic neuronal damage and an increase in synaptic activation of NMDA receptors and cellular injury (Hendricson et al., 2007). The second proposed mechanism is that alcohol activates glial cells through the stimulation of intracellular signaling pathways, which increases the production and release of proinflammatory cytokines, resulting in neuronal damage and ultimately, cell death (Blanco, 2007, Vallés et al., 2004). Findings have shown that the inflammation within the brain has been associated with an increase in quantity and activation of microglia (Lee et al., 1994, Kreutzberg, 1996). Microglial cells are a type of glial cell within the brain that play an important

role in the immune response. *In vitro* studies have also shown that chronic alcohol consumption increases microglia activation and pro-inflammatory cytokine expression. Microglia have various levels of activation, which are associated with different phenotypes, including a resting state, hyper-ramified state, and activated state. The inflammatory cascade resulting from microglia activation can be mediated by estradiol (Vegeto et al., 2001). This review discusses the role of microglia in an inflammatory response from alcohol drinking, key players in the neuroinflammatory response pathway, and sex differences in vulnerability to the effects of alcohol.

Role of microglia in the brain and the development of the brain:

The brain is especially sensitive to substances such as drugs and alcohol during periods of development, including adolescence. During the earlier stages of adolescence, puberty results in the rise of gonadal hormones, and we and others have found that pubertal maturation lags in male rats compared to females (Ojeda & Skinner, 2006; Tavares et al., 2019). In addition to these gonadal hormone changes, there are other structural changes that occur in an individual's brain during adolescent development. White matter has been shown to steadily increase throughout childhood to adolescence, which is due to axons of neurons undergoing myelination during adolescence. The brain's gray matter has also been shown to increase in volume (Blakemore et al., 2010). Chronologically, the brain develops from back to front so that the prefrontal cortex is the last region of the brain to become fully developed. Glial cells are a type of cell that are important for maintaining the function of and protecting neurons in the central nervous system. Specifically, microglia are the resident immune cells in the brain. They are macrophages that play a role in inflammatory signaling. These cells have different morphological states, which are

representative of their level of activation. When they are resting, they have long processes extending from their soma. In a more activated state, the microglial processes become more extended and increase in branching. This state is defined as the "hyper-ramified" state. A third state of activation of microglia is "activated" and this is when microglia take on a more amoeboid phenotype and their processes fully retract (A Karperien et al., 2011). An amoeboid phenotype occurs early in development, and microglia are thought to mature to a more ramified state with the maturation of neurons (Harry & Kraft, 2012, Kaur & Ling, 1991). These cells become activated in response to toxins or damage, and have a role in signaling in an inflammatory cascade, which may be occurring during binge drinking.

Alcohol's effect on microglia and signaling pathways:

Alcohol use is prevalent among adolescents in the U.S. and is one precursor for alcohol use disorder and other alcohol-related problems later in life (Chou and Pickering, 1992). High levels of alcohol consumption can trigger a pro-inflammatory cascade, activating toll-like receptors and the production of cytokines from glial cells. Upon activation, microglia change morphology and proliferate, as well as release oxygen intermediates, nitric oxide, and cytokines, such as IL-1, IL-6, IFN-gamma, and TNFalpha (Benzing et al., 1999, Lively & Schlichter, 2018). Using RNA-sequencing and analyzing differential gene expression of microglia post-chronic alcohol consumption, an overrepresentation of genes involved in "inflammatory response" and "regulation of apoptotic signaling pathway" was found (McCarthy et al., 2018). One specific toll-like receptor, Toll-Like Receptor 4 (TLR4) is critically important in the inflammatory pathway. TLR4s are found in microglia and astrocytes and are capable of activating mitogen activated protein kinase (MAPK) and nuclear factor-κB (NF- κB) pathways, resulting in the

transcription of inflammatory genes. Knocking out the TLR4 receptor through siRNA does not result in activation of microglia, nor the inflammatory response cascade. (Alfonso-Loeches et al., 2010). This effect is also observed in microglia cell cultures (Fernandez-Lizarbe, 2019). Furthermore, neuronal degeneration is observed in male rats from binge exposure to alcohol in the cortex and hippocampus using amino cupric silver staining (Crews et al., 2000; McClain et al., 2011). This neuronal degeneration could be a consequence of the neurotoxins that microglia release, as well as a consequence from the disruption of myelination of neurons, which occurs after voluntary binge drinking during adolescence (Mengler et al., 2014; Vargas et al., 2014; McDougall et al., 2018; Tavares et al, 2019). Alcohol may be interrupting the developmental process of axonal myelination, which would then have long term consequences in adulthood.

Long-Lasting Changes:

Aside from findings that microglia change morphology and increase in quantity, researchers found that even a single four day binge of alcohol led to longer lasting changes in microglia activation in the hippocampus. To investigate survival of cells, dividing cells were labelled with Bromo-deoxy-Uridine (BrdU) and microglia were labelled with Iba-1. Two groups were analyzed, T2 and T30, which were groups euthanized 2 days and 30 days after the last alcohol exposure, respectively. It was found that many cells that had the BrdU label at T2 still existed at T30, suggesting that the immunoreactivity of microglia lasts into early adulthood, which is considered 65 days postnatal for rodents (McClain et al., 2011). The findings of long-lasting changes in the hippocampus from alcohol binges provide some evidence that the hippocampus is a particularly susceptible area of the brain during adolescence and that changes

can affect brain development later in life, thus making individuals who drink during adolescence more susceptible for alcohol use disorder later in adulthood.

Sex Differences:

An important aspect to consider when studying the effects of alcohol and binge-drinking behavior on the brain are individual differences, as well as sex differences. These differences may make some individuals more sensitive than others to the inflammatory effects of alcohol consumption. A sex difference can be seen in the responses to chronic alcohol exposure using a 5-month home cage drinking paradigm where adult female mice had a greater neuroinflammatory response than males (Alfonso-Loeches et al., 2013). Additionally, significant reductions in myelin in the cortex of females were found after chronic alcohol exposure using the same paradigm (Alfonso-Loeches et al., 2012). Notably, we have found evidence for sex differences in the effect of adolescent alcohol on myelinated axons. While adolescent alcohol drinking caused microstructural changes at the Nodes of Ranvier in both sexes, it decreased myelinated fiber density within the mPFC of male, but not female rats (Tavares et al., 2019). A potential explanation for the observed sex differences in sensitivity to alcohol, is that ovarian hormones may be playing a protective role in females.

Conclusion:

Alcohol use disorder is a very serious condition that affects people of all ages and has the potential to lead to mortality. Through binge drinking, microglia become activated and proliferate as the brain's immune defense. Through their phagocytic activity, they eliminate substances that could potentially be toxic to the brain. Another consequence of the activation of

microglia is the release of cytokines and other neurotoxic molecules, which is regulated in part by TLR4 signaling to MAPK and NF- κB for inflammatory gene expression. The activation of microglia can potentially have a harmful effect on the myelination of axons, leaving them susceptible to degeneration. Future research may involve studying how to mitigate the harmful effects of the activation of this inflammatory response to potentially reduce the amount of neurodegeneration. Investigating the effects of alcohol on microglia activation could provide insight into the mechanisms behind the sex differences in decrease in myelination. By gaining a better understanding of how alcohol consumption during adolescence is related to neurodegeneration and neuroinflammation allows researchers to gain insight as to how individuals may be more susceptible for alcohol use disorder or substance abuse in adulthood.

INTRODUCTION

Alcohol use disorder is a medical diagnosis of abnormal alcohol use or dependency, in which people suffering from this disorder have little to no control over their use of alcohol. Dependency on alcohol may begin as early as adolescence, which is a crucial time for development. Drinking during adolescence has been associated with alcohol use related problems later in life (Chou and Pickering, 1992). Adolescence is a time period where individuals are likely to engage in risky behaviors, such as binge drinking and drugs. It is during this time that individuals are most vulnerable to changes in the brain that have long lasting effects into adulthood (McDougall et al., 2018; Mengler et al., 2014; Vargas et al., 2014). The brain is still developing and maturing, including the prefrontal cortex, the region responsible for judgement and decision making, which is considered to be a reason that adolescents are more likely to engage in risky behavior. Our lab has previously found sex differences in the response to alcohol drinking, including a greater loss of myelin of axons in the mPFC specifically in adolescent males (Tavares et al., 2019). The mechanism behind this greater loss of myelination remains unclear. The first step of the neuroinflammatory pathway involves the activation of microglial cells, the resident immune cells of the brain. When activated, microglia cells change phenotype and release cytokines and other neurotoxic molecules. Working closely with my PhD candidate mentor, Andrea Silva-Gotay, the objective of the present study is to test whether differences in the neuroinflammatory response are responsible for the differences in myelination through quantifying microglia activation. We chose to investigate the mPFC because this is the region where we identified a greater loss of myelin in adolescent males from our previous study. In addition to analyzing microglia activation in the mPFC, we also investigated activation in

three regions of the hippocampus because of the earlier development of the hippocampus and its identified susceptibility to changes from alcohol.

My original thesis plan was to quantify protein expression from isolated microglia cells in response to alcohol treatment in vitro. This was an extensive process and because of Covid-19 restrictions, I was unable to be in the lab when UMass went into “High Risk” posture in the fall. Not knowing whether the Covid-19 cases and exposure would increase or decrease, my PI and I decided to transition to make my thesis work remote for the year. This remote work involved taking cropped images, developing the automated analysis program, extracting the measurements for the images, and performing data analyses. Some of the challenges of this work included the time to download and open images as well as scheduling remote Workstation time at the Life Sciences Laboratory because of how often people were now using the Workstations. Switching to a remote thesis this year has been the impetus for me to become more flexible, adaptable, and resilient.

METHODS

Animals:

12 male and 12 female Wistar rats arrived at UMass Amherst on postnatal day (PD) 18 and were weaned from their mothers at PD 21. They were housed with two other rats of the same sex on a 12 hour light/dark cycle with free continuous access to food and water. Each animal was handled daily during the operant training period in an effort to get the rats used to human contact and to minimize confounding effects of stress during the experiment. All procedures were approved by the Institutional Animal Care and Use Committee and performed in accordance with the NIH Guide for the Care and Use of Laboratory Animals.

Operant self-administration:

Adolescent rats were operant trained using sweetened water from PD25-PD28, where one lever press was equivalent to one bout of sweetened water. Between PD28-PD42, the rats had overnight drinking sessions of six 30-minute bouts of access to either sweetened alcohol or sweetened water, depending on if they were assigned to the alcohol or control group. Each group consisted of 6 animals: 6 control males, 6 alcohol males, 6 control females, and 6 alcohol females. 60 minute breaks were included in between the 30 minute drinking intervals, during which the lever was retracted. Rats in the alcohol group could press a lever which delivered 0.1 ml of sweetened alcohol (3% glucose, 0.125% saccharin, 10% w/v alcohol). The control group was delivered 0.1 mL sweetened water (3% glucose, 0.125% saccharin) per lever press. The rats were also provided free access to water and food. A computer was connected to the operant boxes to cap the maximum number of responses per each session to keep the amount of solution consumed by each group similar. After an animal reached the maximum number of responses,

the lever was retracted in order to avoid extinguishing self-administration responses in the control group. This paradigm is shown in **Figure 1.**

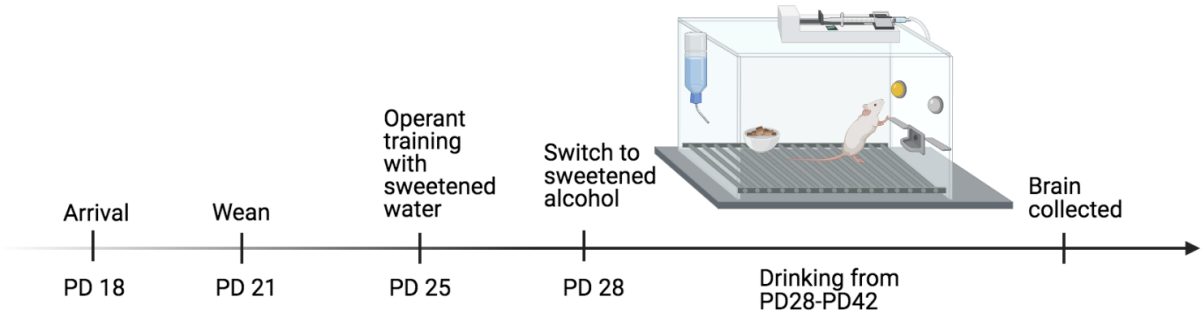

Figure 1. **Timeline of operant self-administration of sweetened alcohol or sweetened water.** Male and female Wistar rats arrived on PD18 and were weaned at PD21. On PD25, rats were trained to use the operant box, which was followed by self-administration of sweetened alcohol or water, depending on the group, from PD28-42. Created with BioRender.com.

Immunohistochemical analysis of microglia:

1) *Perfusions and tissue processing*

Animals were anesthetized with 50 mg/kg sodium pentobarbital and intracardially perfused with room temperature 0.9% saline for 5 minutes, followed by cold 4% paraformaldehyde in 0.1 M sodium tetraborate (pH 9.4) for 20 minutes. The rats were then decapitated and their brains were extracted and fixed in paraformaldehyde solution for 4 hours. Then, the brains were dehydrated in 20% sucrose in phosphate-buffered saline (PBS) solution for approximately 48 hours and were snap frozen. They were stored at -80ºC until they were cut at 35 µm thickness on a freezing microtome. The sections were stored in cryoprotectant at -20ºC.

2) *Immunohistochemistry*

Free-floating sections from both treatment groups were rinsed in 0.05M Tris-buffered saline (TBS; pH 7.2). Sections were rinsed in solution of 10% saline, 1% gelatin, 0.2% sodium azide, and 0.2% Triton-X in 0.05M TBS. Sections were incubated in a blocking buffer (2% normal goat serum, 1% bovine serum albumin, 1% hydrogen peroxide in gel TBS) for 1 hour. Then, sections were incubated for 48 hours at 4ºC with a rabbit polyclonal antibody raised against ionized calcium binding adapter molecule (Iba-1) at a dilution of 1:10000 in 2% normal goat serum and 0.55% Triton X-100 in gel TBS. The sections were then rinsed in TBS and incubated in biotinylated secondary antibody in 2% normal goat serum in gel TBS for 90 min., followed by washed in gel TBS. The sections were then incubated with an avidin-biotin horseradish peroxidase complex for 90 minutes at room temperature, washed in gel TBS, then washed in TBS. The sections were visualized with nickel sulfate and 3,3'-diaminobenzine tetrahydrochloride. After, the sections were rinsed in TBS and mounted on gelatin-coated glass slides and coverslipped. This protocol is represented in **Figure 2.**

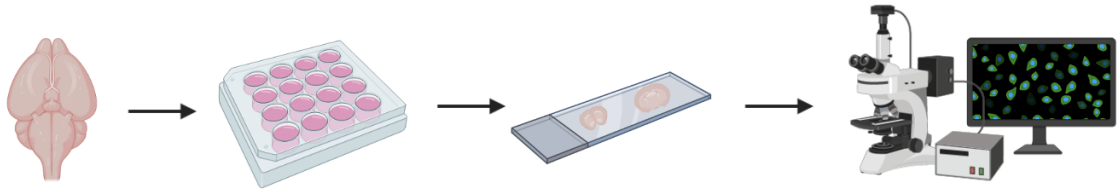

Figure 2. Immunohistochemistry experiment labeling microglia with ionized binding adaptor protein 1 (Iba-1). Brains were extracted and immunohistochemistry was performed on

mPFC and hippocampus tissue. After labeling microglia with Iba-1 and coverslipping, high content microscopic analyses were performed. Created with BioRender.com.

3) *Microscopic analysis*

Whole mPFC and hippocampus sections were imaged at 20x using a Nikon microscope set up with Köhler illumination and using an Andor Zyla 4.2 sCMOS camera. Slides were scanned at 4x to identify tissue location and to find the best plane of focus. After coordinates were determined, z-stacks were taken at 20x magnification, images were stitched, and extended depth of focus was used to find high contrast of each z-stack to assemble in a single plane. Nikon NIS-Elements Advanced Research software was used for automatic cell counts and morphological analyses.

4) *Cell counts and morphological analysis for mPFC*

Automatic cell counts for the mPFC were performed using General Analysis within NIS-Elements Advanced Research Software. To mark cell bodies, images were preprocessed using a Gaussian convolution - size 5 and thresholded using between 0-401 (darkest pixels). Regions were smoothed and cleaned at 4x magnification. Regions of interest were filtered by those with area of greater than or equal to 55 μm². Regions were dilated by 1.3 μm. Objects were then counted. Microglia morphology of the mPFC was analyzed by quantifying the total branching points per microglial cell using General Analysis 3 in NIS-Elements AR. Somas were highlighted using the same steps from the automatic cell counting protocol. Next, microglia ramifications were highlighted through the skeletonize feature. Images were preprocessed to detect valleys with Kernel count of 2x magnification and thresholded using pixels between 249-1253. Regions were smoothed and cleaned at 1x and skeletonized to create a second binary layer. A radius of 15 μm

was chosen to make sure that only the ramifications that belonged to the cells in our sampling area were being measured. For this, the dilate function was used at 15 μm to create a third binary layer. The separate objects function was then used so the overlapping cells were counted as individual objects and not as one. The expanded radius and skeletonized layers were combined to get the quantification of ramifications per cell. The object parenting function and aggregate children function were used to measure the branch points per object.

5) *Cell counts and morphological analysis for hippocampus*

Automatic cell counts and morphological analyses were performed using General Analysis 3 in NIS-Elements AR. Three crops of 500 x 500 pixels from each of the following regions were taken: DG, CA1, and CA2/3, consistent with the sampling method of the hippocampus in literature. To mark cell bodies, images were thresholded between 0-596 (darkest pixels). Regions were smoothed and cleaned at 2x magnification. Cell bodies were filtered by an area of 5.00 μm to 347.09 μm. Regions of interest were filtered by an area with a minimum of 55 μm^2 to infinity. Regions of the somas were dilated to 15.0 μm. The separate objects function was then used to create independent objects if there was overlap. Microglia morphology of the hippocampus was analyzed by quantifying the total branching points per microglial cell. Microglia ramifications were highlighted through the skeletonize feature. For this, images were preprocessed to detect valleys with Kernel count of 2x magnification and thresholded using pixels between 249-1253. Regions were smoothed and cleaned at 1x and skeletonized to create a second binary layer. In order to make sure that only the ramifications that belonged to the cells in

our sampling area were being measured, a radius of 15 μm was picked. For this, the dilate function was used at 15 μm to create a third binary layer. The expanded radius and skeletonized layers were combined and used with the detect branching function to get the quantification of ramifications per cell. The separate objects and Child ID were used to measure branch points per object. Another binary layer was created to give a measurement for circularity and object area. The images were thresholded from 0 to 2000 and filtered by an area of 5 μm to 347.09 μm. The object filter function was used for object area from 70.0 to 1007.068. Circularity and object area functions were then applied, along with aggregate children function. This analysis program is shown below in **Figure 3**.

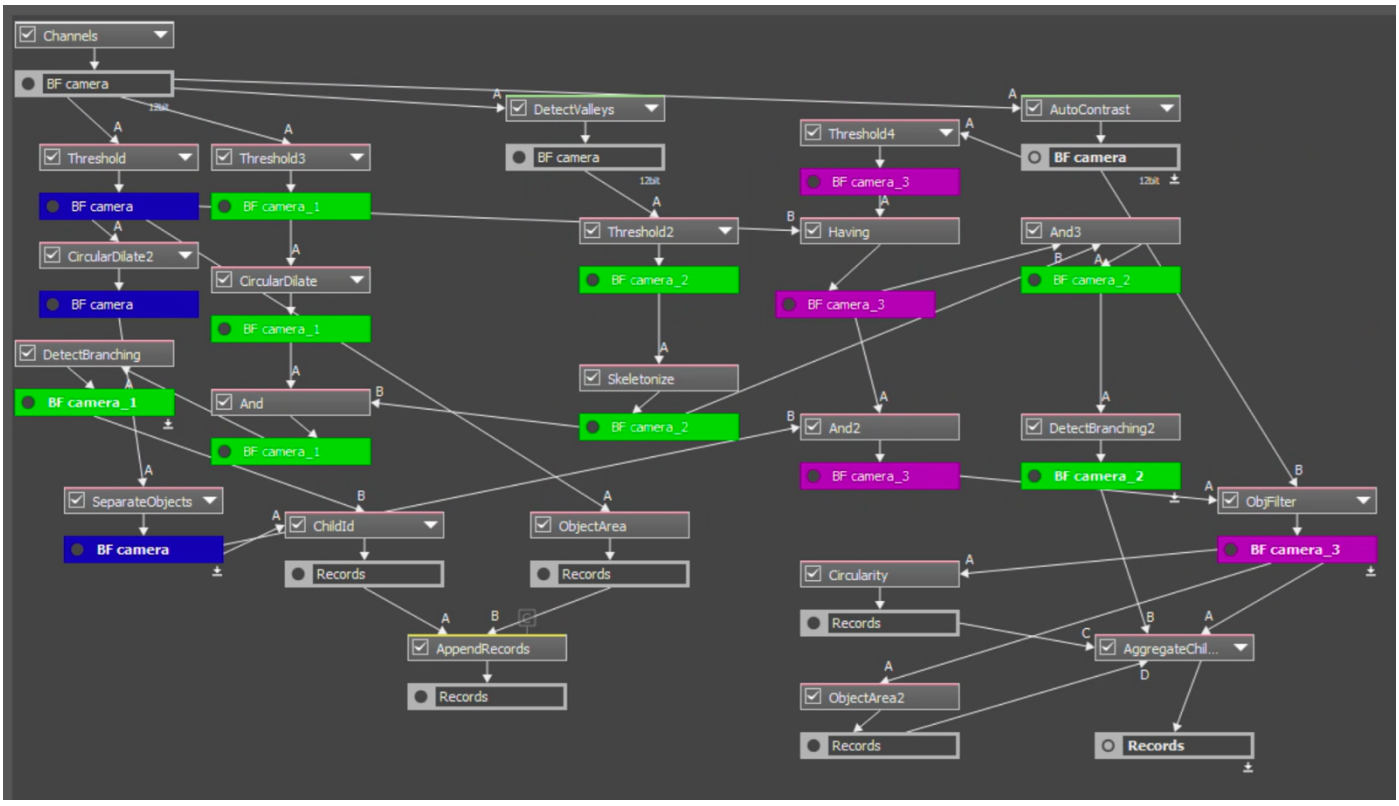

Figure 3. NIS Elements AR General Analysis 3 for hippocampus measurements.

Measurements of cell counts, cell area, circularity, and branching points per cell were obtained from this program.

6) Statistical analysis

Cumulative alcohol intake was analyzed using a mixed-model ANOVA, with sex as a between-subject variable and operant day as a within-subject variable. For the mPFC, control males: n=6, alcohol males: n= 6, control females: n=6, alcohol females: n= 6. Microglia cell counts in the mPFC were analyzed using two-way ANOVAs with sex and treatment as within-subject variables. For the CA1, control males: n=6, alcohol males: n= 3, control females: n=4, alcohol females: n= 5. For the CA2/3, control males: n=6, alcohol males: n= 3, control females: n=5, alcohol females: n= 4. For the DG, control males: n=5, alcohol males: n= 3, control females: n=6, alcohol females: n= 4. For the

hippocampus regions, a Shapiro-Wilk normality test was run for each group to test for normalcy. Microglia cell density was analyzed using an Unpaired Two-Samples Wilcoxin Test. A two-sample Kolmogorov-Smirnov test was used to compare the distributions of total branching points between the alcohol and control group for both the mPFC and hippocampus. Circular phenotypes were analyzed using an Unpaired Two-Samples Wilcoxin Test. Statistical significance was defined as p is less than or equal to 0.05, using two-tailed tests. Error bars in graphs were +SEM. Analyses were performed using R statistical software package (open source from https://www.r-project.org).

RESULTS

Ethanol intake in adolescent male and female rats

First, we looked at amount of alcohol intake in grams/ kilogram of body weight between males and females over the two-week binge period to investigate any sex differences in alcohol consumption (**Figure 4**). We found that males and females consumed very similar amounts of alcohol and there were no significant differences in cumulative alcohol intake during the binge period ($p>0.05$).

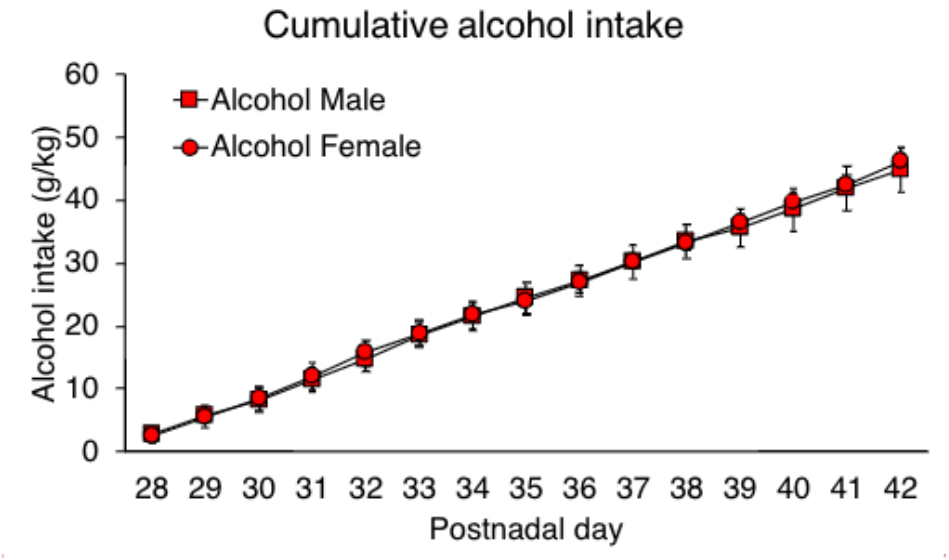

Figure 4. No significant difference in cumulative alcohol intake between males and females. Consumption of alcohol was measured in terms of the rats' body weights. Males and females drank similar amounts of alcohol (control males: n=6, alcohol males: n= 6, control females: n=6, alcohol females: n= 6). Adapted from Silva-Gotay, unpublished.

Microglial cell density in the mPFC

To first look at microglia proliferation and recruitment as an indicator of their activation, we quantified microglia cell density in the mPFC (**Figure 5**). We found that microglia cell density did not change for the alcohol treatment in either males or females.

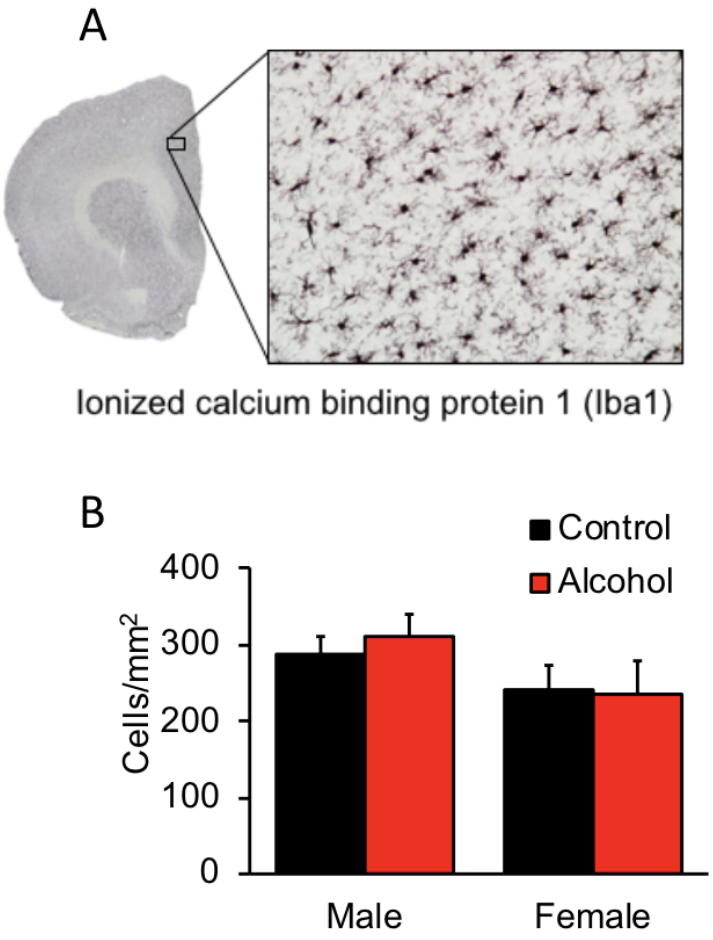

Figure 5. Microglia recruitment in the mPFC as an indicator of the neuroinflammatory response. A) Microglia were labeled with Iba-1 in the mPFC. Microglia cell density (B) did not change between the alcohol and control groups in either males or females (control males: n=6, alcohol males: n= 6, control females: n=6, alcohol females: n= 6). Adapted from Silva-Gotay, unpublished.

Ramifications per microglial cell distribution in the mPFC

To further look at microglia activation, we investigated the distribution of ramifications per microglial cell in the population. Using a General Analysis 3 program in NIS-Elements software, the ramifications were selected and branching points were calculated. Generating a density plot of branching points, we found a shift in the distribution to a state of cells having fewer ramifications in the alcohol group for both males and females (**Figure 6,** $p<0.05$).

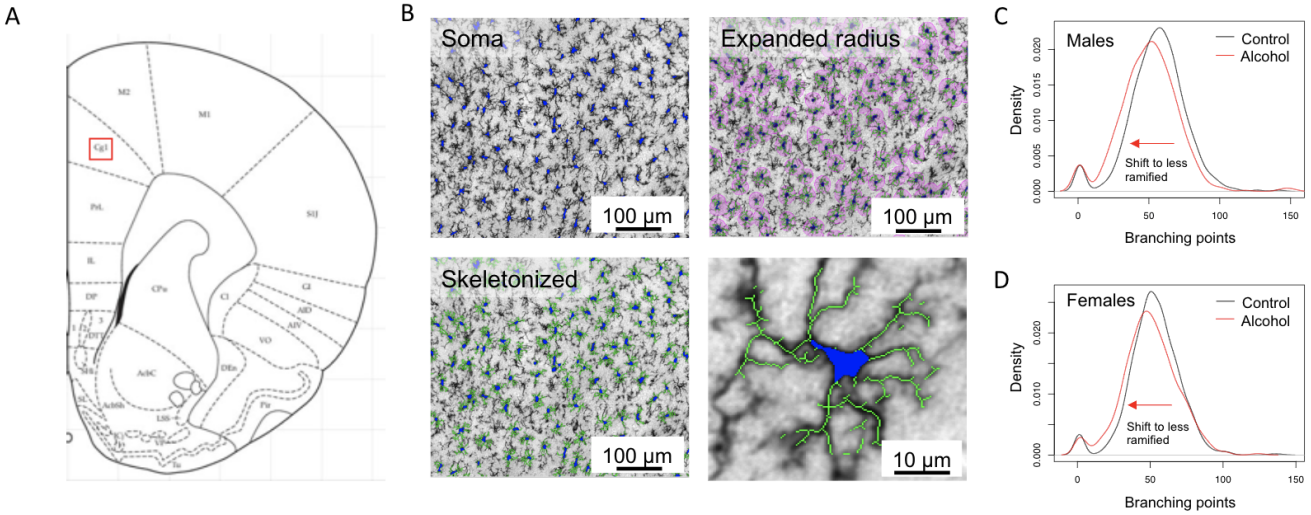

Figure 6. Shift in Microglia Population to a State of Fewer Ramifications. A) The mPFC was the region of interest for this analysis. B) Using an automated analysis program, branching points per cell were obtained for males and females. C) We found a shift in the microglia population to a state of having fewer ramifications per cell in the alcohol group of both males and females (control males: n=6, alcohol males: n= 6, control females: n=6, alcohol females: n= 6).

Microglial cell density in the hippocampus

We investigated changes in microglial cell density in the CA1, CA2/3, and DG (**Figure 7**) as an indicator of activation of microglia. The number of cells in an image was divided by the

area of the cropped image to get cell density. These preliminary results show the microglia cell density decreased for the male alcohol group compared to the control group in the CA1 and DG ($P<0.05$). Contrastingly, the cell density increased in the CA2/3 for the male alcohol group ($p<0.05$). There were no changes in microglia cell density for the females groups.

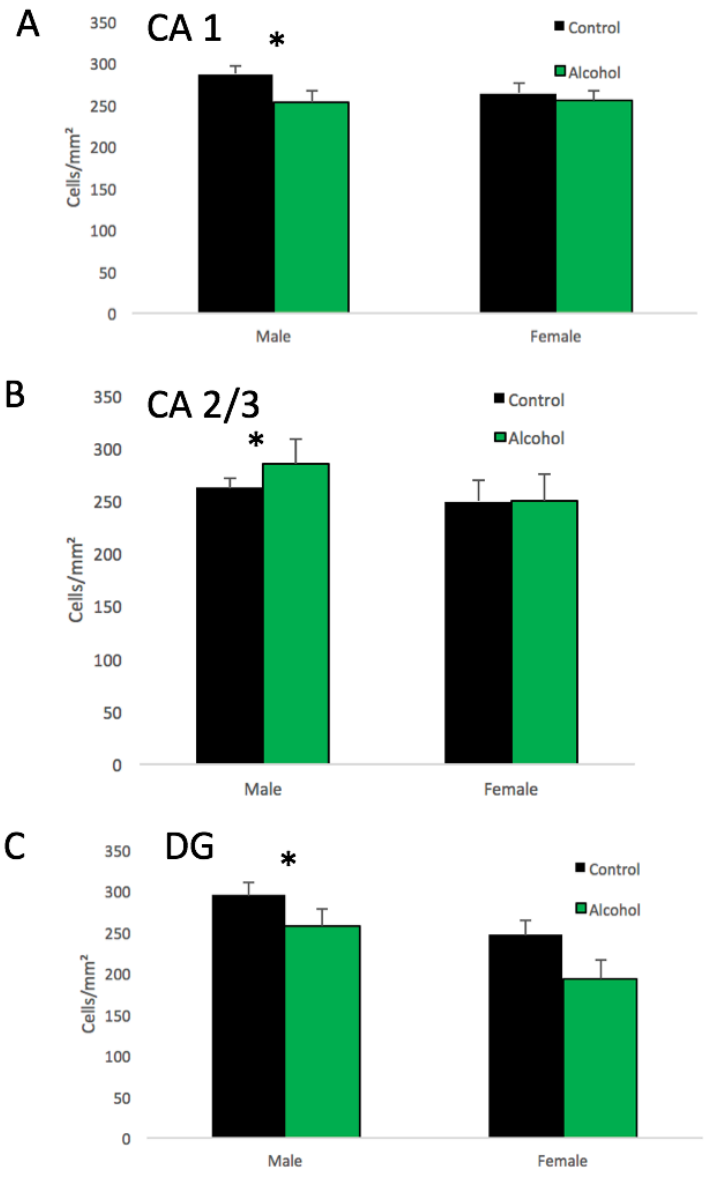

Figure 7. Microglia recruitment in the hippocampus as an indicator of the neuroinflammatory response. Microglial cells labelled with Iba-1 were counted using an automated analysis program in NIS-Elements. A) CA1 microglia cell density for control and alcohol males and females were compared (control males: n=6, alcohol males: n= 3, control females: n=4, alcohol females: n= 5). There was a decrease in density for the male alcohol group. B) CA2/3 microglia cell density for control and alcohol males and females were compared (control males: n=6, alcohol males: n= 3, control females: n=5, alcohol females: n= 4). There was an increase in microglia density in the male alcohol group. C) DG microglia cell density for control and alcohol males and females were compared (control males: n=5, alcohol males: n= 3, control females: n=6, alcohol females: n= 4). Microglia cell density decreased for the male alcohol group.

Ramifications per microglia cell distribution in the hippocampus

We also investigated the distribution of ramifications per microglial cell in the population in the hippocampus in the CA1, CA2/3, and DG regions (**Figure 9**). Using a density plot, we found a shift in the distribution to a state of cells having fewer ramifications in the alcohol group for both males and females in the CA2/3, in males in the CA1, and in females for the CA1 and DG. On the other hand, we found a shift to a state of more ramifications in the DG of alcohol-exposed males. Using Kolmogorov-Smirnov two-sample tests for each region, it was found that the alcohol and control groups' distributions were significantly different for both sexes in every region ($p<0.05$).

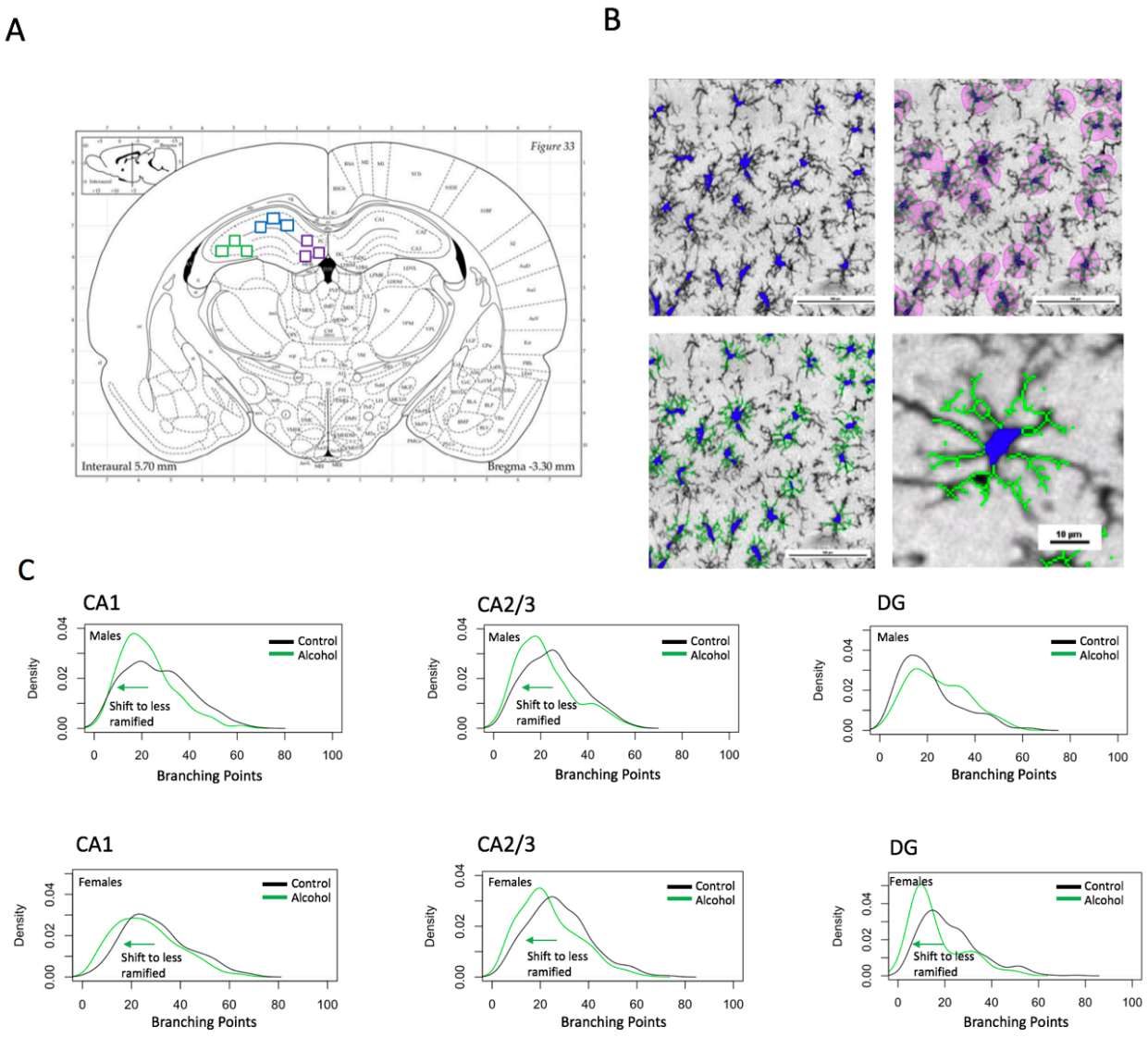

Figure 8. Quantification of ramifications for hippocampus using NIS-Elements Advanced Research software. A) 3 cropped images were taken for each of the following regions for analysis: DG, CA1, and CA2/3. Purple, blue, and green squares represent crops taken from the DG, CA1 and CA2/3, respectively. Using an automated analyses program (B), branching points per microglial cell were obtained for males and females. C) Using the automated analysis program in NIS-Elements AR, we found a shift in the microglia population to a state of having fewer ramifications per cell in the alcohol group in the CA1 and DG of females, and in the CA2/3 of both males and females. For the CA1, control males: n=6, alcohol males: n= 3, control females: n=4, alcohol females: n= 5. For the CA2/3, control males: n=6, alcohol males: n= 3, control females: n=5, alcohol females: n= 4. For the DG, control males: n=5, alcohol males: n= 3, control females: n=6, alcohol females: n= 4.

Microglia circularity measurement in the hippocampus

Circularity was another measurement that we collected from the automated analysis (**Figure 10**). These values were generated on a scale from 0 to 1. Values that were closer to 1 indicated a more circular phenotype. A measurement was provided for each cell that was counted in an image. No significant differences were found for circularity values in this study.

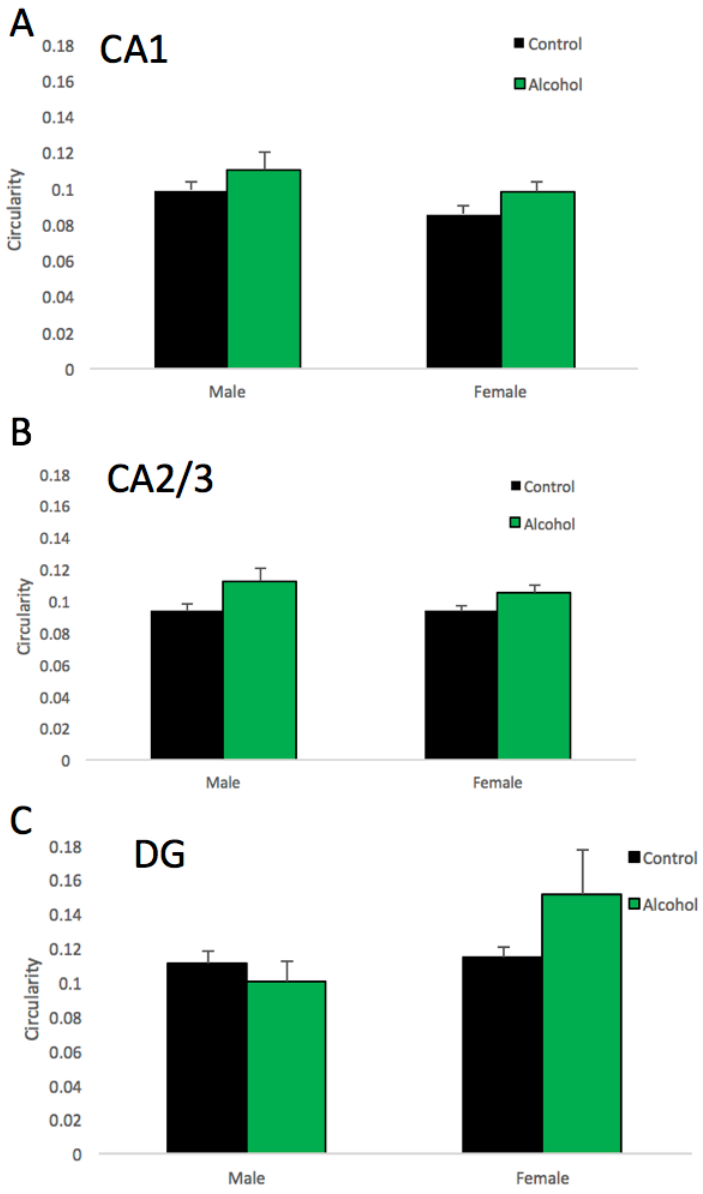

Figure 9. Microglia circularity phenotype as an indicator of activation in the hippocampus. Using NIS-Elements automated analyses, a 0-1 value was generated for each cell. A value closer to 1 indicating a more circular phenotype. This measurement of phenotype was calculated for A) the CA1 (control males: n=6, alcohol males: n= 3, control females: n=4, alcohol females: n= 5), B) the CA2/3 (control males: n=6, alcohol males: n= 3, control females: n=5, alcohol females: n= 4), and C) the DG (control males: n=5, alcohol males: n= 3, control females: n=6, alcohol females: n= 4) for male and female alcohol and control groups. No significant differences in circularity were found.

DISCUSSION

The present study's aim was to investigate the differences in the neuroinflammatory response, characterized by microglia activation, following exposure of adolescent voluntary alcohol drinking in rats. Based on previous findings of a greater loss of myelin in adolescent males after drinking, we hypothesized that adolescent males would have a higher vulnerability to alcohol, characterized by an increase in microglia and changes in morphology. Our findings show that voluntary alcohol drinking during adolescence does not result in microglia proliferation or recruitment in the mPFC. However, preliminary results show there were statistical differences found in the hippocampus in terms of microglia cell density. Interestingly, these differences were only found in males. In the CA1 and DG subregions, microglia density decreased, whereas in the CA2/3, the density increased. One potential explanation for this finding is that the CA2/3 is a more susceptible region to alcohol and microglia are being recruited from the CA1 and the DG to the CA2/3. The finding of increased microglia density in the CA2/3 alone supports a finding from a separate study that ethanol treatment increased toll-like receptor 4 (TLR4), a pattern recognition receptor, expression only in CA2/3 region and not in the CA1 or DG (Li et al., 2019). Another potential explanation for this is that during alcohol intake, microglia die off, and then during abstinence, they proliferate (Crews and Nixon, 2009). The differences in the timing of these events could support the increase in microglia density of the CA2/3 and a decrease in density in the CA1 and DG. These cell density difference findings only in males suggest there may be a sex difference in microglia recruitment in the hippocampus. However, inconsistent findings regarding cell density and a small sample size make forming an all-encompassing conclusion difficult.

We also found that there are changes in the morphology of microglia cells, suggesting that these cells are becoming activated, which is characterized by a decrease in branching points. Statistical analyses showed that the cells from the alcohol group and the control group were from different distributions in both males and females. In all regions and both sexes, except for the DG region in the male alcohol group, the alcohol group's microglia population shifted to a state of fewer ramifications, suggesting they are taking on an activated phenotype. The shift to a state of more ramifications in the DG in the male alcohol group could suggest that the microglia in this group and subregion are becoming "hyper-ramified", which is an intermediate activation state. From this, it can be reasoned that alcohol activates a neuroinflammatory response, regardless of sex, to some extent.

Additional activation characteristics were quantified by using circularity measurements in the hippocampus. We found no significant differences in this measurement in any group. The lack of consistent significant differences in microglial proliferation and similar differences in the decrease in branching between males and females suggest that there may be another mechanism behind the differential myelination of axons. To gain a better understanding of the mechanisms behind the greater extent of loss of myelination of axons in males, differential gene and protein expression should be investigated.

Limitations of the study should be noted. First, the z-stack microscopic imaging resulted in some blurry stitched images of the tissue, so some animals had to be excluded due to poor image quality. This led to decreased sample size of each group and resulted in unequal group sizes. Second, creating an automated analysis program to be near perfect was a challenge because of the variable image quality of the hippocampus regions. Some of the images from each section of the hippocampus were excluded from analyses because the automated program did not

accurately capture the qualities of the microglial cells. After running the analysis program and exporting the data, the images for the hippocampus were given a classification code of 1, 2, or 3, indicating if the analysis program was accurately detecting the cells “good”, “okay” or “poorly”. Using an ANOVA and then a pairwise t-test with a Bonferroni correction method, we found that images coded as 1 were not statistically different from 2. However, images classified as 3 were statistically different from images classified as 1 or 2. Images classified as 3 were excluded from the cell count, ramification, and circularity analyses. In some cases, all three crops from a region of one animal were excluded and in other cases, only 1 or 2 crops from a region of one animal were excluded. This resulted in an even smaller sample size for each group. One potential contributing factor to the poor image quality is that the file size of these images was too big to fit into memory and could not be opened as is. Images had to be downsampled, which allowed opening the image in smaller resolution. Third, I was unable to receive the drinking data to perform analyses on binge number versus microglia cell density in the hippocampus, so this report is lacking correlational analysis on binge number and microglia density.

Future research should further investigate the roles of other inflammatory mediators, such as toll-like receptors and cytokines, as well as gonadal hormones, which may serve as a protective factor in females in order to better understand the differences in vulnerability. Microglia are just one part of the neuroinflammatory response pathway. It would be important to investigate differential expression of other genes and proteins that are later involved in this cascade. Conducting a study where microglia are wiped out could also provide insight as to whether they play a role in the loss of myelin of axons. Gonadal hormones may also play a role in the sex differences in the response to alcohol-induced neuroinflammation. It would be interesting to see the current study replicated with a greater sample size and microscopic imaging

of a higher quality. Additionally, responses to alcohol drinking has also been shown to implicate astrocytes and considering their involvement could provide more insight into the mechanisms behind neuroinflammatory responses. Lastly, it is important to consider whether this neuroinflammatory response is mediating repair or damage.

CONCLUSION

Previous studies have shown that there are sex differences in the microstructural changes that occur in response to alcohol exposure. Specifically, we have previously found evidence for a greater decrease in myelinated fiber density of axons in adolescent males than females. The current study investigates the effects of alcohol consumption on microglia activation, measured by changes in proliferation and morphology, during adolescence in males and females. When microglia become activated, they proliferate, their ramifications become retracted, and they take on a more circular shape. We investigated microglia activation within the medial prefrontal cortex as well as three regions within the hippocampus because of these regions' susceptibility to changes from alcohol. There were no significant differences found in microglia recruitment in the mPFC, but there were differences in cell density in the hippocampus, specifically in males. Additionally, there was a fairly consistent finding of a shift in the distribution of cells towards a state of fewer ramifications in both the hippocampus and the mPFC in both males and females. These findings suggest that microglia activation occurs to a similar extent in both males and females in the mPFC. We cannot confidently make a conclusion to support our hypothesis of greater microglia activation in males exposed to alcohol because of the small sample size and inconsistency of our preliminary results for the hippocampus. Future research could investigate differential gene and protein expression in the neuroinflammatory cascade as well as how hormones may potentially play a protective role in the loss of myelin.

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