Do Circulating Sex Hormones Influence Neuronal Susceptibility to Binge Alcohol?

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To my grandmother, Cynthia McCandless, who is dearly missed but was always my biggest support.

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I wish to offer my thanks to my friends, family, lab members, and my committee who helped and supported me throughout this project.

ABSTRACT

Circulating sex-hormones influence various functions throughout the body, including neuroprotection and neurodegeneration across types of brain injury. Limited evidence exists to suggest whether sex hormones influence neuronal susceptibility to binge alcohol induced brain damage too. Stimulation of the stress response, microglial activation, and loss of neurons in the hippocampus are known outcomes of alcohol exposure. Alcohol injury is a stressor that results in neuroimmune activation and stimulates the hypothalamic-pituitary-adrenal-axis (HPA-axis) responsible for the body's stress response. Sex hormones organize the HPA-axis during development, creating sex-based responses to stress, and are highly influential for activation of this axis throughout adulthood. Females produce a more robust response of cortisol (CORT) from the HPA than males under stressful conditions, this may be linked to increased risk of alcohol injury. Existing studies of gonad- (GDX) and ovariectomy (OVX) at adulthood report alterations in CORT secretion profiles, switching to patterns similar to the opposite intact sex. The removal of circulating estrogens may afford females protection from binge damage but males may lose their testosterone buffer, raising their response to alcohol. To study the relationship between circulating sex hormones and binge-induced brain damage, we performed GDX and OVX in adult rats. Quantification of neuron loss, neuroimmune response, and CORT levels were used to observe the influence of circulating sex hormones on binge alcohol-induced neuronal damage in males and females. We hypothesized binge exposure would produce greater neuron loss, more neuroimmune activation, and higher CORT levels in all binge groups compared to controls. We expected OVX females and intact males

to show similar damage profiles, and that GDX males and intact females would show similar damage profiles. Specifically, we expected higher CORT secretion in GDX males and intact females, compared to OVX females and intact males, accompanied by greater decrement in neurons and an increased neuroimmune response. Examination of the impact of removing circulating sex hormones did not support a role for circulating sex hormones in modulating the HPA-axis and CORT output. Binge exposure led to granule neuron loss in the hippocampus but no effect of removing circulating sex hormones was found in males or females. No effect of binge exposure or circulating sex hormones was found for the neuroimmune response in the hippocampus for either sex, but binge exposure and sex hormones influenced outcomes in the mPFC for males. Neuroimmune response, quantified by number of total, ramified, and activated microglia, was increased in the mPFC from binge exposure in sham males. GDX males showed a decrease in overall neuroimmune response, characterized by loss of ramified microglia, with exposure to binge alcohol. Our findings support the loss of granule neurons from binge exposure and an influence of circulating sex hormones on the mediation of the neuroimmune response to binge alcohol in males but not females.

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Introduction

Sex Hormones

Sex hormones, androgens and estrogens, have differential developmental and functional roles in males and females (Hammes & Levin, 2019). Androgens are known as "male" hormones, with testosterone being the most abundant in circulation, but other androgens include androstenedione, dehydroeiandrosterone (DHEA), DHEA-sulfate, and dihydrotestosterone (DHT) (McEwan & Brinkmann, 2021). Estrogens are typically considered "female" hormones and consist of three types: estrone (E1), estradiol (E2), and estriol (E3). Estradiol is the primary form of estrogen in the body during reproductive years, while estriol takes prevalence during pregnancy in women and estrone after menopause (Hamilton et al., 2017). Testosterone production in the body is found in the male testes, adult female ovaries, as well as the adrenal glands and small levels in other bodily tissues (Naamneh Elzenaty et al., 2022). Estrogen is synthesized in the ovaries of females and the testes of males, though a large portion of male estrogen is converted from testosterone production in the body via the action of aromatase. This is an adrenal enzyme that converts androgens to estrogens (Bakker, 2013). Despite being simplified to "male" or "female" hormones, these sex hormones are found in both sexes and play an integral part in complex biological actions. These hormones are necessary for sexual development and reproduction but have influence all over the body, modulating various functions (C. Chen et al., 2019; Naamneh Elzenaty et al., 2022). Sex hormones are evidenced to modulate the immune system (Bhatia et al., 2014), physical performance (Collado-Boira et al., 2021), the body's response to stressors (Goel et al.,

2014), and functionality in the developing and adult brain (Marrocco & McEwen, 2016).

Sex Hormones and Neuroprotection

As mentioned, sex hormones have numerous functions and studies have reported sex hormones as being neuroprotective in various brain injuries. Sex-hormones were shown to reduce edema after traumatic brain injury (Moulaei et al., 2008; Roof et al., 1992), and pharmacological dosages of estrogen and progesterone appear to be useful for reducing brain edema, treating elevated intercranial pressure, and reducing cerebral perfusion pressure (Shahrokhi et al., 2010). In some studies, females were reported to have less susceptibility to postischemic and posttraumatic brain injury than men and that this is likely due to circulating estrogens and progestins (Roof & Hall, 2000). Further, neurodegenerative diseases such as Parkinson's are more prevalent in men (Gillies et al., 2014; Zárate et al., 2017). On the other hand, evidence suggests women are more vulnerable when it comes to neuroinflammatory disorders, as seen in chronic pain and neuroimmune diseases, with women having higher rates of migraines (Buse et al., 2013), multiple sclerosis (Harbo et al., 2013), and Alzheimer's disease (Andersen et al., 1999). New research focusing on stroke brings to light that individual sex-hormones alone may not be responsible for risk or protection from brain injury. Rather, that the interplay between pituitary hormones that act on the gonads known as gonadotropins, sex-hormones, and the ratio of estrogen to testosterone may contribute (Sohrabji et al., 2019). Age related bodily changes and alterations in sex-hormone levels seem to play an important role in the mediation of brain injury too, as observed in studies of early and late menopausal women (Gleason

et al., 2015; Hodis et al., 2016; Miller et al., 2009). While the existence of sex-based vulnerability to brain injuries exists, the complexities of sex-hormone mediation across these various types of injury are still under investigation. Another type of brain injury where sex-hormones may have influence is in alcohol exposure. It is possible that sex hormones have a role in sex-dependent neural effects of alcohol too.

Binge-alcohol Induced Brain Damage & Sex-based Susceptibility to Alcohol Insult

Binge drinking, defined as a pattern of drinking that elevates blood alcohol concentration to 0.08 g/dl or above, has been linked to illness and dysfunction in the body (*Understanding Binge Drinking | National Institute on Alcohol Abuse and Alcoholism (NIAAA), 2021*). This pattern of intermittent but heavy consumption leads to pancreatic injury (Ren et al., 2016), cardiac abnormalities (Ai et al., 2020), and liver damage (Lamas-Paz et al., 2018) in animal models. Beyond the body, binge exposure takes a toll on the brain. It is tied to increased risk for dementia and stroke, along with decrease in cognitive function (Järvenpää et al., 2005; Ledesma et al., 2021; Sundell et al., 2008). Binge ethanol exposure causes cell death in the olfactory bulb (Obernier et al., 2002) and degeneration of neurons in the dentate gyrus of the hippocampus in rodents (West et al., 2019). In addition to neurodegeneration, binge ethanol produces an increase in neuroinflammation-linked enzymes throughout the brain and stimulates the neuroimmune system (Tajuddin et al., 2014). While it is established that binge ethanol exposure causes damage to the brain, understanding of what influence circulating sex hormones have on this damage is limited. The existing literature investigating the relationship between sex hormones and alcohol revolves around chronic and binge exposure, with a greater emphasis on chronic consumption.

A number of studies provide evidence for disparities in the degree of brain damage from alcohol exposure in men and women. Women are more susceptible to alcohol-induced brain injury under certain conditions, but the mechanisms driving that susceptibility are unclear. Early research comparing brain shrinkage in chronic heavy drinkers as a marker for neuronal damage, found significantly larger amounts of cerebrospinal fluid than in controls, indicating a loss of neural tissue (Jacobson, 1986; Mann et al., 1992). Even though women in the study reported to have had half the number of years of excessive drinking. Similarly, Nixon and colleagues (1995) found the same magnitude of cognitive impairment in alcoholic men and women; again, the women displaying heavy drinking behavior for fewer years. This accelerated progression of neurodegeneration and cognitive impairment, known as "telescoping", has not been consistently observed. Sullivan and colleagues (2010) studied brain volume in men and women with alcohol dependence and found no evidence to suggest sex differences and "telescoping" in women (Sullivan et al., 2010).

We have shown, in a 4-day binge exposure model in rats, that high blood ethanol concentrations lead to significant cell loss in the dentate gyrus of the hippocampus in female rats, but not males (Leasure & Nixon, 2010; Maynard et al., 2018; Maynard & Leasure, 2013). However, in our most recent paper we compared the effects of 3 or 8 weeks of once weekly binge exposure on hippocampal cell loss, astrogliosis, and neuroimmune activation. In both sexes, binge-induced cell loss and microglial morphology showed similar outcomes of binge-induced damage (West et al., 2021). These results are inconsistent with previous findings that sex-based susceptibility to alcohol induced neuronal damage exists but support studies showing

no telescoping. We've speculated that the binge exposure being infrequent and heavy, potential lack of physiological adaptation to the alcohol, and other factors may account for the disparity (Cortez et al., 2020). Circulating sex hormones seem to mediate neuroprotection to alcohol insult, though additional mechanisms beyond the hormones alone likely influence positive or negative outcomes. We believe that studying the effects of circulating sex-hormones on mechanisms known to influence binge alcohol induced neuronal damage may help to better understand the role sex-hormones have in brain injury.

Mechanisms of Binge Induced Damage - HPA-axis, Microglia, & Alcohol

Alcohol stimulates the Hypothalamic-Pituitary-Adrenal-Axis (HPA-axis) responsible for the body's stress response; an axis linked to neuroinflammation upon disruption of its homeostatic functions (Ahmad et al., 2021; Tapp et al., 2019). The HPA-axis functions to allow appropriate responses to stressful stimuli, through the paraventricular nucleus (PVN) of the hypothalamus, the anterior pituitary gland, and the adrenal gland. When a threat is detected, the PVN secretes corticotropin-releasing-hormone (CRH), which in turn stimulates release of adrenocorticotropic hormone (ACTH) from the pituitary. ACTH then signals for the adrenal cortex to release and circulate glucocorticoids (CORT); cortisol in humans and corticosterone in mice and rats. This axis works on feedforward and feedback loops, to maintain homeostasis when a detected threat has passed (Oyola & Handa, 2017; Rivier & Vale, 1985; Ulrich-Lai & Herman, 2009).

Alcohol is a physiological stressor that elicits a heightened HPA response. Results of a 4-day binge exposure study found CORT levels to be additive to alcohol

induced neurodegeneration. Ethanol-incited neurotoxicity in the dentate gyrus (DG) of the hippocampus increased progressively with higher levels of CORT (Cippitelli et al., 2014). Another study confirmed ethanol's ability to stimulate neuron activity in the PVN, leading to CRH activation (Rivier & Lee, 1996). Chronic heavy alcohol consumption, including chronic binge exposure, presents blunted HPA activity after repeated exposure (King et al., 2006; Przybycien-Szymanska et al., 2011; Richardson et al., 2008; Zhou et al., 2000). Further, alcohol may stimulate the relationship between the HPA-axis and neuroimmune reactions. Under healthy conditions, the HPA-axis and immune processes of the body work to maintain homeostasis but alcohol exposure seems to dysregulate this relationship, primarily through cytokine production (Bateman et al., 1989; Haorah et al., 2008; Mandrekar et al., 2009; Rachdaoui & Sarkar, 2017). Pro-inflammatory cytokines are linked to this dysregulation and are stimulants of microglia, neuroimmune cells found to be activated across neuroinflammtory disorders, including in the presence of alcohol (Acaz-Fonseca et al., 2015; Leng & Edison, 2021; West et al., 2021).

Under normal conditions microglial cells maintain a "surveillance" mode, sometimes referred to as "resting" or ramified and hyper-ramified morphology, monitoring the brain to respond to immune needs (Augusto-Oliveira et al., 2019; F. Crews & Vetreno, 2015). These ramified cells are characterized by small cell bodies and long processes with branching. When a change in the environment occurs microglia multiply and move to an activated, or primed, state when responding to neurodegeneration and neuroimmune activation (Perry & Holmes, 2014). These activated morphologies are characterized by larger, darker cell bodies and thicker,

bushier processes, or rounded macrophage-like form with no or few processes (*see Figure 3 in Results section*) (F. Crews & Vetreno, 2015). This activation is interesting, as microglia can transition into two phenotypes: M1, known to be proinflammatory and neurotoxic, or M2, exhibiting anti-inflammatory properties that are neuroprotective (Guo et al., 2022). This priming poises microglia to respond to a secondary inflammatory stimulus, resulting in excessive release of neurotoxic or neuroprotective mediators (Field et al., 2010; Perry & Holmes, 2014). It is important to note that microglial activation can also shift between M1 and M2 categorizations, and the role microglia play in neuroimmune activation and neuroinflammation can be hard to decipher. We have evidenced priming of microglia from binge alcohol, seeing an activated morphology and higher quantity of microglia in females from a 4-day binge (Barton et al., 2017), but seeing an increase in number of activated and total microglia, in both sexes, from 8 weeks of once weekly binge (West et al., 2021). Evidence supports alcohol's impact on HPA activation, as well as how HPA dysregulation contributes to neuroimmune and microglial activation. We believe that interplay between the HPA-axis and circulating sex-hormones may be instrumental in alcohol-induced neuronal damage.

Gonadectomy & Ovariectomy

A common technique implemented to examine the relationship between circulating estrogens, androgens, and HPA activity are gonadectomies – removal of the gonads. Gonadectomized (GDX) male rats have increased levels of CORT and ACTH in response to stress but androgen replacement is effective at reducing the response (Handa et al., 1994). Others have shown ovariectomy (OVX) to reduce HPA

activity while supplemental estradiol can in turn increase levels (Lund et al., 2004). An additional study looking at GDX and OVX versus sham in rats following both noise and immune-mediated stress showed castrated males and sham females to have significantly higher CORT secretion than sham males and OVX females. Further, the ovariectomies reduced CORT levels to those comparable to sham males (Seale et al., 2004). Of note, some studies have found no stimulatory effect of estradiol (Babb et al., 2013; Young et al., 2001).

Sex-Specific HPA-axis Activity

Gonadal hormones are highly influential for sex-based responses to stressors. The general consensus is that androgens, mainly testosterone, are depressants of the stress response (Viau & Meaney, 1996). Conversely, estradiol is not as clear cut and can inhibit or enhance the response (Handa et al., 2009; Zuloaga et al., 2014). For example, CRH from the PVN following stress exposure gives off a larger ACTH response in females compared to males (Le Mevel et al., 1979). CORT levels upon stress increase more and are maintained at high levels for longer durations in female rats (Figueiredo et al., 2002). On the other hand, a few studies have shown a decrease in HPA-axis activity, or no effect, from estradiol (Ochedalski et al., 2007; Young et al., 2001). Some evidence suggests that estradiol could have a modulatory effect on CRH primarily when there is a high stress response (Goel et al., 2014). Potential explanations for discrepancies in estradiol's effects could be due to differences in dose and/or duration of the stressor, or variation in signaling from different estrogen transcription factors (ERs) in the brain (Tsigos & Chrousos, 2002). Another suggestion is that females may have a built-in buffer from their high glucocorticoids

by their high circulating levels of corticosteroid binding globulin, which functions to limit CORT from binding to its receptors; mineralocorticoid (MR) and glucocorticoid (GR) receptors (Henley & Lightman, 2011; McCormick et al., 2002). MR and GR receptors have differing affinity for CORT, MRs mediated by a high affinity and GRs with low. Because of this, MRs generally bind at basal levels of glucocorticoids, and GRs under stress conditions (Reul et al., 1987). However, pathologies have been linked to atypical activity of these receptors, such as overactivation of GR increasing risk for mood disorders or low MR activity associated with depression (Pereira et al., 2010; ter Heegde et al., 2015). Interestingly, female rats have been found to have fewer MR and GR receptors in the pituitary gland than males, potentially establishing sex-based differences in glucocorticoid negative feedback during the stress response (Bangasser, 2013; Turner, 1990). Further research is warranted to understand sex-specific HPA-axis activity and how the relationship affects neuronal outcomes of binge exposure.

Hypothalamo-Pituitary-Gonadal-Axis & Alcohol

Circulating sex-hormones are also linked to elements of the HPA-axis through the hypothalamo-pituitary-gonadal-axis (HPG), which functions to release gonadal hormones by stimulating gonadotropin-releasing hormone (GnRH) from the hypothalamus, which in turn leads to the release of luteinizing hormone (LH) and follicle-stimulating hormone (FSH) from the pituitary gonadotropes. Finally, at the gonads, production and release of sex steroids occur – testosterone, estradiol, progesterone (Acevedo-Rodriguez et al., 2018). Gonadal hormones released from the HPG modulate HPA function (Oyola & Handa, 2017). These hormones act on

corticotropin-releasing hormone (CRH) neurons that are responsible for triggering the cascade of HPA activity during a stress response (Ferguson et al., 2008). Estrogen (ER) and androgen (AR) receptors are differentially expressed in CRH neurons of the PVN and may be involved in sex hormone mediated HPA response (Oyola & Handa, 2017). Acute alcohol exposure has been linked to activation of the HPG in human and animal studies, further supporting an interaction of stress and sex-hormones in the neurodegenerative properties of alcohol (Blaine et al., 2016; O'Dell et al., 2004; Porcu et al., 2004). Testosterone, progesterone, and deoxy-corticosterone, a hormone relevant for stress-related changes in the body, are all released into systemic circulation by the HPG in the presence of acute alcohol (Blaine et al., 2016; *Deoxycorticosterone - an Overview | ScienceDirect Topics*, n.d.; Rupprecht, 2003). Dissecting out how sex and stress hormones interact during binge alcohol exposure may provide a more comprehensive understanding of the mechanisms leading to binge-induced damage.

Organizing Effects of Sex-Hormones on HPA-axis

These sex differences are thought to be programmed by organizational effects of sex-hormones on the neural circuitry of the HPA-axis while the brain is developing (Seale et al., 2005). During development, males are exposed to testosterone release in late gestation and again soon after birth (Weisz & Ward, 1980). It is believed that these surges of testosterone underlie the dampened stress response seen in males and the lack of these surges sets females up for a more reactive HPA response. It is important to note that while testosterone surges are released in males, it is the aromatization of testosterone to estradiol that then masculinizes the male brain. In

females, an important protein known as alpha-fetoprotein circulates and binds any estrogen in circulation to prevent it reaching the brain. Prenatally, females have low circulating levels of testosterone and therefore aromatization does not occur (Gabor, 2018; Nelson, 2000). Gonadectomy soon after birth is reported to produce female-like HPA activity into adulthood (Patchev et al., 1995) and inhibition of the aromatase enzyme that converts testosterone to estradiol in males has similar effects, suggesting the organizational effect these hormones have early in life (Lucion et al., 1996). Treatment with testosterone in females immediately after birth imposes a male-like pattern of CORT secretion (Seale et al., 2005). Testosterone is implicated in having an inhibiting effect on the HPA-axis pre- and early postnatally, which may be important for understanding sex-based susceptibility to neural damage from alcohol under certain circumstances. Study of the interaction between inflammatory cytokines and the HPA have found testosterone to be suppressive, inhibiting the HPA's stress response (Papadopoulos & Wardlaw, 2000). Induction of hypogonadal conditions in men exhibits ramped up basal and peak levels of CORT throughout the day, whereas normal levels of circulating testosterone showed significantly lower CORT levels in men (Rubinow et al., 2005). Testosterone may present a buffer, minimizing secretion of CORT following alcohol exposure. Conversely, activational effects of circulating estrogen in females or the absence of testosterone in males, may lead to increased immune activation and damage in the brain.

Proposal

While we know there is mediation of circulating sex-hormones across types of brain injury, we do not know if they make the brain differentially susceptible to alcohol damage in males and females. We speculate that the interplay between activational effects of circulating sex hormones and CORT derived from the HPA-axis could underlie sex-dependent effects on neuronal damage from alcohol. More particularly, that removal of circulating sex-hormones could potentially shift CORT secretion to patterns of the opposite intact sex, ultimately elucidating if circulating sex hormones influence vulnerability to damage due to alcohol.

The purpose of this study was to investigate if the removal of circulating sex-hormones has an impact on neurodegeneration and neuroimmune activation following exposure to binge alcohol. We will explore this impact by observing the relationship between sex-hormones and the HPA-axis, as a mechanism evidenced to be implicated in neuronal damage and immune activation following binge exposure. This research will help to disentangle questions around if circulating sex-hormones are responsible for sex-based discrepancies in binge-induced damage, or if they play a role in differentially altering HPA-axis function and CORT levels, ultimately leading to alternate profiles of neuronal damage. Further, we assessed if the absence of circulating sex-hormones would produce neuronal damage and neuroimmune profiles that differ from control animals. Neuronal damage was assessed by quantifying neurodegeneration (counting remaining neurons in the dentate gyrus of the hippocampus) and neuroimmune activation (microglial number and morphology, ramified vs. activated, in the hippocampus and medial prefrontal cortex - mPFC). We looked at the role of circulating sex-hormones on binge damage and neuroimmune

activation severity by performing gonad- and ovariectomies, in addition to sham surgeries, to compare binge and control groups. Finally, we quantified CORT levels to determine if there is an underlying relationship between the presence/absence of circulating sex hormones and the secretion of CORT following binge exposure. Our hypotheses were as follows: Exposure to binge alcohol will cause neurodegeneration and neuroimmune activation. Testosterone provides males protection from neurodegeneration and neuroimmune activation of binge exposure and estrogen is a risk factor for females. And finally, that circulating sex hormones influence sex-specific HPA-activity and removal will lead to a shift away from typical male and female CORT levels. Specifically, that binge exposure will produce lower neuron counts in the hippocampus, a greater microglial neuroimmune response (characterized by levels of total and activated cells) in the hippocampus and mPFC, and elicit higher CORT levels in all binge groups in comparison to controls. The hippocampus and mPFC were selected as our lab has repeatedly shown them to be affected by alcohol exposure (West et al., 2018, 2019, 2021). Based on existing research of the relationship between GDX/OVX and CORT secretion, we expected to see differences in damage, neuroimmune response, and CORT levels between OVX binge and intact/sham females, as well as GDX binge, and intact/sham male binge groups. Specifically, that OVX binge animals will have similar CORT and damage profiles to intact males and GDX animals will have similar CORT secretion and damage profiles to intact females. While we expect greater levels of CORT and DG neuron loss, and more activated microglia due to binge exposure in general, we expect to see a shift in higher CORT secretion, greater decrement in DG neurons, and increased

neuroimmune activation in GDX males and intact females, compared to OVX females and intact/sham males.

Materials & Methods

Animals

Sixty-four male and female Long Evans rats were randomly assigned to one of 8 groups for a 5-week experimental protocol: OVX binge (8), OVX control (8), OVX Sham binge (8), OVX Sham control (8), GDX binge (8), GDX control (8), and GDX Sham binge (8), GDX Sham control (8). Breeding for all rats in the study was done in house. Rats were weaned at postnatal (PND) day 21, introduced to the intragastric gavage procedure several times prior to the start of the study, and approximately 63 days of age on the first experimental day. They were routinely handled before and throughout the study to keep them accustomed to experimenters. Rats from all groups were housed in groups of 2 or 3 per cage, per sex, on a reversed light cycle (9AM to 9PM) at 23°C. Gonad- and ovariectomies were performed bilaterally at PND 46 (OVX) and 53 (GDX) to bring them up to the age of sexual maturity (Fuochi et al., 2022) while ensuring time for recovery before starting the binge protocol at 9 weeks of age. 9 weeks of age at the start of binge exposure is consistent with our previously used binge time-frame. Euthanasia and tissue collection was done 24 hours following the final binge dose, for all binge and control animals. All experimental procedures followed the *Guide for the Care and Use of Laboratory Animals* of the National Institutes of Health. The study protocol was approved by the Institutional Animal Care and Use Committee at the University of Houston.

Ovariectomy & Gonadectomy

Bilateral gonad- and ovariectomies, and sham surgeries, were performed on PND 53 (GDX) and 46 (OVX), as mentioned above. Isoflurane was used to anesthetize the animals and slow-release buprenorphine (Ethiqua) was administered prior to the start of surgery. The surgical site was shaved, prepped with betadine and alcohol swabs, and covered with a plastic film to ensure a clean surgical area. All surgical instruments were autoclaved and sterile gloves were used during surgery. GDX were performed by making a small incision from the middle of the scrotum, testes were removed, and incision was closed with sutures. OVX were performed by making two incisions, one on either flank, incising through the muscle wall, gently pulling the ovary through the incision, placing a hemostat and suture between the ovary and uterus, and making a cut just below the ovary. The muscle layer was then sutured, followed by the skin. Following all surgeries, animals were monitored for any indication of pain, discomfort, or improper healing. We did not observe any cases of pain, discomfort, or improper healing. Sham animals followed the same prep and post-surgery check-up procedure and were anesthetized for the same duration as GDX/OVX animals. All animals were fed soy-free chow for the duration of the study, to ensure animals were not exposed to phytoestrogens from soy protein (N. M. Brown & Setchell, 2001). To verify successful removal circulating sex hormones, GDX males were paired with breeder females and behavior was observed for any display of sexual interest. Vaginal swabs were collected from OVX females for 5 days to confirm they were no longer cycling through the typical estrous cycle (Westwood, 2008). While OVX rats did show some remnants of cycling, their smears were characteristic of

those observed in other studies, with consistent leukocyte presence and inconsistent changes through the estrous cycle (Lafuente et al., 1994; MANDL, 1951).

Binge Ethanol Administration

Food, but not water, was removed from all cages 4 hours prior to ethanol administration and returned following experimental procedures. An EtOH dose of 5g/kg (37% ethanol w/v in nutritionally complete diet; vanilla Ensure Plus™) was administered with intragastric gavage, once every 7 days. Control animals received an isocaloric solution that matched the liquid volume of the EtOH animals. Blood was drawn between 60-90 minutes post-gavage, the optimal timeframe for peak intoxication using intragastric gavage (Crews et al. 2006; Kelly et al., 1987), from the saphenous vein of the hind leg. 26G needles and heparinized capillary tubes were used to transfer blood to clear Eppendorf tubes, free of anticoagulant, and allowed to clot at room temperature for 60 minutes before being centrifuged to obtain serum. Blood ethanol concentration (BEC) was then analyzed using 5ul of serum, in triplicates, run against an ethanol standard in a GM7 Analyzer (Analox, MA) as per Analox protocol.

Tissue Collection & Processing

Approximately twenty-four hours following binge dosage on the 5th week, the rats were injected with Fatal PLUS anesthetic. Upon adequate anesthetic administration, confirmed by checking for lack of muscle reflex with a paw pinch, intracardial perfusion was done with cold saline and 4% paraformaldehyde in 0.2 PBS (pH 7.2) until limbs became stiff. The brain of the rat was then carefully extracted, stored in 30% sucrose, and refrigerated. Brain tissue sectioning was done using a Cryostat (HM 525NX, Thermo Scientific, Kalamazoo, MI), at -20°C, in 50µm

intervals. After sectioning, tissue was stored at -20°C in cryoprotectant solution until immunohistochemistry (IHC). The standard IHC protocol used mirrors that used in our previous studies (West et al., 2018; West et al., 2021). Tissue was incubated at 4°C for 72 hours in primary antibodies to stain mature neurons in the dentate gyrus of the hippocampus (guinea pig anti-NeuN, EMD Millipore, Billerica, MA, #ABN90P; 1:1,000) and microglia in the hippocampus and mPFC (rabbit anti-Iba1, Wako USA, Richmond, VA, #019-19741;1:10,000). Biotinylated secondary antibody incubation followed, at room temperature for 24 hours (Jackson ImmunoResearch, West Grove, PA; 1:250). Upon completion of IHC, the tissue was mounted on gelatin-coated glass slides and allowed to dry, covered, in the vent hood overnight. All slides were counterstained in methyl-green, cleared in xylene, and cover slipped. While the tissue was sectioned at 50µm, the average section thickness after IHC, drying, and counterstain was 21.66 µm.

NeuN & Iba1 Quantification

Unbiased quantification of mature neurons and microglial morphology was completed using optical fractionator with automated stereology (StereoInvestigator, MicroBrightField, VT, USA), with the experimenter blinded to condition. For the hippocampus, every 6th section (300 µm apart) between Bregma -1.80 and -5.20 mm and for the mPFC, every sixth section (300 µm apart) between Bregma 3.7 and 1.7 mm (Paxinos and Watson, 2005) was analyzed. The optical fractionator was used to quantify NeuN+ cells in the DG (20 x 20 µm counting frame, 200 x 200 µm grid) and Iba1+ in the hippocampus (280 x 215 µm counting frame, 400 x 400 µm grid) and mPFC (140 x 140 µm counting frame, 400 x 400 µm grid). Estimated count of total

remaining mature neurons throughout the DG were quantified and microglial activation was visually categorized as ramified or partially activated, this was based on established morphology of darker cell bodies and thicker, bushier processes when microglia are partially primed. Average coefficient of error was based on Gunderson and affirmed at less than 0.10.

Corticosterone Quantification

Serum was collected (blood draw procedure follows that outlined in *Binge Ethanol Administration* section) from all rats 60-90 minutes following ethanol administration each week to determine any changes in corticosterone levels from repeated binge ethanol exposure and gonadectomies. Serum from week 1 and week 5 timepoints were analyzed with a 1:80 dilution using corticosterone ELISA kits according to the product instructions (Enzo Life Science; New York, USA) and compared within and between groups.

Experimental Timeline

Statistical Analysis

A priori sample size was determined to be 64 animals, 8 per group, using G-Power analysis. Effect size was calculated from partial n^2 of existing granule neuron data from our lab, α set to .05, power set to 0.9, number of groups to 8, and numerator *df* to 7. Assumptions of normality and homogeneity were not violated. Kolmogorov-Smirnov was used to assess normal distribution and sphericity was not assumed,

Geisser-Greenhouse correction was applied. Data was split by sex to assess each sex independently. Two-way ANOVAs were used to determine significant differences in DG neuron count, total microglial quantification, as well as ramified and activated microglial quantification in the mPFC and hippocampus. Mixed-model ANOVA was used to assess significance of CORT levels across groups; only binge-exposed animals were assessed with surgery or sham surgery as between subject factors and time (week 1 or 5) as within subject factors. One subject was removed from the sham OVX binge group of CORT analysis due to being an extreme outlier, greater than two standard deviations above the mean. Fisher's LSD was used for microglial comparisons to allow for individual assessment of pairwise comparisons within sham and surgery groups. GraphPad Prism (Graphpad Software, San Diego, CA) was used for all statistical analyses. Data are expressed as group mean ± standard error of the mean (SEM) in each graph.

Results

Corticosterone Quantification

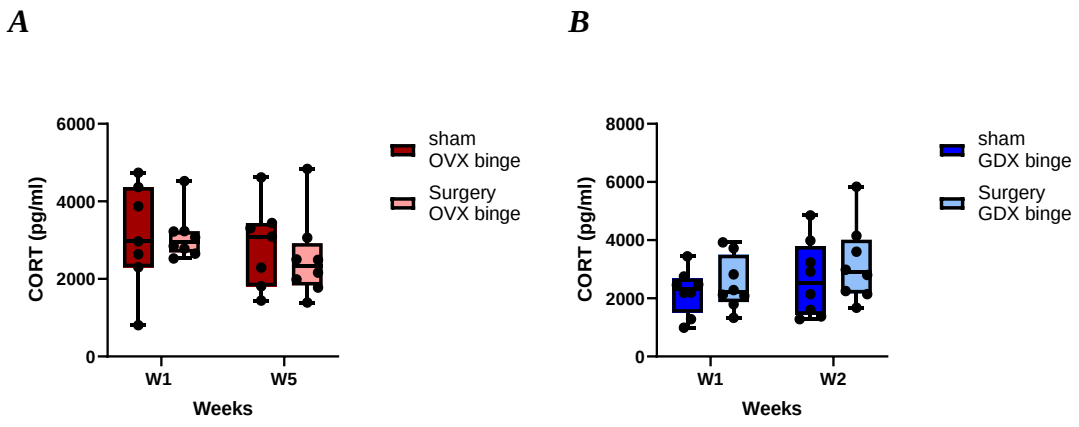


Figure 1. Corticosterone Levels Across Weeks of Binge Exposure. CORT quantification for female (A) and male (B) rats exposed to binge diets, on weeks 1 and 5. No statistical significance was observed.

Analysis of CORT levels for females at weeks 1 and 5 of the study showed no significant main effects of week [F (1, 13) = 1.346, p = 0.2669], surgery [F (1, 13) = 0.1521, p = 0.7028], or interactions [F (1, 13) = 0.2289, p = 0.6403]. CORT levels for males at weeks 1 and 5 of the study showed no significant main effects of week [F (1, 14) = 3.199, p = 0.0953], surgery [F (1, 14) = 0.7474, p = 0.4019], or interactions [F (1, 14) = 0.1242, p = 0.7297]. This suggests CORT levels are not altered by removal of circulating sex hormones, or altered across 5 weeks of repeated exposure.

NeuN+ Granule Cell Quantification

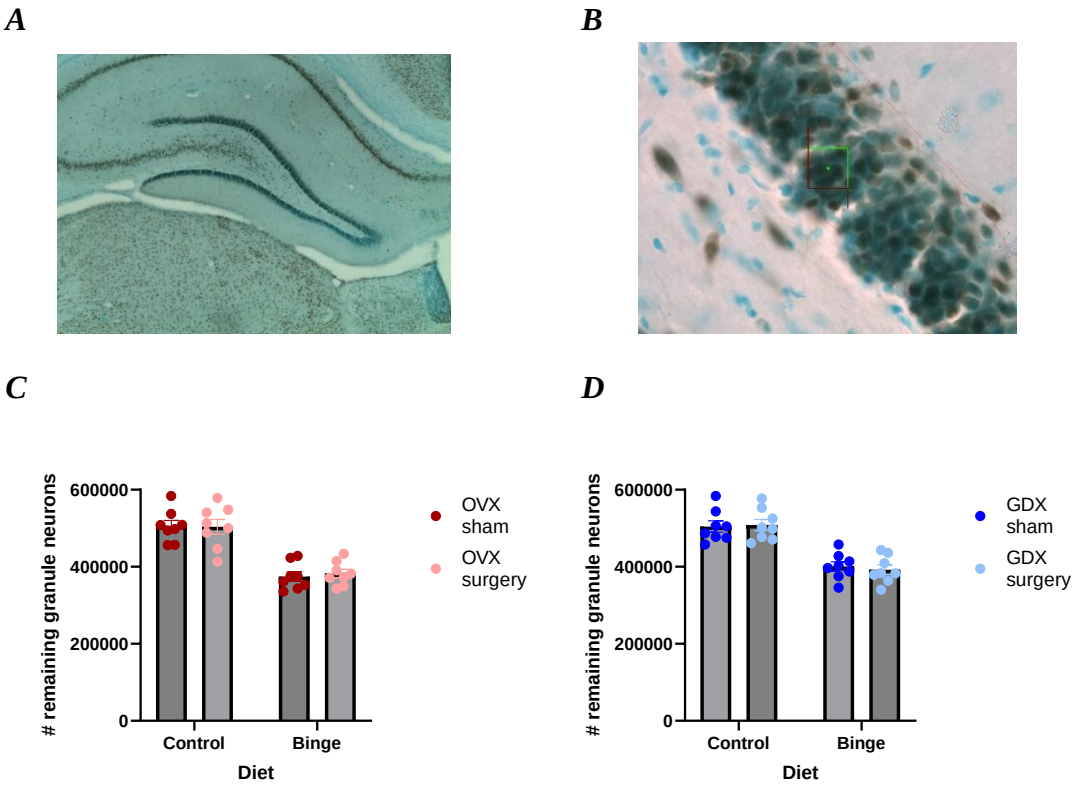
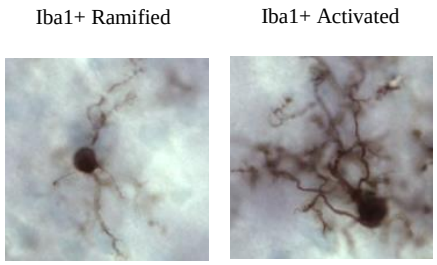


Figure 2. Granule Neuron Quantification in the Dentate Gyrus of the Hippocampus. Representative image of DG of hippocampus at 4x using stereology (A). NeuN + cells in the granule neuron layer of the hippocampal DG, with counting frame for stereology overlaid at 20x (B). A significant main effect of diet exists for females (C) and males (D), with all groups exposed to binge having fewer NeuN+ cells than control diet groups.

There was a statistically significant main effect of diet on the number of NeuN + cells in the dentate gyrus of the hippocampus for females [F (1, 28) = 74.24, $p < 0.0001$] and males [F (1, 28) = 66.84, $p < 0.0001$]. No significant main effects were found for surgery [F (1, 28) = 0.04540, $p = 0.8328$] or any interactions [F (1, 28) = 0.1082, $p = 0.7446$] for females. Males also showed no significant main effect of surgery [F (1, 28) = 0.03470, $p = 0.8536$] or interaction [F (1, 28) = 0.2218, $p = 0.6413$]. These data suggest that 5 weeks of once weekly binge exposure is sufficient to induce cell loss. Further, that removal of circulating gonadal hormones does not alter the damage profiles from that of intact animals.

Iba1+ Microglia Quantification in the mPFC

A



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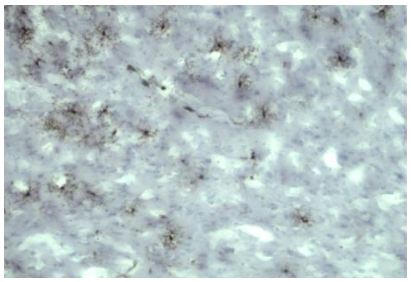

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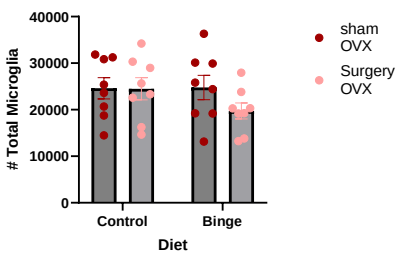

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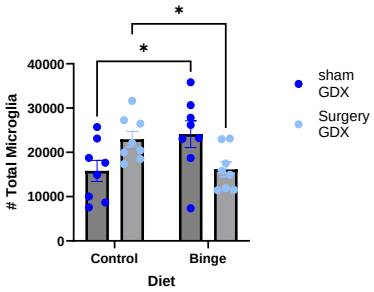


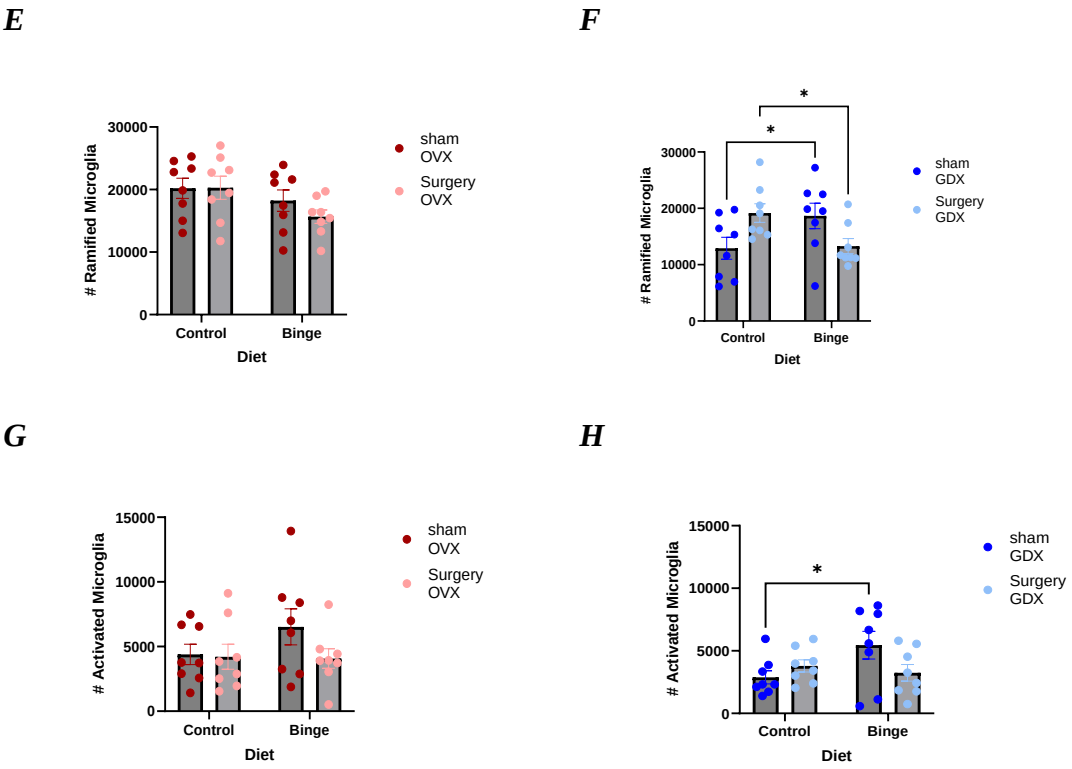


Figure 3. Microglia Quantification in the mPFC. Representative images of ramified and partially activated Iba1+ cells at 40x (A) and microglial density in the mPFC at 10x (B). No significant main effects were observed in females for total (C), or partially activated (G) Iba1+ cells in the mPFC. Sham males exposed to ethanol had an increased immune response with increased total (D) and activated (H) levels of microglia than control shams. Gonadectomized males showed a dramatic decrease in total microglia with binge exposure (D). GDX males showed no change in activated cells across diet conditions (H). Significantly lower numbers of ramified microglia were observed in ovariectomized females (E) and gonadectomized males (F) exposed to binge, while sham females and males showed increased levels with binge exposure.

Statistical analysis of the total number of Iba1+ cells in the mPFC of females showed no significant main effects of surgery [F (1, 28) = 1.279, p = 0.2677], diet [F (1, 28) = 1.021, p = 0.3209], or interactions [F (1, 28) = 1.174, p = 0.2878]. For males, an interaction of surgery x diet was found [F(1, 28) = 11.02, p = 0.0025] but no main effects of surgery [F (1, 28) = 0.03093, p = 0.8617] or diet [F (1, 28) = 0.1072, p = 0.7458]. Selected within group multiple comparisons of the surgery x diet interaction in males showed significant simple main effects for control sham GDX and binge

sham GDX and for control surgery GDX and binge surgery GDX (($p = 0.0155$) and $p = 0.0434$, respectively). Binge sham GDX showed an increase in total microglia count in comparison to sham controls, while binge surgery GDX showed a decrease in total microglia in comparison to surgery controls. These results suggest that exposure to binge resulted in an increased neuroimmune response in sham males but a decrease in neuroimmune response in surgery males, with no effect in females. Further, that removal of circulating sex-hormones had an inverse impact on total microglial number in surgery males exposed to binge in comparison to sham males exposed to binge. But, no effect of removing circulating sex hormones in females.

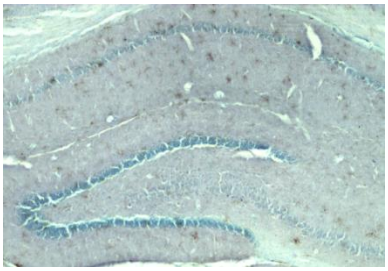
Analysis of ramified microglia in the mPFC of females produced a significant main effect of diet [$F (1, 28) = 4.326$, $p = 0.0468$], but no main effect of surgery [$F (1, 28) = 0.6170$, $p = 0.4388$], or interaction effect [$F (1, 28) = 0.6949$, $p = 0.4116$]. A significant decrease in ramified microglia was observed in the binge surgery females in comparison to the control surgery females. Males had a significant surgery x diet interaction [$F (1, 28) = 10.01$, $p = 0.0037$] but no main effects of surgery [$F (1, 28) = 0.05896$, $p = 0.8099$] or diet [$F (1, 28) = 0.001230$, $p = 0.9723$]. Within group comparisons for control sham GDX and binge sham GDX and control surgery GDX and binge surgery GDX were significant ($p = 0.0353$ and 0.0317 respectively). Binge sham GDX males had a significantly higher amount ramified cells than control sham GDX, while binge surgery GDX had significantly lower ramified cells than control surgery GDX. The findings support that binge exposure had an effect on the neuroimmune response in sham and surgery males, but only surgery females. The

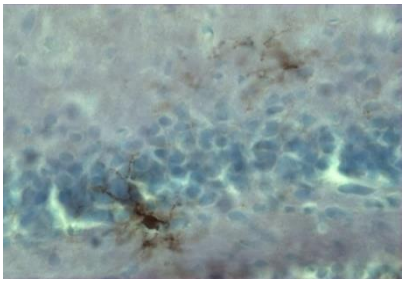
removal of circulating sex hormones induced a reduction of ramified microglia in surgery males, but an increase in ramified microglia in sham males.

Statistical results for the quantification of activated cells in the mPFC showed no significant main effect of surgery [F (1, 28) = 1.697, p = 0.2033], diet [F (1, 28) = 0.9804, p = 0.3306], or interaction [F (1, 28) = 1.256, p = 0.2719] in females. No significant main effects were observed for surgery [F (1, 28) = 0.7756, p = 0.3860] or diet [F (1, 28) = 1.870, p = 0.1824] in males, but a significant interaction [F (1, 28) = 4.512, p = 0.0426] was observed. Simple main effects in males showed significance for control sham GDX and binge sham GDX (p = 0.0199) but not for control surgery GDX and binge surgery GDX (p > 0.05). Sham males showed an increase in activated microglia with binge exposure, while surgery males did not. These results do not support an effect of removing circulating sex-hormones on the number of activated microglia within the mPFC.

Iba1+ Microglia Quantification in the Hippocampus

A ***B***





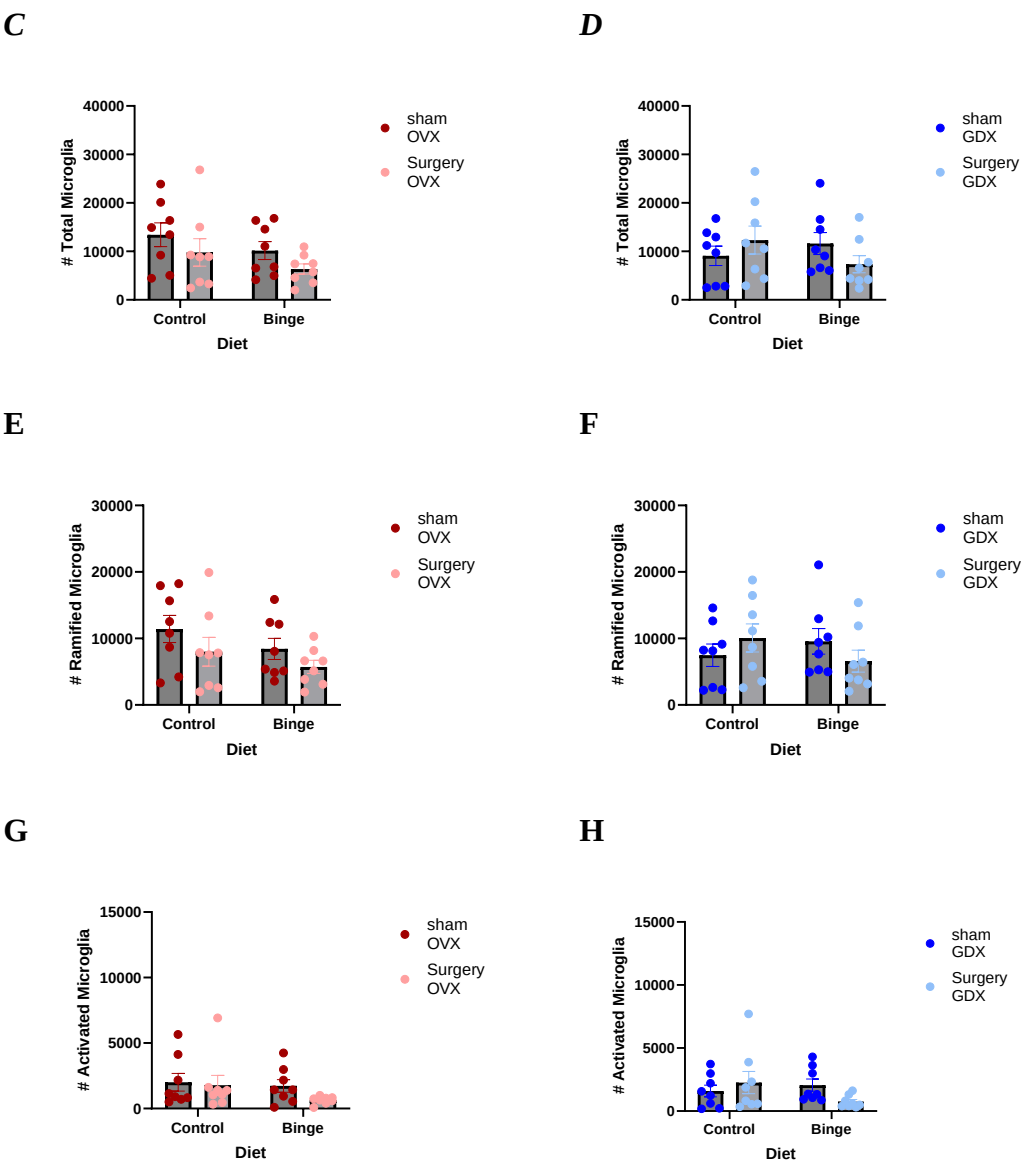


Figure 4. Microglia Quantification in the Hippocampus. Representative images of microglial density in the hippocampus at 4x (A) and 40x (B). No effects of diet or surgery were observed for total microglia in females (C) or males (D), ramified microglia in females (E) or males (F), or activated microglia for females (G) or males (H).

Analysis of total Iba1+ cells in the hippocampus showed no significant main effects in females for surgery [F (1, 28) = 2.983, p = 0.0952], diet [F (1, 28) = 2.414, p = 0.1315], or an interaction [F (1, 28) = 0.001284, p = 0.9717]. No significant main effect on total microglia quantity was found in males for surgery [F (1, 28) = 0.05311, p = 0.8194], diet [F (1, 28) = 0.2899, p = 0.5945], or an interaction [F (1, 28) = 2.746,

p = 0.1087]. Analysis of ramified cells showed no significant main effects of surgery [F (1, 28) = 3.037, p = 0.0924], diet [F (1,28) = 2.255, p = 0.1444], or interaction [F (1, 28) = 0.04086, p = 0.8413] in females and no significant main effect of surgery [F (1,28) = 0.01014, p = 0.9205], diet [F (1,28) = 0.1413, p = 0.7099], or interaction [F (1,28) = 2.249, p = 0.1449] in males. Quantification of activated microglia in the hippocampus did not show significant main effects in females for surgery [F (1, 28) = 1.386, p = 0.2490], diet [F (1, 28) = 1.603, p = 0.2159], or an interaction [F (1, 28) = 0.5957, p = 0.4467]. No significant main effects for surgery [F (1, 28) = 0.3333, p = 0.5683], diet [F (1, 28) = 0.8762, p = 0.3572], or an interaction [F (1, 28) = 3.125, p = 0.0880] was found for males. Collectively, these findings do not support an influence of binge exposure or an impact of circulating sex hormone removal on neuroimmune response in the hippocampus, in males or females.

Discussion

The influence of circulating sex hormones on binge alcohol induced brain injury has been little-studied. The present study investigated the role of circulating sex-hormones and the interplay of the HPA-axis, a mechanism of binge-induced damage, on neurodegeneration and neuroimmune activation following binge exposure in gonadectomized male and female rats. Corticosterone levels did not show significant differences across groups, or over time, for either sex. These data do not support our hypothesis that removal of circulating sex hormones, would affect CORT output and lead to a reversal of CORT levels from that of intact animals of the same sex. In fact, our data do not show a difference in levels between surgical conditions, or from the start to end of the binge exposure (Spencer, 1999). While we do not have

data from control animals, existing studies of serum CORT levels in rats show baseline levels at or below 2000 pg/ml and heightened levels from 3000-6000 pg/ml (Fernández Quezada et al., 2022). It is possible this stressor may not be strong enough across all animals or that some of the animals have a higher threshold and are more resilient to the stress of the binge exposure. Further, we collected serum 60-90 minutes post gavage, as this timeframe has been used to assess CORT levels following ethanol administration in other studies (F. T. Crews et al., 2006; Kelly et al., 1987). Given that CORT elevations are transient, a shorter time frame between ethanol administration and blood collection may have been more appropriate to see true peak levels. Another explanation is that we used intragastric gavage administration of the ethanol, rather than intraperitoneal injection that would allow for direct access to the blood stream and potentially a greater HPA response. Our findings do not support a direct connection between circulating sex hormones and CORT levels. It is possible that the HPA-axis is not a main mechanism responsible for binge-induced damage in the brain, that timing of blood collection is pivotal for an accurate representation of peak CORT, or that administration of the ethanol exposure may matter for CORT levels to have an influence on binge-induced damage in the brain.

As our lab has observed in previous 4-day and 8-week models of binge exposure, we did see a significant decrease in remaining granule neurons in the dentate gyrus of the hippocampus in animals exposed to binge diet. This effect was observed regardless of surgery condition, for each sex. These findings support that 5 weeks of once-weekly binge exposure is sufficient to induce cell loss in the hippocampus of male and female rats. Our results did not support an influence of

removing circulating sex hormones on the remaining neurons in the hippocampal dentate gyrus for either sex. As noted previously, a shorter timeframe between ethanol administration and blood collection may have shown increased CORT levels, but with the current data, it is not possible to point to CORT levels as being responsible for the decrement in neurons. Mechanisms that we did not directly test in this project may be responsible for imposing neurotoxic effects on the granule layer. Binge alcohol exposure has been found to activate the GSK3β protein responsible for regulating neuronal survival and neurogenesis and has been implicated in mediating binge alcohol-induced neuron apoptosis in the hippocampus (Ji et al., 2018). A widely accepted mechanism of neurotoxicity following alcohol exposure is deregulation of neurotransmitter release and synaptic function, believed to lead to imbalance of excitatory/inhibitory currents and eventually, neurotoxicity (F. T. Crews & Nixon, 2009). Exposure to chronic and binge-like alcohol has been shown to increase levels of pro-inflammatory cytokines and reactive oxygen species in the hippocampus, both of which have been linked to neuronal death (Mira et al., 2019).

Microglia play an interesting and complex role in the neuroimmune response to environmental perturbations. Morphological and phenotypical changes in these immune cells allow for diverse pro- and anti-inflammatory neuroimmune responses. While the scope of this study does not assess the phenotype or protein expression of microglia to determine if they are exhibiting neuroprotective or neurotoxic elements, we observed the overall neuroimmune response following our binge model. Our findings in the hippocampus did not support our hypothesis that we would see an increased neuroimmune response across binge groups, or that removal of circulating

sex hormones would have an impact on neuroimmune response. One possible explanation for the lack of binge response in the hippocampus may be the duration of binge exposure. In previous work from our lab, there was a binge induced neuroimmune response in the hippocampus after 8, but not 3, weeks of once weekly exposure (West et al., 2021). The 5 weeks of binge exposure in this model may not be sufficient to induce a robust neuroimmune response in the hippocampus. Regional heterogeneity of microglia has also been reported, with emphasis that microglia differ across brain regions, abundancies, and subtypes – these discrepancies may explain why we saw no effect of binge or sex hormone removal in the hippocampus, but found significant results in the mPFC (Tan et al., 2020).

Our findings within the mPFC show evidence that removal of circulating sex hormones influenced total microglial number, mainly through the number of ramified cells, in males but not females. Binge sham males showed an increased neuroimmune response with exposure, compared to control sham males. The gonadectomized males exhibited the opposite immune reaction, with a decrease in their total microglia quantity with binge exposure. Ramified microglia quantities followed the same pattern, with sham males having an increase when exposed to binge, but surgery animals having loss of ramified cells. This trend stayed true for sham animals for activated microglia but no difference in activated microglia were detected in surgery animals.

Though the existence of a heightened neuroimmune response is frequently proposed as a mechanism for alcohol-induced neuronal damage, deterioration and loss of microglia has recently been reported in some models of binge exposure, and the

loss has occurred in addition to the presence of activated cells (Grifasi et al., 2019; Hu et al., 2020; Marshall et al., 2020). We believe that loss of microglia is reflected here in the GDX males, as displayed by the decrease in ramified cells. Interestingly, these studies found loss of microglia across age groups of rats and mice, from adolescents to aged animals. These paradigms also used more aggressive models of ethanol exposure with 4 days of exposure every eight hours, or 14 days of intermittent exposure. This study used a more clinically-relevant model of binge-like exposure and we do not see this dampening of the neuroimmune response in sham animals who received the binge diet. We believe that the removal of circulating testosterone may be driving the loss of ramified microglia in males in this model, as it is only observed in surgery animals. These findings support the influence of circulating sex-hormones on the neuroimmune response to alcohol in males, but opens speculation as to whether the mechanism of this action is specific to testosterone itself, as it is the main male hormone produced in the gonads.

Evidence exists to support testosterone and estrogen as modulators of microglial activity. Developmental sex-based differences exist in microglial maturation and immune reactivity during pre and postnatal development timeframes, though there are conflicting reports of exactly what those specific differences are (Han et al., 2021; Hanamsagar et al., 2017). Testosterone and estradiol have independently been implicated in altering microglial morphology and proliferation throughout the brain, however testosterone's effects may be mediated by estradiol (Barreto et al., 2007; Villa et al., 2016). The aromatization of testosterone into estrogen may be the route of testosterone's influence on microglial response. The existence of estrogen

receptors (ER) on adult microglia is well established, although they do not express androgen receptors (AR) (C. M. Brown et al., 2007; Handa et al., 1994; Liu et al., 2020). Neuroprotective and neurotoxic effects of estrogens throughout the brain are influenced by the activation of three types of ERs: ERα, ERβ, and the membrane ER, GPER1. How these receptors contribute to neuroprotective versus neurotoxic neuroimmune responses is unclear, but research supports the connection between ERs and microglial function (Acosta-Martínez, 2020). It is possible that alterations to the expression and functions of these receptors, or the ratio of these receptors, due to alterations in estrogen levels may shape the neuroimmune response to binge alcohol exposure.

Given that estrogens are made in the testes or converted through aromatization of testosterone in males, gonadectomy and removal of a large portion of circulating testosterone in our study may have led to overall reduction of estrogen to influence microglia, or alternate ER function and expression than in sham animals. Ultimately, leading to an inability for microglia in the mPFC to regulate the neuroimmune response to ethanol appropriately. We may not have observed the same effect in females because estrogens are made in higher quantities throughout different tissues in females. Higher levels of neurosteroids and precursors to testosterone and estrogen, namely progesterone and DHEA, have been found throughout the female brain under basal conditions in comparison to males (Sze et al., 2018). These neurosteroids are steroids produced in the brain, independent from circulating sex hormones produced at the testes and ovaries (Fester & Rune, 2021). The higher neurosteroid levels may have provided a buffer to the microglial loss seen with binge exposure for females.

Interestingly, a site of the synthesis of estrogen and testosterone neurosteroids is neurons in the hippocampus, which could be another reason we did not see effects within the hippocampus as protection may have been provided due to that region being the site of hormone synthesis (Hojo & Kawato, 2018).

Another potential route of microglial mediation through testosterone removal in males is through AR expression throughout the brain, and the potential for that expression to alter microglial activity through cytokine production. A study of the relationship between AR levels, pro and anti-inflammatory factors, and microglia found AR expression to modulate microglial activation (M. Chen et al., 2021). Specifically, they found that increased AR expression throughout the brain increased microglial activation while a decrease in AR expression promoted a reduced neuroimmune response. Decreased levels of circulating testosterone in our surgery males may have resulted in decreased AR expression in the brain, and therefore a reduced microglial response brought on by AR mediated cytokine activity.

We acknowledge there are limitations to this experiment. We have previously reported binge effects to be cumulative, seeing greater neurodegeneration and neuroimmune activation in 8 weeks of once weekly binge, in comparison to 3 weeks (West et al., 2021). Longer exposure to this 1x per week model may have elicited a neuroimmune response in the hippocampus. Further, we did not collect blood to assess serum CORT in control animals. The addition of control data would have been valuable, allowing for comparison between control and binge animals for a better understanding of CORT trends across the experiment. Finally, the assessment of microglia in this study was limited to the quantification of total cells and the levels of

ramified versus activated cells. This method allowed for a high-level look at the overall neuroimmune response but did not afford the ability to decipher specific neuroprotective or neurotoxic effects. Future studies should aim to dive deeper into the relationship between circulating sex hormones and the HPA-axis, gathering a more complete picture of the alterations in HPA activity and how/where sex hormones may be exerting their effects. Quantifying levels of CRH and ACTH in addition to CORT, along with remaining levels of testosterone and estrogen, would allow for a more granular understanding of the ongoing activity. Moreover, we believe the role of ERs may be pivotal to understanding how circulating sex-hormones mediate damaging and/or neuroprotective effects throughout the brain, especially regarding microglia. Additional research should be done to not only further our knowledge of ERs and microglia, but to understand the mechanisms of microglia deterioration and loss from binge exposure, and to explore the potential actions of sex hormones on that loss.

Conclusions

This study observed the influence of removal of circulating sex-hormones on the neuronal susceptibility to binge alcohol exposure in male and female rats. Our findings support the loss of granule neurons in the hippocampus after 5 weeks of binge exposure, for both sexes. We found no evidence of HPA-axis involvement in this loss, as there were no differences in CORT levels across conditions. Microglial loss in the mPFC was observed in GDX males upon binge exposure. Comparison to sham males with the same binge exposure provides evidence that removal of circulating sex-hormones dampened the neuroimmune response to binge alcohol. Overall, the findings of this study support an influence of circulating sex hormones on the mediation of the

neuroimmune response to binge alcohol exposure in males but not females. We did not find evidence to conclude that circulating sex hormones influence CORT levels following binge or susceptibility to neuronal damage.

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