ABSTRACT

Sex Differences in Ethanol-Induced Inhibition of Hyperpolarization-Activated Current and Excitability of Amygdala Neurons After Traumatic Stress

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The basolateral amygdala (BLA) plays a vital role in integrating sensory information and establishing emotional salience of the environment. Ethanol potentiates GABAergic inhibition in BLA neurons, regulating excitatory transmission and playing an integral role in controlling anxiety-like behaviors. While the effect of ethanol on the amygdala and anxiety-like behaviors is well-established, sex differences in the effects of ethanol on BLA neurophysiology and the effect of ethanol on traumatic stress-induced changes to BLA activity are currently unknown. Understanding the sex-related factors that contribute to BLA neuron membrane properties in stressed animals may point to potential neural mechanisms associated with gender differences in resilience or susceptibility to stress and improve individualized treatment. Ethanol-mediated inhibition is stronger in BLA neurons from males than females and ethanol-mediated inhibition of hyperpolarization-activated, cyclic nucleotide gated current (I_h) is stronger in males, owing in part to the low amplitude of I_h in females in the absence of ethanol. In response to SPS, action potential firing in the BLA did not change between males and females. However, ethanol significantly decreased action potential firing in BLA neurons exposed

to SPS in females only, suggesting that the inhibitory effect of alcohol is greater in females than in males exposed to stress. Ethanol decreased I_h amplitude only in BLA neurons from males exposed to SPS, in part because I_h amplitude is larger in BLA neurons from males than it is in females. Since ethanol reduced BLA excitability only in males, these data suggest that ethanol may be more anxiolytic in males than in females. This difference may contribute to the sex differences in comorbid PTSD and alcohol abuse.

Sex Differences in Ethanol-Induced Inhibition of Hyperpolarization-Activated Current and Excitability of Amygdala Neurons after Traumatic Stress

by

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5-HT	5-Hydroxytryptamine Receptor
ACTH	Adrenocorticotropic Hormone
ANS	Autonomic Nervous System
AUD	Alcohol Use Disorder
AVP	Arginine Vasopressin
BA	Basal Amygdala
BLA	Basolateral Amygdala
BNST	Bed Nucleus of the Stria Terminalis
BOLD	Blood-Oxygen-Level Dependent
Ca^{2+}	Calcium
CeA	Central Amygdala
CORT	Cortisol
CS	Conditioned Stimulus
CRF	Corticotropin-Releasing Factor
DSM-V	Diagnostic and Statistical Manuel of Mental Disorders-V
E	Epinephrine
ERα	Estrogen Receptor-α
ERβ	Estrogen Receptor-β
EtOH	Ethanol
GABA	Gamma-Aminobutyric Acid

HPA	Hypothalamic-Pituitary-Adrenal Axis
I_h	Hyperpolarization-Activated, Cyclic Nucleotide Gated Current
IL	Infralimbic
K^+	Potassium
LA	Lateral Amygdala
LC	Locus Coeruleus
lITC	Lateral Intercalated Cells
LTP	Long-Term Potentiation
MeA	Medial Amygdala
mITC	Medial Intercalate Cells
mPFC	Medial Prefrontal Cortex
NA^+	Sodium
NAcc	Nucleus Accumbens
NMDA	N-Methyl-D-Aspartate Receptor
NE	Norepinephrine
OT	Oxytocin
PFC	Prefrontal Cortex
PL	Prelimbic
PV	Parvalbumin
PTSD	Post-Traumatic Stress Disorder
PVN	Paraventricular Nucleus of the Hypothalamus
rACC	Rostral Anterior Cingulate Cortex
SOM	Somatostatin

SPS	Single Prolonged Stress
USC	Unstressed Control
VGCC	Voltage Gated Calcium Channels
VTA	Ventral Tegmental Area

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DEDICATION

To my family: Benito, Maria and Gabriel Ornelas.

Thank you for always believing in me and having faith that through God,

all things are possible.

CHAPTER ONE

Introduction and Background

Post-Traumatic Stress Disorder

Post-traumatic stress disorder (PTSD) is an anxiety, trauma-and stressor-related disorder that manifests after exposure to potentially life-threatening events such as sexual assault, natural disasters, combat exposure and life-threatening events (DiMauro, Carter, Folk, & Kashdan., 2014). PTSD can range from the past 6 to 12 months to a lifetime prevalence. Using the *Diagnostic and Statistical Manual of Mental Disorders (DSM-V)* diagnostic criteria, a nationally representative sample of U.S. adults reported approximately 8.3% of individuals experience a lifetime prevalence of PTSD (Kilpatrick et al., 2013). Exposure to an extreme traumatic stressor that threatens death and serious injury to one self or another can lead to a constellation of PTSD symptomology including re-experiencing, avoidance and hyperarousal symptoms (American Psychiatric Association [APA], 2013). Considering the types of traumatic events that can lead to PTSD is not exhaustive and includes a multitude of different experiences and types of exposures, development of PTSD is not limited to a specific age group or gender and can occur throughout the lifespan from children and adolescents to adults and the elderly (Stein, Friedman,& Blanco, 2011; APA, 2013; Reynolds, Pietrzak, Mackenzie, Chou, & Sareen, 2016).

Trauma continues to be a threatening and universal aspect of life in the 21st century leading to a growing public health concern (Lang, Campbell, Shanley, Crusto, &

Connell, 2016; Iribarren, Prolo, Neagos, & Chiappelli, 2005). One of the most defining characteristic of PTSD, is it is a disorder of reactivity to traumatic experiences and not altered baseline state as seen in other psychological disorders such as depression (Stein et al., 2011). Increased neuroendocrine response during a traumatic event augments hyperarousal and reactivity when exposed to environmental stimuli that are associated with the traumatic event experienced (De Kloet, Joëls & Holsboer, 2005). Individuals with PTSD have persistent abnormal conditioned fear responses to psychologically-traumatic events (Norrholm, Jovanovic, Olin, Sands, Bradley, & Ressler, 2011; Milad, Orr, Lasko, Chang, Rauch, & Pitman, 2008) that manifests a multitude of symptomology including behavioral avoidance, negative alterations in cognitions and mood, intrusion, and alterations in arousal and reactivity (APA, 2013). Exposure to traumatic events can occur throughout the world; they are unpredictable and cannot be anticipated. PTSD can develop in any age-group and occurs universally amongst any gender, culture, and ethnicity (APA, 2013). Therefore, considering the prevalence and impact PTSD can have throughout the world, understanding the unique characteristics of PTSD across all types of individuals can lead to more specific approaches to assessment, diagnosis, and treatment of PTSD.

Symptom severity of PTSD varies amongst individuals, but can occur after an individual has either witnessed or experienced a traumatic event (APA, 2013). According to the DSM-V, diagnostic criterial of PTSD is characterized by symptoms that cause severe psychological distress or interfere with normal social living (APA, 2013). PTSD symptomology is described by anxiety, hyper-arousal, generalized fear, intrusive memories, and depressive symptoms (Siegmund & Wotjak, 2006; Yehuda & LeDoux,

2007). DSM-V categories of diagnostic criterions include: A) Exposure to actual or threatened death, serious injury, or sexual violence, B) Intrusive symptoms associated with the traumatic event(s), C) Persistent avoidance of stimuli associated with the traumatic event, D) Negative alterations in cognitions and mood associated with traumatic event(s), and E) Alterations in arousal and reactivity associated with traumatic event(s) (APA, 2013). Despite having already established symptom domains for PTSD, the variability in types of traumatic events, greater risk of trauma exposure, and gender-specific PTSD risk factors make it a challenging and complex disorder to study (Olff, Langeland, Draijer, & Gersons, 2007). In addition, biological variability between genders such as sex hormones and autonomic and neuroendocrine responses during stress contribute to gender-differences in PTSD prevalence (Cohen & Yehuda, 2011; Olff et al., 2007). Therefore, it is important to establish research models that assess gender and stress responses to understand biological mechanisms involved in increased prevalence in PTSD amongst females compared to males (Kilpatrick et al., 2013).

Neurobiology of Pathological Anxiety

Stress and anxiety disorders, specifically PTSD, are associated with deficits in neural circuitry that regulates responses to emotional or threat-related stimuli (Buckley, Blanchard & Neill, 2000). Subcortical activity in the amygdala in response to emotional stimuli or fear indicates excessive hyperarousal activity. In addition, the medial prefrontal cortex (mPFC) and ventral-rostral anterior cingulate cortex (rACC) show dysregulation of neural activity in response to fear, leading to impairments in inhibition, hyperarousal and fear responses (Shin et al., 2001; 2005). Impaired neural circuitry between cortical and subcortical networks in response to traumatic events suggests a breakdown in neural

activity leading to hyperexcitability and impaired inhibitory control (Johnstone, Yan, Alwis & Rajan, 2013).

Over-generalized fear is the major symptom of anxiety and stress disorders and occurs when an responses to the traumatic event becomes generalized to situations that should not elicit fear, anxiety or stress but still cause an increase autonomic response (Mahan & Ressler, 2012). The initial traumatic experience produces significant hypothalamic pituitary adrenal axis (HPA) autonomic responses, which secretes catecholamine's epinephrine (E) and norepinephrine (NE) from the adrenal medulla and allows for processing and encoding of fearful and stressful stimuli into distinct memories (Tsigos & Chrousos, 2002). Release of NE in the locus coeruleus (LC) can occur from all types of stressors and projects upwards towards forebrain regions and limbic structures such as the hippocampus (Robertson, Plummer, De Marchena, & Jensen, 2013). Repeated acoustic startle response produces significant NE release in the hippocampus during startle, as well as during re-exposure to footstock context. In addition, these high levels of NE in the hippocampus elicited increased conditioned fear to fearful stimuli and increased extracellular NE levels in the mPFC (Kao, Stalla, Stalla, Wotjak & Anderzhanova, 2015). Innervation of noradrenergic transmission in these regions, specifically the hippocampus, facilitates fear memory acquisition, consolidation of memory for the stressful event, and retrieval off the memory (Fiorenza, Rosa, Izquierdo, & Myskiw, 2012; Zhang, Ásgeirsdóttir, Cohen, Munchow, Barrera & Stackman, 2013). Therefore, the central noradrenergic system plays a vital role in creating a strong and aversive memory of the traumatic experience leading to re-experiencing, flashbacks, and conditioned emotional responses (Southwick, Bremner, Rasmusson, Morgan, Arnsten, &

Charney, 1999). Conditioning to fearful events or experiences occurs when an individual first reacts to an unconditioned stimulus (e.g., traumatic event) with high levels of fear or stress (i.e., unconditioned response) and elicits future fear or arousal responses (i.e., conditioned response) to the memory or associated traumatic-related stimuli (i.e., conditioned stimuli) even after the traumatic event is over (Yehuda & LeDoux, 2007). This strong associative learning to traumatic events and increased autonomic arousal signals a positive feed-forward and feedback loop between central corticotropin-releasing factor (CRF) systems and NE in the locus coeruleus (LC). Feed-forward CRF-NE stress system includes paraventricular nucleus of the hypothalamus (PVN) release of CRF which activates brainstem noradrenergic activity to stimulate LC secretion of NE during stress. Feed-back NE-CRF stress system modulates continued release of CRF by NE from forebrain, central nucleus of the amygdala, and bed nucleus of the stria terminalis and PVN (Koob, 1999). These two modulating systems may explain why conditioned fear responses and consolidated traumatic memory may strengthen people's ability to remember specific details of the event and produce subsequent intrusive recollections of the traumatic experience (Koob, 1999; Southwick et al., 1999).

In addition to the neurocircuitry involved with establishing and consolidating a traumatic memory, cortico-limbic pathways play a vital role in conditioned fear learning and inhibition of fear responses. The primary responsibility of the amygdala is to regulate learned fear responses through top-down control from cortical inputs. Prefrontal cortical fields process and integrate sensory information that is received from all sensory modalities and triggers subcortical inputs specific to environmental and moment-to-moment experiences (Purves et al., 2018). Cortical control of fear in the amygdala is

initiated by prefrontal cortex inputs that regulate aversive conditioning, fear expression and extension. Hübner et al. (2015) optogenetically stimulated mPFC excitatory inputs to the basal amygdala, which targeted a small portion of GABAergic interneurons in the BLA to evoke GABAergic inhibitory feed-forward neurotransmission from the BLA back to the mPFC. In human studies, the ventromedial prefrontal cortex (vmPFC) hypo-activity was correlated with reduction in inhibitory transmission in the amygdala which leads to significant hyperactivation in the amygdala while increasing fear behavior (Milad et al., 2008). Animal studies demonstrate two subregions, the prelimbic (PL) and infralimbic (IL) regions, located in prefrontal cortices implement a dual cortical control of the amygdala-based fear responses, specifically fear expression and extinction. The prelimbic mPFC projects long-range layer 2 pyramidal neurons directly to the BLA and central medial PFC (cmPFC), which activates reciprocal excitatory outputs back to the mPFC, revealing a novel cortico-limbic circuitry involved with controlling emotional behaviors (Little & Carter, 2013). Activity in the PL directly targets the basal amygdala (BA) mediating excitation of fear expression and increasing fear output (McDonald, Mascagni & Guo, 1996; Vertes, 2004). Contrary to the PL, the IL cortex indirectly communicates to the BLA through indirect stimulation of the medial ITC masses (mITC) located in the internal capsule between the BLA and CeA (Strobel, Marek, Gooch, Sullivan & Sah, 2015). Furthermore, indirect inhibitory transmission to the amygdala through the activation of the IL has been shown to play a role in extinction of fear memory (Strobel et al., 2015). Therefore, cortico-limbic top-down, bottom-up control over responding to emotional stimuli reveals reciprocal interconnectivity that influences the outcome of fear and extinction learning. Furthermore, an important key to

understanding the regulation of fear and emotion is to investigate the microcircuits within the amygdala which include distinct populations of principal neuron and local interneuron interactions. Also, gender-specific neurobiological mechanism within the amygdala may contribute to gender-differences in PTSD prevalence rates and vulnerability and susceptibility to traumatic stress and anxiety. In addition, understanding how drugs of abuse, specifically ethanol, effects amygdala microcircuitry between genders can elucidate neurobiological mechanisms associated with alcohol abuse and pathological anxiety between genders.

Gender-Differences in PTSD

Specific populations are at higher risk of developing PTSD based on greater likelihood to experiences trauma. Females are more likely to develop PTSD compared to males in the general population (Kessler, Berglund, Demler, Jin, Merikangas, & Walters, 2005; Kilpatrick et al., 2013). Women are more likely to fall victim to direct interpersonal violence such as childhood physical abuse, aggravated assault, rape and other sexual assault. Also, exposure to physical assault victimization is greater amongst women compared to men (Kilpatrick et al., 2013). Furthermore, in the general population, rape and sexual assault are highest in traumatic events associated with developing PTSD (Kilpatrick, Saunders, Veronen, Best, & Von, 1987). Approximately 20% of rape victims develop PTSD (Kilpatrick et al., 1987) and traumatic exposure to rape and assault account for the highest proportions of PTSD worldwide (Kessler et al., 2014). Interpersonal violence is one of the most traumatizing types of trauma because it includes both physical distress but also emotional distress such as feeling of betrayal (Freyd, 1994), loss of safety and control of one's environment (Janoff-Bulman, 1992).

Therefore, women are at higher risk and more susceptible to experience trauma and develop PTSD symptoms. There are also several possible explanations as to why women are more susceptible to PTSD (Kessler, Crum, Warner, Nelson, Schulenberg, & Anthony, 1997). Gender-specific psychological and psychosocial reactions to trauma such as cognitive appraisal and coping mechanisms may highlight sex-differences in neuroendocrine, biological, and behavioral responses in PTSD (Olff et al., 2007).

An individual's subjective interpretation and evaluation of a traumatic event, known as cognitive appraisal, plays a major role in how the individuals will respond emotionally and behaviorally to trauma, and further augment neuroendocrine responses such as HPA dysregulation and sympathetic nervous system hyperactivity (Olff et al.,2007). Subjective interpretations of trauma including perceptions of threat, danger, harm and loss of control, are significant factors that predict the development of PTSD (McNally, 2003). According to Ehlers and Clark (2000), individuals who negatively appraise a traumatic event as catastrophic are more likely to feel threatened and helpless for a significant period of time after the traumatic event has occurred. These individuals continue to suffer from this heightened sense of fear and threat, which elucidates maladaptive neuroendocrine stress response and dysregulation of the HPA-axis. Women are more likely to appraise stressors as more stressful and traumatic (Tamres, Janicki & Helgeson, 2002), and report feelings of uncontrollability (Mak, Blewitt, & Heaven, 2004) compared to men. According to Larsen and Fitzgerald (2010), perceived control over traumatic recovery and perception that experiencing the same traumatic event in the future is unlikely was associated with fewer PTSD symptoms in women who experience sexual harassment. Perceived control during and traumatic exposure could be associated

with poor coping mechanisms (Schneiderman, Ironson, & Siegel, 2005). Women and men report differences in coping strategies implemented to cope with physical and psychological trauma. Women are more likely to use passive/avoidance and emotion-focused coping mechanisms compared to men who use more active and instrumental behaviors (Pearlin & Schooler, 1978; Ptacek, Smith, & Zanas, 1992; Matud, 2004). In addition, individuals with PTSD and substance use disorders are more likely to engage in emotion-focused and avoidant coping strategies (Staiger et al., 2009). Understanding the relationship between gender-differences in psychosocial and psychobehavioral characteristics of PTSD may elucidate differences in prevalence rates as well as gender differences in neurobiological stress responses and substance abuse.

Gender-Differences in Neuroendocrine Response to Stress

An individual's global response to threat is through activation of the ANS. This physiological reaction is initiated by perception of aversive or threatening situations. During exposure, our physiological response is heightened and we enter a preparatory stage of "fight-or-flight" (Cannon, 1932) in which we will either engage in strenuous efforts to fight through the threat or dangerous situation; or individual's will flee and work to remove themselves from the threat. Whichever decision, the physiological response to stress first starts with mobilization of energy and resources through autonomic and endocrine responses (Miller & O'Callaghan, 2002).

The sympathetic nervous system (SNS) is activated within seconds of exposure to stress and utilizes pre-and post-autonomic ganglia to innervate organs in the abdominal and thoracic viscera, such as the heart, lungs, liver, kidneys, gut, pancreas, bladder and reproductive organs (Gilbey & Spyer, 1993). Postganglionic terminals release

noradrenergic neurotransmitters to synapse with postsynaptic receptors and mediate sympathetic effects in smooth and cardiac muscle and glands. Sympathetic neural activity of these organs allows the body to maximize its metabolic, cardiovascular and respiratory resources to increase chances of survival in threatening situations. In addition, sympathetic preganglionic innervation of the adrenal medulla stimulates release of epinephrine and norepinephrine in to the bloodstream (i.e., sympathetic adrenomedullary system). Both epinephrine and norepinephrine increase cardiovascular function allowing for greater cardiac output and increased blood flow to muscles for strenuous activity and sustained physiological response to stress (Elfvin, Lindh & Hökfelt, 1993; Benarroch, 1994; Purves et al., 2018). Following activation of the sympathetic nervous system, the HPA axis is initiated to control secretion of stress hormones which facilitate glucose metabolism, increase blood flow, and mobilize energy for behavioral responsiveness (Munck, Guyre & Holbrook, 1984).

During an autonomic response to stress, the HPA-axis functions to regulate the secretion of cortisol by the adrenal cortex which is critical not only for the response to stress but termination of the stress response. The cascade of HPA endocrine functioning is initiated in the paraventricular nucleus (PVN) of the hypothalamus which secretes corticotrophin-releasing hormone (CRH) and arginine vasopressin (AVP) to the anterior pituitary gland. CRH stimulates release of stress-induced adrenocorticotropic hormone (ACTH) from the anterior pituitary gland which enters the general circulation system and stimulates the adrenal cortex to secrete glucocorticoids (i.e., cortisol). Secretion of cortisol from the adrenal cortex plays a vital role in regulating glucose metabolism as well as protein breakdown to make use for energy and cardiovascular functioning during

stress. In addition, release of glucocorticoids establishes a negative feedback loop to the anterior pituitary and hypothalamus by binding to glucocorticoid receptors which down regulate CRH release and ACTH to restore the body back to homeostasis. Once the appropriate levels of cortisol have been released from the adrenal cortex, the negative feedback loop controls the termination of further cortisol release. Disruptions of the negative feedback loop can lead to dysregulations of the HPA axis and have serious implications in stress and anxiety disorders (Tsigos & Chrousos, 2002; Purves et al., 2018; Chrousos, 2009; Jones & Moller, 2011).

Gender differences in HPA-axis stress responses may contribute to differences in vulnerability and susceptibility between men and women during a traumatic event. Human and animal research has sought to determine changes in neuroendocrine functioning between males and females to explain higher female susceptibility to stress and stress-related pathology (Kudielka & Kirschbaum, 2005; Stephens, Mahon, McCaul & Wand, 2016). However, there are disparities in findings between rodent and human studies, specifically in basal cortisol/corticosterone levels between males and females (Cohen & Yehuda, 2011). Following a psychosocial stress paradigm, adult men produced greater ACTH and cortisol levels compared to adult women (Uhart, Chong, Oswald, Lin, & Wand, 2006; Stephens et al., 2016). Salivary cortisol and ACTH levels in men showed steeper baseline-to-peak and peak-to-end slopes compared to women suggesting men reflect a faster activation and down regulation of the HPA axis (Stephens et al., 2016). Similarly, adult men exhibit greater cortisol response to a cognitive challenge protocol that has been shown to increase HPA and SNS activity, compared to adult women (Seeman, Singer, Wilkinson & McEwen, 2001). However, animal studies show more

stable but opposing gender differences in stress response compared to human's. Female rodents at baseline show increased ACTH and corticosterone levels (Critchlow, Liebelt, Bar-Sela, Mountcastle, & Lipscomb, 1963), as well as higher and more rapid secretion of ACTH after a physical footshock stressor (Burgess & Handa, 1992). Furthermore, Louvart et al. (2006) exposed female and male rats to a footshock stressor and female rats exerted a greater physiological stress response compared to males. Increase in ACTH and corticosterone levels during baseline and stress in female rodents can be explained by circulating female gonadal steroid, estradiol, which has been shown to significantly influence HPA reactivity to stress in both rodents and humans (Burgess & Handa, 1992; Serova, Harris, Maharjan & Sabban, 2010). Female rats with surgically implanted beta-estradiol capsules showed significantly higher and prolonged corticosterone and ACTH peak levels after footshock compared to males (Burgess & Handa, 1992). Therefore, human studies should account for the female stress response during different stages of the female menstrual cycle to show how females differ in response to stress based on endogenous sex hormone levels. Recently, Wolfram, Bellingrath & Kudielka (2011) revealed female cortical awakening response (CAR) is significantly higher during ovulation, when females have elevated sex steroid levels including estrogen, luteinizing hormone and follicle stimulating hormone. In addition, Juster et al. (2016) demonstrated that sex hormones including testosterone in women and progesterone in men significantly adjusted sex-specific HPA reactivity to a psychosocial stress test as well as diurnal cortisol levels. Stephens et al. (2016) investigated gender-differences in ACTH and cortisol response while monitoring circulating sex hormones. Results indicate men have higher HPA axis responses compared to female cortisol and

ACTH levels but stress levels were measured during the follicular phase of their menstrual cycle when progesterone levels are similar to men. Although the results indicate gender-differences in HPA axis stress response, women may show an increased stress response (i.e., ACTH and cortisol levels) during other stages of their cycle such as the ovulation phase when estrogen is highly elevated (Juster et al. 2016).

Females have greater cyclical variation in neuroendocrine responses due to their reproductive cycle (Taylor, Klein, Lewis, Gruenewald, Gurung, Updegraff, 2000). Fluctuation of estrogen levels occur throughout the female menstrual cycle, with lower levels of estrogen occurring in the follicular phase and high, peak levels occurring immediately before or during the ovulation phase (Kirschbaum, Kudielka, Gaab, Schommer & Hellhammer, 1999). Rodent studies report elevated ACTH and cortisol (CORT) levels in female rats during proestrus when estrogen levels are at high levels (Carey, Deterd, De Koning, Helmerhorst, & De Kloet,1995; Atkinson & Waddell, 1997). Female rats exposed to both acute and chronic stress after receiving estradiol treatment showed increased expression of CRH mRNA in the PVN of the hypothalamus compared to non-treated animals (Lunga & Herbert, 2004). Human studies report increased HPA endocrine response to psychosocial stressor during the luteal phase when estrogen levels by reaching peak levels, suggesting estrogen produces mediating effect of the HPA axis by possibly impairing the negative feedback loop (Kirschbaum et al., 1999).

The primary mechanism of estrogen action on the neuroendocrine response is through estrogen receptors, ERα and ERβ, located throughout the hypothalamus, as well as the hippocampus and other limbic structures which plays a major role in activating the HPA axis (Woolley, 2007). A small portion of ERα receptors are located within the peri-

PVN of the hypothalamus and have been shown to impair glucocorticoid receptor-mediated negative feedback of the HPA axis through reducing GABAergic neuronal transmission within the peri-PVN (Bali & Kovacs, 2003; Weiser & Handa, 2009), a region implicated in HPA axis inhibition (Suzuki & Handa, 2005). Furthermore, Weiser and Handa (2009) reported a multitude of estradiol effects on the neuroendocrine system including increasing plasma CORT and ACTH levels to estradiol in the PVN, which is mediated through an ERα-dependent mechanism. ERα-agonist propylpyrazoletriol produced significantly higher CORT and ACTH levels compared to controls after restraint stress, indicating ERα as the potential receptor in the PVN to regulate glucocorticoid-mediated HPA negative feedback. ERβ receptors have been shown to produce opposite effects of ERα activity by reducing response to stress through oxytocin (OT) and vasopressin (AVP) mediated mechanisms (Alves, Lopez, McEwen & Weiland, 1998). ERβ are expressed on CRH neurons (Miller, Suzuki, Miller, Handa, & Uht, 2004), in addition with OT and AVP neurons in the PVN (Alves et al., 1998). In the PVN, CRH secretion can be inhibited in response to AVP and OT as these neuropeptides colocalize with CRH on PVN neurons (Whitnal, 1989; Alves et al., 1998; Nomura, McKenna, Korach, Pfaff & Ogawa, 2002). This modulatory mechanism has been implemented in human studies which demonstrate high levels of oxytocin are associated with reductions in stress and anxiety (Heinrichs, Baumgartner, Kirschbaum & Ehlert, 2003) and can attenuate amygdala responses to emotional stimuli (Domes, Heinrichs, Gläscher, Büchel, Braus, & Herpertz, 2007). In addition to estrogen regulating HPA axis response to stress, hippocampal-dependent learning and memory are enhanced through circulating female sex hormones.

Animal studies have previously shown high levels of estradiol during the estrus cycle of female rats produce a significant increase in spine density and synapses on apical dendrites on pyramidal neurons within the hippocampus (Woolley & McEwen, 1992). Furthermore, estradiol potentiates intrinsic neuronal excitability by depolarizing hippocampal CA1 neurons and producing spontaneous firing (Wong & Moss, 1991), as well as reducing afterhyperpolarization (AHP) amplitude through reducing Ca^{2+} influx through calcium-activated K^{+} channels (Kumar & Foster, 2002). Therefore, estrogen not only has stimulatory effects on the neuroendocrine system but also hippocampal-dependent memory systems which may explain why females are more susceptible to physiological response to stress and anxiety. The impact of female sex hormones may further explain sex differences in the regulation of stress and have significant implications for understanding gender-specific neurobiological mechanisms that occur during stress.

Implications in human clinical findings reveal important biological explanations for gender-differences in response to stress. First, higher cortisol and ACTH levels, as well as rapid onset and deactivation of the HPA axis in men suggest the glucocorticoid-mediated negative feedback mechanism is fast acting and quickly terminates the stress response bringing the body back to homeostasis. Release of cortisol is short-term, allowing for rapid mobilization of energy and resources to adapt and respond to stress but not prolonged to induce stress-related impairments (Sapolsky, 2000; Papadimitriou & Priftis, 2009; Stephens et al., 2016). Furthermore, increased neuroendocrine functioning in men to acute stress may contribute to men reporting higher levels of perceived control

during stress (Mak, Blewitt, & Heaven, 2004) and less acute emotional (i.e., intense fear, avoidance, helplessness, and panic) and dissociative responses (Bryant & Harvey, 2003).

Women's attenuated HPA axis stress response attributes to a multitude of biological and behavioral implications for PTSD, as well as elevated PTSD risk in women. Olff et al. (2007) theorized that women who engage in avoidant and emotionally numbing type psychological defense mechanisms during stress may induce less activation of the HPA axis and produce lower levels of stress hormones. Furthermore, reported marked differences in cortisol responses to stress between men and women may contribute to experiences of greater fear-provoking danger and lead to emotional responsiveness such as horror, intrusive thoughts and intense fear, which has been reported in women's response to acute stress (Byrant & Harvey, 2003). Taylor et al. (2000) proposed that women approach stressful situations through a "tend-and-befriend" response to protect their offspring from danger or threats to survival compared to males who engage in "fight-or-flight" response to stress. This evolutionary perspective may contribute to gender-differences in sympathetic and HPA axis stress responses.

Gender, Alcohol, and PTSD

Alcohol consumption produces significant positive reinforcing states (i.e., relaxation) and reduces negatives affective states including stress, anxiety, and tension reduction (Cooper, Frone, Russell & Mudar, 1995). It is well established that anxiety disorders, specifically PTSD, are comorbid with alcohol use disorders and dependence (Kessler et al., 1997; Sonne, Back, Zuniga, Randall, & Brady, 2003). According to the self-medication hypothesis, individuals who suffer from psychiatric disorders use abused substances, such as alcohol, as an attempt to decrease psychiatric symptoms (Khantzian,

1985; Miranda, Meyerson, Long, Marx & Simpson, 2002). Alcohol exposure produces significant negative reinforcing effects, such as anxiolysis (Koob & Le Moal, 2008), which plays an important role in the high comorbidity rate of PTSD and alcohol use disorders. Individuals exposed to trauma that have symptoms of PTSD are more motivated to consume alcohol for multiple coping-oriented reasons including managing sleep difficulties (Nishith, Resick & Mueser, 2001) and hyperarousal symptoms of PTSD (Stewart, Conrod, Samoluk, Pihl & Dongier, 2000). Kessler et al. (1997) conducted a national comorbidity study which reported 26.2% of women and 10.3% of men with a lifetime prevalence rate of alcohol dependence (AD) meet the criteria for PTSD. In addition, development of PTSD precedes alcohol dependence in women indicating women become alcohol-dependent more frequently after experiencing traumatic event(s) associated with PTSD (Sonne et al., 2003). In addition to high comorbidity rate, gender differences between prevalence rates amongst alcohol dependence and PTSD, (Kessler et al., 1997) suggest specific psychological and behavioral mechanisms that may influence the onset and presentation of alcohol use and PTSD between genders (Jayawickreme, Yasinski, Williams & Foa, 2012; Lehavot, Stappenbeck, Luterek, Kaysen, & Simpson, 2014).

Individual coping mechanisms not only play a significant role in the neuroendocrine stress response and appraisal of a threat (Olff et al., 2007), but also mediates psychological distress and alcohol use (Grayson & Nolen-Hoeksema, 2005). Women who suffered from childhood sexual assault engage in maladaptive emotional regulation strategies such as excessive alcohol consumption to experience positive emotional states and reduce negative emotions associated with emotional distress

(Grayson & Nolen-Hoeksema, 2005). Alcohol dependence amongst women with comorbid PTSD is associated with greater incidence and intensity of trauma-related avoidance, and greater social impairment (Sonne et al., 2003). Bornovalova, Ouimette, Crawford and Levy (2009) reported women have greater difficulty controlling impulsive behavior with distress, which mediates the relationship between PTSD symptomology and substance use. Men demonstrated less clarity and awareness of emotions during distress, which partially explained the relationship between substance use and stress. In addition to emotional and coping responses, gender differences between alcohol craving and urge to drink following stress is correlated with subjective, behavioral and physiological responses to emotional stress (Chaplin, Hong, Bergquist & Sinha, 2008). Women report greater feelings of sadness and anxiety following emotional stress, as well as greater behavioral and bodily responses and increase heart rate when expressing behavioral emotion compared to men. In accordance with gender differences in HPA axis activity, men display significantly higher diastolic blood pressure during stress compared to women, and alcohol craving was correlated with subjective sadness, anxiety, fear, and behavioral/bodily response in only men. Also, subjective negative emotion for men was significantly correlated with stress-and alcohol-cue related alcohol craving compared to women (Chaplin et al., 2008).

Gender differences in alcohol craving in response to stress (Chaplin et al., 2008), as well as levels of HPA stress hormones (i.e., corticosterone) (Fahlke, Engel, Eriksson, Hard & Söderpalm, 1994) can be explained through experimental evidence indicating that HPA axis response hormones potentiate consumption of alcohol (Fahlke et al., 1994; 2000; Prasad & Prasad, 1995). Rodent and primate animal models have shown that

enhanced glucocorticoid stress response elicits an increased in alcohol consumption at higher quantities (Fahlke et al., 2000; Prasad & Prasad, 1995). In contrast, alcohol induces gender-specific patterns of ACTH and corticosterone release by HPA axis secretion. Ovariectomized female rats administered acute low to moderate ethanol (i.e., 1.5 g/kg) through intraperitoneal (i.p.) injection produce higher ACTH and corticosterone levels compared to male rats. Intact female animals expressed even greater HPA axis stress hormone release during proestrus and estrus cycles (Rivier, 1993), further indicating a modulatory role of sex hormones on responses to stress. Although human and animal research showing alcohol stimulates the HPA system is contradictory to primary effects of alcohol on inhibitory neurotransmission in the brain, research suggests that alcohol stimulating the HPA system is the mechanism involved in repeated alcohol intake promoting addiction (Koob & Le Moal, 2001). In addition, the cellular mechanisms that contribute to alcohol-stress-anxiety interactions indicates acute alcohol exerts significant GABAergic transmission dampening glutamatergic excitatory effects and promoting a decrease in anxiety and fear responses (Silberman, Shi, Brunso-Bechtold, & Weiner, 2008).

Neurobiological Mechanisms Contributing to Alcohol and Anxiolysis

Molecular pharmacological studies have identified specific primary targets of alcohol in the central nervous system including *N*-methyl-D-aspartate (NMDA) (Lovinger, White & Weight, 1989), γ-aminobutyric acid A ($GABA_A$), glycine, 5-hydroxytryptamine-3 ($5\text{-}HT_3$) (Lovinger & Zhou, 1998), and neuronal nicotinic acetylcholine (nACh) receptors, L-type Ca^{2+} channels and G protein-activated inwardly rectifying K^+ channels (Vengeliene, Bilbao, Molander & Spanagel, 2008). Most notable,

specific alcohol-sensitive sites on NMDA and GABA receptors and ion channels play major modulatory roles in neurochemical systems involved with reinforcing effects of alcohol.

Lovinger, White, and Weight (1989) reported ethanol produces significant inhibition to NMDA receptor function in a dose-dependent manner, which reduces the mean open time and opening frequency of NMDA-activated channel currents (Lima-Landman & Albuquerque, 1989; Wright, Peoples & Weight, 1996). Although no consensus has been made for an alcohol binding site, recent literature suggests there are specific alcohol-sensitive proteins located on receptor transmembrane domains of receptor subunits. Ren, Honse & Peoples (2003) conducted a mutagenesis study showing methionine residue on the transmembrane 4 (TM-4) domain of NR2A subunit influences sensitivity of the NMDA receptor-ion channel to ethanol. In addition, site-directed mutagenesis exchanging a phenylalanine residue for alanine in the TM3 of the NR1 subunit significantly reduced ethanol sensitivity of the NMDA receptor (Ronald, Mirshahi & Woodward, 2001). These studies reveal sites of action for ethanol on NMDA receptors, specifically in amino acids located in the TM-3 and 4 domains that influence channel activity.

Another well-known site of action for alcohol is the $GABA_A$ receptor/chloride channel complex which exerts inhibitory transmission throughout the central nervous system. $GABA_A$ is a heteropentameric ligand-gated ion channel that is composed of seven different types of subunits including $\alpha, \beta, \gamma, \delta, \varepsilon, \pi, \theta$, each of which containing a different number of subtypes. The brain and spinal cord is primarily made of up of $GABA_A$ receptors that consists of α_1, β_2, and γ_2 subtypes, assembling a pentameric

complex of two α_1s, two β_2s, and one γ_2 (Squire, Berg, Bloom, Du Lac, Ghosh & Spitzer, 2012). Mihic et al. (1997) reported ethanol allosterically modulated two specific amino acids located in TM-2 and-3 of the $GABA_A$, and subunits α_1 and β_1 exhibited inhibition by ethanol. Low concentrations of ethanol (3mM) that are comparable to human consumption concentrations and low BAC levels target specific GABA receptor subunit expression in both $\alpha_4\beta_3\delta$ and $\alpha_6\beta_3\delta$ resulting in unique sensitivity to ethanol (Wallner, Hanchar & Olsen, 2003).

The role of alcohol-induced modulation and attenuation of GABAergic and glutamatergic neurotransmission is critically involved in the regulation of anxiety-like behaviors. The BLA plays a vital role in integrating sensory information and establishing emotional salience of the environment (Davis, 1992). The neural circuitry between cortical regions and the BLA and central nucleus of the amygdala (CeA) highly regulates both stress and anxiety (Le Doux, 1993; Sah et al., 2003). The majority of neurons in the BLA are excitatory glutamatergic pyramidal neurons which provide excitatory projections to the CeA and cortical structures (Sah et al., 2003). In addition, the BLA also contains a small population of GABAergic interneurons which provide inhibitory regulation of excitatory transmission from the BLA (Washburn & Moises, 1992). Therefore, while glutamatergic input is involved with transmitting excitatory information in response to stress and anxiety, GABAergic transmission is thought to provide inhibitory control and regulate anxiety-like behaviors (Davis, 1992; Menard & Treit, 1999). Alcohol exposure enhances GABA-mediated synaptic transmission, leading to enhanced synaptic inhibition in brain regions dense with GABAergic neurons (Roberto, Madamba, Stouffer, Parsons, & Siggins, 2004; Zhu & Lovinger, 2006). Furthermore,

since one of the primary roles of the amygdala is to regulate responses to fearful stimuli, neurophysiological studies have focused on characterizing the effects of ethanol on neurophysiological properties of neurons in the amygdala. GABAergic inhibition of the BLA occurs through two distinct pathways including GABAergic interneurons located in the BLA (Woodruff & Sah, 2007), and a second inhibitory network called paracapsular intercalated cell masses including both lateral (lITC) and medial (mITC) clusters located in the external and internal capsule borders of the amygdala (Marowsky, Yanagawa, Obata & Vogt, 2005). These two pathways are thought to regulate both feedback and feed-forward inhibition of the BLA. Silberman et al. (2008) showed that ethanol potentiated GABAergic transmission in the BLA through local interneuron and distal lateral paracapsular ITC neuron stimulation, suggesting ethanol enhances GABAergic inhibition on BLA excitatory pyramidal neurons through feed-forward inhibition. Therefore, ethanol exerting inhibitory transmission in the amygdala plays a major role in reducing negative affect and dampening anxiety response to stress and fear behaviors (Spanagel, Montkowski, Allingham, Shoaib, Holsboer, & Landgraf, 1995; Silberman et al., 2008; 2012).

Human studies using fMRI techniques report significant dampening of amygdala dependent anxiety-like responses after administration of acute alcohol (Gilman, Ramchandani, Davis, Bjork & Hommer, 2008; Sripada, Angstadt, McNamara, King & Phan, 2011). During threat or fear detection, the amygdala receives cortical input from the prefrontal cortex to generate a behavioral response to the threat, such as fight or flight (LeDoux, 1993). Impaired activation of this neural circuitry by alcohol may mediate the amygdala's ability to differentiate threating and non-threatening stimuli, attenuating the

emotional response to threat and reduce avoidant behavior (Gilman et al., 2008; Sripada et al., 2011). Gilman et al. (2008) assessed the effects of intravenously acute alcohol on amygdala activation in response to visual threatening stimuli and nonthreatening facial stimuli. Using fMRI (blood-oxygen-level dependent) bold activation measures, acute ethanol attenuated amygdala activation to fearful stimuli to a greater extent than during placebo condition. Alcohol intoxication activated striatal reward areas including nucleus accumbens (NAcc), caudate and putamen demonstrating anxiolytic and reward circuitry interactions in response to emotionally threatening stimuli. Self-reported intoxication was significantly correlated with larger NAcc and caudate BOLD signal activation in response to acute ethanol. Therefore, alcohol regulates rewarding experiences through striatal activation while attenuating threat-detection circuitry in the amygdala leading to negative reinforcing responses. As ethanol potentiates GABAergic inhibition in the amygdala contributing to acute anxiolytic responses to stress and anxiety (Silberman et al., 2008), ethanol also mediates GABAergic inhibition in the mesolimbic dopaminergic system to initiate alcohol reinforcing properties (Spanagel & Weiss, 1999).

The mesolimbic dopamine pathway is a prominent neurological system associated with drug reinforcement, motivational processes, and drug-induced reward. This drug reward pathway originates in the ventral tegmental area (VTA)s and projects dopaminergic excitatory projections to multiple regions including the NAcc and PFC (Koob, 1992). One major contributing mechanism involved with alcohol reinforcement is initiated by GABAergic inhibition on GABA interneurons which synapse on the VTA. During alcohol intoxication, ethanol produces a GABA inhibitory feedback causing disinhibition of GABA interneurons on VTA transmission, resulting in increased

dopaminergic projections from the VTA to NAcc. Increase dopaminergic transmission from VTA to NAcc increases extracellular dopamine (DA) within the NAcc and plays a critical role in the acquisition of alcohol reinforcement (Gessa, Muntoni, Collu, Vargiu & Mereu, 1985; Kalivas, 1993; Kohl, Katner, Chernet & McBride, 1998; Spanagel & Weiss, 1999; Spanagel, 2009).

Gender differences in the effects of acute ethanol on stress-induced neuronal alterations in the amygdala have only recently been examined in cellular and molecular studies. Self-administration of sweetened ethanol during adolescence significantly decreases CRF immunoreactive cells in the central amygdala in females compared to males (Karanikas, Lu & Richardson, 2013). Ethanol consumption during adolescence may impair the neurodevelopment of brain regions and cells associated with response to stress, indicating early consumption in females may increase the risk of developing stress disorders in adulthood. Gender differences in c-Fos expression shows dysregulation of neuronal activity in the central amygdala (CeA) in rats exposed to ethanol and stress, demonstrating distinct gender-dependent responses (Retson, Hoek, Sterling & Van Bockstaele, 2015). During baseline (no stress), female and male rats displayed significant differences in c-Fos (marker of neuronal activity) and ΔFosB (maker of long-term activity) levels following chronic ethanol exposure. Male rats showed significant increases in ΔFosB compared to females and controls; however, c-Fos expression in males was similar compared to controls. In contrast, female rats following chronic ethanol exposure displayed significant increases in c-Fos compared to male ethanol rats. Changes in neuronal and long-term activity in the CeA between males and females indicate males may habituate to increase neuronal activity potentiated by ethanol, while

females continue to show increased amygdala activity in response to ethanol. To assess the gender differences in the effects of ethanol on amygdala activity in response to stress, the same study reported male rats after chronic ethanol exposure and a forced swim test, showed significantly higher c-Fos levels compared to baseline, but similar levels to female ethanol subjects. Results indicate males do not habituate to CeA neuronal activity after stress compared to baseline while exposed to ethanol suggesting separate neural circuitries involved between stress and no-stress situations (Retson et al., 2015). Therefore, while the effect of ethanol on the amygdala and anxiety-like behaviors have been examined, research has yet to establish gender differences in the effects of ethanol on the cellular physiology level in neurobiological regions associated with stress and anxiety levels.

The Basolateral Amygdala

The amygdaloid complex is located in the rostromedial portion of the temporal lobe and is caudally continuous with the uncus of the parahippocampal gyrus. The amygdala plays a significant role in processing memory and attention and pairing somatosensory, visual, and visceral information with emotional context. Kluver and Bucy (1939) were amongst the first to establish the role of amygdala in regulation of emotional behavior. Pronounced behavioral changes, such as reduced fear, rage, and aggression were elicited in rhesus monkeys after removal of the temporal lobes which houses the amygdaloid complex. Early lesions studies in both animal and human studies give evidence the amygdala acts of a subcortical interface structure between cortical input (i.e., prefrontal cortex), limbic system structures (i.e., hippocampus and hypothalamus) and brainstem nuclei (i.e., locus coeruleus), all concerned with cognitive learning and

memory and emotional behavioral responses. (Sah et., 2003; Sotres-Bayon & Quirk, 2010; Marek, Strobel, Bredy & Sah, 2013; Janak & Tye, 2015)

Anatomically, the amydaloid complex is divided into three major functional and anatomical subdivisions: the basolateral, cortical, and central nuclei, all of which have a set of unique connections with each other and other parts of the brain (Sah et al., 2003; Janak & Tye, 2015). Within each nuclei complex, specific cell groups make up each subdivision; for example, the basolateral complex consists of the lateral, basal, and basomedial cell groups. The central nuclei is made up of the lateral and medial central amygdala nuclei (Janak & Tye, 2015), and the cortical nuclei, a more superficial group, contains the nucleus of the lateral olfactory tract, bed nucleus of the accessory olfactory tract, the anterior and posterior cortical nucleus and the periamygdaloid cortex (Sah et al., 2003). Specifically, the BLA and CeA are the primary input and output systems of the amygdala and are interconnected to regulate and initiate physiological responses to fear (Sah et al., 2003; Pape & Pare, 2010). The BLA receives direct and indirect cortical, thalamic, hippocampal, and ITC inputs in which interconnected BLA cell groups process incoming sensory information associated with fear conditioning and learning (Maren & Fanselow, 1995; Strobel et al., 2015). The BLA communicates with the CeA through downstream excitatory principal projection neurons to the lateral CeA, which is reciprocally connected with the medial CeA, the output nuclei of the amygdala (Pare & Duvarci, 2012). Specifically, the CeA projects feed-back information to cortical structures, hypothalamus, and the (bed nucleus of the stria terminalis) BNST. The current project focuses on understanding neuronal excitability in the BLA because it initiates

synaptic interactions between amygdala nuclei and regulates neuronal transmission to the CeA to initiates physiological responses to stress.

The morphology and physiological properties of BLA neurons have been extensively studied and characterized; specific classification of BLA neurons include glutamatergic pyramidal projection neurons that are densely packed with dendritic trees containing numerous spines and axon collaterals which synapse with other BLA neurons, amygdala nuclei or subcortical and cortical projections regions (Sah, Faber, De Armentia, & Power, 2003). Active properties of these neurons displayed broad action potentials and full spike frequency adaptation from a depolarizing stimulus (Faber, Callister & Sah, 2001). In addition to pyramidal neurons, the BLA contains interneurons which utilize GABA as their neurotransmitter and provide significant inhibitory regulation within amygdala microcircuitry (Lang & Paré, 1998) and controls acquired fear (Ehrlich et al., 2009). Local-circuit GABAergic interneurons comprise of approximately 20% of all cells in the BLA and provide both feed-forward and feed-back inhibitory transmission in the amygdala (Woodruff & Sah, 2007; Ehrlich et al., 2009). Microcircuitry within the BLA and feed-forward projecting neurons to CeA plays a vital role in promoting fear and reducing anxiety. In addition, classical conditioning mechanisms within the BLA promote fear learning and extinction (Rodrigues, Schafe & LeDoux, 2004).

The modulation of fear conditioning by the BLA has been predominately explored using rodent models in Pavlovian classical conditioning. The LA, located in the BLA, maintains excitatory neural responses to auditory-tone and nociception-pain pairings (Paré & Collins, 2000). *In vivo* studies report extensive glutamatergic neuronal transmission in the BLA during conditioned fear response paradigms, labeling these

populations of glutamate neurons a 'fear neurons' (Herry, Ciocchi, Senn, Demmou, Müller, & Lüthi, 2008). Continuous pairing of the CS-US stimuli and potentiation of sensory information to the LA significantly enhances synaptic plasticity to produce long-term changes in synaptic properties conveying CS information (Maren & Quirk, 2004). According to the Hebbian plasticity rule, a weaker synapse (i.e., conditioned stimulus) can become strengthened and repeatedly active when the postsynaptic neuron fires in response to a stronger afferent projection carrying unconditioned stimuli information. Therefore, through long-term potentiation, the conditioned stimulus can elicit a conditioned response in the absence of the US by activating and firing neurons in the LA (Janak & Tye, 2015). Within the BLA, long-term synaptic plasticity is first initiated by thalamic and/or cortical inputs containing afferent sensory information. Direct cortical and thalamic projection neurons release glutamate from the presynaptic terminal to bind to NMDA receptors postsynaptically or excitatory stimulation of Ca^{2+} dependent voltage gated calcium channels (VGCC) to produce LTP. In addition, cortical input can indirectly activate BLA projection neurons through thalamic glutamatergic excitatory transmission on NMDA receptors located on cortical presynaptic terminals. This concurrent thalamic activation allows for depolarization of cortical inputs and release of glutamate onto (α-amino-3-hydroxy-5methyl-4-isoxazolepropionic acid) AMPA receptors within BLA principal neurons and evoked BLA responsiveness to fear conditioned stimulus. Therefore, these cellular mechanisms that form sustained excitation of BLA principal neurons play a major role in the biological mechanisms underlying fear learning (Keele et al., 2007).

New neurotechnologies such as optogenetics have recently been used to activate neurons in the amygdala corresponding to the CS and US presentation to elicit a conditioned fear response. Kwon et al. (2014) optogenetically photostimulated auditory presynaptic inputs to LA, a region of the BLA, to act as a CS and produce fear conditioning. Channelrhodopsin (ChR2) -based photostimulation of the medial geniculate nucleus (MGm) and ventral secondary auditory cortex evoke presynaptic auditory transmission to the LA to synapse with target principal neurons within the LA. *In vitro* whole-cell patch-clamp electrophysiological recordings during photostimulation found enhanced action potential and excitatory postsynaptic currents in the LA resulting from presynaptic input from auditory cortex and MGm. To examine fear conditioning with optogenetic stimulation of LA, a series of photostimulated auditory presynaptic inputs to the LA was paired with a footshock. Retention tests with conditioned stimulus (i.e., photostimulation) alone produced significant freezing and fear responses indicating that activation of auditory projections to LA neurons not only act as a conditioned stimulus but also elicit fear conditioning and long-term associative memory (Kwon, Nakajima, Kim, Jeong, Augustine, & Han, 2014).

The regulation of sensory information flow to and from the amygdala represents a dynamic interplay between varieties of neuronal connections within the basolateral amygdala (Lang & Paré, 1998). During stress and anxiety, the primary role of the amygdala is to receive sensory input, regulate synaptic transmission associated with the input, and produce outward, feed-back stimulation to gate the responsiveness to the threatening or fearful stimuli (Sah et al., 2003). The BLA contains distinct population of interneurons which regulate excitatory afferent projections to the BLA and promote

GABAergic inhibition onto excitatory pyramidal neurons exiting the BLA (Lang & Paré, 1998; Wolff et al., 2014). These subpopulations of interneurons represent important microcircuitry within the BLA to suppress and control fear responses and fear conditioning. Recently, Wolff et al. (2014) examined the role of parvalbumin (PV) and somatostatin (SOM) expressing BLA interneurons in acquisition of fear learning. As previously stated, one of the major roles of interneurons is to exert GABAergic inhibitory transmission onto excitatory principal neurons in the BLA to gate amygdala-dependent fear responses (Sah et al., 2003; Janak & Tye, 2015). Optogenetic stimulation of PV expressing interneurons during fear conditioning paradigm containing a footshock (CS) with auditory tone (CS) pairing produced significant reductions in conditioned fear induced freezing responses to CS (Wolff et al., 2015). While PV activity during fear conditioning reduces freezing responses and excitatory footshock responses during retrieval, footshocks during conditioning inhibited PV cell activity. Inhibition of BLA interneurons by US during conditioning lead to increased principal neuron excitation and enhanced conditioned fear response to footshock. In addition to this disinhibited BLA principal neuron components, optogenetic activation of PV interneurons during the CS alone after US-CS fear conditioning inhibits SOM interneurons which also synapse onto BLA principal neurons; this manipulation causes disinhibition of principal neurons by SOM and overrides both dendritic inhibitory synapses from SOM interneuron on principal neurons. Therefore, fear conditioning within the BLA is regulated by complex interneuron microcircuitry producing somatodendritic inhibition onto principal neurons; in addition, stimulation of PV interneurons during exposure to conditioned fearful stimulus elicits a disinhibitory response on to SOM neurons allowing for increased

excitatory output from BLA principal neurons and enhanced fear responsiveness (Wolff et al., 2014). Internal BLA interneuron represent an important gating mechanism which regulates sensory information flow from cortical and subcortical information to the BLA and outward flow of processed information to CeA or back to cortical regions. Therefore, understanding the mechanism in which ethanol potentiates inhibitory transmission onto excitatory glutamatergic BLA neurons after stress may provide evidence for gender-specific pharmacological treatment for alcohol use problems and PTSD.

Amygdala Physiology, Acute Ethanol and Stress

In the United States, alcohol use disorder (AUD) is a growing public health concern with approximately 15 million adults ages 18 and older diagnosed with AUD (SAMHSA, 2015). Alcohol consumption produces significant positive reinforcing states such as relaxation, and reduces negative affective states including relief of stress and anxiety (Cooper et al., 1995). Individuals with stress-related and anxiety disorders, including post-traumatic stress disorder (PTSD), are motivated to consume alcohol to manage sleep difficulties (Nishith, Resnick, & Mueser, 2001) and reduce hyperarousal (Stewart et al., 2000). In addition, development of PTSD precedes alcohol dependence in women compared to men (Sonne et al., 2003) and women with PTSD and alcohol use disorders (AUDs) are more likely to consume alcohol to alleviate symptoms of stress and anxiety (Lehavot et al., 2014). When examining alcohol consumption only, males have a slightly higher consumption rate compared to females (males: 56.22%; females: 47.4%). However, in adolescence this gender gap is reduced, and males and females exhibit the same alcohol use rate of 9% (Center for Behavioral Health Statistics and Quality, 2016). From a biological perspective, females and males significantly differ in acute and long-

term effects of alcohol on brain (Mann et al., 2005), endocrine (Rivier, 1993)), and metabolism (Sutker et al., 1983). However, we do not know sex-related differences in the physiological mechanisms underlying the discrepancy in prevalence rates of AUD in males and females. While the anxiolytic effects of ethanol are well known, sex differences in the effects of ethanol on membrane physiology in areas of the brain that regulate emotion/anxiety, such as the basolateral amygdala (BLA), are currently unknown. Therefore, determining sex differences in the physiological mechanisms of alcohol on neurons in the BLA may elucidate the mechanisms important in sex disparities in alcohol use disorders.

The basolateral amygdala (BLA) plays a vital role in integrating sensory information and establishing the emotional salience of environmental stimuli. Together with the medial and orbital prefrontal cortex (Porrino et al., 1981), the BLA and central nucleus of the amygdala (CeA) regulate both stress and anxiety (LeDoux, 2000; Sah et al., 2003). Furthermore, the BLA plays a crucial role in drug dependence and ethanol-reinforcing actions (Koob & Le Moal, 2001). Glutamatergic pyramidal neurons located in the BLA provide major excitatory input to the CeA and the nucleus accumbens (Perra et al., 2008). Electrophysiological studies have shown that acute ethanol inhibits spontaneous firing rates in BLA projection neurons (Perra et al., 2008), leading to reduced glutamatergic output of the BLA. Therefore, considering the critical role of the BLA in regulating stress, anxiety, and substance abuse, it is important to understand specific neurophysiological mechanisms that drive excitability of the BLA and determine how ethanol modulates these cellular mechanisms differently in males and females.

Although there are a range of firing patterns, BLA pyramidal neurons are recognized physiologically by the presence of spike frequency adaptation and/or strong action potential accommodation in the presence of a depolarizing stimuli (Sah et al., 2003; Keele & Randall, 2003). Increasing accommodation in the BLA may lead to a reduction in excitatory glutamatergic transmission and reduce emotional behavior associated with stress and anxiety. In addition to depolarization-activated mechanisms such as calcium-activated potassium conductances (Faber & Sah, 2005) alter firing patterns, hyperpolarization-activated mechanisms also affect neuronal firing. The hyperpolarization-activated cyclic nucleotide dependent non-selective cationic current (I_h) is a slow inward current mediated by a mix of Na^+ influx and K^+ efflux. I_h may regulate neuronal excitability by bringing the cell back to a more depolarized state to allow further action potential firing (Robinson & Siegelbaum, 2002). I_h has been found throughout the central nervous system, including CA1 hippocampal pyramidal neurons (Lörincz et al., 2002), thalamacortical relay neurons (McCormick & Bal, 1997), and the basolateral amygdala (BLA) (Herman & Keele, 2007). Within the thalamus, activation of I_h generates slow depolarization of thalamacocortical neurons triggering calcium-dependent spike firing (McCormick & Bal, 1997). In the medial amygdala (MeA), inhibition of I_h promotes an increase of excitatory glutamatergic transmission on to GABAergic interneurons in the amygdala which release inhibitory transmission onto pyramidal-like neurons (Zhang et al., 2016). Together, this suggests that I_h in the amygdala may have regulatory control over neuronal excitability and have a critical role in controlling amygdala-dependent behaviors such as fear/anxiety and substance abuse. While the effect of ethanol on the amygdala and fear behaviors is well-established, sex

differences in the effects of ethanol on BLA neurophysiology and the effect of traumatic stress on ethanol-induced changes to BLA activity are currently unknown. Uncovering sex differences in BLA neuronal membrane properties in stressed animals may reveal potential neural mechanisms associated with resilience or susceptibility to stress and improve individualized treatment.

Single-Prolonged Stress: An Animal Model of PTSD

Animal models have become increasingly important in stress and anxiety research because we can create stress models to induce symptomology of stress and anxiety disorders found in humans, and further understand the neurobiology and brain mechanisms associated with these specific disorders. The BLA has been a primary area of research in electrophysiology studies because it becomes significantly elevated in activity in response to stress (Keele, Hughes, Blakeley, & Herman, 2007; Hetzel and Rosenkranz, 2014). Recently, Hetzel and Rosenkranz (2014) examined the effects of repeated restraint stress in adolescent and adult male rats BLA neuronal excitability. In adolescent rats, restraint stress increased both BA and LA neuronal excitability, or increased mean action potentials during depolarization steps. In addition to Hetzel and Rosenkranz (2014), previous studies found that a single 1 hr restraint stress procedure increased neuronal hyperexcitability in LA neurons (Guo et al., 2012). While these findings provide evidence as to how physical or psychological stressors may affect mechanisms important in regulating stress, conclusions from single-model stress paradigms remain limited in their ability to understand neurobiology and physiological aspects of PTSD in humans.

To further understand the neurobiology involved with PTSD, animal models of PTSD have been developed to mimic the pathophysiological and behavioral changes that occur in humans (Yamamoto et al., 2009). An animal model of PTSD has been established in which animals are exposed to a single-prolonged stress (SPS) paradigm (Liberzon, Krstov, & Young, 1997). Liberzon et al. (1997) first developed the SPS model to show a single-day multimodal stress procedure in rodents can develop similar PTSD symptomology found humans including overactive and abnormal HPA-axis negative feedback mechanisms, shown through increased plasma ACTH after SPS exposure. Later studies prove that the SPS model causes changes in brain mechanisms similar to PTSD individuals in rodents including reduced GABAergic levels in the hippocampus (Harvey, Oosthuizen, Brand, Wegener, & Stein, 2004), increased contextual freezing after contextual fear conditioning (Takahashi et al., 2006; Perrine et al., 2016), amplifies acoustic startle response (Khan & Liberzon, 2004), longer immobilization time as an indicator of fear responses to trauma-related stimuli (Wu et al., 2016), and impaired fear extinction after contextual fear conditioning (Yamamoto, Morinobu, Takei, Fuchikami, Matsuki, Yamawaki, & Liberzon, 2008; Perrine et al., 2016). Recently, Perrine et al (2016) conducted an extensive study on neurobiological, neuroendocrine, and behavioral abnormalities exhibited in PTSD and reported mice administered subcutaneous (s.c.) injection of dexamethasone after SPS significantly suppressed plasma CORT levels suggesting SPS affects HPA-axis activity to a greater extent than controls and SPS animals are more sensitive to dexamethasone treatment. Glutamate levels in the PFC were also significantly reduced in SPS mice indicating SPS can alter cortical neuronal excitability during stress. Finally, a single study has recently exposed female rats to SPS

and examined cued fear extinction retention effects and glucocorticoid receptors in the dorsal and ventral hippocampus. Interestingly, female subjects exhibited dorsal hippocampus up regulation of GR level and no cued fear extinction retention deficits. This may suggest females are resilient to the SPS model.

These stressed induced impairments are similar to human PTSD symptomology and changes in brain mechanisms reported in humans. The SPS model consists of three behavioral stress paradigms including 2 hour restraint, a 20 minute forced swim test, and loss of consciousness by anesthesia following forced swim test (Liberzon et al., 1997). Animals are allowed a seven day recuperation period after the final stress paradigm; this recuperation period allows for neuropathological changes to occur in response to SPS (Yamamoto et al., 2009). One of the benefits of the SPS model is its short duration time, but severe, multimodal procedures are able to elicit neuroendocrine and neurobiology changes found in PTSD. Therefore, to take advantage of techniques that are advantageous for understanding brain mechanisms associated with PTSD, the SPS model should be implicated in whole-cell electrophysiology research to investigate the underlying neuronal mechanisms, specifically in the amygdala, that are associated with regulation of stress responses. To date, there are limited studies examining the effects of SPS on amygdala functioning, and to the best our knowledge no study has looked at SPS-induced hyperexcitability in the BLA between genders and in the presence of acute ethanol. Therefore, with the establishment of a PTSD animal model, we can establish the effects of ethanol on PTSD neural circuitry in the BLA and understand pathophysiological and behavioral changes that are associated with gender-differences in PTSD.

Summary and Significance

Understanding the neurophysiological effects of acute ethanol on both male and female BLA neurons may lead to more effective treatment strategies for anxiety disorders such as post-traumatic stress disorder (PTSD), where alcohol use disorders are highly comorbid. Individual responses to the negative consequences of stress vary (Rutter, 1985), and the female stress response biologically differs from the male stress response (Olff et al., 2007). Understanding the sex-related factors that contribute to BLA neuron membrane properties in stressed animals may point to potential neural mechanisms associated with gender differences in resilience or susceptibility to stress, and potentially improve individualized treatment.

The repeated application of a stress paradigm in rodents produces significant BLA neuronal hyperexcitability and is likely to contribute to amygdala-dependent anxiety and stress behaviors (Hetzel & Rosenkranz, 2014). Additionally, ethanol potentiates GABAergic inhibition in BLA neurons, thereby regulating excitatory transmission and playing an integral role in controlling anxiety-like behaviors (Silberman et al., 2008). However, the utility of SPS, an animal model of PTSD, and the interaction between stress and alcohol between genders on BLA neuronal excitability using this model has yet to in determined. Considering that the prevalence of PTSD and comorbid alcohol abuse has been shown to vary between genders (Sonne et al., 2003; Najavits et al., 1997), it follows that understanding the neurophysiological basis of sex differences in amygdala physiology following exposure to stress and ethanol. Therefore, there is an *urgent* need to fill this gap in our fundamental understanding in order to enhance the chance of

developing more effective and gender-specific clinical prevention and treatment strategies for individuals with PTSD and alcohol abuse.

The *long-term goal* of this line of research is to determine the cellular mechanisms that underlie gender differences in increased alcohol abuse in anxiety disorders. The *specific objective* of this project is to determine how the SPS model affects BLA physiology and how stress-induced changes in BLA neurophysiology are altered by ethanol in males and females. The *rationale* for this study is that determining how ethanol affects SPS-induced hyperexcitability in BLA neurons will yield important new information about comorbid anxiety and substance abuse disorders and reveal important differences between males and females.

CHAPTER TWO

Materials and Methods

Animals

All animal procedures were conducted according to a protocol approved by the Institutional Animal Care and Use Committee of Baylor University. Male and female Sprague-Dawley rats approximately 60-120g were group housed with their littermates and weaned on day postnatal day 21 (P21). Animals had ad libitum access to food and water and were maintained on a 12-hour light/dark cycle. To control for litter effects, only one animal of either sex was assigned to either the control or SPS group.

Single-Prolonged Stress

Procedure

Both male and female rats underwent SPS. Date of SPS Day 1 ranged from PD 25-50. Animals in the SPS group were subjected to 2 hour whole body restraint in an animal holder, followed immediately by a 20 minute forced swim test. Exactly 15 min after completion of the swim stress, animals lost consciousness by inhalation of ether (Liberzon et al., 1999, Ding, Han, & Shi, 2010; Ganon-Elazar & Akirav, 2012).

Restraint was accomplished by immobilizing animals inside a cylindrical plastic tube (Med Associates, Inc.) that was 7.5 cm in diameter x 19 cm long. Restraint tubes were placed on a hard surface atop a clean, dry absorbent pad to absorb urine. Animals were confined individually and continuously for 2 h in the restraint tubes (Crawley et al., 2003). Forced swim stress consisted of placing animals in a plastic cylinder 50.5 cm tall x

20 cm in diameter that had been filled with tap water to a depth of 28 cm and left to reach room temperature of approximately 22° C. Animals were placed in the water facing the wall of the cylinder and left to swim for 20 min. After forced swim, animals were gently removed and placed into a dry bath towel to dry the entire body surface of the animal then placed in a clean, dry cage. Loss of consciousness was accomplished by exposing animals to diethyl ether. Ether (3mL) was pipetted onto a cotton ball and placed in a 50 mL centrifuge tube that was placed inside a plastic cylinder containing a slotted keeper to retain the animal within the tube. The animal was gently placed into the tube and exposed to the ether-soaked cotton ball for 5 min. Animals were then removed from the tube and placed in their home cage to recover from loss of consciousness. Following SPS, animals were individually housed where they remained for seven days to allow for neuropathological changes to occur in response to SPS (Yamamoto et al., 2009). Unstressed control (USC) rats were keep in identical cages and undisturbed except for normal husbandry.

Restraint Stressor

Animals were immobilized inside a cylindrical plastic tube (Med Associates, Inc.), 7.5-cm diameter, 19 cm long. Restraint tubes were placed on a hard surface atop a clean, dry absorbent pad to absorb urine. Subjects were confined individually and continuously for 2 h in the cylindrical plastic tubes. The subjects were allowed to walk into the open end of the restraint tube, then a slotted keeper was inserted into the tube so that the animals could neither depart the tube nor contort end-for-end within the enclosure. Subjects were not allowed to exhibit freedom of movement; if the animal moved or was able to move, the slotted keeper advanced one position to press more

firmly against the subject. At the conclusion of restraint, the subjects were removed by withdrawing the slotted keeper and allowing the subject to back out of the tube, or the researcher grasped the base of the tail and gently withdrew the animal. Restraint tubes were washed and thoroughly dried prior to reuse in order to avoid transmission of olfactory cues.

Forced Swim Stressor

Immediately following the duration of restraint stress, animals were placed in a cylindrical plastic container 50.5 cm tall x 20 cm in diameter. The cylinder was is filled with tap water to a depth of 28 cm and left to reach room temperature of approximately 22° C. Animals were then placed in the water, facing the wall of the cylinder, and left to swim for 20 min. After duration of swim test, animals are gently removed from the tube and placed into a dry bath towel to dry the entire body surface of the animal. Following the duration of the forced swim stressor, animals were allotted a 15 min recuperation period. During the 15 min recuperation period, animals were kept in their animal cage in which they recovered in after the swim test.

Loss of Consciousness Stressor

During the 15 min recuperation period, researchers prepared the ether anesthetic exposure. Either was pipetted onto a cotton ball which was placed inside an open test tube. The test tube was placed inside an air-tight cylindrical tube for approximately 10 min. All holes or places where air could escape from the cylindrical tube were cover with tape. After the duration of the 15 min recuperation period, the animal was removed from the home cage and exposed to the ether for approximately 5 min or until loss of

consciousness. After 5 min ether exposure the animal was removed from the cylindrical tube, placed on their back in the home cage and loss of consciousness was determined through loss of righting reflex by plac (Ganon-Elazar & Akirav, 2012).

Tissue and Slice Preparation

Brain slices were prepared for electrophysiological recording from USC and SPS animals 8 days after the SPS procedure. Rat brains were removed through rapid decapitation and placed in cold (4°C) low calcium artificial cerebrospinal fluid (low-Ca^{2+} aCSF) containing (in mM): NaCl (104), KCl (4.7), $MgCl_2$ (6), NaH_2PO^4 (1.2), $CaCl_2$ (0.5), glucose (11.5), and $NaHCO_3$ (25), aerated with 95% O_2 5% CO_2 mix. Brains were blocked by two coronal cuts (one anterior to the cerebellum, one posterior to the optic chiasm), and one horizontal cut to remove the cortex superior to the hippocampus. A midsagittal cut separated the hemispheres. Slices (500μm) containing the amygdala were made using a vibrotome. Slices remained in low Ca^{2+} aCSF for at least one hour to acclimate to room temperature before recording (Keele et al., 1997; Keele & Randall, 2003).

Electrophysiological Recording

Recording Rig Setup

Brain slices containing the BLA were transferred to a recording chamber where they were continuously superfused with control aCSF that contained (in mM), NaCl (117), KCl (4.7), $MgCl_2$ (1.2), NaH_2PO4 (1.2), $CaCl_2$ (2.5), glucose (11.5), and $NaHCO_3$ (25), aerated with 95% O2 5% CO_2.

Recording electrodes having 2-5 MΩ tip resistance were pulled from borosilicate glass capillary tubing (Drummond Scientific, Broomall, PA) using a Flaming-Brown puller (Sutter Instruments, Foster City, CA). The recording electrode internal solution consisted of (in mM): potassium-gluconate (122), NaCL (5.0), $MgCl_2$ (2.0), $CaCl_2$ (0.3), EGTA (1.0), HEPES (10.0), Na_2ATP (5.0), Na_3GTP (0.4). The blind approach (Blanton et al., 1989) was used to conduct whole-cell recordings from BLA neurons. After obtaining a tight seal (resistance >1 GΩ) the neuronal membrane was ruptured and neurons were voltage-clamped at a holding potential of -60 mV. All data were collected using pClamp software (v9.x; Molecular Devices, Foster City, CA). Only neurons that exhibited a resting membrane potential (RMP) of at least -50 mV and action potentials that overshoot 0 mV were included in the data. Recordings were performed in both current and voltage clamp.

Experimental Protocols

Current/Voltage Protocol. Current/voltage relationship was obtained in voltage clamp mode from a holding potential (V_h) of -60 mV by applying a series of depolarizing and hyperpolarizing steps between -40 and -120 mV (400 ms duration). The steady-state membrane resistance was determined from a fit of the linear region of the I/V relationship, evoked by voltage steps between -50 to -70 mV. Resting membrane potential (RMP) was also measured from fitting the linear region of the current-voltage relationships as the membrane potential where I = 0.

H-Current Protocol. The hyperpolarization-activated, cyclic nucleotide dependent current (H-current, I_h) was determined using voltage clamp recordings. I_h was elicited from a holding potential of -40 mV by applying a series of hyperpolarizing voltage steps between -40 mV and -110 mV (2 s duration). I_h was calculated by subtracting instantaneous current from steady state current ($I_{ss} – I_i = I_h$). Instantaneous current was measured immediately following the decay of the capacitive transient current; approximately 5 - 20 ms after the step-change in Vm. Steady state was measured at the end of each trace. The voltage-dependence of I_h activation was determined by normalizing the amplitude of I_h evoked at each hyperpolarizing step (I_o) to the maximum current elicited from hyperpolarization to -110 mV (I_{max}). I_o/I_{max} was plotted as a function of membrane potential, and the data were fit to the Boltzmann equation $Y = A+ (B-A)/(1+e^{[(V1/2-Vm)/k]})$, where Y is the normalized current Io/Imax, A is the maximum conductance (constrained to A = 1); B is the minimum conductance (constrained to B = 0), $V_{1/2}$ is the voltage at which the conductance is half-activated, and k is the steepness of the curve. Data were fit using a least squares nonlinear regression with Prism (ver. 5, GraphPad Software, La Jolla, CA). Goodness of fit was determined by calculating the total variance (R^2) of I_0/I_{max} explained by the fit ($R^2 = 1 – SS_{fit}/SS_T$, where SS_{fit} is the sum of squares of the best-fit nonlinear regression and SS_T is the total sum of squares).

Accommodation Protocol. Current clamp recordings were used to determine the active properties of BLA neurons. Action potential threshold was determined in current clamp mode from a membrane potential of -60 mV. Small depolarizing current steps (10 ms duration) of increasing amplitude were delivered until the first single action potential

was elicited. Action potential accommodation was determined by applying depolarizing current steps between -100 and 600 pA (600 ms duration). Accommodation was quantified as the number of spikes elicited during each depolarizing current step. The time constant for membrane charging (τ_m) was also determined in current clamp by an exponential fit of the initial 30 ms voltage response to injecting 50 pA hyperpolarizating current.

Ethanol Superfusion. EtOH (30 mM) was added to the aCSF bath solution and applied by superfusion. Passive and active membrane properties of BLA neurons were recorded prior to the superfusion of ethanol (control) and again following at least 10 min of ethanol superfusion.

Estrous Cycle Monitoring. Vaginal smears were obtained from female rats in both the control and SPS conditions to confirm stage of estrous cycle. For controls, samples were taken immediately before tissue collection and in SPS-treated females, samples were taken on Day 8 immediately prior to tissue collection for electrophysiological recordings.

Statistical Analysis. The effect of ethanol on BLA excitability was determined by analysis of variance (ANOVA) using JMP 13 (SAS, Cary, NC). Main effects of sex and ethanol, as well as sex x gender interaction were calculated by two-way ANOVA where the ethanol condition was treated as a repeated measure. To determine ethanol-induced changes in neuronal excitability and I_h amplitude in SPS animals, we applied ANOVA

with the following factors (levels): factor 1 is sex (male, female), factor 2 is ethanol treatment (Control, Ethanol) and treated as a repeated measure. To determine SPS-induced changes in neuronal excitability and I_h amplitude, data were analyzed by analysis of variance (ANOVA) with the following factors (levels): factor 1 is sex (male, female), factor 2 is SPS (Control, SPS). Post-hoc comparisons were evaluatedd by Student's t-test, corrected for multiple comparisons. All data are expressed as mean ± standard error of the mean (SEM). The acceptable type I error rate was $p < 0.05$.

CHAPTER THREE

Results

Sex Differences in Ethanol-Induced Inhibition of Action Potential Firing

Neuronal excitability of basolateral amygdala (BLA) neurons was determined in brain slices from both male and female rats (figure 1). Excitability was measured in current clamp mode as the number of action potential spikes in response to depolarizing current injection. A series of depolarizing steps (200-450 pA, 600 ms) was delivered in normal aCSF (Control) and again during superfusion of ethanol (EtOH 30 mM). Representative recordings obtained in control aCSF are shown in figures 1A (male) and figure 1D (female). Representative recordings obtained during the superfusion of EtOH (30 mM) are shown in figure 1B (male) and figure 1D (female). Two-way ANOVA revealed a significant main effect of ethanol ($F(1,66)=30.9$, $p < 0.0001$) and a significant gender x ethanol interaction ($F(1,66)=4.1$, $p < 0.05$). There was no significant main effect of gender ($p > 0.05$). Post-hoc comparisons (Student's t-test) revealed that ethanol (30mM) significantly decreased the number of action potentials elicited in BLA neurons from male rats only (figure 1C; $t(5)=2.23$, $p < 0.05$), and not in BLA neurons from female rats (figure 1F; $p > 0.05$). The EtOH-induced inhibition of excitability occurred without changes in resting membrane potential (RMP), input resistance (R_{in}), or the membrane time constant (τ_m) (Table 1). Two-way ANOVA reveal no significant main effects or significant gender x EtOH inter actions on any of these resting membrane properties.

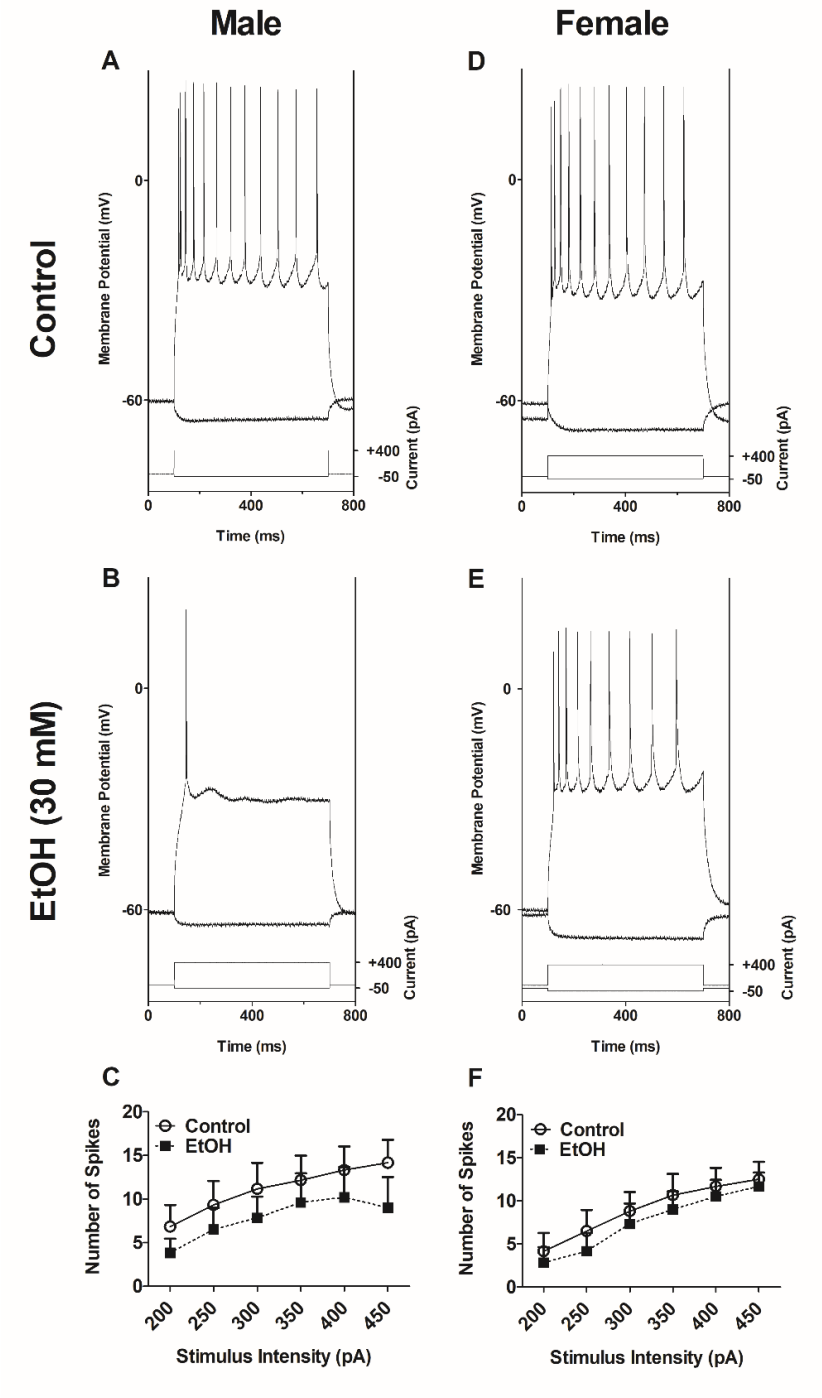

Figure 1. Ethanol (EtOH) decreases neuronal excitability of basolateral amygdala (BLA) neurons from male (A – C), but not female (D – E) rats. Representative action potential firing in BLA neurons before (Control, **A** and **D**) and during superfusion of EtOH (30 mM, **B** and **E**). Action potentials were elicited from a holding potential of -60 mV by a series of depolarizing current step. Responses to 400 pA depolarizing current steps are shown. Summary data from all neurons are shown in **C** (males, N = 6) and **F** (females, N = 6). Data are shown as mean ± S.E.M.

Table 1. Mean RMP, R_{in}, and τ

Treatment	RMP (mV)		R_{in} (MΩ)		τ (ms)	
	Male	Female	Male	Female	Male	Female
Control	-66 ± 4	-58 ± 3	191 ± 62	146 ± 24	20 ± 3	17 ± 3
EtOH	-63 ± 6	-58 ± 2	174 ± 72	166 ± 34	17 ± 5	17 ± 3

Notes 1. Ethanol (EtOH, 30 mM) had no effect on resting membrane potential (RMP) or electrotonic properties of BLA neurons from male and female rats. For RMP, there was no significant main effect of gender ($F(1,8) = 0.2$, $p > 0.05$), no significant main effect of EtOH ($F(1,8) = 1.34$, $p > 0.05$), and no significant gender x EtOH interaction ($F(1,8) = 0.05$, $p > 0.05$). For input resistance (R_{in}), there was no significant main effect of gender ($F(1,8) = 0.01$, $p > 0.05$), no significant main effect of EtOH ($F(1,8) = 0.28$, $p > 0.05$), and no significant gender x EtOH interaction ($F(1,8) = 0.14$, $p > 0.05$). For tau (τ), there was no significant main effect of gender ($F(1,8) = 0.32$, $p > 0.05$), no significant main effect of EtOH ($F(1,8) = 0.15$, $p > 0.05$), and no significant gender x EtOH interaction ($F(1,8) = 0.09$, $p > 0.05$).

Ethanol Inhibits Hyperpolarization-Activated Current (I_h) in BLA Neurons from Males but Not from Females

The inhibition of action potential firing produced by EtOH occurred without changes in resting membrane properties, suggesting that the cellular mechanism underlying the inhibitory effect of EtOH is not active at rest. We therefore determined the effect of EtOH on the hyperpolarization-activated, cyclic nucleotide-gated cation current, I_h. I_h was elicited in voltage clamp mode by a series of hyperpolarizing steps (2 s duration) from a holding potential of -40 mV. I_h was elicited in BLA neurons from both males and females, in control aCSF and during the superfusion of EtOH (30 mM). Results are shown in figure 2. Two-way ANOVA revealed significant main effects of both gender ($F(1,9) = 7.60$, $p < 0.05$) and EtOH ($F(1,9) = 8.112$ $p < 0.05$), and no significant gender x EtOH interaction ($F(1,9) = 0.82$, $p > 0.05$). Post-hoc comparisons showed that I_h amplitude in BLA neurons from male rats was significantly larger than I_h in neurons from females ($t(7) = 2.31$, $p < 0.05$). In BLA neurons from male rats, EtOH (30 mM) significantly decreased I_h amplitude in 5 of 6 neurons examined from 143 ± 42

pA in control to 63 ± 22 pA in the presence of EtOH ($t(7) = 2.54$, $p < 0.05$). Ethanol decreased I_h amplitude in BLA neurons from female rats from 63 ± 12 pA to 13.8 ± 7 pA, but this was not significant ($p > 0.05$).

To further define further the cellular mechanism of EtOH-induced inhibition of I_h, we constructed activation curves (Figure 2C). The I_h current amplitude elicited at each voltage step (I_o) was normalized to the maximal current (I_{max}) elicited from hyperpolarization to -110 mV. I_o/I_{max} was fit with the Boltzman equation (see Methods). In BLA neurons from male rats, the activation data were well fit with Boltzman equation (Control: $r^2 = 0.8$; EtOH $r^2 = 0.7$). The activation curves show the conductance underlying I_h begins to open with hyperpolarization to approximately -70 mV. In control aCSF, the half-maximal activation ($V_{1/2}$) was 89 ± 2 mV. In the presence of EtOH (30 mM), $V_{1/2}$ was slightly hyperpolarized to 92 ± 2 mV ($t(5) = 1.48$, $p > 0.05$). In BLA neurons from female rats, $V_{1/2}$ was -80 ± 5 mV (data not shown), but these data were not well-fit using the Boltzman equation ($r^2 = 0.4$). We did not construct an activation curve for I_h in the presence of EtOH recorded in neurons from females because the current amplitude was not consistently observed in these conditions. Together, these data show that EtOH inhibits I_h specifically in BLA neurons from males without changing the voltage-dependence of the underlying conductance.

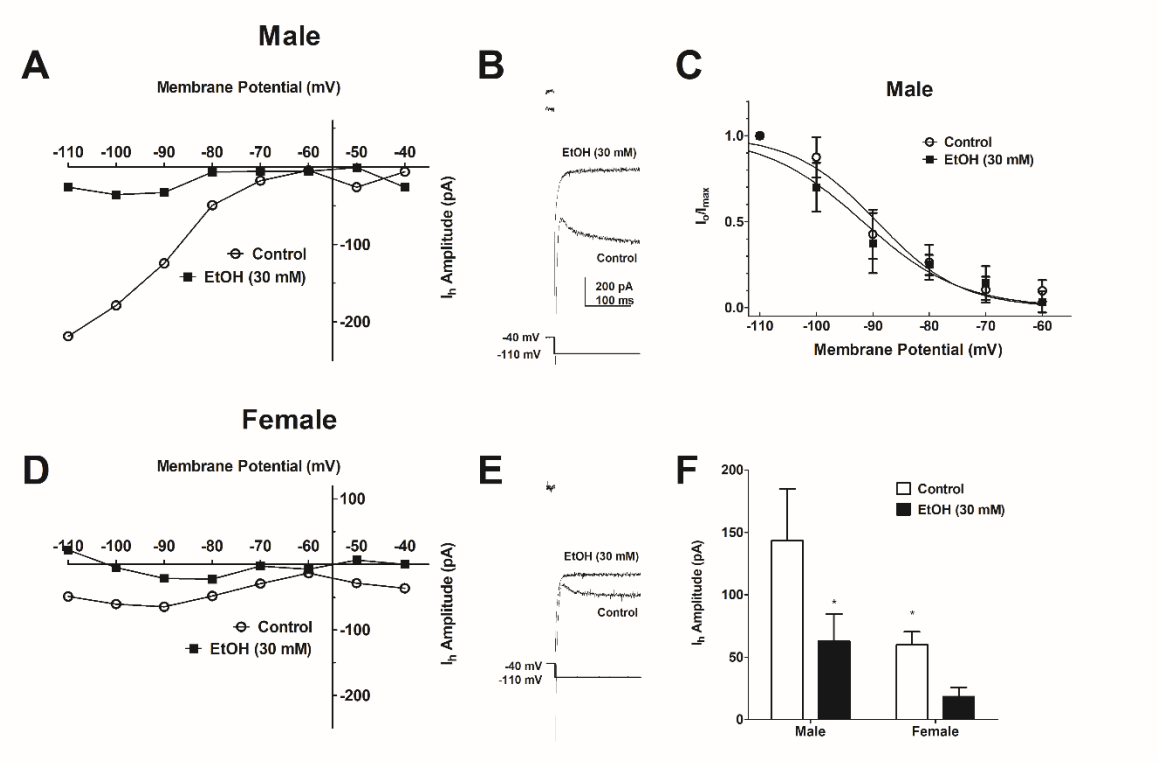

Figure 2. Ethanol decreased the amplitude of the hyperpolarization-activated current (I_h) in BLA neurons from both male and female rats. I_h was elicited from a holding potential of -40 mV by a series of hyperpolarizing voltage steps in 10 mV increments (2 s duration) to -110 mV. **A.** Current–voltage relationships of I_h obtained before (Control, ○) and during ethanol (30 mM; ■) in a typical BLA neuron from a male rat. **B.** Representative recordings of I_h elicited upon hyperpolarization to -110 mV before (Control) and during EtOH (30 mM) from the same neuron shown in A. **C**. Activation curves of I_h before and during superfusion of EtOH (30 mM) show no EtOH-induced change in voltage dependence of the underlying conductance. Current-voltage relationships and representative I_h recordings in BLA neurons from a female are shown in **D** and **E**, respectively. Scale in B is the same in E. **F**. The amplitude of I_h evoked upon hyperpolarization to -110 mV in males (left, N = 5) and females (right, N = 6) before (Control, open bars) and during superfusion of EtOH (30 mM, filled bars). Data are shown as mean ± S.E.M. * = $p < 0.05$ compared to male/control (ANOVA followed by Student's t test. See text for details).

Single-Prolonged Stress Does Not Alter Passive Membrane Properties of BLA Neurons from Either Male or Female Rats

SPS produced no changes in resting membrane potential (RMP), input resistance (R_{in}), or the membrane time constant (τ_m) (Table 2). Two-way ANOVA reveal no significant main effects or significant Sex x SPS interactions on any of the resting membrane properties examined. For RMP, there was no significant main effect of sex ($F(1,36) = 0.01$, $p > 0.05$) or SPS ($F(1,36) = 0.57$, $p > 0.05$), and no sex x SPS interaction

($F(1,36) = 1.13$, $p > 0.05$). For input resistance (Rin), there was no significant main effect of sex ($F(1,36) = 0.54$, $p > 0.05$) or SPS ($F(1,36) = 2.18$, $p > 0.05$), and no sex x SPS interaction ($F(1,36) = 0.08$, $p > 0.05$). For tau (τ), there was no main effect of sex ($F(1,36) = 3.03$, $p > 0.05$) or SPS ($F(1,36) = 2.57$, $p > 0.05$), and no sex x SPS interaction ($F(1,36) = 0.11$, $p > 0.05$). These data suggest SPS does not change electrotonic properties of BLA neurons in either male or female rats.

Table 2. Mean RMP, R_{in}, and τ

Treatment	**RMP (mV)**		**R_{in} (MΩ)**		**τ (ms)**	
	Male	Female	Male	Female	Male	Female
Control	-61 ± 3	-57 ± 2	172 ± 25	160 ± 14	21 ± 3	16 ± 2
SPS	-55 ± 4	-58 ± 4	143 ± 21	120 ± 26	16 ± 2	13 ± 2

Notes 2. Single prolonged stress (SPS) had no conclusive effect on resting properties of BLA neurons from male and female rats. Data shown are membrane potential (RMP), input resistance (R_{in}), or the membrane time constant (tau, τ) for Male and Female rats in both the Control and SPS conditions. ANOVA revealed no significant main effects or interaction for any of the variables examined. See text for details. Data are shown as mean ± SEM from 20 BLA neurons from males (Control, N = 13; SPS, N = 7) and 20 BLA neurons from females (Control, N = 12; SPS, N = 8).

Single-Prolonged Stress Does Not Alter Action Potential Firing of BLA Neurons from Either Male or Female Rats

Neuronal excitability of basolateral amygdala (BLA) neurons was determined in brain slices from both male and female rats (figure 3). Excitability was measured in current clamp mode as the number of action potential spikes in response to depolarizing current injection. Representative recordings obtained in control aCSF are shown in figures 3A (male) and figure 3D (female). Representative recordings obtained in rats exposed to single prolonged stress (SPS) are shown in figure 3B (male) and figure 3D (female). Two-way ANOVA revealed no significant main effect of gender ($F(1,36)=1.24$,

p 0.05) or stress (F(1,36)=2.48, p>0.05) and no significant gender x stress interaction F(1,36)=1.03, p>0.05).

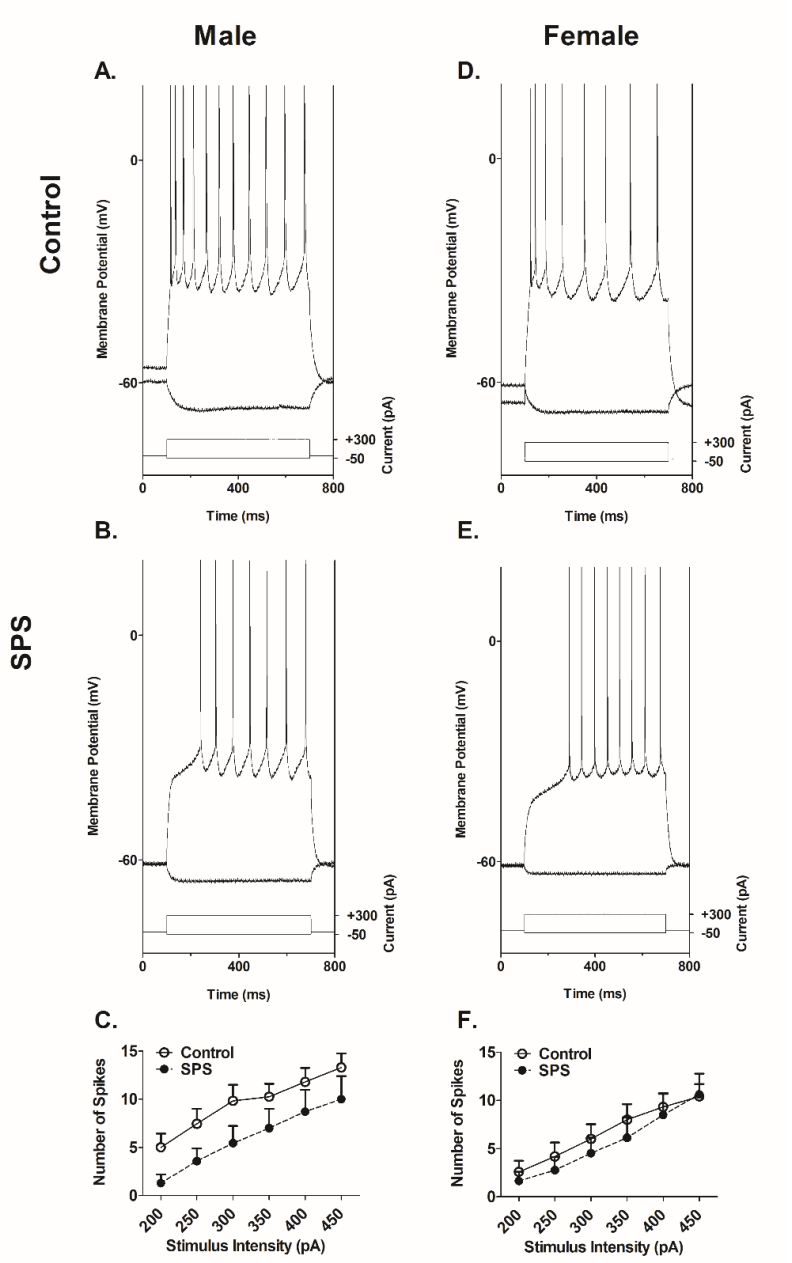

Figure 3. Single prolonged stress (SPS) did not change neuronal excitability of basolateral amygdala (BLA) neurons from male (A-C) or female (D-E) rats. Representative action potential firing in BLA neurons from rats in the unstressed control (Control, **A** and **D**) and single prolonged stress (SPS, **B** and **E**) conditions. Action potentials were elicited form a holding potential of -60 mV by a series of depolarizing current steps. Responses to 300 pA depolarizing current are shown in A and B (Male) and D and E (Female). Peaks of the action potentials are truncated at +30 mV. Summary data from all neurons are show in **C** (Males: Control, N = 13; SPS, N = 7) and **F** (Females: Control, N = 12; SPS, N = 8). Data are show as mean ± S.E.M. Two-way ANOVA revealed no significant main effects of sex or SPS, and no sex x SPS interaction (see text for details).

Sex Differences in Hyperpolarization-Activated Current (I_h) in BLA Neurons from Males and Females

To further examine further the active properties of BLA neurons in response to SPS, we determined the effect of SPS on the hyperpolarization-activated, cyclic nucleotide-gated cation current, I_h (figure 4). I_h was elicited in voltage clamp mode by a series of hyperpolarizing steps (2 s duration) from a holding potential of -40 mV. Two-way ANOVA revealed significant main effects of sex ($F(1,34) = 6.03$, $p < 0.05$) but no main effect of SPS ($F(1,34) = 0.43$, $p > 0.05$), and no significant sex x SPS interaction ($F(1,34) = 0.01$, $p > 0.05$). Post-hoc comparisons showed that I_h amplitude in BLA neurons from male rats was significantly larger than I_h in neurons from females (Figure 4F) (male: 94 ± 27 pA; female: 34 ± 9 pA; $t(33) = 2.07$, $p < 0.05$). We also examined the voltage-dependence of activation of I_h in BLA neurons from male rats in both Control and SPS conditions (Figure 4C). The I_h current amplitude elicited at each voltage step (I_o) was normalized to the maximal current (I_{max}) elicited from hyperpolarization to -110 mV. I_o/I_{max} was fit with the Boltzman equation (see Methods). In BLA neurons from male rats, the goodness of fit of activation data were $r^2 = 0.5$ for Control and $r^2 = 0.2$ for SPS. The activation curves show the conductance underlying I_h begins to open with hyperpolarization to approximately-70 mV. In BLA neurons from male rats, the half-maximal voltage of activation ($V_{1/2}$) was -87 ± 2 mV in Control rats, and -83 ± 4 mV in neurons from SPS-treated rats ($t(17) = 0.58$, $P > 0.05$). Voltage activation curves for I_h in neurons from female rats was not determined because the current amplitude was not consistently large. Together, these data confirm our previous findings that I_h is smaller in BLA neurons from female rats, and extend these finding by showing that SPS does not conclusively inhibit I_h.

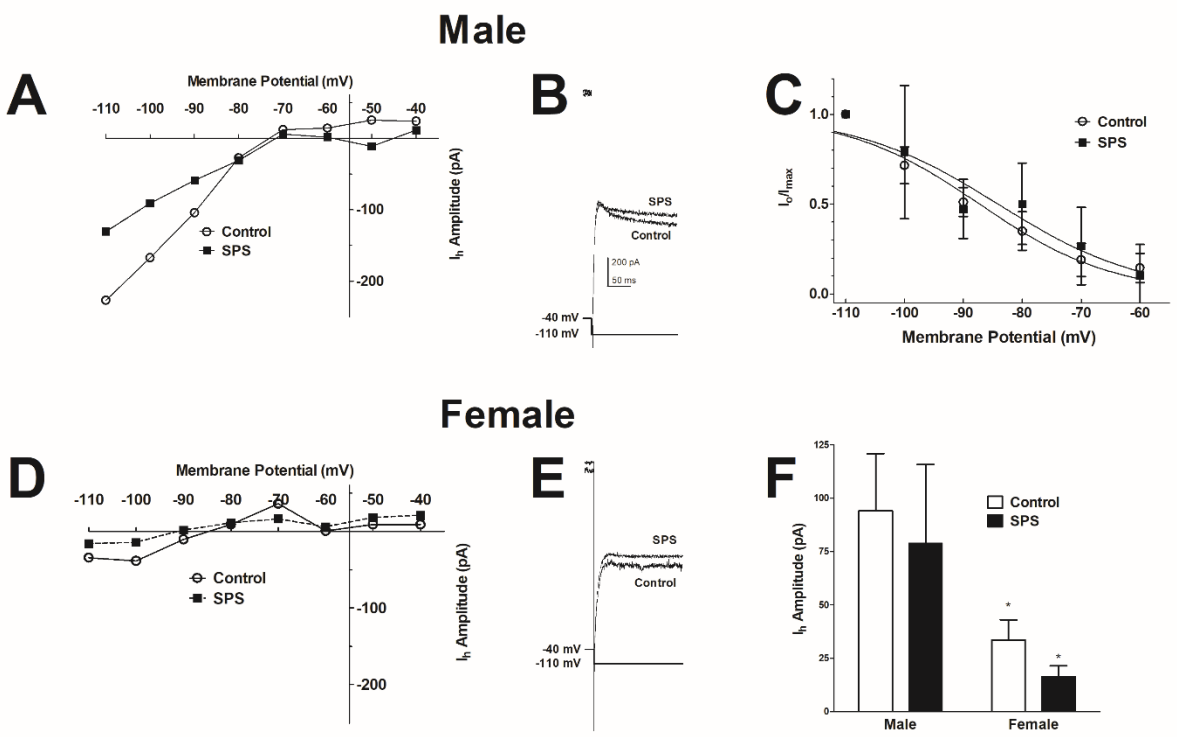

Figure 4. SPS does not alter the amplitude of the hyperpolarization-activated current (I_h) in BLA neurons from either male or female rats. I_h was elicited from a holding potential of -40 mV by a series of hyperpolarizing voltage steps in 10 mV increments (2 s duration) to -110 mV. **A**. Current-voltage relationships of I_h obtained from representative BLA neurons from male rats in the unstressed control (Control, ○) and single prolonged stress (SPS, ●) conditions. **B.** Representative recordings of I_h elicited upon hyperpolarization to -110 mV in BLA neurons from male SPS or control male rats. **C.** Activation curves of I_h in Control and SPS show no SPS-induced change in voltage-dependence of the conductance underlying I_h. Current-voltage relationships and representative I_h recordings in BLA neurons from female Control and SPS rats are show in **D** and **E**, respectively. Scale in B is the same in E. **F**. The amplitude of I_h evoked upon hyperpolarization to -110 mV in males (left; Control, N = 13, open bars; SPS, N = 7, filled bars) and females (right; Control, N = 12; SPS N = 6). Data are shown as mean ± S.E.M. * = $p < 0.05$, compared to male/control condition (ANOVA followed by Student's t test. See text for details).

Ethanol (EtOH) Does Not Affect Passive Membrane Properties of BLA Neurons from SPS-treated Rats

Ethanol-induced changes in resting membrane potential (RMP), input resistance (R_{in}), and the membrane time constant (τ_m) were determined in BLA neurons from both male and female SPS-treated rats (Table 3). Two-way ANOVA revealed no significant main effects or significant Sex x SPS interactions on any of the resting membrane properties examined. For RMP, there was no significant main effect of sex ($F(1,12) =$

0.25, $p > 0.05$) or EtOH ($F(1,12) = 3.0$, $p > 0.05$), and no significant sex x EtOH interaction ($F(1,12) = 0.42$, $p > 0.05$). For input resistance (R_{in}), there was no significant main effect of sex ($F(1,12) = 1.05$, $p > 0.05$) or EtOH ($F(1,12) = 0.04$, $p > 0.05$), and no significant gender x EtOH interaction ($F(1,12) = 0.23$, $p > 0.23$). For the membrane time constant Tau (τ), there was no significant main effect of gender ($F(1,12) = 1.46$, $p > 0.05$) or EtOH ($F(1,12) = 1.8$, $p > 0.05$), and no significant gender x EtOH interaction ($F(1,12) = 0.89$, $p > 0.05$). These data suggest that EtOH does not change electrotonic properties of BLA neurons from either male or female SPS-treated rats.

Table 3. Mean RMP, R_{in}, and τ.

Treatment	**RMP (mV)**		**R_{in} (MΩ)**		**τ (ms)**	
	Male	Female	Male	Female	Male	Female
Control	-64 ± 4	-63 ± 2	117 ± 9	135 ± 28	21 ± 39	14 ± 3
EtOH	-52 ± 8	-58 ± 5	108 ± 8	156 ± 39	14 ± 3	13 ± 3

Notes 3. Ethanol (EtOH, 30 mM) had no conclusive effect on membrane properties of BLA neurons from SPS-treated male or female rat. Data shown are membrane potential (RMP), input resistance (R_{in}), and the membrane time constant (tau, τ) for Male and Female rats, both before (Control) and during superfusion of ethanol (EtOH, 30 mM). ANOVA revealed no significant main effects or interaction for any of the variables examined. See text for details. Data are shown as mean ± SEM from N = 3 BLA neurons from SPS-treated male rats, and N = 5 BLA neurons from SPS-treated female rats.

Ethanol Decreases Neuronal Excitability in BLA Neurons from SPS-treated Rats Differently in Males and Females

While the effect of ethanol on the amygdala and fear behaviors is well-established, sex differences in the effects of ethanol on BLA neurophysiology and the effect of traumatic stress on ethanol-induced changes to BLA activity are currently unknown. Therefore, we examined the effects of acute ethanol on stress-induced changes in BLA excitability (Figure 3). Ethanol (30 mM) decreases action potential firing in SPS male (Figures 5A and 5B) and female (Figures 5D and 5E) rats following depolarizing

current steps (200-450 pA, 600 ms). There was no significant main effect of sex (p > 0.05). However, there was a significant main effect of ethanol (F(1,66)=12.51, p < 0.01). Superfusion of ethanol (30mM) significantly decreased spike firing in BLA neurons from females at larger depolarizing steps. EtOH also decreased firing at smaller depolarizing steps in BLA neurons from males but this was not significant (p > 0.05). There was no sex x EtOH interaction (F(1,66)=1.19, p > 0.05). These data show that EtOH-induced inhibition of BLA excitability is stronger in SPS-treated female than in male rats.

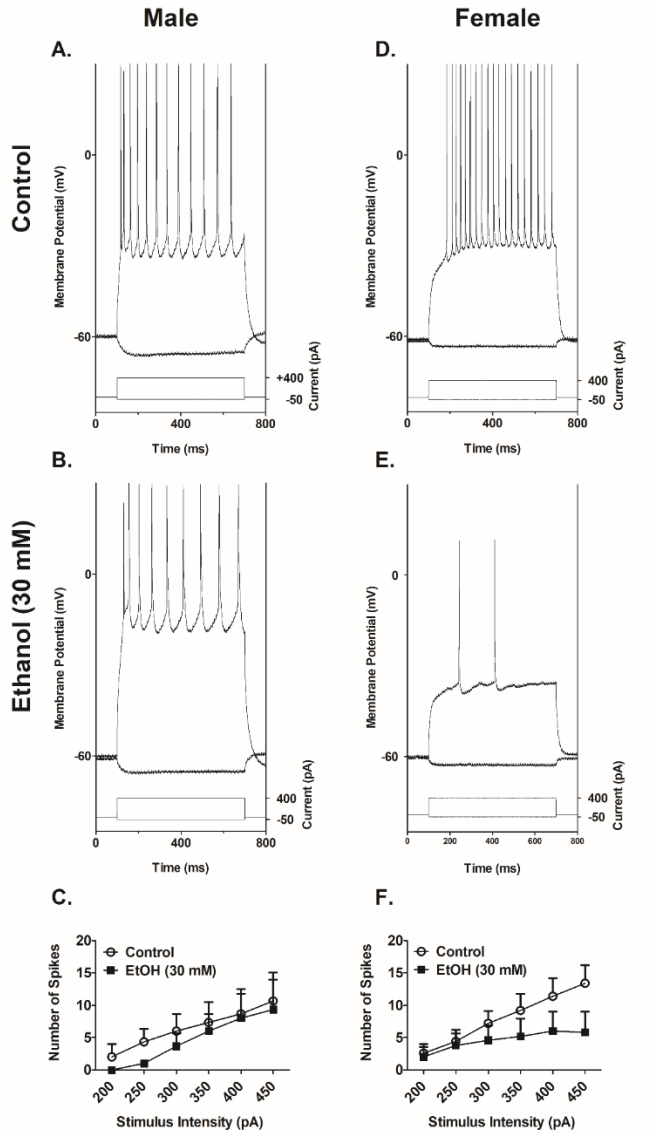

Figure 5: Ethanol (EtOH) decreases neuronal excitability of basolateral amygdala (BLA) neurons from SPS female (D – F), but not male (A - C) rats. Representative action potential firing in BLA neurons from SPS-treated rats before (Control, **A** and **D**) and during superfusion of EtOH (30 mM, **B** and **E**). Action potentials were elicited from a holding potential of -60 mV by a series of depolarizing current steps. Responses to 400 pA depolarizing current are shown in BLA neurons from male (A and B) and female rats (D and E). Peaks of the action potentials are truncated at +30 mV. Summary data from all neurons are shown in **C** (Males, N = 3) and **F** (Females, N = 5). Data are shown as mean ± S.E.M. Two-way ANOVA showed a significant main effect of EtOH, but not main effect of sex and no sex x EtOH interaction (see text for details).

Ethanol Inhibits Hyperpolarization-Activated Current (I_h) in BLA Neurons from SPS-treated Rats

Previously we showed that EtOH reduces hyperpolarization-activated, cyclic nucleotide-gated cation current (I_h) in the BLA. In the present study, we examined EtOH-induced inhibition on I_h in BLA neurons from SPS-treated rats to determine sex differences in BLA excitability following acute ethanol. I_h was elicited in voltage clamp mode by a series of hyperpolarizing steps (2 s duration) from a holding potential of -40 mV. I_h was elicited in BLA neurons from both SPS-treated male and female rats, before (Control) during superfusion of EtOH (30mM). Results are shown in figure 6. Ethanol decreases I_h amplitude in BLA neurons from both SPS-treated male and female rats. Two-way ANOVA revealed a significant main effect of EtOH ($F(1,6) = 278.8$, $p < 0.0001$) and a significant sex x EtOH interaction ($F(1,6) = 211.9$, $p < 0.0001$). There was no main effect of sex ($p = 0.07$). Post-hoc comparison showed that I_h amplitude in BLA neurons from female rats was significantly smaller than I_h in neurons from males ($t(5) = 3.58$, $p < 0.05$). In BLA neurons from SPS-treated male rats, EtOH (30mM) significantly decreased I_h amplitude from 171 ± 46 pA in control to 53 ± 51 pA (n=3) in the presence of EtOH ($t(1,5)= 20.67$, $p < 0.001$). Ethanol similarly decreased I_h amplitude in BLA neurons from female rats from 24 ± 3 pA to 16 ± 6 pA (n=4 out of 5), but this was not significant ($p > 0.05$). We also examined the voltage-dependence of activation of I_h in BLA neurons from SPS-treated male rats before (Control) and during superfusion of EtOH (30 mM; Figure 6C). The goodness of fit of activation data to the Boltzmann equation were $r^2= 0.8$ for Control and $r^2= 0.3$ during superfusion of EtOH. $V_{1/2}$ = -89 ± 2 mV in Control (N = 3), and $V_{1/2}$ = ~ -100 mV in the presence of EtOH (30 mM). Voltage activation curves for I_h in neurons from SPS-treated female rats were not

determined. Together, these data show that following SPS, EtOH inhibits I_h selectively in BLA neurons from SPS-treated male rats. Further, these data show that EtOH-induced inhibition of I_h may be due to a hyperpolarizing shift in the voltage-dependence of activation.

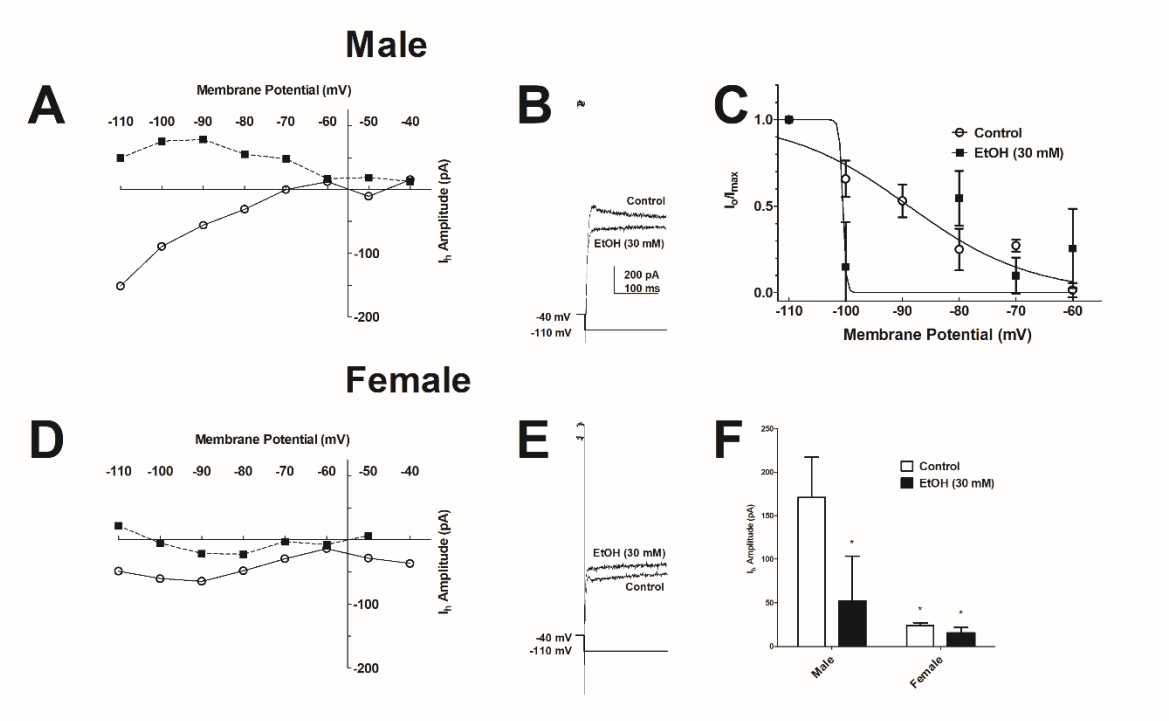

Figure 6. Ethanol decreased the amplitude of the hyperpolarization-activated current (I_h) in BLA neurons from SPS-treated male but not SPS-treated female rats. I_h was elicited from a holding potential of -40 mV by a series of hyperpolarizing voltage steps in 10 mV increments (2 s duration) to -110 mV. **A.** Current–voltage relationships of I_h obtained before (Control, ○) and during ethanol (EtOH 30 mM; ■) in a typical BLA neuron from a male rat. **B.** Representative recordings of I_h elicited upon hyperpolarization to -110 mV before (Control) and during EtOH (30 mM) from the same neuron shown in A. **C**. Activation curves of I_h before and during superfusion of EtOH (30 mM) show no EtOH-induced change in voltage dependence of the conductance underlying I_h. Current-voltage relationships and representative I_h recordings in BLA neurons from a female are shown in **D** and **E** respectively. Scale in B is the same in E. **F**. The amplitude of I_h evoked upon hyperpolarization to -110 mV in males (left, N = 3) and females (right, N = 4 out of 5) before (Control, open bars) and during superfusion of EtOH (30 mV, filled bars). Data are shown as mean ± S.E.M. * = $p < 0.05$ compared to Male Control condition (ANOVA followed by Student's t test. See text for details).

CHAPTER FOUR

Discussion

In the current study we show distinct sex differences in neuronal excitability of the basolateral amygdala (BLA). Ethanol inhibits action potential firing and hyperpolarization-activated current (I_h) in BLA neurons from males more than in females, indicating sex differences in ethanol-induced inhibition of BLA neuronal excitability. Furthermore, ethanol inhibits I_h in males, without affecting the voltage dependence of activation. The peak amplitude of I_h recorded in BLA pyramidal neurons from males is significantly larger than I_h recorded in BLA neurons from females. The sex difference in amplitude suggests I_h plays a reduced role in modifying neuronal activity in the BLA of females. Finally, ethanol-induced inhibition of action potential firing and I_h amplitude occurred without changes in RMP or passive membrane properties, suggesting that the cellular mechanisms underlying the inhibitory effect of ethanol results from direct effects on HCN channels underlying I_h. The sex differences in ethanol-induced inhibition of BLA excitability may play a role in sex differences in alcohol use disorders.

Sex differences in physiological properties of BLA neurons and their response to acute ethanol have not been well-established. Since the BLA has a critical role in fear/anxiety, drug dependence and the reinforcing actions of ethanol (Koob & Le Moal, 2001), these findings are important in determining the role of the BLA in both positive and negative affective states. Within the BLA, glutamatergic pyramidal neurons drive

excitatory transmission to the central amygdala (CeA) and nucleus accumbens (Perra et al., 2008), providing a vital role in regulating stress, anxiety and fear behavior. The active properties of neurons included in our analyses displayed broad action potentials and full spike frequency adaptation from a depolarizing stimulus, strongly suggesting they are excitatory projection neurons (Faber, Callister & Sah, 2001). Previous studies have shown that ethanol modulates glutamatergic activity in males, through distinct GABAergic pathways that synapse on to projection neurons in the BLA (Marowsky et al., 2005; Zhu & Lovinger, 2006; Silberman et al., 2008). Also, acute ethanol inhibits spontaneous action potential firing in BLA projection neurons (Perra et al., 2008), leading to reduced glutamatergic output of the BLA. However, few if any studies have determined firing properties of BLA neurons from females or determined the effect of acute ethanol on BLA pyramidal neurons in females. In the central amygdala (CeA), the main output nucleus of the amygdala, ethanol produces differences in neuronal inhibition. Specifically, in both the medial (CeM) and lateral (CeL) nuclei, ethanol more strongly reduces excitatory postsynaptic potentials (EPSPs) in males than in females (Logrip et al., 2017), suggesting sex differences in sensitivity and responsivity to acute ethanol. Sex differences in c-Fos expression show dysregulation of neuronal activity in CeA in rats exposed to ethanol (Retson et al., 2015). Following chronic ethanol exposure female rats display a significant increase in c-Fos compared to male ethanol-treated rats suggesting that females may have more prolonged responses to ethanol that do males. Overall, our results extend previous findings by discovering a novel mechanism of ethanol-induced inhibition in the BLA by comparing the effects of ethanol in neurons from males and females.

We examined the role of I_h on BLA pyramidal neurons and the inhibitory effects of ethanol on I_h in male and female rats. There was a clear sex difference in I_h amplitude in BLA neurons, in which males exhibited significantly larger I_h amplitudes compared to females. In adult male rats inhibition of I_h in the medial amygdala (MeA) promotes an increase of excitatory glutamatergic transmission on to GABAergic interneurons in the amygdala which release inhibitory transmission onto pyramidal-like neurons (Zhang et al., 2016). Conversely, pharmacologically blocking I_h in the BLA increases membrane excitability by reducing spontaneous inhibitory postsynaptic currents (Park et al., 2011). However, physiological properties of I_h in the BLA of females has not been previously determined. Most studies that examine I_h in females are typically examining sex-specific effects of I_h on cardiovascular and hormonal functioning. I_h has been found in baroreceptor neurons, which play a major role in blood pressure and baroreflex function. Specifically, I_h shifts the membrane potential to a more depolarized state towards the action potential firing threshold and induces spontaneous firing (Han et al., 2014). 17-β-Estradiol, a female hormone, facilitates the role of I_h in membrane excitability by enhancing the role of I_h in preserving afterhyperpolarization in baroreceptive neurons and sustaining the neuronal firing rate (He et al., 2015). In female rats, I_h has been found in gonadotropin-releasing hormone (GnRH)-containing neurons of the hypothalamus and may be involved with burst secretion of GnRH (Arroyo et al., 2006). However, within the amygdala, I_h has yet to be compared in males and females. Considering I_h contributes to neuronal activity in other brain areas, understanding the differing role of I_h in BLA neurons of males and females improves our understanding of the sex-related differences

cellular mechanisms underlying membrane excitability and ensuing amygdala-dependent behaviors.

We found that ethanol decreased the amplitude of I_h in BLA neurons from both male and female rats. Females overall have small amplitude I_h in the BLA, suggesting that females may have different or additional physiological processes controlling excitability. I_h has been implicated in the control of neuronal excitability in several brain regions. Similarly, the inhibitory influence of ethanol varies in cell type and brain region being examined. Purkinje cells located in the cerebellum show large I_h amplitudes in response to postnatal exposure to ethanol (Light et al, 2015). In midbrain dopaminergic neurons, ethanol also increases I_h amplitudes which is thought to influence ethanol's ability to increase firing rates in dopamine (DA) neurons in the ventral tegmental area (VTA) (Tateno & Robison, 2015) and promote reinforcing actions of ethanol. In GABAergic interneurons of the hippocampus, ethanol increases I_h amplitude, which may play a role in ethanol-induced increase of spontaneous firing of GABAergic neurons (Yan et al, 2009). However, the role of ethanol in suppressing neuronal excitability in females has not been determined. Therefore, this study is one of the first to observe acute ethanol-induced changes in I_h in neurons from females. Although previous studies report that ethanol increases I_h amplitude (Light et al., 2015; Tateno & Robinson, 2015; Yan et al., 2009), we show that BLA pyramidal neurons reduce action potential firing in response to ethanol by inhibiting I_h amplitude. These novel findings of sex differences in ethanol-induced inhibition in the BLA may be important in the mechanisms underlying sex differences in alcohol abuse and alcohol use disorders.

In the current study, we also sought to determine sex differences in BLA membrane excitability in response to single prolonged stress (SPS) and acute ethanol. SPS does not alter action potential firing in BLA pyramidal neurons from either male or female rats. In addition, SPS does not produces changes to hyperpolarization-activated current (I_h) in either males or females; however, BLA neurons in males overall have larger I_h amplitudes compared to females, suggesting I_h plays a reduced role in modifying neuronal activity in the amygdala in females. In response to acute ethanol, females exposed to SPS exhibited greater ethanol-induced inhibition on action potential firing compared to SPS-treated males. However, I_h was reduced by ethanol only in SPS-treated males and not females exposed to stress. These results suggest SPS alone does not affect action potential firing or I_h in either males or females, but ethanol-induced inhibition of BLA excitability is greater in SPS-treated females than in SPS-treated males. The mechanism underlying this disparity remains to be shown.

The SPS model produces overactive and abnormal HPA axis-feedback (Liberzon et al., 1997), amplified acoustic startle response (Khan & Liberzon, 2004), and fear responses to trauma-related stimuli (Wu et al., 2016). We therefore expected to find increased excitability in BLA neurons from SPS-treated rats. The BLA has been implicated in regulating anxiety-like behavior through direct excitatory projections to the central nucleus of the amygdala, in conjunction with top-down control provided by the mPFC. Recently, SPS produced suppression of long-term potentiation (LTP) in external capsule-lateral amygdala (EC-LA) synapses potentially producing disinhibition in pyramidal neurons in the LA that leads to increased contextual fear condition (Kohda et al., 2007). Although overactive amygdala activity has been shown in people with PTSD

(Badura-Brack et al., 2018) and in animals exposed to stress (Hetzel & Rosenkranz, 2014), previous research has also shown SPS produces neuronal apoptosis in the amygdala (Liu et al., 2010) which may underlie decreased amygdala volume in individuals with PTSD (Karl et al., 2006). Furthermore, increased apoptosis could result in reduced action potential firing and decreased excitability of the BLA. SPS-induced changes in BLA excitability have been largely unexplored. Therefore, the data produced here represents an early investigation of SPS-induced changes in neuronal excitability of the BLA in both males and females.

SPS did not affect I_h amplitudes in BLA neurons from either males or females, but we did replicate previous findings showing that I_h is smaller in BLA neurons from females. Although I_h has been shown to play a role in membrane excitability in males, the small amplitude of I_h shown in BLA neurons from females suggests I_h is not a primary mechanism involved in controlling activity of the amygdala in females. Few, if any studies have examined sex differences in SPS and the BLA. However, previous research has shown female rats are more resilient to exposure to the SPS model. For example, SPS-treated female rats exhibit less glucocorticoid expression in the ventral hippocampus and greater retention of cued fear extinction compared to males (Keller et al., 2015). We did not observe sex differences in BLA excitability in response to SPS, suggesting that SPS-induced changes in BLA excitability are not likely to contribute extensively to sex differences in other SPS-induced changes.

The current study also examined neurophysiological effects of acute ethanol on basolateral amygdala (BLA) neurons in both male and females exposed to SPS. While the effect of ethanol on the amygdala and stress/anxiety is well established, sex differences in

the effects of ethanol on BLA neurophysiology and the effect of traumatic stress on ethanol-induced changes to BLA activity are currently unknown. Interestingly, ethanol-induced inhibition of action potential firing was greater in females exposed to SPS than in males. We have previously investigated the acute effects of ethanol in unstressed (control) rats. Previous results showed that ethanol-mediated inhibition is greater in males than in females. In both the medial (CeM) and lateral (CeL) nuclei, ethanol more strongly reduces excitatory postsynaptic potentials (EPSPs) in males than in females (Logrip et al., 2007), suggesting sex differences in sensitivity and responsivity to acute ethanol. However, in the current study we extend those findings by determining the effect of acute ethanol in SPS-treated rats where we showed that ethanol has the opposite effect on excitability in SPS-treated rats, namely that ethanol-induced inhibition of BLA excitability is greater in females than in males.

We also showed that I_h is inhibited by ethanol more strongly in BLA neurons from SPS-treated males than SPS-treated females. We expected to find that either SPS and/or ethanol would have congruent effects on excitability and I_h, namely that inhibition of excitability would also occur with lower amplitudes of I_h. However, our data show that I_h is reduced by ethanol in SPS-treated males, but that BLA excitability is unchanged by ethanol; and conversely that excitability is inhibited by ethanol in SPS-treated females but I_h amplitude is not reduced by ethanol in SPS-treated females. This discrepancy, was unexpected, but may suggest novel sex-dependent effects of I_h on the regulation of neuronal excitability in the BLA. Previous studies examining I_h in females typically examined sex-specific effects of I_h on cardiovascular and hormonal functioning (Arroyo et al., 2006; Han et al., 2015; He et al., 2015). However, within the amygdala, limited

research on I_h in males and females has not been reported. Understanding the differing role of I_h in BLA neurons improves our understanding of the sex-related differences cellular mechanisms underlying membrane excitability and ensuing amygdala-dependent behaviors. These data are consistent with a growing body of data showing that females with PTSD and alcohol use disorders are more likely to consume alcohol to alleviate symptoms of stress and anxiety (Lehavot et al., 2014). We suggest that the anxiolytic properties of ethanol may involve the inhibition of excitability and I_h in BLA neurons in male rats, but ethanol may be less anxiolytic in females because the inhibitory effect of ethanol on I_h is reduced by the initial low amplitude I_h observed in BLA neurons of females. This may contribute to increased ethanol consumption in females. These data are consistent with other findings that, in adolescent female rats, preference for alcohol and alcohol intake are increased after exposure to restraint stress (Wille-Bille et al., 2017). Furthermore, adolescent female rats with greater endogenous anxiety engage in more ethanol drinking and have stronger preference for alcohol (Acevedo et al., 2016) indicating age-related factors in alcohol-stress-anxiety interactions. Altogether, these data suggest that there are neurobiological mechanisms of vulnerability to ethanol during stressful events that are different in males and females, and that sex differences in ethanol-induced inhibition of I_h may contribute to sex differences in vulnerability to alcohol abuse following traumatic stress.

Conclusions

The current study shows distinct sex differences in neurophysiological responses in BLA pyramidal neurons to acute ethanol exposure. Previous data have shown ethanol inhibits neuronal excitably in the BLA, but sex differences in I_h and its role in ethanol-

mediated inhibition have not been determined. Our data show that ethanol has a larger inhibitory effect on I_h in BLA neurons from males than from females. This may suggest males are more vulnerable to the inhibitory effects of acute ethanol. Lower ethanol-induced inhibition in females may be associated with lower alcohol abuse since a primary mechanism of controlling amygdala excitability and the anxiolytic effects of ethanol is not available in females. Ethanol may be a more efficacious anxiolytic in males and contribute to the higher prevalence rate of AUD in males. Furthermore, this study is the first to examine I_h in the BLA in males and females. We have shown that I_h amplitude is larger in males than in females. Considering I_h is a cellular mechanism that exerts a key role in modulating membrane excitability, sex differences in I_h may also contribute to sex differences in alcohol efficacy abuse and alcohol use disorders. Our data show significant differences in neuronal excitability in males and females and addresses a critical gap in determining the physiological mechanisms of acute ethanol on amygdala neurons from females. This study makes a significant contribution in overcoming current gender barriers in neurophysiological studies and investigate important differences between males and females in cellular mechanisms contributing to alcohol abuse and alcoholism.

Furthermore, the current study examined sex differences in the effects of ethanol on BLA neurophysiology and the effect of traumatic stress on ethanol-induced changes to BLA activity. Determining the physiological properties of amygdala neurons in the SPS model has not been widely accomplished in either male or female rats. This is therefore one of the first explorations of the effects of SPS neuronal excitability in the BLA. We have shown distinct sex differences in BLA membrane excitability in SPS-treated male and female rats in response to ethanol. Ethanol reduced action potential firing in the BLA

more in females compared to males, suggesting greater ethanol-induced inhibition in females exposed to traumatic stress. Furthermore, this study is one of the first to examine I_h in the BLA in males and females exposed to SPS and ethanol. Similar to our previous work, males exhibit significantly larger I_h amplitudes compared to females in response to stress and ethanol. Small I_h amplitudes in females may be associated with less neuronal excitability in response to ethanol. We propose that in the female brain, ethanol reduces BLA excitability helping to control anxiety-like behavior and potentially leading to the individual consuming more alcohol to further alleviate stress and anxiety. Overall our data makes a significant contribution to bridging the current gender gap in neurophysiological studies and investigating important differences between genders in cellular mechanisms contributing to alcohol abuse, stress and anxiety disorders.

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