THE INTERACTION OF PROGESTERONE AND ALLOPREGNANOLONE WITH FEAR MEMORIES

A Dissertation

by

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ABSTRACT

Sex differences in stress and anxiety disorders are well reported in the clinical population. For instance, women are twice as likely to be diagnosed with post-traumatic stress disorder (PTSD) compared to men. This difference is most profound during the reproductive years suggesting a role for sex hormones and their metabolites in regulating fear and anxiety. Previous studies suggest sex differences also occur in rodents, which is modulated by gonadal hormones. Despite the majority of clinical cases occurring in women, most rodent studies only include male subjects. An understanding of what contributes to this disparity is necessary for sex-specific therapies and interventions. Here, we use Pavlovian fear conditioning, a learned task in which male rats display higher fear levels compared to females, to understand the neurobiological basis for sex differences in fear and anxiety. Specifically, we focus on allopregnanolone (ALLO), a metabolite of progesterone and potent allosteric modulator of $GABA_A$ receptors and its effects in the bed nucleus of the stria terminalis (BNST) in male and female rats. The BNST is a sexually dimorphic brain region and site of hormonal modulation that has been implicated in contextual fear. Raising ALLO levels in the BNST of male rats successfully impaired contextual fear and blocking ALLO in the BNST of female rats successfully increased contextual fear. However, impairing contextual fear via ALLO in male rats proved to be the result of state-dependent learning, which was unique to the BNST as global impairments in both contextual and cued fear to ALLO were found in the basolateral amygdala. In females, hormonal changes across the rodent estrous cycle

follow similar patterns to that of the menstrual cycle in women and provide a naturalistic model to observe changes in fear responses at the cellular level when ALLO levels are high or low due to progesterone fluctuations. Results suggest naturally circulating progesterone may also contribute to state-dependent contextual fear; however, ovariectomized rats administered progesterone do not. The work presented here aims to elucidate the mechanisms underlying ALLO's influence on sex differences in fear and anxiety and contribute to the growing body of literature on women's health.

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TABLE OF CONTENTS

Page

Page

LIST OF FIGURES

Page

LIST OF TABLES

Page

CHAPTER I

INTRODUCTION

Sex Differences in Fear and Anxiety

Anxiety is a normal response to threat, novelty, or stress in everyday life. However, when anxious behavior persists in the absence of threatening or stressful stimuli, various psychiatric disorders may arise. Anxiety disorders are among the most common mental disorders in the U.S. with an estimated lifetime prevalence of 31% (Kessler et al., 2009) and a 12-month prevalence rate of 18.1% of which 22.8% are considered serious in terms of severity (Kessler et al., 2005b). Unsurprisingly, given the high number of diagnoses, anxiety disorders have devastating effects on society including decreased work performance, medical costs, and mortality when presented with comorbid disease (Greenberg et al., 1999; Smoller et al., 2007).

Importantly, there is a considerable sex difference in the rates of diagnoses with those for women far exceeding those for men (Kessler et al., 1994; 1995; 2006; Tolin and Foa, 2006). Overall, the lifetime prevalence rate of any anxiety disorder is 30.5% in women compared to 19.2% in men (Kessler et al., 1994). This pattern is consistent over a wide variety of anxiety and fear-related disorders including panic disorder, agoraphobia, social phobia, generalized anxiety disorder, and posttraumatic stress disorder (PTSD). In particular, women are twice as likely to be diagnosed with PTSD compared to men. These findings are not due to women witnessing traumatic events more than men: in fact, men and women do not differ in exposure to a traumatic event.

Overall, the greatest indicator of development of PTSD was preexisting anxiety disorders (Breslau et al., 1997). This suggests that women are more susceptible to prolonged negative emotional reactions to stressful and traumatic life events that ultimately may lead to anxiety disorders. Many overlapping factors may contribute to this susceptibility, including sex differences in the structure and function of the brain, the hypothalamic-pituitary-gonadal axis, the hypothalamic-pituitary-adrenal axis (HPA), and memories of emotional events (Canli et al., 2002; Goel et al., 2014; Ingalhalikar et al., 2014; Toufexis et al., 2014).

Fear and anxiety studies in rodents also show sex differences. Sex differences related to anxiety in rodents, however, are less clear. On a number of tests including the elevated plus maze, light-dark box, open field test, Vogel punished drinking test, and contextual fear conditioning, female rodents actually have decreased fear and anxiety behavior compared to males (Barker and Galea, 2010; Johnston and File, 1991; Marcondes et al., 2001; Maren et al., 1994; Markus and Zecevic, 1997; Ramos et al., 2002; Steenbergen et al., 1990; van Haaren et al., 1990; Zimmerberg and Farley, 1993). However, other studies have found no sex differences or the opposite pattern (Chang et al., 2009; Frick et al., 2000; Johnston and File, 1991; Marcondes et al., 2001; Nomikos and Spyraki, 1988; Pryce et al., 1999). Many factors may contribute to this including time of testing (Marcondes et al., 2001), testing conditions (Mora et al., 1996), and whether or not the estrous cycle was considered (Markus and Zecevic, 1997).

In the laboratory, certain aspects of PTSD can be modeled using Pavlovian fear conditioning, including learned fear behaviors, extinction of fear, and generalization

(Milad and Quirk, 2012; VanElzakker et al., 2014). A previously innocuous cue (conditioned stimulus, CS) is paired with an aversive stimulus, such as a footshock (unconditioned stimulus, US). After repeated pairings, the CS alone predicts the US and results in fear-like behavior. Specifically, freezing behavior is used as an index of fear by measuring immobility aside from what is necessary for respiration. Conditioned fear develops not only to the specific CS, but also to the distinct context in which training occurs as characterized by particular odors, lighting, or structural constraints within the environment. Freezing to the context in the absence of aversive stimuli is a measure of contextual fear whereas freezing to presentations of the CS in a novel context is a measure of cued fear.

Hormonal Control of Fear

Menstrual Cycle vs. Estrous Cycle

While initially there may seem to be a discrepancy between human clinical data and rodent studies of fear and anxiety (where women have higher rates of anxiety and female rodents overall show decreased anxiety), there are nonetheless many similarities. First, women and female rodents share similarities in the cyclical fluctuations of gonadal hormones over time. In humans, the menstrual cycle, averaging 28 days, begins in the follicular stage when levels of progesterone and estrogen are low. Estrogen levels spike causing a surge in luteinizing hormone (LH) and follicle-stimulating hormone (FSH), which stimulates ovulation. After ovulation, estrogen levels remain elevated and

progesterone levels rise, leading to the luteal phase. Estrogen and progesterone levels then diminish and the cycle repeats (McLachlan et al., 1987).

While the rodent estrous cycle is different from the menstrual cycle, the fluctuations in hormones are relatively similar, making rodents a useful model for studying the effects of hormone level changes on behavior. The 4-5 day estrous cycle consists of proestrus, estrus, metestrus, and diestrus stages (in 5-day cycles, diestrus or estrus may last for two days). Low hormone levels correspond with the follicular phase in humans and high hormone levels correspond with the mid-luteal phase when estrogen and progesterone are elevated (Emanuele et al., 2002). Estrogen levels remain low in the diestrus phase while progesterone has a slight rise in the early part of diestrus. During proestrus, estrogen levels rise followed by a surge in LH and FSH, stimulating ovulation. After the rise in estrogen, progesterone is acutely elevated and then drops and the cycle repeats (Smith et al., 1975). Given the relatively similar hormonal profiles in both humans and rodents, the shortened rodent cycle provides an ideal model for study due to its brevity. Caution must be given to the specific time periods that testing occurs as well as the light cycle; simple factors such as these can drastically change behavioral outcomes. Estrous cycle stage is easily determined in rodents via vaginal smearing and cellular profiling (Goldman et al., 2007). However, simply testing subjects at different stages of the estrous cycle does not necessarily answer the question of the role of hormones in behavioral tests of anxiety. Because estrogen and progesterone rise and fall in relatively the same time periods, further analysis must be done to decipher the role of each of these hormones and its metabolites individually.

Because of similarities in cycling hormones between humans and rodents, comparisons can be made between low levels of hormones (diestrus), and high levels of hormones (proestrus). Similarities exist between the two in that low levels of hormones corresponds with impairments of fear inhibition (Glover et al., 2013) and decreased extinction of fear in both humans (Glover et al., 2012; Milad et al., 2010; Zeidan et al., 2011) and rodents (Milad et al., 2009). Overall anxiety behaviors are also decreased during periods of elevated hormones across both the estrous and menstrual cycle (Llaneza and Frye, 2009; Marcondes et al., 2001; Pigott, 2003; Wegerer et al., 2014). During menopause (51-55 years of age), women have higher rates of PTSD. However, lower levels of PTSD are observed during postmenopausal periods (Ditlevsen and Elklit, 2010), further supporting the role in fluctuating hormones contributing to greater PTSD diagnosis. Therefore, it may not be absolute levels of hormones during different stages of the cycle but the constant fluctuations and change in hormone supply that contributes to sex differences. It is likely not one specific hormone that modulates fear memories, but rather a combination of each and its metabolites that affect behavior.

Hormones & Sex Differences in Fear Conditioning

Earlier work first established a sex difference in rodents on fear conditioning (Maren et al., 1994). Initially, sex differences were found in the magnitude of induced long-term potentiation (LTP) in the dentate gyrus, a component of the hippocampal system. After high-frequency stimulation of the performant path (the major afferent input of the dentate gyrus), females displayed weaker LTP than that in males. Due to the

role of hippocampal LTP in contextual learning, sex differences were examined in both hippocampus-dependent and hippocampus-independent forms of fear conditioning. Robust sex differences were observed in contextual fear conditioning, a hippocampus dependent task. At both low and high shock intensities, male rats showed greater freezing behavior compared to female rats. In contrast, male and female rats did not show differences in the amount of freezing to an auditory CS after either 1 or 3 trials. However, when tested in the same context used for conditioning, males again showed greater freezing behavior (Maren et al., 1994).

After this seminal discovery, subsequent work sought to determine whether gonadal steroids contributed to these sex differences. In the first set of experiments, male rats were castrated to examine the effects of circulating testosterone on contextual fear. No differences were reported during acquisition or extinction of fear between castrated males, castrated males with testosterone replacement, or unoperated controls. Furthermore, when compared to females, both castrated and intact males had higher freezing levels compared to females (Anagnostaras et al., 1998). Therefore, activational effects of testosterone do not mediate sex differences in contextual fear. In contrast, it was found that ovariectomized female rats froze at comparable levels to males, both of which were higher than sham-operated females following contextual fear conditioning. Estrogen replacement in ovariectomized females reduced freezing to that seen in sham operated groups (Gupta et al., 2001). When examining estrogen's effects on LTP, it was found to reduce hippocampal LTP (Gupta et al., 2001). Together, this body of work established a sex difference in fear conditioning between male and female rodents and

identified a role for ovarian steroids in mediating this observed difference. The work in this dissertation therefore focuses on the contributions of female sex hormones, particularly estrogen, progesterone, and their metabolites and their influence on fear conditioning.

Estrogen

Synthesis

Estrogen is synthesized from locally produced androgens through the enzymatic actions of aromatase (Naftolin et al., 1975). Specifically, estrone is synthesized from androstenedione and estradiol from testosterone. Estrone can be converted to estradiol and vice versa via 17-hydroxysteroid-dehydrogenase, and then can be converted to estriol. Estrone is primarily secreted during menopause and estriol during pregnancy (Gruber et al., 2002). Estradiol is the most dominant and potent estrogen acting at estrogen receptors during the reproductive years and therefore will be the focus of this section. In premenopausal females, the majority of estrogen is synthesized from the ovaries, which then enters the bloodstream to affect target organs. Males also produce estrogen in peripheral tissue including the testes, although at much lower levels compared to females (Brodie and Inkster, 1993). Aromatase activity has also been found in both male and female rodent, primate, and human brains, suggesting local synthesis and availability (Naftolin et al., 1975; Roselli and Resko, 2001; Stanić et al., 2014). Importantly, aromatase activity has been localized to key brain regions implicated in anxiety including the hypothalamus, amygdala, bed nucleus of the stria terminalis,

hippocampus, and cingulate cortex (Hojo et al., 2004; Jakab et al., 1993; Kretz et al., 2004; Tabatadze et al., 2014). The availability of locally synthesized estradiol suggests it may modulate anxious behavior.

Mechanisms of action

Estrogen exerts its effects at a number of different receptors to affect behavior. Classically, estrogen was thought to act on nuclear receptors ERα and ERβ to initiate transcription of genes (Green et al., 1986; Kuiper et al., 1996). This effect is considered to be a long-term, genomic effect that could take anywhere from hours to days. Due to the lipophilic nature of estrogens, they readily cross the cell membrane and act as ligands to ERα and ERβ in both the cytoplasm and nucleus. Once activated, the receptors translocate to the nuclear DNA domain and bind to estrogen-response-elements (ERE) to alter the rate of gene expression through the recruitment of coactivator or corepressor proteins (Gruber et al., 2002). Evidence suggests estrogen receptors may also be located on the cell surface (Mitterling et al., 2010; Spencer et al., 2008). Both ERα and ERβ are present in the limbic system although the distribution is not always overlapping (Cover et al., 2014; Osterlund et al., 1998; Shughrue et al., 1997). Additionally, receptors are noted to change over the course of the estrous cycle and after ovariectomy (Bowlby et al., 2015; Haywood et al., 1999; Mitterling et al., 2010; Shughrue et al., 1992). When considering the endogenous or exogenous actions of hormone administration on anxiety, it is therefore important to consider the diverse and changing receptor profiles.

More recently, a novel receptor with high affinity for estrogen was discovered. GPR30 is a G-protein coupled receptor (GPCR) that evokes rapid, nongenomic effects,

unlike the classical nuclear receptors. Current research has begun to elucidate the role of this receptor in behavioral and emotional processes (Hammond et al., 2009). GPR30 has been found in cell membranes as well as intracellularly in the endoplasmic reticulum. Unlike many other GPCRs, estrogen binds to GPR30 intracellularly (Revankar et al., 2007). Binding of estrogen to this receptor initiates a number of intracellular signaling cascades including mobilizing calcium stores, cyclic adenosine monophosphate (cAMP), mitogen-activated protein kinases (MAPKs) and phosphatidylinositol 3-kinases (PI3Ks) (Prossnitz et al., 2008). Mouse studies found GPR30 labeling in regions implicated in fear and anxiety including the hippocampus, amygdala, BNST, and hypothalamus (Hazell et al., 2009). As research progresses, estrogen's effects on fear and anxiety can no longer be strictly defined to the genomic actions of classical estrogen receptors, ERα and ERβ, but must also include the rapid actions at GPR30 and membrane bound ERs.

Estrogen and fear

It is well established that estrogen affects learning in many different paradigms and has been found to both facilitate and impair fear learning. In support of anxiolytic actions of estrogen, cycling rats in proestrus display reduced fear, but enhanced learning, in the two-way avoidance paradigm (Sfikakis et al., 1978) as well as OVX rats chronically treated with estradiol (Singh et al., 1994). However, in opposition, Diaz-Veliz et al. (1989; 1991; 2000) found estrogen to increase fear on the two-way avoidance task and decrease learning in cycling and OVX rats. Learning was strengthened in diestrus animals and decreased in proestrus (Diaz-Veliz et al., 1989). In OVX rats, learning was enhanced and estrogen administration decreased acquisition (Diaz-Veliz et

al., 1991; 2000). More research is necessary to understand estrogen's role in this paradigm. In fear conditioning models, rats in proestrus froze significantly less to contextual fear compared to estrus rats and males, but showed no difference to cued fear (Markus and Zecevic, 1997). Furthermore, when ovarian supply of estrogen is eliminated via OVX, rats displayed levels of context fear comparable to males, and estrogen administration prior to conditioning reduced freezing (Gupta et al., 2001). There are reports, however, of no effect as well with administration of exogenous estrogen to GDX and OVX rats on light-enhanced startle or CRF-enhanced startle (Toufexis et al., 2005; 2004) or across the estrous cycle and in OVX rats with estrogen replacement in context fear conditioning (Chang et al., 2009). An increase in context and cued fear related to estrogen was also found (Morgan and Pfaff, 2001; Jasnow et al., 2006). ERβ-KO mice had reduced context fear in females and reduced context and cued fear in males, suggesting a possible mechanism for this effect (Day et al., 2005). Similar increases in fear were also found on tests of fear-potentiated startle (Hiroi and Neumaier, 2006). Interestingly, sex differences were found in passive avoidance with estrogen enhancing retention in males, but inhibiting retention in females (Mora et al., 1996; Vázquez-Pereyra et al., 1995). Estrogen was also found to increase generalization of fear responses in females which may be mediated by actions of ERβ (Lynch et al., 2013; 2014).

Research on the role of estrogen in extinction learning, on the other hand, has been surprisingly consistent. Sex differences were absent in conditioning, extinction, and recall until estrous cycle was taken into consideration. Rats show enhanced

extinction during the proestrus phase compared to metestrus rats and male rats as seen in the recall test 24 hours later (Milad et al., 2009; Rey et al., 2014). The deficit seen in metestrus rats was rescued via exogenous administration of estrogen immediately after extinction learning, but not after delayed administration which corresponded with increased *fos* in the IL and decreased *fos* expression in the amygdala (Milad et al., 2009; Zeidan et al., 2011). Furthermore, blocking estrogen receptors prior to extinction learning impaired consolidation (Milad et al., 2009). In addition to naturally cycling

Task	Hormonal State	Effects on Fear	Study
Extinction	Proestrus	decrease freezing	Milad et al., 2009
	Metestrus	increase freezing	
Extinction	Estrus	increase freezing	Rey et al., 2014
	Metestrus	increase freezing	
	Diestrus	increase freezing	
	Proestrus	decrease freezing	
Extinction	OVX + estrogen	decrease freezing	Chang et al., 2009
Extinction	OVX + estrogen	decrease freezing	Gupta et al., 2001

Table 1 Summary of estrogen's effects on fear extinction.

rats, ovariectomized rats administered estradiol directly into the hippocampus also display enhanced extinction learning (Chang et al., 2009). Similar effects were also seen when estrogen receptor agonists were administered to rats on hormonal contraceptives with reduced estradiol levels (Graham and Milad, 2013). Estrogen does not appear to only be important for extinction learning in females; in males, inhibition of estradiol

synthesis during extinction training impairs fear extinction, which can be reversed by co-administration of estradiol (Graham and Milad, 2014). Further examination reveals a specific role of ERβ in mediating enhanced extinction learning. Administration of ERβ selective agonists specifically facilitated this effect (Chang et al., 2009; Zeidan et al., 2011).

Estrogen clearly enhances extinction learning and it appears to be mediated by ERβ not ERα. However, the confounding literature on other learning tasks related to fear and anxiety creates a more complicated picture. Enhanced extinction learning is associated with decrease fear, yet in a number of studies discussed, estrogen increased fear (Hiroi and Neumaier, 2006; Jasnow et al., 2006; Morgan and Pfaff, 2001). Increased fear after acquisition suggests enhanced fear learning. Estrogen therefore may not simply cause increases or decreases in freezing behavior, but enhance the learning mechanisms that are responsible for the presentation of fear. A number of these studies used OVX female rats, which discounts the possibility of fluctuating hormones in mediating these effects. In addition, Toufexis et al. (2006) used a discrimination learning paradigm to further assess the role of ERα and ERβ and found opposing roles with increased responding to ERα agonists on both excitatory and inhibitory signals and ERβ agonists having little effect on any cues (Toufexis et al., 2006). Complexity of the learning tasks and receptor distribution may therefore contribute to the varying effects seen in tasks such as the two-way avoidance test.

Humans

Because of the inherent limitations in directly manipulating estrogen levels in healthy cycling humans, studies generally focus on periods in the reproductive cycle when estrogen levels are high (late follicular or luteal phase) or low (early follicular phase). Higher estrogen levels in the menstrual cycle of females appears to enhance fear recognition, but not to other emotional cues (Pearson and Lewis, 2005). Much research has focused on the extinction of fear in humans with findings similar to rodent studies. Males had greater conditioned responses compared to females in both the early and late follicular stage. No differences between sexes and hormone state occurred during extinction learning, but during recall, only males and females with low estrogen (early follicular) had impaired learning (Milad et al., 2006). Females with high estrogen showed enhanced extinction memory as measured by skin conductance response and this was also true when fear conditioning was preceded by stress (Antov and Stockhorst, 2014; Milad et al., 2010; Wegerer et al., 2014), which was at least in part mediated by ventromedial prefront cortex (vmPFC) and amygdala activity (Zeidan et al., 2011). In addition, women on hormonal contraceptives, which represents lower circulating estradiol, also had impaired extinction learning as was seen in rodent studies (Graham and Milad, 2013). This was explored further in a discrimination task in which fMRI data revealed increased differential activation in regions critical to fear and extinction such as the amygdala, anterior cingulate, and vmPFC (Merz et al., 2012). Unlike rodents, hormonal contributions in human populations living with anxiety-related disorders can be assessed. For instance, in a traumatized population half of whom had PTSD, women

with low estrogen and women with high estrogen levels had similar levels of fear conditioning on a fear-potentiated startle task, but during extinction, startle was higher in those with PTSD; this difference was absent in the high estrogen group (Glover et al., 2012). Furthermore a comparison of healthy controls and a traumatized group found impaired fear inhibition in healthy and traumatized individuals with low estrogen levels on a discrimination task (Glover et al., 2013). This suggests that estrogen, in part, can mediate the difference seen between those who proceed to develop a disorder following a traumatic event and those that are exposed to traumatic events but can recover.

Progesterone

Synthesis

Along with estrogen, progesterone is produced in significant quantities in the ovaries, with the biggest surge occurring during the luteal phase in humans and during proestrus in rats. Progesterone, however, is not exclusive to females. The adrenal glands produce progesterone in both males and females (Herrera et al., 2016; Holzbauer et al., 1969; Kalra and Kalra, 1977). Synthesis also occurs in the central nervous system in both sexes, thus making it a neurosteroid. Progesterone is synthesized from its precursor pregnenolone via 3β-hydroxysteroid dehydrogenase (3β-HSD), which has ubiquitous expression in the rodent and human brain (Guennoun et al., 1995; Ibanez et al., 2003; Inoue et al., 2002). Allopregnanolone (ALLO) is the most studied metabolite of progesterone regarding fear and anxiety and is synthesized by the sequential actions of two enzymes. First, progesterone is irreversibly converted to 5α-dihydroprogesterone

(5α-DHP) via 5α-reductase. 5α-DHP is further reduced to ALLO via 3α-hydroxysteroid-dehydrogenase which in turn can be converted back to 5α-DHP via short-chain dehydrogenase reductases (Belyaeva et al., 2007). Enzymes necessary for ALLO synthesis have been localized to numerous brain regions suggesting local availability and modulation of neuronal activation through ALLO actions (Agís-Balboa et al., 2006; Melcangi et al., 1993; 1998). ALLO fluctuates in levels according to the menstrual and estrous cycle in a similar fashion to its precursor, however, ALLO levels have been found to be higher in the brain than in the plasma (Bixo et al., 1997; Corpéchot et al., 1993; Droogleever Fortuyn et al., 2004).

Mechanisms of action

Progesterone binds to a number of receptors to produce both genomic and non-genomic actions. Classically, progesterone binds to intracellular receptors causing conformational changes and release of chaperone proteins to allow progesterone binding with progesterone response elements at target genes for gene upregulation or downregulation. Progesterone receptors (PR) come in two isoforms, PR-A and PR-B, which may have differing roles (Jacobsen and Horwitz, 2012). Both are widely expressed within the brain and do not appear to have neuronal specificity. Specifically, PRs are expressed in the hippocampus, frontal cortex, and amygdala with especially high levels of expression in the BNST (Guerra-Araiza et al., 2001; 2002; Kato et al., 1993). Importantly, PR expression fluctuates with the estrous cycle in certain brain regions such as the hypothalamus and frontal cortex, but not in the hippocampus (Guerra-Araiza et al., 2000; 2003). Even though classical PRs are thought to be genomic in nature, there is

evidence of non-genomic actions of PRs via Src kinase (Boonyaratanakornkit et al., 2001). PR-A and PR-B are not the only binding sites of progesterone. Although many nongenomic actions of progesterone are attributed to its metabolite, ALLO, there is still evidence of progesterone actions at membrane bound receptors. Recent advances have identified a number of membrane receptors specific to progesterone although their localization and function in the central nervous system is only just beginning to be understood. One such class of receptors is progesterone receptor membrane component 1 and 2 (Pgrmc1, Pgrmc2), which are located throughout the brain, including the limbic region (Intlekofer and Petersen, 2011; Krebs et al., 2000). Studies suggest Pgrmc1 cannot act independently, but requires serpine mRNA binding protein 1 (Serbp1) to act as a binding partner (Peluso et al., 2006). In addition, receptors in the PAQR family have been identified as potential targets: Paqr7 and Paqr8 (mPRα and mPRβ) which are coupled to G-proteins and regulate both cAMP and MAPK (Tang et al., 2005; Thomas et al., 2007; Zhu et al., 2003). Although brain analyses have identified these receptors, their exact role in neurons is still being questioned (Fernandes et al., 2008). Future research is necessary to elucidate the role of these novel progesterone receptors in fear and anxiety.

Allopregnanolone does not act at classical progesterone receptors, but instead acts as a potent positive allosteric modulator of $GABA_A$ receptors (Majewska et al., 1986). At low concentrations, ALLO promotes the open state of the $GABA_A$ ion channel and at high concentrations can directly activate the receptor in the absence of GABA (Callachan et al., 1987). Recent work has localized the binding site of ALLO to

the α-subunit transmembrane domains and a site between α and β subunits when directly activating the receptor (Hosie et al., 2006; 2009). There is a wide variation in subunit compositions of $GABA_A$ receptors as well as localization of $GABA_A$ receptors to either the synapse or extrasynaptic space. ALLO appears to have effects at multiple subunit compositions as well as at synaptic and extrasynaptic $GABA_A$ receptors, however different concentrations may be necessary for a response. The response of ALLO at $GABA_A$ receptors can be heterogeneous; ALLO actions at $GABA_A$ receptors in one brain region may not have identical responses in other brain regions or in other neurons in the same region, which may be due in part to subunit composition (Belelli and Lambert, 2005). Despite the differences in subunit composition, overwhelming evidence points to ALLO having much stronger effects at δ subunit-containing $GABA_A$ receptors (Belelli et al., 2002; Wohlfarth et al., 2002). Relatively low concentrations of ALLO are needed to modulate these receptors. Delta subunit-containing receptors are extrasynaptic and produce a tonic inhibitory tone on the cell as opposed to a phasic tone on synaptic receptors. Subunit composition changes over the course of the estrous cycle, such as in the hippocampus (Maguire et al., 2005), allow ALLO to modulate inhibitory tone at varying degrees within the brain. In addition to $GABA_A$ receptors, ALLO may also act on mPRα although this has only been studied in neuronal cell lines (Thomas and Pang, 2012) and also on pregnane xenobiotic receptor (PXR; Lamba et al., 2004).

Progesterone and fear

On conditioned measures of fear, progesterone and ALLO have been shown to both increase and decrease fear, although a closer examination of these data may account

for this discrepancy. Fear conditioning literature in rats and mice have varying results with progesterone on freezing behavior, however, there are consistent results regarding its effects on context versus cued fear. In a number of different studies, progesterone's effects on cued fear appear to be nonexistent. For instance, when comparing fear potentiated startle (FPS) to corticotropin-releasing factor-enhanced startle (CRF-enhanced startle), an effect of progesterone was only observed in the CRF-enhanced startle, a task which is thought to be contextual in nature as opposed to the FPS, which is more cue-dependent (Toufexis et al., 2004). Furthermore, progesterone's effects at reducing CRF-enhanced startle are thought to be due to ALLO, as ALLO had similar effects and medroxyprogesterone, which cannot be metabolized to ALLO blocked the effect (Toufexis et al., 2004). A similar effect was seen in male mice administered ALLO systemically prior to context pre-exposure; during context testing, those that received ALLO had decreased freezing whereas no effects were observed during tone testing (Rabinowitz et al., 2014). Therefore, ALLO appears to selectively reduce contextual fear. In contrast to this, OVX mice treated with progesterone after training showed increased contextual and cued fear (Frye and Walf, 2008), suggesting progesterone and ALLO may have different roles in conditioned fear regarding consolidation versus expression.

Regarding extinction learning, female rats that were extinguished during the proestrus phase consolidated the memory better than those in metestrus as seen by reduced freezing on the recall test the following day (Milad et al., 2009). While this could be due to both estrogen or progesterone, as exogenously administering estrogen or

progesterone during metestrus reversed this, blocking progesterone receptors during proestrus eliminated the decreased freezing during recall, suggesting progesterone is in some way involved (Milad et al., 2009). However, in an OVX model, timing of progesterone administration appears to be critical; progesterone administered six hours prior to extinction enhanced recall, but impaired it when administered 24 hours prior. In further support, blocking progesterone receptors in proestrus during extinction prevented the increase in fear during recall as seen during metestrus (Graham and Daher, 2015).

Progesterone withdrawal has also been linked to anxiety. In both withdrawal paradigms after exogenous progesterone administration and in natural fluctuations across the estrous cycle, declining progesterone is associated with an anxiogenic response (Koonce et al., 2012; Lofgren et al., 2006; Mora et al., 1996; Smith et al., 1998). Although these tests were not specific to conditioned fear, they suggest fluctuating progesterone levels change anxiety profiles. Mechanistically, this has been linked to changes in $GABA_A$ subunit expression, suggesting a role for ALLO (Griffiths and Lovick, 2005; Lovick et al., 2005; Smith et al., 1998).

The socially isolated male mouse has served as a model for PTSD as evidenced by its increase in anxiety and aggressive behavior. Disruption of ALLO levels have specifically been linked to this effect (Pinna et al., 2003). Decreased ALLO levels coincided with decreased levels of 5α-reductase in crucial corticolimbic regions (Agís-Balboa et al., 2007). Once again, these irregularities in ALLO levels correspond to increased contextual fear with no effects on cued fear which specifically correlated with decreased 5α-reductase in the amygdala and hippocampus (Pibiri et al., 2008). Further

supporting a beneficial role of ALLO in treating PTSD, ganaxolone, a synthetic version of ALLO has been shown to facilitate extinction of context fear in this model (Pinna and Rasmusson, 2014) and many drugs have been shown to be effective through upregulation of ALLO (Ugale et al., 2007; Uzunov et al., 1996). Together, progesterone and its metabolite ALLO appear to preferentially affect contextual fear processing, although the exact mechanisms behind this, remain to be discovered.

Humans

The influence of progesterone and ALLO on both healthy individuals and those diagnosed with psychiatric disorders mirrors many of the studies done in rodent models. In humans, progesterone rises during the luteal phase and tapers off to low levels in the follicular phase. Many anxiety-related symptoms are reported in women during this time of hormonal flux in healthy subjects and clinical patients (Breier et al., 1986; Gonda et al., 2008). Similar to the literature on progesterone withdrawal in rodents, diminishing levels of progesterone may also contribute to increased anxiety in humans. This is particularly important in premenstrual syndrome (PMS) and its extreme variant premenstrual dysphoric disorder (PMDD), both of which involve anxiety symptoms (Angst et al., 2001; Vickers and McNally, 2004). In patients with PMS, ALLO levels during the luteal phase were lower than healthy controls and progesterone levels were lower in both the luteal and follicular phases (Monteleone et al., 2000), but rising ALLO levels also correlated with adverse moods such as irritability (Backstrom et al., 2003). These discrepancies suggest that absolute levels of ALLO may not be the contributing factor, but overall sensitivity changes to $GABA_A$ receptors as subunits change (Epperson

et al., 2002). This is further supported by the receptor subunit changes observed in animal models. Interestingly, progesterone administered to healthy subjects during the follicular phase when levels are typically low resulted in increased amygdala activity (van Wingen et al., 2008). Increased amygdala activity is thought to correlate with increased anxiety; however, these results could be due to a sudden increase in ALLO levels at a time when this does not naturally occur.

Human studies of extinction learning, which largely model therapies for PTSD, do not convincingly show a role for progesterone. In fact, levels of progesterone did not correlate with fear acquisition, extinction, or recall (Milad et al., 2010; Wegerer et al., 2014). However, patients with PTSD have lowered ALLO levels compared to controls (Rasmusson et al., 2006). In clinical populations, there also appears to be a dysfunction in GABAergic neurotransmission, specifically with a decrease in GABA levels (Vaiva et al., 2004) and decreased activity of benzodiazepines (Bremner et al., 2000). One hypothesis for the lack of success with benzodiazepines could be due to changes in $GABA_A$ receptor subunit expression that do not bind benzodiazepines, but still would be responsive to ALLO. Because of the ineffectiveness of benzodiazepines in treating PTSD (Davidson, 2004), efforts should be made to consider allopregnanolone or its synthetic analogues in the treatment of PTSD.

Neuroanatomical Substrates for Sex Differences in Fear and Anxiety

Amygdala

Seminal work identifying the role of the amygdala in fear behavior came from Kluver and Bucy (1937). Removing the medial temporal lobes in rhesus monkeys resulted in an absence of fear, anger, and aggression. Before surgery, animals were fearful of their handlers, but after removal of the temporal lobes, they readily approached humans and objects without hesitation (Kluver and Bucy, 1937). The lesions in Kluver and Bucy's work, however, encompassed more area than just the amygdala. Later work by Weiskrantz (1956) demonstrated that the loss of fear was a result of damage specific to the amygdala. These initial studies provided evidence of the role of the temporal lobe, specifically the amygdala, in regulating fear behaviors and spawned decades of research that continues today. The amygdala is composed of two main divisions: the lateral (LA), basolateral (BL), and basomedial (BM) nuclei, together referred to as the basolateral amygdala (BLA), and the central amygdala (CEA) composed of the lateral and medial nuclei (Krettek and Price, 1978a; McDonald, 1982). Through its afferent sensory information from auditory, visual, and somatosensory inputs, the BLA, particularly the LA, is responsible for processing CS-US associations and subsequent fear expression (Fanselow and Ledoux, 1999; Maren, 1999a). The CEA, on the other hand, is generally believed to be the output region of the amygdala to brain regions important for the fear response including the periaqueductal gray for freezing responses and the hypothalamus for stress responses (Ciocchi et al., 2010; Gray and Magnuson, 1987; Gray et al., 1989). The BLA is predominantly composed of two different neurons: pyramidal neurons and

interneurons. Glutamatergic pyramidal neurons form connections within the BLA as well as with other regions in the amygdala and outside. GABAergic interneurons mostly form local connections within the BLA (Bienvenu et al., 2012; Pape and Pare, 2010; Polepalli et al., 2010; Wolff et al., 2014). The CEA, is mostly made up of GABAergic interneurons that contain a variety of peptides including CRH which are responsible for both local control and external connections (Davis et al., 1994; McDonald, 1985; Pape and Pare, 2010).

Synaptic plasticity within the BLA is necessary for conditioned fear (An et al., 2012; Herry et al., 2008; Quirk et al., 1995) and numerous studies using lesions or reversible inactivation have identified the BLA as the site of CS-US convergence along with other indices of fear such as fear potentiated startle. Blocking BLA actions prior to acquisition or expression of both contextual and cued fear impairs freezing behavior (Campeau and Davis, 1995; Gale et al., 2004; Goosens and Maren, 2001; Helmstetter and Bellgowan, 1994; Maren et al., 1996a). Indeed, disrupting the acquisition of conditioned fear can still be observed at one month up to a year post-training (Gale et al., 2004; Maren et al., 1996a). The CEA may also be involved in fear acquisition insofar as CEA inactivation prior to training hinders fear learning (Ciocchi et al., 2010). Furthermore, with overtraining, rodents with BLA lesions are still able to acquire conditioned fear, which in part is mediated by the CEA (Zimmerman et al., 2007). Consistent with the role of the CEA as the output region of the amygdala, lesions of the CEA block fear expression to contextual and cued fear (Goosens and Maren, 2001;

Nader et al., 2001), however, the CEA is implicated in fear-potentiated startle (resembling cued fear), but not light-enhanced startle (Walker and Davis, 1997).

While the amygdala, including both the BLA and CEA, is important for both the acquisition and expression of fear behavior, it has not received significant attention as a locus for hormone action in mediating sex differences. It does contain estrogen and progesterone receptors (Cover et al., 2014; Guerra-Araiza et al., 2003; Hagihara et al., 1992; Laflamme et al., 1998). However, ERα receptors and ERβ receptors predominate in different nuclei (Laflamme et al., 1998; Osterlund et al., 1998). Necessary enzymes for ALLO production are also present (Pibiri et al., 2008) and direct administration of ALLO into the CEA decreases anxiety on a variety of tasks (Akwa et al., 1999).

Although initial experiments exploring sex differences in fear did not focus on the amygdala, more recent work has begun to examine its effects. First, prolonged exposure to estrogen following OVX facilitates both contextual and cued fear conditioning which is associated with increased CRH mRNA expression in the central amygdala (Jasnow et al., 2006). Female rats have greater context generalization of fear to similar contexts compared to males and this also correlated with greater activation in the amygdala of females (Keiser et al., 2017). Estrogen may also play a role in modulating neuronal activity and synaptic plasticity in the amygdala following extinction in both rodents and humans (Zeidan et al., 2011). Mechanistically, sex differences may be attributed to differences in functional connectivity (Engman et al., 2016) or differences in synaptic plasticity due to aromatase activity within the amygdala (Bender et al., 2017).

Hippocampus

Due to the initial discovery of sex differences in contextual fear conditioning, much research has focused on the contribution of the hippocampus to sexually dimorphic fear behavior. The hippocampus is believed to be responsible for encoding contextual information, but not specifically creating the context-US relationship. For instance, hippocampal post-training lesions cause a deficit in freezing to the original training context (Frankland et al., 1998; Maren et al., 1997; Phillips and Ledoux, 1992; Selden et al., 1991), although pre-training lesions do not always cause a deficit (Frankland et al., 1998; Maren et al., 1997). Although the hippocampus is not typically associated with cued fear conditioning, following extinction of cued fear, it is involved in context-dependent fear renewal. Lesions prior to fear acquisition and after extinction training blocked renewal (Ji and Maren, 2005; 2008). Thus, the hippocampus is important for encoding contextual fear memories.

Contextual representations formed in the hippocampus are relayed directly or indirectly to the BLA (Maren and Fanselow, 1995; Pitkänen et al., 2000). Connections from the dorsal hippocampus to the BLA are important for the expression of contextual fear (de Oliveira Coelho et al., 2013; Huff and Rudy, 2004; Lesting et al., 2011; Maren and Hobin, 2007). In addition, the ventral hippocampus, which has significant reciprocal connections with the amygdala (Canteras and Swanson, 1992) is also involved. Reversible and irreversible inactivation of the ventral hippocampus impairs conditioning to contextual and auditory stimuli as well as memory retrieval (Bannerman et al., 2003; Bast et al., 2001; Hobin et al., 2006; Maren, 1999c; Maren and Holt, 2004).

Optogenetic studies suggest that BLA inputs to the ventral hippocampus promote an anxiogenic tone whereas inhibiting this projection is anxiolytic (Felix-Ortiz et al., 2013). Therefore, the hippocampus plays a critical role in contextual encoding and through its projections with the amygdala contributes to fear behavior.

Importantly, gonadal steroids heavily influence the hippocampus. Progesterone and estrogen receptors are both expressed (Hagihara et al., 1992; Osterlund et al., 1998), but ERβ receptors, important for fear and anxiety, are more prevalent than ERα receptors (Foster, 2012; Mitra et al., 2003). Estrogen is also known to change hippocampal morphology, which may affect its role in fear behavior. For instance, changes in hormone levels across the estrous cycle influence the density of spines in the hippocampus (Woolley and McEwen, 1993; Woolley et al., 1990) and loss of hormones after ovariectomy results in a loss of spine density (Woolley and McEwen, 1993). Changing spine density is also important in learning and LTP (Engert and Bonhoeffer, 1999; Parnass et al., 2000; Trachtenberg et al., 2002). In the hippocampus, estrogen-induced LTP is dependent on NMDA receptors (Smith and McMahon, 2006). Indeed, sex differences were first observed in dorsal hippocampal LTP with males presenting stronger LTP, which correlates with greater contextual freezing responses (Maren et al., 1994). This was later found to be modulated by estrogen (Gupta et al., 2001). The ventral hippocampus also shows sex differences in extracellular signal-regulated kinase (ERK) phosphorylation with higher levels in males following fear conditioning (Gresack et al., 2009).

Fear extinction is believed to be a form of hippocampal-dependent learning, such that hormone actions in the hippocampus are relevant for fear extinction as well. In female OVX rats, intra-hippocampal estrogen, enhanced extinction, which was found to be dependent on ERβ (Chang et al., 2009). Clinically, high estrogen in women is also associated with increased hippocampal activation during extinction recall which corresponds with increased extinction retention compared to low estrogen groups (Zeidan et al., 2011).

The evidence above suggests a critical role for involvement of the hippocampus in contextual fear and provides support for the role of hormones in mediating this effect. Although there is compelling evidence for the hippocampus to mediate sex differences in fear, other brain regions that have received less attention may be important for hormonal regulation and contextual fear, such as the BNST.

Bed Nucleus of the Stria Terminalis

Circuitry

The BNST is situated in a unique position to affect fear through its connections with the amygdala, hippocampus, prefrontal cortex, and hypothalamus. However, due to the complexity of the many different subnuclei that compose the region, it remains poorly understood. The BNST is part of the extended amygdala, ventral to the lateral septal area, and dorsal to the hypothalamic preoptic area. It connects with both the basolateral and central amygdala (BLA and CEA, respectively), ventral hippocampus (VH), prefrontal cortex (PFC), and hypothalamus (Figure 1; Dong et al., 2001a; 2001b;

Dong and Swanson, 2004), positioning it to affect freezing behavior with both afferent and efferent connections.

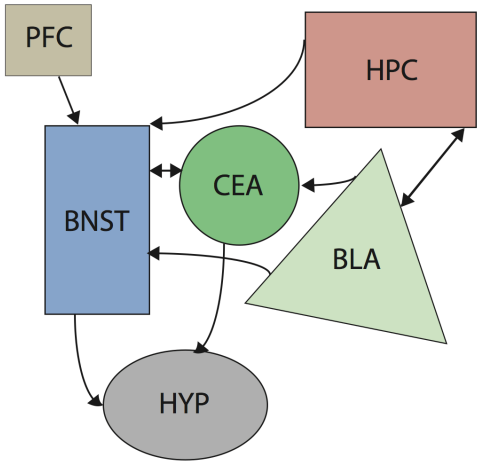

Figure 1 Fear conditioning circuitry.
Prefrontal cortex (PFC); hippocampus (HPC); bed nucleus of the stria terminalis (BNST); central amygdala (CEA); basolateral amygdala (BLA); hypothalamus (HYP).

Specifically, the intricate connections from the amygdala differentially target the BNST. The basal nuclei projects to the BNST, however the basomedial region preferentially targets the anteromedial region of the BNST (BNST-AM) whereas the basolateral region preferentially targets the anterolateral region of BNST (BNST-AL) (Dong et al., 2001a; Krettek and Price, 1978b). Interestingly, the projections from the basolateral region to the BNST-AL exclude the oval nuclei (BNST-OV; Dong et al., 2001a), but BLA to BNST-anterodorsal pathway appears to produce an anxiolytic tone (Crowley et al., 2016; Kim et al., 2013). The CEA and the BNST have long been known to be anatomically and neurochemically similar. Both receive glutamatergic inputs from the BLA (Dong et al., 2001a; Krettek and Price, 1978b) and largely project to the same

regions of the hypothalamus except the BNST projects to the paraventricular nucleus of the hypothalamus and the CEA does not (Dong et al., 2001b; Dong and Swanson, 2006; Prewitt and Herman, 1998). There are also reciprocal projections between the two regions such that the CEA sends GABAergic projections mainly to the BNST-AL and the BNST sends projections back to the CEA via the BNST-AL and BNST-AV regions (Dong et al., 2001b; Dong and Swanson, 2004; Gungor et al., 2015).

Outside of the amygdala and relevant to fear circuitry, the BNST (dorsalmedial and fusiform nuclei) receives glutamatergic projections from the infralimbic cortex (Dong et al., 2001a; Radley et al., 2009) whereas it receives glutamatergic projections from the ventral subiculum mainly to the BNST-AM (Cullinan et al., 1993; Dong et al., 2001a; Radley and Sawchenko, 2011). Within the BNST there are vast intrinsic connections as well (Turesson et al., 2013).

Contextual fear

Importantly, the BNST is implicated in contextual fear. Both electrolytic lesions and reversible inactivation of the BNST selectively impairs contextual fear, but has no effect on auditory cued fear (Hammack et al., 2004; Resstel et al., 2008; Sullivan et al., 2004; Zimmerman and Maren, 2011). This distinction may depend on the length of the auditory cues as very long cues depend on BNST activity (Waddell et al., 2006; Walker et al., 2009) as well as when the US is delivered (Hammack et al., 2015). Inactivating the BNST also impairs fear to unconditioned stimuli such as bright lights (Walker and Davis, 1997), and predator odor (Fendt et al., 2003; Xu et al., 2012) where the threat is

present throughout the entire test. Therefore, the BNST appears to be important for contextual information, however what constitutes contextual information is subjective.

Neuroendocrine properties

The BNST regulates a number of sexually dimorphic behaviors including reproductive (Claro et al., 1995; Liu et al., 1997) and maternal (Numan, 1996; Numan and Numan, 1995) behaviors. As such, it is considered a site of hormonal regulation. Progesterone, estrogen, and androgen receptors are all represented across sexes in the rodent BNST (Auger and de Vries, 2002; Laflamme et al., 1998; Roselli, 1991) with equal levels of estrogen and progesterone receptors in male and female rats and a greater number of androgen receptors in males (Roselli, 1991). Immunostaining also suggests the presence of ALLO in this region (Saalmann et al., 2007). Additionally, the BNST-OV and BNST-FU of the anterior BNST are rich with CRF neurons (Phelix and Paull, 1990), which may exert an anxiogenic influence (Daniel and Rainnie, 2016; Lee and Davis, 1997). Lastly, the BNST has direct projections to CRH containing neurons in the paraventricular nucleus, situating it to play a major role in regulation of HPA response to stress (Dong et al., 2001b; Dong and Swanson, 2006). Many projections from the BNST to the PVN are GABAergic, suggesting some nuclei may inhibit the stress response (Cullinan et al., 1993), whereas others, particularly in the ventral BNST may activate the PVN via CRH projecting or glutamatergic neurons (Choi et al., 2007). Given the neuroendocrine properties of the BNST and its role in contextual fear, it is poised to be a key locus of action in mediating sex differences seen in fear and anxiety.

State Dependence: A Potential Mechanism of Hormone Action

Another possible role for hormones in mediating fear memories is through state-dependent effects. Here, memories are more robust when recalled in the same state in which they were learned (Overton, 1991). This raises the question if hormones create interoceptive context cues in addition to exteroceptive context cues such as lighting, odors, and physical boundaries. Little research has explored the state-dependent effects of hormones, however exogenous progesterone administration following ovariectomy produced state-dependent effects in passive avoidance whereas naturally cycling hormones did not (Ebner et al., 1981). The interaction of hormone states during learning and testing may be critical to understanding its role. Contextual fear is sensitive to exteroceptive context shifts and therefore may also be subject to interoceptive context shifts as well.

Specific Aims

The central goal of this dissertation is to explore the role of ALLO in mediating sex differences in fear behavior via its actions in the BNST. Following fear conditioning in rodents, male rats display enhanced contextual fear in comparison to females. ALLO, which binds to $GABA_A$ receptors, has pronounced anxiolytic effects on a variety of behaviors, including contextual fear. Females have higher circulating levels of ALLO compared to males through its conversion from progesterone. The BNST may be a locus of ALLO action given its important role in contextual fear and hormonal modulation. In Chapter 2, to test the hypothesis that ALLO modulates contextual fear via the BNST, we

injected ALLO into the BNST of male rats and blocked the synthesis or binding of ALLO in the BNST of female rats prior to testing for contextual fear or fear to a discrete stimulus. This reversed the normal pattern of behavior in which males had reduced contextual fear and females had increased contextual fear. As expected, neither manipulation affected cued fear. The experimental design in these studies did not account for the possible role of state-dependent effects. Thus, in Chapter 3, we explored the role of ALLO in producing state-dependent effects via the BNST or the BLA. Surprisingly, ALLO resulted in state-dependent effects on contextual fear when administered to the BNST of male rats. Consistent with prior literature, intra-BLA ALLO did not confer state-dependent effects. Because this neurosteroid produced state-dependent effects in males, in Chapter 4, we sought to find out if progesterone, the precursor to ALLO, is associated with state-dependent effects in females. First, we used naturally cycling female rats and explored the role of the BNST on state-dependent contextual fear by measuring neuronal activation, as assessed by c-fos. Secondly, we used an OVX model with progesterone replacement to explore possible state-dependent effects on both contextual and cued fear. Interestingly, naturally cycling progesterone levels results in asymmetrical state-dependent contextual fear, however, this did not appear to match c-fos patterns in the BNST. The OVX model, however, produced state-dependent fear to cued stimuli but not context. Together, these findings suggest hormones and their metabolites can confer state-dependent effects on fear learning; not only is hormonal state either during training or during testing important, but the concordance between the two states matters.

CHAPTER II

ALLOPREGNANOLONE IN THE BED NUCLEUS OF THE STRIA TERMINALIS MODULATES CONTEXTUAL FEAR IN RATS*

Overview

Trauma- and stress-related disorders are among the most common types of mental illness affecting the U.S. population. For many of these disorders, there is a striking sex difference in lifetime prevalence; for instance, women are twice as likely as men to be affected by posttraumatic stress disorder (PTSD). Gonadal steroids and their metabolites have been implicated in sex differences in fear and anxiety. One example, allopregnanolone (ALLO), is a neuroactive metabolite of progesterone that allosterically enhances $GABA_A$ receptor activity and has anxiolytic effects. Like other ovarian hormones, it not only occurs at different levels in males and females but also fluctuates over the female reproductive cycle. One brain structure that may be involved in neuroactive steroid regulation of fear and anxiety is the bed nucleus of the stria terminalis (BNST). To explore this question, we examined the consequences of augmenting or reducing ALLO activity in the BNST on the expression of Pavlovian fear conditioning in rats. In Experiment 1, intra-BNST infusions of ALLO in male rats suppressed freezing behavior (a fear response) to the conditioned context, but did not

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influence freezing to a discrete tone conditioned stimulus (CS). In Experiment 2, intra-BNST infusion of either finasteride (FIN), an inhibitor of ALLO synthesis, or 17-phenyl-(3α,5α)-androst-16-en-3-ol, an ALLO antagonist, in female rats enhanced contextual freezing; neither treatment affected freezing to the tone CS. These findings support a role for ALLO in modulating contextual fear via the BNST and suggest that sex differences in fear and anxiety could arise from differential steroid regulation of BNST function. The susceptibility of women to disorders such as PTSD may be linked to cyclic declines in neuroactive steroid activity within fear circuitry.

Introduction

In the U.S., the lifetime prevalence of trauma- and stress-related disorders is 60% greater for women compared to men (Kessler et al., 2005a). Indeed, posttraumatic stress disorder (PTSD) is twice as likely to occur in women. Sex differences in fear and anxiety have also been reported in non-human species. One well-established behavioral paradigm that models aspects of PTSD, Pavlovian fear conditioning, has revealed sex differences in rodents (Aguilar et al., 2003; Barker and Galea, 2010; Chang et al., 2009; Daviu et al., 2014; Gresack et al., 2009; Kudo et al., 2004; Maren et al., 1994; Markus and Zecevic, 1997; Wiltgen et al., 2001) as well as in humans (Grillon, 2008; Lebron-Milad et al., 2012; Milad et al., 2006). In this procedure, a neutral conditioned stimulus (CS, tone) that has been paired with an aversive unconditioned stimulus (US, footshock) comes to elicit conditioned fear responses, including freezing, increases in acoustic startle, and changes in heart rate and blood pressure (Fanselow and Poulos, 2005;

LeDoux, 2000; Maren, 2001). After fear conditioning, male and female rats show a dramatic sex difference in levels of contextual freezing: females express significantly lower levels of freezing in the conditioning context compared to males (Barker and Galea, 2010; Maren et al., 1994; Markus and Zecevic, 1997). Interestingly, males and females exhibit similar levels of freezing during conditioning and during the expression of fear to the discrete CS (Barker and Galea, 2010; Maren et al., 1994; Markus and Zecevic, 1997).

One potential neural substrate for this sex difference in contextual fear is the bed nucleus of the stria terminalis (BNST). The BNST receives input from multiple limbic structures involved in emotional processing and sends output directly to the hub of the hypothalamic-pituitary-axis, the paraventricular nucleus of the hypothalamus (Crestani et al., 2013). BNST lesions or inactivation selectively impair freezing to shock-associated contexts, but not auditory CSs (Hammack et al., 2004; Resstel et al., 2008; Sullivan et al., 2004; Zimmerman and Maren, 2011). In addition, BNST inactivation reduces light- (Walker and Davis, 1997) and corticotropin-releasing hormone (CRH)-enhanced (Lee and Davis, 1997) startle, two forms of startle potentiation argued to reflect contextual fear. BNST lesions also reduce fear to long-duration auditory CSs that mimic the temporal properties of contextual stimuli (Waddell et al., 2006). Moreover, recent optogenetic approaches have shown that discrete neural circuits within the BNST are involved in regulating responses to aversive contexts (Jennings et al., 2013; Kim et al., 2013). Collectively, these data suggest that the BNST is central for the expression of conditional fear responses, including freezing, to aversive contexts.

Given its role in reproductive (Claro et al., 1995; Liu et al., 1997) and maternal (Numan, 1996; Numan and Numan, 1995) behaviors, the BNST is a prime site for hormonal modulation. BNST neurons have receptors for estrogen (Laflamme et al., 1998), progesterone (Auger and de Vries, 2002), and androgens; levels of estrogen and progesterone receptors are similar between male and female rats whereas levels of androgen receptors are greater in males (Roselli, 1991). Indeed, hormonal modulation of BNST activity in fear conditioning has been implicated by studies of intact cycling females and ovariectomized females treated with gonadal steroids. For example, natural fluctuations in ovarian steroids across the estrous cycle are associated with differences in the expression of contextual fear such that rats in proestrus, when progesterone and estradiol levels are highest, show the lowest level of fear compared to males and females in estrus (Markus and Zecevic, 1997). Moreover, systemic administration of either progesterone or its neuroactive metabolite, allopregnanolone (ALLO), impairs CRH-enhanced increases in acoustic startle, a form of startle that is mediated by the BNST (Toufexis et al., 2004). A potent allosteric potentiator of $GABA_A$ receptors (Majewska et al., 1986), ALLO has been linked to reduced anxiety in a variety of other behavioral paradigms in which female rodents appear less anxious than males (Frye et al., 2000; Hughes et al., 2004), including the elevated plus maze (Bitran et al., 1991), the defensive burying test (Picazo and Fernandez-Guasti, 1995), the light/dark transition test (Wieland et al., 1991), and the open field test (Wieland et al., 1991). Although ALLO can be synthesized within the gonads, adrenal glands, and brains of both male and female rats, it is found in higher concentrations in female brains and plasma (Corpéchot et al., 1993).

In females, ALLO levels in whole brain and plasma are 8- to 10-fold higher than in males (Cheney et al., 1995; Corpéchot et al., 1993; Purdy et al., 1990).

Collectively, this work suggests that ALLO may regulate fear and anxiety by modulating neuronal activity in the BNST. To explore this question, we assessed whether augmenting ALLO activity in the BNST of male rats would decrease contextual freezing (Experiment 1) and, conversely, whether reduction of ALLO activity in the BNST of female rats would increase contextual freezing (Experiment 2). Males received intra-BNST infusions of ALLO whereas females received infusions of either an ALLO synthesis inhibitor, finasteride (FIN; Finn et al., 2006), or a selective antagonist, 17-phenyl-(3α,5α)-androst-16-en-3-ol (17-PA; Mennerick et al., 2004; Kelley et al., 2007). Importantly, this set of experiments examines whether hypothesized sex differences in endogenous intra-BNST ALLO (i.e., low endogenous ALLO activity in males and high endogenous ALLO activity in females) contribute to the contextual fear phenotype in each sex. We now show that manipulations of ALLO activity within the BNST affect the expression of contextual fear in both male and female rats, a finding that supports neuroactive steroid modulation of the BNST in fear and anxiety.

Materials and Methods

Animals

Male and female Long-Evans rats (200–224 g; Blue Spruce) were obtained from a commercial supplier (Harlan Laboratories, Indianapolis, IN, USA). At the time of

arrival, rats were individually housed in clear plastic cages; males and females were kept in separate rooms. Lights were maintained on a 14:10 h light:dark cycle (lights on at 7:00 A.M.) with access to food and water ad libitum. Upon arrival, each rat was handled for 20 s each day for five consecutive days to acclimate to the experimenter. All experiments were carried out in accordance with guidelines approved by the Institutional Animal Care and Use Committees at Texas A&M University.

Behavioral Apparatus

All behavioral procedures occurred in eight identical observation chambers (30 × 24 × 21 cm; Med Associates, St. Albans, VT, USA) composed of aluminum sidewalls and Plexiglas ceiling, rear wall, and front door. One sidewall contained a speaker for CS delivery and the other contained an incandescent house light. Chamber floors consisted of 19 stainless steel rods (4 mm in diameter) spaced 1.5 cm apart (center to center) for footshock US delivery. The rods were wired to a shock source and solid-state grid scrambler (Med Associates). Beneath the rods was a removable stainless steel tray. In addition, each chamber was positioned in a sound-attenuating cabinet equipped with a ventilation fan to provide background noise (65 dB).

The behavioral procedures were conducted in two distinct contexts. For “context A” (conditioning and context test), the chambers were cleaned with 1% acetic acid; the pans beneath the floors were also rinsed in acetic acid. The house lights (15 W) in the chambers were lit and the cabinet doors were left open. White fluorescent room lights, the computer monitor, and cabinet fans were on. Animals were transported to and from

the vivarium in white plastic boxes. For "context B" (tone test), the chambers were cleaned with 1% ammonium hydroxide. The house lights were turned off and the cabinet doors were closed. Red fluorescent lights illuminated the room, the computer monitor was turned off, and the cabinet fans were on. Animals were transported to and from the vivarium in black plastic boxes.

Locomotor activity was measured by recording the displacement of the load cell platform located underneath each chamber (Maren, 1998). Prior to the experiment, each load cell amplifier was calibrated to a fixed chamber displacement with the output of each amplifier set to a specific gain to detect immobility. The output of the load cell amplifier was digitized such that one observation every 200 ms for each rat was recorded via the Threshold Activity software (Med Associates). Freezing behavior was derived from the locomotor activity as previously described (Maren, 1998).

Surgery

Male rats were anesthetized with ketamine (100 mg/kg body weight; i.p.) and xylazine (10 mg/kg body weight; i.p.) and treated with atropine sulfate (0.04 mg/kg body weight, i.p.). Female rats were anesthetized with ketamine (60 mg/kg body weight; i.p.) and xylazine (8 mg/kg body weight; i.p.) and treated with atropine sulfate (0.04 mg/kg body weight; i.p.). After shaving the top of the head, each rat was secured in a stereotaxic apparatus (David Kopf Instruments, Tujunga, CA, USA). The scalp was incised and retracted. Head position was adjusted to align lambda and bregma in the same horizontal plane. Small burr holes (1 mm in diameter) were drilled bilaterally into

the skull for placement of guide cannulae (stainless steel, 26-gauge, 9 mm below pedestal; Plastics One, Roanoke, VA, USA) directed towards the BNST (0.0 mm AP and 2.7 mm ML relative to bregma, −6.9 mm DV from dura at a 10° angle from vertical towards the midline). In addition, three burr holes were drilled for the placement of anchoring screws. After implantation of guide cannulae, dental acrylic was applied to affix the cannulae to the skull. After surgery, dummy cannulae (33-gauge, 9 mm with 1-mm projection; Plastics One) were inserted into the guide cannulae. Rats were allowed to recover for 1 week before behavioral procedures. To habituate the animals to the infusion procedures, rats were individually transported in white 5-gal buckets lined with bedding to the infusion room (within the vivarium) and received dummy changes on two separate days during the recovery period.

Vaginal Smears

Vaginal smears were obtained from all female rats for at least eight consecutive days to ensure normal cycling; estrous cycle phase was not used as a variable in the analysis due to sample size. To track estrous cycle phase, vaginal smears were taken daily with cotton swabs moistened with distilled water between 8:00 and 10:00 A.M. starting the day of surgery and continuing throughout the behavioral procedures (allowing for at least 1 h between smears and behavior). Cells were visualized under a light microscope at 100× and characterized according to Goldman et al. (2007). One rat with an irregular cycle was excluded from the analysis.

Drugs

All drugs were prepared in a 30% (w/v) solution of the complexing agent hydroxypropyl-β-cyclodextrin (VEH; Sigma-Aldrich, St. Louis, MO, USA) in purified water. Allopregnanolone or (3α,5α)-3-hydroxy-pregnan-20-one (ALLO; R&D Systems, Minneapolis, MN, USA) was solubilized in VEH (8 mg/ml). The 5α-reductase inhibitor commonly known as finasteride, (5α,17β)-N-(1,1-dimethylethyl)-3-oxo-4-azaandrost-1-ene-17-carboxamide (FIN; Sigma-Aldrich), was solubilized in VEH (10 mg/ml). The steroid antagonist, 17-phenyl-(3α,5α)-androst-16-en-3-ol (17-PA; R&D Systems) was solubilized in VEH (3.5 mg/ml). Infusions were made with 10-µl Hamilton syringes mounted into an infusion pump (KD Scientific, Hollistan, MA, USA) and connected to internal cannulae (33-gauge, 9 mm with 1-mm projection; Plastics One) with either polyethylene tubing (PE-20, Braintree Scientific, Braintree, MA, USA) for VEH or polytetrafluoroethylene tubing (PTFE; 28-gauge, SAI Infusion Technologies, Lake Villa, IL, USA) for drugs. PTFE tubing was used to minimize drug loss due to nonspecific binding.

Procedures

Experiment 1: effects of ALLO on the expression of contextual and cued fear in male rats

For the ALLO study, 31 male Long-Evans rats were housed and cannulated as described above. On Day 1, rats were transported to the laboratory and placed in the conditioning chambers (context A) for training. After a 3-min baseline period, rats were presented with five tone (CS; 10 s, 80 dB, 2 kHz)-shock (US; 2 s, 1 mA) pairings in

which the tone was immediately followed by the shock. There was a 1-min inter-trial interval (ITI) between each tone-shock pairing followed by a 1-min wait period after the final shock. On Day 2, 24 h later, squads of 4 rats were transported to the infusion room in white 5-gal buckets lined with bedding. Rats received bilateral intra-BNST infusions (0.25 μl at 0.25 μl/min) of either VEH (n = 15) or ALLO (2 μg/side; n = 16). The dosage and timing of ALLO infusions were based on previous reports of behavioral effects resulting from intracranial infusions (Akwa et al., 1999; Bitran et al., 1991; Engin and Treit, 2007); BNST infusion volumes were based on previous work from our laboratory (Zimmerman and Maren, 2011). After the 1-min infusion, internal cannulae were left in place for 2 min to allow for drug diffusion and then replaced with clean dummy cannulae. Context testing began ten minutes after the start of infusions. Rats were placed in the conditioning chambers (context A) for a 10-min context test in which no tones or shocks were delivered. On Day 3, rats were infused in the same manner with the same drug as on Day 2 and, at 10 min after the start of infusions, were placed in a novel context (context B) for a tone test. The tone test consisted of a 3-min baseline period followed by four tone (CS; 10 s, 80 dB, 2 kHz) presentations with a 1-min ITI and a 1-min wait period after the last tone. The conditioning and testing procedures (including the order of context and tone tests) were patterned after the experimental designs used in many of our studies (Maren, 1998; 1999b; Maren et al., 1997; Zimmerman and Maren, 2011), including work revealing sex differences in the expression of contextual fear (Maren et al., 1994).

Experiment 2: effects of FIN and 17-PA on the expression of contextual and cued fear in female rats

Seventy-six female Long-Evans rats were housed and cannulated as described above. On Day 1, rats were transported to the laboratory, placed in the conditioning chambers (context A) and trained in the same manner as in Experiment 1. On Day 2, squads of 8 rats were transported to the infusion room in white 5-gal buckets lined with bedding. Rats received bilateral intra-BNST infusions (0.25 µl at 0.25 µl/min) of VEH, FIN (2.5 µg/side), or 17-PA (0.875 µg/side). The doses of FIN and 17-PA were based on previous reports (Frye and Vongher, 2001; Frye and Walf, 2002; Kelley et al., 2007; Rhodes and Frye, 2001; Svensson et al., 2013; Walf et al., 2006). After the 1-min infusion, internal cannulae were left in place for 2 min to allow for drug diffusion and then replaced with clean dummy cannulae. Animals receiving FIN infusions (and a subset of VEH controls) were returned to their home cages for 2 h prior to retrieval testing to allow sufficient time for 5α-reductase inhibition (Frye and Walf, 2002; Rhodes and Frye, 2001; Walf et al., 2006). Rats in the 17-PA group (and a subset of VEH controls) were tested 10 min after their infusions (Svensson et al., 2013). For the context testing, rats were transported to the conditioning chambers (context A) for a 10-min context test as described in Experiment 1. On Day 3, rats were infused with the same drug as on Day 2 and were transported to a novel context (context B) for a tone test as described in Experiment 1. One rat from the FIN group was excluded due to acyclicity and a squad of rats (4 VEH and 4 17-PA) was excluded due to an equipment malfunction. Data from VEH controls for the FIN and 17-PA groups were collapsed for

analysis as they did not differ. This left group sizes of: VEH (n = 26), FIN (n = 15), and 17-PA (n = 12).

Histology

After behavioral testing, all rats were overdosed with pentobarbital (100 mg/kg) and transcardially perfused with 0.9% saline followed by 10% formalin. Brains were rapidly dissected and post-fixed in 10% formalin for 24 h before transfer to a 30% sucrose-formalin solution. Brains were sectioned at 40 μm on a cryostat at a constant temperature of −20°C and mounted on subbed slides with 70% ethanol. Sections were stained with 0.25% thionin to visualize cannulae placements within the BNST.

Data Analysis

In all experiments, the percentage of freezing behavior was averaged across 1-min blocks in each behavioral session (the 10-s CS periods were excluded from the analysis of the conditioning and tone test sessions) and analyzed using analysis of variance (ANOVA). We hypothesized that across the context tests, ALLO would reduce freezing in males and FIN and 17-PA would increase freezing in females. Therefore, a priori planned comparisons using Fisher's PLSD test were computed after significant effects in the ANOVA. All data are represented as means ± SEMs.

Results

Intra-BNST ALLO Infusion and Contextual Fear in Male Rats

Histology

Of 31 male rats receiving cannulae, nine were excluded because their cannulae were not centered in the BNST. This yielded the following group sizes: VEH (n = 9) and ALLO (n = 13). As shown in Figure 2A, the majority of cannula placements were centered at 0.26 mm caudal to bregma, although placements both rostral and caudal to that level were similarly represented.

Behavior

Acquisition of fear conditioning did not differ between male groups assigned to receive infusions of VEH or ALLO (Figure 3A). Levels of freezing were low (<10%) prior to the start of training and increased across the five training trials similarly for all animals. A repeated measures ANOVA with a between-subjects variable of drug group (VEH or ALLO) showed a significant main effect of trial number (TRIAL) on the level of freezing expressed ($F_{(5,100)}$ = 18.0, $p < 0.0001$) but neither the main effect of drug group ($F_{(1,20)}$ = 1.88, p = 0.19) nor the interaction between drug group and trial number ($F_{(5,100)}$ = 0.30, p = 0.91) was found to be significant. Thus, all male rats acquired conditioned fear at similar rates and levels prior to their drug manipulations.

Intra-BNST ALLO infusions 10 min prior to testing resulted in a decreased level of freezing to the conditioned context for male rats (Figure 3B). A repeated measures ANOVA with a between-subjects variable of drug group (VEH or ALLO) showed that

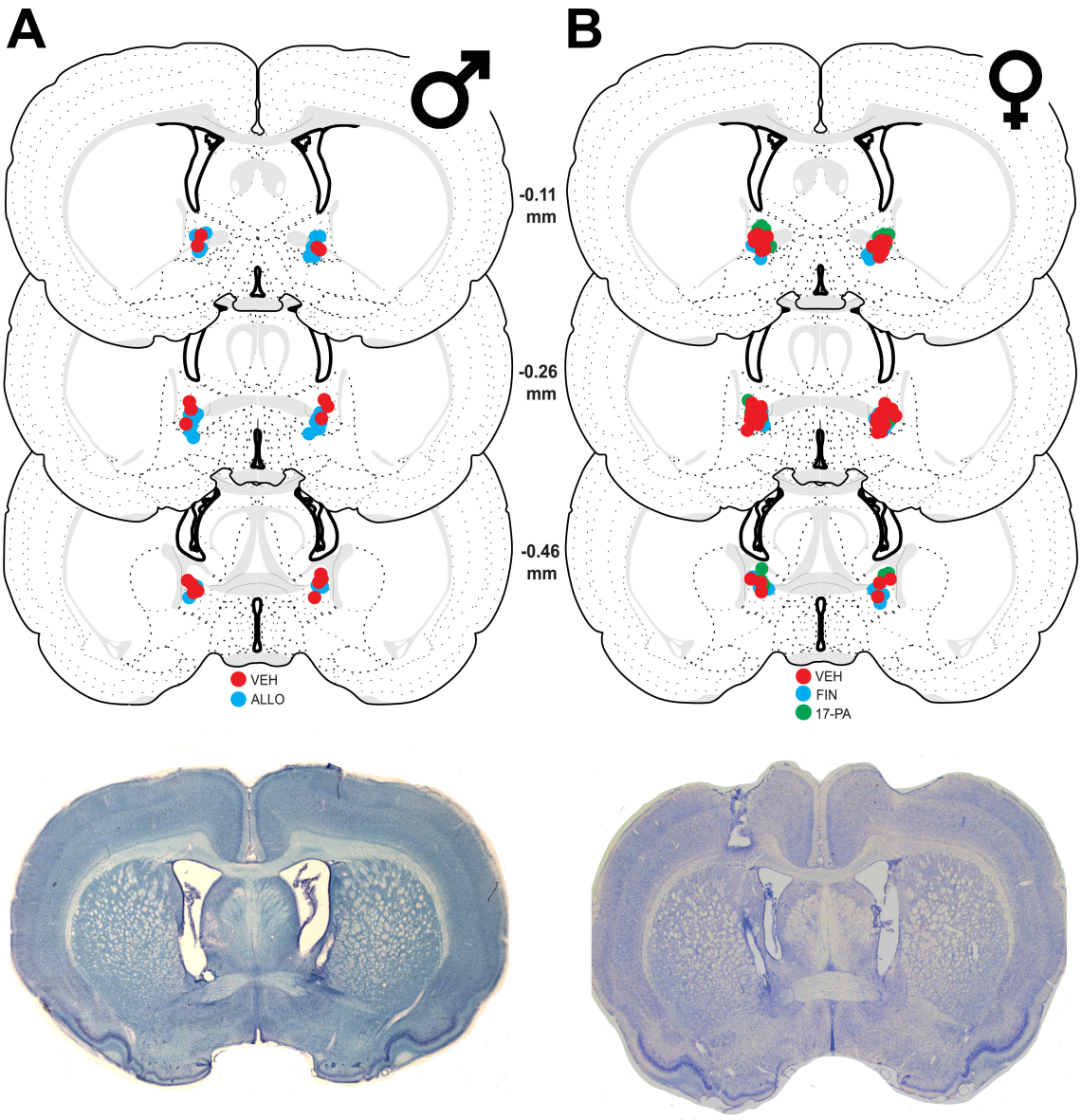

Figure 2 Schematic coronal sections showing cannula placements in the bed nucleus of the stria terminalis (BNST).
Cannula placements are indicated for infusions of vehicle (VEH), allopregnanolone (ALLO), finasteride (FIN), and 17-phenyl-(3α,5α)-androst-16-en-3-ol (17-PA). Representative thionin-stained sections are shown below each set. **(A)** Infusion sites for VEH (red circles) and ALLO (blue circles) in male rats with photomicrograph of a representative section. **(B)** Infusion sites for VEH (red circles), FIN (blue circles), and 17-PA (green circles) in female rats with photomicrograph of a representative section. Coronal brain section images adapted from Swanson (2003).

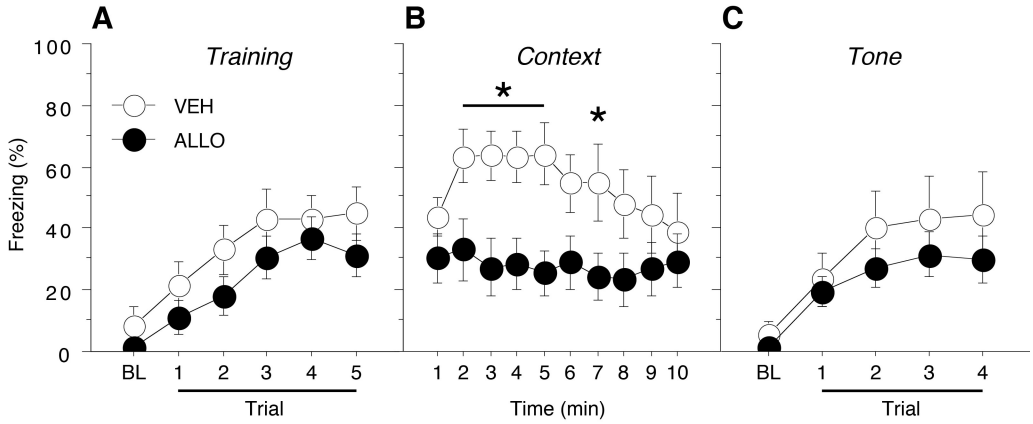

Figure 3 Conditioned freezing in male rats receiving pre-test infusions of ALLO into the BNST.

(A) Mean percentage of freezing (±SEM) during the five-trial training session (data are shown with a 3-min pre-trial period followed by five tone-shock pairings). Freezing was quantified before the first conditioning trial (baseline, BL) and during the 1-min period after each conditioning trial. **(B)** Mean percentage of freezing (±SEM) to context over 10 min 1 day after training. **(C)** Mean percentage of freezing (± SEM) to four auditory conditioned stimulus (CS) presentations in a novel context 2 days after training. Freezing was quantified before the first tone trial (baseline, BL) and during the 1-min period after each tone trial. *$p < 0.05$ ALLO vs. VEH.

intra-BNST infusion of ALLO suppressed freezing throughout the 10-min context test. The analysis revealed significant main effects of drug group ($F_{(1,20)} = 5.22$, $p < 0.05$) and time (min 1–10; $F_{(9,180)} = 2.08$, $p < 0.05$). In addition, there was a significant interaction between drug group and time ($F_{(9,180)} = 1.99$, $p < 0.05$). Planned comparisons ($p < 0.05$) of the average freezing during each minute of the context test revealed that ALLO significantly reduced freezing during minutes 2–5 and minute 7 of the test (Figure 3B). These data indicate that acute ALLO administration in the BNST suppressed the expression of contextual fear in male rats.

By contrast, pre-test infusion of ALLO into the BNST did not affect conditional freezing during the tone test (Figure 3C). A repeated measures ANOVA with a between-

subjects variable of drug group (VEH or ALLO) and a within-subject variable of trial number (TRIAL) revealed a main effect of trial ($F_{(4,80)} = 16.9$, $p < 0.0001$), but there was neither a significant main effect of drug group ($F_{(1,20)} = 0.97$, $p = 0.34$) nor an interaction between drug group and trial ($F_{(4,80)} = 0.50$, $p = 0.73$). These data indicate that acute ALLO administration in the BNST produces a selective reduction in contextual freezing in male rats.

Intra-BNST Infusion of FIN or 17-PA and Expression of Contextual Fear in Female Rats

Histology

Of the 67 cycling female rats, 14 were excluded because their cannulae either missed the target or were not patent (resulting in a unilateral infusion). This yielded the following group sizes: VEH ($n = 26$), FIN ($n = 15$), and 17-PA ($n = 12$). As shown in Figure 2B, the majority of cannula placements were centered 0.26 mm caudal to bregma although there were many placements rostral and caudal to that level.

Behavior

Acquisition of fear conditioning did not differ between the females assigned to receive VEH, FIN, or 17-PA (Figure 4A). Levels of freezing were low prior to the start of training (<5%) and increased across the five training trials similarly for all animals. A repeated measures ANOVA with a between-subjects variable of drug group (VEH, FIN, or 17-PA) and a within-subject variable of trial (TRIAL) showed a main effect of trial ($F_{(5,250)} = 24.7$, $p < 0.0001$). There was neither a significant main effect of group

($F_{(2,50)} = 0.31$, $p = 0.74$) nor an interaction between drug group and trial ($F_{(10,250)} = 0.98$, $p = 0.46$). Thus, female rats from all groups acquired conditioned fear at similar rates and levels prior to their drug manipulations.

As shown in Figure 4B, intra-BNST infusions of either FIN or 17-PA prior to testing (2 h or 10 min, respectively) modestly, but significantly, increased levels of freezing to the conditioned context in female rats. A repeated measures ANOVA with a between-subjects variable of drug group (VEH, FIN, or 17-PA) and a within-subject variable of time (min 1–10) revealed a significant main effect of time ($F_{(9,450)} = 14.6$, $p < 0.0001$) and a significant group X time interaction ($F_{(18,450)} = 1.81$, $p < 0.05$). There was no significant main effect of drug group ($F_{(2,50)} = 1.36$, $p = 0.27$). Inspection of Figure 4B suggested that the significant group X time interaction in the ANOVA was due to increases in freezing produced by intra-BNST infusions of FIN and 17-PA in the early minutes of the context test. This impression was confirmed by planned comparisons ($p < 0.05$) of average freezing during each minute of the context text. These comparisons revealed that FIN increased freezing relative to VEH-treated rats during the first 2 min of the test; 17-PA increased freezing relative to VEH controls in minutes 2–4 of the context test. These data indicate that intra-BNST infusion of an ALLO synthesis inhibitor (FIN) or an ALLO antagonist (17-PA) enhanced the expression of contextual freezing in females.

As shown in Figure 4C, pre-test intra-BNST infusions of either FIN or 17-PA did not affect freezing during the tone test. A repeated measures ANOVA with a between-subjects variable of drug group (VEH, FIN, or 17-PA) and a within-subject variable of

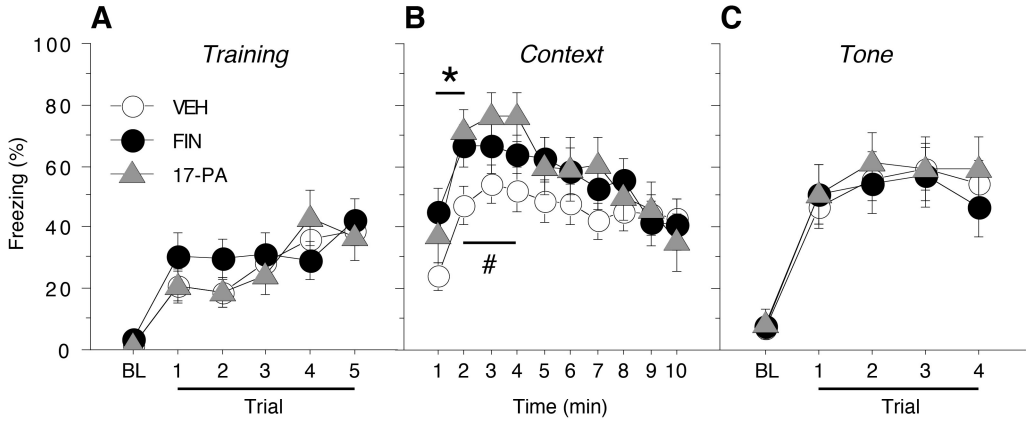

Figure 4 Conditioned freezing in female rats receiving pre-test infusions of either FIN or 17-PA into the BNST.
(A) Mean percentage of freezing (± SEM) for all females during the five-trial training session (data are shown with a 3-min pre-trial period followed by five tone-shock pairings). Freezing was quantified before the first conditioning trial (baseline, BL) and during the 1-min period after each conditioning trial. **(B)** Mean percentage of freezing (± SEM) to context over 10 min 1 day after training. **(C)** Mean percentage of freezing (± SEM) to four auditory CS presentations in a novel context 2 days after training. Freezing was quantified before the first tone trial (baseline, BL) and during the 1-min period after each tone trial. *$p < 0.05$ FIN vs. VEH, #$p < 0.05$ 17-PA vs. VEH.

trial number (TRIAL) showed a main effect of trial number ($F_{(4,200)} = 68.8$, $p < 0.0001$). There was neither a significant main effect of drug group ($F_{(2,50)} = 0.09$, $p = 0.92$) nor an interaction between drug group and trial number ($F_{(8,200)} = 0.44$, $p = 0.90$). These data reveal that intra-BNST infusions of FIN or 17-PA produce a selective enhancement of contextual freezing in female rats.

Discussion

The present results reveal that manipulations of ALLO activity within the BNST modulate the expression of conditioned contextual fear in rats. Consistent with our hypothesis, endogenous ALLO activity in males and females appears to contribute to the

contextual fear phenotype in each sex. For males, in which brain ALLO levels are relatively low, augmenting BNST ALLO levels suppressed the expression of contextual freezing. In contrast, for females, in which brain ALLO levels are relatively high, reducing intra-BNST ALLO activity enhanced the expression of contextual freezing. The effects we observed were not likely due to nonspecific sensorimotor effects of the BNST drug manipulations insofar as freezing to the auditory CS was unaffected by all treatments. Together, our results suggest that ALLO in the BNST may be a critical modulator of fear behavior in both male and female rats.

By utilizing ALLO infusions into the brain, our findings extend previous work linking circulating gonadal steroid levels to contextual fear in female rats (Markus and Zecevic, 1997; Toufexis et al., 2004). In these studies, intact female rats that were conditioned and tested during proestrus, when progesterone levels are high, exhibited lower levels of contextual freezing compared to those trained and tested in other phases of the estrous cycle (Markus and Zecevic, 1997). In addition, ovariectomized female rats treated with systemic ALLO showed a significant reduction in CRH-enhanced but not fear-potentiated acoustic startle (Toufexis et al., 2004). The former is a test of "sustained" or contextual fear whereas the latter is a test of "phasic" or cued fear (Davis et al., 2010). Similar reductions have been observed in ovariectomized females treated either acutely or chronically with progesterone (Toufexis et al., 2004). By contrast, systemic treatment of ovariectomized females with medroxy-progesterone acetate, a synthetic steroid that binds to the progesterone receptor but is not metabolized to ALLO, did not affect levels of CRH-induced startle (Toufexis et al., 2004). Altogether, these

data suggest that the reduced levels of context-dependent fear observed in females with elevated circulating progesterone levels may be due to the actions of its metabolite, ALLO, within the BNST.

Additional evidence supporting ALLO modulation of contextual fear can be found in studies of socially isolated mice. Male mice that have been socially isolated for three to four weeks show elevated levels of context- but not cue-specific fear (Pibiri et al., 2008). Interestingly, this change in conditioned fear behavior coincides with decreases in ALLO and 5α-reductase mRNA within components of the fear circuitry (medial prefrontal cortex, hippocampus, and basolateral amygdala). Group-housed mice showed similar increases in contextual fear following pharmacological blockade of 5α-reductase. This work lends support to the idea that reduced 5α-reductase activity in fear circuits can contribute to reduced ALLO levels in brain and concomitantly elevated contextual fear. We cannot, however, exclude the possibility that the early effects of FIN on contextual freezing in females may be due to reduced levels of the GABAergic potentiators, (3α,5α,17β)-androstane-3, 17-diol (3α-androstanediol) and (3α,5β)-3, 21-dihydroxypregnan-20-one (tetrahydrodeoxycorticosterone or THDOC), as 5α-reductase is involved in the synthesis of these compounds from 17-hydroxyandrostan-3-one (dihydrotestosterone or DHT) and 11-deoxycorticosterone (deoxycorticosterone or DOC), respectively (Reddy, 2010).

The results of the current study are consistent with the widely held view that the BNST is critical for the expression of contextual fear responses (Sullivan et al., 2004; Zimmerman and Maren, 2011) and support a role for GABAergic synaptic transmission

therein. Previous work involving infusions of the GABAergic agonist, muscimol, (Fendt et al., 2003) and a GABA synthesis inhibitor (Sajdyk et al., 2008) has underscored the importance of the inhibitory neurotransmitter in regulating fear and anxiety via the BNST. Here we show for the first time that ALLO modulation of $GABA_A$ receptors in the BNST may be involved in the regulation of contextual fear. While its use in behavioral paradigms is quite new, 17-PA has been well-characterized by *in vitro* and *in vivo* studies for its selective antagonism of ALLO action at $GABA_A$ receptors (Kelley et al., 2007; Mennerick et al., 2004; Svensson et al., 2013). Although its antagonism of 3α-androstanediol has not been examined, 17-PA has been shown to be ineffective against 5β-reduced steroids, barbiturates, and benzodiazepines and only partially effective against THDOC (Kelley et al., 2007; Mennerick et al., 2004). Thus, the effects of the 5α-reductase inhibitor, FIN, on contextual fear in females when considered in conjunction with those of 17-PA provide support for locally available ALLO acting at $GABA_A$ receptors within the BNST.

Recent human studies support links between ALLO, the BNST, and dysfunctional anxiety. Decreased levels of ALLO in CSF have been reported in premenopausal women diagnosed with PTSD compared to healthy individuals although it was not known whether this deficiency was pre-existing (Rasmusson et al., 2006). A context-specific role for the BNST in conditioned fear may apply to humans. Imaging studies have shown that nonhuman primates with trait anxious temperament have increased resting metabolism in the BNST (Oler et al., 2009) and that trait anxious human subjects exhibit exaggerated activity in the BNST during threat monitoring

(Somerville et al., 2010), suggesting that "hypervigilant threat monitoring" (akin to contextual fear) may be a BNST-dependent process.

Nonetheless, the rodent data present a paradox: female rats with presumably higher levels of circulating ALLO exhibit less contextual fear and extinguish their fear responses faster than males, a pattern of results that seemingly contradicts that observed in human females (Chang et al., 2009; Gupta et al., 2001; Maren et al., 1994). Namely, although women cyclically attain higher circulating levels of ALLO than men (Genazzani et al., 1998), they exhibit a greater susceptibility to PTSD (Kessler et al., 2005a). One answer to this paradox may lie in the relative propensity of males and females to extinguish fear once it is acquired. For example, studies in both rats (Chang et al., 2009; Gupta et al., 2001; Milad et al., 2009; Zeidan et al., 2011) and humans (Milad et al., 2006; 2009; 2010) reveal that low but not high estrogen levels in females retard the extinction of conditioned fear below that observed in males. Deficits in fear extinction are widely believed to contribute to the maintenance of PTSD (Maren et al., 2013; Pitman et al., 2012) and the regulation of fear extinction by gonadal steroids may be critical to increasing the vulnerability of both female rats and humans to persistent and pathological fear responding. Although it is not known how progesterone and its metabolites contribute to fear extinction, it is also possible that low levels of ALLO produce a resistance to extinction that contributes to enduring fear responses thought to underlie PTSD (Pinna, 2014). In socially isolated male mice, systemic administration of ganaxolone, a synthetic analog of ALLO, has been shown to facilitate extinction of contextual fear (Pinna and Rasmusson, 2014). Thus, cyclical variation in neuroactive

steroid levels in female rats and humans might promote vulnerabilities to fear extinction, thereby contributing to disorders such as PTSD. It should be noted that ganaxolone is currently in clinical trials for treatment of PTSD (NCT01339689).

In summary, our findings suggest that ALLO within the BNST modulates contextual fear in both male and female rats. Although we cannot speak to the relative contribution of different ALLO sources (peripheral vs. brain), immunolabeling studies have shown high levels of cellular staining in the rat BNST by an anti-ALLO antibody (Cook et al., 2014; Saalmann et al., 2007), supporting the local availability of this progesterone metabolite. As previously suggested by systemic administration, ALLO promotes anxiolytic behavior in response to fear-conditioned contexts and may account for sex differences observed in contextual fear. In women, deficiencies or large variations in circulating and brain ALLO over the reproductive cycle may contribute to increased vulnerability for anxiety disorders such as PTSD. Further work on the cellular and molecular mechanisms of ALLO action in the BNST may yield insight on determinants of susceptibility to anxiety disorders both within and across genders and inform on potential avenues of pharmacological intervention.

CHAPTER III

ALLOPREGNANOLONE INDUCES STATE-DEPENDENT FEAR VIA THE BED NUCLEUS OF THE STRIA TERMINALIS*

Overview

Gonadal steroids and their metabolites have been shown to be important modulators of emotional behavior. Allopregnanolone (ALLO), for example, is a metabolite of progesterone that has been linked to anxiety-related disorders such as posttraumatic stress disorder. In rodents, it has been shown to reduce anxiety in a number of behavioral paradigms including Pavlovian fear conditioning. We have recently found that expression of conditioned contextual (but not auditory) freezing in rats can be suppressed by infusion of ALLO into the bed nucleus of the stria terminalis (BNST). To further explore the nature of this effect, we infused ALLO into the BNST of male rats prior to both conditioning and testing. We found that suppression of contextual fear occurred when the hormone was present during either conditioning or testing but not during both procedures, suggesting that ALLO acts in a state-dependent manner within the BNST. A shift in interoceptive context during testing for animals conditioned under ALLO provided further support for this mechanism of hormonal action on contextual fear. Interestingly, infusions of ALLO into the basolateral

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amygdala produced a state-independent suppression of both conditioned contextual and auditory freezing. Altogether, these results suggest that ALLO can influence the acquisition and expression of fear memories by both state-dependent and state-independent mechanisms.

Introduction

The importance of sex steroid hormones in anxiety-related disorders has gained recent support from both preclinical and clinical studies (Glover et al., 2015; Maeng and Milad, 2015). Estrogen has been shown to affect different aspects of fear learning including acquisition of contextual fear (Barha et al., 2010), facilitation of fear extinction in female rats (Chang et al., 2009; Gupta et al., 2001; Milad et al., 2009) and humans (Milad et al., 2009; 2010; Zeidan et al., 2011) as well as modulation of fear generalization in male and female rats (Lynch et al., 2014; 2016). Progesterone has mixed effects on fear extinction; it is facilitatory in some instances (rats; Milad et al., 2009) and impairing in others (humans; Pineles et al., 2016). Support for a role of androgens in conditioned fear has also varied across studies. Castration with or without testosterone replacement had no effect on the acquisition, expression, or extinction of conditioned contextual fear in male rats (Anagnostaras et al., 1998). In contrast, when light-enhanced startle (similar to contextual fear) and fear-enhanced startle (similar to cued fear) were examined in male rats, androgens were found to suppress the former but not the latter (Toufexis et al., 2005).

Modulation of conditioned fear processes may involve steroid metabolites as well. Allopregnanolone (ALLO), a major metabolite of progesterone, has been associated with decreased levels of context-related fear (Pibiri et al., 2008; Toufexis et al., 2004) and its synthetic analogue, ganaxolone, has been shown to facilitate extinction in mice (Pinna and Rasmusson, 2014). We have recently shown that local ALLO activity in the bed nucleus of the stria terminalis (BNST) modulates the expression of conditioned contextual fear (Nagaya et al., 2015). In male rats, increased ALLO activity via local infusion suppressed context-dependent freezing whereas in female rats, decreased ALLO activity via pharmacological means resulted in enhanced freezing. Given that ALLO has been shown to be a potent allosteric potentiator of $GABA_A$ receptor (GABAR) currents (Majewska et al., 1986), one possible mechanism of ALLO action on contextual fear may involve GABARs within the BNST.

The importance of GABARs in regulating contextual fear has been recently demonstrated by studies of fear acquisition and expression following intra-hippocampal infusion of gaboxadol (Jovasevic et al., 2015). Interestingly, this work revealed that activation of extrasynaptic GABARs within the hippocampus produces state-dependent contextual fear. That is, intra-hippocampal gaboxadol infusions before either fear conditioning or retention testing blunted the expression of contextual fear in mice. However, gaboxadol infusions before both conditioning and testing resulted in high levels of conditioned freezing—indicating a state-dependent effect of GABAR modulation. ALLO shares similarities with gaboxadol in that it promotes GABAergic tone especially at extrasynaptic GABARs (Brown et al., 2002; Mortensen et al., 2012).

As such, we explored the mechanism by which ALLO affects conditioned fear by administering it into the BNST or the BLA prior to acquisition, expression, or both.

Materials and Methods

Subjects

Adult male Long-Evans rats (200-224 g at arrival; Blue Spruce) were purchased from a commercial supplier (Envigo, Indianapolis, IN, USA). Rats were individually housed in clear plastic cages and maintained on a 14:10 h light:dark cycle (lights on at 7:00 AM) in a temperature- and humidity-controlled vivarium with unrestricted access to food and water. All experiments occurred during the light phase. Upon arrival, rats were handled for five consecutive days for approximately 20 s each day to acclimate to the experimenter. All procedures were performed in accordance with the guidelines approved by the Texas A&M University Institutional Animal Care and Use Committees.

Behavioral Apparatus

All behavioral sessions occurred in eight identical observation chambers (30 x 24 x 21 cm; Med Associates, St. Albans, VT, USA) in a single testing room. Each chamber consisted of two aluminum sidewalls, a Plexiglas ceiling, rear wall, and hinged front door. A speaker for delivery of the auditory conditioned stimulus (CS) was mounted on one sidewall and an incandescent house light (15 W) was mounted on the other. The floor of each chamber consisted of 19 stainless steel rods (4 mm in diameter) spaced 1.5 cm apart (center to center) for delivery of the footshock unconditioned stimulus (US).

Rods were connected to a shock source and solid-state grid scrambler (Med Associates). A removable stainless steel tray underneath the rods was used to manipulate contexts with odors. Each chamber was situated inside a sound-attenuating cabinet equipped with a ventilation fan to provide background noise (65 dB).

Behavioral procedures were conducted using two distinct contexts. For "Context A", white fluorescent room lights, cabinet fans, and house lights were all on and cabinet doors left open. Each chamber was cleaned with 1% acetic acid; a small amount of liquid remained in the tray beneath the floor rods. Subjects were transported to and from the vivarium in white plastic boxes and the computer monitor in the room was left on. For "Context B", red fluorescent room lights and cabinet fans were on whereas house lights were off and cabinet doors kept closed. Each chamber was cleaned with 1% ammonium hydroxide; a small amount of liquid remained in the tray beneath the floor rods. Subjects were transported to and from the vivarium in black plastic boxes and the computer monitor in the room was turned off.

Each chamber rested on a load cell to measure locomotor activity via Threshold Activity Software (Med Associates). Before the experiment, each load cell was calibrated to a specific displacement with the output of each amplifier set to a specific gain. The results from this output were digitized at 5 Hz, such that one observation every 200 ms was recorded. Freezing was only detected if the rat was immobile for, at minimum, 1 s.

Surgery

Rats were anesthetized with isoflurane (5% induction, 2% maintenance). The top of the head was shaved and the rat was secured in the stereotaxic apparatus (David Kopf Instruments, Tujunga, CA, USA) and given a subcutaneous injection of lidocaine before incision. After incision and retraction of the scalp, the head was adjusted such that lambda and bregma were aligned in the same horizontal plane. Three small holes were drilled for placement of anchoring screws and two small holes were drilled for bilateral implantation of guide cannulae (stainless steel, 26-gauge, 9 mm for the BNST, 11 mm for the BLA; Plastics One, Roanoke, VA, USA) attached to internal cannulae (33-gauge, 9 or 11 mm with 1-mm projection; Plastics One) directed at the BNST (0.0 mm AP, ±2.7 mm ML, -6.9 mm DV from dura at a 10° angle towards the midline) and BLA (-2.9 mm AP, ±4.9 mm ML, -7.4 mm DV from dura). Dental acrylic was applied around the anchoring screws and guide cannulae. After surgery, internal cannulae were removed and replaced with dummy cannulae, projecting 1 mm beyond the guide cannulae (33-gauge; Plastics One). Rats received one week of recovery prior to the start of behavioral procedures. To habituate the animals to the infusion procedure, rats were transported in white, 5-gal buckets lined with bedding to the infusion room. Rats were gently restrained and dummy cannulae were changed twice prior to the start of behavioral experiments.

Drugs

Allopregnanolone or (3α,5α)-3-hydroxy-pregnan-20-one (ALLO; R&D Systems, Minneapolis, MN, USA) was solubilized (8 mg/ml) in 30% (w/v) hydroxypropyl-β-cyclodextrin in purified water (VEH; Sigma-Aldrich, St. Louis, MO, USA).

Infusions

All rats were individually transported from their home cages to the infusion room in 5-gal white buckets and dummy cannulae were removed. Infusions were made with internal cannulae secured to either polyethylene tubing (PE-20, Braintree Scientific, Braintree, MA, USA) for VEH or polytetrafluoroethylene tubing (PTFE; 28-gauge, SAI Infusion Technologies, Lake Villa, IL, USA) for ALLO. Tubing was connected to 10-µl Hamilton syringes mounted on an infusion pump (KD Scientific, Hollistan, MA, USA). Bilateral infusions of VEH and ALLO were made at a rate 0.25 µl/min for a total volume of 0.25 µl in the BNST and 0.3 µl in the BLA. Internal cannulae were left in place for a 2 min post-infusion period to allow for drug diffusion. Fresh dummy cannulae were then inserted and rats remained in buckets until 10 min post-infusion after which they were transported to the testing rooms in plastic boxes for behavior. Previous work in our laboratory has found reliable effects of ALLO at this dosage without producing sedation (Nagaya et al., 2015).

Experiment 1: effects of intra-BNST ALLO on the acquisition and expression of contextual and cued fear

Rats were housed and implanted with cannulae targeting the BNST as described above. The behavioral experiment followed a 2 X 2 factorial design consisting of four different groups: V/V, V/A, A/A, and A/V (V= VEH, A=ALLO; the first letter signifies drug state on conditioning day and the second letter signifies drug state on testing days). On Day 1, rats were transported to the infusion room and received bilateral infusions of VEH or ALLO (2 μg/side) targeting the BNST. Ten minutes following the start of the infusion, rats were transported in white plastic boxes to the observation chambers for conditioning (Context A). Conditioning consisted of a 3-min baseline period, followed by five tone (CS; 10 s, 80 dB, 2kHz) -shock (US; 2 s, 1 mA) pairings in which the tone immediately followed the shock. A 1-min inter-trial interval (ITI) separated each tone-shock pairing. Following the last conditioning trial, there was a 1-min waiting period after which rats were returned to their home cages. Contextual and cued fear testing occurred over Days 2 and 3. Half of the animals were counterbalanced for order of context and cued tests. As previously shown (Nagaya et al., 2015), the suppressive effect of intra-BNST ALLO on freezing was limited to context exposure. Therefore, the remaining half of the animals only received context tests. On Day 2, rats were infused in the same manner as on Day 1 with either the same drug or a different drug. Ten minutes after infusion, rats were transported to either the conditioning context (Context A) for context testing or a novel context (Context B) for cued fear testing. Context testing consisted of a 10-min test with no tones or shocks given. Cued fear testing consisted of

a 3-min baseline period followed by four CS presentations with a 1-min ITI and a 1-min period after the final tone. On Day 3, rats were infused in the same manner and with the same drug as on Day 2. After 10 min, rats were transported to the testing room for either context or cued fear testing.

Experiment 2: effects of intra-BLA ALLO on the acquisition and expression of contextual and cued fear

Rats were housed and implanted with cannulae targeting the BLA as described above. Similar to Experiment 1, this experiment followed a 2 X 2 factorial design yielding groups conditioned under VEH or ALLO (2.4 μg/side) and tested in either the same or different drug state. All subjects were tested for both context and cued fear with the order of testing counterbalanced across groups. On Day 1, rats were infused with either VEH or ALLO and then conditioned in Context A. On Days 2 and 3, rats were given either context (Context A) or cued fear (Context B) testing. On Day 2, rats were infused with either the same or different drug as on conditioning day. On Day 3, rats received the same drug as on Day 2.

Experiment 3: contribution of intra-BNST ALLO to interoceptive and exteroceptive states

Subjects were housed and cannulated as described above. Unlike Experiments 1 and 2, this experiment involved animals that were all conditioned under the same context (Context A) and drug state (ALLO) and then tested in context and drug states that were either the same as those at conditioning or different, yielding the following groups with different shift conditions for context testing: None (no shift), Context (context shift

only), Drug (drug shift only), and Context & drug (context and drug shift). On Day 1, rats were transported to the infusion room and received bilateral infusions of ALLO (2 μg/side) into the BNST in the same manner as previously described. Ten minutes after the infusion, rats were transported to the conditioning room (Context A) and trained under the same conditions as in Experiment 1. On Day 2, rats were infused with either VEH or ALLO. After 10 min, rats were placed in either the conditioning context (Context A) or a novel context (Context B). As in the previous experiments, context testing consisted of a 10-min test with no tone or shock presentations. Subjects were not tested for cued fear.

Histology

After all behavioral testing, animals were overdosed with sodium pentobarbital (100 mg/kg) and perfused transcardially with 0.9% saline and 10% formalin. Brains were rapidly extracted and post-fixed for 24 h in 10% formalin at 4 °C before being transferred to a 30% sucrose-formalin solution. Using a cryostat set at -20 °C, brains were sectioned at 40 μm and mounted onto gelatin-subbed slides with 70% ethanol. Sections were stained with 0.25% thionin and imaged at 10X (Leica Microsystems, Buffalo Grove, IL) to ensure proper cannula placement for each experiment.

Data Analysis

The percentage of freezing behavior during conditioning was averaged across the 3-min pre-CS baseline period, the 1-min ITI for each trial, and the final 1-min post-trial

period. For context tests, mean of percentage freezing was averaged across the entire 10-min period. For cued tests, freezing was computed as the mean of percentage freezing during the 1-min ITI for each trial and the final 1-min post-trial period. All data were analyzed with repeated or factorial analysis of variance (ANOVA) and represented as means ± SEMs ($\alpha = 0.05$). In the case of significant F ratios, Fisher's protected least significant difference (PLSD) post hoc comparisons were performed. Effect size estimates were calculated using $\eta 2$ for ANOVAs (ratio of effect variance to total variance) and Cohen's d for pairwise comparisons (http://www.campbellcollaboration.org/escalc/html/EffectSizeCalculator-SMD1.php).

Results

Effects of intra-BNST ALLO on the acquisition and expression of contextual and cued fear

Histology

A photomicrograph of a thionin-stained brain section shows the representative bilateral placement of cannulae targeted to the anterior BNST (Figure 5A). Figure 5B depicts the cannula placements for all subjects included in Experiments 1 and 3. For Experiment 1, 64 rats were implanted; nine were excluded due to improper cannula placement. Cannula damage during experimental procedures resulted in the exclusion of two subjects and an additional four did not survive the study. This yielded the following group sizes: V/V ($n = 12$), A/A ($n = 11$), A/V ($n = 14$), and V/A ($n = 12$). All subjects included had tip placements localized to the anterior BNST, both dorsal and ventral to

the anterior commissure. Cannula placements for Experiment 1 were distributed from 0.00 to -0.46 mm relative to bregma, with the majority occurring between -0.11 and -0.26 mm.

Behavior

Groups receiving VEH or ALLO prior to acquisition started with low baseline levels of freezing which then increased during conditioning trials (Figure 5C). Throughout conditioning, ALLO-treated subjects exhibited increased levels of freezing relative to VEH-treated animals. A repeated measures ANOVA showed a significant main effect of drug state ($F_{(1,47)} = 8.1$, $p < 0.01$, $\eta^2 = 0.147$) and trial number ($F_{(5,235)} = 48$, $p<0.0001$, $\eta^2 = 0.496$) on freezing. There was no significant interaction between drug state and trial number ($F_{(5,235)} = 1.3$, $p = 0.26$, $\eta^2 = 0.014$). These data indicate that pre-training infusions of ALLO into the BNST facilitate acquisition of fear conditioning.

Context testing (Figure 5D) revealed elevated levels of freezing in subjects trained and tested in the same drug state (V/V and A/A) and reduced levels of freezing in subjects trained and tested in different drug states (V/A and A/V). A factorial ANOVA showed no significant main effect of training state ($F_{(1,45)} = 0.60$, $p = 0.44$, $\eta^2 = 0.001$) or testing state ($F_{(1,45)} = 0.16$, $p = 0.69$, $\eta^2 = 0.003$) on context-specific freezing. There was, however, a significant interaction between training and testing states ($F_{(1,45)} = 15$, $p < 0.001$, $\eta^2 = 0.247$). *Post hoc* comparisons revealed significant differences in percentage freezing between the A/A group and the A/V ($p < 0.01$, $d = 1.16$) and V/A ($p < 0.05$, $d = 1.03$) groups. Percentage freezing for the V/V group was also found to be significantly different from the A/V ($p < 0.01$, $d = 1.21$) and V/A ($p < 0.05$, $d = 1.06$)

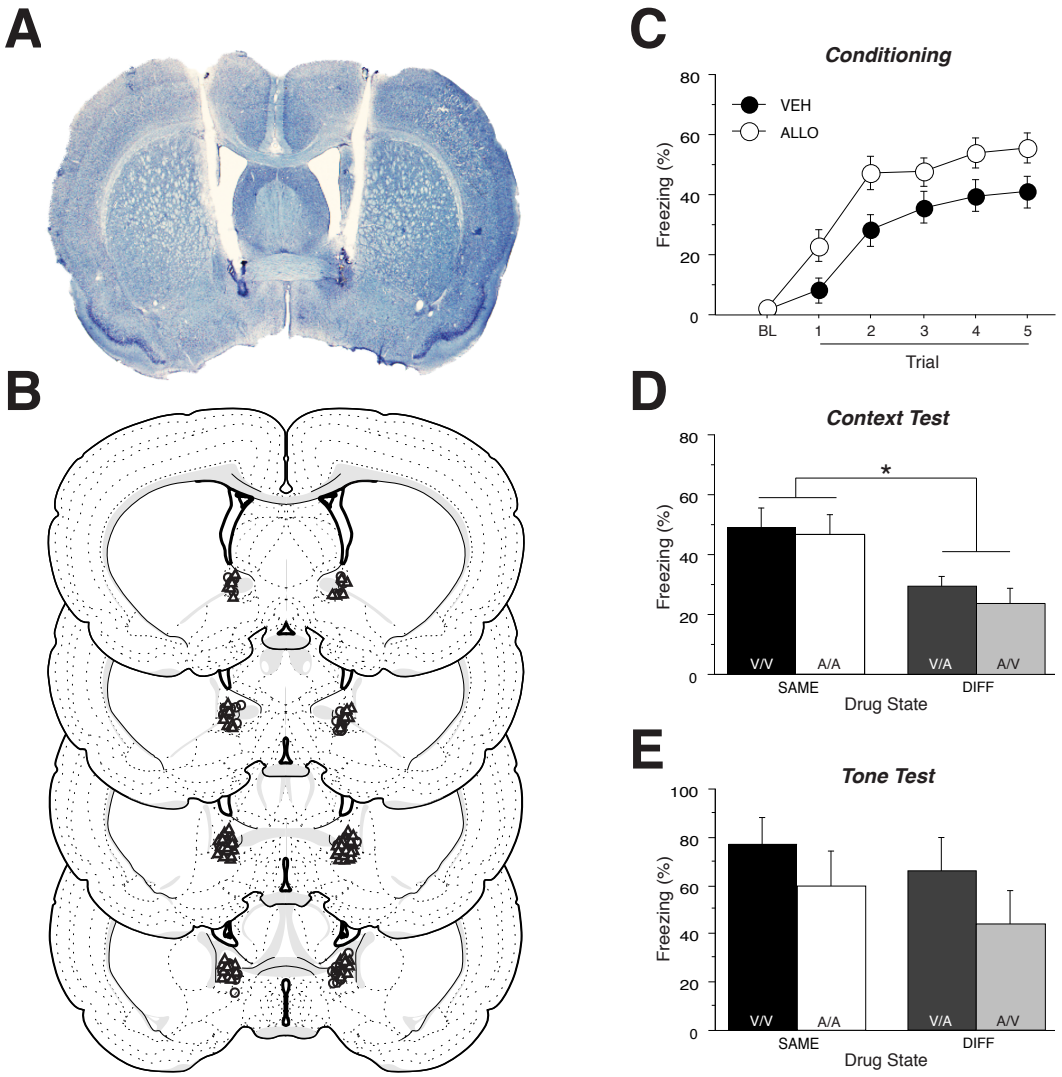

Figure 5 Effects of intra-BNST ALLO infusions on the acquisition and expression of contextual and cued fear.

A) Representative thionin-stained coronal section showing cannula placements. **B)** Schematic coronal sections showing infusion sites for vehicle (VEH) or allopregnanolone (ALLO). Experiment 1 sites are indicated by circles and Experiment 3 sites are indicated by triangles. Coronal brain section images are adapted from Swanson (2003). **C)** Mean percentage of freezing (± SEM) during the conditioning session (data are shown for a 3-min pre-trial period followed by five tone-shock pairings). Freezing was quantified before the first trial (baseline, BL) and during the 1-min period after each trial. **D)** Mean percentage of freezing (± SEM) averaged across the 10-min context test. The drug states for each group are indicated by a label on each bar indicating the drug infused before conditioning followed by the drug infused before testing (V for VEH, A for ALLO). The V/V group differed from the V/A ($p < 0.01$) and A/V ($p < 0.05$) groups. The A/A group differed from the V/A ($p < 0.05$) and A/V ($p < 0.01$) groups. **E)** Mean percentage of freezing (± SEM) averaged across each 1-min period after four tone presentations.

groups. These data indicate that training and testing under the same drug state results in the expression of higher levels of contextual fear.

In contrast, differences in drug state between training and testing did not affect freezing in response to cued fear (Figure 5E). A factorial ANOVA with training state and testing state as between-subjects variables and trial number as the within-subject variable revealed no significant main effect of training state ($F_{(1,19)} = 1.9$, $p = 0.18$, $\eta^2 = 0.089$) or testing state ($F_{(1,19)} = 0.032$, $p = 0.86$, $\eta^2 = 0.001$) and no significant interaction between training state and testing state ($F_{(1,19)} = 0.86$, $p = 0.37$, $\eta^2 = 0.039$). These data indicate that drug state during training or testing does not influence the expression of cued fear.

Effects of intra-BLA ALLO on the acquisition and expression of contextual and cued fear

Histology

A photomicrograph of a thionin-stained brain section shows the representative bilateral placement of cannulae targeted to the BLA (Figure 6A). Figure 6B depicts cannula placements for all subjects used in the analysis for Experiment 2. Of the 64 rats implanted, 12 were excluded due to improper cannula placement. Additional subjects were excluded due to loss of head caps ($n = 2$) and blocked cannulae ($n = 2$). This yielded the following group sizes: V/V ($n = 14$), A/A ($n = 11$), A/V ($n = 10$), and V/A ($n = 13$). All subjects included had tip placements localized to the BLA. Cannula

placements for Experiment 2 were distributed from -2.45 to -3.70 mm relative to bregma, with the majority between -2.85 and -3.25 mm.

Behavior

During conditioning, initial freezing for VEH- and ALLO-treated animals was negligible and then steadily rose across tone-shock presentations (Figure 6C). A repeated measures ANOVA with a between-subjects variable of drug group revealed a significant main effect of trial ($F_{(5,230)} = 53$, $p < 0.0001$, $\eta^2 = 0.527$) but neither an effect of drug state prior to training ($F_{(1,46)} = 1.1$, $p = 0.3$, $\eta^2 = 0.023$) nor an interaction between the two ($F_{(5, 230)} = 1.6$, $p = 0.17$, $\eta^2 = 0.016$). Therefore, regardless of drug manipulation prior to training, all subjects acquired conditioned fear at similar rates.

Context testing showed that subjects receiving ALLO prior to training (A/V), testing (V/A), or both (A/A) had reduced freezing compared to those receiving VEH prior to both training and testing (V/V; Figure 6D). A factorial ANOVA with between-subjects variables of training state and testing state revealed a significant main effect of testing state ($F_{(1,44)} = 12$, $p < 0.01$, $\eta^2 = 0.187$), but not training state ($F_{(1,44)} = 0.64$, $p = 0.43$, $\eta^2 = 0.01$). There was, however, a significant interaction between training state and testing state ($F_{(1,44)} = 8.8$, $p < 0.01$, $\eta^2 = 0.134$). *Post hoc* analysis revealed significant differences between the V/V group and all other groups ($p < 0.05$, $d = 1.04$ for A/V; $p < 0.01$, $d = 1.24$ for A/A; $p < 0.0001$, $d = 2.24$ for V/A).

Similar results were found with drug infusions prior to cued fear testing; animals treated with VEH prior to both training and testing had elevated levels of freezing

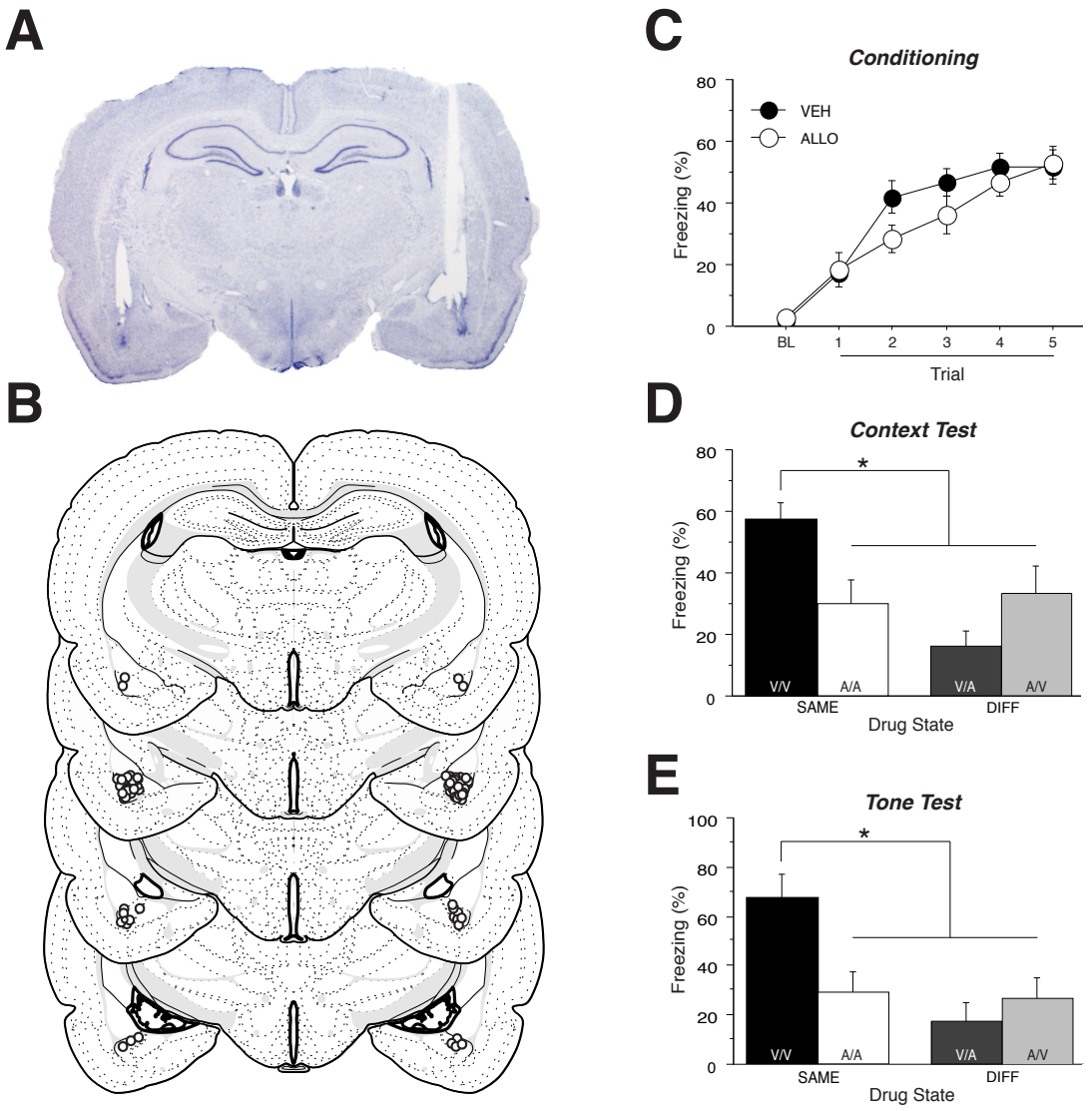

Figure 6 Effects of intra-BLA ALLO infusions on the acquisition and expression of contextual and cued fear.

A) Representative thionin-stained coronal section showing cannula placements. **B)** Schematic coronal sections showing infusion sites for vehicle (VEH) or allopregnanolone (ALLO). Experiment 2 sites are indicated by circles. Coronal brain section images are adapted from Swanson (2003). **C)** Mean percentage of freezing (± SEM) during the conditioning session (data are shown for a 3-min pre-trial period followed by five tone-shock pairings). Freezing was quantified before the first trial (baseline, BL) and during the 1-min period after each trial. **D)** Mean percentage of freezing (± SEM) averaged across the 10-min context test. The drug states for each group are indicated by a label on each bar indicating the drug infused before conditioning followed by the drug infused before testing (V for VEH, A for ALLO). The V/V group differed from the A/A ($p < 0.01$), V/A ($p < 0.0001$), and A/V ($p < 0.05$) groups. **E)** Mean percentage of freezing (± SEM) averaged across each 1-min period after four tone presentations. The V/V group differed from the A/A ($p < 0.01$), V/A ($p < 0.0001$), and A/V ($p < 0.01$) groups.

compared to other groups (Figure 6E). A factorial ANOVA revealed a significant main effect of testing state ($F_{(1,44)} = 7.4$, $p < 0.01$, $\eta^2 = 0.117$), but not training state ($F_{(1,44)} = 2.7$, $p = 0.11$, $\eta^2 = 0.042$). A significant interaction between training state and testing state was also found ($F_{(1,44)} = 9.0$, $p < 0.01$, $\eta^2 = 0.143$). *Post hoc* analysis revealed significant differences between the V/V group and all other groups ($p < 0.01$, $d = 1.19$ for A/A; $p < 0.01$, $d = 1.26$ for A/V; $p < 0.0001$, $d = 1.56$ for V/A).

Contribution of intra-BNST ALLO to interoceptive and exteroceptive states

Histology

Cannula placements for all subjects in Experiment 3 are depicted in Figure 5B. Of the 64 rats implanted, eight were excluded due to improper cannula placement. This yielded the following group sizes: None ($n = 12$), Context ($n = 16$), Drug ($n = 12$), and Context & drug ($n = 16$). All subjects included had tip placements localized to the anterior BNST. Cannula placements for Experiment 3 were distributed from 0.00 to -0.46 mm from bregma, with the majority between -0.11 and -0.26 mm.

Behavior

In order to explore the possible contribution of intra-BNST ALLO to interoceptive and exteroceptive aspects of conditioned context, the design illustrated in Figure 7A was devised for Experiment 3. All animals were initially infused with ALLO and then conditioned in Context A. The following day, subjects were tested under one of four assigned context shift test conditions: 1) the same drug state and context (None), 2) the same drug state but different context (Context), 3) the same context but different

drug state (Drug), or 4) different context and drug state (Context & drug). Acquisition of fear conditioning did not differ between subjects assigned to these groups (data not shown). Baseline freezing was low across all groups and as seen in ALLO-treated animals from Experiment 1, rats exhibited robust freezing after the onset of tone-shock trials. A repeated measures ANOVA with a between-subjects variable of assigned context shift test condition (None, Context, Drug, or Context & drug) and a within-subject variable of trial number revealed a significant main effect of trial number ($F_{(5,260)} = 53$, $p < 0.0001$, $\eta^2 = 0.493$) but no significant main effect ($F_{(3,52)} = 1.4$, $p = 0.26$, $\eta^2 = 0.074$) or interaction of context test assignment ($F_{(15,260)} = 0.99$, $p > 0.05$, $\eta^2 = 0.027$). Therefore, subjects in all assigned test groups acquired conditioned fear similarly across the five trials.

During context testing, changes in both context and drug state as well as a change in drug state alone had suppressive effects on freezing behavior (Figure 7B). There was a significant main effect of both test context ($F_{(1,52)} = 4.1$, $p < 0.05$, $\eta^2 = 0.065$) and test drug state ($F_{(1,52)} = 6.5$, $p < 0.05$, $\eta^2 = 0.103$), but no interaction between the two factors ($F_{(1, 52)} = 0.47$, $p = 0.50$, $\eta^2 = 0.007$). *Post hoc* analysis revealed a significant difference between None and Drug groups ($p < 0.05$, $d = 0.99$) and None and Context & drug groups ($p < 0.01$, $d = 1.46$). In addition, a trend towards a significant difference between percentage freezing expressed by None and Context groups ($p = 0.06$, $d = 0.78$) was obtained. These data indicate that a change in the interoceptive drug state of the BNST at test can impair recall of a fear-conditioned exteroceptive context.

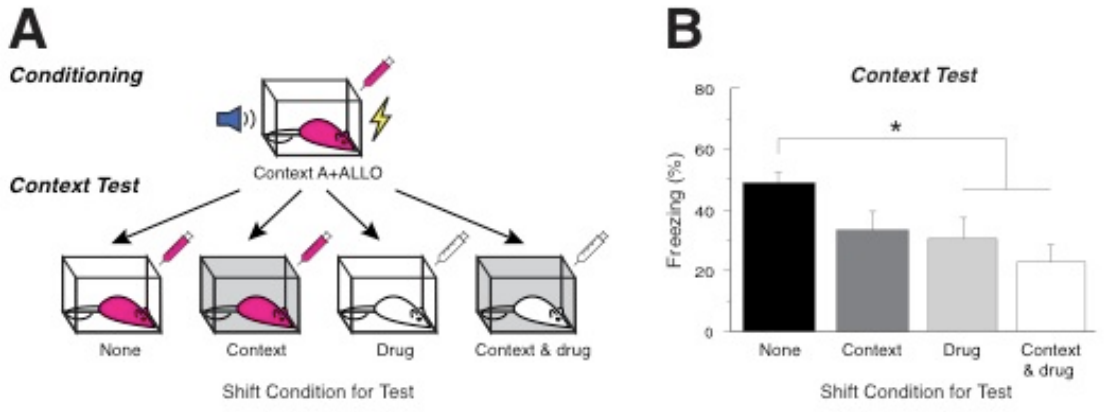

Figure 7 Contribution of intra-BNST ALLO to interoceptive and exteroceptive states.
A) Schematic diagram showing the design for Experiment 3. For conditioning, all animals were in the same interoceptive (infused with allopregnanolone; ALLO) and exteroceptive (Context A) states. For context testing, animals were assigned to one of four groups with varying degrees of shift in interoceptive and exteroceptive states: 1) the None group had no shifts, 2) the Context group had a shift in exteroceptive state (infused with ALLO, exposed to Context B), 3) the Drug group had a shift in interoceptive state (infused with vehicle, exposed to Context A), and 4) the Context & drug group had shifts in both states (infused with vehicle, exposed to Context B). **B)** Mean percentage of freezing (± SEM) averaged across the 10-min context test. The None group differed from the Drug ($p < 0.05$) and Context & drug groups ($p < 0.01$).

Discussion

The present study demonstrates that intra-BNST administration of ALLO results in state-dependent contextual fear in male rats. Thus, our previous finding that intra-BNST ALLO suppresses contextual fear (Nagaya et al., 2015) may not be due to a simple pharmacological impairment of context-specific fear expression. Rather, our current data suggest that intra-BNST ALLO may induce an interoceptive state that becomes conditioned to exteroceptive aversive stimuli. This effect of ALLO was unique to the BNST, insofar as ALLO infusions into the BLA produced a state-independent impairment in both the acquisition and expression of conditional freezing to context and

cue. The state-dependent modulation of contextual fear by intra-BNST ALLO suggests a novel mechanism by which gonadal steroids and their metabolites might influence the acquisition and expression of fear memories.

In addition to producing state-dependent contextual fear, we found, unexpectedly, that ALLO infused into the BNST prior to conditioning facilitated the acquisition of post-shock freezing. The increase in post-shock freezing after ALLO infusion was unique to the BNST and, did not necessarily result in increased contextual fear—in fact, the opposite was true when VEH rather than ALLO was administered prior to testing. Infusion of ALLO or other GABAR modulators into the BNST prior to fear conditioning have not been reported, although chemical lesions of the BNST have been found to either impair fear to context-like, long-duration stimuli (Waddell et al., 2006) or have no effect on contextual fear (LeDoux et al., 1988). The increased level of freezing we observed in ALLO-treated animals during acquisition could arise from enhanced synaptic inhibition within the BNST resulting in 1) increased activity in anxiogenic circuits and/or, 2) facilitated acquisition of a US association with the drug-induced interoceptive state. ALLO infusion into the BNST may shift GABAergic inhibition such that anxiogenesis is promoted during fear conditioning. Optogenetic and *in vitro* slice recording studies support the presence of heterogeneous subnuclei within the BNST that have opposing functions in regulating anxiety (Gungor et al., 2015; Jennings et al., 2013; Kim et al., 2013). GABAergic inputs from the lateral portion of the central amygdala (CeL) have been proposed to inhibit neurons in the anterolateral portion of the BNST (BNST-AL), which in turn, inhibit neurons in the anteromedial portion of the BNST

(BNST-AM) and modulate fear (Gungor and Pare, 2014; Haufler et al., 2013). Infused ALLO may facilitate CeL inhibition and thereby increase disinhibition of BNST-AM neurons that generate fear. Alternatively, fear acquisition in ALLO-treated animals may be enhanced by the 10 min pre-conditioning exposure to ALLO, which could promote greater salience between the US (footshock) and interoceptive state rather than the tone CS or context. In other words, ALLO within the BNST may serve as a 'cue' that becomes associated with footshock (Fanselow, 1980), thereby facilitating acquisition in ALLO-treated but not VEH-treated animals. Indeed, the greater level of freezing induced by ALLO infusion is apparent after the first conditioning trial (Figure 5C).

Of course, it is of considerable interest that despite increasing post-shock freezing, ALLO in the BNST produced marked deficits in contextual freezing during retention testing when infused either before conditioning or before testing. While this might suggest a general inhibitory effect of GABAR modulation on the acquisition and expression of fear, animals for which ALLO was infused prior to both fear conditioning *and* retention testing exhibited high levels of conditioned freezing. This suggests that impairments in contextual freezing in animals experiencing differing drug states were due to state-dependent generalization deficits. In particular, we suggest that a shift in the interoceptive context between conditioning 'on drug' and testing 'off drug' (and vice versa) reduced contextual fear. To our knowledge, this is the first time state-dependent drug effects have been reported for the BNST. Not surprisingly, given that the BNST has been predominantly implicated in contextual fear, state dependence was not observed for cued fear (Hammack et al., 2004; Resstel et al., 2008; Sullivan et al., 2004;

Zimmerman and Maren, 2011). State-dependent learning occurs when the learned event is more strongly recalled when training and testing occur in the same "state" (Overton, 1991). The state may refer to endogenous cues that create an interoceptive context resulting from drug consumption or alteration of brain excitation through receptor modulation as opposed to external contextual attributes such as lighting or odor.

The present finding that the BNST is involved in a state-dependent form of contextual fear is somewhat surprising and casts previous work on its role in fear learning and expression in a different light. For example, in Experiment 3, matching of the interoceptive and exteroceptive contexts resulted in the greatest degree of expressed fear, whereas testing under different interoceptive and exteroceptive contexts resulted in the lowest degree of expressed fear. Not surprisingly, animals trained and tested with either the same interoceptive or the same exteroceptive context showed intermediate levels of fear. Differences in freezing of animals tested in the same drug state but different contexts approached significance ($p = 0.06$; Figure 7B). These results suggest that the interoceptive (e.g., hormonal) contextual representation generated by the BNST may carry as much weight as the exteroceptive representation of spatial context generated by the hippocampus. In other words, the level of activation within the BNST may serve as a critical component to the overall "context" encoded during fear acquisition.

The ability of ALLO to induce state dependence in contextual fear learning is perhaps not surprising when the precedence of GABAR modulators in state-dependent learning is considered. Studies involving active avoidance in the T-maze have shown

that barbiturates, such as pentobarbital, and steroids, such as hydroxydione and progesterone, can induce state-dependent learning upon systemic administration (Overton, 1964; Stewart et al., 1967). Passive avoidance has also been shown to be state-dependent in ovariectomized females treated with progesterone (Ebner et al., 1981), which can be metabolized to ALLO, and in intact males treated with diazepam or muscimol (Nakagawa et al., 1993). More recently, Jovasevic et al. (2015) have demonstrated that state-dependent contextual fear learning can be induced by altering interoceptive state with gaboxadol infusion into the hippocampus prior to conditioning, suggesting that activation of extrasynaptic GABARs can "gate" access to contextual fear memory (Holmes and Chen, 2015).

Although the effects we observed of intra-BLA ALLO infusion on acquisition and expression of contextual and cued fear are distinctly different from those observed for the BNST, they are in concordance with previous studies involving pharmacological blockade of synaptic activity within the BLA (Fanselow and Kim, 1994; Harris and Westbrook, 1998; Helmstetter and Bellgowan, 1994; Maren and Holt, 2004; Maren et al., 1996b; Muller et al., 1997). They are also consistent with ALLO acting to increase GABAergic tone and inhibit activity within the BLA. Helmstetter and Bellgowan (1994) found that infusions of muscimol (prior to conditioning or testing) produce deficits in the expression of conditioned contextual fear (cued fear was not examined), suggesting that essential neural activity within the BLA is required for both acquisition and expression of conditioned contextual fear. Similarly, Maren and Holt (2004) reported deficits in both conditioned contextual and cued fear with pre-conditioning and

pre-testing infusion of muscimol into the BLA. Such effects of pharmacological suppression of synaptic transmission are not limited to GABAR agonists but have also been observed with GABAR potentiators such as midazolam, a benzodiazepine (Harris and Westbrook, 1998). The effects of ALLO infusion into the BLA are also very similar to the effects of APV infusion into the BLA, except that ALLO did not attenuate freezing during acquisition of contextual fear whereas APV significantly decreased freezing during conditioning (Maren et al., 1996b) and during testing (Fanselow and Kim, 1994; Maren et al., 1996b). The effects of APV were not due to state-dependent decrements in generalization, were evident when infusions were given before conditioning, testing, or both, and did not affect consolidation. Thus, the Maren et al. (1996b) study supports the role of NMDA receptors in the acquisition and expression of conditioned contextual fear. Interestingly, the effects we observed of ALLO infusions prior to conditioning and testing on contextual and cued fear look very similar to those seen by Muller et al. (1997). They found that muscimol infusions into the lateral and basal amygdala suppress both context- and cue-dependent freezing when administered before conditioning, testing, or both. When considered along with previous reports, our present findings on the suppressive effects of intra-BLA ALLO on contextual and cued fear suggest that ALLO, like muscimol and APV, is blocking synaptic transmission essential to acquisition and expression of conditioned fear. Given that ALLO was not administered post-training, we cannot directly address whether or not consolidation was affected.

We acknowledge that the effects of ALLO we observed could involve molecular mechanisms other than GABAR potentiation. ALLO may modulate other ionotropic neurotransmitter receptors (5-HT3 and NMDA; Frye et al., 2014) or act at nonclassical steroid receptors (membrane progesterone and pregnane xenobiotic; Porcu et al., 2016). In addition, local availability of metabolic enzymes could convert ALLO into its precursor, dihydroprogesterone, which acts at intracellular progesterone receptors (Rupprecht et al., 1993), or its 3β epimer, isoallopregnanolone, which can antagonize ALLO-mediated potentiation of GABARs (Johansson et al., 2016).

Conclusions

We have presented evidence that local infusion of ALLO into the BNST generates state-dependent contextual fear in male rats. This finding strongly suggests that the effects of intra-BNST ALLO we recently observed (Nagaya et al., 2015) are due to impaired retrieval of conditioned contextual fear due to ALLO's role as an interoceptive component of the conditioned context. It also suggests that previously reported deficits in contextual freezing following pre-test administration of ALLO and other GABAergic modulators are due to the pharmacologically induced state-dependent nature of contextual fear learning. Additionally, pre-training administration of GABAergic modulators may similarly produce deficits in performance if they are absent during testing. The implications of our work with regards to anxiety-related disorders are that levels of endogenous gonadal steroids and their metabolites may shape CS-US associations during acquisition and serve as an important component to retrieval of

conditioned context-specific fear. Consideration of hormonally induced interoceptive state may contribute to more effective treatment of disorders such as PTSD.

CHAPTER IV

DIFFERENTIAL EFFECTS OF PROGESTERONE ON CONDITIONED FEAR IN CYCLING AND OVARIECTOMIZED FEMALE RATS

Overview

Women are more susceptible to stress and trauma-related disorders, suggesting a role for ovarian hormones in modulating fear and anxiety. In both human and rodent studies, estrogen and progesterone have been shown to influence fear learning during acquisition, expression, and extinction. Recently, we have shown that allopregnanolone (ALLO), a progesterone (PROG) metabolite and $GABA_A$ receptor potentiator, can modulate conditioned contextual fear through its actions in the bed nucleus of the stria terminalis (BNST). In male rats, local administration of ALLO into the BNST appears to confer state-dependence to fear learning such that subjects conditioned and tested under ALLO have higher levels of fear than those conditioned or tested without ALLO. In females, ALLO levels follow the fluctuations in PROG during the estrous cycle. In order to determine whether natural fluctuations of PROG are associated with state-dependent contextual fear in females, animals received Pavlovian fear conditioning during one of two estrous cycle phases: either late diestrus (DI; low PROG) or late proestrus (PRO; high PROG). Following conditioning, animals were tested for contextual fear in either the same or different hormonal state. C-fos immunoreactivity was used to assess neuronal activation within the BNST following testing under low and high PROG cycle phases. We found that subjects conditioned and tested in PRO had

elevated levels of contextual fear compared to subjects conditioned in DI and tested in PRO, suggesting asymmetric state-dependent effects of PROG on fear learning. This effect did not carry over to an ovariectomized model with PROG replacement. Together, these results provide evidence for state-dependent effects of PROG on contextual fear in naturally cycling female rats.

Introduction

It is well-established that context affects aversive memories in both rodent and human studies (Maren et al., 2013). During all aspects of Pavlovian fear conditioning including acquisition, extinction, recall, and reinstatement, context encoding and context shifts affect memory (Bouton, 2002; Bouton and Bolles, 1979; Bouton and King, 1983; Matus-Amat et al., 2004; Westbrook et al., 2002). Typically, contextual stimuli are thought to involve external cues such as lighting, scent, or environmental constraints, however, interoceptive contextual cues including satiety, stress, and drug-induced or hormonal state may be just as important.

In particular, gonadal steroid hormones and their metabolites are known to affect aversive memories (Chang et al., 2009; Gupta et al., 2001; Milad et al., 2009; Nagaya et al., 2015; Toufexis et al., 2004) and may serve as internal contextual cues. Gonadal steroids have been shown to enhance or dampen fear memories during acquisition, extinction, and testing, however, the concordance between hormonal states during conditioning and testing has received less attention. Specifically, differences in hormonal state between conditioning and training can lead to apparent learning deficits

due to state-dependent learning, a phenomenon in which memories are better recalled when retrieved in the same state in which they were acquired (Overton, 1964; 1991).

State-dependent learning has been reported in both humans and animal models (Gill et al., 2015; Goodwin et al., 1969; Overton, 1966; Rezayof et al., 2008; Rosa et al., 2014). While most studies of state-dependent learning have focused on systemic effects, recent work suggests both the hippocampus (Jovasevic et al., 2015) and the bed nucleus of the stria terminalis (BNST; Acca et al., 2017) are particular sites that mediate these effects, whereas the BLA does not (Acca et al., 2017; Helmstetter and Bellgowan, 1994). Furthermore, the hippocampus encodes interoceptive cues through ethanol drinking in mice during fear conditioning (Yoo et al., 2017). Both the hippocampus (Holland and Bouton, 1999; Maren et al., 1997; Matus-Amat et al., 2004; Phillips and Ledoux, 1992) and the BNST (Hammack et al., 2004; Nagaya et al., 2015; Sullivan et al., 2004; Zimmerman and Maren, 2011) are important loci for contextual processing in fear conditioning and therefore may also be critical regions for interoceptive contextual processing as well.

Recently, we showed that allopregnanolone (ALLO) in the BNST of male rats results in state-dependent contextual fear (Acca et al., 2017). ALLO is a metabolite of progesterone (PROG) and a potent potentiator of $GABA_A$ receptors. ALLO levels fluctuate along with PROG levels across the estrous cycle in female rodents (Corpéchot et al., 1993) suggesting that PROG may also contribute to state-dependent memory. To our knowledge, no previous study has directly examined possible state-dependent effects of PROG on fear conditioning in naturally cycling rats or in ovariectomized (OVX) rats

given exogenous PROG. Here, we sought to determine if high and low PROG levels within the estrous cycle in female rats contribute to state-dependent contextual fear and differential neuronal activation within the BNST. Furthermore, we explored if this same learning mechanism applied to OVX subjects with exogenous PROG replacement.

Materials and Methods

Subjects

Female Long-Evans rats (200-224 g upon arrival; Blue Spruce) were purchased from a commercial supplier (Envigo, Indianapolis, IN, USA). Rats were individually housed in clear plastic cages on a 14:10 h light:dark cycle (lights off at 5:00 P.M.) in a temperature and humidity controlled room with unrestricted access to food and water. For 5 consecutive days, rats were handled for roughly 20 s to habituate them to the female experimenters. All behavioral procedures took place in the late afternoon during the light cycle. Experiments were carried out in accordance with guidelines approved by the Institutional Animal Care and Use committees at Texas A&M University.

Behavioral Apparatus

All behavioral procedures occurred in 8 identical observation chambers (30 x 24 x 21 cm; Med Associates, St. Albans, VT, USA) composed of Plexiglas rear wall, ceiling, and front-hinged door and 2 aluminum sides. One sidewall contained a speaker for delivery of the auditory stimulus and the opposite sidewall held an incandescent light. The floor was composed of 19 stainless steel rods (4 mm in diameter) spaced 1.5

cm apart (center to center). Rods were connected to a shock source and solid-state grid scrambler (Med Associates) for shock delivery. Underneath the rods was a removable stainless steel tray for odor manipulations. Spy cameras were mounted above each chamber for live monitoring of rodent behavior. All observation chambers were housed in sound-attenuating cabinets with fans to provide background noise (65 dB).

Locomotor activity was measured via Threshold Activity Software (Med Associates). Each chamber was situated on a load cell that was previously calibrated to a specific displacement to convert movement into an electrical signal. Signals were digitized at 5 Hz yielding one observation every 200 ms. Freezing, or immobility aside from that which is necessary for respiration, was only scored if the rat was immobile for at least 1 s.

Behavioral procedures were conducted in one of two contexts. Context "A", used for conditioning and context testing, consisted of each chamber being wiped down with 1% acetic acid with a small amount left in the removable tray beneath the rods. White fluorescent room lights, chamber lights, and fans were all on, and cabinet doors were left open. Rats were transported to and from the vivarium in white plastic boxes. Context "B", used for tone testing, was cleaned in a similar fashion but with 1% ammonium hydroxide. Red fluorescent lighting was used in the room, but chamber lights remained off and cabinet doors were closed. Fans remained on to mask background noise. Black plastic boxes were used to transport rats to and from the vivarium for behavioral testing.

Experiment 1: Effects of Estrous Cycle Phase on the Acquisition and Expression of Contextual Fear

Estrous cycle tracking

After 5 days of acclimation to the housing conditions, vaginal smears were obtained daily at 11 A.M. for at least 10 consecutive days to ensure normal cycling. Cotton swabs moistened with distilled water were used and cells were visualized under a light microscope at 100× and characterized according to (Goldman et al., 2007). Cycle tracking continued throughout behavioral testing and rats with irregular cycles were excluded from the analysis.

Behavioral procedure

In order to examine state-dependent effects of estrous cycle phase on contextual fear, a 2 × 2 × 2 factorial design was used. Late proestrus (PRO) was selected for high circulating PROG levels and late diestrus (DI) was selected for low circulating PROG levels. Subjects in the Shock group were conditioned in either PRO or DI and then allowed to cycle until they reached that same phase or a different phase for contextual fear testing, thus yielding the following groups: P/P, D/D, P/D, and D/P (P = PRO, D = DI; the first letter indicates the estrous cycle phase on conditioning day and the second letter indicates the estrous cycle phase on testing day). For conditioning, rats were transported to the behavior room in white plastic boxes (Context A). Conditioning consisted of a 3-min baseline period, followed by 5 tone (CS; 10 s, 80 dB, 2kHz)-shock (US; 2 s, 1 mA) pairings in which the shock immediately followed the tone. A 1-min inter-trial interval (ITI) was interspersed between each CS-US pairing, including a 1-min

period following the last shock. Immediately after conditioning, rats were transported back to their home cages in white plastic boxes. Estrous cycle continued to be tracked until rats were in either the same or different cycle phase (3-6 days) for context testing. Context testing occurred in Context A and rats were transported to the conditioning chambers again in white plastic boxes. Freezing behavior was assessed over a 10-min period in the absence of any tones or shocks. Immediately following context testing rats were returned to their home cages. A No Shock group was included as a control for c-Fos immunoreactivity. No Shock subjects received the same treatment as the Shock subjects except that during conditioning they did not receive shocks. All other experimental procedures were identical. Due to the complexity of matching estrous cycles in this design and our focus on contextual fear processing, tone testing was not performed.

Immunohistochemistry

Ninety minutes after context testing, rats were overdosed with sodium pentobarbital (100 mg/kg), subjected to cardiac puncture for blood sampling (see below), and transcardially perfused with 0.9% saline and 10% formalin. Brains were rapidly dissected and stored for 18 h in 10% formalin at 4 °C before being transferred to 30% sucrose-formalin. Coronal brain sections (40 μm) were made on a cryostat maintained at -20 °C and stored in phosphate-buffered saline (pH 7.4) with 0.01% sodium azide (VWR, Radnor, PA, USA) until processing.

Immunohistochemistry was performed on free-floating brain sections. Sections were first washed in Tris-buffered saline (TBS; pH 7.4) followed by 0.3% hydrogen

peroxide (H_2O_2; Sigma-Aldrich, St. Louis, MO, USA) in TBS. After three washes, samples were incubated overnight with rabbit anti-c-Fos polyclonal antibody (ABE457**;** EMD Millipore, Billerica, MA, USA) diluted 1:10,000 in 0.1 % Tween-20 in TBS (TBST). The next day following serial washes, sections were incubated for 1 h in biotinylated donkey anti-rabbit secondary antibody (1:1,000 in TBST, Jackson ImmunoResearch Laboratories, West Grove, PA, USA). Upon completion, sections were washed and further incubated for 45 min in avidin-biotin complex (ABC; 1:1,000 in TBST; VECTASTAIN ABC Elite HRP kit, Vector Laboratories, Burlingame, CA, USA). Slices were then stained for 10 min with a combination of 0.025% 3,3'-diaminobenzidine tetrahydrochloride hydrate (Sigma-Aldrich), 0.5% nickel ammonium sulfate (Sigma-Aldrich) and 0.015% H_2O_2. After washes, sections were mounted onto gelatin-subbed slides.

Image analysis

Two images for the anterior BNST approximately -0.11 and -0.26 posterior to bregma (Figure 8), according to Swanson Brain Maps (2003), were captured for quantification using a Zeiss Axio Imager 2 microscope. All images were taken at 10x magnification. Using representative sections from the brain map, nuclei of interest were outlined and standardized sections were isolated from two images. Using ImageJ (NIH) automated counting feature, c-Fos positive cell counts for the oval, fusiform, medial, and lateral nuclei were recorded in isolated sections, averaged, and normalized to area.

Hormone assay

Prior to transcardial perfusion, blood samples (approximately 2 ml) were collected via cardiac puncture and allowed to sit at room temperature for 45 min.

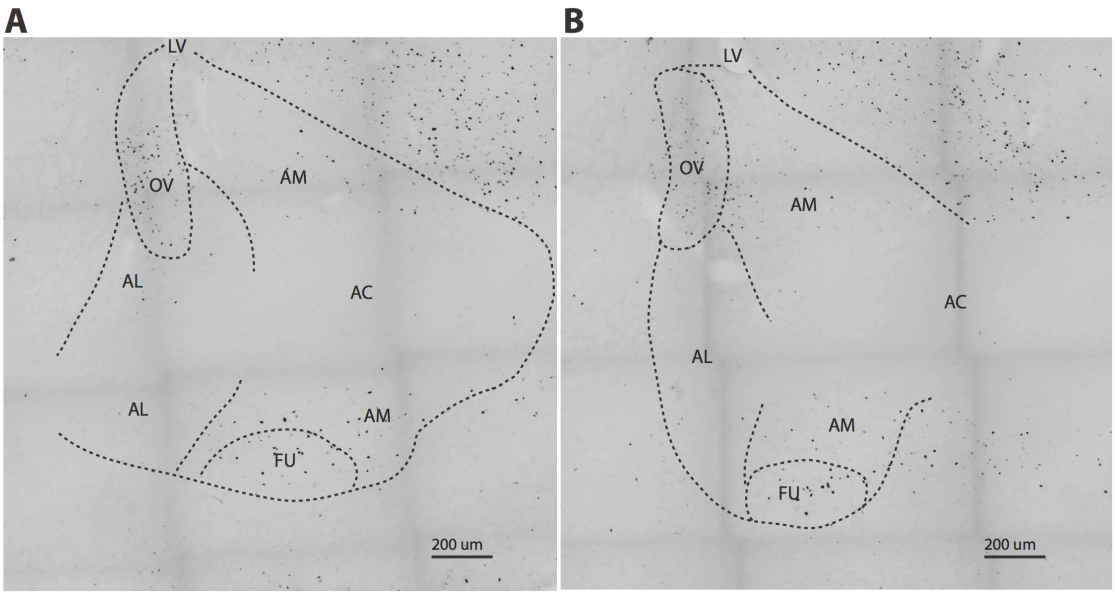

Figure 8 Representative c-Fos images in the BNST.
A) Approximately -0.11posterior to bregma. **B)** Approximately -0.26 posterior to bregma. Lateral Ventricle (LV); Anterior Commissure (AC); BNST-OV (OV); BNST-AM (AM); BNST-AL (AL); BNST-FU (FU).

Samples then were centrifuged at room temperature for 15 min at 3,000 RPM and plasma was collected and stored at -80 °C until processing. Progesterone levels were assayed using a progesterone ELISA kit (sensitivity 8.57 pg/ml, Enzo Life Sciences, Farmingdale, NY, USA) and read on a Multimode Plate Reader (PerkinElmer, Shelton, CT, USA). All samples were run in duplicate at a 1:200 dilution.

Data analysis

For conditioning, percentage freezing behavior was averaged across the 3-min baseline period, the 1-min ITI, and the 1-min post-trial period. Freezing was averaged across the entire 10-min context test. Behavioral and immunohistochemical data were analyzed using repeated measures or factorial analysis of variance (ANOVA) and presented as means ± SEMs ($\alpha = 0.05$). For *post hoc* examination, Fisher's protected least significant difference (PLSD) was chosen. Hormone data were analyzed by an independent *t*-test and presented as means ± SDs ($\alpha = 0.05$).

Experiment 2: Effects of Exogenous PROG on the Acquisition and Expression of Contextual and Cued Fear in OVX Rats

Surgical procedures

Briefly, rats were anesthetized with isoflurane (5% induction, 2% maintenance). They were bilaterally ovariectomized using standard aseptic surgical techniques. A single dorsal skin incision was made and bilateral muscle incisions exposed the ovaries. On each side, the uterine horn was ligated just below the ovary and the ovary removed. The muscle incisions were closed with absorbable suture and skin incisions were closed with surgical staples. Rats were monitored daily during a one-week recovery period prior to the start of behavior.

Drugs

For subcutaneous injections, progesterone (PROG; 4 mg/ml, Sigma-Aldrich) was prepared fresh in sesame oil (VEH, Sigma-Aldrich) on Day 1 on constant heat with continuous stirring and then stored at room temperature and used for Days 2 and 3.

Behavioral procedures

In order to examine state-dependent effects of exogenous PROG on conditioned fear in OVX females, a 2 × 2 factorial design was used. No Shock controls were excluded from this experiment, as we were only interested in the behavioral effects of OVX females with PROG replacement. In Experiment 1, we particularly chose late proestrus in order to capture the point in the estrous cycle where PROG levels were the highest, and subsequently ALLO levels. Therefore, in Experiment 2 we focused solely on PROG replacement to isolate the possible effects to PROG and not a combination of estrogen and PROG. OVX females received one of four different combinations of drug injections prior to conditioning and testing as follows: Pr/Pr, V/V, Pr/V, and V/Pr (Pr = PROG, V = VEH; the first letter indicates the drug given on conditioning day and the second letter indicates the drug given on testing days). PROG (4 mg/kg) or VEH (1 ml/kg) was subcutaneously injected into the scruff of the neck 1 h prior to behavioral testing. PROG doses were chosen from previous studies that reported physiological levels of circulating PROG as seen during the proestrus phase of the estrous cycle (Llaneza and Frye, 2009). Conditioning occurred on Day 1 in Context A following procedures identical to those used in Experiment 1. Twenty-four hours later, rats were injected with either the same or different drug as Day 1 and tested for contextual fear.

On Day 3, rats were injected with the same drug they received prior to context testing and were transported for tone testing in black plastic boxes. For tone testing, animals were placed in Context B and monitored for a 3-min baseline period followed by 4 CS presentations with a 1-min ITI and a 1-min post period after the last CS.

Hormone assay

Immediately after tone testing on Day 3, rats were overdosed with sodium pentobarbital and blood samples were collected via cardiac puncture. Progesterone levels were assessed as described for Experiment 1.

Data analysis

Data were analyzed in an identical manner to Experiment 1. For tone testing, percentage freezing behavior during the 1-min ITI period between each tone presentation and after the final fourth tone presentation were averaged.

Results

Experiment 1: Effects of Estrous Cycle Phase on the Acquisition and Expression of Contextual Fear

Behavior

No Shock control animals had less than 10% freezing levels during conditioning (data not shown). PRO and DI subjects did not differ in acquisition of fear. All subjects had little to no freezing behavior prior to the first shock with increased freezing over the 5 trials (Figure 9). A repeated measures ANOVA with a between-subjects factor of training state (PRO or DI) showed a significant main effect of trial number ($F_{(5, 250)}$ =

32.01, $p < 0.0001$) on freezing. There was no significant difference between estrous cycle phases ($F_{(1, 50)} = 0.08$, $p = 0.77$) or an interaction between estrous cycle phase and trial number ($F_{(5, 250)} = 0.16$, $p = 0.98$). Thus, each group acquired similar levels of fear as indexed by freezing behavior.

No Shock controls maintained freezing levels below 10% throughout context testing (data not shown). Subjects in PRO only during testing (D/P) showed reduced levels of freezing compared to those conditioned and tested in PRO (P/P), those conditioned and tested in DI (D/D), and those in DI only during testing (P/D) (Figure 9). A factorial ANOVA revealed a significant main effect of cycle phase at conditioning ($F_{(1, 48)} = 4.293$, $p = 0.04$), but not cycle phase at testing ($F_{(1, 48)} = 1.33$, $p = 0.26$). There was also a significant interaction between cycle phase at conditioning and at testing ($F_{(1, 48)} = 6.03$, $p = 0.02$). *Post hoc* analysis showed significant differences between P/P and D/P ($p < 0.01$), P/D and D/P ($p < 0.05$), and D/D and D/P ($p < 0.05$). Together, these data suggest only subjects trained in DI and tested during PRO had reduced levels of contextual fear.

Fos analysis

We next examined Fos expression in the anterolateral (BNST-AL), anteromedial (BNST-AM), oval (BNST-OV), and fusiform (BNST-FU) nuclei of the BNST (Figure 10) due to previous results suggesting its role in state-dependent context fear memories following ALLO administration in male rats (Acca et al., 2017). Surprisingly, there was no significant main effect of footshock group (No Shock vs. Shock) in any region (BNST-AL, $F_{(1,89)} = 0.37$, $p > 0.05$; BNST-AM, $F_{(1,89)} = 0.34$, $p > 0.05$; BNST-OV,

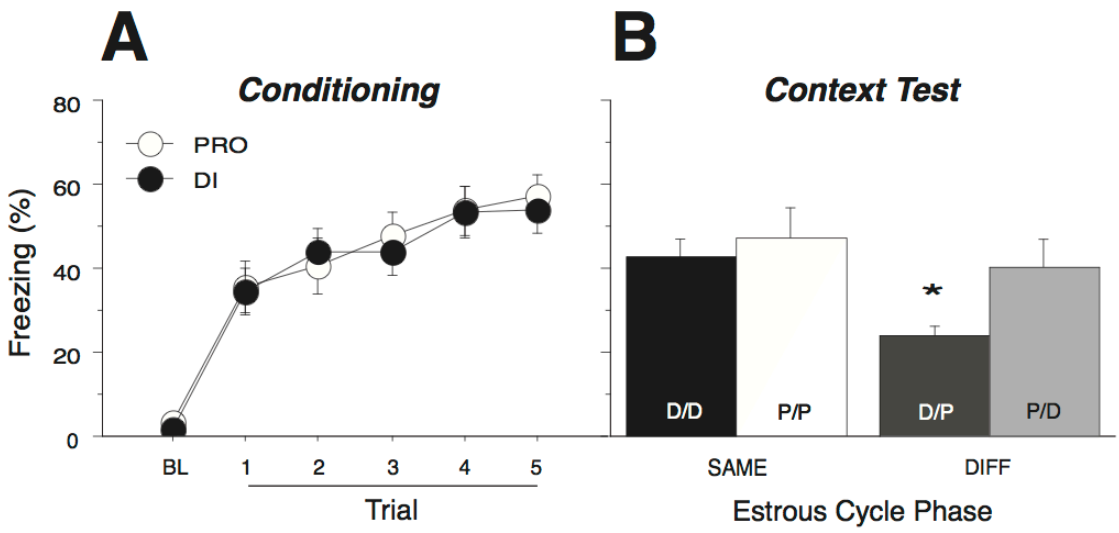

Figure 9 Effects of estrous cycle phase on the acquisition and expression of contextual fear.
A) Mean percentage of freezing (± SEM) during the conditioning session (data are shown for a 3-min pre-trial period followed by five tone-shock pairings) for animals in proestrus (PRO, open circles) or diestrus (DI, filled circles). Freezing was quantified before the first trial (baseline, BL) and during the 1-min period after each trial. **B)** Mean percentage of freezing (± SEM) averaged across the 10-min context test. Estrous cycle phases are indicated by D for DI and P for PRO with conditioning phase followed by testing phase. The D/P group differed from the D/D group ($p = 0.01$), the P/P group ($p = 0.001$), and the P/D group ($p = 0.02$). Group sizes consisted of 11 in D/D, 13 in P/P, 17 in D/P, and 11 in P/D.

$F_{(1,89)} = 0.84$, $p > 0.05$; BNST-FU, $F_{(1,89)} = 0.38$, $p > 0.05$). BNST-AL showed a significant main effect of cycle phase at conditioning ($F_{(1,89)} = 5.49$, $p = 0.02$) and post hoc analysis revealed a significant difference between those conditioned in PRO and those conditioned in DI ($p < 0.05$). There was also a significant interaction between footshock group and cycle phase at testing ($F_{(1,89)} = 6.30$, $p = 0.01$). A significant main effect of cycle phase at testing in BNST-FU ($F_{(1,89)} = 5.61$, $p = 0.02$) was found, but no effect of cycle phase at conditioning ($F_{(1,89)} = 0.06$, $p > 0.05$). There were also no significant main effects or interactions for both BNST-OV and BNST-AM.

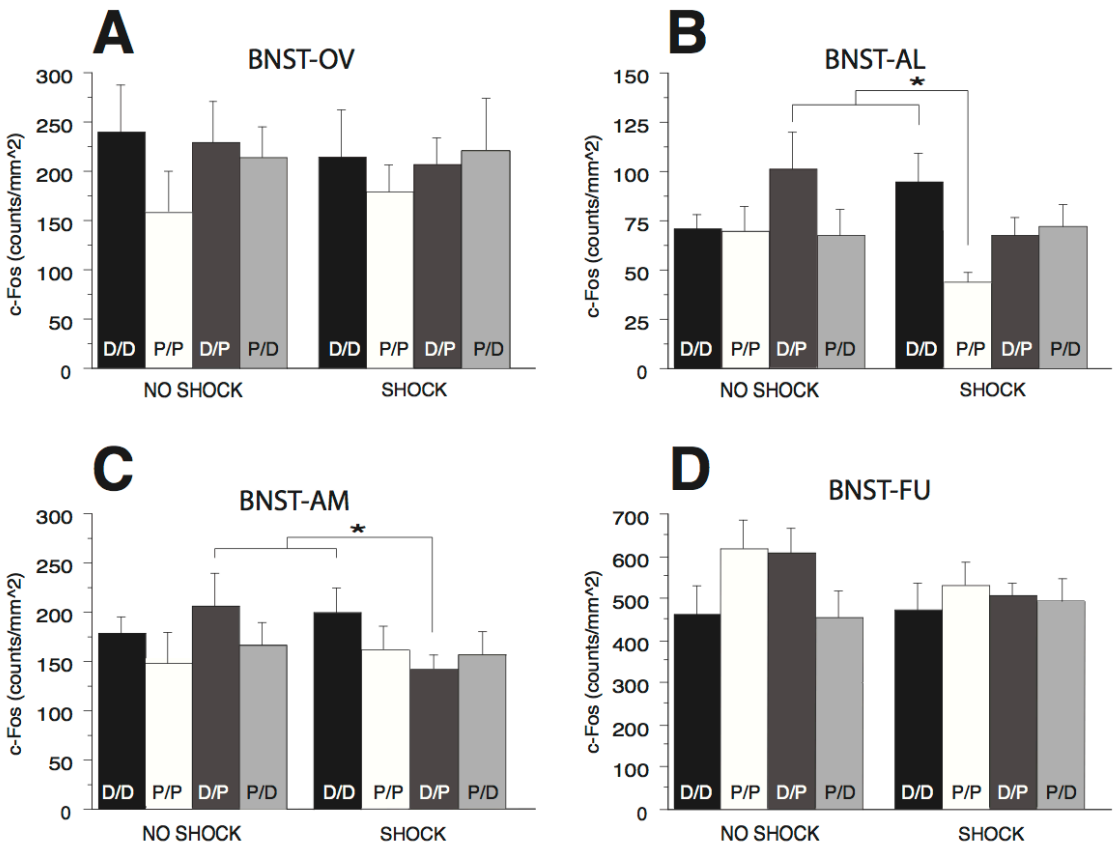

Figure 10 Effects of estrous cycle phase on c-Fos expression in the BNST following context testing.

Estrous cycle phases are indicated by D for diestrus (DI) and P for proestrus (PRO) with conditioning phase followed by testing phase. **A)** Mean (±SEM) cell counts/mm^2 for Fos-positive neurons in the oval nucleus of the BNST (BNST-OV). **B)** Mean (±SEM) cell counts/mm^2 for Fos-positive neurons in the anterolateral nucleus of the BNST (BNST-AL). No Shock D/P and Shock D/D groups differed from the Shock P/P group ($p < 0.01$). **C)** Mean (±SEM) cell counts/mm^2 for Fos-positive neurons in the anteromedial nucleus of the BNST (BNST-AM). No Shock D/P and Shock D/D groups differed from the Shock D/P group ($p < 0.05$). **D)** Mean (±SEM) cell counts/mm^2 for Fos-positive neurons in the fusiform nucleus of the BNST (BNST-FU).

Hormone assay

On testing day, plasma progesterone levels were significantly higher in animals in PRO compared to animals in DI ($t_{(95)} = 21.23$, $p < 0.0001$), thus supporting the cycle phase assignments based on estrous cycle tracking (Table 1).

Experiment	Group	Mean PROG Levels (ng/mL)	Standard Deviation
1 cycling rats	DI	10.57	5.8
	PRO	48.18	10.76
2 OVX rats	VEH	10.07	3.44
	PROG	44.55	9.4

Table 2 Mean plasma PROG levels following context testing in Experiment 1 and tone testing in Experiment 2.
In Experiment 1, mean plasma PROG levels were significantly different between DI and PRO groups ($p < 0.001$). In Experiment 2, mean plasma PROG levels were significantly different between VEH and PROG groups ($p < 0.001$).

Experiment 2: Effects of Exogenous PROG on the Acquisition and Expression of Contextual and Cued Fear in OVX Rats

Behavior

Acquisition of fear did not differ between PROG and VEH subjects (Figure 11A). Levels of freezing remained low prior to the first shock and increased similarly across all 5 trials. A repeated measures ANOVA revealed no significant difference between drug treatment groups during conditioning ($F_{(1, 46)} = 0.04$, $p = 0.85$). There was, however, a significant main effect of trial number ($F_{(5, 230)} = 31.64$, $p < 0.0001$). No

interaction between the two was found ($F_{(5, 230)} = 0.37$, $p = 0.87$). Thus all subjects, regardless of drug treatment acquired fear in a similar fashion.

Context testing revealed no significant main effects of drug group for conditioning or testing, nor an interaction between the two (Figure 11B; drug at conditioning, $F_{(1,44)} = 3.67$, $p = 0.06$; drug at testing, $F_{(1, 44)} = 0.11$, $p = 0.74$; interaction, $F_{(1, 44)} = 1.45$, $p = 0.23$). *Post hoc* analysis did reveal a significant difference between subjects conditioned and tested on VEH (V/V) and those conditioned on PROG and tested on VEH (Pr/V; $p < 0.05$). Tone testing (Figure 11C) revealed no significant main effect of drug group at conditioning ($F_{(1,44)} = 0.71$, $p = 0.40$) or drug group at testing ($F_{(1,44)} = 0.93$, $p = 0.34$).

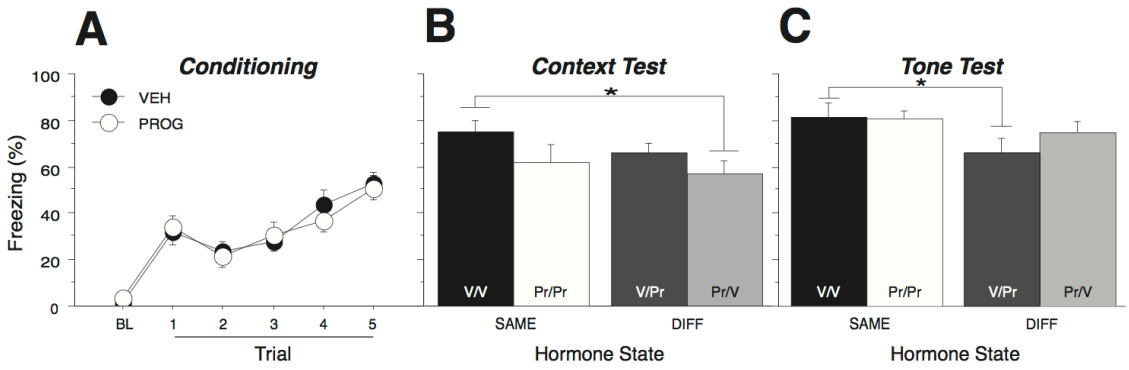

Figure 11 Effects of exogenous PROG on the acquisition and expression of contextual and cued fear in OVX female rats.
A) Mean percentage of freezing (± SEM) during the conditioning session (data are shown for a 3-min pre-trial period followed by five tone-shock pairings) for animals treated with vehicle (VEH, filled circles) or progesterone (PROG, open circles). Freezing was quantified before the first trial (baseline, BL) and during the 1-min period after each trial. **B)** Mean percentage of freezing (± SEM) averaged across the 10-min context test. Drug treatment groups are indicated by V for VEH and Pr for PROG with conditioning treatment followed by testing treatment. The V/V group differed from the Pr/V ($p = 0.03$) group. **C)** Mean percentage of freezing (± SEM) averaged across each 1-min period after four tone presentations. The V/V group differed from the V/Pr group ($p = 0.04$). All groups consisted of 12 subjects.

An interaction between the two fell just short of significance ($F_{(1,44)} = 3.91$, $p = 0.054$). *Post hoc* analysis revealed a significant difference between subjects conditioned and tested on VEH (V/V) and those conditioned on VEH and tested on PROG (V/Pr; $p < 0.05$) and a trend towards significance between subjects conditioned and tested on PROG (Pr/Pr) and those conditioned on VEH and tested on PROG (V/Pr; $p = 0.052$).

Hormone assay

Subjects receiving subcutaneous injections of PROG had significantly higher levels of plasma PROG compared to VEH injected subjects ($t_{(46)} = -16.88$, $p < 0.0001$).

Discussion

The results presented here suggest naturally fluctuating PROG levels across the estrous cycle can contribute to state-dependent contextual fear in female rats. This finding is largely driven by high levels of freezing in the group conditioned and tested in DI (D/D) compared to the low freezing levels in the group conditioned in DI and tested in PRO (D/P). For the first time, this suggests naturally cycling levels of PROG may act as an interoceptive contextual cue that becomes associated with the exteroceptive aversive context following Pavlovian fear conditioning. In an OVX model with exogenous PROG administration, hormone treatment was not associated with state dependence in contextual fear. However, for cued fear, the decreased freezing levels in the group conditioned under VEH and tested under PROG (V/Pr) compared to those in the group conditioned and tested under VEH (V/V) suggest that a state-dependent mechanism is possible. PROG administration after OVX had slight reductions in

freezing across all groups during contextual fear testing regardless of when it was administered. In contrast, following cued fear testing, subjects that only received PROG prior to testing (V/Pr) had reduced freezing. In all cases, however, freezing levels were elevated above 50% throughout testing, suggesting robust fear recall in all groups. While OVX models are valuable, they do not necessarily replicate intact cycling states. These models negate the impact of fluctuating hormones across the estrous cycle and instead give fixed amounts of hormones. Therefore, it cannot be assumed that OVX experiments will model naturally cycling experiments.

Previously, we found male rats infused with ALLO in the BNST prior to conditioning resulted in enhanced freezing during acquisition (Acca et al., 2017). Because PROG is a precursor to ALLO, elevated PROG levels in females could have also resulted in increased freezing during acquisition. However, high PROG levels during conditioning in naturally cycling and OVX female rats did not affect freezing during fear acquisition. PROG levels in PRO groups and PROG groups would be expected to be within normally elevated physiological levels during acquisition and therefore supraphysiological levels of PROG or ALLO may be needed for this enhanced freezing effect. In addition, the experiments presented here focus on systemic changes in hormone levels that are more likely to affect the whole brain whereas previously only the BNST was targeted. Competing actions of hormones in different brain regions may have erased this effect.

Upon first glance, our results from naturally cycling PROG levels in female rats on contextual fear follow existing literature. Previous studies involving tests of fear and

anxiety suggest animals tested in PRO or OVX with hormone replacement have reduced fear levels compared to other estrous cycle stages or no hormone replacement (Frye et al., 2000; Gupta et al., 2001; Marcondes et al., 2001; Milad et al., 2009; Toufexis et al., 2004). However, in this case not all subjects tested in PRO had decreased freezing; those conditioned and tested in PRO had elevated levels of fear, suggesting state-dependent effects. These effects were asymmetrical, as those trained in PRO and tested in DI maintained elevated freezing levels. Clinically, asymmetrical state-dependent effects have also been reported with memory recall after alcohol consumption (Goodwin et al., 1969). Gonadal steroid hormones, therefore, can act as interoceptive cues such that memories are better recalled when in the same hormone state as when the memory was learned. It is not just hormonal state during testing that matters, but an interaction between hormone levels during both conditioning and testing.

To our knowledge this is the first time state-dependent effects of naturally cycling hormones on contextual fear conditioning have been reported. Previously, similar studies using Pavlovian fear conditioning followed by passive avoidance testing in both naturally cycling females or in same or different stages of pregnancy, found no evidence for state-dependent effects. However, in an OVX model with PROG administration, asymmetrical state-dependent effects were found after a 1-day interval between training and testing, but not at 7 days (Ebner et al., 1981). The difference in results found in Ebner et al. (1981) and here could be due to a number of factors. First, passive avoidance testing was used instead of fear recall and secondly, different stages of the estrous cycle were examined, PRO and estrus. In another study in which both

conditioning and testing occurred either during PRO or estrus, lower levels of contextual fear were observed in PRO animals compared to estrus animals (Markus and Zecevic, 1997). However, in this study, there was no comparison made to mismatched cycle phases during conditioning and testing and differences in rat strains and duration of time between conditioning and testing are all possible factors. Time between conditioning and testing in our study was less than a week whereas in Markus & Zecevic (1997), two weeks elapsed between conditioning and testing. This, along with the results from Ebner et al. (1981), raises interesting questions as to how salient interoceptive cues remain over time.

Given the behavioral effects of circulating PROG, we were surprised to find no differences in c-fos expression in the BNST between conditioned and tested subjects and those that never received shocks (CS- groups) but otherwise experienced the same behavioral tests. Previous studies in female rats involving restraint stress found differences in Fos reactivity in BNST, specifically in BNST-OV and BNST-FU (Sterrenburg et al., 2012). Even though rats may not be learning a tone-shock association in a distinct context in the CS- control group, they are still learning and acquiring contextual information. Using a home control may have revealed greater differences because Fos would have been assessed outside of the conditioning context.

Many subnuclei within BNST did not reveal c-fos differences between groups with matched and mismatched estrous cycle phases during conditioning and testing, which may be due to the diverse subregions and intricate competing connections. For instance, BNST-OV is thought to exert an anxiogenic profile through its rich

corticotropin releasing factor (CRF) neurons (Kim et al., 2013; Lee and Davis, 1997; Sahuque et al., 2006), even though BNST-OV neurons are also GABAergic (Dabrowska et al., 2013). In support of this, it was recently shown that optogenetically silencing projections from CRF-containing neurons in the lateral region of the central amygdala to CRF-containing neurons in the lateral region of BNST during training, reduced fear retention (Asok et al., 2017). Although no differences were found in our study, more precise targeting of specific neuronal types may have yielded differences. In the ventral BNST, BNST-FU also contains CRF neurons (Phelix and Paull, 1990) along with glutamatergic cells (Csáki et al., 2000), however, most neurons in this area are GABAergic (Radley et al., 2009). Despite this, the minority glutamatergic cells in the ventral BNST appear to have excitatory affects on the hypothalamus (Choi et al., 2007) and thus drive anxiety behaviors. There are differing reports on the role of BNST-AM in anxiety. BLA inputs to BNST-AM drive anxiolytic behavior on the elevated plus maze (Kim et al., 2013) whereas in another study, BNST-AM neurons fired more during freezing behavior in response to CS presentations and contextual fear (Haufler et al., 2013). BNST is also comprised of multiple intrinsic connections which further confound its role in anxiety behavior (Dong et al., 2001b; Dong and Swanson, 2004; 2006).

Interestingly, BNST-AL revealed differences with those trained in DI having greater Fos expression compared to those trained in PRO yet behaviorally, PRO trained subjects had slightly higher fear levels compared to DI trained subjects as evidenced by an overall significant main effect of cycle phase at conditioning. This is in line with

previously described work that suggests BNST-AL is anxiolytic (as reviewed in Gungor and Pare, 2016, although see Gungor and Pare, 2014; Sink et al., 2011). Our analysis parsed BNST-OV from BNST-AL, and revealed no such differences in BNST-OV. Given the intricate connections both within and outside this region along with the differences in neuronal subtypes, solely measuring neuronal activation may not be sufficient to detect state-dependent effects of hormonal actions within BNST. Recent work found state-dependent effects of gaboxadol in the dorsal hippocampus were mediated by miR-33, which may be another mechanism to explore in the BNST (Jovasevic et al., 2015). Our previous results found state-dependent actions of supraphysiological levels of ALLO in the BNST of male rats (Acca et al., 2017) and although the current results of Fos patterns in BNST did not correlate with freezing behavior, it does not exclude its role in mediating the observed behavior seen here in females. It should be noted that other brain regions including the prefrontal cortex, ventral hippocampus, BLA, and CEA also showed no differences across groups in Fos immunoreactivity (data not shown).

We replicated our naturally cycling design with OVX rats with PROG or VEH treatment prior to conditioning as well as contextual and cued fear testing and did not find the same state-dependent effects as we did with intact female rats across the estrous cycle. Estrous cycles were not tracked in Experiment 2 between arrival and surgery and given the short interval between OVX and behavior, residual hormones may still be in circulation and therefore all subjects may not have been in the same physiological state at the onset of behavior. Ovariectomy results in a loss of fluctuating hormone levels

across the estrous cycle and accompanying that, a loss in fluctuating receptor profiles. For instance, $GABA_A$ receptor subtypes fluctuate over the estrous cycle with changing hormone levels (Clark et al., 1998; Maguire et al., 2005) and in response to estrogen following OVX (Herbison and Fénelon, 1995). In addition, estrogen is known to prime PROG receptors (Parsons et al., 1982) and in our OVX model with PROG replacement, PROG receptors may not be in abundance. Given our experimental design, we cannot conclude that the actions of PROG in intact or OVX rats are the product of PROG or its conversion to any of its metabolites, including ALLO. Further work is needed to specify this.

The work presented here suggests PROG, particularly in intact females, can contribute to state-dependent contextual and cued fear. Currently most studies do not consider the interaction of hormonal state during conditioning and testing, yet our work provides evidence of its importance. Clinically, this may have important implications when considering exposure therapy in female patients.

CHAPTER V

CONCLUSIONS

Summary of Results

The work presented here stems from earlier research demonstrating that male rats have greater levels of contextual fear compared to female rats (Maren et al., 1994). This, along with evidence of sex differences in fear and anxiety disorders in clinical populations, suggests hormones and their metabolites may influence fear. Chapter 1 introduces this phenomenon and details previous research on gonadal hormones and their effects of fear behavior in both human and animal studies. In particular, this dissertation focuses on progesterone (PROG) and its metabolite, allopregnanolone (ALLO). PROG, which is less studied in the fear literature compared to estrogen, fluctuates throughout the estrous cycle with highest levels observed during proestrus. However, much of its actions in fear and anxiety behavior are attributed to its metabolite, ALLO. Through its actions at $GABA_A$ receptors, ALLO has well established anxiolytic properties (Akwa et al., 1999; Bitran et al., 1993; Frye and Walf, 2002) on a number of different tasks. Furthermore, PROG and ALLO appear to preferentially affect contextual fear as opposed to cued fear (Pibiri et al., 2008; Rabinowitz et al., 2014; Toufexis et al., 2004). Due to its anxiolytic properties and modulation of contextual fear, ALLO may therefore play an important role in mediating the observed sex differences. I focused the efforts here on analyzing the effects of ALLO in the BNST because of its role in contextual fear and as a site of hormonal

regulation. The overarching goal was to examine the effects of ALLO in the BNST on mediating observed sex differences seen in fear conditioning.

In Chapter 2, I successfully demonstrated that ALLO's actions in the BNST affect contextual fear in both males and females. The opposite hormonal state was created in each sex by increasing BNST ALLO levels in males and decreasing BNST ALLO levels in females to reverse the typical behavioral profile. Following fear conditioning, administration of intra-BNST ALLO in males significantly reduced contextual fear but had no effect on cued fear. Conversely, in females, blocking the synthesis of ALLO or binding of ALLO to $GABA_A$ receptors increased contextual fear, but again had no effect on cued fear. Given prior research on the role of the BNST in contextual versus short duration cued fear, the lack of effects during tone testing was not surprising. However, the bi-directional role ALLO played in the BNST suggests it is a site of hormonal regulation of contextual fear and may, in part, mediate the observed sex differences in contextual fear in rats. ALLO levels are higher in females throughout most of the estrous cycle compared to males and proestrus, when PROG levels and subsequently ALLO levels are higher, may act as an anxiolytic period in female rats. However, the effects of ALLO in male rats was possibly due to state-dependent mechanisms as subjects were trained in the absence of drug and only received ALLO or VEH infusions prior to testing. This led to testing for state-dependent effects of intra-BNST ALLO infusions on contextual fear in Chapter 3.

Surprisingly, ALLO infusions into the BNST resulted in state-dependent contextual fear in male rats. State-dependent effects have not previously been reported

with hormones or their metabolites in the BNST; only the hippocampus had previously been identified as a specific brain region that mediates state-dependent memories (Jovasevic et al., 2015). To ensure this was specific to the BNST and not to ALLO itself, the same experiment was replicated in the BLA, a region that previously has been shown to be state-independent with other drugs, including GABAergic modulators. ALLO effects in the BLA proved to be state-independent and affected both contextual and cued fear. Therefore, state-dependent effects of ALLO are unique to the BNST. In an experiment examining the effects of external vs. internal context shifts, both appeared to have equal influence of freezing behavior. This was unexpected as it was thought that external contextual cues are more salient than internal contextual cues.

The results from Chapter 3 prompted me to analyze the state-dependent effects of naturally occurring hormonal fluctuations in female rats. Specifically, I focused on PROG because it is the precursor to ALLO. Cycling PROG levels in female rats results in asymmetrical state-dependent effects on contextual fear that are driven by high fear levels in animals trained and tested in high PROG states and low fear levels in animals trained in a low PROG state and tested in a high PROG state. Previous literature suggests that proestrus, when ovarian steroid levels are high, is a period of lower fear and anxiety, however, the present results show that hormone state during both training and testing are important. The behavioral effects, however, could not be localized to the BNST through mapping of neuronal activation patterns, although there were effects of training state in the BNST-AL. More work is necessary to identify if the state-dependent effects of PROG on contextual fear are mediated by the BNST or other brain regions. In

an OVX model, PROG treatment was associated with state-dependent effects on cued but not contextual fear.

The work presented here began by examining the effects of ALLO in the BNST in male and female rats and has evolved to uncover a novel role of hormones in fear memories. As such, it has strong implications on how future experiments will be designed and conducted. Taking into consideration hormonal state during training or testing alone is not sufficient; the interaction between to the two is just as important. This provides novel evidence for the role of hormones and their metabolites in creating internal contextual cues that affect the recall of memories.

BNST and Contextual Fear, Expanded

Prior research identified the BNST as important for contextual fear, but not cued fear. The work presented here, along with other recent research presents evidence that how the field defines and separates contextual vs. cued fear needs to be reevaluated. Initially, it was first proposed that the BNST is not involved in discrete cues such as the short duration cues used in the work here (Gewirtz et al., 1998; Ledoux et al., 1988). Instead, it was involved in contextual fear responses (Hammack et al., 2004; Sullivan et al., 2004; Zimmerman and Maren, 2011). What constitutes a context, however, is a more critical question. Long duration cues are affected by BNST manipulations (Waddell et al., 2006; Walker et al., 2009) and when the shock is presented in the first minute of contextual fear conditioning as opposed to after 10-min context exposure, inactivation of the BNST does not affect freezing behavior (Hammack et al., 2015).

Together, this suggests that the BNST is not strictly involved in contextual fear over cued fear, but that the timing and duration of the CS is important. One interpretation may be that the longer a discrete cue is presented, the more likely it is to be incorporated as part of the external context; shocks early on during training were not affected by BNST manipulations (Waddell et al., 2006). Either way, Hammack et al. (2015) suggests stimulus duration is necessary for BNST recruitment during contextual fear processing. In the work here, neurosteroids in the BNST are present throughout the entire context test in Chapter 2 and throughout training and testing in Chapter 3, therefore they create an interoceptive context that lasts the duration of the behavioral task.

There are reports though of BNST manipulations that do affect short duration cued fear. For instance, intra-BNST muscimol injections enhanced fear potentiated startle (Meloni et al., 2006) and BNST recordings found neuronal responses in the BNST-AL and BNST-AM to discrete CSs (Haufler et al., 2013). Clearly, more work is needed to examine the direct role of the BNST in mediating fear responses, including its novel role in state-dependent memories. The BNST is comprised of heterogeneous neuronal subtypes with diverse receptors. Calcitonin gene-related peptide (CGRP), corticotrophin releasing factor (CRF), and pituitary adenylate cyclase-activating polypeptide (PACAP) have all been shown to affect anxiety behaviors (Hammack et al., 2010; Lee and Davis, 1997; Sink et al., 2013). Given recent technological advancements in the field, more precise manipulations of neuronal subtypes can be targeted to understand the more detailed role the BNST plays in fear and anxiety. The state-

dependent effects of ALLO are likely mediated through its actions at $GABA_A$ receptors and therefore increase the inhibitory tone on the BNST, leading to disinhibition due to the majority of GABAergic neurons in this area. However, because the ALLO manipulations here globally affected the BNST and were not specifically targeting neuronal subtypes, it is unclear if inhibiting specific subtypes contributes to state-dependent learning or if global disinhibition affected local circuitry. As more research is done to examine the specific role of the BNST in fear and anxiety, attention must be paid to state-dependent effects and how interoceptive states contribute.

Hormones, Metabolites, and State-Dependent Learning

The results presented here found state-dependent effects of ALLO in male rats and of PROG in naturally cycling female rats, which raises an interesting role for hormones in memory processing. Hormone states are known to affect memory in a variety of tasks in both human and rodent studies. Outside of fear conditioning, PROG has been shown to enhance consolidation of memories in the Morris water maze (Frye et al., 2009). However, when given prior to training, ALLO impairs learning (Johansson et al., 2002). Estrogen dose-dependently affects cognitive performance with high levels impairing and low levels facilitating working memory performance (Holmes et al., 2002; Wide et al., 2004). Of course, given the outcome of the present work it is possible that the impairments in these tasks represent state-dependent generalization deficits.

Within the domain of aversive learning, recent work has implicated gonadal steroids in the extinction of fear. During extinction, the CS is presented in the absence

of the US and after repeated presentations the CR gradually fades. Numerous studies demonstrate that extinction results in a new memory and is not simply an erasure of the original fear memory (Maren, 2011). Indeed, extinction memories are context dependent (Maren et al., 2013). Although most studies only consider exteroceptive context, extinction memories may be susceptible to interoceptive state-dependent effects as well. Currently, research on hormones in extinction learning suggest that extinction learning is optimal in proestrus, when estrogen and PROG levels are elevated compared to metestrus (Milad et al., 2009). Similarly, OVX rats treated with estradiol had decreased freezing during both context testing and extinction trials (Chang et al., 2009; Gupta et al., 2001). However, these designs involve extinguishing and testing fear in different hormonal states that are associated with different interoceptive contexts. It is possible that these studies underestimate the magnitude of extinction, because of the interoceptive context shift in between extinction training and the retention test.

Clinical research also demonstrates differences in fear extinction across the menstrual cycle in women with and without a clinical diagnosis. Higher estrogen levels are associated with more robust extinction memory (Milad et al., 2010; Wegerer et al., 2014) and fear inhibition (Glover et al., 2013). However, a recent report suggests the effects of menstrual cycle phase on extinction learning may depend on if the subjects have been diagnosed with PTSD. Impaired retention of extinction learning was found in the midluteal phase of the menstrual cycle in women with PTSD when estrogen and PROG levels are high, whereas those without a diagnosis of PTSD had greater extinction retention in this phase (Pineles et al., 2016). The experimental design in these

experiments does not allow for testing of state-dependent memory. Of course, the human menstrual cycle is much longer than that in the rat. It remains to be seen if the state-dependent effects of cycling hormones on contextual fear are as robust across the human menstrual cycle. It is well known that fear memories persist even after long passages of time, yet it is not known how robust interoceptive context shifts affect retention across these intervals. Therefore, cycling gonadal hormones may not show state-dependent effects on fear learning due to the longer cycle length. Regardless, understanding the role of gonadal hormones in state-dependent memories, particularly in cycling women is critical for developing better sex-specific therapeutic options for treating fear and anxiety disorders in women. If hormonal state-dependent fear memories are present in clinical populations, attention must be given to when the trauma occurred and how that interacts with hormone state during therapeutic interventions such as exposure therapy.

Potential mechanisms for state dependence

The state-dependent effects localized to the BNST in Chapter 3 are likely due to the actions of ALLO at $GABA_A$ receptors, however the mechanisms underlying it remain unknown. In the hippocampus, miR-33 was found to play a role in the state-dependent effects of gaboxadol on contextual fear (Jovasevic et al., 2015). Given both ALLO and gaboxadol act at $GABA_A$ receptors, it is reasonable to speculate that ALLO may act as an interoceptive cue in the dorsal hippocampus as well. However, on a variety of behavioral tasks, intra-hippocampal ALLO did not have any effect (Engin and Treit, 2007; Mòdol et al., 2011, although see Frye and Walf, 2002). These tasks, aside

from passive avoidance, are innate behavioral tasks and therefore missing the learning component that is essential for state-dependent effects. Ethanol also acts as an interoceptive cue encoded by the hippocampus. Shifting interoceptive states between extinction and fear relapse resulted in a return of fear that was reversed with AMPAR antagonists (Yoo et al., 2017). Although this study did not explicitly test for state-dependent effects, ethanol has been reported to induce state-dependent learning albeit this was in systemic studies (Nakagawa and Iwasaki, 1995; 1996). Therefore, the control of $GABA_A$ receptor expression through miR-33 and also AMPA receptors within the hippocampus may underlie interoceptive context cues.

The BNST is rich in neuromodulators such as CRF, dopamine, norepinephrine, serotonin, pituitary adenylate cylase-activating peptide (PACAP), and calcitonin gene related peptide (CGRP; Daniel and Rainnie, 2016; Hammack et al., 2009; Sink et al., 2013). Whether other peptides such as these also contribute to state-dependent effects remains to be explored. It is unclear how the BNST encodes interoceptive contexts and given its diversity, it is interesting to speculate if other peptides in this area would have similar results. Indeed, norepinephrine has been implicated in state-dependent effects on the extinction of inhibitory avoidance (Rosa et al., 2014). With supraphysiological levels of ALLO, there is probably a global inhibitory tone on the BNST following administration, which may be necessary for encoding of interoceptive cues; it is unclear how "strong" of a shift is needed to result in state-dependent effects. However, more minute changes with some of the neuromodulators mentioned above that regularly affect BNST activity may also constitute interoceptive shifts.

Incorporating Sex Differences in Fear Research

Although the work here evolved to examine the role of hormones in state-dependent fear memories, evidence still suggests that gonadal hormones contribute to and mediate sex differences in fear and anxiety. However, recent reports from combat veterans are beginning to report smaller sex differences in the rates of PTSD compared to earlier work. Given changes in military protocol, women now face more combat than before. When examining combat-related stressors to post-deployment mental health, women did not fair worse compared to men (Vogt et al., 2011). Furthermore, another study did not find sex differences in PTSD following deployment (Street et al., 2013). Yet among healthcare professionals in the military, women were more likely to report PTSD symptoms compared to men (Gibbons et al., 2012). As participation of women in combat areas increases, more research will be needed to see if sex differences still exist in this setting, or if there are underlying differences in trauma type or readjustment once they return that contribute (Gilmore et al., 2016; Smith et al., 2017). Regardless, outside military-specific studies, sex differences are still robust with women far exceeding men across the lifespan (Ditlevsen and Elklit, 2010).

Because NIH now mandates research in both males and females, even at the preclinical level, it is important to ensure studies are conducted with an appreciation of cyclical hormonal effects on learning and memory. Because most existing literature that formed the basis of the field was done in males, it is important to make sure assumptions exist when including females. For instance, Pavlovian fear conditioning uses freezing behavior as a measure of fear and all other movement outside that suggests an absence of

fear. Gruene and colleagues (2015) identified a novel active fear response which they coined "darting" in which female rats are more likely to quickly "dart" across the behavioral chamber as a fear response, instead of freezing. Darting behavior increases with conditioning trials and decreases throughout extinction, all characteristics of learned fear behavior (Gruene et al., 2015). Given that female rodents are known to generally be more active than males (Fernandes et al., 1999; File, 2001), it is not surprising that different strategies might be utilized between sexes when responding to threat. While the female rodent work conducted in this dissertation did not display darting behavior, it is still important to consider.

Future Directions

Here we show clear state-dependent effects on contextual fear via intra-BNST ALLO, but not via intra-BLA ALLO. Recent work demonstrates the dorsal hippocampus is also involved in state-dependent contextual fear via gaboxadol (Jovasevic et al., 2015). ALLO has anxiolytic actions in the dorsal hippocampus as well (Bitran et al., 2000; Mòdol et al., 2011). Therefore it would be worthwhile to examine its role in this brain region as well. Jovasevic et al. (2015) identified miR-33, a micro-RNA that regulates different GABA related proteins, as influential for maintaining the state-dependent effects. The same mechanisms may be conserved in the BNST and given the diverse $GABA_A$ subtypes in the BNST (Pirker et al., 2000; Wisden et al., 1992), it may involve similar mechanisms as the hippocampus.

Our results on state-dependent contextual fear in intact females only consider the role of PROG. While the surge in PROG follows the rise and fall of estrogen, the studies here do not consider the effects of estrogen. In the OVX model with PROG replacement, results did not replicate the naturally cycling model. With only exogenous PROG administration, estrogen levels are presumably very low. Therefore, estrogen most likely contributes in some manner to the state-dependent effects on contextual fear in female rats. OVX studies using estrogen in place of PROG or both hormones to mimic natural fluctuations would be useful to more fully appreciate this effect. Both intact and OVX models also do not account for the differential actions of PROG vs. ALLO. To differentiate between the two, blocking the synthesis of ALLO from PROG or the binding of ALLO at $GABA_A$ receptors as done in Chapter 2 are possible options.

ALLO, however, does mediate contextual fear in females via the BNST as seen in Chapter 2. By reducing ALLO levels or its binding with $GABA_A$ receptors, contextual fear was enhanced. Given the disruption in ALLO levels in patients with PTSD (Rasmusson et al., 2006), a further understanding of the role ALLO plays in fear and anxiety is critical. $GABA_A$ receptor subunits change across the estrous cycle, yet exactly how they change, and where the changes are located within the BNST remain to be discovered. As a more detailed understanding of the circuitry of the BNST evolves, a more precise understanding of how ALLO affects neuronal activity can be determined.

In summary, more work is needed to understand how hormones and their metabolites influence fear. The sex differences in human psychiatric diagnoses and abundant research in both rodent and clinical models identify a role for hormones and

their metabolites in contributing to fear and anxiety. With more research, sex-specific therapies can be developed to ease the burden on individuals and society.

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