ESTRADIOL MODULATION OF THE RENIN ANGIOTENSIN SYSTEM AND THE REGULATION OF FEAR EXTINCTION

by

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Low estradiol levels during extinction training lead to poor extinction consolidation and increased fear during extinction recall; however, the mechanism by which this occurs has not been identified. The renin angiotensin system (RAS), which is often studied in the context of blood pressure regulation and cardiovascular function, has recently been associated with the stress response and stress related pathologies. Antagonists of the angiotensin II type 1 receptor (AT1R), which are commonly prescribed to treat hypertension, reduce symptoms of posttraumatic stress disorder (PTSD) in humans and enhance extinction consolidation in male mice. Since estradiol downregulates many components of the RAS, including AT1R, we hypothesized that estradiol modulates the RAS to affect fear extinction consolidation. We predicted that high estradiol levels during extinction training lead to downregulation of RAS components and enhanced extinction consolidation. We show for the first time that systemic administration of AT1R antagonist losartan prior to extinction training reverses the extinction consolidation deficit found in female rats taking a hormonal contraceptive (HC), which reduces estradiol levels. We also found that female rats that receive ovariectomy (OVX) surgery have a deficit in extinction consolidation compared to sham-operated proestrus females, and that systemic treatment with losartan prior to extinction training rescues the deficit in OVX females. Finally, we explore potential mechanisms for how estradiol is regulating the RAS to affect fear extinction consolidation. While differences in RAS components have been extensively studied in

OVX females, no studies have examined how HC treatment affects RAS components or how AT1R levels differ between males and females in the brain. We found that OVX females have increased AT1R ligand binding compared to intact proestrus females in the pituitary gland and ventral subiculum. We also found that HC-treated females have increased circulating angiotensin II (Ang II) peptide levels compared to proestrus females. Our findings have significant clinical implications, suggesting that patients with anxiety disorders such as PTSD should take an AT1R antagonist, especially if they have low estradiol levels, prior to an exposure therapy session to improve treatment outcome.

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LIST OF ABBREVIATIONS

ACE – Angiotensin converting enzyme

ACTH -- Adrenocorticotropic hormone

Ang 1-7 – Angiotensin-(1-7)

Ang II – Angiotensin II

Ang IV – Angiotensin IV

AT1R – Angiotensin II type 1 receptor

AT2R – Angiotensin II type 2 receptor

BBB – Blood brain barrier

BLA – Basolateral nucleus of the amygdala

BM – Basomedial nucleus

C – Celsius

CeA – Central nucleus of the amygdala

CR – Conditioned response

CRF – Corticotropin-releasing factor

CS – Conditioned stimulus

DMSO – Dimethyl sulfoxide

ER – Estrogen receptor

ET – Exposure therapy

HC – Hormonal contraceptive

HPA – Hypothalamic-pituitary-adrenal

ICV -- Intracerebroventricular

IL – Infralimbic cortex

i.m. – Intramuscular

i.p. -- Intraperitoneal

ITI – Inter-trial interval

ITC – Intercalated cells

LA – Lateral nucleus

MasR – Mas receptor

MnPO – Median preoptic nucleus

mPFC – Medial prefrontal cortex

NMDA – *N*-methyl-D-aspartate

OVX -- Ovariectomy

PL – Prelimbic cortex

PTSD – Posttraumatic stress disorder

PVN – Paraventricular nucleus of the hypothalamus

RAS – Renin angiotensin system

s.c. -- Subcutaneous

SFO – Subfornical organ

SSRI – Selective serotonin reuptake inhibitor

UR – Unconditioned response

US – Unconditioned stimulus

vmPFC – Ventromedial prefrontal cortex

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1.0 INTRODUCTION

The formation of memories, or information that is retained through learning (Abel, 1997), is essential for survival and can be used to guide future behavior (Taylor and Torregrossa, 2015). There are many processes involved in the formation of memories, including acquisition (encoding), consolidation, and retrieval. Acquisition refers to the learning of newly processed information (Kandel, 2000, Abel and Lattal, 2001). Once a new memory has been acquired, consolidation begins, where the new memory is converted from its labile state into a more stable state for storage into long-term memory (Kandel, 2000, Nader et al., 2000, Taylor and Torregrossa, 2015). Consolidation occurs over a period of several hours, where gene expression, protein synthesis, and *N*-methyl-D-aspartate (NMDA) receptor function are necessary for successful memory consolidation (Abel and Lattal, 2001, Taylor and Torregrossa, 2015). Finally, retrieval refers to recalling the stored memory after a period of time has passed in order to influence behavior (Kandel, 2000, Taylor and Torregrossa, 2015). Retrieval is most successful when the memory is recalled in the same context and with the same cues that were present during the initial encoding (Kandel, 2000). Although memory formation is essential for guiding behavior, in the case of maladaptive memory disorders such as posttraumatic stress disorder (PTSD), disruptions in these memory processes can be extremely problematic for daily functioning and often hinder treatment.

In the remainder of the introduction, I will review why PTSD is classified as a maladaptive memory disorder and how women are understudied when it comes to better understanding mechanisms involved in extinguishing fear memories, despite being more than twice as likely to develop PTSD (Kessler et al., 1995). I will highlight the importance of hormone levels, specifically estradiol, in regulating the consolidation of fear extinction memories. While it is currently unknown how estradiol regulates fear extinction consolidation, it is known that estradiol modulates the renin angiotensin system (RAS), which has recently emerged as a key mediator in the stress response. Interestingly, negative regulation of the RAS has been previously shown to reduce symptoms associated with PTSD. This thesis uses behavioral and molecular approaches to examine how estradiol modulates the RAS to affect fear extinction consolidation, which we hope will fill a critical gap in the current literature and provide better treatment options for people – especially women – who are diagnosed with PTSD.

1.1 PTSD: A MALADAPTIVE MEMORY DISORDER

PTSD is a devastating psychiatric illness, which leads to impairment in social and occupational functioning (APA, 2013). For diagnosis, patients need to develop the following characteristic symptoms after exposure to one or more traumatic events, where the symptoms occur within 3 months of trauma and persist for longer than one month: intrusion symptoms associated with the trauma, persistent avoidance of stimuli associated with the trauma, negative alterations in cognitions and mood, and alterations in arousal and reactivity (APA, 2013). PTSD has an estimated lifetime prevalence of 7.8% in the United States (Kessler et al., 1995), with the highest rates found in patients who have survived rape, military combat and captivity, and ethnically or

politically motivated imprisonment and genocide (APA, 2013). Although approximately 80% of people in the United States will experience at least 1 traumatic event in their lifetime, less than 10% of people with trauma exposure will go on to develop PTSD (Breslau, 2009). PTSD is far more prevalent in females compared to males, with women being more than twice as likely to be diagnosed with PTSD (Kessler et al., 1995). While more research needs to be done to determine the direct cause of female vulnerability in PTSD, many factors can be ruled out. A meta analysis examining sex specific risk of traumatic events and PTSD found that large sex differences in PTSD prevalence and severity remain even when controlling for type of traumatic event, specifically, the increased occurrence of sexual violence in females compared to males (Tolin and Foa, 2006). In addition, prior traumatic experiences, preexisting mood disorders, and sex-related bias in reporting have been eliminated as potential causes for female vulnerability in developing PTSD (Breslau, 2009).

Maladaptive memory processes are at the core of PTSD symptomatology. For instance, the patient may have more readily acquired the memory due to genetic or environmental factors, or perhaps the patient consolidated the memory more strongly, leading to an engram that is more stable than in healthy subjects (Taylor and Torregrossa, 2015, van Marle, 2015). In addition, intrusion symptoms point to a problem with retrieval, since the patient is recalling the memory associated with trauma in non-threatening situations (Chiamulera et al., 2014, Taylor and Torregrossa, 2015). Treatment for PTSD often focuses on disrupting these maladaptive memory processes through behavioral therapy sessions or pharmacological treatment.

1.1.1 Treatment strategies for PTSD

The recommended first-line treatment for PTSD is currently psychotherapy (McFarlane et al., 2017). Exposure therapy (ET), which is classified as a type of trauma-focused cognitive behavioral therapy, has been identified as one of the most effective ways to treat patients who are diagnosed with PTSD (Barlow, 2002, Taylor et al., 2003, McLean and Foa, 2011, Rauch et al., 2012, Shalev et al., 2012). There is evidence to support the use of ET to treat PTSD regardless of the type of trauma experienced and other comorbid conditions (Defense, 2010, van Minnen et al., 2015). According to the emotional processing theory, trauma survivors with PTSD have two basic dysfunctional thoughts: 1) the world is completely dangerous, and 2) the survivor will believe that he/she is totally incompetent (Rauch, 2006). Treatment for PTSD must therefore activate the fear memories (through exposure to feared stimuli, or "triggers") and assist the patient in receiving information that disconfirms these dysfunctional thoughts, which will ultimately reduce PTSD symptoms (Rauch, 2006).

ET accomplishes this through 4 main components, including psychoeducation, in vivo exposure, imaginal exposure, and emotional processing (Rauch et al., 2012). Patients typically attend 9-12 sessions that are 60-90 minutes in length (Harvey et al., 2003). Psychoeducation occurs during the first few sessions, and it focuses on the patient's experience with their PTSD symptoms. During these sessions, emphasis is placed on the necessity of confronting the triggers to help reduce PTSD symptoms (Rauch et al., 2012). In vivo exposure involves confronting people, places, and things that trigger fear, while imaginal exposure occurs when the patient revisits the trauma by describing it in the present tense and engaging with the emotional content of the memory (Rauch et al., 2012). Finally, during emotional processing sessions, the therapist has an open-ended discussion with the patient to assist in processing the emotional content of the

memory (Rauch et al., 2012). Through exposing patients to their triggers in multiple ways and allowing them to emotionally process their traumatic memories, dysfunctional thinking is lessened and the patients will have reduced PTSD symptoms over time.

There are currently no pharmacological treatments that have been specifically developed for reducing symptoms of PTSD, and all available options are FDA-approved drugs to treat other disorders (McFarlane et al., 2017). Although more studies are needed to better assess treatment options for PTSD patients, some research has been done on selective serotonin reuptake inhibitors (SSRIs), propranolol, and benzodiazepines (Howlett and Stein, 2016). Overall, SSRIs are typically the first drugs prescribed by doctors when medication is considered; however, evidence on efficacy of SSRIs in treating PTSD is mixed, with some studies reporting no effect (Shalev et al., 2012). Other reviews highlight benefits of using SSRIs to treat patients with PTSD (Jonas et al., 2013). Although propranolol has been shown to reduce PTSD rates and symptoms in some populations of trauma-exposed patients (Vaiva et al., 2003), other studies have found no effect of propranolol (Stein et al., 2007, Hoge et al., 2012). There is promise for propranolol treatment when it is administered during a time when the patient is actively recalling the traumatic event, suggesting that propranolol may be more effective when the traumatic memory is reactivated (Brunet et al., 2008, Poundja et al., 2012). Benzodiazepine use among US veterans with PTSD is rapidly declining, and the Department of Defense is recommending against the use of benzodiazepines (Defense, 2010, Lund et al., 2012). While the use of pharmacological treatments can be beneficial, especially in combination with psychotherapy, the use of medication alone is not recommended as a routine first-line treatment (Forbes et al., 2007).

While there is some inconsistency in the literature, some studies have shown that subgroups of patients with PTSD have reduced cortisol levels (Mason et al., 1986, Yehuda,

2002, Yehuda and Seckl, 2011). In addition, studies that measured cortisol levels following a traumatic event report that lower levels of cortisol after the traumatic event predicted PTSD symptoms one month later (McFarlane et al., 1997, Delahanty et al., 2000). Thus, increasing cortisol levels following a traumatic event may be a potential treatment for those at risk of developing PTSD. Stress level doses of hydrocortisone, which is pharmaceutical cortisol, have been found to reduce the incidence of PTSD in patients with septic shock compared to septic shock patients that did not receive hydrocortisone treatment (Schelling et al., 1999, Schelling et al., 2001). Hydrocortisone treatment has also been found to reduce chronic stress and PTSD symptoms in patients with cardiac surgery (Schelling et al., 2004) and in patients with traumatic injuries (Delahanty et al., 2013). While hydrocortisone administration in trauma-exposed patients seems like a promising treatment strategy, further research needs to be done to determine which patient population would benefit the most from this treatment.

1.1.2 Importance of studying fear processes in females

Despite the fact that women are more than twice as likely to develop an anxiety disorder compared to men (Kessler et al., 1995), most research focusing on understanding the mechanisms underlying fear extinction focus primarily on males (Cover et al., 2014). This disparity may be due in part to the difficulty of tracking the estrous cycle throughout an entire behavioral study (Maeng et al., 2015). Studies that do include females often do not clearly report how estrous cycle phase affects behavioral results (Maeng et al., 2015). In addition to the increase in PTSD prevalence, women with PTSD also tend to experience greater symptom severity compared to men, establishing a critical need for females to be included in these types of studies (APA, 2013). For instance, women diagnosed with PTSD tend to experience symptoms

for a longer duration compared to males (APA, 2013), and chronicity of the disease is associated with higher incidence of comorbid disorders (Seedat et al., 2005). In addition, some studies have shown that women with PTSD have significantly reduced quality of life compared to men (Holbrook et al., 2002). Because women have increased risk of developing PTSD and tend to have greater symptom severity, poor quality of life, and longer symptom duration compared to males, it is critical that more research is dedicated to better understanding the mechanisms underlying fear extinction in females so that more efficacious treatments can be developed.

1.2 HOW ARE RODENTS USED TO STUDY FEAR LEARNING AND EXTINCTION?

1.2.1 Associative learning and Pavlovian conditioning

In order to survive in an ever-changing world, it is essential for both animals and humans to learn relationships between events and stimuli in their environment, often referred to as associative learning (Thompson, 1986, Le Pelley, 2004). Patients with PTSD have deficits in associative learning, which often leads to strong, intrusive fear memories that are difficult to extinguish (J. Gayle Beck, 2012, Lissek and van Meurs, 2015). There are two forms of associative learning, instrumental conditioning and classical conditioning, which is often referred to as Pavlovian conditioning (Thompson, 1986). Instrumental conditioning refers to a learning process in which behavior is maintained by reinforcement or punishment (Staddon and Cerutti, 2003). Pavlovian conditioning involves pairing an innocuous stimulus with a biologically relevant unconditioned stimulus (US), which naturally produces an unconditioned response (UR) on its own. After

multiple pairings of the US and innocuous stimulus, the innocuous stimulus becomes associated with US and is referred to as the conditioned stimulus (CS), where presentation of the CS alone can lead to a conditioned response (CR). While Ivan Pavlov performed some of the best-known classical conditioning experiments to study the physiology of digestion of dogs in 1927 (Pavlov, 1927), the same principles are often applied to a paradigm known as Pavlovian fear conditioning, which is a classic model that is used to examine the neurobiological mechanisms underlying learning and memory processes related to fear expression and fear-related anxiety disorders, such as PTSD (LeDoux, 1994, Ledoux and Muller, 1997, LeDoux, 2000, Flandreau and Toth, 2017).

1.2.2 Pavlovian fear conditioning and extinction training

The Pavlovian fear conditioning paradigm has been widely used in animal models to study the neurobiology of normal and pathological fear (Bowers and Ressler, 2015). In rats, an innocuous stimulus, such as a light or tone, is paired with an aversive US, which is typically a mild electric footshock. After at least one (LeDoux, 2000), but typically multiple pairings of the innocuous stimulus and the US, the rat will begin to associate the innocuous stimulus, which becomes the CS, with the footshock. Presentation of the CS alone will result in a CR, which is the absence of movement other than breathing (often referred to as freezing), an innate response to danger in rodents (Bolles, 1980). In addition to defensive behavior, rats will exhibit other autonomic and endocrine responses indicative of fear or stress when the CS is presented (Ledoux and Muller, 1997, LeDoux, 2000), such as increased heart rate and blood pressure (Cohen and Randall, 1984, LeDoux et al., 1984) and elevated levels of corticosterone in plasma (Van de Kar et al., 1991). Freezing is the most common, easily quantifiable way to measure fear-related behavior to the CS in rodents (Morrison and Ressler, 2014). Like other types of learning, fear conditioning consists

of three phases, including acquisition, consolidation, and retrieval (Abel and Lattal, 2001). The acquisition phase occurs during the conditioning session, where freezing increases as the rat learns that the once innocuous stimulus predicts the US. Following the session, consolidation occurs, which lasts for several hours, where physiological and molecular processes are involved in stabilizing the fear memory for long-term storage. Assuming that the fear memory was properly consolidated, presentations of the CS after the conditioning session will result in retrieval (also commonly referred to as recall) of the fear memory, resulting in high levels of freezing.

The expression of conditioned fear can be altered using other learning and memory paradigms. Learned fear can be extinguished by administering an extinction training session, which occurs after the conditioning session, where the CS is presented multiple times in the absence of the US. Extinction training also consists of three phases, including acquisition, consolidation, and recall (Quirk and Mueller, 2008). During extinction acquisition, the CS is presented multiple times in the absence of the US, leading to a gradual reduction in freezing across the session as the rat learns that the CS no longer predicts the US. Similar to consolidation after the fear conditioning session, extinction consolidation occurs immediately after extinction acquisition and lasts for several hours, where the extinction memory is stabilized for long-term storage (Quirk and Mueller, 2008). Recall occurs when the CS is presented at a later time. Low levels of freezing are indicative of good consolidation of the extinction memory. Alternatively, high levels of freezing during recall indicate that there may have been a disruption during extinction consolidation or extinction recall itself (Quirk and Mueller, 2008).

Extinction is widely believed to involve new learning rather than erasure of the original fear learning (Bouton, 2004). Because extinction does not eliminate fear memories, these

memories can be very difficult to extinguish long term. In addition, CR can sometimes occur even after extinction training has taken place in processes such as renewal, spontaneous recovery, and reinstatement (Bouton, 2002, 2004, Morrison and Ressler, 2014). Renewal refers to the occurrence of the CR to the CS when it is presented in a context that differs from the extinction training context. Spontaneous recovery is the phenomenon where the CR will occur if the CS is presented in the extinction context after sufficient time has passed since extinction training occurred. Finally, reinstatement can occur when an extinguished response reappears upon exposure to the US. These processes should highlight that extinction is extremely context dependent, which can lead to complications in treatment of PTSD with exposure therapy (Bouton, 2002, Myers and Davis, 2007). Because extinction does not erase the original fear memory and is often not permanent, finding a way to enhance extinction consolidation and/or extinction retrieval across context and time could potentially lead to reduced fear memories and better quality of life for patients with PTSD (Craske et al., 2008).

Although standard fear conditioning does not encompass all of the long-term characteristics and symptoms of PTSD (Flandreau and Toth, 2017), this procedure has high face validity and can be easily utilized to better understand mechanisms underlying fear learning and extinction (Morrison and Ressler, 2014, Flandreau and Toth, 2017), which can ultimately lead to identifying better treatments for fear-related disorders. For instance, only one CS-US pairing, or traumatic event, in rodents is enough to induce strong fear responses to the CS alone (Flandreau and Toth, 2017). In addition, extinction training in rodents has many similarities to exposure therapy (Morrison and Ressler, 2014), which is commonly used to treat human patients with PTSD (Barlow, 2002, Taylor et al., 2003, McLean and Foa, 2011, Rauch et al., 2012, Shalev et al., 2012), who often have deficits in fear extinction consolidation (Milad et al., 2008, Milad et

al., 2009b). From a practical standpoint, fear behavior is easily quantifiable and reproducible, with an apparatus that is easily modified based on the environment and cues needed for the session (Morrison and Ressler, 2014, Flandreau and Toth, 2017). Therefore, the fear conditioning paradigm provides a reliable way for investigators to study fear learning and extinction in rodents that is highly relevant to fear disorders in humans.

1.3 FEAR CIRCUITRY

1.3.1 Amygdala

It has been well established that the amygdala plays a critical role in the formation of fear memories in the Pavlovian fear conditioning paradigm. There are four major nuclei of the amygdala, including the lateral nucleus (LA), basolateral nucleus of the amygdala (BLA), basomedial nucleus (BM), and central nucleus of the amygdala (CeA) (LeDoux, 2000). Information from the CS and US converge onto the LA, and the LA projects to the BLA and the BM (Pitkanen et al., 1997). All three nuclei project to the CeA (Pitkanen et al., 1997), which is the major output nucleus of the amygdala, and the CeA projects to various brain regions and controls many conditioned fear responses, including defensive behavior, autonomic arousal, hypoalgesia, reflex potentiation, and stress hormone release (Figure 1.1; (LeDoux et al., 1988, LeDoux, 2000)).

Many studies have used electrolytic lesions or pharmacological inactivation to examine the role of various amygaloid nuclei in auditory and contextual fear conditioning paradigms. A

few studies are highlighted below. One study found that pre-training bilateral electrolytic lesions to the amygdala, including the LA, BLA, and CE, disrupted conditioning to both the auditory CS

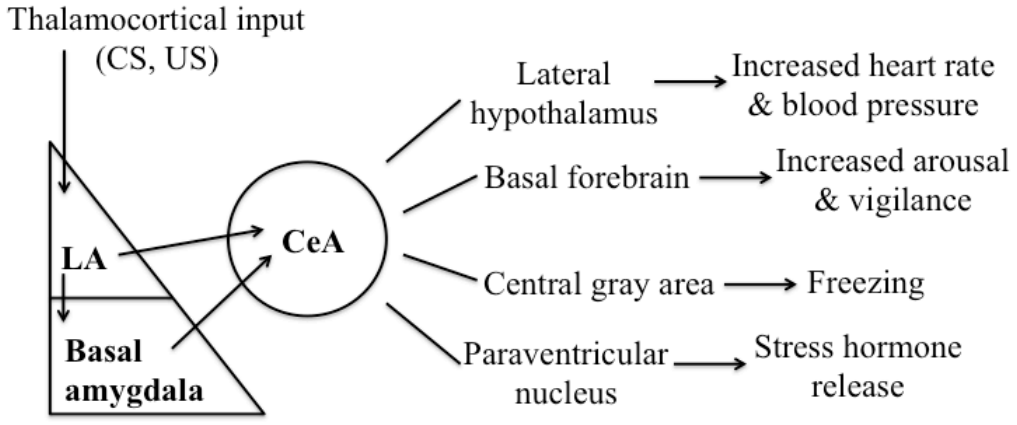

Figure 1.1 Amygdala circuitry and the fear response.

The LA receives information from the CS and the US, which projects to the BLA and BM (labeled basal amygdala here). All three nuclei project to the CeA, which projects to many brain regions to elicit conditioned fear responses. Figure modified from (Parsons and Ressler, 2013, Singewald et al., 2015).

and contextual stimuli (Phillips and LeDoux, 1992). A similar study used muscimol, a $GABA_A$ agonist, to temporarily inactivate LA, BLA, and BM. They found that an infusion of muscimol prior to training interfered with acquisition in both cued and contextual fear conditioning paradigms (Muller et al., 1997). In addition, rats infused with muscimol prior to testing had reduced freezing to both the tone and context, suggesting that LA and basal nuclei are critical for the expression of conditioned fear memories (Muller et al., 1997). A similar study focused more closely on the role of specific amygdaloid nuclei in contextual and auditory fear conditioning and found that a pre-training electrolytic lesion of the LA, CE, or basal nuclei (anterior portion only) reduced freezing to the CS in both paradigms (Goosens and Maren, 2001). Lesions in posterior portions of basal amygdala nuclei did not impair conditioned fear responses. Finally, studies have found that damage to outputs of the CE affect fear responses (LeDoux, 2000). CE projects to many regions, including the lateral hypothalamus, basal forebrain, central gray area, bed

nucleus of the stria terminalis, and paraventricular nucleus of the hypothalamus (LeDoux et al., 1988, Parsons and Ressler, 2013). Damage to the lateral hypothalamus affected conditioned mean arterial pressure response but not freezing behavior, while damage to the midbrain central gray region disrupted conditioned freezing but not the conditioned autonomic response (LeDoux et al., 1988). Damage to the bed nucleus of the stria terminalis affected neither conditioned freezing nor autonomic responses.

1.3.2 Medial prefrontal cortex (mPFC) interactions with the amygdala

The mPFC plays an important role in expression and extinction of conditioned fear. The mPFC of rodents can be subdivided into the prelimbic (PL) and infralimbic (IL) cortices, and these two regions have both been shown to project to the basal nuclei of the amygdala (Vertes, 2004). IL has also been found to project to the intercalated cells (ITCs), GABAergic neuron clusters found between the BLA and CeA, where disinhibition of the IL cortex resulted in significantly increased Fos-immunoreactive cells in ITCs (Berretta et al., 2005). Many studies have shown that these two regions of the mPFC play different roles in the regulation of fear, with the PL promoting the expression of conditioned fear and activation of the IL leading to extinction of conditioned fear (Figure 1.2; also reviewed in(Peters et al., 2009, Orsini and Maren, 2012)). These regions are reviewed in detail below, with an explanation of how each of these regions could be interacting with the amygdala to regulate conditioned fear.

Activity in the PL has been implicated with expression of conditioned fear in many studies. Recordings from neurons in the PL revealed that activity was positively correlated with freezing responses to the CS across both conditioning and extinction procedures (Burgos-Robles et al., 2009). In addition, this group also found significantly increased activity in PL neurons in

rats that failed to recall extinction. Pharmacological inactivation has demonstrated the PL is necessary for the expression of fear (Sierra-Mercado et al., 2011) in conditioned fear paradigms, and that the PL is not implicated in innate fear (Corcoran and Quirk, 2007). Microstimulation of the PL, but not the dorsal anterior cingulate or the medial precentral cortices, during CS presentations in the extinction training session resulted in significant increases in freezing during extinction training and extinction recall (Vidal-Gonzalez et al., 2006). Due to the role of the PL in fear expression, it is projected that neurons in the PL activate neurons in the basal amygdala, which activate neurons in the CeA to elicit conditioned fear responses (Vidal-Gonzalez et al., 2006, Peters et al., 2009, Orsini and Maren, 2012).

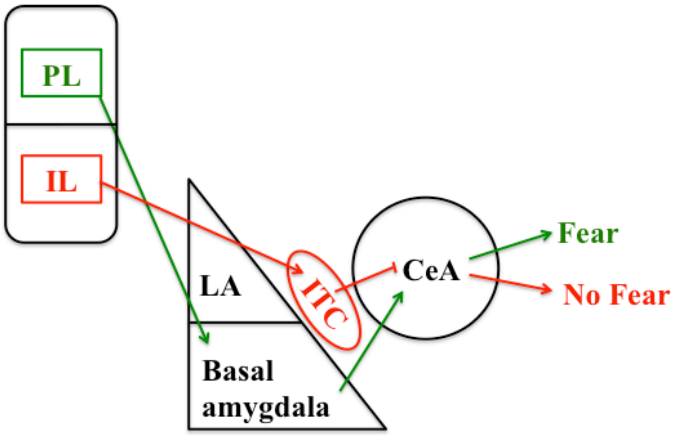

Figure 1.2 Role of PL and IL in expression of conditioned fear and fear extinction.

PL promotes expression of conditioned fear responses: PL projects to the basal amygdala, which activates neurons in the CeA. The CeA projects to many other brain regions to elicit fear. IL promotes extinction of conditioned fear: IL activates ITCs, which inhibit CeA activity, leading to decreased conditioned fear response. Figure modified from (Peters et al., 2009).

In contrast to neurons in the PL, neurons in the IL fire specifically during CS presentations during extinction recall, and change in IL tone response is negatively correlated with freezing to the tone during extinction recall (Milad and Quirk, 2002). Inactivation of the IL prior to extinction training with muscimol resulted in impaired extinction acquisition and recall

(Sierra-Mercado et al., 2011). In addition, NMDA receptor antagonists infused into the IL disrupted extinction recall (Burgos-Robles et al., 2007, Sotres-Bayon and Quirk, 2010). Many studies have shown that electrical stimulation of the IL during tone presentations of the extinction training session result in reduced freezing during extinction acquisition and extinction recall (Milad and Quirk, 2002, Milad et al., 2004, Vidal-Gonzalez et al., 2006). Since the IL has been shown to project to the ITCs (Berretta et al., 2005), which are necessary for fear extinction (Likhtik et al., 2008), it is postulated that IL activates ITCs. The ITCs then inhibit the CeA, and conditioned fear responses are abated (Vidal-Gonzalez et al., 2006, Peters et al., 2009, Orsini and Maren, 2012).

1.3.3 The role of the hippocampus in contextual learning

The hippocampus provides important contextual information in fear conditioning paradigms and projects to the LA, basal amygdala, and mPFC (Sotres-Bayon et al., 2006, Orsini and Maren, 2012). Bilateral lesions of the hippocampus have been shown to interfere with conditioning in contextual (Phillips and LeDoux, 1992) and trace (McEchron et al., 1998) rather than cued fear conditioning paradigms. Since the hippocampus does not play a critical role in cued fear conditioning, which is the only fear conditioning paradigm used in this thesis, it will not be covered in detail in this section. For a detailed review on the role of the hippocampus in conditioned fear paradigms, please refer to (Maren et al., 2013).

1.4 ESTRADIOL LEVELS REGULATE LEARNING AND MEMORY PROCESSES

1.4.1 Synthesis and actions of estrogens

The synthesis of estrogens occurs via a process called aromatization, where aromatase cleaves a carbon from an androgen precursor. Figure 1.3 shows the conversion of C19 androgen precursors to C18 estrogens. Aromatase is found throughout the body, including the placenta, ovarian granulosa cells, skin, and adipose tissue (Nelson and Bulun, 2001). Aromatase is also found in various cell types within the brain, including neurons and astrocytes. The production of estrogens in the brain, which occurs via aromatization of androgen precursors, have been shown to regulate reproductive behaviors, neurogenesis, and cognition (Garcia-Segura, 2008). As shown in Figure 1.1, there are three types of estrogens: estrone, estradiol, and estriol. Estrone and estriol are most prominent in post-menopausal and pregnant women, respectively, while estradiol levels are highest in pre-menopausal women who are not pregnant (Cui et al., 2013). Circulating estradiol levels in premenopausal women peak during the pre-ovulatory phase, and are approximately 19-140 pg/mL, 110-410 pg/mL, and 19-160 pg/mL during the follicular phase, pre-ovulatory phase, and luteal phase, respectively (Stricker et al., 2006). During classical (genomic) signaling, estradiol moves through the lipid bilayer to bind to intracellular estrogen receptors (ER; α and β subtypes). ERα and ERβ are found throughout the brain, in regions including the amygdala, hippocampus, hypothalamic nuclei, and prefrontal cortex (Shughrue et al., 1997a, Shughrue et al., 1997b, Cui et al., 2013). The estrogen bound ER complex then translocates to the nucleus, where it binds to the estrogen response element on DNA and initiates gene transcription and protein synthesis. Estrogen can also signal through membrane-bound receptors (e.g. G-protein-coupled ERs) to initiate signaling cascades, which leads to more rapid physiological responses.

For a more detailed description of estrogen signaling pathways, please review (Heldring, 2007, Cui et al., 2013, Frick et al., 2015).

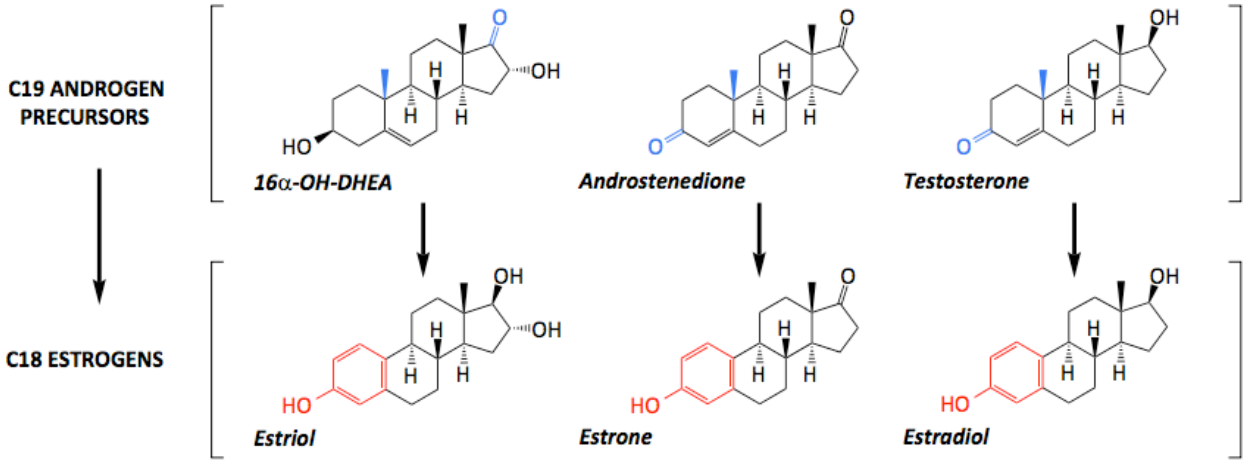

Figure 1.3 Estrogen synthesis from androgen precursors.

Aromatization of androgen precursors to estrogens. Three types of estrogens exist, but the focus of this document will be on estradiol, which is the most prevalent estrogen in premenopausal women who are not pregnant. Figure from (Azcoitia et al., 2011).

1.4.2 Estradiol affects the consolidation of fear extinction memory

Although women are nearly twice as likely to develop an anxiety disorder, such as PTSD, compared to men (Kessler et al., 1995), there are relatively few studies in the literature that focus on better understanding the mechanisms underlying fear extinction in females (Lebron-Milad and Milad, 2012, Maeng et al., 2015). However, recent studies have indicated that levels of estradiol on the day of extinction training affect the consolidation of fear extinction memories. Figure 1.4A depicts how levels of estradiol change across the menstrual cycle in women. In one study, healthy men and women were compared in a two-day fear conditioning paradigm, where skin conductance response was used to measure physiological arousal. While no differences in

fear extinction consolidation were detected when men were compared to women, distinct differences were found when women were separated into high or low levels of estradiol at the time of extinction training (Milad et al., 2010). Women with low estradiol levels during extinction training had poor extinction consolidation and significantly increased fear during extinction recall compared to women with high estradiol levels and men (Milad et al., 2010). However, when women in the early follicular (low estradiol) phase received an oral estrogen tablet 30 minutes prior to extinction training, their extinction consolidation significantly improved, resulting in significantly reduced fear during extinction recall compared to women with low estradiol levels that were treated with placebo (Graham and Milad, 2013). Similarly, studies of healthy women taking combined monophasic hormonal contraceptives (HC) found that these women have poor extinction consolidation and significantly elevated fear during extinction recall compared to naturally cycling healthy females with high circulating levels of estradiol (Graham and Milad, 2013). Studies examining changes in blood oxygen level-dependent signal found that women with high estradiol levels during extinction had significantly higher activation in the ventromedial prefrontal cortex (vmPFC), which has previously been positively correlated with extinction retention (Milad et al., 2007), and the amygdala during extinction recall compared to women with low levels of estradiol (Zeidan et al., 2011). Taken together, these studies suggest that low estradiol levels on the day of extinction training impair extinction consolidation, resulting in increased fear and reduced activation in the vmPFC and amygdala during extinction recall compared to women with high estradiol levels. These findings indicate that interventions for PTSD, such as exposure therapy, should occur during times when patients have high circulating levels of estradiol.

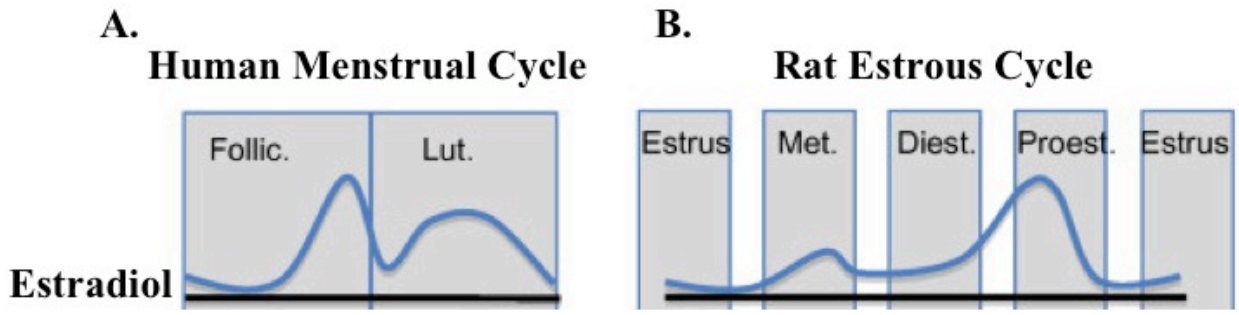

Figure 1.4 Levels of estradiol change across the menstrual cycle in women and estrous cycle of female rodents.

A.) Estradiol in females is low at the beginning of the follicular phase and peaks during the late follicular phase. Estradiol levels drop rapidly and peak again in the middle of the luteal phase. B.) Estradiol levels reach their peak during the proestrus phase of the estrous cycle in rodents. Figure from (Lebron-Milad and Milad, 2012).

Similarly to humans, several studies have shown that naturally cycling female rats with low circulating estradiol levels (metestrus phase) during extinction training have poor extinction consolidation and increased fear during extinction recall compared to naturally cycling female rats with high circulating estradiol levels (proestrus phase) (Milad et al., 2009a, Zeidan et al., 2011). Figure 1.4B shows how levels of estradiol change across the estrous cycle in female rodents. Deficits in extinction consolidation are also found when female rats are treated with HC levonorgestrel, a progestin commonly used in HCs that has been shown to lower levels of estradiol (Graham and Milad, 2013), 4 days prior to and throughout the Pavlovian fear conditioning paradigm (Graham and Milad, 2013). Impairments in extinction consolidation can be eliminated by administering systemic estradiol 30 minutes before (Milad et al., 2009a) or immediately after (Zeidan et al., 2011) the extinction training session in naturally cycling female rats with low estradiol levels. Additionally, naturally cycling metestrus females treated with an ERβ agonist, but not an ERα agonist, 30 minutes prior to extinction training did not have impairments in extinction consolidation (Zeidan et al., 2011). Surprisingly, both ERα and ERβ

agonists were able to reduce the deficits in extinction consolidation in female rats treated with HC, which may be due to changes in ER expression in HC-treated rats versus freely cycling rats that have not been fully investigated (Graham and Milad, 2013). Interestingly, estrogen levels during extinction training are also important for males, as blockade of estradiol synthesis from testosterone 30 minutes before or immediately following extinction training results in extinction consolidation deficits (Graham and Milad, 2014). Taken together, these findings, which are summarized in Table 1.1, suggest that estradiol is needed in both sexes to facilitate the consolidation of extinction and prevent deficits in the recall of extinction memory. Both preclinical and clinical evidence suggest that high estradiol levels during extinction training are necessary for consolidation of extinction and good recall of extinction memory; however, the mechanism by which this occurs remains unclear.

Table 1.1 Summary of how estradiol levels affect fear extinction consolidation.

Summary of clinical and pre-clinical findings examining how estradiol levels during extinction training affect fear during extinction recall.

Groups	Hormonal Manipulation	Fear Levels During Extinction Recall	Reference
Men; Women with ⇑ or ⇓ estradiol levels	Naturally cycling women had ⇑ or ⇓ levels of estradiol during extinction training	Women with ⇓ estradiol had ⇑ fear vs. women with high estradiol and men	(Milad et al., 2010)
Women with ⇓ estradiol	Women with ⇓ estradiol treated with oral estrogen tablet or placebo 30 minutes before extinction training	Women with ⇓ estradiol that were treated with estrogen tablet had ⇓ fear vs. placebo-treated women	(Graham and Milad, 2013)
HC-treated women or naturally cycling women	Women either were taking HC or were naturally cycling	HC-treated women have ⇑ fear vs. naturally cycling women with high estradiol	(Graham and Milad, 2013)
Naturally cycling female rats	Naturally cycling female rats have ⇑ or ⇓ estradiol levels during extinction training	Female rats with ⇓ estradiol levels have ⇑ fear vs. females with ⇑ estradiol	(Milad et al., 2009a, Zeidan et al., 2011)
Naturally cycling female rats with ⇓ estradiol	Female rats with ⇓ estradiol treated with estradiol, ERβ agonist, or vehicle 30 minutes before or immediately after extinction training	Females with ⇓ estradiol treated with estradiol or ERβ agonist had ⇓ fear vs. vehicle-treated ⇓ estradiol females	(Milad et al., 2009a, Zeidan et al., 2012)
HC- and vehicle-treated female rats	Rats were chronically injected with HC or vehicle	HC-treated females have ⇑ fear vs. vehicle-treated ⇑ estradiol females	(Graham and Milad, 2013)
HC-treated female rats	HC-treated females were given ERα agonist, ERβ agonist, or vehicle before extinction training	HC females treated with an ERα or ERβ agonist had ⇓ fear vs. HC vehicle-treated females	(Graham and Milad, 2013)
Male rats	Treated with aromatase inhibitor or vehicle 30 minutes before or immediately after extinction training	Males treated with aromatase inhibitor had ⇑ fear vs. vehicle-treated males	(Graham and Milad, 2014)

1.4.3 How does ovariectomy (OVX) impact fear behavior?

While Milad and colleagues have suggested that low estradiol levels, rather than progesterone, during extinction training result in impaired extinction consolidation and increased fear during recall (Milad et al., 2010), the cyclic nature of ovarian hormones can often make it difficult to determine the exact role that gonadal hormones have on behavior. One way to circumvent this problem is to surgically remove the ovaries and add back the hormones of interest in a controlled way to physiologically mimic those of naturally cycling females during a particular phase of the estrous cycle. Given the recent studies surrounding gonadal hormones and fear extinction consolidation, it would seem as though there would be many studies that would have already addressed this particular question. However, most studies that examine the role of OVX in females have only examined the effect of gonadal hormones on fear acquisition in a cued fear conditioning paradigm, and the majority of studies looking at fear extinction do so using a contextual fear conditioning paradigm. Although results from these studies, which are summarized in Table 1.2 and described in detail below, are somewhat mixed, they further support the findings from previous studies indicating that hormones do play a role in fear-related behaviors.

A number of studies have examined sex differences in the contextual fear conditioning paradigms using OVX female rats. For instance, one study found no significant differences during the conditioning session of the contextual fear conditioning paradigm between males, sham-operated females, and OVX females (Gupta et al., 2001). However, during the context extinction test, sham-operated females exhibited significantly lower freezing levels during

extinction days 3 and 4 compared to males and OVX females (Gupta et al., 2001). This group also found that administering estradiol to OVX females in such a way that it recapitulates the hormonal pattern of normally cycling female rats results in significantly less freezing during a context extinction test compared to OVX vehicle-treated females (Gupta et al., 2001). Another study found that systemic treatment with estradiol or progesterone, which closely mimicked levels found in normally cycling proestrus or estrus females, had no effect on freezing behavior 24 hours following contextual fear conditioning (Chang et al., 2009). However, during contextual fear extinction, which occurred 4 consecutive days following the 24-hour test, OVX females treated with estradiol displayed significantly less freezing compared to OVX vehicle- and progesterone-treated females (Chang et al., 2009). Systemic administration or infusion of ERβ agonist into the dorsal hippocampus of OVX females prior to extinction significantly reduces freezing during the contextual fear extinction session, suggesting that the effects of estradiol on fear extinction are mediated by ERβ rather than ERα (Chang et al., 2009). While some studies in mice report opposite findings, with OVX estradiol-treated females freezing more to presentations of the CS in a novel environment (Morgan and Pfaff, 2001, Jasnow et al., 2006) or to a context 24 hours after conditioning (Jasnow et al., 2006), this could be due to the use of different estradiol doses and treatment timeframes or differences in fear conditioning paradigms.

Although there were no studies in the literature examining sex differences during fear extinction consolidation at the time the experiments in this thesis were performed, there has been a study published recently that examines the effect of gonadal hormones on fear extinction consolidation in a cued fear conditioning paradigm. This study administered estradiol and progesterone similarly to another study testing the effects of gonadal hormones on dendritic spine density in CA1 hippocampal pyramidal cells (Woolley and McEwen, 1993), where

hormone levels used were similar to those of naturally cycling female rats during proestrus. While administration of estradiol had no effect during extinction training, OVX estradiol-treated females exhibited significantly less freezing during extinction recall compared to OVX vehicle-treated females (Graham and Daher, 2016). Administration of progesterone in OVX estradiol-treated females resulted in a further reduction in freezing during extinction recall if it was given 6 hours prior to extinction training but increased freezing to the level of OVX vehicle-treated females if it was administered 24 hours prior to extinction training (Graham and Daher, 2016). Finally, this group found that blocking progesterone receptor activation during proestrus prevented the deficit in fear extinction consolidation in females that had extinction training during the metestrus phase of the estrous cycle (Graham and Daher, 2016). This is the first study that attempts to test the role that estradiol and progesterone have during fear extinction consolidation using OVX females. Consistent with studies from Milad and colleagues in naturally cycling women and female rats, these results imply that estradiol is responsible for enhancing fear extinction consolidation in females during proestrus, while progesterone seems to impair fear extinction consolidation when extinction training occurs during metestrus.

Table 1.2 Summary of fear conditioning studies in OVX females.

This table summarizes results from studies done in OVX female rats in cued and contextual fear conditioning paradigms.

Groups	Type of Fear Conditioning Paradigm	Results	Reference
Males, sham-operated females, OVX females	Contextual	Sham-operated females extinguished fear at faster rate than males and OVX females	(Gupta et al., 2001)
OVX females treated with estradiol or vehicle	Contextual	OVX estradiol-treated females had ⇓ fear during extinction vs. OVX vehicle-treated females	(Gupta et al., 2001)
OVX females treated with estradiol, progesterone, or vehicle	Contextual	OVX estradiol-treated females had ⇓ fear during fear extinction	(Chang et al., 2009)
OVX females treated with estradiol or vehicle	Cued	OVX estradiol-treated females displayed ⇑ fear to presentations of CS in a novel environment	(Morgan and Pfaff, 2001, Jasnow et al., 2006)
OVX females treated with estradiol or vehicle	Contextual	OVX estradiol-treated females displayed ⇑ fear to conditioning chamber 24 hours after fear conditioning	(Jasnow et al., 2006)
OVX females treated with estradiol or vehicle	Cued	OVX estradiol-treated females had ⇓ fear during extinction recall vs. OVX vehicle-treated females	(Graham and Daher, 2016)
OVX estradiol-treated females also treated with progesterone 6 hours or 24 hours prior to extinction training	Cued	Progesterone treatment given 6 hours prior to extinction training further ⇓ fear during extinction recall in estradiol-treated OVX females, but ⇑ fear during extinction recall if given 24 hours prior to extinction training	(Graham and Daher, 2016)

1.5 RENIN ANGIOTENSIN SYSTEM (RAS)

1.5.1 The RAS – Discovery, hypertensive and anti-hypertensive axes, and main functions

Components of the RAS were first discovered in the periphery in the 1940s (Braun-Menendez et al., 1940, Page and Helmer, 1940). In the periphery, angiotensinogen is cleaved by renin to form the inactive precursor, angiotensin I (Ang I). Ang I is converted to angiotensin II (Ang II) via the angiotensin converting enzyme (ACE), and Ang II binds to the angiotensin II type I receptor (AT1R), which mediates the hypertensive effects of the RAS. AT1R are located throughout the brain, kidney, adrenal gland, and heart (Allen et al., 2000). In addition, both Ang I and Ang II can be converted to angiotensin-(1-7) (Ang 1-7), which acts on the Mas receptors (MasR) and is thought to mediate the antihypertensive effects of the RAS through the bradykinin-nitric oxide pathway (Iwai and Horiuchi, 2009, Xu et al., 2011). Since its initial discovery, many studies have found that all components of the RAS in the central nervous system (Bunnemann et al., 1992, McKinley et al., 2003, Saavedra, 2005), though the enzymatic mechanism for the formation of Ang II is unclear, and requires further elucidation (Saavedra, 2005). While the RAS has been widely studied in terms of blood pressure regulation and cardiovascular function, many recent studies have identified the RAS as a mediator of the stress response and stress-related pathologies (Yang et al., 1996, Pavlatou et al., 2008, Krause et al., 2011, Saavedra et al., 2011, Khoury et al., 2012, Saavedra, 2012, Marvar et al., 2013, Hurt et al., 2015, Nylocks et al., 2015, Wang et al., 2016, de Kloet et al., 2017). The sections below will highlight how blockade of

AT1R affects anxiety- and fear-related behavior and discuss a major modulator of the RAS -- estradiol.

1.6 BENEFICIAL EFFECTS OF NEGATIVE REGULATION OF THE RAS

1.6.1 Anxiolytic and cognitive effects of AT1R antagonists

Many studies have found that treatment with an AT1R antagonist reduces anxiety- and depressive-like behavior in rodents. Anxiolytic actions of AT1R antagonists were first presented by Barnes and colleagues, who found that mice orally treated with AT1R antagonist losartan 45 minutes prior to testing had significantly increased latency of moving from the aversive light to the dark chamber in the light/dark aversion test and increased number of rears performed in the light chamber (Barnes et al., 1990). Oral administration of losartan one hour before elevated plus maze (EPM) testing resulted in significantly increased time spent in the open arms and increased open arm entries, where no significant effects of losartan were observed on locomotor behavior (Kaiser et al., 1992). Similar effects in the EPM were found when rats were treated chronically with AT1R antagonist candesartan (Saavedra, 2005, Saavedra et al., 2006). In the forced swim test, which is commonly used for the evaluation of antidepressant drugs, male mice subcutaneously treated with losartan 1 hour prior to testing spent significantly less time being immobile, indicating that losartan possesses antidepressant properties (Gard et al., 1999). Finally, in male bred low responder rats, chronic treatment with an AT1R antagonist was able to reverse anhedonic behavior in rats exposed to unpredictable chronic mild stress to the level of non-stressed controls (Stedenfeld, 2011; unpublished).

AT1R antagonists have also been shown to improve cognitive function in human studies. In a population of healthy young adults, losartan was found to improve memory in a prospective memory task, and it also reversed scopolamine-induced deficits in other tests of memory (Mechaeil R, 2007). In elderly patients with hypertension, losartan treatment improved word list memory and recall (Fogari et al., 2003). In 30-73 year old patients with hypertension, 26 months of losartan treatment significantly improved cognitive functioning and quality of life (Tedesco et al., 1999). The improvement in quality of life of hypertensive patients taking AT1R antagonists has also been reported in other studies (Weber, 2005).

1.6.2 Negative regulation of the RAS affects fear behavior

In addition to anxiolytic properties, AT1R antagonists have been found to reduce PTSD symptoms and enhance fear extinction consolidation in males. Highly traumatized patients taking ACE inhibitors or AT1R antagonists, which are both commonly prescribed for hypertension, were found to have reduced PTSD symptom severity, lower hyperarousal scores, and decreased intrusive thoughts in a retrospective study (Khoury et al., 2012). Blood pressure medications that act independently of the RAS had no effect on these measures. Similarly, treatment with losartan, an AT1R antagonist, prior to extinction training enhanced extinction consolidation and decreased freezing during extinction recall in male mice (Marvar et al., 2013). No effect of losartan on blood pressure or baseline anxiety was found. In an effort to explain how AT1R antagonists are modulating fear behavior, the Ressler lab explored how the deletion of AT1R from corticotropin-releasing factor (CRF) expressing cells would affect fear behavior, since previous studies report that the CRF response to isolation stress is blunted by the inhibition of central AT1Rs (Armando et al., 2007). Mice with the AT1R knockout in CRF-expressing cells

were found to have reduced fear during the extinction training session (Hurt et al., 2015). Knockout of AT1R in CRF-expressing cells did not lead to alterations in baseline blood pressure, heart rate, anxiety-like behavior in the EPM, or locomotor activity in the open field compared to wildtype mice (Hurt et al., 2015). Thus, activity of AT1Rs on CRF-expressing cells may be acting to modulate the expression of conditioned fear, but further study is needed. Together, these studies suggest that negative regulation of the hypertensive axis of the RAS reduces symptoms associated with PTSD and enhances extinction consolidation in male mice.

1.7 ESTRADIOL MODULATION OF RAS COMPONENTS

Many studies confirm that estradiol downregulates several components of the RAS (reviewed in (Fischer et al., 2002)). This section will highlight how estradiol modulates the hypertensive components of the RAS, ultimately showing that estradiol suppresses the RAS (for an overview, refer to Figure 1.5).

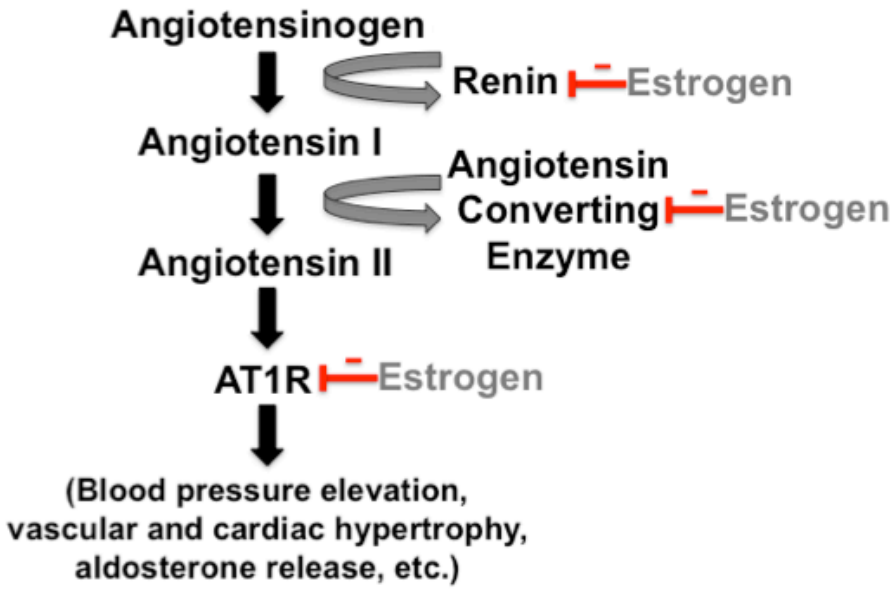

Figure 1.5 Estrogen regulation of the RAS.

Estrogen downregulates components of the RAS, including renin, ACE, and AT1R. Modified from (Fischer et al., 2002).

1.7.1 AT1Rs are downregulated by estradiol

A few studies have shown that there are changes in the concentration of AT1Rs across the estrous cycle in some tissues using quantitative autoradiography. For instance, in the anterior pituitary lobe, freely cycling female rats in metestrus (lowest levels of estradiol) had significantly elevated AT1R ligand binding compared to rats in proestrus (highest levels of estradiol) (Seltzer et al., 1992). Similar results were found in the dorsomedial arcuate nucleus, with significantly higher AT1R ligand binding detected in estrus (low estradiol state) compared to females in proestrus that had the lowest levels of AT1R ligand binding (Seltzer et al., 1993). Thus, freely cycling rats in the proestrus phase of the estrus cycle, characterized as a high estradiol state, have significantly reduced AT1R ligand binding compared to rats in low estradiol states in the anterior pituitary gland and the dorsomedial arcuate nucleus.

While estrous cycle regulation of AT1Rs has not been extensively explored, many studies have looked at the effects of OVX on AT1R levels in both central and peripheral tissues of female rats. AT1b receptor mRNA levels were significantly reduced in the pituitary gland of OVX estradiol-treated females compared to OVX vehicle-treated females (Kakar et al., 1992). Another study reported significant reductions in mRNA levels of AT1a and AT1b receptor subtypes in the pituitary gland of OVX estradiol-treated females (Kisley et al., 1999, Wu et al., 2003a). Similar reductions in AT1R mRNA were found in hypothalamic-thalamic-septal tissue samples of OVX estradiol-treated females (Kisley et al., 1999). OVX estradiol-treated females are reported to have reductions in AT1R ligand binding in the pituitary gland, subfornical organ (SFO), paraventricular nucleus of the hypothalamus (PVN), median preoptic nucleus (MnPO), and vascular organ of the lamina terminalis compared to OVX vehicle-treated females (Seltzer et al., 1992, Kisley et al., 1999, Dean et al., 2005). Peripherally, AT1R mRNA levels in aortic tissue of OVX females were significantly elevated compared to sham operated controls, and treatment with estrogen in OVX females was found to reverse this effect (Nickenig et al., 1998). Similar results in AT1R mRNA levels were found in vascular smooth muscle cells in culture following a 12-hour incubation with estradiol (Nickenig et al., 1998). Estrogen treatment in OVX females has been found to reduce AT1R ligand binding in the adrenal glands, heart, kidney, and abdominal aorta (Wu et al., 2003b, Dean et al., 2005). While the effect of estradiol on AT1R levels is dose dependent, we can conclude that physiological levels of estradiol typically reduce AT1R mRNA expression and ligand binding in many tissues. These results are summarized in Table 1.3.

Table 1.3 Summary of estradiol modulation of AT1Rs.

This table summarizes results from studies examining how estradiol affects levels of the AT1R.

Groups	Region(s)	Results	Reference
Metestrus vs. Proestrus females	Anterior pituitary lobe	Metestrus females had ⇑ AT1R ligand binding vs. proestrus females	(Seltzer et al., 1992)
Estrus vs. Proestrus females	Dorsomedial arcuate nucleus	Females in estrus had ⇑ AT1R ligand binding vs. proestrus females	(Seltzer et al., 1993)
OVX estradiol-treated and OVX vehicle-treated females	Pituitary gland; hypothalamic-thalamic-septal tissue samples	AT1a and AT1b mRNA levels ⇓ in OVX estradiol-treated females	(Kakar et al., 1992, Kisley et al., 1999, Wu et al., 2003a)
OVX estradiol-treated and OVX vehicle-treated females	Pituitary gland, SFO, PVN, MnPO, vascular organ of the lamina terminalis	OVX estradiol-treated females have ⇓ AT1R ligand binding vs. OVX vehicle-treated females	(Seltzer et al., 1992, Kisley et al., 1999, Dean et al., 2005)
OVX estradiol-treated and OVX vehicle-treated females	Adrenal glands, heart, kidney, abdominal aorta	OVX estradiol-treated females have ⇓ AT1R ligand binding vs. OVX vehicle-treated females	(Wu et al., 2003b, Dean et al., 2005)

1.7.2 Other RAS components modulated by estradiol

While fairly consistent findings on the effect of estradiol on AT1R levels have been reported, the effects of estradiol on other components of the RAS, which are summarized in Table 1.4, are mixed, especially in the clinical literature. Studies in postmenopausal women have shown that treatment with estrogen results in increased levels of circulating angiotensinogen (Seely et al., 2004, Harvey et al., 2005), also referred to as renin substrate, which is predominately synthesized in the liver. mRNA levels of angiotensinogen were also found to be increased in the pituitary gland of OVX females treated with estradiol (Kisley et al., 1999). These findings are

not surprising, as synthesis of angiotensinogen is stimulated by an estrogen response element in its gene promoter (Clauser et al., 1989). Postmenopausal women taking an estrogen/progestagen hormone replacement therapy were found to have reduced ACE activity following 6 months of treatment compared to controls (Proudler et al., 1995). Similar findings were reported in OVX female rats, with estradiol treatment reducing ACE mRNA concentrations in the kidney, lung, and aorta (Gallagher et al., 1999). OVX estradiol-treated female rats also have reduced ACE binding density in the anterior pituitary, SFO, PVN, and MnPO (Seltzer et al., 1992, Dean et al., 2005). Finally, ACE activity is reportedly reduced in OVX estradiol-treated females in the anterior pituitary, kidney, and lung (Seltzer et al., 1992, Gallagher et al., 1999). Thus, estradiol treatment has been shown to upregulate angiotensinogen and downregulate ACE across many studies.

There is some uncertainty in the literature on how hormone levels regulate Ang II and other RAS components. One study reported reductions in circulating Ang II in intact female rats compared to males (Pendergrass et al., 2008). Another study found reductions in adrenal Ang II levels of OVX estradiol-treated females compared to OVX vehicle-treated females (Wu et al., 2003b). Young, healthy combined oral contraceptive users, who had low estradiol levels that are similar to those found in the early follicular phase (Mishell et al., 1972), were found to have increased baseline Ang II, aldosterone, and Ang I (Kang et al., 2001, Ahmed et al., 2004). While these studies suggest an inverse relationship between estradiol and Ang II, other studies have reported a direct relationship between estradiol and Ang II. One study found that women taking a combined oral contraceptive had significant reductions in Ang II levels (Wiegratz et al., 2003). In freely cycling female rats, central Ang II levels were significantly elevated in proestrus compared to metestrus (Phillips et al., 1995). In normotensive postmenopausal women, treatment

with estradiol has been found increase levels of circulating Ang II (Seely et al., 2004, Harvey et al., 2005). Because the literature regarding the relationship between estradiol and Ang II is mixed, more work is needed to clarify how estradiol levels affect Ang II.

Table 1.4 Summary of estradiol modulation of RAS components.

This table summarizes results from studies examining how estradiol affects components of the RAS.

Groups	Component of RAS	Results	Reference
Postmenopausal women	Angiotensinogen	Treatment with estrogen results in ⇑ circulating levels	(Seely et al., 2004, Harvey et al., 2005)
OVX estradiol-treated and OVX vehicle-treated females	Angiotensinogen	mRNA levels ⇑ in pituitary of OVX estradiol-treated females	(Kisley et al., 1999)
Postmenopausal women	ACE	Women on hormonal replacement therapy have ⇓ ACE activity	(Proudler et al., 1995)
OVX estradiol-treated and OVX vehicle-treated females	ACE	ACE activity ⇓ in OVX estradiol-treated females in anterior pituitary, kidney, and lung	(Seltzer et al., 1992, Gallagher et al., 1999)
OVX estradiol-treated and OVX vehicle-treated females	ACE	mRNA concentrations in kidney, lung, and aorta ⇓ in OVX estradiol-treated females	(Gallagher et al., 1999)
OVX estradiol-treated and OVX vehicle-treated females	ACE	Binding density in anterior pituitary, SFO, PVN, MnPO is ⇓ in OVX estradiol-treated females	(Seltzer et al., 1992, Dean et al., 2005)
Intact male vs. intact female rats	Ang II	⇓ circulating Ang II found in intact females	(Pendergrass et al., 2008)
OVX estradiol-treated and OVX vehicle-treated females	Ang II	⇓ adrenal Ang II in OVX estradiol-treated females	(Wu et al., 2003b)
Women taking combined oral contraceptives (with low estradiol levels) vs. non-users	Ang II, Ang I, aldosterone	Oral contraceptive users had ⇑ Ang II, Ang I and aldosterone vs. non-users	(Kang et al., 2001, Ahmed et al., 2004)
Women taking oral contraceptives vs. non-users	Ang II	Oral contraceptive users had ⇓ levels vs. non-users	(Wiegratz et al., 2003)
Freely cycling female rats	Ang II	Levels ⇑ in proestrus vs. metestrus	(Phillips et al., 1995)
Postmenopausal women	Ang II	Estradiol-treated women had ⇑ circulating Ang II	(Seely et al., 2004, Harvey et al., 2005)

1.8 GOALS AND RELEVANCE OF THIS DISSERTATION

Previous studies have demonstrated that high estradiol levels during extinction training lead to enhanced extinction consolidation and reduced fear during extinction recall (Milad et al., 2009a, Milad et al., 2010, Zeidan et al., 2011, Lebron-Milad et al., 2012b, Graham and Milad, 2013, 2014). However, the mechanism by which this occurs has not been identified, and the major goal of this thesis was to test how estradiol modulates fear memories. Because the RAS has recently been implicated in the stress response and stress related pathologies (Saavedra et al., 2006, Saavedra and Benicky, 2007, Saavedra et al., 2011, Saavedra, 2012, de Kloet et al., 2017), we focused on how estradiol interactions with the RAS may be regulating fear (refer to Figure 1.6 for an overview of what is known in the literature and our hypothesis). Previous studies have demonstrated that patients taking AT1R antagonists for hypertension have reduced PTSD symptoms (Khoury et al., 2012) and systemic administration of an AT1R antagonist prior to extinction training resulted in enhanced extinction consolidation in male mice (Marvar et al., 2013). Estrogen is a well known modulator of the RAS, downregulating many components including renin, ACE, and AT1R (Nickenig et al., 1998, Brosnihan et al., 1999, Gallagher et al., 1999, Fischer et al., 2002, Wu et al., 2003a, Wu et al., 2003b, Dean et al., 2005, Xu et al., 2008). Taking all of this together, our hypothesis was that estradiol is modulating the RAS to affect fear extinction consolidation. We predict that estradiol is downregulating components of the RAS to improve fear extinction consolidation and reduce fear during extinction recall. In **Chapter 2**, we test whether AT1R antagonist losartan can enhance extinction consolidation in HC-treated female rats with low estradiol levels and how AT1R expression and binding differ between females with high and low levels of estradiol. While estradiol modulation of fear extinction memories has been extensively tested, surprisingly, during the time these studies were being

performed, no previous studies had examined the effect of OVX on fear extinction consolidation in a cued fear conditioning paradigm. Thus, in **Chapter 3** we test whether OVX impairs fear extinction consolidation, and if losartan can improve fear extinction consolidation in OVX females. In addition, we further test how various hormone manipulations affect components of the RAS, which have not been fully characterized.

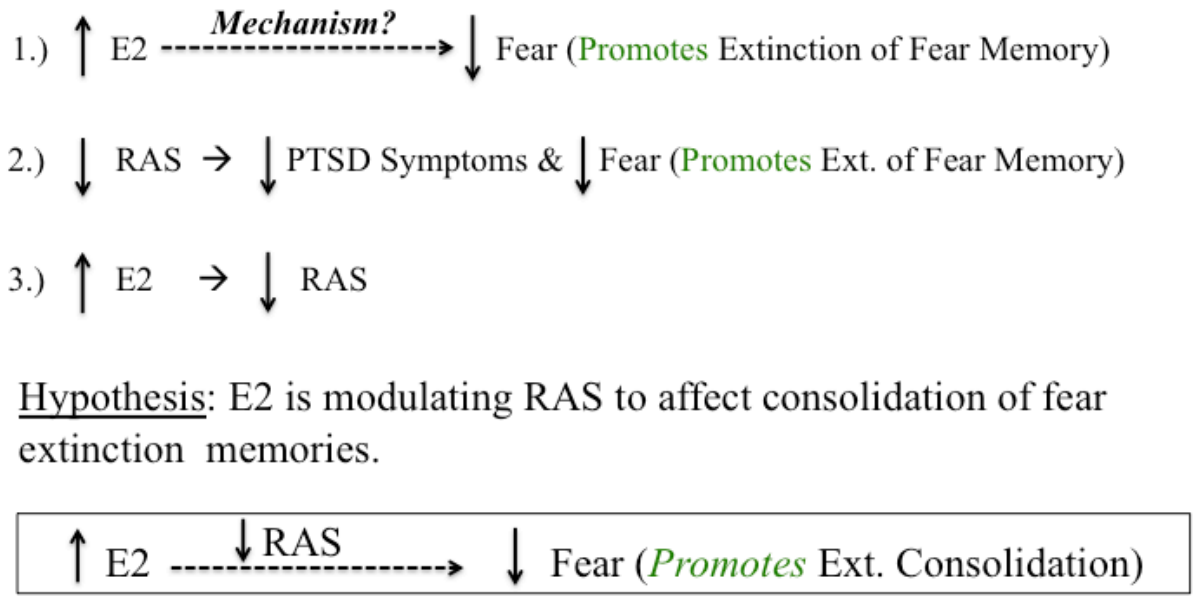

Figure 1.6 How are fear memories modulated in females?

Diagram of our hypothesis and prediction, based on previous findings in the literature. Previous studies have demonstrated that high estradiol (abbreviated E2 in this figure) levels during extinction training enhance fear extinction consolidation, but the mechanism has not been identified. Inhibiting the RAS, which has been implicated in the stress response and stress related pathologies, has been found to reduce symptoms of PTSD and enhance fear extinction consolidation in male mice. Since estradiol is a well-known modulator of the RAS, downregulating many of its components, our hypothesis is that estradiol is modulating the RAS to affect the consolidation of fear extinction memories. Specifically, we predict that having high levels of estradiol during extinction training will downregulate components of the RAS and enhance extinction consolidation, reducing fear during extinction recall.

2.0 AT1R ANTAGONIST LOSARTAN RESCUES EXTINCTION CONSOLIDATION DEFICIT IN FEMALES WITH LOW ESTRADIOL LEVELS

2.1 INTRODUCTION

Posttraumatic stress disorder (PTSD) is a devastating illness, with an estimated lifetime prevalence of 7.8% in the United States (Kessler et al., 1995). Although women are more than twice as likely to be diagnosed with PTSD and other anxiety disorders compared to men (Kessler et al., 1995), few studies focus on better understanding mechanisms underlying fear extinction in women (Lebron-Milad and Milad, 2012). Examining the effect of sex on treatment (exposure therapy) outcome in human subjects has been difficult due to differences between treatment studies (Foa, 2000). However, many studies report that women are able to recall an emotional event more readily than men (Seidlitz and Diener, 1998, Davis, 1999, Bauer et al., 2003, Rubin et al., 2008), suggesting that hormone levels may be contributing to the formation of memories for emotional events. Importantly, estradiol levels are shown to affect the consolidation of fear extinction memories. Specifically, low estradiol levels during extinction training lead to deficits in the consolidation of fear extinction memory, resulting in persistent fear expression during extinction recall in women and female rats (Milad et al., 2010, Zeidan et al., 2011, Lebron-Milad et al., 2012b, Graham and Milad, 2013, Cover et al., 2014, Hwang et al., 2015, Maeng et al., 2017). Similar effects are evident following systemic inhibition of aromatase, the enzyme that

converts testosterone into estradiol, prior to or immediately after extinction training in male rats, which suggests that estradiol is critical for extinction consolidation in both sexes (Graham and Milad, 2014). However, the precise mechanism for this phenomenon is currently unknown.

One potential target in understanding how estradiol is modulating the consolidation of fear extinction memories is to examine the renin angiotensin system (RAS), which plays a critical role in modulating sympathetic nervous system tone and cardiovascular health. A retrospective study examining a highly traumatized population of patients found that those who were taking blood pressure medications that act on the RAS had reduced PTSD symptom severity, lower hyperarousal, and decreased intrusive thoughts compared to patients taking blood pressure medications that act independently of the RAS (Khoury et al., 2012). In addition, male mice treated with angiotensin II type I receptor (AT1R) antagonist losartan prior to extinction training had decreased fear during extinction recall compared to vehicle-treated males (Marvar et al., 2013). Thus, drugs that negatively regulate the hypertensive axis of the RAS reduce symptoms of PTSD in human subjects and enhance fear extinction consolidation in male mice. Notably, estrogen downregulates the hypertensive axis of the RAS, reducing AT1R expression and enzymes involved in the synthesis of angiotensin II (Ang II), the endogenous ligand for the AT1R (Nickenig et al., 1998, Brosnihan et al., 1999, Fischer et al., 2002). Reducing circulating estradiol levels in female rodents via ovariectomy (OVX) significantly increases AT1R binding in both central and peripheral tissues, which is rescued by estradiol treatment (Nickenig et al., 1998, Wu et al., 2003a, Wu et al., 2003b, Dean et al., 2005, Rogers et al., 2007). Although many studies examine the effect of estradiol on Ang II levels, the results are mixed (Zakheim et al., 1976, Kang et al., 2001, Zheng et al., 2002, Wiegratz et al., 2003, Reyes-Engel et al., 2006, Sullivan et al., 2007, Pendergrass et al., 2008, Xu et al., 2008, O'Hagan et al., 2012), suggesting

that estradiol may be differentially regulating Ang II and AT1R levels. Though estradiol regulation of the RAS has been extensively studied, it is unclear if this interaction is involved in regulating the consolidation of fear extinction memories, leaving this as an important and open area of investigation.

Since both estradiol and the RAS are involved in the consolidation of fear extinction memories, and estradiol is known to downregulate the RAS, we hypothesize that estradiol is modulating the hypertensive axis of the RAS to affect the consolidation of fear extinction memory. To test this hypothesis, freely cycling female rats with high estradiol levels were compared to those with low levels of estradiol to test the effect of AT1R antagonist, losartan, on extinction consolidation. Further, the precise mechanism by which estradiol is interacting with the RAS to enhance the consolidation of extinction memory was explored by measuring central AT1R levels and by determining concentration of circulating Ang II in serum of female rats with high and low levels of estradiol.

2.2 MATERIALS AND METHODS

2.2.1 Animals

Adult female Sprague Dawley rats (Harlan/Envigo, Frederick, MD) aged 60-65 days were used for all studies. Rats were socially housed 2-3 per cage in plastic cages (40 x 22 x 19 cm) in a temperature- and humidity-controlled room with a regular light-dark cycle, and food and water were provided *ad libitum*. All experimental manipulations were performed during the light cycle

and were carried out in accordance with the policies implemented by the University of Pittsburgh Institutional Animal Care and Use Committee.

2.2.2 Drugs

Levonorgestrel (Sigma-Aldrich; 1362602), a progestin-only hormonal contraceptive (HC), was dissolved at a concentration of 0.25 mg/mL in a 1:1 solution of deionized water and dimethyl sulfoxide (DMSO; Fisher Scientific; BP231-1) and administered subcutaneously (s.c.) at a dose of 0.5mg/kg/day (Graham and Milad, 2013) (Experiments 1 & 3). Losartan potassium (Sigma-Aldrich; 61188), an AT1R antagonist, was dissolved in sterile saline and injected intraperitoneally (i.p.) at doses of 3 mg/kg or 10 mg/kg (Marvar et al., 2013) (Experiment 1). Ang II (Sigma-Aldrich; A9525), the endogenous ligand for the AT1R, was reconstituted in sterile saline and injected i.p. at doses of 30 μg/kg or 100 μg/kg (Fregly and Thrasher, 1978) (Experiment 2).

2.2.3 Fear conditioning protocol

Apparatus and Stimuli: Behavioral testing occurred in operant conditioning boxes contained within sound-attenuating chambers (Med Associates, Inc., St. Albans, VT). Boxes were equipped with a shock generator, house light, tone generator, and an exhaust fan. Two different contexts were used for behavioral testing. Fear conditioning occurred in Context A and consisted of rod floors, two nosepoke apertures, and the odor of Accel disinfectant sprayed into the floor pan. Extinction training and extinction recall testing occurred in Context B, which consisted of grid floors, five nosepoke apertures, and an almond odor sprayed into the floor tray. *Fear*

Conditioning Procedure: Rats were placed in an operant chamber (Context A) and were given 3 minutes to acclimate before 5 tones lasting 10 seconds each, with a 1 minute inter-trial interval (ITI), were presented, where each tone co-terminated with a 1 mA, 1 second foot shock. Rats were placed back in their home cage following testing. *Extinction training and Extinction Recall Procedures*: Extinction training occurred 24 hours following the fear conditioning procedure, and rats were placed into a different operant chamber (Context B). Following a 3-minute acclimation period, 30 tones were presented (10 seconds, 1 minute ITI) in the absence of foot shock. Following the session, estrous cycle was monitored via vaginal lavage, and rats were returned to their home cage. Extinction recall was tested 24 hours after extinction training. Rats were placed into Context B and presented with 30 tone-only presentations and returned to their home cage following testing. *Behavioral assessment*: All behavioral testing was video recorded. A trained, blinded scorer assessed freezing (fear) behavior, defined as the lack of movement other than breathing, for rats during all testing phases. The amount of time (seconds) the rats spent freezing during each tone presentation was recorded. *Estrous cycle monitoring*: Estrous cycle was monitored immediately after extinction training. A pipette tip containing 150 μL of saline was gently inserted into the vagina of the rat and saline was flushed into the vaginal canal. The saline was pipetted onto a clean microscope slide, and a coverslip was placed on the slide. This process was repeated for each rat. Slides were examined under a light microscope, and estrous cycle phase was determined by observation of cellular morphology according to published protocols (Goldman et al., 2007).

2.2.4 Experiment 1: Effect of systemic losartan on extinction consolidation in HC- and vehicle-treated rats

Behavior: Rats received either HC or vehicle treatment four days prior to and throughout the fear conditioning paradigm. The fear conditioning procedure was administered. Immediately before extinction training, rats were treated with 0, 3, or 10 mg/kg losartan (i.p.). Estrous cycle was monitored after the extinction session, and it was confirmed that HC-treated females were in low estradiol states (estrus, metestrus, diestrus) and vehicle-treated females were in high estradiol states (proestrus) during the time of extinction. Extinction recall occurred 24 hours later. Rats were sacrificed via rapid decapitation, and brains were collected for qPCR analysis of RAS genes. *qPCR:* Flash frozen brains were sectioned and tissue punches of the following brain regions were taken from HC- (n = 10) and vehicle-treated (n = 10) rats: prelimbic (PL) cortex, infralimbic (IL) cortex, basolateral amygdala (BLA), central nucleus of the amygdala (CeA), dorsal hippocampus, and ventral hippocampus. Total RNA extraction was performed using brain tissue punches with an AllPrep DNA/RNA Micro kit (Qiagen). cDNA was generated with the Quanta BioSciences qScript cDNA system, and qPCR was performed based on three internal controls, as previously described (Guilloux et al., 2011). Samples from 4 rats (2 HC, 2 vehicle) were run per 96-well plate, with the 2 genes of interest and 3 internal controls run in quadruplicate per sample. Figure 2.1A highlights the details of this experiment.

2.2.5 Experiment 2: Effect of systemic Ang II on extinction consolidation in freely cycling female rats

Behavior: Freely cycling rats were exposed to the fear conditioning procedure, as shown in Figure 2.1B. Ang II (30 μg/kg or 100 μg/kg, i.p.) or vehicle was administered 5 minutes pre-extinction session or immediately post-extinction session. Vaginal lavage was used to monitor estrous cycle immediately after the extinction session to ensure all females were in a high estradiol phase (proestrus) during the time of extinction. Extinction recall occurred 24 hours later. Following behavioral testing, rats were sacrificed via rapid decapitation.

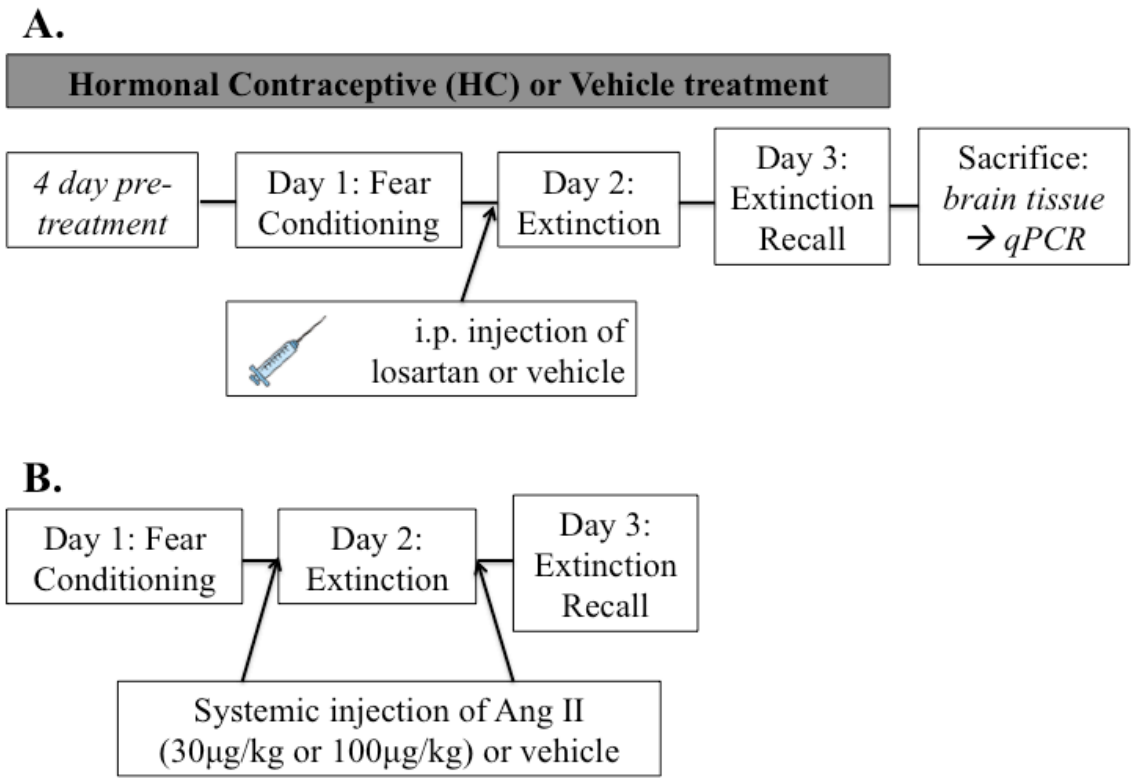

Figure 2.1 Experimental timeline for Experiments 1 and 2.

A.) Experiment 1: Rats were treated with HC or vehicle 4 days prior to and throughout the fear conditioning paradigm. An i.p. injection of vehicle or losartan was given before the extinction session, and extinction recall was tested 24 hours later. Rats were sacrificed the day after extinction recall, and brain tissue was used for qPCR analysis of RAS genes. B.) Experiment 2: Freely cycling rats were subjected to the fear conditioning paradigm. An i.p. injection of Ang II or vehicle was given 5 minutes before or immediately following the extinction session. Extinction recall was tested 24 hours later.

2.2.6 Experiment 3: Effect of HC treatment on AT1R binding via AT1R autoradiography

Rats were treated with HC or vehicle for 5 days, which represents the typical treatment period with HC prior to the extinction training session. No behavioral testing was conducted in this experiment. Rats were sacrificed via rapid decapitation on the following day, which was

equivalent to the extinction training day in the behavioral experiments, and corresponds with the day at which estradiol levels are known to regulate the efficacy of extinction consolidation. Brains were collected, flash frozen, and stored at -80° Celsius (C). Trunk blood was also collected for measurement of estradiol and Ang II in serum via commercially available ELISA kits (Calbiotech Mouse/Rat Estradiol ELISA, ES180S-100; Raybiotech Rat ANGII EIA, P01015). Brains were removed from storage, cut on the cryostat at 20 μM, mounted onto slides (6 slices mounted per slide, in series of sequential sets of five), and stored at -80° C. Samples were shipped on dry ice to Dr. Robert Speth, who performed AT1R autoradiography as previously described (Linares et al., 2016). Briefly, 2 slides from each series were taken from the -80°C freezer, and one slide was labeled as 'total' treatment and the other slide was labeled 'non-specific' treatment. All slides were mounted into commercially available slide grips and inverted into Coplin jars, which contained 35-40 mL of solution (10 μM final concentrations) of angiotensin II type 2 receptor (AT2R) antagonist and AT1R antagonist in assay medium buffer in the 'non-specific treatment' jar and 10 μM AT2R antagonist in assay medium buffer in the 'total treatment' jar. Once the 30 minute pre-incubation step was completed, slides were transferred to incubation slide mailers, which contained 10 mL of assay medium buffer with a predetermined concentration of ^{125}I-SI-Ang II (Linares et al., 2016) along with the respective inhibitors described in the pre-incubation step. Slides were incubated for 60-90 minutes at room temperature. Slides were then returned to the slide grips, blotted, rinsed twice with distilled water, and rinsed four times (1 minute each) with assay medium buffer. Slides were swirled in 4 separate containers of ice-cold distilled water and blow dried with cool air for 4 minutes. Slides were mounted with the tissue side facing up to cardboard for X-ray film apposition with double sided tape. A minimum of one ^{125}Iodine calibration standard slide was also mounted to each

cardboard. The cardboard with the slides was transferred to a darkroom and placed in a strap-back X-ray cassette. The lights were turned off and the safelight was turned on. X-ray film was obtained and placed shiny side up on top of the slides in the cassette. The cassette was closed, and the slides were exposed for several days at -20° C. Cassettes were removed from the freezer and the films were placed in developer solution for 2 minutes, followed by stop bath for 30 seconds, and finally fixer solution for 5 minutes. Films were placed in a tray with running water for 20 minutes and then placed into Photoflo for 10 seconds. Films were hung up to dry. Slides and films were scanned into the computer, and Image J was used to perform densitometric analyses of ^{125}I-SI Ang II binding (fmol/g) of samples from multiple brain regions of interest relative to standards. Sample values were interpolated from standards fit to a centered third order polynomial curve. Non-specific binding was subtracted from specific binding, and these values were averaged for each brain region.

2.2.7 Statistical Analyses

Statistical analyses were conducted using SPSS Software for Mac. For Experiment 1, a three-way mixed factorial ANOVA was used to analyze behavioral data, with HC (0 vs. 0.5 mg/kg) and losartan (0, 3, 10 mg/kg) treatment as between-subjects factors and CS presentation (data are averaged into blocks of 5 CS presentations each for extinction and extinction recall, for a total of 6 CS blocks per session) as the within-subjects factor for fear conditioning, extinction, and extinction recall. Bonferroni post-hoc comparisons were performed when appropriate. qPCR replicates were averaged and transformed into relative expression levels ($2^{-\Delta\Delta Ct}$), such that higher values represent greater relative expression. Differences in AT1R and Mas receptor (MasR) expression as a function of HC treatment were analyzed by independent samples t-tests. In

Experiment 2, a two-way ANOVA was used to analyze behavioral data, with Ang II (0, 30 μg/kg, or 100 μg/kg) as the between-subjects factor and tone presentation (6 CS blocks) as the within-subjects factor for fear conditioning, extinction, and extinction recall. If females were not in proestrus on the extinction day, they were excluded from the study. In Experiment 3, differences in circulating estradiol, circulating Ang II, and ^{125}I-SI Ang II binding as a function of HC treatment were analyzed by independent samples t-tests.

2.3 RESULTS

2.3.1 Systemic losartan enhances extinction consolidation in HC-treated rats

The purpose of this experiment was to test whether a single systemic dose of AT1R antagonist losartan administered prior to extinction training would rescue the extinction consolidation deficits that have previously been reported in HC-treated rats with low estradiol levels (Graham and Milad, 2013). We predicted that losartan would have minimal to no effect in vehicle-treated rats with high estradiol levels, since we expect that these rats will already have very low levels of freezing during extinction recall. No significant differences were observed between HC- and vehicle-treated groups during conditioning, $p>0.05$ (Figure 2.2). A significant main effect of CS presentation was uncovered [$F(4,132)=166.43$, $p<0.001$], where rats exhibited significantly less freezing during CS presentations 1 and 2 compared to CS presentations 3-5. This suggests that both groups acquired fear comparably during the conditioning session. A CS presentation x HC treatment interaction was also found [$F(4,132)=2.98$, $p<0.05$]; however, post hoc analyses found

no significant differences in levels of freezing for each CS presentation between HC- and vehicle-treated groups, $p>0.05$.

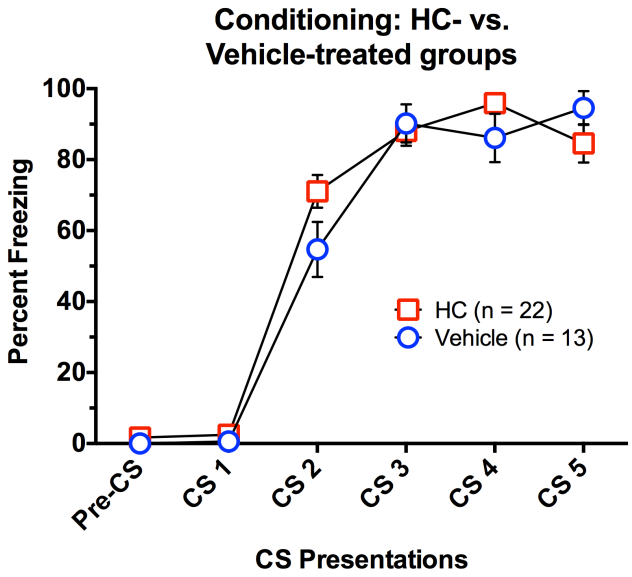

Figure 2.2 HC treatment had no effect on fear acquisition during conditioning.

Mean (±SEM) freezing levels are shown across the fear conditioning session. No significant differences were found between HC- and vehicle-treated females.

A main effect of HC treatment was found during extinction training [$F(1,79)=11.95$, $p=0.001$], where vehicle-treated rats (Figure 2.3A) had significantly lower levels of freezing compared to HC-treated rats. This finding has been reported in other studies (Graham and Milad, 2013), and it could indicate that HC treatment results in some resistance to extinction. A significant main effect of CS block was also found [$F(5,395)=137.16$, $p<0.001$], where all blocks significantly differed from each other and the highest and lowest levels of freezing were in CS Block 1 and CS Block 6, respectively. This finding was expected, and demonstrates that extinction was acquired across the session. No significant effects of losartan were found during extinction training in vehicle-treated (Figure 2.3B) or HC-treated (Figure 2.3C) females.

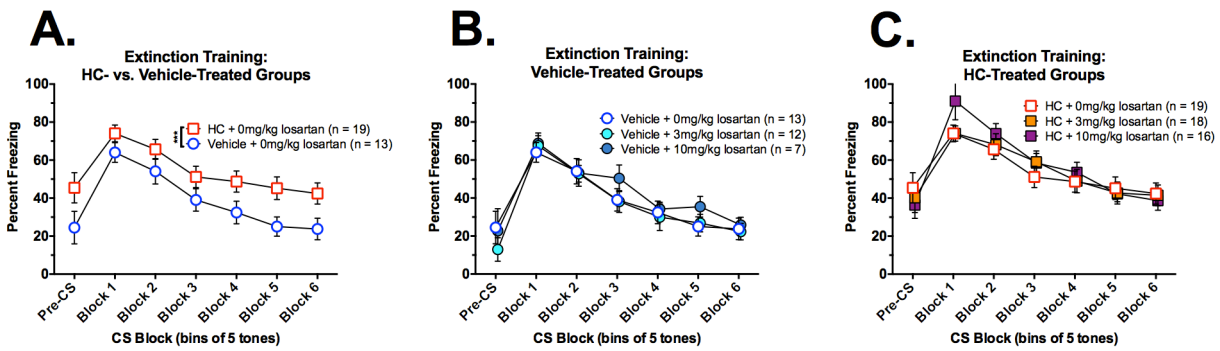

Figure 2.3 HC treatment results in increased levels of freezing during extinction training.

Mean (±SEM) freezing levels are shown across the extinction training session. A.) A main effect of HC treatment was found, where HC-treated females had significantly higher freezing levels compared to vehicle-treated control females, ***p=0.001. B.) Losartan treatment alone had no effect on freezing levels during extinction in control rats. C.) Losartan treatment did not affect freezing levels in HC-treated females.

During extinction recall, a main effect of CS Block was found [$F(5,395)=50.96$, $p<0.001$], where significantly higher levels of freezing were detected in CS Blocks 1 and 2 compared to CS Blocks 3-6. A main effect of HC treatment was also found [$F(1,79)=11.05$, $p=0.001$]; replicating prior studies showing that HC-treated groups demonstrated significantly higher freezing than vehicle-treated groups (Figure 2.4A). A CS block x HC treatment interaction [$F(5,395)=7.75$, $p<0.001$] revealed that the HC-treated groups had higher levels of freezing compared to vehicle-treated groups across CS Blocks 1-4. A trend of losartan treatment (0, 3, or 10 mg/kg) was also found ($p=0.109$). The 3mg/kg dose was based on a previous study showing enhanced extinction consolidation in male mice (Marvar et al., 2013), while the 10mg/kg dose was selected based on the hypothesis that a higher dose of losartan may be necessary to overcome the HC deficit. Because of this *a priori* hypothesis that losartan would reduce freezing levels during extinction recall in HC-treated rats, and HC treatment resulted in significantly increased freezing during extinction recall compared to vehicle treatment, subsequent ANOVAs were run to determine the effect of losartan treatment on the HC-treated

groups and vehicle-treated groups separately. Losartan treatment alone had no effect on freezing (Figure 2.4B; $p>0.05$), which was expected, given the already very low levels of freezing in high estradiol control rats. However, there was a trend for losartan treatment to dose-dependently reduce freezing in the HC-treated groups ($p=0.10$). Exploratory 2x6 ANOVAs failed to detect any differences between the 3 mg/kg losartan dose and any other dose tested. However, consistent with the hypothesis that larger losartan doses would be needed to surmount the HC-related extinction consolidation deficit, we found that the 10 mg/kg dose of losartan normalized freezing in HC-treated females, evidenced by significant decreases in freezing compared to the 0 mg/kg losartan group [$F(1,33)=4.45$, $p<0.05$] (Figure 2.4C).

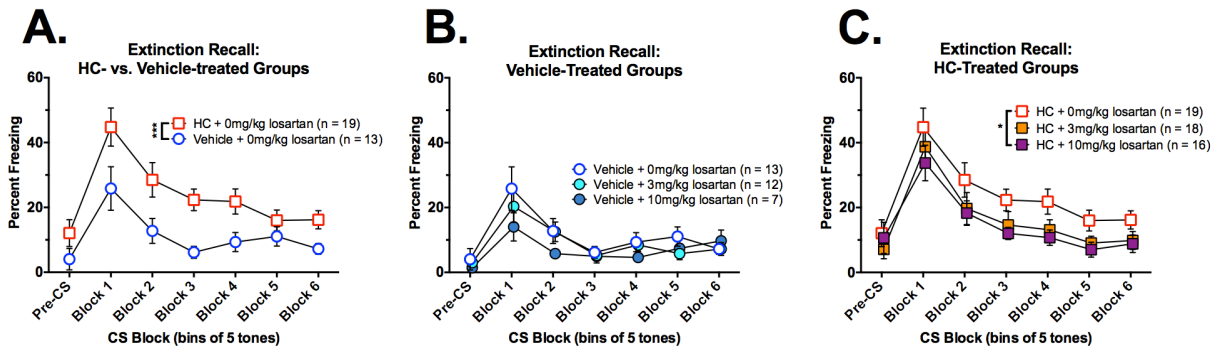

Figure 2.4 HC treatment results in elevated freezing levels during extinction recall, which can be rescued by systemic treatment with AT1R antagonist losartan.

Mean (±SEM) freezing levels are shown across the extinction recall session. A.) HC-treated females displayed significantly higher levels of freezing compared to vehicle-treated control females during extinction recall, ***$p=0.001$. B.) Losartan treatment alone had no effect on freezing during extinction recall in high estradiol control females. C.) The 10mg/kg dose of losartan significantly reduced freezing during extinction recall in HC-treated females compared to HC-treated females in the 0mg/kg losartan group, *$p<0.05$.

2.3.2 Systemic Ang II does not significantly alter freezing levels during extinction recall in freely cycling female rats with high estradiol levels

Next, we wanted to test whether we could impair extinction consolidation in freely cycling female rats by systemically administering Ang II, the endogenous ligand for the AT1R, prior to or immediately following extinction training. No significant differences were found between prospective treatment groups during fear conditioning (Figure 2.5A; $p>0.05$). A significant main effect of CS presentation was detected [$F(4,152)=80.38$, $p<0.001$]. Rats had significantly less freezing during CS presentations 1 and 2 compared to CS presentations 3-5, suggesting that all rats acquired fear during the conditioning session comparably.

A significant main effect of CS Block was detected during extinction training [$F(5,195)=22.834$, $p<0.001$]. Significantly higher freezing levels were found in CS Block 1 compared to all other CS Blocks, demonstrating that freezing levels decreased across the extinction session. No differences were detected between treatment groups (Figure 2.5B).

No significant differences were found between treatment groups during extinction recall (Figure 2.5C). However, pre-session treatment with 30ug/kg Ang II non-significantly increased freezing compared to the vehicle-treated females in proestrus.

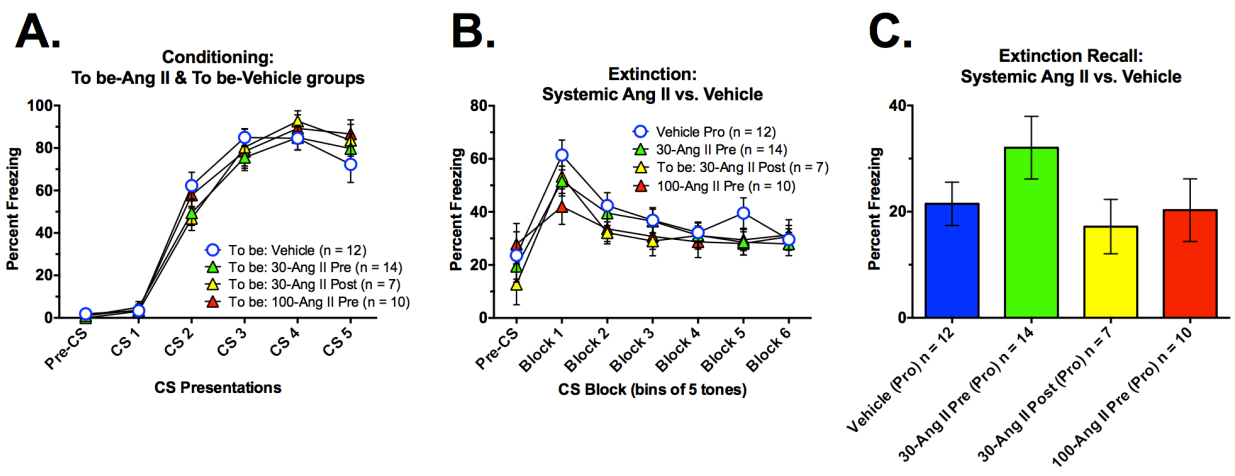

Figure 2.5 Systemic Ang II treatment does not significantly alter freezing levels during extinction training or extinction recall.

Mean (±SEM) freezing levels are shown across A.) fear conditioning, B.) extinction training, and C.) extinction recall (average of first three tones of extinction recall for each group is shown). No significant effects of Ang II treatment were found during any of the behavioral sessions; however, pre-session treatment with 30 ug/kg Ang II resulted in a non-significant elevation in freezing during extinction recall compared to the vehicle-treated proestrus females.

2.3.3 HC treatment results in low circulating estradiol levels and increased levels of Ang II compared to vehicle-treated females in proestrus, but no differences found in AT1R or MasR mRNA expression or central AT1R ligand binding

We wanted to examine the mechanism by which estradiol regulation of the RAS may be altering extinction consolidation. To do this, we first verified that our HC treatment was significantly reducing central estradiol levels compared to vehicle-treated females in proestrus. Circulating levels of estradiol were significantly reduced in HC-treated females compared to vehicle-treated proestrus females, [$t(7.22) = 3.258$, $p = 0.01$] (Figure 2.6A), which is what we expected to find based on previous studies (Graham and Milad, 2013). Next, we wanted to determine whether

circulating levels of Ang II were different between treatment groups. We predicted that HC-treated females with low estradiol levels would have significantly higher levels of Ang II. Consistent with our prediction, we found that circulating Ang II levels were significantly elevated in HC-treated females compared to vehicle-treated females with high estradiol levels, [$t(18.586) = 2.286$, $p < 0.05$] (Figure 2.6B).

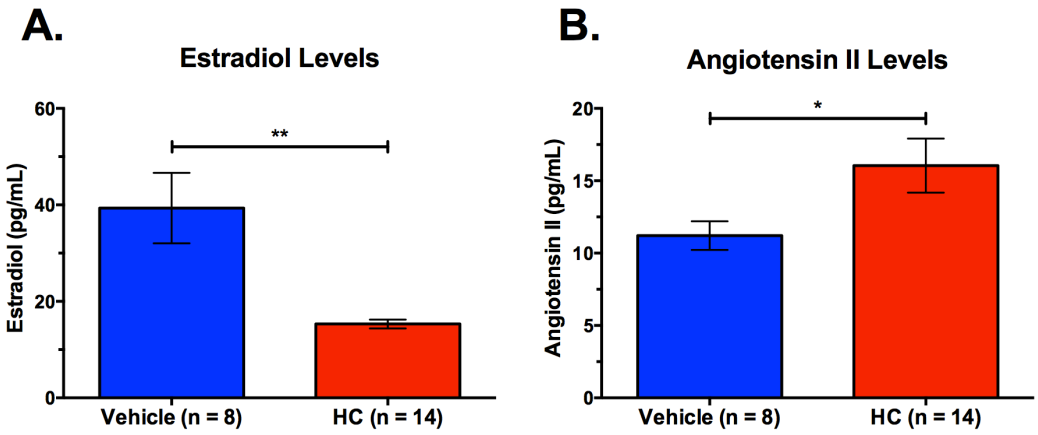

Figure 2.6 HC-treated females with low estradiol levels have significantly elevated Ang II levels.

A.) Estradiol levels are significantly elevated in proestrus vehicle-treated females compared to HC-treated females, ***p*=0.01. B.) Vehicle-treated females with high estradiol levels have significantly lower Ang II levels compared to HC-treated females, **p*<0.05.

We next compared central AT1R and MasR mRNA expression levels in HC- and vehicle-treated groups. Independent samples t-tests found no significant differences between HC- and vehicle-treated groups in AT1R or MasR mRNA expression for the PL, IL, BLA, CeA, dorsal hippocampus, or ventral hippocampus, $p>0.05$ (Figure 2.7A-F & 2.8A-F, respectively).

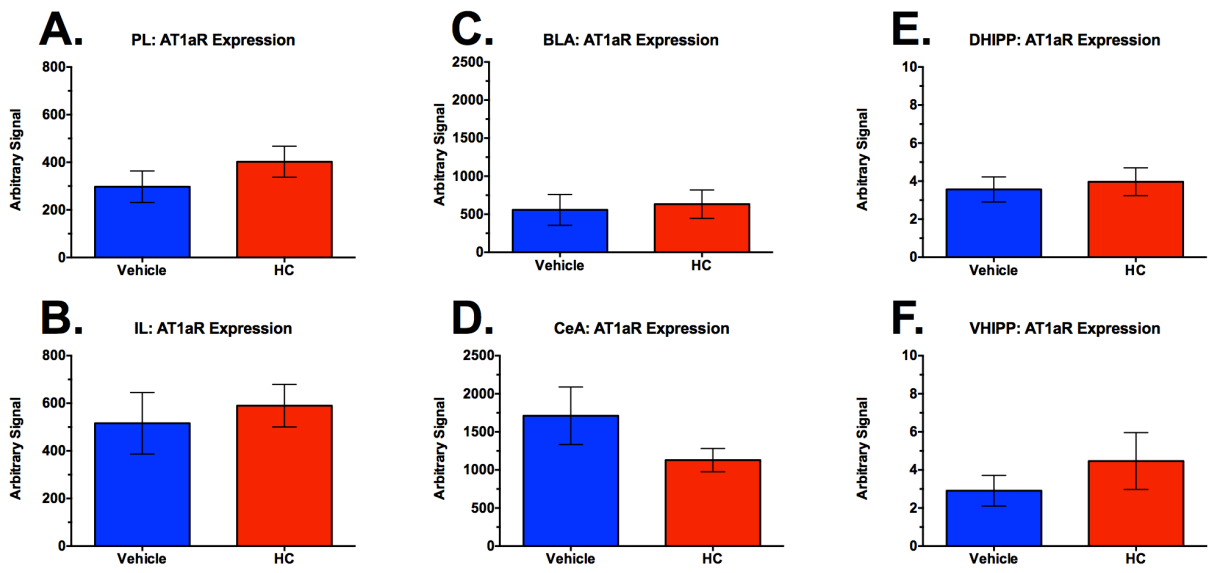

Figure 2.7 Central AT1aR mRNA expression does not significantly differ between HC- and vehicle-treated groups.

Independent samples t-tests revealed no significant differences in AT1aR mRNA expression between HC- and vehicle-treated groups in the A.) PL, B.) IL, C.) BLA, D.) CeA, E.) dorsal hippocampus, or F.) ventral hippocampus.

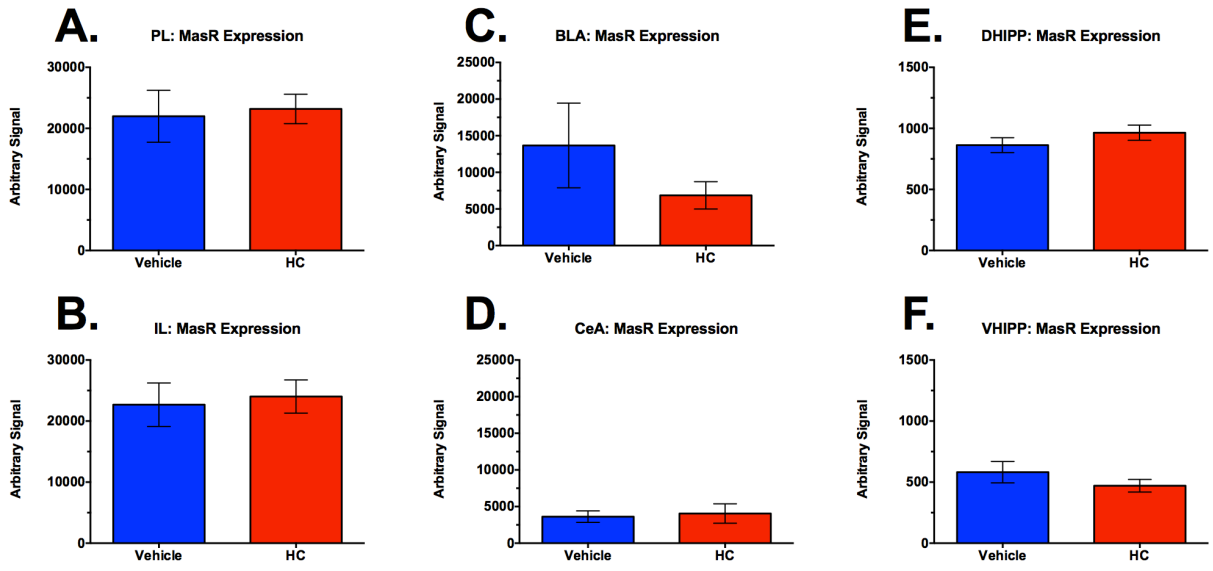

Figure 2.8 Central MasR mRNA expression does not significantly differ between HC- and vehicle-treated groups.

No significant differences in MasR mRNA expression were found between HC- and vehicle-treated groups in the A.) PL, B.) IL, C.) BLA, D.) CeA, E.) dorsal hippocampus, or F.) ventral hippocampus.

Because some studies suggest that estradiol affects AT1R levels through a post-transcriptional mechanism (Krishnamurthi et al., 1999, Wu et al., 2003a), we next tested whether AT1R ligand binding differed between HC-treated females and vehicle-treated proestrus females. Independent samples t-tests revealed no significant differences in AT1R ligand binding between HC- and vehicle-treated groups for the IL, PVN, anterior piriform cortex, posterior piriform cortex, BLA, or ventral subiculum, $p>0.05$ (Fig. 2.9A-F, respectively).

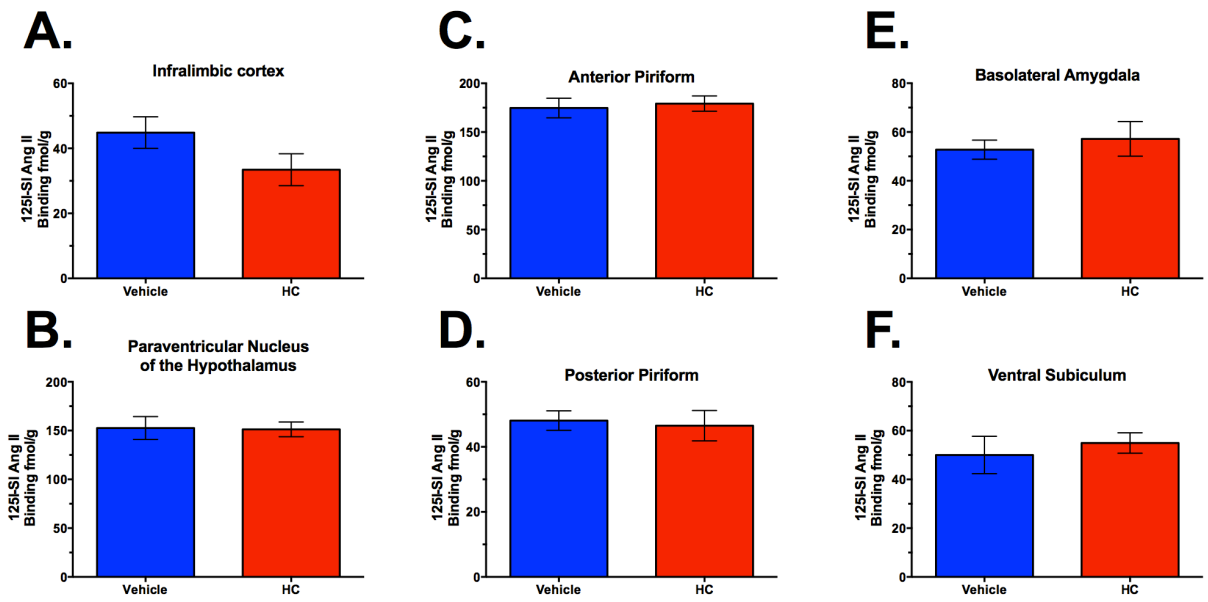

Figure 2.9 AT1R ligand binding did not significantly differ between HC- and vehicle-treated groups.

No significant differences in AT1R ligand binding were detected between HC- and vehicle-treated groups in the A.) infralimbic cortex, B.) PVN, C.) anterior piriform cortex, D.) posterior piriform cortex, E.) BLA, or F.) ventral subiculum.

2.4 DISCUSSION

In the present experiment, we tested whether AT1R antagonist losartan, which has previously been shown to enhance extinction consolidation in male mice (Marvar et al., 2013), would rescue the fear extinction consolidation deficit that has previously been reported in females with low estradiol levels (Graham and Milad, 2013). We replicated findings from previously published studies showing that low levels of estradiol in female rats, via daily systemic administration of HC levonorgestrel, increased freezing during extinction recall (Graham and Milad, 2013). We are the first to find that systemic treatment with AT1R antagonist losartan enhanced extinction consolidation in HC-treated females, shown by decreased levels of freezing during extinction recall. While systemic treatment with Ang II, the endogenous ligand for the AT1R, did not significantly alter freezing levels during extinction training or recall, proestrus females treated with a low dose of Ang II prior to extinction training had a non-significant rise in freezing during extinction recall compared to vehicle-treated proestrus females. No differences were detected between HC- and vehicle-treated groups in MasR mRNA expression, AT1R mRNA expression, or AT1R ligand binding; however, we did find that HC-treated females with reduced circulating estradiol levels had elevated Ang II plasma levels. These findings indicate that females with low estradiol as a function of HC have increased Ang II levels and impaired extinction consolidation, and that blocking AT1R can reduce freezing during extinction recall to the level of vehicle-treated females in proestrus. Together, this suggests that women with low estradiol levels during the time of exposure therapy, who typically have poor consolidation of extinction memories, may benefit from treatment with an AT1R antagonist.

2.4.1 Treatment with HC results in impaired extinction consolidation and increased freezing during extinction recall

Only one other lab has examined the effect of levonorgestrel on fear-related behaviors in female rats. While Graham and colleagues found that HC-treatment did not affect fear acquisition during conditioning, they did find that HC-treated rats had significantly elevated freezing levels compared to vehicle-treated proestrus females during extinction training (Graham and Milad, 2013). Similarly, in the present study we report no differences between HC- and vehicle-treated groups during fear conditioning. However, a main effect of HC treatment was found during extinction training, where HC-treated females had significantly increased freezing levels compared to vehicle-treated proestrus females. Because freezing levels between HC- and vehicle-treated females were similar at the beginning of the extinction training session and differ towards the end of extinction, these results indicate that the HC-treated females had some resistance to extinction, rather than stronger consolidation of the initial fear memory. Losartan treatment did not significantly alter freezing levels in HC-treated or control rats during the extinction session, which is consistent with prior reports in male mice (Marvar et al., 2013).

During extinction recall, we were able to replicate findings from a previously published study (Graham and Milad, 2013), where HC-treated females, who were confirmed to have significantly reduced estradiol levels compared to proestrus vehicle-treated females, had significantly elevated levels of freezing compared to vehicle-treated proestrus females. However, due to the differences in freezing levels that were observed between HC- and vehicle-treated groups during extinction training the previous day, it is difficult to clearly interpret whether the deficits found during extinction recall in HC-treated females are due to deficits in extinction acquisition or extinction consolidation. Previously published findings indicate that HC treatment

resulted in similar deficits during extinction acquisition (Graham and Milad, 2013); however, Graham and Milad found no significant differences between HC- and vehicle-treated groups during the last block of 5 tones of the extinction training session. In the present study, we found differences between HC- and vehicle-treated groups that primarily occurred towards the end of the extinction training session, where significant differences were found between groups during the last block of extinction training. In order to more clearly determine the effect that estradiol has on extinction consolidation, it would be necessary to manipulate estradiol during this window of time and determine the effect on freezing levels during the extinction recall session. Milad and colleagues have done these experiments and have found that metestrus (low estradiol) females systemically treated with estradiol (Milad et al., 2009a, Graham and Milad, 2013) or an ERβ (but not α) agonist (Zeidan et al., 2011) before or immediately following extinction training had significantly reduced freezing during extinction recall compared to vehicle-treated females in metestrus. While these experiments clearly point to an effect of estradiol on extinction consolidation, due to the fact that HC treatment is administered chronically in the present study, the effect of HC on the extinction consolidation window is less clear.

2.4.2 AT1R antagonist losartan dose-dependently rescues the extinction recall deficit in HC-treated females

We were able to show for the first time that HC-treated females systemically treated with a 10 mg/kg dose of losartan prior to the extinction training session had significantly reduced freezing levels during extinction recall compared to HC-treated females that were given 0mg/kg losartan. Because losartan treatment did not significantly alter freezing levels during extinction acquisition, we can conclude that losartan treatment enhanced extinction consolidation in the

HC-treated females. No effect of losartan was found in vehicle-treated proestrus females, though freezing levels for vehicle-treated groups was already very low, which makes it difficult to detect a further reduction in freezing due to losartan treatment. Although we did not directly test how this dose of losartan affects anxiety-like behavior and locomotor activity, findings in this experiment are similar to a previously published study showing that a similar dose of systemic losartan given prior to extinction training enhanced extinction consolidation in male mice without affecting baseline anxiety, blood pressure, and distance traveled in an open field (Marvar et al., 2013). Similarly, trauma-exposed patients who took AT1R antagonists to treat hypertension reported reduced symptoms of PTSD compared to subjects taking blood pressure medications that act independently of the RAS (Khoury et al., 2012). Our findings suggest that a 10mg/kg dose of losartan, a drug commonly prescribed to treat hypertension, can rescue deficits in extinction consolidation in patients undergoing exposure therapy in low estradiol states. This would improve treatment outcome for patients who have fear and anxiety disorders, leading to reduced fear outside the treatment clinic.

2.4.3 The effect of systemic Ang II on extinction consolidation in proestrus females

Since systemic administration of an AT1R antagonist rescued deficits in female rats with low circulating estradiol, we next wanted to test whether systemically administering Ang II, the endogenous agonist of the AT1R, would result in impairments in extinction consolidation in rats during the proestrus (high estradiol) phase of the estrous cycle. No significant differences were found between treatment groups for extinction training or extinction recall. However, a non-significant rise in freezing was found during extinction recall in females treated with 30 μg/kg Ang II prior to the extinction session compared to vehicle-treated females. While the lack of a

significant effect of Ang II treatment was unexpected, there are many factors could be contributing to this effect. First, the systemic dose that was used may need to be altered. While there are studies examining the effect of central Ang II in fear-related paradigms in male rats (Tsuda et al., 1992, de Souza et al., 2004, Marinzalda Mde et al., 2014), none have examined the effect of systemic Ang II administration on fear in females. However, similar doses of systemic Ang II to what was used in the present study have been found to produce increases in water intake three hours after administration in female rats (Fregly and Thrasher, 1978). Another factor contributing to the lack of significant results could be that the systemically administered Ang II did not have enough time to pass the blood brain barrier (BBB) and act centrally, or that not enough peptide was able to cross the BBB to have an effect on fear-related behavior. While there is some controversy over whether Ang II can cross the BBB (Roncevic, 2012), there are studies showing that Ang II can acquire access to the brain through various mechanisms (Rose and Audus, 1998, Paton et al., 2008, Biancardi et al., 2014). However, the rate at which these processes occur in adult female Sprague Dawley rats has not yet been determined. Finally, it is important to consider timing of Ang II administration, as studies testing the effects of Ang II in the passive avoidance paradigm have found mixed results depending on when Ang II was administered during training and testing (de Souza et al., 2004). To circumvent these problems, future experiments will centrally administer Ang II to test whether treatment disrupts extinction consolidation in female rats with high circulating estradiol levels.

2.4.4 Increased Ang II levels in HC-treated females may contribute to impaired extinction consolidation

Finally, we wanted to determine the mechanism by which estradiol was regulating the RAS to affect fear extinction consolidation. Since HC treatment results in impaired extinction consolidation, which can be rescued by treatment with losartan, we first examined mRNA expression levels of the AT1R and the MasR, which mediate the hypertensive and antihypertensive effects of the RAS, respectively (Iwai and Horiuchi, 2009). We examined many brain regions associated with fear in HC- and vehicle-treated females, including PL, IL, BLA, CeA, dorsal hippocampus, and ventral hippocampus. No significant differences were found between HC- and vehicle-treated groups in AT1R or MasR mRNA expression. These results were initially unexpected, as other studies had found reduced AT1R mRNA expression in the pituitary gland and aorta of OVX estradiol-treated female rats (Nickenig et al., 1998, Wu et al., 2003a). However, it should be noted that for this particular study, estrous cycle was not monitored on the day of sacrifice. While we can be fairly confident that HC-treated females all had low levels of estradiol, it is possible that some females in the vehicle-treated group did not have high estradiol, which could impact our findings. In addition, some studies have indicated that estradiol affects AT1R levels through a post-transcriptional mechanism (Krishnamurthi et al., 1999, Wu et al., 2003a). Therefore, we decided to move forward by comparing AT1R ligand binding between HC- and vehicle-treated females with confirmed low and high levels of estradiol on the day of sacrifice, respectively. We found no significant differences between groups in AT1R ligand binding in brain regions associated with fear and AT1R hotspots, including the IL, PVN, anterior piriform cortex, posterior piriform cortex, BLA, or ventral subiculum. Because previously published studies have found that estradiol treatment decreases

AT1R ligand binding in OVX rats in the adrenal glands, heart, SFO, PVN, MnPO, organ of the lamina terminalis, kidney, and abdominal aorta (Wu et al., 2003b, Dean et al., 2005), we had predicted to find significantly less AT1R ligand binding in the vehicle-treated (proestrus) females. Since it is unclear why HC-treated females with confirmed low estradiol levels do not have significantly elevated levels of AT1R ligand binding compared to vehicle-treated females, our future studies more closely examine how hormone manipulations affect AT1R ligand binding in both central and peripheral tissues (**Chapter 3**).

Although AT1R mRNA expression and ligand binding were not altered in HC-treated females, we did find a moderate but significant increase in Ang II levels in HC-treated females compared to vehicle-treated proestrus females. Many other studies have examined how hormone manipulations affect plasma or cortical Ang II levels; however, the findings in the literature are mixed. One study found no significant differences in plasma or cortical Ang II between male and female Lewis rats (Pendergrass et al., 2008). Similarly, no significant differences in plasma Ang II were found between men and women in their early twenties; however, males had significantly elevated Ang 1-7 compared to females (Reyes-Engel et al., 2006). Surprisingly, one study examining plasma Ang II levels in OVX female rats found that this group had significantly reduced plasma Ang II, and treatment with estradiol reversed this effect (Xu et al., 2008). In women taking hormonal contraceptives that contain both estrogen and progesterone, levels of plasma Ang II were found to be significantly elevated compared to healthy females that were not taking hormonal contraceptives and males (Zakheim et al., 1976, Kang et al., 2001). Finally, postmenopausal women taking an estrogen supplement were found to have reduced plasma Ang II levels; however, no effect on plasma Ang II levels were found if women were taking an estrogen plus progesterone supplement (Zheng et al., 2002). Since findings on circulating Ang II

levels are inconsistent across studies, we hope that our findings help to clarify the role that estradiol levels have on Ang II peptide levels. We acknowledge that the methodology used for measuring Ang II peptide levels in this study can detect other angiotensin peptides, such as Ang III and Ang IV; therefore, in future studies, we will measure various angiotensin peptides using liquid chromatography-mass spectrometry (LC-MS), a highly sensitive technique that is capable of measuring angiotensin peptides in biological samples (Cui et al., 2007, Ali et al., 2014).

In conclusion, we have replicated that HC-treated females with low estradiol levels on the day of extinction training have impaired extinction consolidation and increased freezing during extinction recall, which can be rescued with systemic administration of a 10 mg/kg dose of losartan. While no changes in AT1R mRNA expression or AT1R ligand binding were found between HC- and vehicle-treated groups in any of the brain regions that were tested, preliminary data indicates that HC-treated females have a moderate but significant increase in Ang II levels. Future studies will further explore how HC treatment affects AT1R ligand binding, since this has not been fully characterized. In addition, we plan to test how estradiol affects central activation of AT1Rs by measuring components of the intracellular signaling pathway known to be affected when Ang II binds to AT1Rs in neurons, including phospholipase C and calcium/calmodulin-dependent protein kinase II.

3.0 AT1R ANTAGONIST LOSARTAN RESCUES EXTINCTION CONSOLIDATION DEFICIT FOUND IN OVARIECTOMIZED (OVX) FEMALE RATS

3.1 INTRODUCTION

Estradiol has been implicated in many learning and memory processes, such as working memory performance and tests of cognitive functioning, and estradiol also affects anxiety- and depressive-like behavior (Walf and Frye, 2006, Pompili et al., 2012, Luine, 2014, Frick et al., 2015). Recently, studies have shown that estradiol levels regulate fear extinction consolidation. Studies examining freely cycling women and female rats indicate that when estradiol levels are low during the extinction training session, fear extinction consolidation is impaired and fear is significantly elevated during extinction recall (Milad et al., 2009a, Milad et al., 2010, Zeidan et al., 2011, Lebron-Milad et al., 2012a, Lebron-Milad et al., 2012b, Graham and Milad, 2013, 2014). In addition, female rats treated with hormonal contraceptive (HC) levonorgestrel, a progestin that reduces estradiol levels, have poor extinction consolidation and increased fear during extinction recall (Graham and Milad, 2013) (see **Chapter 2** for replication of these findings). Interestingly, at the time these studies were done, there were no studies that had examined the effects of OVX on fear extinction consolidation in a cued fear conditioning paradigm. Instead, many studies have focused on how OVX affects fear behavior in a contextual fear conditioning paradigm, and those studies that did test the effects of OVX on cued fear

conditioning did not test fear extinction recall (Morgan and Pfaff, 2001, Jasnow et al., 2006, Frye and Walf, 2008, McDermott et al., 2015). Therefore, we wanted to test whether OVX female rats would have impaired extinction consolidation compared to sham-operated proestrus controls in a cued fear conditioning paradigm.

The mechanism by which estradiol affects fear extinction consolidation is currently unknown. One potential candidate that requires further investigation is the renin angiotensin system (RAS), since 1) negative regulation of the RAS has been shown to reduce symptoms of PTSD (Khoury et al., 2012) and enhance fear extinction consolidation in male mice (Marvar et al., 2013), and 2) estrogen has been shown to negatively regulate the hypertensive axis of the RAS (Fischer et al., 2002). Figure 1.5 displays the hypertensive axis of the RAS and how estrogen affects each component of the system. Overall, estrogen downregulates the hypertensive components of the RAS, and it is well documented in the literature that estradiol downregulates angiotensin II type I receptors (AT1R) (Nickenig et al., 1998, Brosnihan et al., 1999, Fischer et al., 2002). OVX female rats treated with physiological doses of estradiol have significantly reduced adrenal AT1R levels, adrenal angiotensin II (Ang II) levels, and AT1R mRNA expression in the pituitary gland (Wu et al., 2003a, Wu et al., 2003b). In addition, other studies have shown that OVX females treated with physiological doses of estradiol had significantly reduced AT1R ligand binding in the heart, kidney, adrenal glands, abdominal aorta, SFO, PVN, MnPO, and vascular organ of the lamina terminalis compared to OVX females treated with vehicle (Dean et al., 2005, Rogers et al., 2007).

While many studies in the literature highlight the effects of OVX on AT1R ligand binding in central and peripheral tissues, less is known about how other hormone manipulations affect AT1R levels. For instance, no studies have tested the effects of HC levonorgestrel, which

clamps estradiol at a low level (similar to a freely cycling metestrus female (Graham and Milad, 2013)) and is known to impair fear extinction consolidation, on AT1R mRNA expression or ligand binding in either peripheral or central tissues. We previously compared AT1R mRNA expression and AT1R ligand binding levels in HC- and vehicle-treated females for central tissues only (**Chapter 2**). While no significant differences were detected, peripheral tissues have not been examined. In addition, while there has been some work comparing AT1R ligand binding between males and females in the periphery (Rogers et al., 2007, Sullivan, 2008), the literature is lacking a direct comparison between normotensive male and female AT1R ligand binding in the brain. Thus, we wanted to determine how these various hormone manipulations affect AT1R ligand binding in both central and peripheral tissues.

The purpose of these experiments was 1) to test how OVX affects the consolidation of fear extinction memories in a cued fear conditioning paradigm and 2) to test how AT1R ligand binding in central and peripheral tissues of HC-treated females, OVX females, and males compare to AT1R ligand binding in intact females with high circulating estradiol levels. In Experiment 1 we performed OVX or sham surgery on female rats and administered a cued fear conditioning paradigm. We hypothesized that OVX surgery would alter fear extinction consolidation, with the prediction that OVX females would have impaired extinction consolidation and increased fear during extinction recall compared to sham-operated proestrus controls. In Experiment 2, we wanted to test whether systemic treatment with losartan would reverse the extinction consolidation deficit observed in OVX females. We hypothesized that treatment with losartan would affect fear extinction consolidation in OVX females, with the prediction that OVX losartan-treated females would have significantly reduced freezing during extinction recall compared to OVX vehicle-treated females. Finally, in Experiment 3 we

examined how hormone manipulations affect AT1R ligand binding in central and peripheral tissues of male and female rats. We specifically wanted to follow up with our previous AT1R ligand binding analyses to test how HC levonorgestrel affects AT1R levels both centrally and peripherally, which has not been examined. We also wanted to determine whether there were differences in central AT1R ligand binding between males and females, since previous studies have only examined differences in peripheral AT1R levels between these two groups. Finally, we plan to examine differences between OVX and intact (high estradiol) females as a positive control, since it is well established how OVX affects peripheral and central AT1R ligand binding. We hypothesized that estradiol levels would alter AT1R binding levels, with the prediction that low estradiol levels would lead to an increase in AT1R receptor levels.

3.2 METHODS

3.2.1 Experiment 1: Effect of OVX on fear extinction consolidation

Subjects: Adult female Sprague Dawley rats (Envigo, Frederick, MD) aged 60-65 days were used, as previously described (**Chapter 2**). Rats were pair housed in a temperature- and humidity-controlled room with a regular light-dark cycle. Food and water were available *ad libitum*, and experiments were performed during the light cycle. Experimental manipulations were carried out in accordance with the University of Pittsburgh Institutional Animal Care and Use Committee.

Experimental Design: All rats received either bilateral OVX or sham surgery. To follow a similar timeline to hormonal contraceptive treatment (described in **Chapter 2**), behavioral

procedures began on the 5th day after surgery. Rats were sacrificed the day after extinction recall. Figure 3.1A shows the experimental timeline.

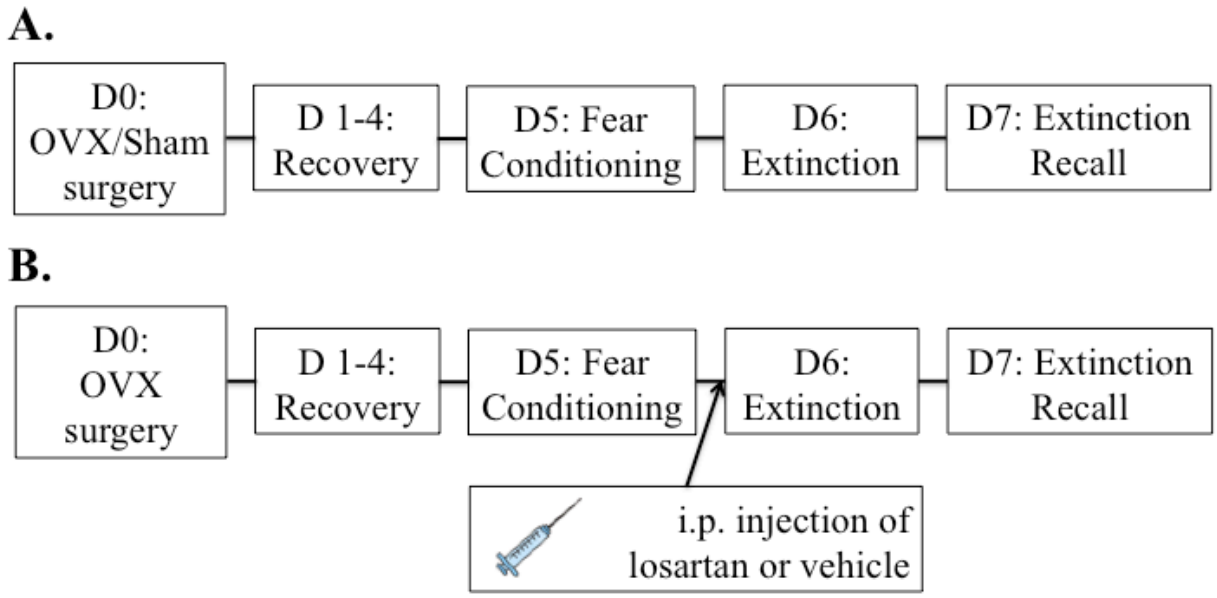

Figure 3.1 Experimental design for OVX behavioral studies.

A.) Experiment 1: Female rats received OVX or sham surgery, and recovery occurred the following 4 days. The cued fear conditioning paradigm was administered starting on the 5th day following surgery. B.) Experiment 2: All females received bilateral OVX surgery, and the fear conditioning paradigm was administered after a short recovery period. An i.p. injection of losartan or vehicle was administered 5 minutes prior to extinction training, and extinction recall was tested 24 hours later.

OVX surgery: Isoflurane was used to fully anesthetize each rat prior to surgery. Rats received an analgesic (Rimadyl, 5 mg/kg, s.c.) and lactated Ringer's (3 mL; s.c.). For the OVX surgery, the incision site was cleaned with betadine and alcohol, and a scalpel was used to make a small incision on the ventral abdomen. The muscle wall was separated from the abdominal cavity using hemostats. An incision was made in the abdominal cavity, and the ovaries were localized and gently externalized with tweezers. Adipose tissue was gently removed from the ovaries and fallopian tubes, and each fallopian tube was clamped tightly with suture thread just below the ovary. Each ovary was removed by using scissors to cut in between the suture and the

ovary. The remaining fallopian tubes were returned to the abdominal cavity. Ethicon perma-hand silk (3-0; 684-H) was used to suture the abdominal cavity and staples were used to close the incision. Neosporin was applied to the incision site, and Rimadyl was administered for the first two days following surgery. Rats in the sham treatment group received the same surgical procedure, with the exception of excision of the ovaries. Staples were removed prior to behavioral testing procedures.

Fear Conditioning Protocol: Behavioral testing occurred in operant conditioning boxes contained within sound-attenuating chambers (for more details, see **Chapter 2**). Fear conditioning occurred in Context A and consisted of rod floors, two nosepoke apertures, and Accel disinfectant was sprayed into the floor tray. Extinction training and extinction recall testing occurred in Context B, which consisted of grid floors, five nosepoke apertures, and almond odor was sprayed into the floor tray. Fear Conditioning Procedure: Rats were placed in an operant chamber (Context A) and were given 3 minutes to acclimate before 5 tones (10 seconds, 1 minute ITI) were presented, where each tone co-terminated with a foot shock (1 mA; 1 second). Extinction training and Extinction Recall Procedures: Extinction training occurred in Context B 24 hours following the fear conditioning procedure. Following a 3-minute acclimation period, 30 tones were presented (10 seconds, 1 minute ITI) in the absence of foot shock. Estrous cycle was monitored after the session via vaginal lavage (see description below), and rats were returned to their home cage. Extinction recall was tested in Context B 24 hours after extinction training. Rats were presented with 30 tone-only presentations and returned to their home cage. Behavioral assessment: Behavioral testing for all testing phases was video recorded, and a blinded scorer assessed freezing (fear) behavior during tone presentations as previously

described (**Chapter 2**). The amount of time (seconds) the rats spent freezing during each tone presentation was recorded.

Estrous cycle monitoring: Immediately following extinction training, a pipette tip containing saline was used to flush the vaginal canal of each rat (as described in **Chapter 2**). The sample was then pipetted onto a clean microscope slide, which was covered with a coverslip. Slides were examined under a light microscope, and estrous cycle phase was determined by observation of cellular morphology according to published protocols (Goldman et al., 2007). Females in the sham-operated group were in proestrus at the time of extinction training.

Sacrifice: The day after extinction recall, rats were sacrificed via rapid decapitation. Trunk blood was collected and stored at -20° C. Brains were collected, flash frozen, and stored at -80° C.

3.2.2 Experiment 2: Effect of systemic losartan on extinction consolidation in OVX females

The experimental procedures used in Experiment 2 were the same as those previously described in Experiment 1 of Chapter 3, with the following changes. All rats received bilateral OVX surgery, and the fear conditioning paradigm was administered after a short recovery period. Losartan potassium (Sigma-Aldrich; 61188), an AT1R antagonist, was dissolved in sterile saline. Each rat received an i.p. injection of 10 mg/kg losartan or saline 5 minutes before extinction training. Extinction recall was tested 24 hours later, and rats were sacrificed the day after testing. Figure 3.1B shows the experimental timeline.

3.2.3 Experiment 3: Effect of hormone manipulations on AT1R binding

Subjects: Adult male and female Sprague Dawley rats (Envigo, Frederick, MD) aged 60-65 days were used. Rats were maintained under the same conditions as described in Experiment 1 of Chapter 3, and experimental manipulations were carried out in accordance with the University of Pittsburgh Institutional Animal Care and Use Committee.

Injections: Rats received daily injections of vehicle or levonorgestrel (Sigma-Aldrich; 1362602), a progestin-only HC, which was dissolved at a concentration of 0.25 mg/mL in a 1:1 solution of deionized water and dimethyl sulfoxide (DMSO; Fisher Scientific; BP231-1) and administered s.c. at a dose of 0.5 mg/kg/day (Graham and Milad, 2013). Rats were treated with HC or vehicle for 5 days, which represents the typical treatment period with HC prior to the extinction training session. No behavioral testing was conducted in this experiment.

Experimental Design: Approximately half of the female rats received either bilateral OVX or sham surgery, as described in Experiment 1. The rest of the rats were kept in the housing room. The day after surgery, all rats received daily s.c. injections of hormonal contraceptive (HC) or vehicle for 5 days. Rats were sacrificed the following day (females were lavaged prior to sacrifice), which would typically represent the extinction training day, when estradiol levels have been shown to be important for fear extinction consolidation. Females in the intact vehicle and sham-operated groups were in proestrus at the time of sacrifice. Central (brain and pituitary gland) and peripheral tissues (liver, kidneys, and adrenal glands) were collected and stored at -80°C to measure AT1R ligand binding via autoradiography and blood was collected and stored at -80°C to measure angiotensin peptide levels. Refer to Figure 3.2 for an experimental timeline. Brains were removed from the freezer, cut on the cryostat at 20 μM and

mounted onto slides in sequential sets of five. Slices were allowed to dry on the slides for approximately 1 hour, at which point they were placed into slide boxes and stored at -80° C. Slides and peripheral organs were shipped on dry ice to Dr. Robert Speth, who performed AT1R autoradiography as previously described (Linares et al., 2016).

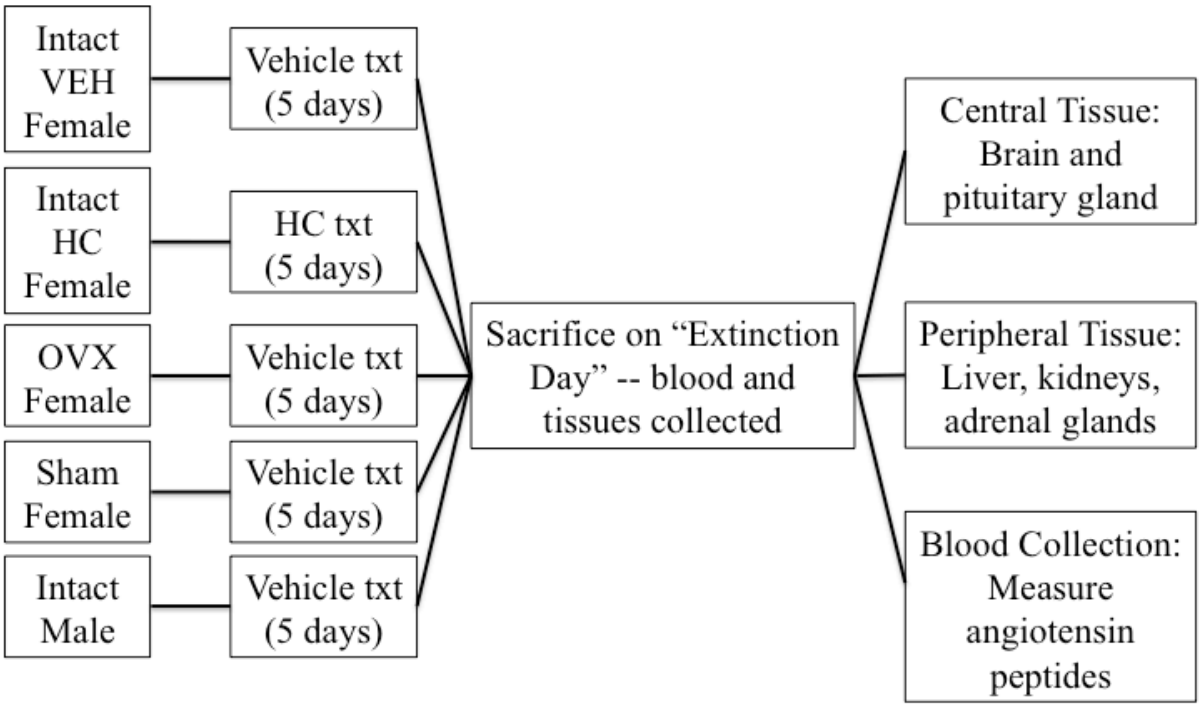

Figure 3.2 Experimental design for AT1R autoradiography study.

Intact males (n = 10), intact females (n = 9), OVX females (n = 9), and sham females (n = 8) were treated with vehicle (s.c.) for 5 days, and a group of intact females were treated with HC (n = 10; s.c.) for the same duration of time. Six days after the start of injections, female rats were lavaged and all rats were rapidly decapitated. Females in the intact vehicle and sham-operated groups were in proestrus at the time of sacrifice. Central and peripheral tissues were collected for further analysis via AT1R autoradiography, and blood was collected to measure angiotensin peptide levels.

AT1R Autoradiography: Two slides from each series were taken from the -80°C freezer, and one slide was labeled as 'total' treatment and the other slide was labeled 'non-specific' treatment. All slides were mounted into commercially available slide grips, and slide grips were

inverted and placed into pre-incubation Coplin jars that were filled with 35-40 mL of assay medium buffer (AM5) for 30 minutes at room temperature. Non-specific treatment Coplin jars also contained 10 μM final concentrations of AT2R antagonist PD123319 and AT1R antagonist losartan, while the total treatment Coplin jars contained only 10 μM of PD123319. Slides were then transferred to incubation slide mailers, which contain 10 mL of AM5 plus ^{125}I-SI-Ang II with the respective inhibitors described above, for 60-90 minutes at room temperature. Slides were then placed back into the slide grips, blotted, and rinsed by swirling in 400 mL of distilled water in two separate containers for 1-2 seconds. Slides were then transferred into four Coplin jars containing 35-40 mL of AM5 for 1 minute each, and promptly swirled in 4 changes of ice-cold distilled water for 1-2 seconds. Slides were then blow dried with cool air until dry and placed on a paper towel. Slides were mounted onto cardboard for apposition to X-ray film. Tissues were facing up, and double sided tape was used to adhere slides to the cardboard. At least one ^{125}Iodine calibration standard slide was also mounted to the cardboard.

The cardboard where slides were mounted was placed into a strap-back X-ray cassette. In a darkroom, the lights were turned off and the safelight was turned on. X-ray film was placed (shiny side up with jagged edge on the bottom right corner) on top of the slides in the cassette. The cassette was closed and locked to seal out the light. Slides were exposed for several days at -20° C. Each film was scanned by placing the film (shiny side down, with the jagged edge on the bottom left corner) on a proprietary scanner that was capable of transmitting film density information without any distortion in the imaging system computer. Image J was used to perform densitometric analyses of ^{125}I-SI Ang II binding (fmol/g) of samples from multiple brain regions of interest relative to standards. Sample values were interpolated from standards fit to a centered

third order polynomial curve. Non-specific binding was subtracted from specific binding, and these values were averaged for each brain region.

Statistical Analysis: Statistical analyses were conducted using SPSS Software for Mac. For behavioral data in Experiment 1, a two-way mixed factorial ANOVA was used to analyze behavioral data, with surgery (OVX vs. sham) treatment as between-subjects factor and tone presentation (6 CS Blocks) as the within-subjects factor for fear conditioning, extinction, and extinction recall. Rats in the sham-operated group were excluded from the analysis if they were not in proestrus at the time of extinction training. A two-way mixed factorial ANOVA was used to analyze behavioral data in Experiment 2, with treatment (vehicle vs. losartan) as the between-subjects factor and tone presentation (6 CS Blocks) as the within-subjects factor for all testing days. Bonferroni post-hoc comparisons were performed when appropriate. Because we had a priori hypotheses regarding AT1R ligand binding between intact males vs. intact females, intact females vs. HC females, and intact females vs. OVX females, differences in binding were compared via independent samples t-tests.

3.3 RESULTS

3.3.1 Experiment 1: Females with ovariectomy (OVX) have impaired fear extinction consolidation compared to sham-operated females in proestrus

The purpose of this experiment was to test how OVX surgery affects fear extinction consolidation compared to sham-operated females in proestrus. Because low estrogen levels during extinction training have been found to impair fear extinction consolidation (Milad et al.,

2009a, Milad et al., 2010, Zeidan et al., 2011, Lebron-Milad et al., 2012b, Graham and Milad, 2013, 2014), we predicted that females with OVX surgery would have impaired fear extinction consolidation and display increased freezing during extinction recall compared to sham-operated females with high estradiol levels. Consistent with other studies in the literature, there were no significant differences found between groups during fear conditioning, $p>0.05$ (Figure 3.3A). A significant main effect of CS presentation was found [$F(4,76)=75.0$, $p<0.001$], where rats exhibited significantly less freezing during the first CS presentation compared to all other CS presentations in the session. These data indicate that fear to the tone was comparably acquired by both groups across the session.

There was a trend for OVX females to exhibit higher levels of freezing during the extinction training session compared to sham operated controls, $p=0.051$ (Figure 3.3B). Since OVX females display high levels of freezing to the tone throughout the extinction session, this could indicate that this group has some resistance to extinction training. Similar results have been found in rats given HC treatment to reduce levels of estradiol, where HC-treated females have a tendency to freeze more during extinction training compared to vehicle-treated females with high estradiol levels (Graham and Milad, 2013) (also, refer to **Chapter 2**). A significant main effect of CS Block was also found [$F(5,95)=36.75$, $p<0.001$], where freezing during CS Blocks 1-4 was significantly higher than freezing during CS Blocks 5 and 6. This indicates that rats extinguished their fear to the tone across the extinction training session. A CS Block by treatment interaction was also uncovered, [$F(5,95)=2.39$, $p<0.05$]. Post hoc analyses revealed that freezing was significantly higher in OVX females compared to sham-operated females for CS Blocks 3 and 4.

A significant main effect of treatment was found during extinction recall [$F(1,19)=6.32$, $p<0.05$], where OVX females had significantly higher levels of freezing compared to sham-operated females (Figure 3.3C). This finding is very similar to the previously published findings in hormonal contraceptive treated rats (Graham and Milad, 2013), which was replicated in **Chapter 2**, and to a very recently published study using OVX rats in a cued fear conditioning paradigm (Graham and Daher, 2016). A significant main effect of CS Block was also found [$F(5,95)=26.03$, $p<0.001$]. Freezing levels in CS Block 1 were significantly higher than freezing levels in CS Blocks 2-6.

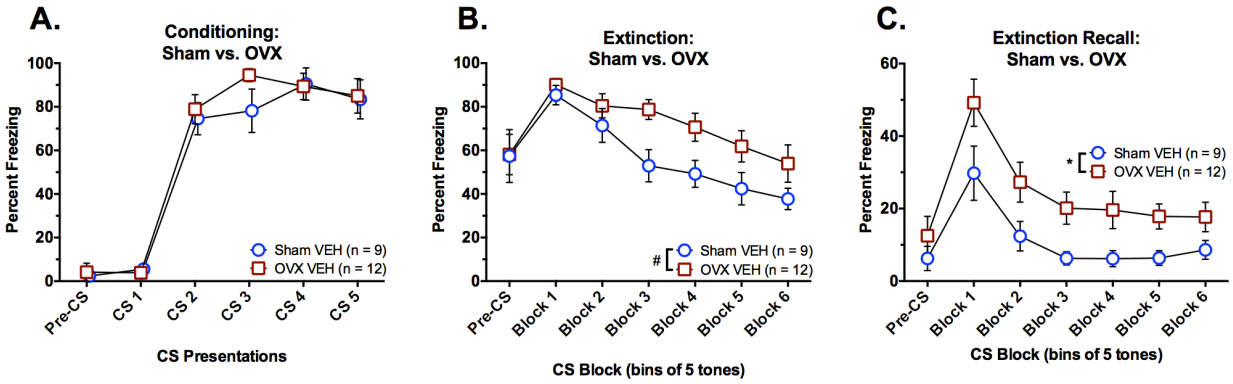

Figure 3.3 Effects of OVX on fear extinction consolidation.

A.) Freezing behavior did not differ between groups during the fear conditioning session. B.) A trend for increased freezing was found in OVX females during the extinction training session compared to sham operated vehicle-treated females (in proestrus at the time of extinction), #$p<0.10$. These data suggest that OVX females may be somewhat resistant to extinction. C.) OVX females spent significantly more time freezing compared to sham-operated females during the extinction recall session (*$p<0.05$), indicating that extinction consolidation is disrupted in the OVX females.

3.3.2 Experiment 2: Systemic losartan treatment rescues fear extinction consolidation deficit in OVX females

The purpose of this experiment was to test whether systemic treatment with AT1R antagonist losartan would enhance fear extinction consolidation in OVX females. Since systemic losartan treatment was shown to enhance extinction consolidation in HC-treated females with low levels of estradiol (**Chapter 2**), we predicted that systemic losartan treatment would enhance extinction consolidation in OVX females. No significant differences were found between groups during fear conditioning, $p>0.05$ (Figure 3.4A). A significant main effect of CS presentation was found [$F(4,84)=79.969$, $p<0.001$], where freezing during the first CS presentation was significantly lower than freezing levels for CS presentations 2-5. This indicates that fear to the CS presentations was comparably acquired by both groups across the conditioning session.

A significant main effect of treatment was found during extinction, [$F(1,21)=5.789$, $p<0.05$], where vehicle-treated OVX females spent significantly more time freezing compared to losartan-treated OVX females (Figure 3.4B). This difference in freezing between groups was mostly observed during the beginning of the extinction session, and by the end of the extinction session (Block 6), there was no significant difference between groups, $p>0.05$. A significant main effect of CS block was found [$F(5,105)=20.671$, $p<0.001$], where freezing during CS Block 1 was significantly higher compared to all other blocks in the extinction session.

A significant main effect of losartan treatment was found during the extinction recall session [$F(1,21)=6.223$, $p<0.05$], where OVX losartan-treated females exhibited significantly less freezing compared to OVX vehicle-treated females (Figure 3.4C). Thus, we have shown that systemic treatment with losartan rescues extinction consolidation deficits in both HC-treated females (**Chapter 2**) and OVX females. A significant main effect of CS block was found

[$F(5,105)=28.473$, $p<0.001$], where freezing levels during Block 1 were significantly higher compared to all other CS blocks in the session.

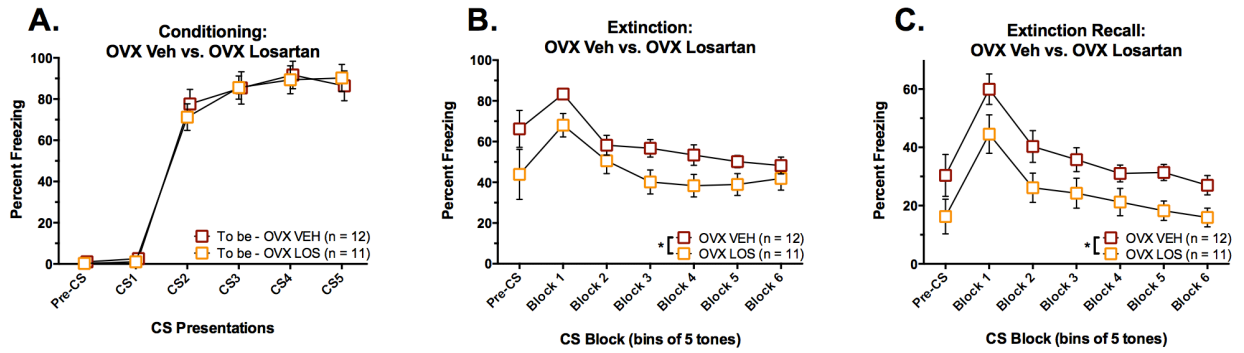

Figure 3.4 Systemic treatment with AT1R antagonist losartan rescues extinction consolidation deficit in OVX females.

A.) During conditioning, no differences in freezing were found between treatment groups. B.) Losartan-treated OVX females spent significantly less time freezing during the extinction session compared to OVX vehicle-treated females, *$p<0.05$; however, no significant differences in freezing were observed between groups during the last CS block, $p>0.05$. C.) Freezing levels were significantly reduced in OVX losartan-treated females during the extinction recall session compared to the OVX vehicle-treated group, *$p<0.05$. This indicates that losartan treatment enhanced extinction consolidation in OVX females.

3.3.3 Experiment 3: OVX females have significantly elevated AT1R ligand binding compared to intact proestrus females in the pituitary gland and ventral subiculum

Because we wanted to compare AT1R ligand binding in males, HC-treated females, and OVX females to AT1R ligand binding in intact proestrus females, t-tests were used to compare sample means for these comparisons. No significant differences were found between intact proestrus females and any other treatment group in the anterior and posterior piriform cortices (Figure 3.5A & B, respectively). AT1R ligand binding in intact proestrus females also did not differ in the BLA compared to any other treatment group (Figure 3.5C). Males had a trend level increase

in AT1R ligand binding in the PVN compared to intact proestrus females (p<0.10; Figure 3.5D). OVX females had significantly higher AT1R ligand binding levels in the pituitary gland [t(19)=2.933, p<0.01] and ventral subiculum [t(22)=1.804, p<0.05] compared to intact proestrus females, (Figure 3.5E & F, respectively). Due to high variability in peripheral tissue samples, no significant differences were found between intact proestrus females and any other treatment group in the adrenal gland or kidney (Figure 3.6A & B, respectively). However, males had a trend level decrease in AT1R ligand binding compared to intact proestrus females, (p<0.10; Figure 3.6C).

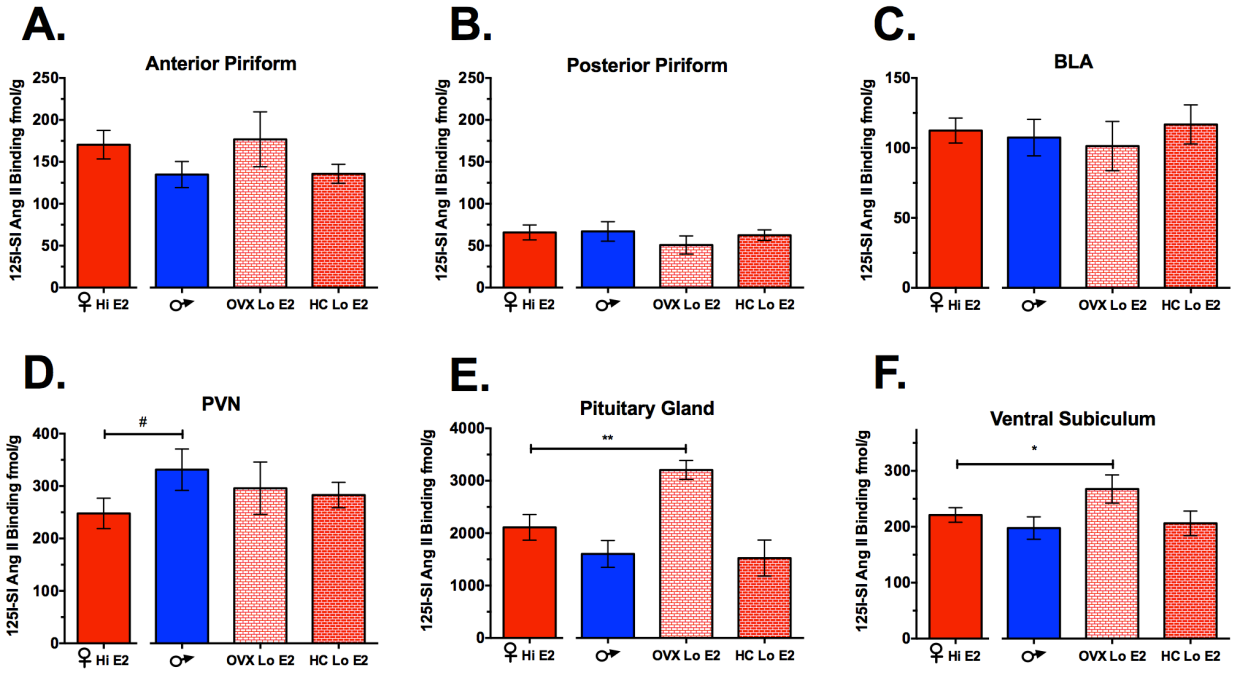

Figure 3.5 OVX females have significantly higher AT1R ligand binding in the pituitary gland and ventral subiculum compared to intact proestrus females.

No significant differences were found between intact proestrus females and any other treatment group in the A.) anterior piriform cortex, B.) posterior piriform cortex, or C.) BLA. D.) Males had a trend for increased AT1R binding in the PVN compared to intact proestrus females, #p<0.10. E.) OVX females had significantly higher AT1R ligand binding levels in the pituitary gland (**p<0.01) and F.) ventral subiculum (*p<0.05) compared to intact proestrus females.

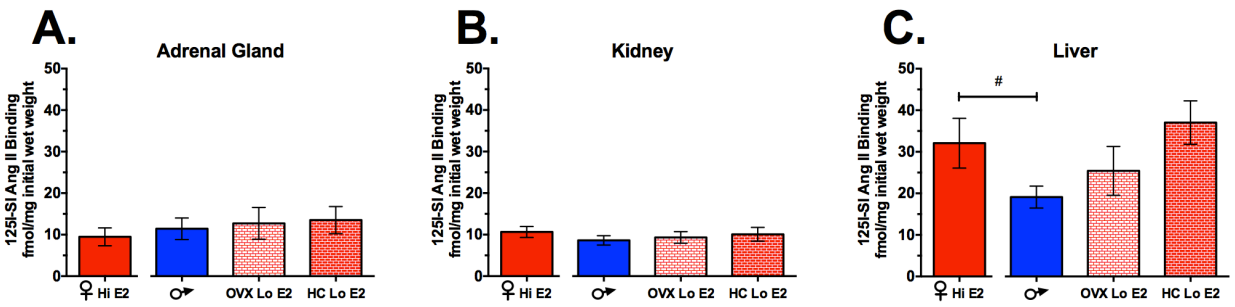

Figure 3.6 AT1R ligand binding does not significantly differ in peripheral tissues between intact proestrus females and all other groups.

No significant differences in AT1R ligand binding were detected in the A.) adrenal gland or B.) kidney. C.) In the liver, there was a trend for males to have less AT1R binding compared to intact proestrus females, #$p<0.10$.

3.4 DISCUSSION

Since no previous studies have tested how OVX affects extinction consolidation, this was an important gap in the literature that needed to be addressed, given the established role of estradiol on fear extinction. Thus, in Experiment 1 we tested whether OVX females would have impaired extinction consolidation compared to intact sham-operated proestrus females. Our main finding was that OVX females exhibited significantly elevated freezing during extinction recall compared to sham-operated controls with high estradiol levels. Because AT1R antagonism has been shown in my previous studies to enhance extinction consolidation in females with low levels of estradiol (**Chapter 2**), in Experiment 2 we wanted to test whether losartan would rescue the deficit in extinction consolidation in OVX females. Similar to my results from Chapter 2, we found that losartan treatment in OVX females resulted in enhanced extinction consolidation and reduced freezing during extinction recall compared to OVX vehicle-treated females. Finally, we wanted to further test how estradiol is modulating the RAS to affect fear extinction consolidation. We tested how AT1R ligand binding in intact proestrus females compares to AT1R ligand binding in males, OVX females, and HC-treated females in both central and peripheral tissues. This experiment will provide further information on the mechanism by which estradiol is modulating RAS components to affect fear-related behavior.

3.4.1 OVX and cued fear conditioning: What is currently known?

Despite the fact that many studies have shown that estradiol levels on the day of extinction training affect extinction consolidation (Milad et al., 2009a, Milad et al., 2010, Zeidan et al., 2011, Lebron-Milad et al., 2012b, Graham and Milad, 2013, 2014), none have ever

examined the effects of OVX on fear extinction consolidation. Instead, to ensure that females had low circulating levels of estradiol at the time of extinction training, previous studies have 1) monitored the estrous cycle and tested females during metestrus (Milad et al., 2009a), 2) chronically treated females with hormonal contraceptive (HC) levonorgestrel (Graham and Milad, 2013), or 3) systemically administered an estrogen receptor antagonist in females with high circulating levels of estradiol (Milad et al., 2009a). Although there are many studies that have tested how OVX affects behavior in fear-related paradigms, the majority of studies in the literature only focus on how OVX affects fear acquisition. These studies report no significant differences in fear acquisition between OVX vehicle- and estradiol-treated females in cued fear conditioning (Morgan and Pfaff, 2001), contextual fear conditioning (Gupta et al., 2001, Chang et al., 2009), and the shock probe paradigms (Gervais et al., 2014). The few studies that have examined the effects of OVX on fear extinction have done so in a contextual fear conditioning paradigm, and these studies have found that OVX estradiol-treated females spend significantly less time freezing across the extinction session compared to OVX vehicle-treated females (Gupta et al., 2001, Chang et al., 2009). Given that no prior studies have examined how OVX affects extinction consolidation in a cued fear conditioning paradigm at the time our experiments were conducted, this seemed like an important gap in the literature to address, especially given that many studies have found that low estradiol levels during extinction training result in poor extinction consolidation and increased fear during extinction recall.

3.4.2 OVX leads to disrupted extinction consolidation and increased fear during extinction recall

We found no significant differences in fear acquisition between OVX and sham-operated proestrus females, which is consistent with prior reports (Gupta et al., 2001, Morgan and Pfaff, 2001, Chang et al., 2009, Gervais et al., 2014). During extinction, we found a trend for increased freezing in the OVX group, which is consistent with previous findings in HC-treated females from our lab (**Chapter 2**) and other labs (Graham and Milad, 2013). This could indicate that chronically lowering estradiol levels results in some resistance to extinction, as indicated in the current study by increased levels of freezing in the OVX females towards to end of the extinction session compared to the sham-operated proestrus females. During extinction recall, OVX females displayed significantly higher levels of freezing compared to sham-operated proestrus controls, which is consistent with prior studies showing that lowering estradiol levels during extinction training results in an extinction consolidation deficit (Milad et al., 2009a, Milad et al., 2010, Zeidan et al., 2011, Lebron-Milad et al., 2012b, Graham and Milad, 2013, 2014). However, it should be noted that OVX surgery results in reduction of both estradiol and progesterone. In order to determine which hormone is primarily responsible for modulating fear extinction consolidation, another experiment would need to be run to test how treating OVX females with estradiol or progesterone would affect extinction consolidation.

While no studies had tested the effects of OVX on extinction consolidation in a cued fear conditioning paradigm at the time we were running Experiment 1, a recent study has examined the role of sex hormones on fear extinction consolidation by treating OVX females prior to extinction training with estradiol only or in combination with progesterone (Graham and Daher, 2016). This group found that treatment with estradiol alone improved extinction consolidation in

OVX females compared to the OVX vehicle-treated group. They also found that treatment with progesterone 6 hours prior to extinction training in OVX estradiol-treated females potentiated the effect of estradiol on fear extinction consolidation; however, treatment with progesterone 24 hours prior to extinction training in OVX estradiol-treated females completely abolished the effects of estradiol, resulting in fear levels similar to vehicle-treated OVX females during extinction recall (Graham and Daher, 2016). Previous studies have found that when OVX females are treated with estradiol, this results in increased hippocampal dendritic spine density (Woolley and McEwen, 1993). Additionally, when progesterone is given to OVX females that are also treated with estradiol, spine density further increases within 6 hours of progesterone treatment and decreases to basal levels 24 hours after progesterone treatment (Woolley and McEwen, 1993). Since poor extinction consolidation in OVX females treated with estradiol and progesterone occurred when hippocampal spine density was expected to be lowest and good extinction consolidation occurred when spine density was expected to peak, this suggests that hormone alterations in hippocampal dendritic spine density may affect fear extinction consolidation, although this mechanism needs to be directly tested (Graham and Daher, 2016). This was the first study to examine the effects of each hormone on fear extinction consolidation, and importantly we can conclude from this study that estradiol is the hormone providing the enhancement in fear extinction consolidation during proestrus, while progesterone either improves or abolishes the effects of estradiol, depending on when it is administered (Graham and Daher, 2016). Thus, we can conclude that the extinction consolidation deficit we observed in OVX females in Experiment 1 was most likely due to the lack of estradiol in the OVX females, rather than low progesterone levels.

3.4.3 AT1R antagonist losartan rescues extinction consolidation deficit in OVX females

The renin angiotensin system (RAS), which is typically studied in the context of blood pressure regulation and cardiovascular function, has recently been implicated in stress responding and has also been linked to stress-related pathologies (de Kloet et al., 2017). A recent retrospective study has found that a highly traumatized population of patients taking AT1R antagonists or angiotensin converting enzyme inhibitors to regulate blood pressure have significantly reduced symptoms of posttraumatic stress disorder (PTSD) compared to patients taking blood pressure medications that act independently of the RAS (Khoury et al., 2012). In addition, systemic administration of AT1R antagonist losartan has been shown to enhance extinction consolidation both in male mice (Marvar et al., 2013) and in female rats that had been chronically treated with HC levonorgestrel (**Chapter 2**). Thus, in Experiment 2, we wanted to test whether systemic treatment with losartan would enhance extinction consolidation in OVX females, which have previously been shown to have deficits in extinction consolidation (Experiment 1; (Graham and Daher, 2016)). We found that OVX losartan-treated females had significantly reduced freezing during extinction recall compared to OVX vehicle-treated females. We are the first to show that systemic treatment with AT1R antagonist losartan prior to extinction training enhances extinction consolidation in both HC-treated (**Chapter 2**) and OVX females, which both exhibit extinction consolidation deficits.

3.4.4 OVX females have increased AT1R ligand binding in the pituitary gland and ventral subiculum compared to intact proestrus females

Many studies have shown that OVX alters AT1R ligand binding compared to OVX estradiol-treated females. For instance, OVX estradiol-treated females have significantly reduced AT1R ligand binding in the adrenal glands, heart, SFO, PVN, MnPO, vascular organ of the lamina terminalis, kidney, abdominal aorta, and pituitary gland compared to OVX vehicle-treated females (Seltzer et al., 1992, Kisley et al., 1999, Wu et al., 2003b, Dean et al., 2005). However, no one has tested how treatment with HC levonorgestrel, which has been shown to reduce estradiol levels and impair fear extinction consolidation (**Chapter 2**; (Graham and Milad, 2013)), affects components of the RAS. A better understanding of how HC treatment is affecting the RAS could help clarify the mechanism by which low estradiol levels impair fear extinction memories in HC-treated females. We predicted that AT1R ligand binding would be significantly elevated in regions associated with fear and stress responding in HC-treated females compared to intact proestrus females. In addition, no studies have tested differences in central AT1R binding between normotensive male and female rodents, although some studies have shown that males have elevated AT1R levels in the periphery compared to females (Rogers et al., 2007, Sullivan, 2008). While males generally have lower circulating estradiol levels compared to females, testosterone can be aromatized to estradiol in many tissues including the brain (Gillies and McArthur, 2010). Therefore, we predicted that males would have increased AT1R ligand binding in peripheral tissues compared to intact proestrus females, and that AT1R ligand binding in central tissues would be comparable to intact proestrus females.

The major findings from the AT1R autoradiography studies (Experiment 3) are that OVX females have significantly increased AT1R ligand binding in the pituitary gland and ventral

subiculum compared to intact proestrus females. Although the anterior and posterior pituitary gland were analyzed together in this study, previous studies have shown that AT1R are primarily found in the anterior pituitary gland (Hauger et al., 1982), which is part of the hypothalamic pituitary adrenal (HPA) axis. In vitro, Ang II has been shown to stimulate the release of adrenocorticotropic hormone (ACTH), which is a hormone secreted by the anterior pituitary gland in response to a biological stressor, in rat anterior pituitary cells (Gaillard et al., 1981). In vivo, Ang II has been shown to increase levels of ACTH, though some studies suggest that this is mediated through Ang II increasing corticotropin-releasing hormone (CRH), rather than directly acting on the anterior pituitary gland (Ganong, 1993). Interestingly, OVX females also had significantly increased AT1R ligand binding in the ventral subiculum, a structure that has been shown to inhibit the HPA axis (Herman et al., 1995, Herman et al., 1998). While the effects of angiotensin signaling on the pituitary gland and ventral subiculum needs further exploration, it is possible that activation of AT1Rs in these regions during extinction training could increase HPA axis activity, which has been shown to affect extinction consolidation. In a rodent model of PTSD, elevated HPA axis activity has been associated with poor extinction consolidation, while dampening HPA axis activity enhances extinction consolidation (Andero et al., 2011, Sawamura et al., 2016). Therefore, during extinction training, which is a time when rodents behaviorally exhibit high levels of fear, OVX females with low estradiol levels and increased AT1R levels in the pituitary gland and ventral subiculum may exhibit increased AT1R activation in these regions which could lead to increased HPA axis activity. This may result in impaired extinction consolidation, which could be reversed by pre-extinction session treatment with an AT1R antagonist. Future studies should test how AT1R activation in the pituitary gland and ventral

subiculum affects HPA axis activity, and whether antagonizing AT1R in these regions reverses the deficit found in extinction consolidation in OVX females.

No significant differences in AT1R ligand binding were found between groups in anterior or posterior piriform cortices, which are AT1R hotspot regions. The anterior piriform cortex plays an important role in odor memory (Wilson, 1998), and in some studies the amygdala has been shown to project to the piriform cortex (Majak et al., 2004). No significant differences in AT1R ligand binding were found between groups in the BLA, which may not be surprising, as other studies have reported that there is low AT1R binding density in this region (Hurt et al., 2015). While levels of AT1R ligand binding in males were similar to intact proestrus females in most central regions that were tested, males did have a trend for increased AT1R ligand binding in the PVN, which is part of the HPA axis and is involved in the release of CRH during times of stress (Herman et al., 2016), compared to intact proestrus females. AT1R ligand binding did not significantly differ between HC-treated females and intact proestrus females, which may be due to various factors. While this was certainly unexpected, at the time of this autoradiography study, a parallel behavioral study found no effect of HC treatment on extinction consolidation. Therefore, it may be difficult to draw too many conclusions from the HC-treated group in this study.

Some of the samples that were collected for peripheral AT1R autoradiography analysis had to be excluded due to the data being extreme outliers. This led to a significant loss of sample size in each group. With the sample size that is left, we are currently underpowered to detect significant differences between groups. A power analysis was run on this data, and approximately 25 samples per group would be needed to achieve 80% power for a one-tailed t-test at 5% significance level for the adrenal gland dataset. We found no significant differences in

AT1R ligand binding in the adrenal gland between groups, which was surprising, given that other studies have reported that estradiol regulates AT1R levels in this region (Wu et al., 2003a, Dean et al., 2005). There were no significant differences between groups in AT1R ligand binding in the kidney, which is inconsistent with other studies (Armando et al., 2002, Rogers et al., 2007). However, studies in the literature typically examine AT1R ligand binding in the glomeruli, while we were looking at binding levels in both the cortex and medulla of the kidney, which could explain why we failed to detect differences between groups. Interestingly, we found that males have a trend for reduced AT1R levels in the liver compared to intact proestrus females, which is inconsistent with other studies showing that treatment of rat liver cells with an estrogen metabolite results in significantly reduced AT1R (Koganti et al., 2012).

Although we collaborated with several labs in an attempt to measure Ang II peptide levels in plasma of rodents in Experiment 3, this was not possible for many reasons. Please refer to General Discussion section 4.3.1 for more details.

3.4.5 Summary and Conclusions

In conclusion, our findings in this study have important implications for treatment of fear and anxiety disorders, particularly for women, who are nearly twice as likely to develop an anxiety disorder such as PTSD compared to men (Kessler et al., 1995). We have found that systemic administration of AT1R antagonist losartan, which is commonly used for treatment of hypertension (Ripley and Hirsch, 2010), prior to extinction training can reverse the deficit induced by low estradiol levels in HC-treated females (**Chapter 2**) and OVX females (**Chapter 3**). We have also found that AT1R ligand binding is elevated in the pituitary gland and ventral subiculum, both of which are known to affect HPA axis activity, in OVX females. During

extinction training, AT1R activation in these regions may lead to increased HPA axis activity and impaired extinction consolidation. Blockade of AT1Rs prior to extinction training could dampen HPA axis activity, which we predict will lead to improved extinction consolidation, as suppressing the HPA axis has been shown to enhance extinction consolidation in rodent models of PTSD (Sawamura et al., 2016).

4.0 GENERAL DISCUSSION

4.1 SUMMARY OF FINDINGS

4.1.1 Importance of studying fear extinction mechanisms in females

PTSD is a devastating illness, which often results in social and occupational impairments, as well as deficits in other areas of functioning (APA, 2013). Thus, early intervention strategies are preferred, so that patients do not develop chronic PTSD and can live a fully functioning life (Foa, 2000). Females are more than twice as likely to develop anxiety disorders such as PTSD compared to men (Kessler et al., 1995), even when controlling for factors such as type of traumatic event, prior traumatic experiences, preexisting mood disorders, and sex differences in reporting (Tolin and Foa, 2006, Breslau, 2009). One possible explanation for the higher prevalence of PTSD diagnosis in females may be at least partly attributed to differences in fear extinction processes between males and females (Maeng et al., 2015); however, females have largely been excluded in the fear extinction literature (Cover et al., 2014). This may be due, in part, to the challenges of monitoring the estrous cycle across studies and accounting for sex hormone effects on behavior (Maeng et al., 2015). It is important that we better understand the mechanisms underlying fear extinction in females so that better treatments can be developed for women who are diagnosed with PTSD.

4.1.2 Estradiol modulates fear extinction consolidation: Could the RAS be involved?

Recently, many studies have examined the role that gonadal hormone levels on the day of extinction training play in the consolidation of fear extinction memories. Specifically, these studies have found that low estradiol levels on the day of extinction training lead to poor extinction consolidation and increased fear during extinction recall (Milad et al., 2009a, Milad et al., 2010, Zeidan et al., 2011, Lebron-Milad et al., 2012b, Lebron-Milad and Milad, 2012, Graham and Milad, 2013, Cover et al., 2014, Graham and Milad, 2014, Maeng et al., 2015). We were able to replicate findings from these studies in **Chapter 2**, where females treated with HC levonorgestrel, which reduces estradiol levels to that of metestrus females (Graham and Milad, 2013), had significantly elevated freezing during extinction recall compared to vehicle-treated females in proestrus. However, information regarding the mechanism by which estradiol is mediating these effects is largely unknown. The main goal of this thesis was to explore the mechanism by which estradiol was regulating fear extinction consolidation. Because the renin angiotensin system (RAS) has been implicated in stress related pathologies (Yang et al., 1996, Saavedra, 2005, de Kloet et al., 2017), and estradiol is a well-known modulator of the RAS, the central hypothesis of these studies was that estradiol modulates the RAS to affect fear extinction consolidation (refer to Figure 1.6 for a model of the central hypothesis). Because AT1R antagonists have previously been shown to reduce anxiety (Saavedra, 2005, Wang et al., 2016), improve affect (Pavlatou et al., 2008), reduce PTSD symptoms (Khoury et al., 2012, Nylocks et al., 2015), and enhance extinction consolidation in male mice (Marvar et al., 2013), we first wanted to test whether systemic administration of AT1R antagonist losartan would improve fear extinction consolidation in HC-treated females. We are the first to show that systemic treatment with losartan prior to extinction training enhances extinction consolidation and reduces freezing

during extinction recall in HC-treated females (**Chapter 2**). This finding suggests that administering an AT1R antagonist can improve extinction consolidation in females with low levels of estradiol, who typically have impairments in fear extinction consolidation.

4.1.3 OVX impairs extinction consolidation in a cued fear conditioning paradigm, and treatment with an AT1R antagonist reverses the deficit

Despite the fact that many studies have investigated the effects of estradiol levels on fear behavior, no studies had examined how OVX affects extinction consolidation in a cued fear conditioning paradigm at the time our experiments were conducted. Many studies have tested how OVX affects behavior in contextual fear conditioning paradigms (Gupta et al., 2001, Chang et al., 2009), and those studies that have tested the effects of OVX on fear behavior in a cued fear conditioning paradigm often only look at the initial conditioning day (Morgan and Pfaff, 2001, Jasnow et al., 2006). Therefore, this was a gap in the literature that needed to be filled. Consistent with previous studies, we report that OVX does not significantly alter fear acquisition. However, we show that OVX significantly impairs extinction consolidation in a cued fear conditioning paradigm, resulting in elevated levels of freezing during extinction recall compared to intact sham-operated proestrus females (**Chapter 3**). This confirms previous findings from Milad and colleagues who suggest that estradiol is important for extinction consolidation (Milad et al., 2009a, Milad et al., 2010, Zeidan et al., 2011, Lebron-Milad et al., 2012b, Lebron-Milad and Milad, 2012, Graham and Milad, 2013, 2014, Maeng et al., 2015, Maeng et al., 2017). Recently, a similar study has been published showing that treatment with estradiol improves fear extinction consolidation in OVX females, and that progesterone either augments this effect or completely abolishes it, depending on time of treatment (Graham and

Daher, 2016). Thus, this study further confirms the role of estradiol in the consolidation of fear extinction memories.

Since OVX resulted in fear extinction consolidation deficits similar to those observed in our HC-treated females (**Chapter 2**), we next tested whether systemic losartan treatment prior to extinction training would improve fear extinction consolidation in OVX females. We are the first to show that systemic losartan prior to extinction training results in improved extinction consolidation and decreased fear during extinction recall in OVX females compared to OVX vehicle-treated females (**Chapter 3**). Thus, we have now shown that systemic treatment with losartan improves extinction consolidation deficits that result from low estradiol levels in both HC-treated and OVX females. Our findings suggest that losartan can be administered prior to an exposure therapy session to enhance fear extinction consolidation and improve treatment outcome in females that have low circulating estradiol levels.

4.1.4 Hormone manipulations affect components of the RAS

Because we have shown that treatment with an AT1R antagonist improves extinction consolidation in females with low estradiol, we next wanted to test how low estradiol levels affect components of the RAS. Understanding how hormonal manipulations affect the RAS is important, as this could give us a better understanding of the mechanism behind the regulation of fear extinction consolidation by estradiol. We specifically wanted to test how AT1R levels are affected in HC-treated and OVX females, which both exhibit extinction consolidation deficits. Since estradiol is known to downregulate components of the RAS, including AT1R (Fischer et al., 2002), we predicted that HC-treated and OVX females, which both have low estradiol levels, would have increased AT1R ligand binding compared to intact proestrus females with high

circulating estradiol levels. In addition, upon further examination of the literature, only one study to our knowledge has compared AT1R binding levels between normotensive males and females in the brain (Yu et al., 2010). However, results from this study should be interpreted with caution, as Western Blots were used to measure AT1R levels, and there are currently no specific antibodies for the AT1R (Benicky et al., 2012). Therefore, we also wanted to compare central AT1R binding levels between males and females, with the prediction that males and females in proestrus would have similar AT1R ligand binding levels in the brain, since males can aromatize testosterone to estradiol in many tissues, including the brain (Gillies and McArthur, 2010). The main findings from the AT1R autoradiography studies were that OVX females had significantly increased AT1R ligand binding in the pituitary gland and ventral subiculum compared to intact females in proestrus. Our findings suggest that AT1R activation in these regions, which have been shown to play a role in HPA axis regulation, in OVX females may lead to impairments in extinction consolidation.

4.2 HOW IS ESTRADIOL MODULATING THE RAS TO REGULATE FEAR EXTINCTION CONSOLIDATION, AND HOW MIGHT AT1R ANTAGONISM BE REVERSING EXTINCTION CONSOLIDATION DEFICITS IN FEMALES WITH LOW CIRCULATING ESTRADIOL LEVELS?

4.2.1 OVX females have increased AT1R ligand binding in the pituitary gland and ventral subiculum, and AT1R activation in these regions may increase HPA axis activity and impair extinction consolidation

Recent studies have shown that elevated HPA axis activity is associated with poor extinction consolidation in a rodent model of PTSD, and that dampening HPA axis activity in these rodents enhances extinction consolidation (Andero et al., 2011, Sawamura et al., 2016). Interestingly, the main findings of our AT1R autoradiography studies revealed that OVX females had significantly elevated AT1R ligand binding in the pituitary gland and ventral subiculum, both regions that affect HPA axis activity, compared to intact proestrus females. In the pituitary gland, AT1R are primarily found in the anterior rather than posterior pituitary gland (Hauger et al., 1982), and the anterior pituitary gland is part of the HPA axis and releases ACTH in response to stressors. While in vitro studies suggest that Ang II can directly stimulate release of ACTH from pituitary cells (Gaillard et al., 1981), in vivo studies suggest that Ang II most likely mediates the increase in ACTH through its actions in the circumventricular organs (Ganong, 1993), though further study is needed. Interestingly, we also found that AT1R ligand binding in OVX females was increased in the ventral subiculum, another brain region that plays a role in modulating the HPA axis (Herman and Mueller, 2006). Lesions of the ventral subiculum have been found to enhance CRF mRNA and CRF peptide expression in the PVN and elevate corticosterone levels following restraint stress (Herman et al., 1995, Herman et al., 1998). While the role of angiotensin

signaling in the ventral subiculum is currently unknown, this needs to be explored in future studies. It is possible that Ang II signaling in this region could interfere with the ventral subiculum inhibition of the HPA axis of females with low estradiol, which would lead to increased HPA axis activity and poor extinction consolidation.

During extinction training, a time in which fear responses are high, we propose that Ang II, which is increased both in the brain and periphery in response to stressors (Saavedra and Benicky, 2007, Saavedra et al., 2011), binds to AT1Rs in the pituitary gland and ventral subiculum, which results in increased HPA axis activity, and poor extinction consolidation in OVX females. We propose that treatment with an AT1R antagonist prior to the extinction training session in OVX females will block AT1Rs in these regions and blunt the neuroendocrine response to stress, thereby enhancing extinction consolidation and reducing fear during extinction recall. Future studies will explore how components of the HPA axis (i.e. CRH, ACTH, corticosterone) are affected in OVX vehicle-treated and OVX losartan-treated females during the extinction consolidation window, and whether AT1R blockade in the pituitary gland or ventral subiculum reverses the deficit in extinction consolidation in OVX females.

4.3 FUTURE DIRECTIONS

4.3.1 Measurement of angiotensin peptides in plasma

In order to better understand how estradiol is regulating the RAS, it is important to measure circulating angiotensin peptide levels. Our data indicate that HC treatment does not significantly alter AT1R ligand binding; however, our preliminary data indicate that HC treatment affects

circulating Ang II peptide levels. We found that HC-treated females had significantly elevated levels of Ang II compared to vehicle-treated proestrus females with high circulating levels of estradiol (**Chapter 2**). However, this kit is potentially detecting other angiotensin peptides in our plasma samples in addition to Ang II, including angiotensin I, angiotensin III, angiotensin IV and angiotensinogen. Due to the concentrations of angiotensin II in plasma being very small, accurately measuring Ang II and other angiotensin peptides in plasma can be a challenge (Kobori et al., 2007, Ali et al., 2014). Many previously published studies have used high performance liquid chromatography, which has been shown to be a necessary step for more accurate measurement of Ang II (Bragat et al., 1997), followed by radioimmunoassay (Ali et al., 2014). Recently, other studies have determined that angiotensin peptides can be separated by mass difference in biological samples using mass spectrometry (Cui et al., 2007, Ali et al., 2014).

Our lab has been in collaboration with the Mass Spectrometry Center at the Medical College of Wisconsin, which has developed a protocol for measuring angiotensin peptides via mass spectrometry (Cui et al., 2007). We are currently sending plasma samples to the Mass Spectrometry Center at the Medical College of Wisconsin so that they will be able to measure angiotensin peptides in my plasma samples. We understand that this data will be an important addition to the AT1R ligand binding data that I have already collected and analyzed, as this will help us to create a clearer picture of how levels of estradiol affect RAS components. The AT1R ligand binding data indicate that males have a trend for increased AT1R ligand binding in the PVN, while OVX females have significantly increased AT1R ligand binding in the ventral subiculum and the pituitary gland. In Chapter 1, treatment with HC resulted in increased Ang II peptide levels, though we are awaiting confirmation on this piece of data with mass spectrometry.

4.3.2 Is losartan acting centrally to enhance extinction consolidation?

In addition to the work that I described in Chapters 2 & 3, I spent a considerable portion of time trying to determine where losartan was acting in the brain to enhance fear extinction consolidation in females with low estradiol levels. These studies were conducted primarily on HC-treated females, and they are described in detail in Appendices A and B. In summary, it was very difficult for us to test where losartan was acting in the brain to reduce fear during extinction recall in our HC-treated females, as the extinction consolidation deficit that was typically observed in HC-treated females was abolished by intracranial surgery. This happened in several experiments over a period of several months, so the lack of an extinction consolidation deficit in HC-treated females following intracranial surgery was not simply due to a coincidental finding (**Appendix A**). To further examine how surgery exposure was affecting fear behavior in our HC-treated females, we examined how exposure to ketamine, which we use for all intracranial surgeries, affected fear extinction consolidation in a cued fear conditioning paradigm. Ketamine has been recently found to blunt the effects of an inescapable stressor when given up to two weeks prior to stress exposure (Amat et al., 2016). Interestingly, our initial findings indicated that just being exposed to the surgery room (without anesthesia exposure) for a few hours was sufficient to abolish the deficit in extinction consolidation we typically observe in HC-treated females. However, follow up studies testing the effects of surgery room exposure on fear extinction consolidation were inconclusive (**Appendix B**). While it is currently unclear why surgery room exposure affects fear extinction consolidation in our HC-treated females, future studies conducted in female rats may want to ensure that exposure to surgery is not driving behavioral effects.

Determining where losartan is acting to enhance fear extinction consolidation – centrally, peripherally, or both – would enable us to better understand the mechanism by which AT1R antagonism affects fear in females with low estradiol levels. One way to circumvent the issues we encountered in our previous studies (**Appendices A & B**) would be to test the effects of central losartan in OVX females. While it is not clear how intracranial surgery will affect extinction consolidation in OVX females, our data seem to suggest that the intracranial surgical procedure itself does not affect extinction consolidation in HC-treated females. Since OVX females (who are exposed to surgery in our surgery suite) exhibit an extinction consolidation deficit, we are optimistic about the possibility of testing where losartan is acting centrally to enhance fear extinction consolidation. Based on our findings in **Chapter 3**, we would be interested in targeting the pituitary gland (this is possible with an intrapituitary infusion (Nikitovitch-Winer, 1962)) and ventral subiculum.

4.4 CLINICAL IMPLICATIONS AND CONCLUSIONS: HOW CAN WE IMPROVE TREATMENT OF ANXIETY-RELATED DISORDERS SUCH AS PTSD?

4.4.1 Monitoring estradiol levels in females

Previous studies indicate that low estradiol levels during extinction training lead to poor extinction consolidation and increased fear during extinction recall in both women and female rats (Milad et al., 2009a, Milad et al., 2010, Zeidan et al., 2011, Lebron-Milad et al., 2012b, Lebron-Milad and Milad, 2012, Graham and Milad, 2013, Maeng et al., 2015, Graham and Daher, 2016, Maeng et al., 2017). We have replicated these findings in HC-treated (confirmed

low estradiol) and OVX females (**Chapters 2 & 3**). Thus, it is very important that females have *high* levels of estradiol during the time of treatment with exposure therapy, which is currently the most effective therapy for treatment of PTSD (Foa, 2000, Barlow, 2002) and has many similarities to extinction training (Rothbaum and Davis, 2003, Craske et al., 2008). Monitoring estradiol levels has been done in other labs (Sakuragi et al., 1981, Milad et al., 2010, Roos et al., 2015), and this procedure should be feasible for treatment clinics. Aromatase inhibitors, which are prescribed in men to increase levels of testosterone (Winer et al., 2005, de Ronde and de Jong, 2011), should be ceased prior to exposure therapy, as male rats treated with an aromatase inhibitor before or immediately after extinction training exhibited significant impairments in fear extinction consolidation (Graham and Milad, 2014). It may also be beneficial in some cases to treat patients with estradiol prior to an exposure therapy session, as preclinical studies suggest that treatment with estradiol or an ERβ agonist in females with low estradiol before or immediately after extinction training results in reduced fear during extinction recall (Milad et al., 2009a, Zeidan et al., 2011, Graham and Milad, 2013).

4.4.2 AT1R antagonists can be used in the clinic to improve treatment outcomes in patients that have low estradiol levels

We are the first to show that systemic treatment with AT1R antagonist losartan prior to extinction training results in significant improvements in fear extinction consolidation in both HC-treated females and OVX females (**Chapters 2 & 3**, respectively). Therefore, treatment with an AT1R antagonist, which has been previously shown to reduce PTSD symptoms in highly traumatized patients with hypertension and increase extinction consolidation in male mice (Khoury et al., 2012, Marvar et al., 2013), should be considered if exposure therapy will occur

when patients have low levels of estradiol. Treatment with an AT1R antagonist in patients with low estradiol levels should enhance extinction consolidation and reduce responses to fear-related cues outside the treatment clinic. While it would not be harmful for patients with high estradiol levels to take an AT1R antagonist prior to exposure therapy, this treatment may not have much benefit for the patient. Females with high circulating estradiol levels that were treated with losartan in our studies (**Chapter 2**) did not show significantly reduced freezing during extinction recall, as fear levels in high estradiol females were already very low. Recent studies provide genetic evidence that some individuals may respond better to AT1R antagonists as treatment for PTSD, so identifying individuals that might benefit the most from this treatment is critical (Nylocks et al., 2015). Because the Food and Drug Administration has already approved the use of AT1R antagonists, such as losartan, in humans (Ripley and Hirsch, 2010), AT1R antagonists could be used in clinics today to improve treatment outcomes in men and women undergoing exposure therapy to treat PTSD.

APPENDIX A

INTRACRANIAL SURGERY ABOLISHES EXTINCTION CONSOLIDATION DEFICIT IN HC-TREATED FEMALES

A.1 INTRODUCTION

Posttraumatic stress disorder (PTSD) is a devastating illness, where core symptoms include persistent intrusive memories of the trauma, increased arousal, and avoidance of people, places, and things associated with the traumatic event (Kessler et al., 1995). Although one of the most successful ways to treat PTSD is exposure therapy (Foa, 2000, Barlow, 2002), studies have shown that patients with PTSD have impaired consolidation of the extinction memory (Milad et al., 2008, Milad et al., 2009b), meaning that patients will continue to experience fear and anxiety outside the treatment context. In order to develop better treatments for PTSD, it is important that the mechanisms underlying fear extinction are fully understood, especially in women, who are diagnosed with the disorder at nearly twice the rate as men (Kessler et al., 1995).

Recent studies have revealed that hormone levels affect fear extinction consolidation. Specifically, if estradiol levels are low during extinction training, this will result in impaired extinction consolidation and increased fear during extinction recall in both women and female rats (Milad et al., 2009a, Milad et al., 2010, Zeidan et al., 2011, Lebron-Milad et al., 2012b,

Graham and Milad, 2013, Maeng et al., 2017). Treatment with oral estradiol or an estrogen receptor agonist prior to extinction training in women or female rats with low estradiol results in significantly improved extinction consolidation and reduced fear during extinction recall compared to low estradiol vehicle-treated females (Graham and Milad, 2013). Interestingly, similar findings have been published in male rats, where males have impaired extinction consolidation when the conversion of testosterone to estradiol is blocked prior to the extinction training session (Graham and Milad, 2014). Although estradiol levels play a significant role in fear extinction consolidation, the mechanism underlying this process has not been uncovered.

The renin angiotensin system (RAS), which has recently emerged as a key mediator of the stress response and stress-related pathologies (de Kloet et al., 2017), may play an important role in how estradiol is modulating fear extinction consolidation. While the RAS has been thoroughly studied in the context of blood pressure regulation and cardiovascular function, RAS activation has also been associated with fear-related behavior. For instance, when an AT1R antagonist losartan is administered systemically prior to extinction training in male mice, fear is reduced the following day during extinction recall compared to the vehicle-treated group (Marvar et al., 2013). Similarly, a retrospective study in a highly traumatized population of human subjects found that patients taking angiotensin II type 1 receptor (AT1R) antagonists or angiotensin converting enzyme (ACE) inhibitors to treat hypertension reported reduced PTSD-related symptoms compared to subjects taking blood pressure medications that act independently of the RAS (Khoury et al., 2012). Thus, negative regulation of the RAS seems to enhance fear extinction consolidation in male mice and reduce PTSD-related symptoms in humans. Estradiol is well-known modulator of the RAS, downregulating many of its components including AT1R and the ACE (Nickenig et al., 1998, Brosnihan et al., 1999, Fischer et al., 2002). While estradiol

regulation of the RAS has been thoroughly studied, further investigation is needed to test how estradiol modulation of the RAS alters fear.

We have previously found that systemic administration of AT1R antagonist losartan enhances extinction consolidation and reduces fear during extinction recall in hormonal contraceptive (HC) treated female rats with low estradiol levels compared to vehicle-treated females with high estradiol levels (**Chapter 2**). We next wanted to test whether losartan was acting centrally to mediate these effects. Thus, an infusion of losartan was delivered into the basolateral amygdala (BLA; Experiment 1) or the ventricular system (Experiment 2) of females with low circulating estradiol levels prior to or immediately following the extinction training session. In addition, while circulating levels of estradiol are known to affect fear extinction consolidation, it is unclear how central estradiol levels regulate fear memories. Thus, in Experiment 3 an intracerebroventricular (ICV) infusion of an estrogen receptor antagonist was administered to rats with high levels of circulating estradiol immediately following extinction training.

A.2 METHODS

Subjects

Adult female Sprague Dawley rats aged 60-65 days were socially housed with a regular light-dark cycle, where food and water were provided *ad libitum*. Experimental procedures were performed during the light cycle and were carried out in accordance with the policies implemented by the University of Pittsburgh Institutional Animal Care and Use Committee.

Drugs

Levonorgestrel, a HC, was dissolved in a 1:1 solution of deionized water and dimethyl sulfoxide (DMSO) and administered subcutaneously at a dose of 0.5mg/kg/day. Losartan potassium, an AT1R antagonist, was dissolved in sterile saline and administered via an intracranial infusion (doses and regions vary; see details in experiments below). Fulvestrant, and estrogen receptor antagonist, was dissolved in a 1:1 solution of DMSO and 0.9% saline and administered via an intracranial infusion at a dose of 50 μg (0.5 μL/side).

Surgical procedures

Ketamine hydrochloride (85 mg/kg, i.m.) and xylazine hydrochloride (5 mg/kg, i.m.) were used to anesthetize each rat. Rats also received an analgesic (Rimadyl, 5 mg/kg, s.c.) and lactated Ringer's (3 mL, s.c.). After the rat was placed into a stereotaxic instrument, betadine and 70% ethanol were used to clean the incision site. For targerting of the BLA, two stainless steel guide cannula were implanted (AP -2.8, ML ± 5.0, DV -7.9, relative to bregma (Paxinos and Watson, 2007)) and secured to the skull surface with 3 small screws and dental acrylic resin. For ICV targeting, a single stainless steel guide was implanted and secured to the skull surface (AP -1.0 mm, ML +1.6 mm, DV -3.0 mm, relative to bregma (Paxinos and Watson, 2007)). A dummy cannulae that was the length of the guide cannulae was inserted into the guide cannulae to maintain patency. Rimadyl (5 mg/kg, s.c.) was administered for the first two days following surgery.

Fear Conditioning Protocol

Apparatus and Stimuli: Behavioral testing occurred in Operant Test Chambers contained within sound-proof cubicles, equipped with a house light, tone generator, and an exhaust fan

(Med Associates, Inc., St. Albans, VT). The fear conditioning chambers consisted of rod floors, two nosepoke apertures, and Accel disinfectant was sprayed into the floor tray (context A). Chambers used for extinction and extinction recall consisted of grid floors, five nosepoke apertures, and almond scent was sprayed into the floor tray prior to testing (context B).

Fear Conditioning Procedure: Rats were placed in an operant chamber (context A) and given 3 minutes to acclimate before 5 tones (10 seconds each, 1 minute ITI) were presented, where each tone co-terminated with a 1 mA foot shock. Immediately following testing, rats were placed in their home cage and left undisturbed until testing the next day.

Extinction training and Extinction Recall Procedures: Extinction training occurred 24 hours following the fear conditioning procedure, and rats were placed into a different operant chamber (context B). Following a 3-minute acclimation period, 30 tones were presented (10 seconds, 1 minute ITI) in the absence of footshock. Following the session, estrous cycle was monitored via vaginal lavage (see details below), and rats were returned to their home cage. Extinction recall was tested 24 hours after extinction training. Rats were placed into context B and presented with 30 tone-only presentations and returned to their home cage following testing.

Behavioral assessment: All behavioral testing was video recorded. A trained, blinded scorer assessed freezing (fear) behavior, defined as the lack of movement other than breathing, for rats during all testing phases. The amount of time (seconds) the rats spent freezing during each tone presentation was recorded.

Estrous cycle monitoring

Estrous cycle was monitored immediately after extinction training. A pipette tip containing 150 μL of saline was gently inserted into the vagina of the rat and saline was flushed into the vaginal canal. The sample was pipetted onto a clean microscope slide, and a coverslip

was placed on the slide. Slides from each rat were examined under a light microscope, and estrous cycle phase was determined by observation of cellular morphology according to published protocols (Goldman et al., 2007).

Histological Analysis

After experiments were completed, rats with cannula in the BLA were sacrificed via rapid decapitation. Brains were collected and placed in 10% formalin for 72 hours and then transferred to 30% sucrose for at least 72 hours. Brains were sectioned coronally on a cryostat, and 50μm sections containing the BLA were collected onto slides. A light microscope was used to help identify infusion placements, and animals with infusions outside of the BLA were excluded from the study. For rats with ICV cannula, rats were euthanized with CO_2, and 10 μL of bromophenol blue was infused into the guide cannula. The brain was then taken out of the skull, and a razor blade was used to cut the brain coronally at the level of the guide cannula. Any rat with bromophenol blue outside the ventricular system was excluded from the study.

Experiment 1: Effect of intracranial infusion of losartan into the BLA on extinction consolidation in HC-treated females. Rats underwent intracranial surgery to implant guide cannulae (28 gauge; Plastics One) bilaterally into the BLA. After recovering from surgery, all rats received daily injections of HC 4 days prior to and throughout the fear conditioning paradigm. The cued fear conditioning paradigm was administered. Rats received bilateral infusions of losartan (50 pmol; 0.5 μL/side) or vehicle into the BLA immediately after the extinction training session. Extinction recall was tested 24 hours later. Refer to Figure A.1A for a timeline of the study.

Experiment 2: Effect of ICV losartan on extinction consolidation in HC-treated female rats. All rats received ICV cannula implantation. After recovery, rats received daily injections of

HC 4 days prior to and throughout the fear conditioning paradigm. The fear conditioning paradigm was administered. Prior to extinction training, rats were infused with losartan (100 μg; 10 μL) or vehicle. A separate group of animals received an infusion of losartan (10 μg or 100 μg; 10 μL) or vehicle immediately following the extinction training session. Extinction recall was tested 24 hours later. Refer to Figure A.1B for an experimental timeline.

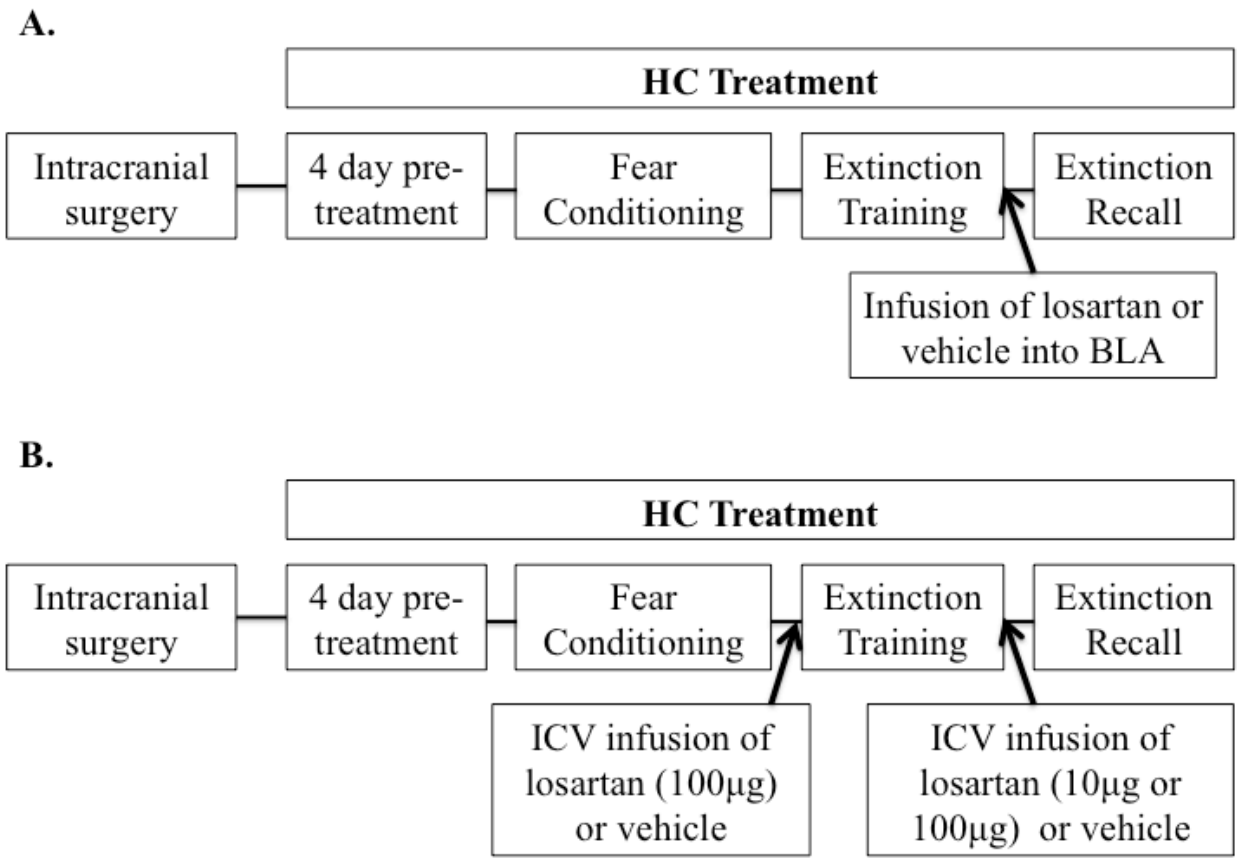

Figure A.1 Timeline for Experiments 1 & 2.

A.) Rats were implanted with cannula in the BLA. Following recovery, rats were treated daily with HC 4 days prior to and throughout the fear conditioning paradigm. Rats received infusions of losartan or vehicle into the BLA immediately following extinction training. B.) Rats underwent ICV cannulation surgery and were treated daily with HC after recovery 4 days prior to and throughout the fear conditioning paradigm. ICV infusions of losartan or vehicle occurring either immediately before or after the extinction training session.

Experiment 3: Effect of ICV fulvestrant on extinction consolidation in freely cycling female rats. All rats received ICV cannula implantation. After recovery, the fear conditioning paradigm was administered to freely cycling female rats (Figure A.2). Fulvestrant (50 μg; 10 μL) or vehicle was infused immediately following the extinction training session, and extinction recall was tested 24 hours later. All females were in proestrus at the time of extinction training.

Statistical Analysis

Statistical analyses were conducted using SPSS Software for Mac. A repeated measures ANOVA was used to analyze all behavioral data. Bonferroni post-hoc comparisons were performed when appropriate.

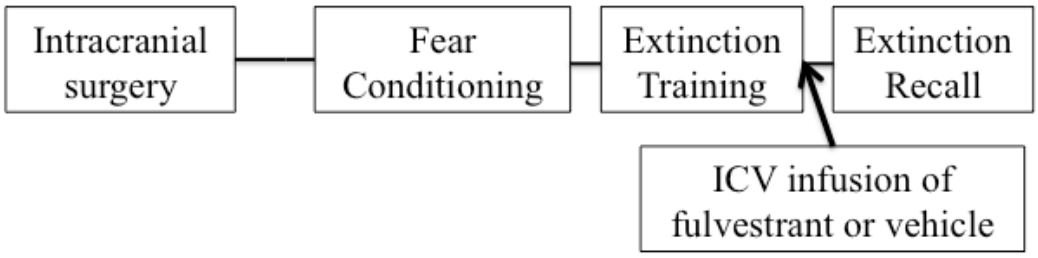

Figure A.2 Timeline for Experiment 3.

Rats underwent ICV cannulation surgery. The fear conditioning paradigm was administered to freely cycling female rats, and rats were infused with fulvestrant or vehicle immediately following extinction training.

A.3 RESULTS

Experiment 1: Effect of BLA infusion of losartan on extinction consolidation in HC-treated females. The purpose of this experiment was to test whether bilateral infusions of losartan into the BLA would enhance fear extinction consolidation in rats with low estradiol levels. Since

systemic losartan treatment was able to reverse the extinction consolidation deficits that were found in HC-treated females, we predicted that treatment with losartan in the BLA, a region that has been shown to be important for extinction memory consolidation (Berlau and McGaugh, 2006), would enhance extinction consolidation in HC-treated females. No significant differences in freezing were found between treatment groups during fear conditioning, $p>0.05$ (Figure A.3a). A significant main effect of CS presentation was found [$F(4,64)=49.426$, $p<0.001$], where freezing during CS presentations 3-5 was significantly higher than freezing during CS presentations 1 and 2. These findings indicate that fear to the CS presentations was acquired comparably across the session.

No differences in freezing were found between groups during the extinction session, $p>0.05$ (Figure A.3b). A significant main effect of CS block was detected [$F(5,80)=9.223$, $p<0.001$], where freezing to the tone was significantly reduced during the final blocks of the extinction session compared to freezing levels at the beginning of the session. During extinction recall, a main effect of CS block was found [$F(5,80)=7.162$, $p<0.001$], where freezing during CS blocks 3-5 was significantly less than freezing during CS block 1. Surprisingly, HC-treated females that were treated with vehicle prior to extinction training had low levels of freezing during extinction recall, so no significant effect of losartan treatment was found, $p>0.05$ (Figure A.3c).

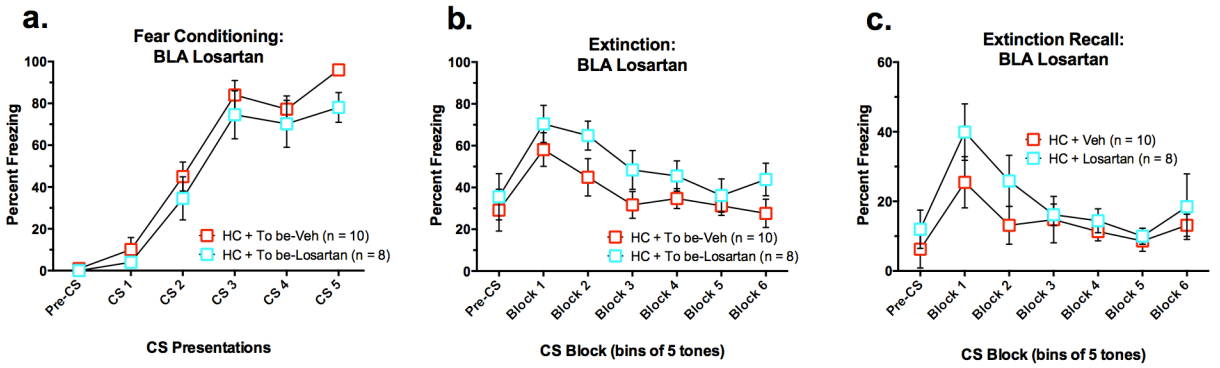

Figure A.3 Post-extinction session treatment with losartan in the BLA has no effect on extinction consolidation in HC-treated females.

a.) There were no significant differences in freezing between groups during the fear conditioning session. b.) No differences in freezing were observed between groups during the extinction session. Estrous cycle was monitored via vaginal lavage immediately after extinction, and all HC-treated females were confirmed to be in low estradiol phases (estrus, metestrus, or diestrus). c.) Losartan treatment did not significantly alter fear extinction consolidation in HC-treated females, as both treatment groups displayed similar amounts of freezing during extinction recall.

Experiment 2: Effect of ICV losartan on extinction consolidation in HC-treated female rats. Since losartan treatment did not significantly alter fear extinction consolidation when it was administered in the BLA, we next tested whether losartan treatment would enhance extinction consolidation if it were given ICV in HC-treated females. Although no differences in freezing were found between groups during fear conditioning ($p>0.05$; Figure A.4a), a main effect of CS presentation was detected [$F(4,92)=82.427$, $p<0.001$]. Females spent significantly less time freezing during CS presentations 1 and 2 compared to CS presentations 3-5.

No significant differences in freezing were found between treatment groups during the extinction session ($p>0.05$; Figure A.4b). However, a significant main effect of CS block was found [$F(5,115)=21.339$, $p<0.001$]. Rats spent significantly less time freezing during the final blocks of the extinction session compared to the beginning of the session. Similarly, a main effect of CS block was detected during extinction recall [$F(5,115)=10.310$, $p<0.001$], where the

amount of time spent freezing during the final blocks of the session was significantly less than freezing levels during the beginning of the session. Due to the low levels of freezing in all HC-treated groups during extinction recall, no significant main effect of losartan treatment was found, $p>0.05$ (Figure A.4c).

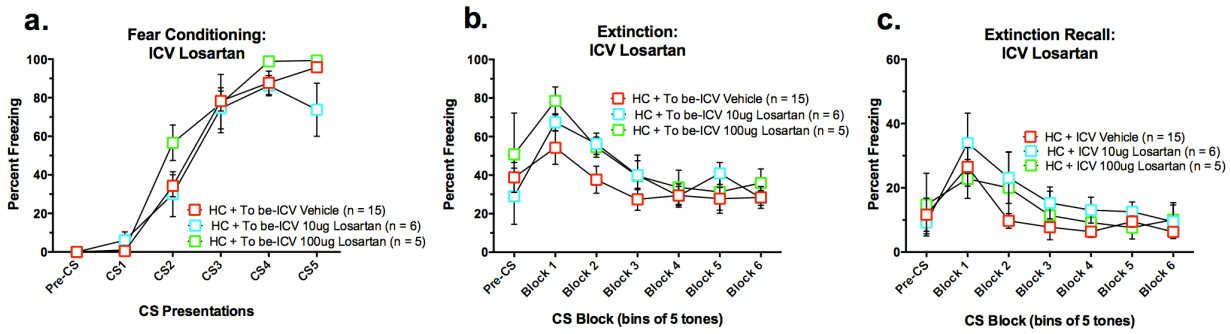

Figure A.4 Post-extinction session ICV treatment with losartan has no effect on extinction consolidation.

a.) All treatment groups acquired fear comparably during the fear conditioning session. b.) No significant differences in freezing were observed between groups during the extinction session. c.) All treatment groups froze similarly during extinction recall, indicating that losartan treatment had no effect on extinction consolidation.

Experiment 3: Effect of ICV fulvestrant on extinction consolidation in freely cycling female rats with high estradiol levels. While studies have shown that systemic estradiol levels on the day of extinction training affect extinction consolidation (Milad et al., 2009a, Milad et al., 2010, Zeidan et al., 2011, Lebron-Milad et al., 2012b, Lebron-Milad and Milad, 2012, Graham and Milad, 2013, 2014), there have been no studies that test how central estradiol levels impact fear extinction consolidation. Thus, we tested how a post-extinction session ICV infusion of estrogen receptor antagonist fulvestrant would affect fear extinction consolidation. We predicted that treatment with fulvestrant would impair extinction consolidation in females with high estradiol, resulting in increased fear during extinction recall.

No differences were found between groups during fear conditioning ($p>0.05$; Figure A.5a); however, there was a main effect of CS presentation [$F(4,40)=53.777$; $p<0.001$]. Freezing

during the first CS presentation was significantly lower compared to CS presentations 2-5. A significant main effect of CS block was found during extinction training [$F(5,50)=29.128$, $p<0.001$], where freezing during blocks 1 and 2 was significantly higher than the remaining blocks in the session. No significant differences in freezing were found between groups during the extinction session, $p>0.05$ (Figure A.5b). There was no significant main effect of fulvestrant treatment during extinction recall ($p>0.05$; Figure A.5c), which may be partly due to the high levels of freezing in the vehicle-treated proestrus females. Although a significant main effect of CS block was found [$F(5,50)=5.238$; $p=0.001$], post hoc analyses revealed no significant differences in levels of freezing between CS blocks.

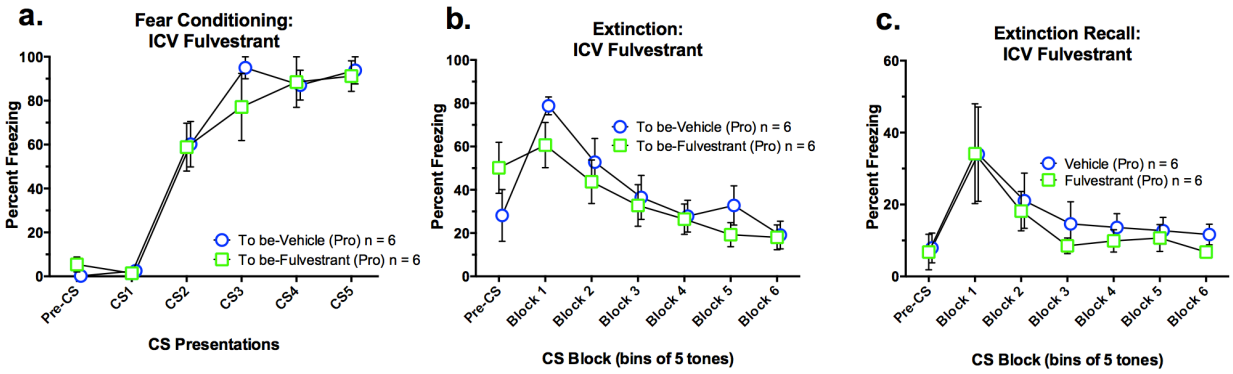

Figure A.5 ICV fulvestrant treatment administered post-extinction session had no effect on extinction consolidation in proestrus female rats.

a.) There were no significant differences in freezing between groups during fear conditioning. b.) No significant differences in freezing levels were detected between groups during the extinction session. c.) The amount of time spent freezing did not differ between groups during extinction recall, indicating that fulvestrant did not have an effect on fear extinction consolidation.

A.4 DISCUSSION

We predicted that an intracranial infusion of losartan would enhance extinction consolidation and reduce freezing during extinction recall in HC-treated females compared to vehicle-treated HC females. We also predicted that an intracranial infusion of estrogen receptor antagonist, fulvestrant, would impair extinction consolidation and increase freezing during extinction recall in female rats with high circulating levels of estradiol. However, we found that losartan was unable to significantly reduce freezing during extinction recall in HC-treated females when it was administered intracranially (BLA or ICV). In addition, an ICV infusion of fulvestrant was unable to produce a deficit in females with high circulating levels of estradiol. In all three studies, the 'control' group seemed to be producing unexpected results. Vehicle-treated HC females exhibited less freezing behavior than what we typically observe in surgery naïve HC-treated females during extinction recall, and vehicle-treated females with high circulating estradiol levels had slightly higher and more variable levels of freezing during extinction recall compared to surgery naïve vehicle-treated females. These data are summarized in Figure A.6.

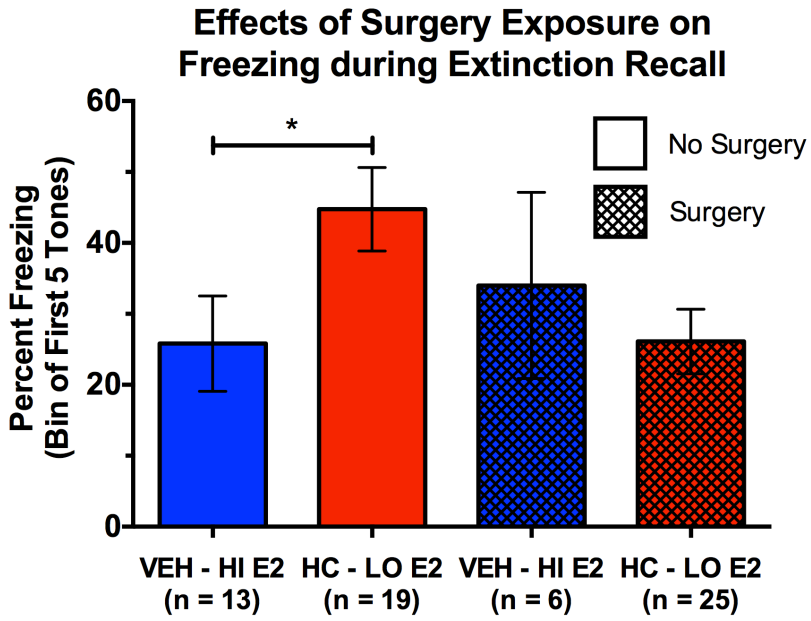

Figure A.6 Surgery exposure alters fear expression during extinction recall.

Surgery naïve HC-treated females have significantly higher levels of freezing during extinction recall compared to surgery naïve vehicle-treated females with high circulating estradiol levels. However, in females that received intracranial surgery prior to behavioral testing, HC-treated females no longer display a deficit in extinction consolidation and have similar levels of freezing as vehicle-treated females without prior surgery.

Since many of our studies involving intracranial surgery yielded unexpected results, especially in HC-treated females, it seemed that there was some component of the intracranial surgery was altering fear-related behavior when it was tested weeks later. We initially thought that ketamine, which is the anesthetic used for all intracranial surgeries described thus far, may be having an effect on fear-related behavior. A recent study has shown that systemic administration of ketamine enhances extinction consolidation and reduces freezing during extinction recall when it is given 24 hours before the extinction session (Girgenti et al., 2017). Similar to this study, many other studies in the literature have examined the fast-acting effects of ketamine, such as its rapid antidepressant actions (Machado-Vieira et al., 2009, Salvadore and Singh, 2013, Abdallah et al., 2015). However, the immediate effects of ketamine on behavior are

not as relevant for our studies, since ketamine administration occurred at least 1-2 weeks prior to fear behavior testing.

Although the fast-acting effects of systemic ketamine have been widely studied, a few studies have shown that ketamine has prophylactic effects when given 1-2 weeks prior to a stressor. Amat and colleagues found that an intraperitoneal (i.p.) injection of ketamine (10mg/kg) administered 2 hours, 1 week, or 2 weeks prior to an inescapable, uncontrollable tail shock stressor prevented the typical decrease in social exploration during the juvenile social investigation test and blunted the increase in extracellular levels of serotonin in the BLA that are typically increased when exposed to the tail shock stressor (Amat et al., 2016). A more recent study has shown that an i.p. injection of ketamine (30mg/kg) given 1 week prior to a contextual fear conditioning (CFC) paradigm significantly reduced freezing during an extinction session administered 4 days after the CFC procedure compared to vehicle-treated animals (McGowan et al., 2017). Ketamine administered 1 hour or 1 month prior to CFC did not produce the same effects during extinction (McGowan et al., 2017). While no studies have tested the prophylactic effects of ketamine exposure two weeks prior to a cued fear conditioning paradigm, these studies suggest that ketamine can affect fear and anxiety-like behaviors when given 1-2 weeks prior to testing.

Even though the anesthetic dose of ketamine administered in our studies was much higher than what was given in previous studies, it is unclear whether this higher dose of ketamine given up to two weeks prior to the cued fear conditioning paradigm will affect fear-related behavior. Also, most studies examining the effects of ketamine have been done in males, so it would be interesting to test how ketamine exposure affects fear-related behavior in females.

Future studies will explore how exposure to an anesthetic dose of ketamine affects behavior two weeks later in the cued fear conditioning paradigm.

APPENDIX B

THE EFFECTS OF ANESTHESIA AND SURGERY ROOM EXPOSURE ON FEAR BEHAVIOR

B.1 INTRODUCTION

Many studies have reported that hormone levels during extinction training play a significant role in how effectively fear extinction memories are consolidated. Specifically, in both women and female rats, low estradiol levels on the day of extinction lead to poor extinction consolidation and increased fear during extinction recall (Milad et al., 2009a, Milad et al., 2010, Zeidan et al., 2011, Lebron-Milad et al., 2012b, Lebron-Milad and Milad, 2012, Graham and Milad, 2013, Cover et al., 2014, Graham and Milad, 2014, Maeng et al., 2017). Similar findings have been reported in males, where blocking the conversion of testosterone to estradiol just before or immediately after extinction training results in impaired extinction consolidation and elevated fear during recall (Graham and Milad, 2014). Our lab has been able to replicate these findings in females treated with a progestin-only hormonal contraceptive (HC) that downregulates estradiol levels, where HC-treated females have impaired fear extinction consolidation and increased fear during extinction recall compared to vehicle-treated females with high estradiol levels (Graham and Milad, 2013) (also, refer to **Chapter 2**). Our lab is the first to show that HC-treated females

that receive systemic treatment with losartan, an angiotensin II type I receptor (AT1R) antagonist, prior to the extinction training session have improved extinction consolidation and reduced fear during extinction recall (**Chapter 2**). One of the goals of future studies was to test where losartan was acting centrally to improve extinction consolidation in HC-treated females. To allow for site-specific drug delivery into the brain, cannulae were surgically implanted into the appropriate brain region 1-2 weeks prior to any drug treatments or behavior. After the recovery period, HC treatment was administered 4 days prior to and throughout the fear conditioning paradigm, and losartan treatment was centrally infused before or immediately following the extinction training session (**Appendix A**).

Although we were able to replicate extinction consolidation deficits in HC-treated females without prior surgery, we found in several studies that HC-treated females that received intracranial surgery prior to hormone treatment did not display extinction consolidation deficits and exhibited low levels of freezing during extinction recall. Ketamine, which is the anesthesia used in our lab for all intracranial surgeries, has recently been shown to blunt both behavioral and neurochemical effects of an inescapable, uncontrollable tail shock stressor when it is given via an intraperitoneal injection up to two weeks prior to the stressor (Amat et al., 2016). The purpose of this experiment was to determine whether intramuscular (i.m.) ketamine could be contributing to the lack of extinction consolidation deficit in HC-treated females. We also tested how isoflurane, an inhalational anesthetic, would impact fear extinction consolidation, with the plan to move forward using isoflurane for future studies involving intracranial surgery if HC-treated females had similar deficits to females without surgery. We predicted that ketamine exposed HC-treated females would not display an extinction consolidation deficit and that this group would have low freezing during extinction recall. This study also included a group of

females that were exposed to the surgery room that received no anesthesia exposure and would later be subdivided into HC- and vehicle-treated groups. We predicted that the HC-treated group that was not exposed to anesthesia would have impaired extinction consolidation and increased fear during extinction recall compared to the no anesthesia vehicle-treated group.

B.2 METHODS

Refer to A.2 Methods for general methods procedures.

Experiment 1: Effect of anesthesia exposure on extinction consolidation in HC- and vehicle-treated female rats. All rats were taken to the surgery suite, where they received one of three possible treatments. One group of rats received an i.m. injection of saline into each hind leg, the second group was given intramuscular injections of ketamine and xylazine (same as described in surgical procedures section), and the third group was given an i.m. injection of saline into each leg and immediately exposed to isoflurane anesthesia for approximately 45 minutes. Rats remained in the surgery room for 2-3 hours after treatment and then were taken back to the housing room. Rats were weighed each day for the next five days, which represents the typical recovery period from surgery. Rats were then treated with HC or vehicle 4 days prior to and throughout the fear conditioning paradigm. Refer to Figure B.1 for an experimental timeline.

Experiment 2: Effect of surgery room exposure on extinction consolidation in HC- and vehicle-treated female rats. One group of rats was carted to the surgery suite, while the other group of rats was left undisturbed in the housing room. In the surgery suite, each rat received an i.m. injection of saline into each leg and was placed in a clean cage with her cage mate for 2 hours. Rats were then carted back to the housing room, where all rats remained undisturbed. Six

days following the exposure, all rats received daily injections of HC or vehicle 4 days prior to and throughout the fear conditioning paradigm. Refer Figure B.2 for an experimental timeline.

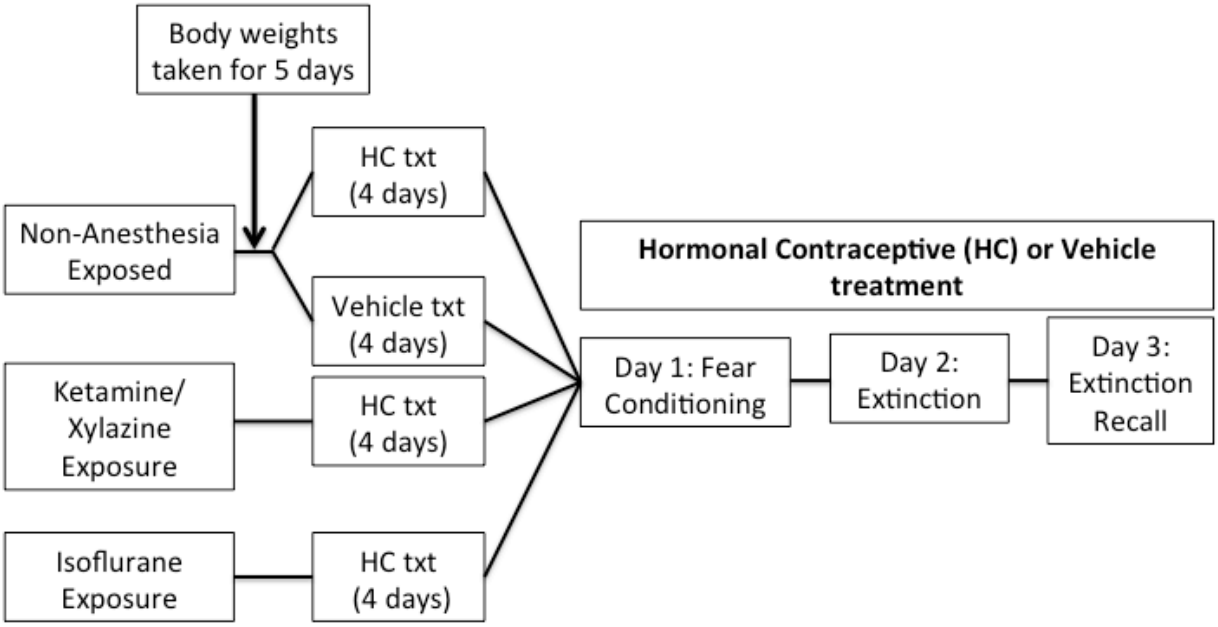

Figure B.1 Timeline for Experiment 1.

In the surgery suite, rats were either given an i.m. injection of saline into each leg, an i.m. injection of ketamine and xylazine, or exposed to i.m. saline injections and isoflurane anesthesia. Rats remained in the surgery suite for 2-3 hours after treatment and were taken back to the housing room. Rats received daily HC or vehicle treatment 4 days prior to and throughout the fear conditioning paradigm.

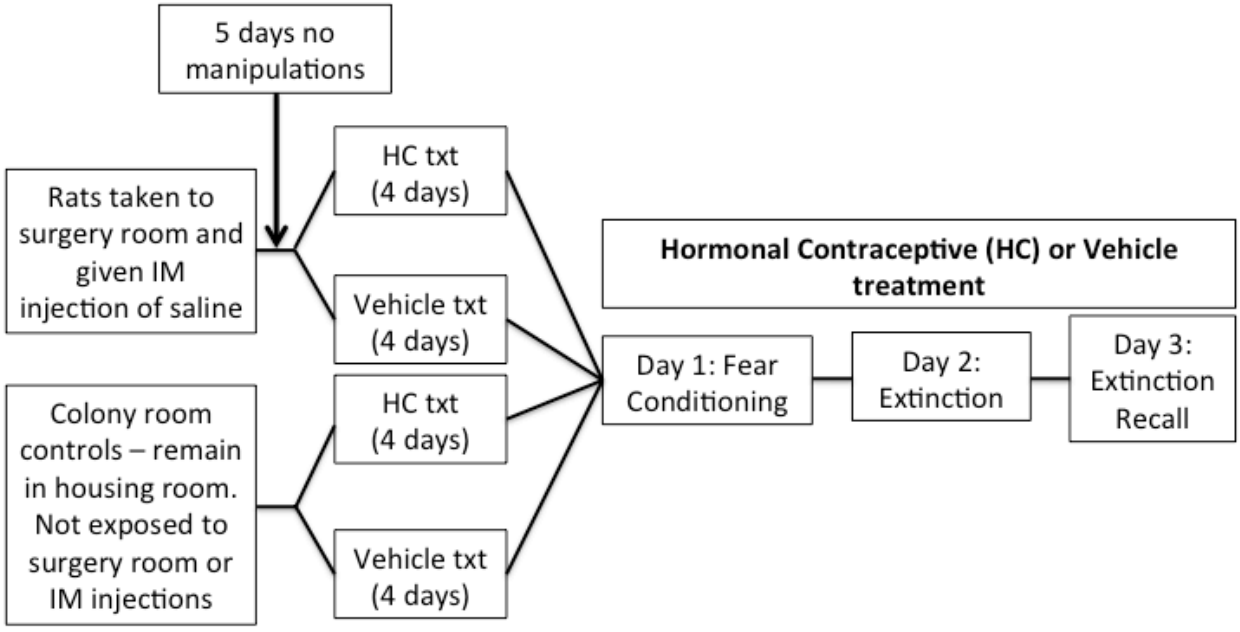

Figure B.2 Timeline for Experiment 2.

One group of rats was taken to the surgery room and given an i.m. injection of saline into each leg, while the other group was left undisturbed in the housing room. Rats were treated daily with HC or vehicle 4 days prior to and throughout the fear conditioning paradigm.

B.3 RESULTS

Experiment 1: Effect of anesthesia exposure on extinction consolidation in HC- and vehicle-treated female rats. The purpose of this study was to test how exposure to anesthesia affects fear extinction consolidation in HC- and vehicle-treated females. We predicted that ketamine exposure would abolish the extinction consolidation deficit that is typically observed in HC-treated rats, and that this group would have reduced fear during extinction recall. We also predicted that HC-treated females that were exposed to the surgery suite, but not anesthesia, would exhibit increased fear during extinction recall compared to vehicle-treated rats that were

not exposed to anesthesia. No significant differences in the amount of time spent freezing were detected between treatment groups during fear conditioning, $p>0.05$ (Figure B.3a). There was a significant main effect of CS presentation [$F(4,120)=113.061$, $p<0.001$], where rats froze less during the first two CS presentations compared to CS presentations 3-5. During extinction, no significant effects of treatment were uncovered, $p>0.05$ (Figure B.3b). A main effect of CS block was found [$F(5,150)=85.789$, $p<0.001$], where freezing tended to decrease across the extinction session. No significant main effect of anesthesia or HC treatment was found during extinction recall $p>0.05$ (Figure B.3c). A main effect of CS block was found [$F(5,150)=14.190$, $p<0.001$], where freezing tended to decrease across the session. Although a CS block by anesthesia exposure interaction was found, post hoc analyses revealed no significant differences in the amount of time spend freezing for each CS presentation between anesthesia exposure groups, $p>0.05$.

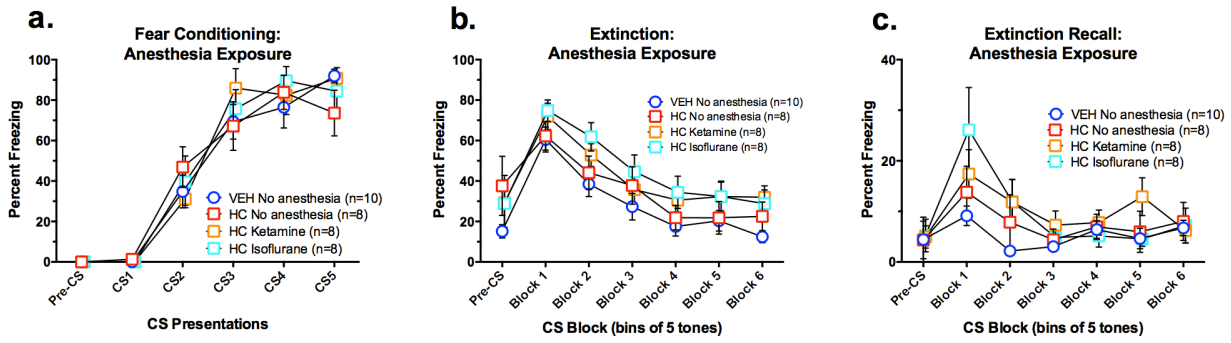

Figure B.3 Effects of anesthesia exposure on the fear conditioning protocol.

a.) There were no significant differences between groups during fear conditioning. b.) No significant effects of treatment were found during the extinction session. c.) HC-treated females exposed to anesthesia did not exhibit the impairment in extinction consolidation that has been reported in females with low estradiol levels. Strangely, exposure to the surgery room itself seems to abolish the HC deficit in rats that were exposed to the surgery room but not exposed to anesthesia.

Experiment 2: Effect of surgery room exposure on extinction consolidation in HC- and vehicle-treated female rats. In Experiment 1, we found that HC-treated females exposed to the surgery suite, but not anesthesia, did not display the extinction consolidation deficit that we typically observe in non-surgery exposed females. Therefore, we decided to test how exposure to the surgery room only would affect extinction consolidation compared to surgery suite naïve females. We predicted that HC-treated females without surgery room exposure would display a deficit in extinction consolidation and increased freezing during extinction recall, and this effect would be abolished in HC-treated females that were exposed to the surgery suite.

There was a significant main effect of HC treatment found during fear conditioning, [$F(1,34)=13.249$, $p=0.001$]. HC-treated females spent significantly more time freezing compared to vehicle-treated females. An HC treatment by surgery room exposure interaction was also uncovered, [$F(1,34)=8.98$, $p<0.01$]. In surgery room naïve females, HC treated females froze significantly more than vehicle treated females, [$F(1,15)=20.975$, $p<0.001$]. No significant effect of HC treatment was found in surgery room exposed females. Finally, a significant main effect of CS presentation was found [$F(4,136)=141.574$, $p<0.001$], where freezing to the first two tone presentations was significantly lower compared to CS presentations 3-5 (Figure B.4a).

A significant HC by surgery room exposure interaction was found during the extinction session, [$F(1,34)=4.88$, $p<0.05$]. There was a trend for surgery room naïve HC-treated females to spend more time freezing compared to vehicle-treated surgery room naïve females, $p=0.098$. Post hoc analyses revealed no significant differences in the amount of time spent freezing between surgery room exposed HC- and vehicle-treated females. A CS block by HC treatment interaction was also found during the extinction session, [$F(5,170)=2.664$, $p<0.05$]. However, post hoc analyses only revealed that there was a trend for increased freezing in HC-treated

females during CS block 1, p=0.075. Finally, a significant main effect of CS block was found [F(5,170)=47.353, p<0.001], where freezing was significantly reduced for the last CS blocks of the extinction session (Figure B.4b).

During extinction recall, a significant main effect of CS block was found, [F(5,145)=9.762, p<0.001]. Freezing during block 1 was significantly higher than freezing during the rest of the CS blocks (Figure B.4c). No significant main effects of treatment were found for extinction recall.

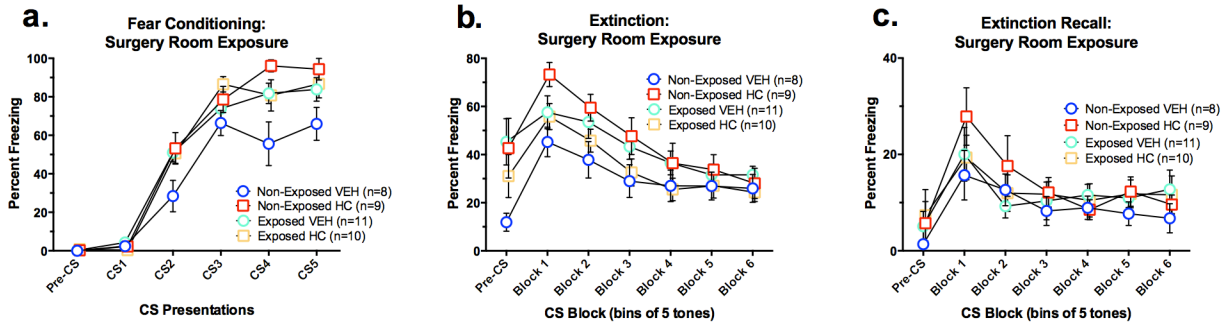

Figure B.4 Effects of surgery room exposure on the fear conditioning protocol.

a.) During fear conditioning, HC-treated females spent significantly more time freezing compared to vehicle-treated females. This effect was driven by the surgery room naïve vehicle-treated group, which exhibited significantly less freezing than surgery room naïve HC-treated group. b.) A trend for increased freezing was found in the surgery room naïve HC-treated group compared to the surgery room naïve vehicle-treated group. However, this was probably due to the vehicle-treated group not acquiring fear as well as the HC-treated group during the fear conditioning session. c.) HC treatment did not result in an extinction consolidation deficit in the surgery room exposed or surgery room naïve groups. However, data from the surgery room naïve groups are difficult to interpret due to differences in fear acquisition during fear conditioning.

B.4 DISCUSSION

Many studies have demonstrated that ketamine has fast-acting antidepressant effects (Machado-Vieira et al., 2009, Salvadore and Singh, 2013, Abdallah et al., 2015); however, there are a few studies showing that an intraperitoneal injection of a ketamine (10-30 mg/kg) can blunt the effects of an inescapable tail shock stressor and enhance extinction in a contextual fear conditioning paradigm if given 1-2 weeks prior to testing (Amat et al., 2016, McGowan et al., 2017). Since prior experiments involving intracranial surgery had yielded unexpected results, particularly in HC-treated females, we decided to test how the anesthetic dose of ketamine we use in our intracranial surgeries was affecting behavior in HC- and vehicle-treated female rats during the fear conditioning paradigm. In Experiment 1, we predicted that HC-treated rats exposed to ketamine would not exhibit a deficit in extinction consolidation and that this group would have low freezing levels during extinction recall. We also predicted that isoflurane would not affect fear-related behavior and that HC-treated rats exposed to isoflurane would have increased freezing during extinction recall. Finally, we predicted that HC- and vehicle-treated anesthesia naïve females that were only exposed to the surgery room would display similar behavior to HC- and vehicle-treated females in previously published studies (Graham and Milad, 2013) and our initial findings (**Chapter 2**), where anesthesia naïve vehicle-treated rats with high circulating levels of estradiol would exhibit significantly less freezing during extinction recall compared to the anesthesia naïve HC-treated females. Unexpectedly, we found that all HC-treated groups, regardless of anesthesia treatment, did not display a deficit in extinction consolidation. Instead, all HC-treated rats had low levels of freezing that were similar to the vehicle-treated females with high circulating levels of estradiol. The results from this study suggest that just exposing rats to the surgery room alone was sufficient to abolish the deficit in

extinction consolidation that is typically observed in HC-treated females that have no prior anesthesia or surgery exposure. Thus, these results indicate that the surgery room itself may be acting as a stressor, which could lead to changes in fear-related behavior when it is tested weeks later.

There are a few studies in the literature indicating that exposure to a stressor hours or days before testing has an impact on fear-related behavior. One study found that women with high estradiol levels had improved extinction recall when fear conditioning was preceded by exposure to a stressor, while the opposite was true for women with low estradiol, who had poor extinction retention when they were exposed to a stressor prior to fear conditioning (Antov and Stockhorst, 2014). Another study examined how a two-hour restraint stress exposure two days prior to a contextual fear conditioning paradigm affected fear behavior in male rats. This study found that stress exposure increased freezing during the retention test administered 24 hours following conditioning, which indicates that stress exposure enhances contextual fear memory consolidation (Cordero et al., 2003). While no differences were found between stress and non-stress exposed rats in the cued fear conditioning paradigm, extinction recall was never tested (Cordero et al., 2003). Finally, an intraperitoneal injection of corticosterone (CORT) immediately following contextual fear conditioning enhanced fear memory consolidation in females with high estradiol and impaired fear memory consolidation in females with low estradiol (Kashefi and Rashidy-Pour, 2014). While these studies highlight the effects of stress on behavior during the fear conditioning paradigm, no one has examined the long term effects of stress on extinction consolidation using a cued fear conditioning paradigm. Since the results from Experiment 1 demonstrated that exposure to the surgery room may be altering fear behavior in the cued fear conditioning paradigm weeks later, our goal for Experiment 2 was to compare

females exposed to the surgery room to surgery room-naïve females, with the prediction that the HC deficit we observe in surgery naïve females would be abolished in the females who had prior surgery room exposure.

In Experiment 2, no significant differences in freezing were found between surgery room exposed HC- and vehicle-treated groups during extinction recall, which supports our hypothesis. However, there were also no significant differences in freezing levels during extinction recall between HC- and vehicle-treated surgery room-naïve females. Data from the surgery room-naïve groups is difficult to interpret, due to the vehicle-treated group exhibiting significantly less freezing during conditioning compared to the HC-treated group. These results were surprising, as findings from our lab and other labs have indicated that HC- and vehicle-treated females acquire fear similarly during the conditioning session (Graham and Milad, 2013) (**Chapter 2**). Because these findings in the surgery room-naïve group make our data difficult to interpret, follow up experiments should be done to confirm that a deficit in extinction consolidation is found in the HC-treated surgery room-naïve females and to test how surgery room exposure affects extinction consolidation in HC-treated females.

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