Sex Differences in Retrieval of Context Fear: Behavioral and Neural Mechanisms

by

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DEDICATION

In loving memory of the amazing women who raised me, my mom Michelle Lynn Schmeling and my grandma Carol Marguerite Winn. You always deserved the world but you gave it to me instead, this is for you.

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ABSTRACT

The behavioral and neural mechanisms underlying consolidation and retrieval of fear-related memories have been defined over many years. However, the majority of established theories of learning and memory have been based on data derived from predominantly male animals. Throughout this dissertation, I examined how females differ from males behaviorally and in brain regions engaged during retrieval of context fear. In chapter 2, I assessed whether males and females differ in foreground context fear conditioning, generalization to a similar, safe context and determined the neural correlates of retrieval in males and females. I found that females showed higher levels of freezing behavior than males and exhibited greater generalized context fear. Further, I demonstrated that ventral hippocampus is required for retrieval of context fear memories in males and females but that males preferentially engaged dorsal hippocampus following retrieval of context fear memory whereas females preferentially engaged ventral hippocampus and amygdala. If males and females differ in context fear conditioning and engagement of brain regions when memory is recalled at recent time points, they may also differ in retrieval of remote context fear. Therefore, in chapter 3 I examined sex differences in retrieval of remote context fear and determined neural correlates of remote retrieval. I found that females but not males trained with background fear conditioning (tone), show reduced freezing to the context at remote time points. Further, I demonstrated that as in retrieval of recent context fear memories, females preferentially engaged ventral hippocampus and basal amygdala, and also engaged retrosplenial cortex after retrieval of background context fear memory compared with

males. Together, chapter 2 and 3 suggest that retrieval and its neural correlates differ between males and females at both recent and remote time points. Therefore, it is likely that males and females engage in distinct cognitive strategies to retrieve context fear. One way to assess sex differences in strategy of retrieval is to examine whether the context-shock association is similarly retrieved in both sexes. In chapter 4 I examined this using a blocking task and extinction paradigm. I show that despite strong context fear memory in both sexes, only males showed blocking to the tone. In extinction, sex differences in patterns of freezing were observed within session where females start off lower in freezing at the beginning of each session and show an increase in freezing throughout the session. While chapters 2-4 provide data to suggest that females differ in hippocampal mechanisms normally activated by retrieval in males, it still remains unclear as to which mechanisms females engage to retrieve context fear memories that may be separate from males. In chapter 5 I use RNA-sequencing as an unbiased approach to identify differential expression of genes in ventral hippocampus of males and females following retrieval and during consolidation of context fear compared with naïve. Despite similar behavior between the sexes during learning and retrieval, I identify a diverse transcriptional profile in ventral hippocampus of males and females following retrieval, during consolidation and at baseline. Collectively, these data determine sex-specific mechanisms associated with retrieval of a context fear memory and move the field closer from pointing out where males and females differ in learning and memory to understanding and defining *how* and *why*.

Chapter I

Introduction

Basic cognitive functions that are critical to survival such as learning and memory are different in males and females and involve sex-specific underlying mechanisms (Mizuno and Giese, 2010; Simpson and Kelly, 2012; Keiser and Tronson, 2015). Sex differences in basic cognitive processes are not surprising given strikingly different susceptibilities to disorders of memory in men and women. For an example, posttraumatic stress disorder (PTSD) is almost 3 times more frequent in women than men (Kessler et al., 1995, 2012).

While great strides have been made in understanding and characterizing the functional role of structures important in learning and memory such as hippocampus in males, a basic understanding of this region and the underlying molecular mechanisms that mediate learning and memory in females is absent. The chapters outlined in this dissertation seek to aid in filling this gap by identifying the ways in which fear-related memory may differ in males and females via 3 primary aims: 1) Examine sex differences in generalization and retrieval of context fear-related memory, 2) Determine the role of dorsal and ventral hippocampus and basal amygdala during retrieval of context fear, 3) Identify sex differences in underlying hippocampal molecular mechanisms activated by retrieval and consolidation of context fear. Understanding how fear memories are encoded and retrieved in both sexes will positively aid in our ability to prevent and improve such impairments in cognitive function in men and women. Some sections below have been adapted from (Keiser and Tronson, 2015).

Sex Differences in Natural Environment

The discovery of sex differences in memory raise a variety of important questions such as: whether functional differences between the sexes are due to natural selection because of the effects of different benefits to specific types of memory, or whether the memory differences result from other aspects of development? Silverman & Eals (1992) pose a theory titled: The Hunter-Gatherer theory of spatial sex differences. The theory concluded that this sex difference may not be due to levels of ability, but to different experience between the sexes. This theory states that the critical piece in the selection of human spatial abilities was due to the division of labor that took place during the Pleistocene era (Silverman et al., 1992). During this era, women played a strong role in gathering and planting food and men were the hunters. Thus, it is not surprising then, that, according to this theory, men perform better than women in many spatial memory tasks. Following the theory of selection for hunting and spatial ability in males, the next question was raised, that perhaps then, gathering was equally selected for in females. In order to ask this question, we must first observe what skills may be required in a good gatherer. Finding plants that are edible and locating them again after the season has ended and a new season has begun may be an important quality and may thus, reflect the ability of women to perform better than men in object placement tasks (Postma et al., 2004) or tasks involving specific attention to a cue or landmark (Saucier et al., 2002; Chai and Jacobs, 2010). Women are shown to perform better than men in tasks that require fast perceptual speed (Kimura, 1999), a trait that would be optimal and necessary in effective gathering strategies.

When studying sex differences, it is important to also understand and consider how the ecological habitats and daily functions may differ within a species. This insight will aid in understanding the natural behavior of males and females, providing a better framework for

interpreting behavioral data collected in the lab. Male and female mice, at an early age differ in responsibility and task, mice live in large communal groups involving a dominant male, several females that will breed with the male and many subordinate males (Reimer and Petras, 1967; Bronson, 1979; Lonstein and De Vries, 2000). Daily functions will likely differ between the sexes with females predominantly caring for offspring given the high percentage of subordinate males in a group. Thus, it is likely that knowledge both acquired and remembered will also differ between the sexes and may involve sex- specific strategies that will be better suited for their needs and environment. For an example, it may be differentially advantageous to pay attention to specific cues such as those in males that may allow for access to a female and in females, cues that may allow for protection of their pups.

When assessing sex differences in behavior, it is important to keep in mind that a similar behavior between males and females may not yield a similar outcome and should be interpreted as such, as different behaviors may be engaged to reach a goal of similar significance and importantly, similar behavioral outcomes between the sexes may still involve different neural mechanisms (for review see Becker and Koob, 2016).

Sex Differences in Memory

Sex differences have been demonstrated in memory formation and the molecular mechanisms that underlie memory function (Shors et al., 2000; Mizuno and Giese, 2010). Human and animal studies yield sex differences in behavioral memory tasks such as, but not limited to, spatial navigation (Maguire et al., 1999), working memory (Talarowska et. al, 2013), autobiographical memory (Pillemer et al., 2003) and verbal memory (Lejbak et al., 2011). Understanding these differences in memory is imperative for developing more effective treatments for women suffering from memory-related disorders such as PTSD and Alzheimer's

disease. The majority of research on learning and memory primarily utilized male animals and as much as 42% of neuroscience and physiology scientific journals fail to report the sex of the animals that were studied (Beery and Zucker, 2011); these findings result in clinical information for the purpose of generalizing to both men *and* women (Zucker and Beery, 2010). With many lines of research revealing sex-specific memory processes, it is becoming increasingly evident that utilizing only male animals does not equally reflect and represent the female population and has resulted in devastating effects including adverse drug reactions in women, especially from antibacterial and anti-inflammatory medications tested primarily in men (Zopf et al., 2009).

In the non-disordered population, males and females often perform equally well across a wide variety of memory tasks, yet use different strategies to reach the same goal. In spatial tasks such as the Morris water maze, for example, males have been shown to largely outperform females, as indicated by their ability to more quickly find the hidden platform in the murky water compared with females. However, males rely predominantly on distal cues, whereas females rely on landmarks or proximal cues (Rodríguez et al., 2011; Bettis and Jacobs, 2013; Keeley et al., 2013; Shah et al., 2013); thus, when a landmark such as a visual wall cue is added, sex differences in performance are abolished. Therefore, when females are able to use their preferred strategy (when a landmark is present), sex differences are abolished (Saucier et al., 2002; Chai and Jacobs, 2010). When assessing sex differences in memory, it is important to examine more than the end behavioral outcome, but additionally how each sex has reached that outcome.

Performance of a memory task may involve recruitment of different neural circuits in males and females. In memory tests with an emotional component, men and women show differences in amygdala lateralization for emotion-related information (Gasbarri et al., 2007; Cahill, 2011), and females show increased recruitment of hippocampal circuitry for cues with an

emotional component (Bellace et al., 2013). Therefore, the strength or type of emotion felt during acquisition or consolidation of memory likely reflects circuitry engaged and may thus have different consequences in males and females if emotion differs during memory acquisition. Recruitment of different neural circuits in males and females may be another case in which memory performance may be similar between the sexes, but the strategy and circuity involved in the initial encoding of the memory is different.

While the majority of research examining sex differences in memory has primarily focused on spatial tasks, sex differences in behavior, circuitry and molecular mechanisms underlying fear memory have been observed. However, less that 2% of research studies assessing fear memory in the form of context fear conditioning and extinction of context fear report use of female animals (Lebron-Milad and Milad, 2012). Further, sex differences underlying fear memory has largely focused on the acquisition and consolidation phase of memory, far less is known on how males and females may differ in retrieval of a fear- related memory and the circuitry and mechanisms that mediate this process. Below, I will first review foundational literature in males on fear-related memory and discuss what is known and absent on sex differences in fear memory acquisition and consolidation.

Studying Fear Memory

In studies assessing fear-related memory in male rodents, Pavlovian context fear conditioning is a common paradigm that is used. In this paradigm, a rodent receives a foot shock in the context and will later be tested for freezing behavior to measure fear-related memory. Freezing behavior is defined in the form of a crouching posture and consists of lack of movement other than respiration (Blanchard and Blanchard, 1969). Importantly, a representation of a context must first be learned and established before it can be connected and associated with an

aversive stimulus, such as a foot shock (Fanselow, 1986, 1990; Wiltgen et al., 2001; Frankland et al., 2004). Context pre-exposure facilitation effect states that mice or rats that are immediately shocked in a context will not learn to associate that context with shock, however, if given time to explore the context the day before being immediately shocked, animals will display strong fear memory, in the form of freezing (Fanselow, 1990). Studies utilizing both sexes have revealed sex differences in acquisition of context representations, with lower levels of freezing in females compared with males when given little time to learn about the context before the onset of shock, however, when given more time to explore the context prior to the onset of shock, sex differences in freezing are abolished (Wiltgen et al., 2001), suggesting sex differences in obtaining a representation of context.

Given the strong and quick behavioral freezing response that results from one context-shock pairing, context fear conditioning is a convenient paradigm for assessing fear memory. One way of examining strength and accuracy of a context representation associated with the aversive shock is via testing freezing in both the fear conditioned context and a novel context (generalization) (Lehmann et al., 2009; Wang et al., 2009; Lynch et al., 2013). Studies using males have noted generalization of fear to novel contexts in contextual fear conditioning when tested at later, but not earlier time points (Biedenkapp and Rudy, 2007; Wiltgen and Silva, 2007) and one study found sex differences in generalization of fear to novel contexts in a passive avoidance task (Lynch et al., 2013). In this task, animals are trained to avoid entering a context paired with foot shock and are tested for avoidance of this, as well as a similar context (the generalization context). Females, but not males generalize fear to this similar context at a later time point of 7 days after training, suggesting that females show more rapid loss of context specificity with time (Lynch et al., 2013).

When assessing sex differences in context fear conditioning, the dominant view for some time has been that males show higher levels of freezing behavior compared with females. However, sex differences in fear conditioning are more complex than a failure of females to acquire context representations or context-shock associations. In support of this, a large number of research studies report no sex differences in context fear conditioning (Wiltgen et al., 2001; Kosten et al., 2006; Dachtler et al., 2011), others show greater fear conditioning in females compared with males (Ris et al., 2005; Moore et al., 2010) and others reveal sex differences emerging as a result of specific strain used (Pryce et al., 1999) or training protocol (Wiltgen et al., 2001). These findings speak to a need for assessment of more than one construct when assessing sex differences in fear- related memory. In fact, there is evidence for sex differences in how fear memory is expressed with females showing displays of a more active, darting response when tested for fear memory of a shock-paired cue, whereas males primarily show a passive "freezing" response (Gruene et al., 2015). Findings such as these suggest that behavioral strategies to express fear memory likely differ in males and females.

It is still largely unknown as to how males and females learn or retrieve context fear and how mechanisms engaged during these processes may differ. The following section will review brain regions critical for acquisition and consolidation of context fear memory and will assess how their engagement and neural mechanisms may differ in males and females.

Role of Hippocampus and Amygdala in Context Fear Memory

The hippocampus is thought to play an important role in compiling distinct features of a fear conditioning chamber (floor, lights, sounds, colors, odor) into a single representation defined as *context* (Rudy and O'Reilly, 1999; Reilly and Rudy, 2001; Matus-amat et al., 2004). Damage to the hippocampus in males results in impairments in contextual conditioning (Selden et al.,

1991; Kim and Fanselow, 1992; Phillips and Ledoux, 1992; Kim and Davis, 1993; Maren and Fanselow, 1997; Frankland et al., 1998; Anagnostaras et al., 1999; Antoniadis and Mcdonald, 2000; Debiec et al., 2002; Lehmann et al., 2007; Sutherland et al., 2008), but does not impair tone fear (Kim and Fanselow, 1992). Therefore, hippocampus is critical in formation of a context representation which will later be associated with shock.

While dorsal hippocampus appears to be critical for successful context fear conditioning, its role is time-limited. Inactivation of hippocampus in males results in disruption of recently acquired context fear memories, but not memories acquired at earlier or remote time points (Kim and Fanselow, 1992; Anagnostaras et al., 1999; Squire et al., 2004; Bayley et al., 2005). Thus, recently acquired memories become hippocampus- independent with time and are thought to be stored in more cortical regions, a process termed systems consolidation (Kim and Fanselow, 1992; Alvarez and Squire, 1994; Squire and Alvarez, 1995; Anagnostaras et al., 1999; Frankland et al., 2004; Wiltgen et al., 2004; Frankland and Bontempi, 2005; Sutherland and Lehmann, 2011; Wiltgen and Tanaka, 2013). Inactivation of these cortical regions during retrieval have resulted in impairments that are specific to remote memory (Bontempi et al., 1999; Frankland et al., 2004). Given that males have been studied almost exclusively to build this foundation on systems consolidation and the role of hippocampus, how females perform at remote time points and the regions they engage during this time remain an open question.

In recently acquired context fear memories damage to hippocampus results in impairment of context fear, however, compensation by other cortical brain regions allow for context fear conditioning to still be acquired with sufficient over training. Though, even with over-training and despite strong memory for the fear conditioning context, discrimination between the context that was paired with shock and a similar environment is impaired (Frankland et al., 1998). Thus

pointing to dorsal hippocampus as a key regulator of context discrimination. Given the critical role of dorsal hippocampus in males for context fear discrimination, quicker impairments in context fear discrimination (Lynch et al., 2013) and slower acquisition of context representation in females (Wiltgen et al., 2001), might engagement of hippocampus in females also differ from that of males?

Studies assessing females as well as males in fear conditioning, suggest that hippocampus may be more strongly activated by males. This can be observed with higher levels of hippocampal LTP in males compared with females at 35 and 60 days of age (Maren et al., 1994), and following acquisition of context fear, higher levels of hippocampal pCREB (Kudo et al., 2004) and pERK (Gresack et al., 2009) are observed in males compared with females, both kinases and transcription factors that play a necessary role in acquisition of context fear memory. Importantly however, the lack of hippocampal engagement by females may not reflect an impairment in acquisition of context fear. The critical role of hippocampus in acquisition and consolidation of context fear conditioning has been largely defined in males, the regions, circuits and mechanisms that females engage to learn context fear may likely differ than that of males, while the behavioral performance between the sexes may or may not be the same. The regions and mechanisms that mediate fear memory in females remain an open question, but there is evidence for female-specific molecular mechanisms.

Sex differences in context learning may also arise from differences in affective processing. In memory tasks involving an emotional component in humans, sex differences are observed in amygdala lateralization, with women showing preferential activation of left amygdala and men, of right (Gasbarri et al., 2007; Cahill, 2011). Thus, sex differences in factors such as emotional engagement or perhaps use of cognitive strategy during learning or memory

recall may influence neural circuitry such as amygdala activation.

In context fear conditioning, context information from the hippocampus is projected to the amygdala, which plays a role in regulating the emotional aspect in fear-related memories and is an important anatomical site of CS-US convergence (Phillips and Ledoux, 1992; Kim and Davis, 1993; Wilensky et al., 1999; LeDoux, 2000; Fanselow and Dale, 2003; Zelikowsky et al., 2014). Lesions to the amygdala result in disrupted fear memory for both the context and a cue such as a tone that was also associated with foot shock (Phillips and Ledoux, 1992; Kochli et al., 2015). Lesions to hippocampus however, leave memory for a specific cue such as a tone unimpaired, while clearly impairing memory for the context (Phillips and Ledoux, 1992; Anagnostaras et al., 1999).

Unlike dorsal hippocampus, ventral hippocampus receives reciprocal projections to and from the amygdala (Pitkanen et al., 2000; Kishi et al., 2006) and ventral hippocampus is critical for displays of defensive responses such as freezing (Zhang et al., 2001). In males, projections from the ventral hippocampus to basal amygdala are important in mediating context fear, while projections to central amygdala are critical in cued fear (Xu et al., 2016), further demonstrating the importance of a ventral hippocampal-basal amygdala interconnection in context fear memory. Pre and post training inactivation of ventral hippocampus in the form of pharmacological manipulations (Zhang et al., 2001) or lesions (Maren and Holt, 2004; Trivedi and Coover, 2004), has resulted in impaired fear conditioning. However, little is known about their role in retrieval of fear memories and how males and females may differentially engage this region. A study in male mice may suggest a role for ventral hippocampus in retrieval of fear memory as evidenced by pre-retrieval inactivation of ventral hippocampus resulting in reduced expression of a generalized fear memory (Cullen et al., 2015). Given sex differences in context

generalization (Lynch et al., 2013; Keiser et al., 2017) and the critical role of ventral hippocampus in retrieval of a generalized memory (Cullen et al., 2015), sex differences may also be present in the way in which ventral hippocampus is engaged and in the molecular mechanisms activated by retrieval of a context fear memory.

Molecular Mechanisms Underlying Memory Formation

There are many coordinated processes that are required for synaptic plasticity and memory formation. These include signaling via neurotransmitter release and receptor activation, calcium and second messenger signaling, transcription of genes, de novo protein synthesis, and long-lasting histone modifications that alter subsequent gene expression. Receptors are trafficked in and out of the membrane, proteins are ubiquitinated and broken down, and scaffolding and cytoskeletal proteins are reorganized.

These basic categories of mechanisms are consistent across many forms of synaptic plasticity, from synapse development and pruning during development, to all types of memory formation; as such they are likely to be the same in males and females. Yet there is substantial redundancy in how each of these steps is instantiated. For example, gene transcription can be induced by many different transcription factors, and multiple signaling pathways converge to activate the same transcription factors. Whether the precise signaling mechanisms are the same in males and females is less clear.

Many of the specific signaling pathways required for memory formation, in particular the transcription factor cre-response element binding protein (CREB) activation via extracellular signaling regulated kinase 1 and 2 (ERK1/2), protein kinase A (PKA), calcium calmodulin kinases (CaMKII, CaMKIV), and mammalian target of rapomycin- AKT (mTOR-AKT) pathways, have been well defined in male rodents, and are described in detail elsewhere

(Tronson and Taylor, 2007; Johansen et al., 2011). Briefly, during a learning experience, glutamatergic signaling at both a-amino-3-hydroxy- 5-methyl-4-isoxazolepropionic acid (AMPA) and *N*-methyl-d-aspartate (NMDA) receptors triggers calcium influx that leads to calcium dependent signaling via calmodulin (CaM), CaMKII (Silva et al., 1992; Lucchesi et al., 2011), CaMKIV (Kang et al., 2001), ERK1/2 (Sananbenesi et al., 2002; Shalin et al., 2004), protein kinase C (PKC), and protein kinase M zeta (PKMz) (Sacktor, 2011) activity. G-protein coupled receptors additionally result in activation of PKA (Abel et al., 1997) and the mTOR-AKT (Horwood et al., 2006; Jobim et al., 2012) pathways. These signaling cascades mediate ongoing receptor activity and trafficking (Malenka, 2003; Rumpel et al., 2005) at the synapse, and transduce the synaptic signals to the nucleus, activating transcription factors that include CREB (Kogan et al., 1996; Pittenger et al., 2002; Josselyn et al., 2004; Alberini, 2009) and cofactors such as CREB Binding Protein (CBP) and p300 (Alarco et al., 2004; Maurice et al., 2008), resulting in transcription of immediate early genes (i.e.g.) such as cFos (Radulovic et al., 1998), early growth response protein 1 (Egr1, Zif268), activity-regulated cytoskeleton-associated protein (Arc, Arg3.1) (Guzowski et al., 2000), and other new proteins required for synaptic strengthening. Histone modifications, including changes in histone acetylation and methylation, are induced via activation of histone deacetylases, histone acetylases, and histone demethylases (Lubin and Sweatt, 2007; Tsai et al., 2009; Penney and Tsai, 2014).

Structural elements in and around the synapse are also modified so that spines and synapses can become plastic. ERK, PKA, and other kinases regulate destabilization and restabilization of dendritic spines and synapses by triggering changes in scaffolding protein interactions (Colledge et al., 2000; Moita et al., 2002; Gao et al., 2013), breakdown of proteins (Artinian et al., 2008; Jarome et al., 2011), modification of adhesion molecules that link the pre-

and postsynapses (Schrick et al., 2007), loosening of the perineuronal net (Kaczmarek and Lapinska-dzwonek, 2002; Stawarski et al., 2014; Tsilibary et al., 2014), and cytoskeleton reorganization (Emes and Grant, 2012). These signaling, structural, and epigenetic alterations together mediate lasting stability of synapses and play an integral role in the cellular storage of memories.

The idea that sex differences in molecular mechanisms of memory formation exist runs counter to the notion that these pathways represent fundamental processes, and small changes in the patterns of kinase activity result in engagement of alternative downstream mechanisms and can dramatically change the outcome. Yet, despite the gross similarities in the mechanisms underlying memory formation, there also exist striking sex differences at all levels, from receptor involvement, kinase signaling, transcription factors, and gene expression (See Figure 1.1; Table 1.1). It remains possible, therefore, that alternative signal transduction pathways may result in broadly similar memory in males and females. Although pharmacological manipulations that result in differential activation of signal transduction pathways in males and females may indeed be due to pharmacodynamics, or the effect of drugs on the body, it is also equally likely that differential responses to drug treatments may result in sex differences in pharmacokinetics, or how the body affects the drug (for review see Soldin & Mattison, 2009; Gandhi et al., 2004). For example, sex differences in pharmacodynamics have been observed in gastric alcohol dehydrogenase activity, which is higher in males (Parlesak et al., 2002), resulting in a lower threshold for toxicity in women compared with men (Baraona et al., 2001). Therefore, it is important to note that sex differences in mechanism may be the result of pharmacodynamics or pharmacokinetic effects. The subsequent sections will discuss sex differences in molecular mechanisms of memory that have been identified to date.

Sex Differences in Molecular Mechanisms

AMPA Receptors

In GluA1 constitutive knockout mice, male animals, but not females, exhibit impaired context fear conditioning (Dachtler et al., 2011). This effect was neither due to locomotor or nociceptive effects, nor learning deficits as male and female GluR1 knockout (KO) animals showed similar freezing levels immediately after foot shock during fear conditioning. Thus, GluA1 has a sex-specific requirement in context fear conditioning (Dachtler et al., 2011). Supporting a differential role of GluA1 in males and females, distinct patterns of activation of cFos in response to novelty in GluA1 KO mice have been observed. Hippocampal cFos is markedly more pronounced in males, whereas lateral septum cFos is more pronounced in females (Procaccini et al., 2013). These results suggest that not only does GluA1 play different roles in learning and memory, but also supports differential recruitment of neural circuits in males and females during memory formation.

Further supporting sex differences in AMPA receptor function, GluA1, 2, and 4 subunits are differentially expressed in memory-related brain regions. Female mice have higher expression of GluA1 in dorsal hippocampus, amygdala, and medial prefrontal cortex, and lower GluA2 and GluA4 expression in dorsal hippocampus and medial prefrontal cortex compared with males (Katsouli et al., 2014). It is surprising that females show more GluA1 expression in hippocampus (Katsouli et al., 2014) but fewer impairments of hippocampal-dependent memory when deleted (Dachtler et al., 2011). It remains possible that compensatory mechanisms, and not the role of GluA1 per se, mediate the differential effect of GluA1 knockout in male and female mice.

There is clear evidence that AMPA receptor expression and function show some sex-

specific effects in learning and memory processes. This suggests that intracellular signaling mechanisms activated by AMPA receptors will also show marked differences between males and females. AMPA receptors and consequent downstream signaling are not sufficient, however, and additional glutamatergic receptors, in particular NMDA receptors, are required for successful memory formation.

NMDA Receptors

Sex differences in memory as a consequence of NMDA receptor manipulation have been observed in both humans and animal models. In general, NMDA receptors are critical for learning in both male and female animals; however, there remains differential activation and performance in NDMA-dependent tasks. For example, men are more vulnerable to amnestic effects of ketamine than women, despite an overall lower behavioral sensitivity to NMDA antagonists (Morgan et al., 2006). In rodents, context fear conditioning and NMDA-dependent LTP is lower in females than males, suggesting limited activation of NMDARs in females (Maren et al., 1994). Similarly, enhancing GluN1 subunit function with d-cycloserine enhances trace eyeblink conditioning in male, but not female, animals (Waddell et al., 2010).

The differential role of NMDARs in male and female synaptic plasticity is also evident during aging, where females exhibit less of an increase in adulthood compared with males (Maren et al., 1994) and LTP in female animals remains intact during aging, whereas declines are observed in males, correlating with a decrease in NR2A subunits (Monfort and Felipo, 2007). Together, these effects may reflect differential levels of hippocampal GluN1 subunits in the hippocampus of female and male animals (Monfort and Felipo, 2007), or differential efficacy of intracellular signaling downstream of NMDARs.

There is additional evidence that modulation of NMDARs has very different outcomes on

neuronal plasticity in males and females. Notably, stress causes an NMDA- dependent decrease in dendritic spines in males, and an NMDA-dependent increase in females (Shors et al., 2004). Although this effect is mediated, in part, by sex differences in hypothalamic–pituitary–adrenal axis signaling it also suggests that the downstream effectors of NMDAR activity exert very different effects in males and females.

GABA Receptors

Concomitant with the differences in glutamatergic excitatory transmission, sex differences have also been observed in inhibitory transmission, specifically gamma-amino-butyric acid (GABA) receptors. GABA receptor activation inhibits memory formation, and pharmacological inhibition (Brioni et al., 1989) or genetic knockdown (Collinson et al., 2002) of GABA receptors results in increased memory formation. There are striking sex differences in the role of δ, α4, α5, and γ2 $GABA_A$ subunits in fear conditioning. Genetic deletion of δ-$GABA_A$ receptor subunits profoundly enhances trace fear conditioning in female, but not male, mice, but does not affect delay conditioning or context conditioning in either males or females (Wiltgen et al., 2005).

In contrast, both male and female α4-$GABA_A$ KO mice show enhanced fear conditioning. Male and female α4-$GABA_A$ KO mice also show opposite patterns of freezing to context after delay and trace tone fear conditioning, where males exhibit increased freezing to context in delay but not trace tone conditioning, and females have increased context freezing after trace, but not delay conditioning (Moore et al., 2010). Similarly, loss of α5-$GABA_A$ subunits in the hippocampus leads to a female-specific enhancement of trace fear conditioning. In these α5-$GABA_A$ H105R mutant animals, delay conditioning was intact and extinction impaired in both sexes (Yee et al., 2004).

Consistent with differential roles of $GABA_A$ receptors in males and females, constitutive phosphorylation of γ2-$GABA_A$ receptor subunit causes sex-specific changes in expression of α4- and δ-$GABA_A$ subunits and increased tonic currents in the hippocampus only in female mice (Nani et al., 2013). GABAergic modulation of memory therefore requires sex-specific recruitment of GABA receptor subunits. This suggests that females require differential activation of signaling mechanisms downstream of GABA receptors during memory formation. Consistent with this possibility, previous work has demonstrated sex differences in subunit composition and pharmacology (Gulinello and Smith, 2003) and the role of androgens (Nuñez and McCarthy, 2008), progesterone (Andrade et al., 2012), and estrogens (Nunez and McCarthy, 2009) on GABAergic functions. The divergent roles of glutamatergic and GABAergic receptors in memory formation supports the hypothesis that memory processes are differentially regulated in males and females. Furthermore, this suggests that downstream effectors including endocannabinoids (Huang and Woolley, 2012), kinase signaling cascades, and transcriptional regulation will be markedly different in males and females during memory formation.

Calcium-dependent signaling

NMDA receptors and GluA1 play key roles in memory consolidation, in part due to their calcium permeability, and calcium influx appears to have differential roles in memory formation in males and females. In addition to sex differences in NMDAR and GluA1 manipulations, males are more sensitive than females to enhancement of memory by nonspecific blockade of calcium channels (Wilmott and Thompson, 2013), suggesting that calcium signaling is central to memory formation in males, but that this relationship is less clear in females.

Consistent with the suggestion that calcium signaling has sex-specific roles in learning and memory, calcium calmodulin kinase kinase α and β (CaMKKα and CaMKKβ) have been

shown to be required for context fear conditioning (Blaeser et al., 2006; Mizuno et al., 2006) and spatial or novel object recognition memory (Peters et al., 2003; Mizuno et al., 2007; Bachstetter et al., 2014), respectively. Neither CaMKKα nor β are required for memory formation in female animals (Mizuno et al., 2006, 2007). Therefore, CaMKKα and β are the first identified male-specific mechanisms of memory formation.

The transcriptional targets of CaMKKα/β also differ between males and females. In males, CaMKKβ knockout results in decreased *Srp20* and increased *Psf* messenger RNA expression in males, but not in females (Antunes-Martins et al., 2007). This suggests that the mechanisms controlling transcription of these genes differ between the sexes. Moreover, upregulation of *Srp20* after context fear conditioning or Morris water maze training is male-specific, and upregulation of *Psf* is stronger in males than females (Antunes-Martins et al., 2007). Thus, calcium signaling via CaMKKα and β, leading to transcriptional regulation of splicing factors *Srp20* and *Psf*, is required for hippocampal- dependent memory formation in males, but not females. Similarly, glycosylphosphatidylinositol anchor attachment protein (GAA1) is unregulated after spatial and contextual fear conditioning, but only in male animals (Mizuno et al., 2007). The importance of *Srp20*, *Psf*, and GAA1 in memory, and their contribution to sex differences in mechanisms of plasticity, is not yet known.

In contrast, there is little evidence for sex differences in other components of the CaMKKα/β signaling pathway. The immediate downstream substrates of CaMKKα and β are CaMKI and CaMKIV, respectively, both of which play key roles in memory consolidation, with no evidence for a differential role in males and females (Takao et al., 2010). This leaves the question of how CaMKKα/β differentially regulates transcription. One possibility is that calcium-dependent signaling, and CaMKKα/β specifically, is coupled to different downstream

effectors. Indeed, CaMKKα/β also couples to the adenosine monophosphate-activated kinase (AMPK) signaling pathway (Birnbaum, 2005; Hawley et al., 2005; Hurley et al., 2005), and CaMKKα phosphorylates and activates Akt (protein kinase B, PKB) (Yano et al., 1998). Alternate signaling pathways may thus be recruited in males compared with females as a consequence of CaMKKα/β activation during memory formation.

Calcium-dependent signaling culminates in the activation of CREB via CaMKK and CaMKIV, and via CaMKII. Sex differences in CaMKK signaling and transcription additionally suggest a differential role of CREB in males and females. In particular, CaMKKβ is required for CREB activation in the hippocampus in male mice (Peters et al., 2003). Therefore, the failure of CaMKKβ deletion to impair memory in females strongly implicates CaMKKβ-CREB as a male-specific mechanism for memory formation. The signaling pathways that mediate this role in females are yet to be identified.

CREB

There is mixed evidence for a diverging role of CREB in males and females. Loss-of-function CREB transgenic manipulations lead to memory impairments in both male and female mice (Kogan et al., 1996; Pittenger et al., 2002). In contrast, direct and indirect evidence suggests a differential role of the transcription factor CREB in memory formation in males and females. After context fear conditioning, males show higher levels of CREB phosphorylation (Kudo et al., 2004) in the CA1 area of the hippocampus, but not the dentate gyrus or CA3. This suggests that CREB is more readily induced in males compared with females, and further, CREB may be more important in memory processes in males. In contrast, in CREB-deficient mutants ($CREB^{\alpha\delta-}$), females show greater spatial memory impairments compared with males during aging (Hebda-Bauer et al., 2007). Given that the latter study observed sex differences only in

aged mice, it is possible that these results reflect more general susceptibility to memory impairments in females compared with males.

Indirect evidence for sex differences in the role of CREB in memory processes comes from analysis of upstream kinases and downstream CREB-dependent transcription during memory formation in male and female animals. As described previously, CaMKKα/β activation is a critical part of calcium signaling that culminates in CREB- mediated transcription in males, but not females. Alternative pathways for CREB activation include the extracellular regulated kinase/mitogen activated protein kinase (ERK/ MAPK) pathway and PKA. Although PKA appears to be required for fear memory formation in both males and females (Abel et al., 1997), ERK is differentially activated after context fear conditioning, with males but not females showing significant ERK phosphorylation in ventral hippocampus (Gresack et al., 2009). The roles of these kinases vary depending on memory task. Substantial sex differences in PKA activity are observed during reward-related tasks (Becker et al., 2007; Iñiguez et al., 2012) and after retrieval of a spatial memory (Iñiguez et al., 2012), whereas ERK is similarly activated in both male and female animals in these tasks (Nygard et al., 2014). It is not sufficient, therefore, to think of signaling proteins or pathways as male- or female-specific. Rather, the pathways activated reflect the information processing in that task, suggesting differential information processing in males and females may drive differential recruitment of signal transduction pathways.

CREB-dependent gene expression also shows differential patterns in male and female animals. Brain-derived neurotrophic factor is more highly expressed in males compared with females whereas other transcripts, including nerve growth factor IB, show similar expression after a learning experience (Mizuno et al., 2006). There are many mechanisms by which

transcription may differ despite activation of a transcription factor. Co- activators (e.g., CBP and P300) (Maurice et al., 2008) and transcriptional repressors (e.g., activator protein 1, AP-1) are key components of transcriptional regulation (Guedea et al., 2011). There is evidence for sex differences in AP-1 binding to DNA (Zhu and Pfaff, 1998), suggesting that beyond transcription factors, regulators of transcription may be important targets for sex differences research. At this time, the contribution of AP-1 and other transcriptional regulators to differential CREB-related transcription in males and females remains unknown.

Conclusion

In sum, although much is still unknown about specific mechanisms mediating fear-related memory, it is clear thus far that significant sex differences are present in the components that make up such a memory. This is observed with sex differences in learning about a context or environment before it is paired with shock, strategies that are used to navigate spatial environments, discrimination between shock-paired and safe contexts and in how a fear memory is behaviorally expressed. Sex-specific strategies to both learn and express fear are likely adaptive to suit the differential needs of males and females. Additionally, the engagement of structures known to be critical for context fear memory in males differ in females during consolidation of context fear and there are sex differences present in molecular mechanisms underlying memory formation at every level of intracellular signaling, from receptors, to second messengers, transcription factors, gene expression, and histone modifications. At this stage, we have only small snapshots of individual proteins, and sometimes pathways. Despite these current limitations, several things are clear: males and females utilize different cognitive strategies, neural circuits, and molecular mechanisms during memory tasks. Such differences have been largely identified during acquisition or consolidation of fear memory. Experiments illustrated in

this dissertation will aid in filling our gap in understanding sex differences in retrieval of context fear memory and such studies will be summarized below. Understanding how males and females differ during retrieval and the structures and molecular mechanisms activated by retrieval will be essential for understanding susceptibility to disorders of memory and developing targeted treatments for males and females.

Summary of Current Studies

CHAPTER 2: SEX DIFFEREENCES IN GENERALIZATION AND RETRIEVAL OF CONTEXT FEAR MEMORY

The goal of these experiments was to determine whether males and females differ in context fear conditioning and context generalization, as well as how the sexes differ in activation and requirement of hippocampus and basal amygdala after memory retrieval. We used foreground (unsignaled) fear conditioning to examine sex differences in generalization, and context fear conditioning after pre-exposure to the training context to manipulate strength of context representations. To control for nonspecific effects of handling and exploration of a novel context on context generalization, we used pre-exposure to a similar context. We demonstrated that females exhibited higher levels of freezing behavior compared to males. Additionally, females but not males generalize fear to similar contexts. Pre-exposure to the training context reduced generalization in females (Keiser et al., 2017). During retrieval of context fear, males show significantly greater levels of cFos positive cells in dorsal hippocampus compared with naïve, whereas females do not show an increase in number of cFos positive cells following retrieval in this region. In contrast, Arc levels were higher in dorsal hippocampus in females compared with males. Female mice showed higher levels of cFos in basal amygdala and ventral hippocampus after retrieval of context fear. Given apparent sex differences in mechanism within

hippocampus, we next assessed whether dorsal and ventral hippocampus is required for retrieval of context fear. Pre-retrieval infusion of muscimol targeting ventral hippocampus resulted in reduced freezing to the fear-conditioned context in both sexes. However, this same manipulation targeting dorsal hippocampus prior to retrieval did not change levels of freezing in males or females. These data suggest a critical role for ventral hippocampus in males and females in retrieval of a context fear memory but that this process likely involves sex-specific molecular mechanisms. Overall, these data suggest sex-specific molecular mechanisms as a consequence of context fear memory retrieval. Our findings open the possibility that females use an amygdalar-dependent strategy in retrieval of foreground context fear. Given competition between hippocampus and basal amygdala in context fear conditioning (Biedenkapp and Rudy, 2009) and the importance of basal amygdala in context fear conditioning (Matus-Amat et al., 2007; Amano et al., 2012; Jin and Maren, 2015), females might shift towards basal amygdala activation during memory retrieval. Sex-biased patterns of hippocampal and amygdalar mechanisms during retrieval may thereby result in greater generalization of context fear in females compared with males. The next chapter aims to further identify differences in the utility of hippocampus in context fear memory and how this may differ in males and females with training protocol used in recent vs remote context fear memory.

CHAPTER 3: TRAINING PROTOCOL MATTERS: SEX DIFFERENCES IN RETRIEVAL OF RECENT VS REMOTE CONTEXT FEAR MEMORY

In males, the specificity of retrieval of context information diminishes at remote time points (Wiltgen and Silva, 2007; Wiltgen et al., 2010) but freezing in the trained context is not reduced. If males and females use different strategies to retrieve context fear memories during recent time points, there may also be differences in retrieval of remote context fear. The goal of

this study was to determine sex differences in retrieval of remote context fear memories and whether remote context retrieval involves differential recruitment of brain regions in males and females. Male and female mice were trained in background or foreground context fear conditioning and were tested for freezing in the training context the day following training and at a remote time point 8 weeks later. Only females trained with background fear conditioning show reduced freezing to the context at remote time points suggesting that the circuit used by females to learn background fear conditioning does not sustain them into remote retrieval of context fear. Thus, our current data suggest sex differences in recruitment of the context fear memory circuit results in less robust remote context memory in females after background context fear conditioning. To examine the activation of this neural circuit after remote memory retrieval in females compared with males, we used cfos immunohistochemistry. Females showed higher levels of cfos activation in ventral hippocampus, basal amygdala, and retrosplenial cortex after retrieval of background context fear memory compared with males. This pattern of activation is unlikely to explain sex differences in remote context memory retrieval, however, as this pattern was also observed after foreground context retrieval. Furthermore, we observed greater cFos activation in dorsal hippocampus during retrieval of foreground compared with background context fear conditioning in both sexes. It remains unclear whether the observed cfos activity is due to retrieval of context memory, or due to other context-exposure related memory processing. Here we demonstrate decreased remote context memory in females, only in background fear conditioning, and sex specific patterns of cFos activity regardless of training protocol. Given robust differences in behavior and mechanism at retrieval during remote time points in males and females that depend on training protocol (background vs foreground), we next assess retrieval at recent time points. Using background fear conditioning, we assess fear memory at recent time

points (1-2 days after training) to the training context and similar (generalization) context, similar to (Keiser et al., 2017). Interestingly, we did not observe sex differences in fear conditioning and neither males nor females displayed generalized fear to a similar context, however a test order effect for both sexes was noted. These findings are in stark contrast with those observed in Keiser et al., 2017 in which mice were trained with foreground context fear and significant sex differences were observed in context fear conditioning and generalization of context fear. Altogether, these findings highlight an important role of training protocol (background vs foreground) when assessing context fear for recent or remote time points and showcase important sex differences in behavior and mechanism that emerge under specific training conditions. Present findings suggest that males and females may recruit differential circuits during systems consolidation and during retrieval of remote memories.

CHAPTER 4: SEX DIFFERENCES IN COGNITIVE STRATEGY: BLOCKING AND EXCTICTION OF CONTEXT FEAR

The goal of this study was to determine whether a context-shock association is similarly retrieved in males and females and how retrieval has an effect on more complex processes such as learning a new association. We further assess new learning, in the form of extinction and how this may differ behaviorally in males and females. In the blocking experiment, one day after context fear conditioning, a tone shock pairing was presented in the same context; since retrieval of context as a predictor of shock should block learning a new tone-shock association (Kamin, 1968; Wheeler and Miller, 2008), we were able to assess sex differences in retrieval of a context-shock association. Despite strong context fear memory in both sexes, only males showed blocking. These data suggest that males retrieve a context-shock association that prevents further learning, whereas females utilize a different strategy during retrieval of context fear

conditioning. Interestingly, neither sex showed blocking to the context when mice were trained with tone but a new context was presented. Together, these data suggest that males and females may be retrieving qualitatively different context information in context fear memory retrieval, and thereby differentially affecting subsequent learning. Another possibility is that males and females are retrieving similar context information, but using different behavioral strategies during retrieval. In a new set of male and female mice, sex differences in freezing patterns within session were examined across a series of 14 days following context fear conditioning. While males and females did not differ in overall levels of extinction by day, sex differences in patterns of freezing were observed within session. Here, we demonstrate that within session during extinction recall males show a typical decrease in freezing across the 3-minute testing session, whereas females start off lower in freezing at the beginning of the session and show an increase in freezing throughout the session. These findings may imply a difference in cognitive processing or strategy in retrieval of extinction fear.

CHAPTER 5: SEX DIFFERENCES IN HIPPOCAMPAL MOLECULAR MECHANISMS ACTIVATED BY RETRIEVAL OF CONTEXT FEAR

The goal of these studies was to identify sex differences in underlying hippocampal molecular mechanisms activated by retrieval and consolidation of context fear. In order to step away from a strictly male-comparative approach, we employed RNA-sequencing as an unbiased measure to examine regulation of hippocampal gene expression after memory retrieval in females and males. Previous studies in males have determined that consolidation and retrieval of context fear downregulate different genes in hippocampus which play different functions (Peixoto et al., 2015; Poplawski et al., 2016). Here, we show a difference in number of genes differentially expressed between males and females during fear memory recall; of particular

interest, were differential expression of genes during retrieval in females that code for proteins in signaling pathways such at the PI3-AKT signaling pathway known to play a key role in spatial learning and cellular stress response. Collectively, these data demonstrate sex-specific mechanisms associated with retrieval of a context fear memory that may underlie female-specific impairments. Alterations in hippocampal gene expression may contribute to the development of sex –specific memory impairments in disorders such as PTSD.

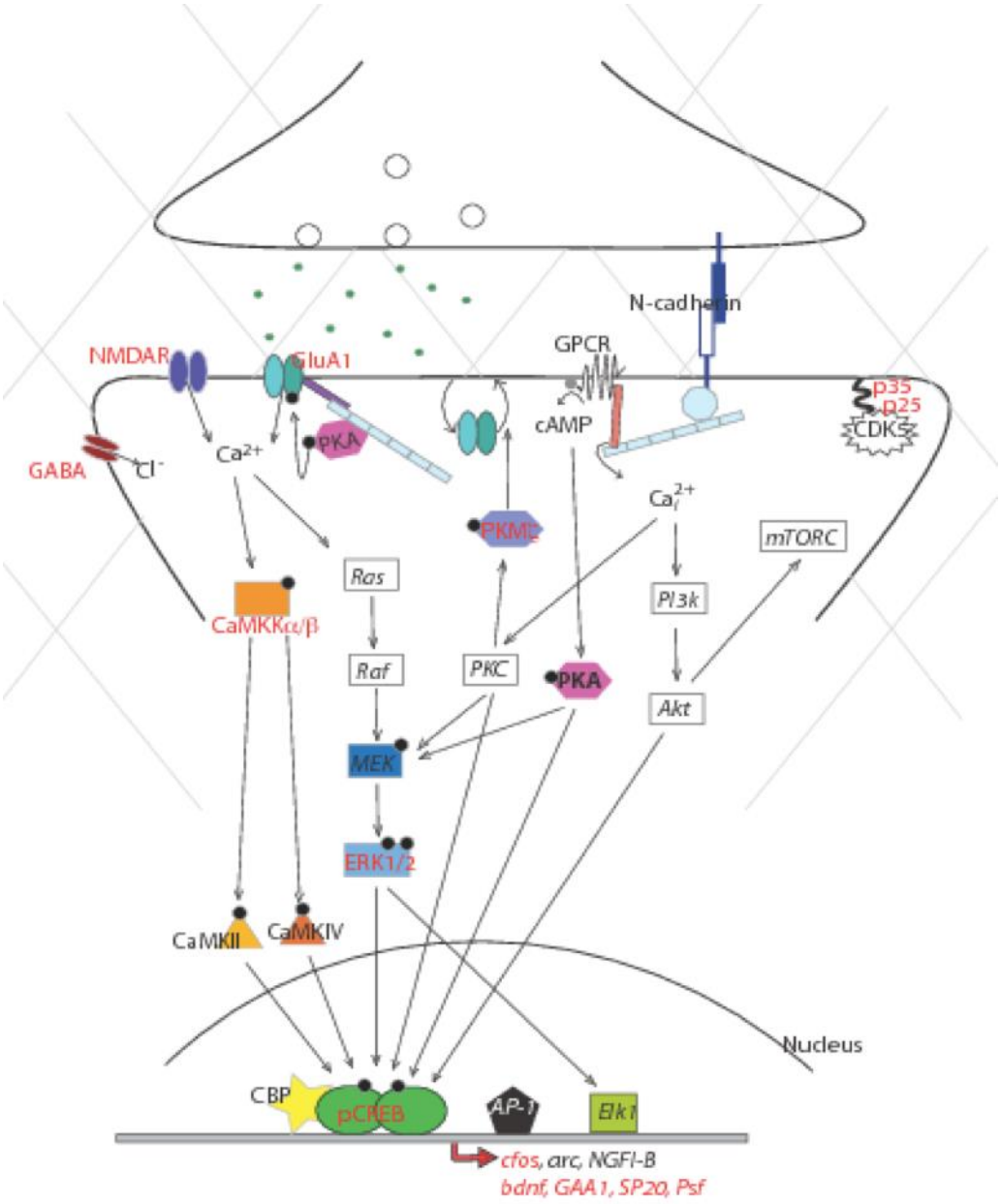

Figure 1.1 ***Summary of signaling mechanisms that mediate molecular mechanisms of memory.*** Text in red represents proteins that are differentially involved in males and females. Key: Red dot, phosphorylation; arrows, demonstrated connections between signaling molecules; pink/purple rectangles, scaffolding proteins; gray grid represents extracellular matrix. NMDA, *N*-methyl-d-aspartate receptor; GluA1, glutamate receptor 1; GABAR, GABA receptor; GPCR, G-protein coupled receptor; Ca^{2+}, calcium, Ca^{2+}, calcium released from internal stores; Cl-chloride ions; AC, adenylyl cyclase; cAMP, cyclic AMP; PKA, protein kinase A; PKC, protein kinase C; Akt, AKT/protein kinase B; mTOR, mammalian target of rapamycin; MEK, mitogen activated protein kinase kinase; ERK, extracellular signal regulated kinase; PKMζ, protein kinase M zeta; PI3K, phosphoinositide 3 kinase; CDK5 cyclin dependent kinase 5; CaMKKα/β, calcium modulated kinase kinase α and β; CaMKII/CaMKIV, calcium modulated kinase II/ IV; Elk1; CPB, CREB binding protein; pCREB, phosphorylated cyclic AMP responsive element binding protein; AP-1, activator protein 1; MMP9, matrix metalloproteinase 9.

Pathway	Protein	Expression/activation	Pharmacological manipulations	Transgenic manipulation
NMDA receptors	GluA1	F > M d.h., mPFC, amyg. (Katsouli et al., 2014)		M impaired CFC F normal CFC (Dachtler et al., 2011)
	GluA2	M > F d.h. mPFC (Katsouli et al., 2014)		
	GluA4	M > F d.h., mPFC (Katsouli et al., 2014)		
	GluN1		M enhanced (Waddell, 2010)	
	GluN2A	F > M d.h. (Monfort and Felipo, 2007)		LTP F > M (Monfort and Felipo, 2007)
	mGluR8			M and F impaired [NLR] F impaired [MWM] (Duvoisin et al., 2010; Iñiguez et al., 2012; Chen et al., 2010; Mendez-Lopez et al., 2009; Shalin et al., 2006; Ter Horst, 2012)
GABA receptors	δ-$GABA_A$			F enhanced [TFC] (Wiltgen et al., 2005)
	α4-$GABA_A$			F enhanced [Context post TFC] M enhanced [Context post TFC] (Moore et al., 2010)
	α5-$GABA_A$			F enhanced [TFC] (Yee et al., 2004)
	γ2-$GABA_A$	F > M (Nani et al., 2013)		F enhanced [IPSP] (Nani et al., 2013)
	PKA	F > M d.h. [MWM] (Iñiguez et al., 2012)		M and F impaired [MWM, NOR, CFC] (Abel et al., 1997)
	ERK1/2	M > F, v.h. [CFC] (Gresack et al., 2009; Antunes-Martins et al., 2007)		
	CaMKKα			M impaired FC, CFC, and MWM (Mizuno et al., 2006)
	CaMKKβ			M impaired MWM; M impaired LTP (Mizuno et al., 2007)

	CBP			M = F [MWM, CFC, NOR] (Chen et al., 2010)
	CREB	M > F CFC, d.h (Kudo et al., 2004)		M and F impaired [MWM, NOR] (Pittenger et al., 2002) M and F impaired (Kogan et al., 1997) F impaired (Hebda-Bauer et al., 2007)
	cFos	F > M [MWM] IL, Ca1, Ca3 (Mendez-Lopez et al., 2009)		
	BDNF	M > F [CFC, HPC] (Mizuno et al., 2006)		
	srp20	M > F [CFC,MWM] (Antunes-Martins et al., 2007)		
	psf	M = F [MWM] (Antunes-Martins et al., 2007)		
	GAA1	M > F d.h. [MWM, CFC] (Mizuno et al., 2007)		
	PKMζ	M > F synaptic localization (Sebastian et al., 2013)		
	NOS			M impaired [LTP] (Dachtler et al., 2012) M and F impaired [CFC] (Kelley et al., 2009)
CDK5	p25			F enhanced [MWM] M and F enhanced [FC] F enhanced [LTP] (Ris et al., 2005)
	p35			F impaired [MWM] (Engmann et al., 2011)
	KSR1			M and F impaired [CFC, PA] (Shalin et al., 2006)
	MR-1			F enhanced CFC; F impaired FC extinction (Ter Horst, 2012)

Table 1.1 ***Differences between male and female protein expression and activation in memory formation.***
Abbreviations: d.h., dorsal hippocampus; v.h., ventral hippocampus; amyg., amygdala; mPFC, medial prefrontal cortex; FC, fear conditioning (cued); CFC, context fear conditioning; TFC, trace fear conditioning; MWM, Morris water maze; LTP, long-term potentiation; NLR, novel location recognition; IPSP, inhibitory postsynaptic potential; nd, no sex difference; MR-1, mineralocorticoid receptor; KSR1, kinase suppressor of Ras 1; NOS, nitric oxide signaling; NOR, novel object recognition; PA, passive avoidance.

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Chapter II

Sex Differences in Generalization and Retrieval of Context Fear Memory

Abstract

Anxiety disorders are commonly associated with increased generalization of fear from a stress- or trauma-associated environment to a neutral context or environment. Differences in context-associated memory in males and females may contribute to increased susceptibility to anxiety disorders in women. Here we examined sex differences in context fear generalization and its neural correlates. We observed higher levels of freezing behavior and more generalization of fear to a similar context in females than males. In addition, context pre-exposure increased fear conditioning in males and decreased generalization in females. Accordingly, males showed stronger cFos activity in dorsal hippocampus during memory retrieval and context generalization, whereas females showed preferential recruitment of ventral hippocampus and basal amygdala. Arc data revealed opposite patterns of activation with greater Arc activity in dorsal hippocampus in females, suggesting that memory retrieval likely involves different signaling mechanisms in males and females. However, pre-retrieval inactivation of ventral hippocampus, but not dorsal hippocampus reveals similar deficits in memory by males and females. Differential competition between hippocampus and amygdala-dependent processes may contribute to sex differences in retrieval of context fear and greater generalization of fear-associated memory.

Introduction

Women are more susceptible to disorders of fear and anxiety than men, with the

prevalence of anxiety disorders and posttraumatic stress disorder (PTSD) two- to three-fold higher in women (Kessler et al., 1995, 2012). Whereas normal fear responses are triggered by trauma-associated contexts, in disorders such as PTSD, fear is also elicited in neutral or safe contexts (Lissek et al., 2010, 2014; Kindt, 2014; Lopresto et al., 2015). Excessive generalization of fear may contribute to increased susceptibility to disorders of fear and anxiety. Therefore, in this project we examined whether females show more generalization of context fear, and how neural correlates of retrieval and generalization differ between females and males.

One potential mechanism for generalization of context- dependent fear is the failure to learn a complete representation of a context associated with an aversive event, resulting in an inability to distinguish between a dangerous context and a neutral place (Westbrook et al., 1994; Rudy and O'Reilly, 1999). Alternatively, failure to retrieve detailed context information at remote time points also results in increased generalization of fear (Wiltgen and Silva, 2007; Wiltgen et al., 2010). Separate studies have demonstrated that females have slower acquisition of context representations (Wiltgen et al., 2001), more rapid loss of context specificity with time (Lynch et al., 2013), and are less adept at pattern separation compared with males (Yagi et al., 2015). Therefore, altered formation or retrieval of context representations in females may result in increased generalization of context fear memories.

Learning about context is a critical contributor to sex differences in context fear conditioning, where males are reported as showing higher levels of freezing behavior (Maren et al., 1994; Gresack et al., 2009; Mizuno et al., 2012) and more activation of hippocampus (Maren et al., 1994; Kudo et al., 2004; Gresack et al., 2009) during acquisition and consolidation of context fear conditioning compared with females. Given the role of dorsal hippocampus in context representations (Biedenkapp and Rudy, 2007; Wiltgen et al., 2010; Zelikowsky et al.,

2014), and the requirement of context representation for context fear conditioning (Barrientos et al., 2002), these findings suggest that males have stronger encoding of context representations and thus more precise context fear memory.

In contrast, additional studies demonstrate that sex differences in context fear conditioning are more complex than a failure of females to acquire context representations or context–shock associations. First, there is limited evidence for higher levels of freezing behavior in males compared with females. Many studies fail to show sex differences in context fear conditioning (Kosten et al., 2006; Dachtler et al., 2011), or only observe sex differences under specific experimental conditions such as very short context exposures (Wiltgen et al., 2001) or in specific strains (Pryce et al., 1999). Other studies show stronger context fear conditioning in females (Ris et al., 2005; Moore et al., 2010), or do not use sex as a variable in analyses (Temme et al., 2014). Second, sex differences in retrieval of fear-associated memories suggest that this process, and not memory formation, causes differential fear responses at test. In females, for example, hippocampal estradiol causes decreased retrieval of hippocampal-dependent memory (Lynch et al., 2014), whereas in males testosterone suppressed amygdala activation (Chen et al., 2014). This pattern of results suggests that females shift away from hippocampal processing during retrieval of fear-associated memories, and males shift toward hippocampal processing. Given competition between hippocampus and basal amygdala in context fear conditioning (Biedenkapp and Rudy, 2009) and the importance of basal amygdala in context fear conditioning (Matus-Amat et al., 2007; Amano et al., 2012; Jin and Maren, 2015), females might shift toward basal amygdala activation during memory retrieval. Sex-biased patterns of hippocampus and amygdala mechanisms during retrieval may thereby result in greater generalization of context fear in females compared with males.

Here we examined whether males and females differ in foreground context fear conditioning and context generalization, as well as how the sexes differ in activation of hippocampus and basal amygdala after memory retrieval. Lastly, we assess the requirement of hippocampus in males and females in context fear memory retrieval. We used foreground context fear conditioning alone to examine sex differences in generalization, and context fear conditioning after pre-exposure to the training context to manipulate the strength of context representations. To control for nonspecific effects of handling and exploration of a novel context on context generalization, we used pre-exposure to a similar context. We demonstrated that in context fear conditioning alone, females exhibited higher levels of freezing behavior and more generalization of context fear than males. Pre-exposure to the training context reduced generalization in females. In addition, we observed weaker cFos activation in dorsal hippocampus, and stronger activation in ventral hippocampus and basal amygdala during memory retrieval in females compared with males. Arc data reveal opposite patterns of activation, with higher levels in the dorsal hippocampus of females compared with males following retrieval. Pharmacological pre-retrieval inactivation of hippocampus reveals similar deficits in memory by males and females when targeting ventral but not dorsal hippocampus.

Methods

ANIMALS

The 9-week-old C57BL/6 mice (96 males, 97 females) from Envigo (Indianapolis, IN) were individually housed throughout experiments with standard diet and water ad libitum. Individual housing in males is required to reduce fighting- induced stress (Meakin et al., 2013), and is consistent with both previous fear conditioning studies (see, eg, Radulovic et al., 1998; Tronson et al., 2009; Tanaka et al., 2014; Van Craenendonck and Ver Donck, 2014) and

University of Michigan Institutional Care and use Committee policies on management of fighting in mice, and does not increase variance in either sex (Prendergast et al., 2014). Because of independent social structures of both male and female mice (Becker and Koob, 2016), individual housing is ecologically appropriate for both sexes. The colony room was adjacent to behavioral testing rooms and maintained at 20 ± 2 °C with a 12 h 0700 : 1900 h light/dark cycle (lights on at 0700 h). All mice were acclimated to the colony room for at least 7 days before experiments began. All experimenters in this study were women (Sorge et al., 2014). The University of Michigan Committee on the Use and Care of Animals approved all experimental methods performed in this research.

APPARATUS

Training and testing conditions were performed in conditioning chambers (9 3/4″ × 12 3/4″ × 9 3/4″; MedAssociates, VT), enclosed in sound-attenuating cubicles, equipped with a NIR camera (VID-CAM-MONO-2A). Grid floor rods were connected to a shock generator. Male and female mice were tested in separate chambers and chambers were cleaned between each animal with either 70% ethanol or 1% acetic acid. Video Freeze software (MedAssociates) automatically scored freezing and locomotor activity. Two experimenters, blind to experimental conditions, hand scored freezing to verify automatic scoring.

Three different contexts were used: (1) Training context: context A (CxtA) consisted of a rectangular box with white walls, lights on, an evenly sized grid floor (36 stainless steel rods, 1/8″ diameter, spaced 1/4″ apart), and 70% ethanol odor. (2) Generalization context: context B (CxtB) had curved white walls, house lights off, the same floors, and ethanol odor as in CxtA. (3) Distinct context: context C (CxtC) consisted of black angled walls, house lights off, staggered grid floors with alternating 1/8″ and 3/16″ grid rods, and 1% acetic acid odor.

CONTEXT FEAR CONDITIONING

Foreground context fear conditioning was conducted as previously described (Tronson et al., 2009). Briefly, mice were placed in CxtA for 3 minutes, followed by delivery of a 2s, 0.8 mA foot shock. Mice were then replaced in their home cage and returned to the colony room.

RETRIEVAL AND GENERALIZATION TESTS

At 24 h after training, mice were placed into CxtA or CxtB for 3 minutes and immediately returned to the colony room. Freezing was assessed during this time. Mice were retested at 24 hour intervals first in the reverse context (CxtB or CxtA) and subsequently in CxtC (ie, test order ABC or BAC; Figure 2.1). All behavioral experiments used a group size of n = 8 males and n = 8 females per test order.

CONTEXT PREEXPOSURE

To determine whether prior exposure to training or test contexts decreased generalization, mice were placed in either CxtA (Figure 2.2) or CxtB (Figure 2.3) for 10 min and returned to their home cage. At 24 h after context exposure, mice were fear conditioned in CxtA and tested in CxtA, B, and C, as described above.

ESTROUS CYCLE

Estrous samples were collected at approximately the same time each day, 1hour before behavioral experiments. To determine estrous phase in female mice, wet vaginal smears were taken in 5µl of distilled deionized water. Fluid was pipetted 3–4 times and dropped on slides. Light microscopy was used to assess vaginal cytology and determine estrous stage (Caligioni, 2009).

DISCRIMINATION SCORE

We calculated a DS to compare context generalization across experiments, taking into

account variability in freezing. We used the following formula: (CxtA−CxtB)/(CxtA−CxtC), where CxtA, CxtB, and CxtC represent freezing in each context. This formula compares the difference in freezing in the similar contexts (CxtA and CxtB) with difference in freezing in distinct contexts (CxtA and CxtC). Because others have demonstrated good discrimination between two distinctive contexts (eg, Cxts A and C) (Wiltgen et al., 2010), freezing in CxtC represents freezing to nonspecific factors including transportation and handling (Rudy and O'Reilly, 2001) that trigger some retrieval of prior experience and thus small increases in freezing. In addition, experimentally, tests in CxtC controlled for different levels of freezing between the sexes in a novel context. Including these data in the discrimination score performs the same function mathematically.

CFOS IMMUNOHISTOCHEMICAL ANALYSIS

Mice were deeply anesthetized (Avertin, 480 mg/kg, i.p.) and transcardially perfused with 4% paraformaldehyde 1 hour after fear conditioning (naive: n = 4 per sex, fear conditioning: male n = 4, female n = 5), or after retrieval in CxtA or CxtB (naive n=4, CxtA n=6, CxtB: n = 6 per sex). Standard immunohistochemistry protocols were used, as previously described (Tronson et al., 2009). Briefly, sections were incubated with anti-cFos (EMD Millipore; 1:6250), anti-Arc (Synaptic systems, 1:2750) goat anti-mouse secondary (VectorLabs; 1 :200) for cFos and goat anti-rabbit (VectorLabs, 1:200) for Arc, and DAB chromogen (Sigma Aldrich, St Louis, MO). Quantification of cFos and Arc+ cells was conducted by investigators blind to experimental group in the following brain regions: dorsal and ventral hippocampal CA1, CA3, DG, and basal amygdala (BA that included both basolateral and basomedial amygdala; see Amano et al, 2012). Sections for all brain regions were cut at 40 μM and selected at the same level (−1.79 mm AP relative to bregma, Paxinos and Franklin, 2013). Regions for quantification were defined by

ImageJ (NIH, Bethesda, MD) and held constant across animals. cFos and Arc+ counts were normalized to the number of cells in naive animals.

STEREOTAXIC SURGICAL PROCEDURES

Avertin (2,2,2-tribromoethanol) was given as an anesthetic and was dissolved in 2-methyl-2-butanol at a dose of 250 mg/kg and was made fresh each day. Carprofen (5 mg/kg, subcutaneous) was given before surgery as an analgesic. Cannulae consisted of a double-guided cannula, dummy, and cap. Using a stereotaxic apparatus (KOPF, stereotaxic alignment system), cannulae were implanted into dorsal (+/-1 mm lateral to bregma; -1.5mm posterior to bregma; 2.00+0.5 mm ventral to dura) or ventral hippocampus (AP -3mm, DV +/- 3.1mm, DV 3.00mm, depth 0.5mm) and were attached to the skull using dental cement (3M Inc.). Mice with implanted cannulae were given 1 week to recover prior to context fear conditioning.

In separate groups of male and female mice, animals received 1 µl of the DREADD virus (AAV-hSyn-HA-hM3D(Gi)-IRES-mCitrine, plasmid #50464, Addgene) or control (pAAV-hSyn-EGFP, plasmid #50465, Addgene) infused slowly over a 5-minute period during surgery. Mice were given 4 weeks to allow for virus expression prior to context fear conditioning. Animals were closely monitored for pain response for days following the surgery.

HIPPOCAMPAL INACTIVATION: MUSCIMOL

On day 1 male and female mice underwent context fear conditioning as described above. On day 2 mice were infused with muscimol (0.5µg/0.25µl/side, Sigma) or artificial cerebral spinal fluid (0.25µl/side, Harvard Apparatus) with Hamilton syringes through the implanted cannulae targeting dorsal or ventral hippocampus by infusion pump at rate of 1µl/min, 10 minutes before retrieval. Disruption of retrieval of context fear was measured by assessing levels

of freezing to the shock-paired context. On day 3, drugs were switched to counterbalance drug infusion order.

HIPPOCAMPAL INACTIVATION: DREADDS

Designer Receptors Exclusively Activated by Designer Drugs (DREADDs) were used as a second measure to assess requirement of hippocampus in retrieval of context fear memory. On day 1 male and female mice underwent context fear conditioning as described above. On day 2 mice were injected intraperitoneally with vehicle (0.5% DMSO in 0.9% saline) or Clozapine-N-oxide (CNO) (3.0 mg/kg, MedChem Express) 45 minutes prior to testing to activate the inhibitory DREADD receptors expressed in dorsal hippocampus. Disruption of retrieval of context fear was measured by assessing levels of freezing to the shock-paired context. On day 3, drugs were switched to counterbalance drug infusion order.

STATISTICAL ANALYSIS

All statistical analyses were conducted using SPSS v23. Three-way repeated measures ANOVA (Context × Order × Sex) was used to determine sex differences in generalization of context fear. Separate two-way analyses were conducted on the discrimination score (Sex × Order), and to compare discrimination scores or freezing of females and males between experiments (Sex × Pre-exposure). For cFos and Arc experiments we used two-way (Sex × Context) ANOVA to determine sex differences in cFos and Arc+ cells after retrieval or consolidation of context fear. A two-way multivariate ANOVA (Estrous × Pre-exposure) was used to determine the effect of estrous on context fear conditioning. A two-way ANOVA (Drug × Testing Order) was used for pharmacological inactivation experiments to determine the effect on retrieval of context fear memory. The post hoc tests with Bonferroni corrections for multiple tests were used to further examine significant effects in each experiment. The 95% confidence

intervals are reported for post hoc tests. Partial η^2 (η^2_p) is reported as effect size estimate (0.01, 0.06, and 0.14 as small, medium, and large, respectively), as calculated by SPSS.

Results

SEX DIFFERENCES IN CONTEXT FEAR CONDITIONING

After single trial context fear conditioning, females showed more freezing in the training context (CxtA) compared with males (Figure 2.1b) (Sex: $F_{(1, 28)} = 12.29$, $P < 0.01$, $\eta^2_p = 0.31$; ABC: $P < 0.05$ (95% CI: 0.86–39.14); BAC: $P < 0.05$ (95% CI: 2.61–40.89)).

ESTROUS CYCLE DID NOT AFFECT FEAR CONDITIONING

Context fear conditioning in females was not affected by estrous phase on training day. In the context fear conditioning alone condition, females showed similar freezing in CxtA ($F(3,12) = 1.50$, $P = 0.265$), CxtB ($F(3,12) = 1.06$, $P = 0.40$), or CxtC ($F(3,12) = 1.04$, $P = 0.41$) regardless of estrous phase (proestrous n = 3, estrous n = 4, metestrous n = 5, diestrous n = 4). To ensure adequate statistical power, we examined the impact of estrous cycle across all behavioral experiments. Freezing was not affected by estrous at training in any test context (Main Effect Estrous: CxtA: $F_{(3, 48)} = 0.643$, $P = 0.59$, CxtB: $F_{(3, 48)} = 1.873$, $P = 0.152$; CxtC: $F_{(3, 48)} = 0.28$, $P = 0.84$) or in any pre-exposure condition (Pre-exposure × Estrous interaction, CxtA: $F_{(6, 48)} = 0.76$, $P = 0.61$; CxtB: $F_{(6, 48)} = 1.74$, $P = 0.14$; CxtC: $F_{(6, 48)} = 1.31$, $P = 0.28$).

SEX DIFFERENCES IN GENERALIZATION OF CONTEXT FEAR

Females showed greater generalization of fear between CxtA and CxtB compared with males (Context × Sex interaction: $F(2,56) = 5.62$, $P < 0.003$, $\eta^2_p = 0.17$), with similar freezing levels in CxtA and CxtB regardless of test order (ABC: $P = 0.073$; BAC: $P = 0.104$), and significantly less freezing in CxtC (ABC: $P < 0.001$ (95% CI: 26.52–49.73); BAC: $P < 0.001$

(95% CI: 24.02–47.23); Figure 2.1b). In contrast, males showed good discrimination of context fear with significantly less freezing in CxtB (ABC: $P < 0.001$ (95% CI: 4.27–28.98); BAC: $P < 0.05$ (95% CI: 1.52–26.23)) and less freezing in CxtC (ABC: $P < 0.01$ (95% CI: 6.52–29.73); BAC: $P < 0.001$ (95% CI: 17.39–40.61)) compared with CxtA (Figure 2.1b).

Both males and females showed low levels of freezing in CxtC, and this did not differ between the sexes (ABC: $P = 1.00$ (95% CI: −15.76 to 15.76); BAC $P = 0.06$ (95% CI: − 0.64 to 30.89); Figure 2.1b). Generalization between similar contexts by females is thus due to its similarity with the training context and not a nonspecific aversion to novel contexts.

To determine whether generalization by females was a function of stronger fear in CxtA, we calculated DS. Here, males had a significantly higher DS (DS = 0.78) than females (DS = 0.1) ($F(1,28) = 5.58$, $P < 0.05$, $\eta^2_p = 0.18$), demonstrating stronger generalization of fear from CxtA to CxtB in females (Figure 2.1c).

PRE-EXPOSURE TO TRAINING CONTEXT REDUCED FEAR GENERALIZATION IN FEMALES

Pre-exposure to the training context decreased generalization of context fear in females. After pre-exposure to CxtA and training in the same context, both male and female mice showed more freezing in CxtA than CxtB or CxtC (Context: $F(2,56) = 249.87$, $P < 0.001$, $\eta^2_p = 0.90$); Figure 2.2b), with no sex differences in freezing or context generalization (Sex $F_{(1, 28)}<1$; Sex × Context $F_{(2, 56)}<1$). Pre-exposure to CxtA resulted in discrimination between CxtA and CxtB by females (ABC: $P < 0.001$ (95% CI: 40.36–60.15); BAC: $P < 0.05$ (95% CI: 0.11–19.90), cf CxtA). Males continued to show little generalization between CxtA and CxtB (ABC: $P < 0.001$ (95% CI: 29.73–49.52); BAC: $P < 0.001$ (95% CI: 15.86–35.65) cf CxtA); Figure 2.2b).

When CxtB was tested before CxtA, both males and females showed stronger context

generalization compared with animals tested in CxtA first (Context × Order: $F_{(2, 56)} = 19.57$, $P < 0.001$, $\eta^2_p = 0.41$), and this effect was more pronounced in females (Context × Sex × Order: $F_{(2, 56)} = 3.97$, $P < 0.05$, $\eta^2_p = 0.12$; Figure 2.2b). Analysis of discrimination scores confirmed these test order effects (Order: $F(1,28) = 37.77$, $P < 0.001$; Females: $P < 0.001$ (95% CI: 0.44–0.91); Males: $P < 0.01$ (95% CI: 0.09–0.55); Sex × Order: $F(1,28) = 4.81$, $P < 0.05$, $\eta^2_p = 0.15$; Figure 2.2c). Compared with the previous experiment, pre-exposure to CxtA significantly increased the discrimination scores of females ($P < 0.05$ (95% CI: 0.08–0.80)) but not males ($P = 0.41$; Figures 2.1c and 2.2c). Pre-exposure to the training context thus reduced context fear generalization in females. Pre-exposure to CxtA eliminated the sex differences in freezing in CxtA (Figure 2.2a), with only males exhibiting more freezing after pre-exposure compared with context fear conditioning alone (2-way ANOVA PreExp: $F(1,60) = 22.51$, $P < 0.001$, $\eta^2_p = 0.27$; Sex: $F(1,60) = 7.34$, $P < 0.01$, $\eta^2_p = 0.11$; PreExp × Sex: $F(1,60) = 6.14$, $P < 0.01$, $\eta^2_p = 0.10$; males: $P < 0.001$ (95% CI: 17.68–40.45); females: $P = 0.114$).

PREEXPOSURE TO CXTB INCREASED GENERALIZATION OF CONTEXT FEAR IN MALES

After pre-exposure to CxtB, there were no sex differences in freezing or context generalization (Context $F(2, 56) = 190.98$, $P < 0.001$, $\eta^2_p = 0.87$; Sex: largest $F(1,28) < 1$; Figure 2.3a). Both males and females showed a small but significant decrease of freezing in CxtB compared with CxtA (Females: ABC: $P < 0.01$ (95% CI: 7.40–29.35), BAC: $P < 0.05$ (95% CI: 1.03–22.98); Males: ABC: $P < 0.01$ (95% CI: 6.90–28.85), BAC: $P < 0.001$ (95% CI: 11.53–33.48)) and substantially less freezing in CxtC (all $P < 0.001$; Figure 2.3b). Discrimination scores did not differ between Context, Order, or Sex (largest $F = 1.02$, $P = 0.32$), demonstrating no sex differences in context generalization after pre-exposure to CxtB (Figure

2.3c).

In comparison to context fear conditioning alone, pre-exposure to CxtB resulted in higher freezing to CxtA, but only in males (PreExp: $F(1,60) = 11.09$, $P < 0.01$, $\eta^2_p = 0.16$; Sex: $F(1,60) = 5.64$, $P < 0.05$, $\eta^2_p = 0.09$; PreExp × Sex: $F_{(1,60)}=7.70$, $P < 0.01$, $\eta^2_p = 0.11$). In males, freezing increased after pre-exposure to CxtB in both CxtA ($P < 0.001$ (95% CI: 13.28–36.22)) and CxtB ($P < 0.001$ (95% CI: 8.35–31.27); Figures 2.1b and 2.3b). Accordingly, context generalization was high in males pre-exposed to CxtB, with lower discrimination scores compared with fear conditioning alone ($P < 0.05$ (95% CI: 0.01–0.736); Figures 2.1c and 2.3c). Pre-exposure to CxtB did not alter generalization in females ($P = 0.29$).

SEX DIFFERENCES IN DORSAL HIPPOCAMPUS CFOS ACTIVATION AFTER CONTEXT FEAR MEMORY RETRIEVAL

We observed more cFos+ cells in males compared with females in CA1 (Figure 2.4a), CA3 (Figure 2.4b), and DG (Figure 2.4c) after retrieval of context fear conditioning with significant main effects of sex in all regions (CA1: $F(1,26) = 4.6$, $P < 0.05$, $\eta^2_p = 0.15$; CA3: $F(1,26) = 2.90$, $P = 0.101$; DG: $F(1,26) = 7.36$, $P < 0.05$, $\eta^2_p = 0.22$) and a main effect of test in CA1 and CA3 (CA1: $F(1,26) = 7.32$, $P < 0.01$, $\eta^2_p = 0.36$; CA3: $F(2,26) = 3.43$, $P < 0.05$, $\eta^2_p = 0.21$; DG: $F(2,26) = 1.16$, $P = 0.33$). Males exhibited strong activation of cFos after test in either training (CxtA) or generalization context (CxtB) compared with naive in both CA1 (CxtA: $P < 0.01$ (95% CI: 1.47–4.26); CxtB: $P < 0.05$ (95% CI: 1.15–3.94)) and CA3 (CxtA: $P < 0.05$ (95% CI: 0.30–3.27); CxtB: $P < 0.05$ (95% CI: 0.09–3.05)). In contrast, females showed a significant increase in cFos only in CA1 after test in CxtA (CA1: CxtA: $P = 0.05$ (95% CI: −0.04 to 5.03); CA3: CxtA $P = 0.27$) but not CxtB (CA1: CxtB: $P = 0.661$; CA3: CxtB: $P = 0.51$). In

CA1, cFos+ cells were similar for both males and females in the training context (CA1: CxtA: P = 0.136), and significantly greater in males in the generalization context (CA1: CxtB: $P < 0.05$ (95% CI: 0.55–5.97)). These findings suggest that CA1 is activated during retrieval of context for both males and females but a similar context recruits cFos in this region only in males (Figure 2.4a).

Similarly, in DG, cFos+ cells were increased in males, only after test in CxtA (Sex: $F(1,26) = 7.36$, $P < 0.05$, $\eta^2_p = 0.22$; Test: $F(2,26) = 1.16$, $P = 0.33$; interaction: $F(2,26) = 1.94$, $P = 0.17$; CxtA: $P < 0.05$ (95% CI: 0.09–1.23); CxtB: $P = 0.27$), whereas females showed no increases in cFos (CxtA: $P = 0.69$; CxtB: $P = 0.54$; Figure 2.4c).

SEX DIFFERENCES IN VENTRAL HIPPOCAMPUS CFOS ACTIVATION AFTER CONTEXT FEAR MEMORY RETRIEVAL

We observed more cFos+ cells in females compared with males in the CA1 region of the ventral hippocampus after retrieval of context fear memory, with a significant main effect of sex ($F(2, 25) = 14.991$, $P < 0.01$), group ($F(2, 25) = 8.638$, $P < 0.01$), and interaction between Group and Sex ($F(2, 25) = 3.744$, $P < 0.05$). Further post-hoc tests revealed that females show greater levels of cFos in ventral CA1 compared with same sex naïve after retrieval, whereas males did not (Males: CxtA: $P = 0.341$, CxtB: $P = 0.391$; Female: CxtA: $P < 0.001$ (95% CI: 6.75-17.24, CxtB: $P < 0.01$ (95% CI: 3.59-17.25), (Figure 2.5). In ventral dentate gyrus (DG), both sexes exhibit an increased level of cFos+ cells during retrieval compared with same sex naïve. We observed a significant main effect of Group ($F(2, 25) = 5.29$, $P < 0.05$, but not Sex ($F(1, 25) = 0.331$, $P = 0.57$), or interaction ($F(2, 25) = 0.1.85$, $P = 0.179$). Further post-hoc tests revealed that both sexes exhibited strong activation of cFos after retrieval compared with same sex naïve in DG (Males: CxtA: $P < 0.05$ (95% CI: 0.06-1.64), CxtB: $P = 0.19$; Females: CxtA: P

= 0.16, CxtB: P < 0.01 (95% CI: 0.25-1.39)). In ventral CA3, neither sex exhibited an increased level of cFos+ cells during retrieval compared with same sex naïve. We did not observe a significant main effect of Group (F (2, 21) = 0.128, P = 0.881, Sex (F (2, 21) = 0.945, P = 0.342), or interaction (F (2, 21) = 0.779, P = 0.472).

SEX DIFFERNCES IN BASAL AMYGDALA CFOS ACTIVATION AFTER CONTEXT FEAR MEMORY RETRIEVAL

In the basal amygdala (Figure 2.4d), females but not males showed an increased number of cFos+ cells (Sex: F (1,26) = 10.74, P < 0.01, η^2_p = 0.29; Condition F (2,26) = 9.19, P < 0.01, η^2_p = 0.41; Interaction: F (2,26) = 2.14, P = 0.14). In females, testing in CxtA (P < 0.001 (95% CI: 1.47–4.26)) or CxtB (P < 0.01 (95% CI: 1.15–3.94)) resulted in increased cFos+ cells compared with naives. In contrast, no such activation was observed in males (CxtA: P = 0.18; CxtB: P = 0.15). After both tests, the number of cFos+ cells in basal amygdala was significantly higher in females compared with males (CxtA: P < 0.01 (95% CI: 0.76–3.26); CxtB: P < 0.05 (95% CI: 0.38–2.88); Figure 2.4d).

Together, these results suggest that males and females differentially recruit fear memory-associated brain regions during retrieval, with males showing preferential cFos activation of hippocampus and females preferentially activating cFos in amygdala.

SEX DIFFERENCES IN DORSAL AND VENTRAL HIPPOCAMPUS ARC ACTIVATION AFTER CONTEXT FEAR MEMORY RETRIEVAL

In the dorsal dentate gyrus region of the hippocampus following retrieval of context fear (Figure 2.6), females but not males showed an increased number of Arc+ cells (Sex F (1, 25) =19.02, P < 0.01; Sex x Condition F (2, 25) = 4.33, P < 0.05; (Males: CxtA: P = 1, CxtB: P = 0.894; Females: CxtA: P < 0.01 (95% CI: 1.01-3.29), CxtB: P < 0.01 (95% CI: 0.62-2.82)). In

the ventral dentate gyrus region of the hippocampus (figure 2.6), both males and females showed an increased number of Arc+ cells (Group ($F(1, 15) = 7.079$, $P < 0.05$); however, there was a trend for higher Arc+ cells in males compared with females (Sex ($F(1, 15) = 0.543$, $P = 0.473$); Group × Sex ($F(1, 15) = 0.543$, $P = 0.473$.

MALES AND FEMALES SHOW SIMILAR CFOS ACTIVATION IN DORSAL HIPPOCAMPUS AND AMYGDALA DURING MEMORY CONSOLIDATION

After training, cFos+ cells were increased in males and females in CA1 (Training: $F(1,14) = 18.92$, $P < 0.01$, $\eta^2_p = 0.58$; Sex: $F_{(1, 14)}<1$; Sex × Training: $F(1,14) <1$; Figure 2.7a) and in basal amygdala (Training: $F(1,14) = 19.55$, $P < 0.01$, $\eta^2_p = 0.58$; Sex: $F(1,14) <1$; Sex × Training: $F(1,14) <1$; Figure 2.7d) after context fear conditioning. The post hoc tests confirmed increased cFos in both females (CA1: $P < 0.01$ (95% CI: 0.70–3.30); BA: $P < 0.01$ (95% CI: 0.69–2.74)) and males (CA1: $P < 0.05$ (95% CI: 0.50–3.41); BA: $P < 0.05$ (95% CI: 0.31–2.60)). Ventral hippocampal cFos and Arc activation was not examined following consolidation. These findings suggest that males and females show similar activation of cFos in hippocampus and amygdala during consolidation of context fear conditioning.

PRE-RETRIEVAL VENTRAL, BUT NOT DORSAL HIPPOCAMPAL INACTIVATION IMPAIRS FREEZING

Males and females show decreased freezing when muscimol was infused into the ventral hippocampus prior to retrieval (Figure 2.8). We observed a main effect of Drug (Males: $F(1, 24) = 8.669$, $P < 0.01$; Females: $F(1, 27) = 4.751$, $P = 0.05$), and no main effect of testing order (Males: $F(1, 24) = 2.234$, $P = 0.148$; Females: $F(1, 27) = 0.061$, $P = 0.807$) or interaction of drug and testing order (Males: $F(1, 24) = 0.006$, $P = 0.941$; Females: $F(1, 27) = 1.204$, $P =$

0.282). Dorsal hippocampal inactivation via muscimol or CNO in DREADD mice did not result in decreased freezing in either sex (see figure 2.9 and 2.10).

Discussion

Here we demonstrated that females show greater generalization of context fear conditioning than males as well as sex- specific patterns of retrieval-induced cFos and Arc in hippocampus and amygdala. Together with previous findings demonstrating that hippocampal activation correlates with less generalization of context fear in males (Wiltgen et al., 2010) because of its role in retrieval of detailed information about context (Gafford et al., 2013), our results suggest that males and females utilize different neural correlates or molecular mechanisms in retrieval of context-associated memories.

Our observation that females showed less context specificity of context fear conditioning than males is consistent with studies demonstrating sex differences in learning and consolidation of context representations (Wiltgen et al., 2001, 2010) and in hippocampal mechanisms of context fear conditioning (Mizuno et al., 2006, 2007; Antunes-Martins et al., 2007; Moore et al., 2010; Dachtler et al., 2011). Our data further demonstrate that sex differences in context fear-associated memory and hippocampal activity occur even when both males and females show strong context fear responses. These findings add to the growing consideration of sex differences as complex and nuanced. Previous studies of context fear conditioning have demonstrated male-specific mechanisms of context fear conditioning (Mizuno et al., 2006; Antunes-Martins et al., 2007; Moore et al., 2010; Dachtler et al., 2011). This is the first paper to observe both male- and female-biased neural correlates of fear-associated memory retrieval, thereby providing some initial insight into how (De Vries, 2004; Maney, 2016) males and females differ in this task.

Sex differences in context fear conditioning in this task cannot be simply attributed to

less efficient formation of context representations in females. Pre-exposure to the training context appeared to enhance context fear conditioning in males, consistent with prior evidence that stronger context representations are required for formation of context–shock associations (Fanselow, 1990; Rudy and O'Reilly, 1999). In contrast, females did not show better context fear conditioning after context pre-exposure, but instead decreased generalization between similar contexts. These results demonstrate that both males and females require additional time to learn detailed representations of context but might use context information in different ways. Whereas females are biased toward increased freezing in ambiguous contexts, males are biased toward increased freezing only in contexts that are strongly associated with aversive events. These findings raise the intriguing possibility that sex differences in generalization between similar contexts are due to differences in retrieval of context information.

Supporting the role of retrieval mechanisms in context generalization, in females, pre-exposure to the training context decreased generalization in a test-order-specific manner—ie, only when tested first in the training context. This has several implications: first, learning about CxtA before context fear conditioning is not sufficient to reduce context generalization as female mice tested in test order CxtB and then CxtA continued to generalize. Second, as females tested in test order CxtA and then CxtB show robust discrimination, retrieval in CxtA and post-retrieval processing such as extinction may contribute to reduced ambiguity and thus decreased fear in subsequent exposures to a similar context.

Counter to our expectations that pre-exposure to CxtB would not affect context generalization, we observed increased generalization in males, suggesting that retrieval of the CxtA representation is less specific than after context fear conditioning alone. This effect is somewhat surprising, as learning about CxtB (with no shock) and CxtA (paired with shock)

should result in no difference in freezing to the generalization context B, and potentially enhance, context discrimination. Our results are consistent, however, with findings that pre-exposure to a context followed by an immediate foot shock in a different box results in conditioned fear to the pre-exposure context (Rudy and O'Reilly, 2001; Bae et al., 2015). Our data extend these findings to show that this overgeneralization effect occurs even when male mice are given sufficient time to form a conjunctive hippocampal representation of the training context. In contrast, pre-exposure to CxtB in males, similarly to CxtA, increased freezing levels to that of females. These data suggest that after pre-exposure to CxtB, males are unable to retrieve a sufficiently distinct memory of the training context to suppress freezing in a similar context.

Our data exclude the possibility that increased generalization by females is due to more defensive behaviors in novel contexts. First, males and females did not differ in freezing to a very different context, despite its novelty. Second, after pre-exposure to the training context, males and females showed equivalent low levels of freezing to the novel CxtB. Third, after pre-exposure to CxtB, both males and females showed strong freezing in CxtB, despite its familiarity. Finally, recent data suggest that in contrast to showing more freezing, females are more likely to show active behavioral responses to shock-associated stimuli (Gruene et al., 2015a). Stronger generalization in females, therefore, cannot be explained by a greater propensity to express freezing responses in novel situations and must therefore be due to sex differences in memory processes.

In accordance with a retrieval-based account of context fear generalization, we observed sex differences in cFos and Arc activation in hippocampus and cFos activation in basal amygdala after memory retrieval, but not consolidation. Specifically, we observed that males had stronger

cFos activation in dorsal hippocampus after tests in both CxtA and CxtB, whereas females had greater ventral hippocampus and amygdala activation. Following retrieval, females showed greater activation of Arc in the dorsal hippocampus compared with males.

That cFos and Arc activity did not correlate with levels of freezing behavior in either males or females is consistent with previous work demonstrating that hippocampal cFos (Tronson et al., 2009; Wiltgen et al., 2010) and Arc (Pevzner and Guzowski, 2014) production does not drive freezing behavior. Sex differences in hippocampal cFos and Arc activation and differing patterns within each brain region suggests that males and females utilize either different underlying molecular mechanisms or an overlapping subset of brain regions in the retrieval of memories for fear-associated contexts.

It is noteworthy that cFos activation does not only reflect memory retrieval. In the hippocampus, cFos has been strongly linked with novelty detection (Radulovic et al., 1998; Radulovic and Tronson, 2010; Yochiy et al., 2012) and habituates with repeated exposure to a context (Radulovic et al., 1998; Tronson et al., 2009). Within this conceptual framework, our data suggest that females retrieve a strong context representation in CxtA, where less hippocampal cFos represents recognition that CxtA is familiar. Furthermore, it suggests that failure to upregulate cFos in CxtB reflects recognition of the similarity between contexts by females, thus triggering fear responses in CxtB. In contrast, robust cFos in both CxtA and CxtB by males suggests that both contexts are perceived as somewhat new, resulting in less freezing compared with females. This interpretation is also consistent with previous findings demonstrating an inverse relationship between hippocampal cFos levels and generalization of context-related fear (Wiltgen et al., 2010), where freezing to a generalization context is high when cFos is low (ie, recognized as similar to the training context), and freezing is low when

cFos is high (ie, the generalization context is perceived as distinct from the training context and therefore novel). This conceptual framework also suggest that males and females are either using different processing strategies or using different circuits or molecular mechanisms during retrieval of context-associated fear.

Given that hippocampus is associated with detailed memory for context (Gafford et al., 2013) whereas amygdala is associated with emotional information (Cahill et al., 2001) and memory 'gist' (Adolphs et al., 2001), these findings also suggest that males and females show biases in retrieval of affective and contextual information. This possibility parallels findings from spatial memory tasks where sex differences are reliably observed in the kind of information and strategies preferentially used (Chai and Jacobs, 2010; Rodríguez et al., 2010; Keeley et al., 2013; Yagi et al., 2015). Whether males and females similarly show different dominant strategies for context fear conditioning remains unknown.

Sex differences in recruitment of brain regions during retrieval may result from differential competition between memory systems. It is well known that during aversive memory tasks, for example, hippocampal activity can result in suppression of amygdala processes, and vice versa (McDonald and White, 1995; Mcintyre et al., 2002; Biedenkapp and Rudy, 2009). In females, activation of amygdala may therefore modulate hippocampal activity, resulting in greater defensive responses and less accurate retrieval of detailed context information. It remains possible that females preferentially activate the ventral hippocampal-basal amygdalar circuit following context fear memory retrieval given greater levels of cFos+ cells in these areas in females compared with males. Recent evidence, however, suggests that (in males) optogenetic stimulation of dentate gyrus cells tagged during consolidation of fear conditioning is sufficient to retrieve a context fear memory (Liu et al., 2012), in part via connections with basal amygdala

(Ryan et al., 2015). That hippocampal–amygdala interactions can either act cooperatively or competitively further suggests that different circuits or molecular mechanisms activated in males and females may result in very different behavioral outcomes.

Despite sex differences in levels of cFos and Arc+ cells in ventral hippocampus following retrieval of context fear, we observed that ventral hippocampus is critical for retrieval in both males and females. This finding is not unexpected given the importance of ventral hippocampus in acquisition and consolidation of context fear in males (Maren and Holt, 2004; Trivedi and Coover, 2004; Zhang et al., 2014). Here, we extend these findings to show a critical role of ventral hippocampus in retrieval of context fear in both sexes. Sex-specific ventral hippocampal immediate early gene patterns following retrieval suggest potential sex differences in upstream signaling cascades that result in differing patterns of cFos expression. Such patterns may also be explained by differences in time course or in cell types involved. Pharmacological or DREADD inactivation of dorsal hippocampus did not disrupt levels of freezing in either sex. This is surprising given the critical role of dorsal hippocampus in contextual fear conditioning in males (Kim and Fanselow, 1992; Phillips and Ledoux, 1992; Kim et al., 1993; Maren and Fanselow, 1997; Frankland et al., 1998; Anagnostaras et al., 1999). While the majority of these studies have focused on manipulations during acquisition or consolidation, it is expected that this region is also critical in the retrieval of context fear. Here, we noted placements surrounding dentate gyrus region of the dorsal hippocampus, however, the majority of studies use placements aimed at the CA1 region. While it is clear that the dorsal dentate gyrus is sufficient to retrieve a context fear memory (Liu et al., 2012), it does not appear to be required to demonstrate fear expression to the training context (Bernier et al., 2017). Additionally, future validation of DREADD virus is needed to make solid claims on ineffective disruption of freezing.

Other brain regions may also be differentially required for retrieval of context fear memories in males and females and involved in competition between memory systems. For example, retrosplenial cortex is required for retrieval of recent and remote context fear memories in males (Corcoran et al., 2011; Cowansage et al., 2013). In addition, anterior cingulate cortex is implicated in retrieval of remote (Frankland et al, 2004), and medial prefrontal cortex (mPFC) in retrieval of recent (Zelikowsky et al., 2013) context fear memories. The role of mPFC in modulation of amygdala activation (Quirk et al., 2003; Sotres-Bayon et al., 2012) and hippocampus (Jin and Maren, 2015), possibly via nucleus reuniens (Xu and Südhof, 2013; Varela et al., 2015), as well as sex differences in mPFC activation during extinction of fear (Gruene et al., 2015b) make this brain region particularly interesting for mediating differential competition between memory systems and context generalization in males and females.

Differential effects of sex and stress hormones on hippocampal and amygdalar function may also mediate sex differences in mechanisms of memory retrieval. In females, hippocampal estrogens regulate generalization of passive avoidance (Lynch et al., 2014, 2016), whereas in males, testosterone decreases amygdala activity during memory retrieval (Chen et al., 2014). Corticosterone alters hippocampus activation (Conrad et al., 2004; Bohacek et al., 2015), competition between memory systems (Beck and Luine, 2010), and is differentially increased during retrieval of fear- associated memories in males and females (Daviu et al., 2014). Sex and stress hormones may thereby contribute to sex differences in retrieval-induced hippocampal and amygdalar mechanisms, and greater generalization of context fear in females.

The initial consolidation of memory determines, in part, which cells store the memory trace (Josselyn et al., 2015) and thus which brain regions are required during memory retrieval, suggesting that sex differences in memory consolidation should also be evident. Nevertheless,

we observed that dorsal hippocampus was activated during consolidation of context fear conditioning in both males and females. This is consistent with findings that both males and females activate hippocampus during consolidation of context fear but recruit different molecular mechanisms in this region (Mizuno et al., 2007; Dachtler et al., 2011; Keiser and Tronson, 2015). It remains unclear how sex differences in mechanisms of memory consolidation contribute to subsequent retrieval and generalization of context fear.

These findings add to growing evidence for sex differences in context fear conditioning and, more generally, sex differences in memory processes. Studies of context fear conditioning have demonstrated lower levels of freezing behavior following context fear conditioning in females compared with males (Maren et al., 1994; Gresack et al., 2009), no differences (Moore et al., 2010; Dachtler et al., 2011), or differences dependent on protocol (Wiltgen et al., 2001). In contrast, we found that females have stronger context fear conditioning and concurrently more context generalization. What is consistent across these studies is that they show differential utilization of hippocampus in females compared with males. This has been exhibited in the form of less hippocampal plasticity (Maren et al., 1994), recruitment of plasticity-related signaling compared with males (Gresack et al., 2009), and more time required for context representations and context fear conditioning (Wiltgen et al., 2001). In these studies, females show less—or delayed—context fear conditioning. Our findings extend this literature to show that males and females differ in context generalization and hippocampal activity during retrieval even under conditions in which females show stronger context fear conditioning than males. Additional studies have demonstrated sex-specific involvement of glutamatergic (Dachtler et al., 2011) and GABAergic (Moore et al., 2010) receptors, and diverging signal transduction mechanisms and gene expression in the hippocampus (Mizuno et al., 2006, 2012; Antunes-Martins et al., 2007)

during learning and consolidation of context fear conditioning. We additionally demonstrate that males and females differentially recruit hippocampal mechanisms and open the possibility that amygdalar-dependent mechanisms also exhibit sex differences in memory retrieval.

Together, our data demonstrate sex differences in retrieval of context fear conditioning, where females show more context generalization and more amygdala and ventral hippocampal cFos activation, and males show strong context discrimination and greater hippocampal cFos activation. Sex differences in memory retrieval have important implications for post-retrieval memory processes, including extinction and memory reconsolidation, and important caveats for their use in treatments for disorders of anxiety and PTSD. Finally, given the role of fear generalization in these disorders (Lissek et al., 2014), our findings suggest that differential circuit activation during anxiety and recall of trauma-associated memories may contribute to increased generalization in women, and higher risk of these disorders in women.

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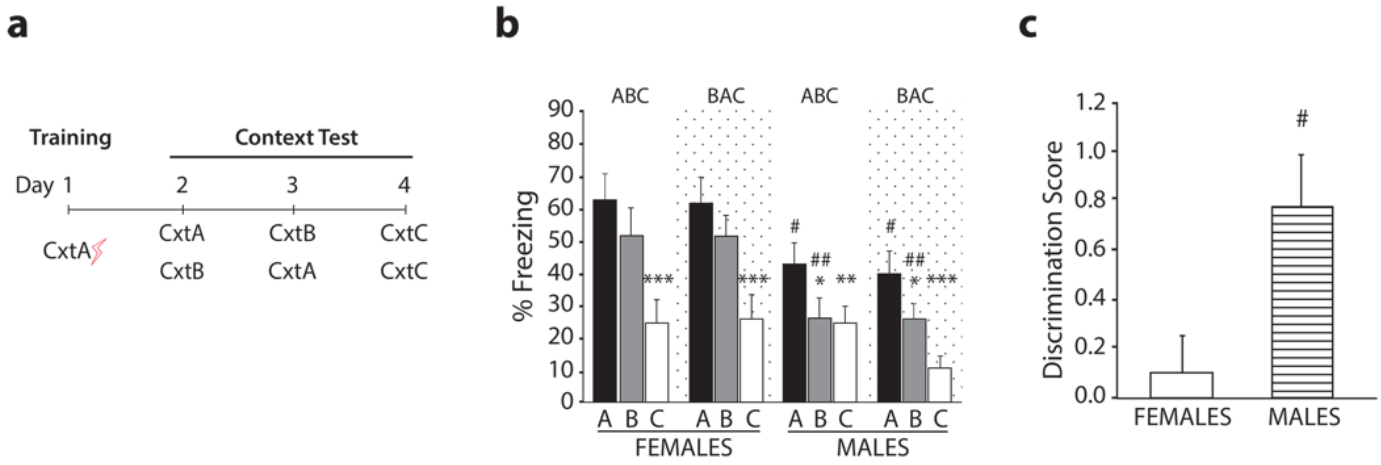

Figure 2.1. ***Females show more generalization of context fear than males.*** (a) Experimental design. (b) Females showed significantly greater freezing than males in the training context (CxtA), and more generalization to CxtB. Both males and females showed low levels of freezing in CxtC. There was no difference in freezing between different test orders (ABC, white background; BAC, dotted background). (c) Males (striped bars) had higher discrimination scores (DS) than females (white bars). Error bars represent SEM. *P<0.05, **P<0.01, ***P<0.001 cf CxtA. $^{\#}$P<0.05, $^{\#\#}$P<0.01 cf females.

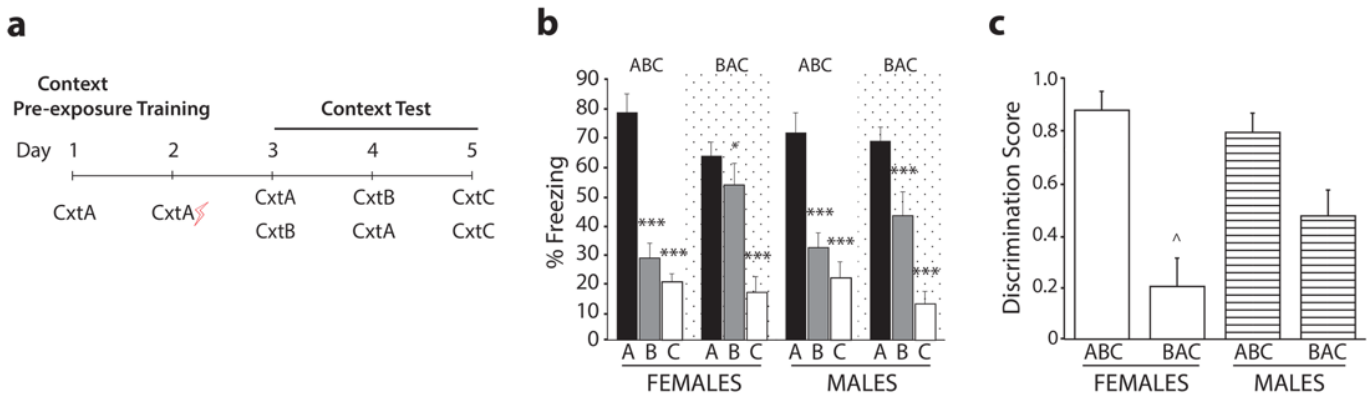

Figure 2.2. ***Preexposure to CxtA reduces generalization of context fear in females.*** (a) Experimental design. (b) Preexposure to CxtA effectively reduced context generalization to CxtB in females, only with ABC test order (ABC, white background; BAC, dotted background). Males showed increased freezing in CxtA compared with non-preexposed males ($P<0.001$). (c) Both males (striped bars) and females (white bars) showed strong discrimination when tested first in CxtA (ABC test order), whereas BAC females showed significantly more generalization. Error bars represent SEM. *$P<0.05$, ***$P<0.001$ cf CxtA. cf ABC test order.

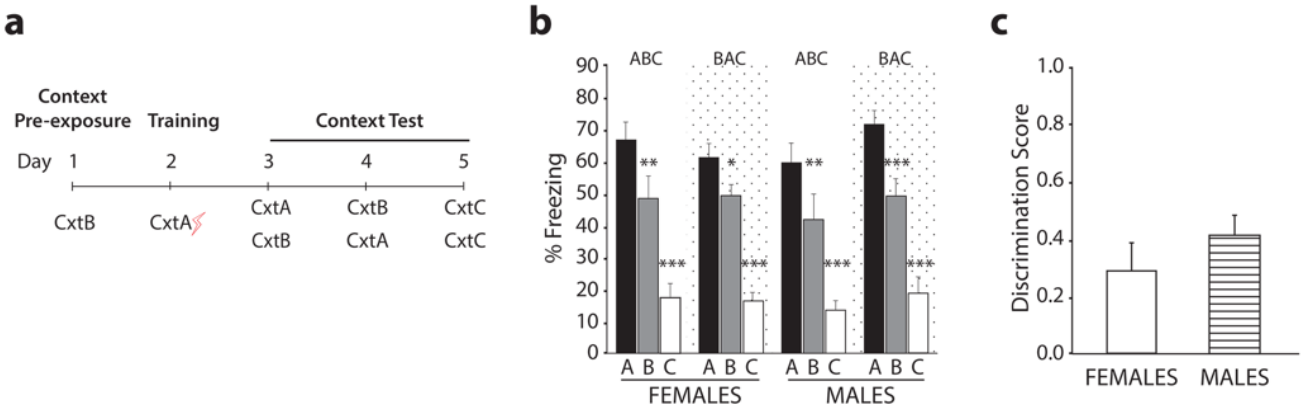

Figure 2.3. ***Preexposure to CxtB increases generalization of context fear in males.*** (a) Experimental design. (b) There were no sex differences in freezing in context. Both males and females showed generalization between CxtA and CxtB. Test order did not affect freezing or generalization (ABC, white background; BAC, dotted background). (c) Discrimination scores did not differ between males (lined bars) and females (white bars). Error bars represent SEM. *P<0.05, **P<0.01, ***P<0.001 cf CxtA.

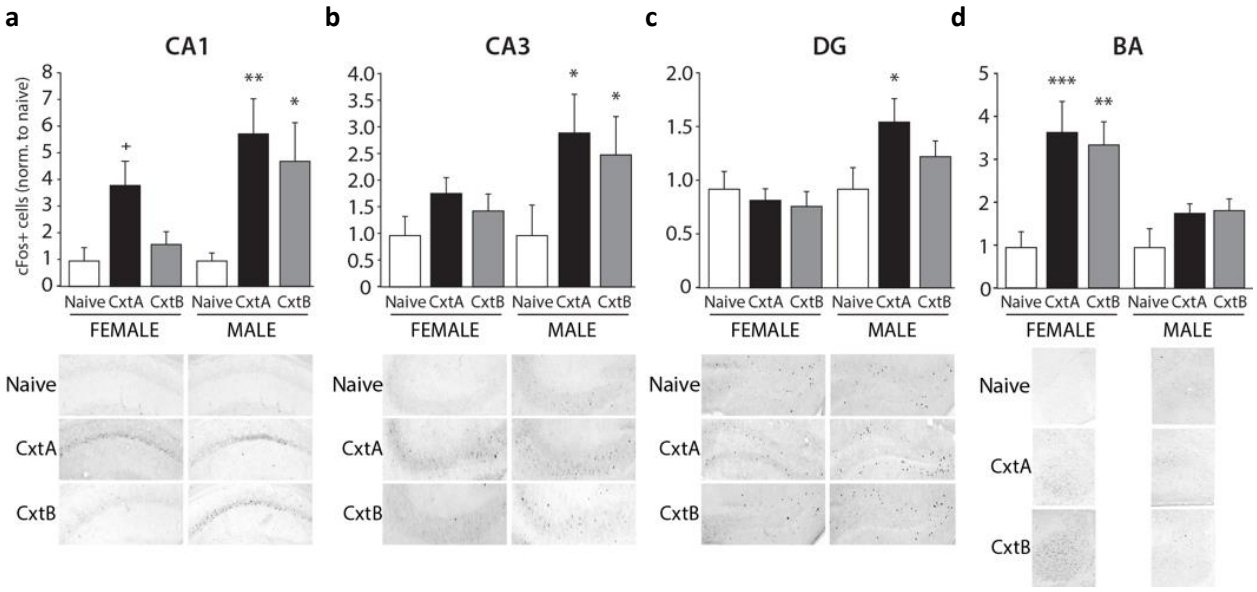

Figure 2.4. ***Sex differences in dorsal hippocampus and amygdala cFos activity during retrieval and generalization of context fear.*** (a) Both males and females show increased cFos+ cells in CA1 following test in CxtA. Only males show increases in CA1 during generalization test in CxtB. (b) Males but not females show increased cFos positive cells in CA3 during tests in CxtA and CxtB. (c) In DG, males but not females show increased cFos+ cells during memory retrieval in CxtA. (d) In basal amygdala, females showed increased cFos+ cells after retrieval in CxtA and generalization test in CxtB. All data are normalized to naive levels. +P = 0.05, *P<0.05, **P<0.01, ***P<0.001 cf same sex naive. Representative images from each group show cFos+ cells.

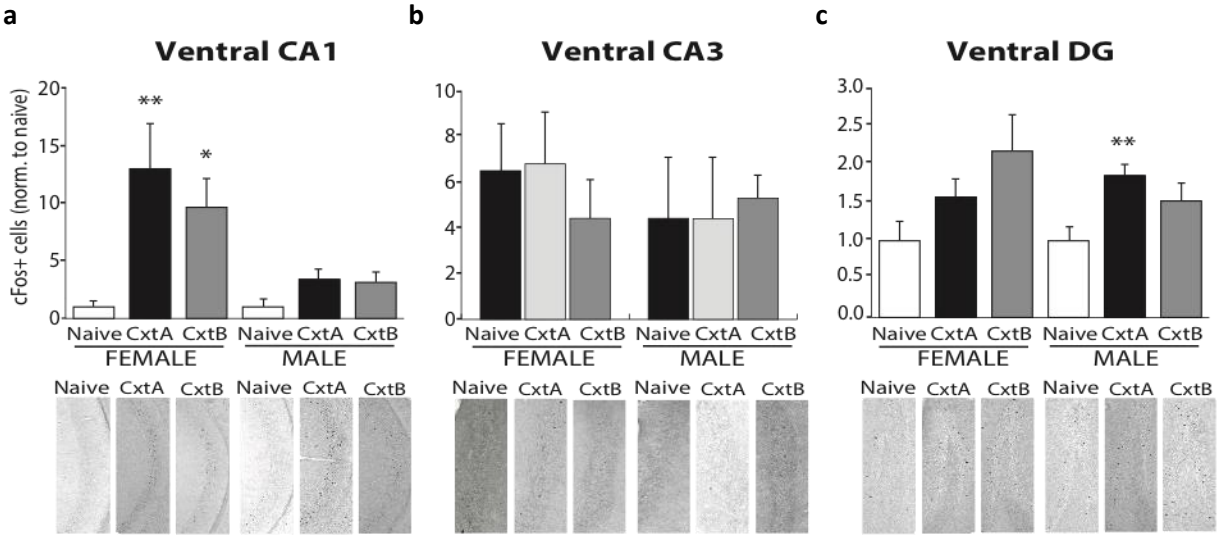

Figure 2.5. ***Sex differences in ventral hippocampus cFos activity during retrieval and generalization of context fear.*** (a) Females but not males show increased cFos+ cells in ventral CA1 following test in CxtA and B. (b) Males nor females show increased cFos positive cells in ventral CA3 during tests in CxtA and CxtB. (c) In ventral DG, males but not females show increased cFos+ cells during memory retrieval in CxtA. *P<0.05, **P<0.01, cf same sex naive. Representative images from each group show cFos+ cells.

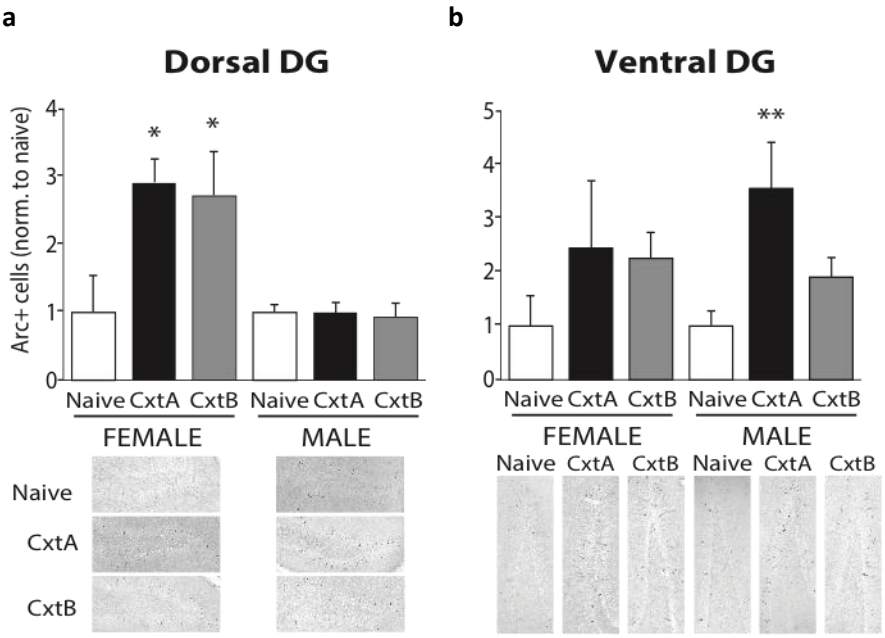

Figure 2.6. ***Sex differences in Arc+ cells in dorsal and ventral hippocampus after retrieval of context fear.*** All data is normalized to naïve levels. Representative images are listed below each graph. (a) Dorsal DG (b) Ventral DG. *p < 0.05, **p<0.01 cf same sex naive.

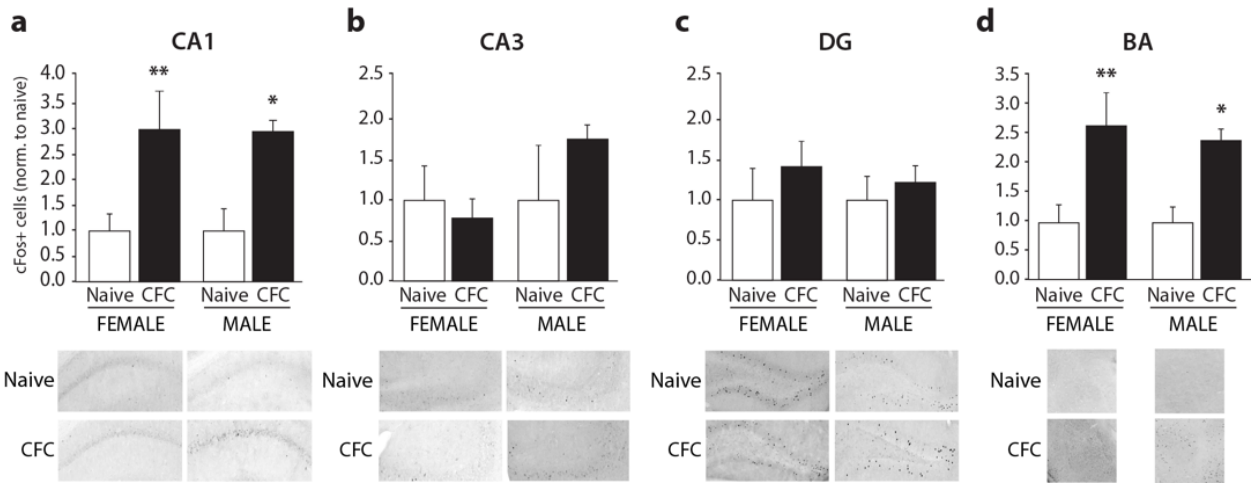

Figure 2.7. ***No sex differences in dorsal hippocampus and amygdala cFos activity during consolidation of context fear conditioning.*** (a) Both males and females show more cFos+ cells in CA1 after context fear conditioning. (b, c) Neither males nor females show elevated cFos in CA3 (b) or DG (c) after fear conditioning. (d) Males and females show similar cFos activation in basal amygdala after context fear conditioning. All data are normalized to naive levels. Error bars indicate SEM. BA, basal amygdala; CFC, context fear conditioning; DG, dentate gyrus. *P<0.05, **P<0.01 cf same sex naive.

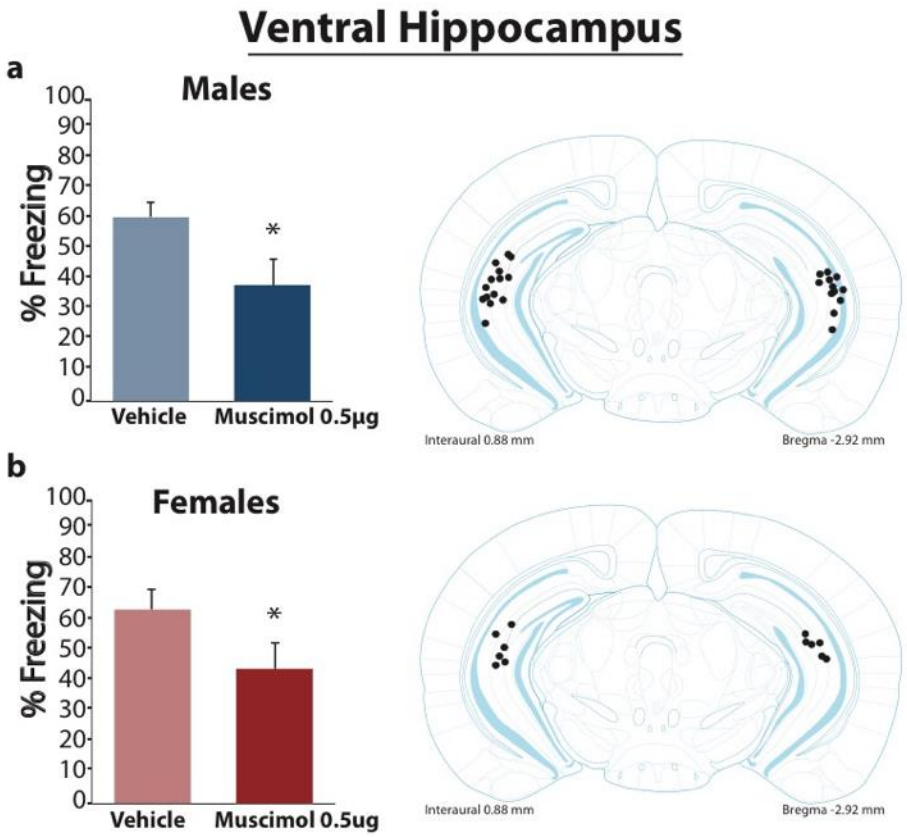

Figure 2.8. ***Pre-retrieval ventral hippocampal inactivation disrupts retrieval in males and females.*** Cannula placements are to the right of graphs. (a) Males (b) Females. *P<0.05, cf vehicle.

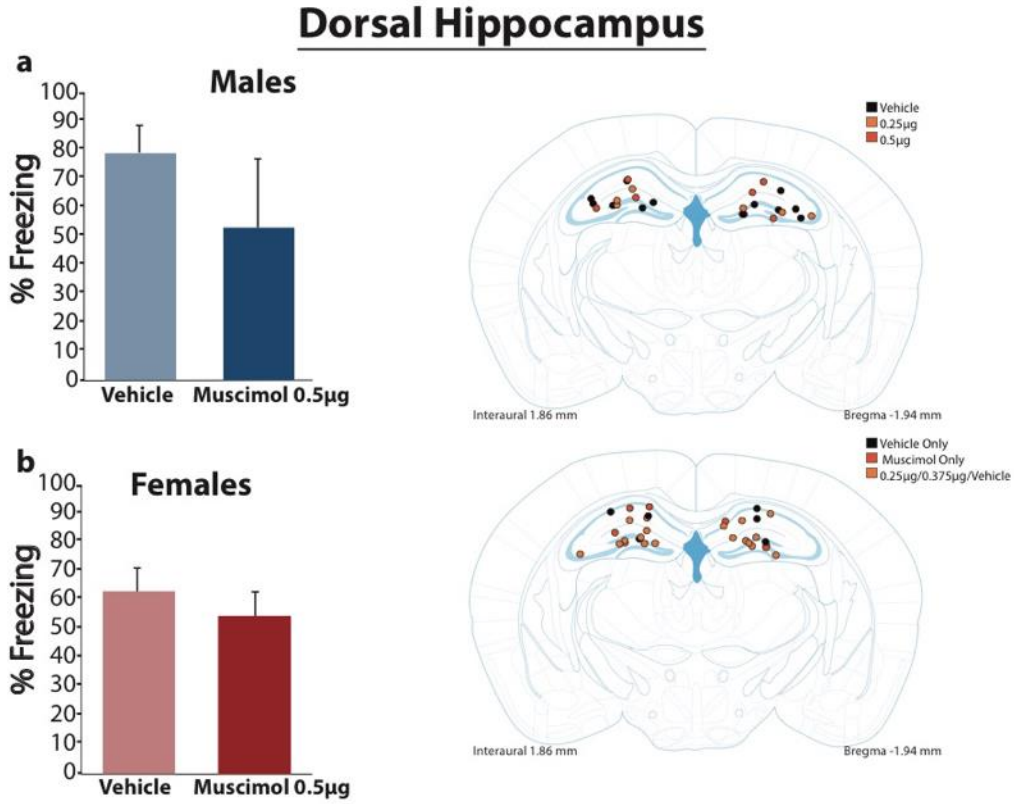

Figure 2.9. ***Pre-retrieval dorsal hippocampal inactivation in males and females.*** Cannula placements are to the right of graphs. (a) Males (b) Females.

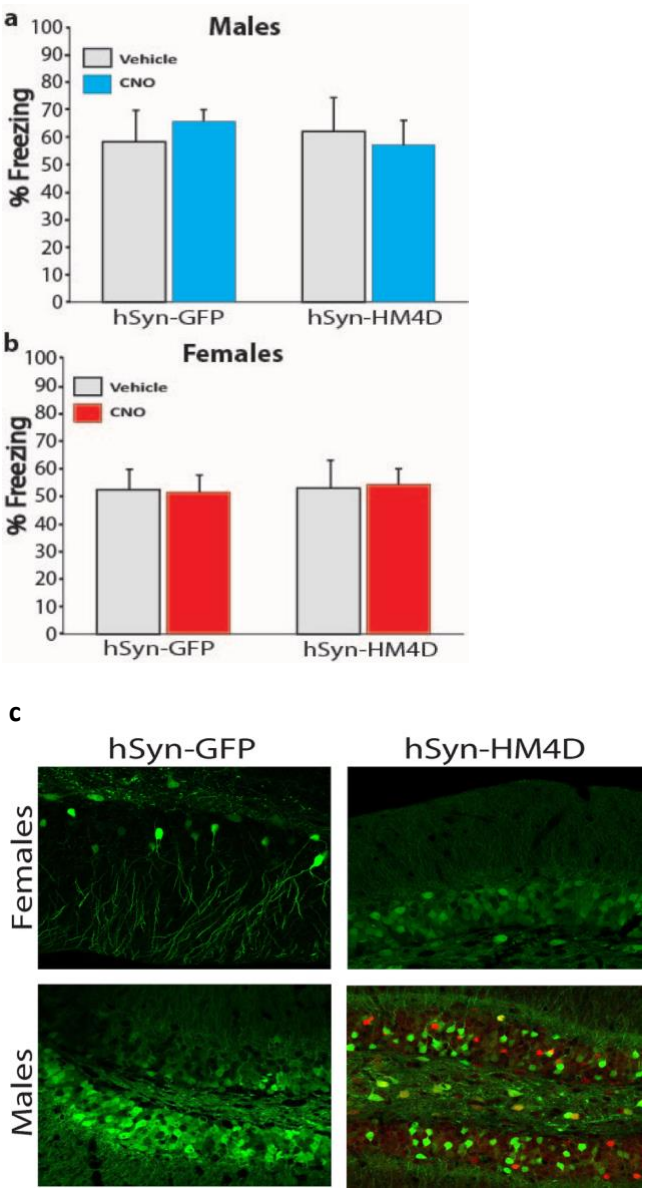

Figure 2.10. ***Pre-retrieval hSyn–HM4D-dependent inactivation of dorsal hippocampus*****.** (a-b) Pre-retrieval hSyn–HM4D-dependent inactivation of dorsal hippocampus does not affect freezing behavior during test in males or females. (c) Representative images show GFP expression. Females (top) and males (bottom); bottom right shows GFP (green) and cFos expression (red).

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Chapter III

Training Protocol Matters: Sex Differences in Retrieval of Recent vs Remote Context Fear Memory

Abstract

Disorders of fear and anxiety including post-traumatic stress disorder (PTSD) are more prevalent in women than in men. Given the long-lasting nature of context fear memory, and the role of circuit dynamics during systems consolidation, we examined sex differences in retrieval of remote context fear memory and its neural correlates. Unlike males, females trained with background context fear conditioning (signaled) showed reduced retrieval of context fear eight weeks after fear conditioning, compared with retrieval of a recent memory. Interestingly, we observed that the female-specific reduction in remote memory was only detected with background fear conditioning, but not foreground context fear conditioning (unsignaled) and go on to show the differential impact training protocol can also have on behavior at recent time points. Retrieval of remote context fear resulted in sex-specific patterns of cFos and Arc activation. Together, these findings are consistent with previous research demonstrating sex differences in remote spatial memory and go on to suggest sex differences in retrieval of context fear at remote time points which depends on training protocol and involves differential utilization of molecular mechanisms by males and females.

Introduction

Post-traumatic stress disorder (PTSD) is characterized by trauma-related memories that persist for many years following the initial traumatic event (Grillon and Morgan, 1999; Solomon

et al., 2009). With women being almost 3 times more likely to develop PTSD (Kessler et al., 1995, 2012), the dynamic nature of these long lasting memories may also differ between males and females both in behavior and mechanism. It has been well studied in males that a fear-related memory has different properties whether it is being retrieved at a recent or remote time point. As memories age they become less hippocampal dependent, a process termed "systems consolidation" (Kim and Fanselow, 1992; Alvarez and Squire, 1994; Squire and Alvarez, 1995; Anagnostaras et al., 1999; Frankland et al., 2004; Squire et al., 2004; Wiltgen et al., 2004; Frankland and Bontempi, 2005; Sutherland and Lehmann, 2011; Wiltgen and Tanaka, 2013); therefore, lesions to the hippocampus at recent, but not remote time points have been shown to impair context fear memory retrieval in males (Kim and Fanselow, 1992; Anagnostaras et al., 1999; Squire et al., 2004; Bayley et al., 2005). While not required for retrieval of remote memory, recent evidence reveals that dorsal CA1 region of the hippocampus still likely plays an important role in retrieval of remote context fear memories (Ocampo et al., 2017). The degree of hippocampal involvement following retrieval of recent and remote context fear in females remains an open question. There is however, evidence to suggest differential underlying neural circuitry in retrieval of recent and remote fear.

We have previously demonstrated strong context fear memory in females but less engagement of hippocampus (Keiser et al., 2017). Additionally, in human studies of spatial navigation, unlike males, a hippocampal-independent strategy is preferred in females (Grön et al., 2000). Other regions known to play a role in learning and memory have also been shown to be differentially required in males and females. Electrolytic lesions of prefrontal cortex result in lower levels of freezing during extinction recall in males but not females (Baran et al., 2010) and unilateral entorhinal cortex lesions impair performance on a spatial working memory Morris

water maze task in males but not females (Roof et al., 1993). Therefore, it is possible that females differentially engage structures such as the hippocampus, as well as require different intracellular mechanisms distinct from males to successfully retrieve recent and remote fear-related memories.

Experiments assessing acquisition or consolidation of context fear have revealed that hippocampal molecular mechanisms activated by males and required for successful memory performance are not equally shared by females. Consolidation of a fear memory is a complex process that includes receptor activation, second messenger and calcium signaling, gene transcription and protein synthesis; all steps which differ between the sexes. At the receptor level, knockdown of GluR1 impairs context fear conditioning in males but not females (Dachtler et al., 2011). Activation of GluR1 receptors often result in the phosphorylation of CaMKKα/β which can lead to activation of Cyclic AMP response element-binding protein (CREB), a necessary transcription factor in memory formation (Dash et al., 1990; Bourtchuladze et al., 1994; Kogan et al., 1996; Guzowski and McGaugh, 1997; Josselyn et al., 2001). Whereas null mutants of both CaMKK α and β result in impaired spatial memory formation in males, neither are required in females (Mizuno et al., 2006, 2007). Following context fear conditioning, males are also reported to show greater levels of hippocampal pCREB compared with females (Kudo et al., 2004). Given that females show differential activation and requirement of members of important signaling pathways defined as critical for males in acquisition and consolidation, it is plausible that males and females may differ in remote context fear memory as well.

Unfortunately, the majority of studies that have assessed sex differences in fear memory have focused on the acquisition or consolidation phase of fear-related memories and have largely focused on retrieval of recent context fear memories. There is however, evidence that males and

females may also differ behaviorally as well as mechanistically at remote time points. A 2013 study by Sebastian and colleagues assessed sex differences in remote long term spatial memory using a radial arm maze task. 30 days post-training females, but not males showed a greater number of reference memory errors compared with their recent test which occurred 1 day after training. This sex difference was evident despite fewer errors in females compared with males at the recent test and training that was aimed towards adequately abolishing sex differences in acquisition. Sex differences were also observed in molecular markers associated with maintenance of long term memory (Sebastian et al., 2013). Here, hippocampal expression patterns of synaptic PKMζ positively correlated with retention test scores in males, but not females. These findings suggest sex differences in maintaining, rather than retaining spatial information and may suggest sex differences in retrieval of remote spatial memories. In males, the specificity of retrieval of context information diminishes at remote time points (Wiltgen and Silva, 2007; Wiltgen et al., 2010) but freezing in the trained context is not reduced. If males and females differ in maintenance of spatial information, there may also be differences in retrieval of remote context fear.

The goal of this study was to determine sex differences in retrieval of remote context fear memories. Here we examined whether males and females differ behaviorally in remote retrieval of context fear as well as how the sexes differ in activation of specific brain regions after remote retrieval of context fear. Male and female mice underwent context fear conditioning and were tested for freezing in the training context the day following training and at a remote time point, 8 weeks later. A separate group of animals were used to assess sex differences in levels of cFos activation in recent vs remote time points. We next assessed retrieval at recent time points. Overall, we showcase important sex differences in behavior and mechanism that emerge under

specific training conditions during retrieval of remote memories. These findings highlight an important role of training protocol when assessing context fear for recent or remote time points.

Methods

ANIMALS

9-week-old C57BL/6 mice (53 males, 69 females) from Envigo (Indianapolis, IN) were used in these experiments. Mice were individually housed throughout experiments with standard diet and water ad libitum. Individual housing in males is necessary to reduce fighting-induced stress (Meakin et al., 2013) and does not increase variance in either sex (Prendergast et al., 2014); Individual housing is consistent with University of Michigan Institutional Care and use Committee policies on management of fighting in mice. Due to independent social structures of both male and female mice (Becker and Koob, 2016), individual housing is ecologically appropriate for both sexes. The colony room was adjacent to behavioral testing rooms and was maintained at 20 ± 2 ℃ with a 12 h 0700: 1900 h light/dark cycle (lights on at 0700 h). Mice were acclimated to the colony room for at least 7 days before experiments began. Experimenters in this study were all women (Sorge et al., 2014). The University of Michigan Committee on the Use and Care of Animals approved all experimental methods performed in this research.

APPARATUS

Mice were trained and tested in conditioning chambers (9 3/4″ × 12 3/4″ × 9 3/4″; MedAssociates, VT), enclosed in sound-attenuating cubicles, equipped with a NIR camera (VID-CAM-MONO-2A). Grid floor rods were connected to a shock generator. Male and female mice were tested in separate chambers which were cleaned between each animal with either 70% ethanol or 1% acetic acid. Freezing and locomotor activity was automatically scored with video freeze software (MedAssociates). Two experimenters, blind to experimental conditions also hand

scored freezing to validate automatic scoring.

Three different contexts were used: (1) Training context: consisted of a rectangular box with white walls, lights on, an evenly sized grid floor (36 stainless steel rods, 1/8″ diameter, spaced 1/4″ apart), and 70% ethanol odor. (2) Distinct context (for testing tone): consisted of black angled walls, house lights off, staggered grid floors with alternating 1/8″ and 3/16″ grid rods, and 1% acetic acid odor. (3) Similar context (for generalization tests): had curved white walls, house lights off, the same floors, and ethanol odor as in the training context.

CONTEXT FEAR CONDITIONING

Foreground context fear conditioning was conducted as previously described (Keiser et al., 2017). Briefly, mice (8 males, 8 females) were placed in the fear conditioning chambers for 3 minutes, followed by delivery of a 2s, 0.8 mA footshock. Mice were then replaced in their home cage and returned to the colony room. For background context fear conditioning (37 males, 52 females/6 naïve per sex), mice were placed in the fear conditioning chambers for 3 minutes, followed by the onset of a 30 second tone; upon termination of the tone mice received a 2s, 0.8 mA foot shock. For generalization experiments, mice were in chambers for 2.5 minutes before the onset of tone.

EXPERIMENTAL DESIGN: REMOTE CONTEXT FEAR (BETWEEN SUBJECTS) TEST

24 h or 8 weeks after background context fear conditioning, female mice (n = 15) were placed into the same training context for 3 minutes. Freezing was assessed during this time. The following day, females were placed in a distinct context that differed in multiple sensory components to test for freezing to the tone. Following 1.5 minutes in the distinct context, a 30 second tone was played followed by a 1-minute inter-trial interval for a total of 5 minutes (3 tones total per test). A separate group of male and female mice (background context fear

conditioning) were used for immunohistochemistry experiments in which brains were taken 1 hour following recent or remote context test (14 males, 14 females).

EXPERIMENTAL DESIGN: REMOTE CONTEXT FEAR (WITHIN SUBJECTS) TEST

*2*4 hours and 8 weeks after background or foreground context fear conditioning, female and male mice were placed into the same training context for 3 minutes (30 male, 30 female). Freezing was assessed during this time. The following day, background trained mice were placed in a distinct context that differed in multiple sensory components to test for freezing to the tone. Following 1.5 minutes in the distinct context, a 30 second tone was played followed by a 1-minute inter-trial interval for a total of 5 minutes (3 tones total per test). 1 day following the tone test, mice were placed again in the training context to test for freezing and brains were taken 1 hour after the test to be used in immunohistochemistry experiments (16 males, 16 females).

EXPERIMENTAL DESIGN: RECENT RETRIEVAL AND GENERALIZATION TESTS

24 hours after background context fear conditioning in a separate group of male and female mice, animals were placed into either the training context or the similar context for 3 minutes to test for freezing and returned immediately to the colony room. Mice were retested at 24 hour intervals first in the reverse context (similar (A) or training context (B)) and subsequently in the distinct context (C) (ie, test order ABC (7 males, 8 females) or BAC (8 males, 8 females); Figure 3.5b). All behavioral experiments used a group size of 8 per sex, per test order.

DISCRIMINATION SCORE

A discrimination score was calculated to account for freezing in all three contexts as previously described (Keiser et al., 2017). Briefly, we used the following formula: (A−B)/(A−C), where A, B, and C represent freezing in each context. This formula compares the difference in

freezing in the training and similar contexts with the difference in freezing in distinct context. Freezing in the distinct context represents freezing to nonspecific factors including transportation and handling (Rudy and O'Reilly, 2001) that trigger some retrieval of prior experience and thus small increases in freezing; including this in the discrimination score controlled for different levels of freezing between the sexes in a novel context.

ESTROUS CYCLE

Estrous samples were collected 1 hour before all behavioral experiments at approximately the same time each day. Wet vaginal smears were taken in 5µl of distilled deionized water to determine estrous phase in female mice. Fluid was pipetted 3–4 times and dropped on slides. Estrous stage was determined with light microscopy by assessing vaginal cytology (Caligioni, 2009). As a second measure of estrous stage determination, the vaginal opening was visually examined to assess characteristics such as swelling and dryness (Byers et al., 2012).

CFOS AND ARC IMMUNOHISTOCHEMICAL ANALYSIS

Mice were deeply anesthetized (Avertin, 480 mg/kg, i.p.) and transcardially perfused with 4% paraformaldehyde 1 h after recent or remote context fear memory retrieval (control/naïve n=8, recent n=7 remote n=15, per sex). Standard immunohistochemistry protocols were used, as previously described (Keiser et al., 2017). Briefly, sections were incubated with anti-cFos (Abcam; 1: 3,000), goat anti-rabbit or mouse secondary (VectorLabs; 1: 200), or anti-Arc (Synaptic Systems; 1: 2,750) and DAB chromogen (Sigma Aldrich, St Louis, MO). Quantification of cFos and Arc+ cells was conducted by experimenters blind to experimental group in the following brain regions: anterior cingulate cortex (ACC), prelimbic cortex (PL), infralimbic cortex (IL), retrosplenial cortex (RSC), dorsal and ventral hippocampal CA1, CA3,

DG, and basal amygdala (BA that included both basolateral and basomedial amygdala; see Amano et al., 2012). Sections for all brain regions were cut at 40 μM and selected at the same level. Regions for quantification were defined by ImageJ (NIH, Bethesda, MD) and held constant across animals. cFos+ and Arc+ counts were normalized to the number of cells in naïve animals.

STATISTICAL ANALYSIS

All statistical analyses were conducted using SPSS v23. Two-way and non-repeated measures ANOVA (Test x Sex) was used to determine sex differences in retrieval of remote context fear memory. For cFos and Arc experiments we used two-way (Sex × Test) ANOVA to determine sex differences in cFos and Arc+ cells after recent and remote context fear memory retrieval. Three-way repeated measures ANOVA (Context × Order × Sex) was used to determine sex differences in generalization of context fear in mice that underwent background context fear conditioning. Separate two-way analyses were conducted on the discrimination score (Sex × Order). A two-way multivariate ANOVA (Estrous × Test) was used to determine the effect of estrous on context fear conditioning. Post hoc tests with Bonferroni corrections for multiple tests were used to further examine significant effects in each experiment. The 95% confidence intervals are reported for post hoc tests. Partial η^2 (η^2_p) is reported as effect size estimate (0.01, 0.06, and 0.14 as small, medium, and large, respectively), as calculated by SPSS.

Results

SEX DIFFERENCES IN REMOTE CONTEXT FEAR

8 weeks following background context fear conditioning, females showed lower levels of freezing compared with females tested 1 day following training day (Figure 3.1b). (Group: $F(1,14) = 9.72$, $P < .01$, $\eta^2_p = 0.45$, (95% CI: 10.02–56.55)). However, freezing to the tone was intact in females in both groups when tested 30 days following the remote test (Figure 3.1c),

(Bin: F (1,13) = 29.29, P < 0.01, η^2_p = 0.69, (95% CI: 30.24–50.01)). 8 weeks following background context fear conditioning, females, but not males showed lower levels of freezing compared with their recent test 1 day following training (Figure 3.1d), (Test x Sex: F (1,13) = 5.64, P < .05, η^2_p = 0.30, (95% CI: 42.41–67.99); recent females compared with remote females P < 0.05). In a second group of mice used for immunohistochemistry, males nor females show different levels of freezing compared with a separate group of same sex mice tested 1 day following training day (Figure 3.1f). (Sex F (1, 24) <1; Test F (1, 24) <1; Test x Sex F (1, 24) <1).

FEMALE-SPECIFIC DECREASE IN REMOTE MEMORY DEPENDS ON TRAINING PROTOCOL

8 weeks following foreground context fear conditioning, males nor females show differences in freezing compared with their recent test 1 day following training day (Figure 3.2b), (Sex F (1, 14) <1; Test F (1, 14) <1; Test x Sex F (1, 14) <1).

ESTROUS CYCLE DID NOT AFFECT FEAR CONDITIONING

Context fear conditioning in females was not affected by estrous phase on training day. Females showed similar freezing to the training context at the remote time point (F (3, 26) = 2.19, P = 0.114) regardless of estrous phase (proestrous n = 10, estrous n = 9, metestrous n = 3, diestrous n = 8). To ensure adequate statistical power, we examined the impact of estrous cycle across all behavioral experiments.

SEX DIFFERENCES IN CFOS AND ARC ACTIVATION FOLLOWING RETRIEVAL OF REMOTE CONTEXT FEAR

We observed more cFos+ cells in females compared with males in basal amygdala (Figure 3.3a) and retrosplenial cortex (RSC) (figure 3.3b) after remote retrieval of context fear

conditioning. A three-way (Sex x Test x Training) ANOVA demonstrated significant main effects of sex in these regions (RSC: $F(1, 23) = 6.75$, $P < 0.05$, $\eta^2_p = 0.23$; basal amygdala: $F(1, 24) = 13.92$, $P < 0.01$, $\eta^2_p = 0.37$) and significant interactions: RSC: (Sex x Test: $F(1, 23) = 8.98$, $P < 0.01$, $\eta^2_p = 0.28$), basal amygdala: (Sex x Test: $F(1, 24) = 16.44$, $P < 0.001$, $\eta^2_p = 0.41$). Females but not males exhibited strong activation of cFos following remote context fear memory retrieval compared with naïve in basal amygdala: $P < 0.001$ (95% CI: 15.55-24.67) and RSC: $P < 0.001$ (95% CI: 17.56-37.73). cFos+ cells were significantly greater in females compared with males following retrieval of remote context fear in RSC: $P < 0.001$ (95% CI: 18.43–60.53) and basal amygdala: $P < 0.001$ (95% CI: 16.91–37.21).

A greater number of cFos+ cells were also observed in males and females combined compared with control after remote retrieval, with significant main effects of test in the following regions: anterior cingulate cortex (ACC): $F(1, 24) = 5.64$, $P < 0.05$, $\eta^2_p = 0.19$; prelimbic cortex: $F(1, 24) = 25.02$, $P < 0.001$, $\eta^2_p = 0.51$; infralimbic cortex $F(1, 24) = 19.03$, $P < 0.001$, $\eta^2_p = 0.44$; RSC: $F(1, 23) = 18.28$, $P < 0.001$, $\eta^2_p = 0.44$; DCA1: $F(1, 23) = 11.14$, $P < 0.01$, $\eta^2_p = 0.32$; DCA3: $F(1, 24) = 25.86$, $P < 0.001$, $\eta^2_p = 0.52$; DDG: $F(1, 24) = 22.73$, $P < 0.001$, $\eta^2_p = 0.49$; basal amygdala: $F(1, 24) = 58.26$, $P < 0.001$, $\eta^2_p = 0.71$; VCA1: $F(1, 24) = 37.71$, $P < 0.001$, $\eta^2_p = 0.61$; VCA3: $F(1, 23) = 29.94$, $P < 0.001$, $\eta^2_p = 0.53$; VDG: $F(1, 24) = 16.08$, $P < 0.01$, $\eta^2_p = 0.40$). Number of cFos+ cells did not differ with training protocol (background or foreground) in any region assessed; Training Paradigm: $F < 1$ (figure 3.3).

Number of Arc+ cells were assessed in regions: RSC, DDG and VDG. We observed more Arc+ cells in females in RSC (figure 3.3c) after remote retrieval of context fear

conditioning. A three-way (Sex x Test x Training) ANOVA demonstrated a significant interaction (Sex x Test: $F(1, 23) = 5.56$, $P < 0.05$, $\eta^2_p = 0.20$), but no main effect of sex. Number of Arc+ cells was significantly greater in females compared with males following retrieval of remote context fear in RSC: $P < 0.05$ (95% CI: 4.23–40.27). Only females exhibited strong activation of Arc following remote context fear memory retrieval compared with naïve in RSC: $P < 0.01$ (95% CI: 16.60–52.65).

A greater number of Arc+ cells were also observed in males and females combined compared with control after remote retrieval in DDG, with a significant main effect of test; (DDG: $F(1, 22) = 10.78$, $P < 0.01$, $\eta^2_p = 0.33$). Neither males nor females showed a significant increase in Arc+ cells in VDG compared with naïve (males: $P = 0.064$, females: $P = 0.154$). Number of Arc+ cells did not differ with training protocol (background or foreground) in all regions assessed; Training Paradigm: $F < 1$ (figure 3.3). Together, these results suggest that males and females differentially recruit fear memory-associated brain regions during remote context fear memory retrieval, with females showing preferential cFos activation of retrosplenial cortex and basal amygdala and Arc activation of retrosplenial cortex.

SEX DIFFERENCES IN CFOS AND ARC ACTIVATION FOLLOWING RECENT VS REMOTE CONTEXT FEAR MEMORY RETRIEVAL (BACKGROUND FEAR CONDITIONING)

We observed more cFos+ cells in females compared with males in Dorsal DG (Figure 3.4a), Basal Amygdala (Figure 3.4b) and Ventral CA1 (Figure 3.4c). A three-way (Sex x Test x Training) ANOVA demonstrated significant main effects of sex in regions (Basal Amygdala: $F(1, 34) = 4.71$, $P < 0.05$, $\eta^2_p = 0.12$ and Ventral CA1: $F(1, 34) = 4.28$, $P < 0.05$, $\eta^2_p = 0.11$) and interactions (Test x Sex) in Dorsal DG: $F(1, 34) = 3.58$, $P < 0.05$, $\eta^2_p = 0.17$). We observed a

greater number of cFos+ cells in females compared with males after remote retrieval of context fear conditioning in Dorsal DG: $P < 0.01$ (95% CI: 2.63–16.80) and Ventral CA1: $P < 0.01$ (95% CI: 3.68–22.17). Males, but not females had greater levels of cFos+ cells following recent context fear memory retrieval compared with naïve in Dorsal DG: $P < 0.01$ (95% CI: 3.41–18.16). Females, but not males had greater levels of cFos+ cells following remote context fear memory retrieval compared with naïve in Dorsal DG: $P < 0.01$ (95% CI: 5.98–20.73).

A greater number of cFos+ cells were also observed in males and females combined compared with control after remote retrieval, with significant main effects of test in the following regions: anterior cingulate cortex (ACC): $F(2, 33) = 4.26$, $P < 0.05$, $\eta^2_p = 0.21$; prelimbic cortex: $F(2, 34) = 15.52$, $P < 0.001$, $\eta^2_p = 0.48$; infralimbic cortex: $F(2, 33) = 22.71$, $P < 0.001$, $\eta^2_p = 0.58$; RSC: $F(2, 34) = 4.95$, $P < 0.05$, $\eta^2_p = 0.23$; DCA3: $F(2, 34) = 7.99$, $P < 0.01$, $\eta^2_p = 0.32$; DDG: $F(2, 34) = 7.79$, $P < 0.01$, $\eta^2_p = 0.31$; basal amygdala: $F(2, 34) = 19.73$, $P < 0.001$, $\eta^2_p = 0.54$; VCA1: $F(2, 33) = 20.97$, $P < 0.001$, $\eta^2_p = 0.55$ and VCA3: $F(2, 34) = 3.92$, $P < 0.05$, $\eta^2_p = 019$). Specifically, males and females had greater levels of cFos+ cells following remote context fear memory retrieval compared with naïve in: (ACC): $P < 0.05$ (95% CI: 0.79–14.21); prelimbic cortex: $P < 0.001$ (95% CI: 7.80–25.16); infralimbic cortex: $P < 0.001$ (95% CI: 17.99–40.70); DCA3: $P < 0.05$ (95% CI: 0.13–8.60); DDG: $P < 0.01$ (95% CI: 1.50–14.64) and VCA3: $P < 0.05$ (95% CI: 0.28–7.06). Following recent context fear memory retrieval, males and females exhibited greater levels of cFos+ cells compared with naïve in: prelimbic cortex: $P < 0.001$ (95% CI: 7.97–25.34); infralimbic cortex: $P < 0.001$ (95% CI: 9.67–32.38); RSC: $P < 0.05$ (95% CI: 5.73–74.11); DCA3: $P < 0.01$ (95% CI: 2.30–10.78) and DDG: $P < 0.05$ (95% CI: 2.93–16.07).

Number of Arc+ cells were counted in the following regions: RSC, DDG and VDG. We observed more overall levels of Arc+ cells in females compared with males in Dorsal DG. A three-way (Sex x Test x Training) ANOVA demonstrated significant main effects of sex in this region (Dorsal DG): $F(1, 29) = 5.27$, $P < 0.05$, $\eta^2_p = 0.15$, but no interaction. There was a main effect of test for the following regions: retrosplenial cortex (RSC): $F(2, 29) = 5.44$, $P < 0.05$, $\eta^2_p = 0.27$ and Dorsal DG: $F(2, 29) = 6.36$, $P < 0.01$, $\eta^2_p = 0.31$. Specifically, both males and females had greater levels of Arc+ cells following recent context fear memory retrieval compared with naïve in: RSC: $P < 0.01$ (95% CI: 9.88–52.49) and Dorsal DG: $P < 0.01$ (95% CI: 1.35–7.66). Both males and females had greater levels of Arc+ cells following remote context fear memory retrieval compared with naïve in: RSC: $P < 0.05$ (95% CI: 7.18–49.49) and Dorsal DG: $P < 0.01$ (95% CI: 1.87–8.14). We observed a greater number of Arc+ cells in females compared with males after remote retrieval of context fear conditioning in Dorsal DG: $P < 0.05$ (95% CI: 0.17–8.83). Together, these results suggest that males and females differentially recruit fear memory-associated brain regions during remote context fear memory retrieval, with greater recruitment of Dorsal DG and VCA1 in females compared with males and greater recruitment of basal amygdala in females compared with males overall.

SIMILARITIES IN GENERALIZATION BETWEEN MALES AND FEMALES WITH BACKGROUND FEAR CONDITIONING

Sex differences in generalization of context fear were not observed in background fear conditioned mice. No main effect of sex was observed ($F_{(1,27)} = 0.215$, $P = 0.646$, $\eta^2 = 0.008$). Both males and females tested in ABC test order exhibited lower levels of freezing in the similar context (females: $P < 0.001$, 95% CI (21.133-44.11), males: $P < 0.01$, 95% CI = (8.143-32.715) and distinct context (females: $P < 0.001$, 95% CI (35.298-60.332), males: $P < 0.001$, 95% CI

(36.688-63.449) compared with the training context (Figure 3.5b). However, an effect of test order was observed ($F(1,27) = 10.157$, $P < 0.01$, $\eta^2 = 0.273$). Both male and female mice did not freeze significantly less in the similar context than the training context when tested in test order BAC (females: $P = 0.171$, 95% CI: (-19.367-3.617), males: $P = 0.416$, 95% CI (-6.867-16.117)), but did freeze significantly less in the distinct context (females: $P < 0.001$, 95% CI (29.793-54.827), males: $P < 0.05$, 95% CI (1.787-26,820)). These results suggest that background fear conditioning results in similar levels of generalization of context fear in males and females; a finding that is in stark contrast to foreground-trained mice in which females but not males generalize context fear (Keiser et al., 2017).

Discussion

Here we demonstrated that males and females differ in both behavior and immediate early gene activation following remote retrieval of a context-fear memory. Specifically, females, but not males trained with background context fear conditioning frequently showed a reduction in remote context fear memory as well as sex-specific patterns of retrieval-induced cFos and Arc activation following remote retrieval of context fear memory. Further, we showed that the female-specific reduction in remote memory depends on training protocol used and demonstrated the impact different training protocols can also have on behavior at recent time points. Together, these findings reveal sex differences in retrieval of context fear at remote time points depend on training protocol used and involves differential molecular mechanisms by males and females.

Our observed finding of sex differences in freezing behavior at a remote time point is consistent with studies demonstrating sex differences in context fear conditioning at recent time points. At recent time points sex differences have been observed in the rate of learning context representations (Wiltgen et al., 2001) which must be established to form a context-shock

association (Fanselow, 1990). Therefore, sex differences in memory at recent time points and in developing a context-shock association may also exist as time passes and memory for context becomes less specific and more generalized (Wiltgen and Silva, 2007; Wiltgen et al., 2010). In fact, we have previously demonstrated greater generalization of context fear by females compared with males at recent time points (Keiser et al., 2017) and others have shown females to be more quick to generalize context fear in a passive avoidance task (Lynch et al., 2013); overall suggesting that females show more rapid loss of context specificity with time. Whether specific elements of the training context diminish at remote time points in females remains an open question, but a possibility given previous data.

A recent paper by Ishikawa et al., 2016 associates forgetting fear- associated memories with an increase in hippocampal neurogenesis in males. They further highlight the importance of forgetting fear-associated memories and provide further implications of this process for treatment of post-traumatic stress disorder (PTSD) (Ishikawa et al., 2016). The specific process mediating forgetting in females is currently unknown, but our data highlight sex differences in molecular mechanisms both when behavior is similar and when behavior is different between the sexes when tested at a remote time point. That a female-specific decrease in remote context fear memory was not observed in every group of mice may be explained by our use of a between subjects design, in which animals are not given a reminder of the context associated with the aversive foot shock. An interesting paper by Ishikawa and colleagues (2016) hypothesizes that re-exposure to a context associated with shock may enable memory to return to a hippocampal-dependent state and allow for enhanced forgetting via an increase in hippocampal neurogenesis. Therefore, it may be possible that this re-exposure of context enhances forgetting in females as well. Similarly, we have previously shown that re-exposure to a fear-paired context, rather than a

novel context is necessary for females, but not males to discriminate between contexts at a recent time point (Keiser et al., 2017). These findings bring to light an intriguing possibility of context re-exposures in females as playing a role in promotion of later forgetting, but further research is needed to confirm this and determine the precise mechanism.

Another important factor mediating the female-specific decrease in remote memory is that of training paradigm. Our finding that only females trained with background context fear conditioning (signaled) but not foreground (unsignaled) show reduced remote context fear memory is novel given few studies comparing such paradigms in remote memory. This paper is the first to use females in such a comparison. Earlier studies in males assessing differences in background vs foreground context fear conditioning suggest differential involvement of hippocampus, with requirement of hippocampus in background but not foreground (Phillips and Ledoux, 1992). We add to this earlier body of work to show that training protocol can have lasting implications for behavior that are sex-specific. Whether this type of training results in stronger hippocampal involvement in females and relates to a decrease in remote context fear is currently unknown. An interesting observation from our data is that females that did not show reduced freezing when tested at a remote compared with recent time point (see Figure 3.2b, Figure 3.4a) show stronger hippocampal recruitment compared with males when tested at a remote time point; stronger hippocampal recruitment was not observed in the group showing reduced levels of freezing at the remote, compared with the recent time point (see Figure 3.3). While remote context fear memories are largely thought to be hippocampal-independent, studies have identified hippocampus as playing a critical role in remote context fear memory retrieval (Goshen et al., 2011). Via temporal inhibition of CA1, initial impairments in remote context fear are observed, but given more time, activity of cortical regions is enhanced and memory

accurately recalled. Suggesting that remote context fear memory retrieval naturally involves hippocampus, but that given the necessity and enough time, can switch to more cortical structures (Goshen et al., 2011). Therefore, a lack of hippocampal engagement in females, may play a role is the reduced freezing observed in females following context fear conditioning at a remote compared with recent time point.

Females showed higher levels of cFos activation in ventral hippocampus, basal amygdala, retrosplenial cortex and dorsal dentate gyrus after remote retrieval of background context fear memory compared with males. This pattern of activation is unlikely to explain sex differences in remote context memory retrieval, however, as this pattern was also observed in females that did not show a reduction in remote context fear, such as those trained with foreground. It remains unclear whether the observed immediate early gene activity is due to retrieval of context memory, or due to other context-exposure related memory processing. Preferential recruitment of basal amygdala and ventral CA1 by females does raise the intriguing possibility of greater engagement or reliance on the VCA1-basal amygdala circuit by females. Unlike dorsal hippocampus, ventral hippocampus has reciprocal connections to amygdala (Phillips and Ledoux, 1992; Kim et al., 1993; Wilensky et al., 1999; Ledoux, 2000; Fanselow and Dale, 2003; Zelikowsky et al., 2014). In males, the VCA1-basal amygdala circuit is noted to play a specific role in retrieval of context but not cued fear (Xu et al., 2016). The degree of engagement and temporal-specific requirement of this circuit during retrieval of remote context fear in males or females has not been well studied and warrants further investigation.

Levels of cFos and Arc+ cells did not mirror female and protocol-specific decreases in remote context fear memory. This finding however is consistent with previous work which demonstrates that hippocampal cFos production does not drive freezing behavior (Tronson et al.,

2009; Wiltgen et al., 2010). Additionally, distinct patterns between immediate early genes is consistent with previous studies (e.g. Frankland et al., 2004). Our findings implicate potential sex differences in molecular mechanisms upstream of cFos and Arc and/or differences in cell types activated following remote context fear memory retrieval.

Females, but not males showed increases in cFos+ cells in retrosplenial cortex following remote context fear memory retrieval compared with control. That males did not show similar increases in cFos+ cells in retrosplenial cortex is surprising given studies implicating stronger activation of EGR1 following remote context fear memory recall (Maviel et al., 2004). Our studies presented here differ however in immediate early genes assessed and in our time frame used for recall of remote context fear memory. The majority of studies that have examined remote context fear memory have focused on a time point of around 30-36 days post-training. Here, we use a longer period of 8 weeks due previous observations in our laboratory of a female-specific reduction in freezing in mice tested 8 weeks following background context fear conditioning (data not shown). Whether immediate early gene activity diminishes as memories become more remote in males requires further study.

We also observed sex differences in cFos+ cells following recent context fear memory retrieval in background-trained mice; notably, males but not females show increases in cFos+ cells in dorsal dentate gyrus compared with naïve, and both sexes show increases in cFos+ cells in basal amygdala following recent retrieval of context fear. These findings differ from prior experiments in our lab examining sex differences in recent retrieval of context fear in which males and females were trained with foreground context fear conditioning (Keiser et al., 2017). Therefore, differences in training protocol may result in differential activation of immediate early genes when assessing sex differences in recent context fear memory retrieval. Additionally,

we observe that training protocol can also have a differential impact on behavior when assessing recent context fear memory retrieval. In stark contrast with Keiser et al., (2017) in which significant sex differences were observed in context fear conditioning and generalization of context fear in mice trained with foreground, here we show no sex differences in freezing in the training context or in generalization of fear between the contexts. While we observed that neither males nor females displayed generalized fear to a similar context when first being tested in the training context, a test order effect for both sexes was noted as both males and females generalize fear to the similar context B when this context is tested first. This finding of greater generalization when context B is tested before the training context is also in line with prior research in males where this same test order effect was observed (Huckleberry et al., 2016). These findings highlight the potential importance of post-retrieval processes such as extinction for reduced freezing to the similar, generalization context in both sexes.

This study demonstrates for the first time, sex differences in remote context fear memory retrieval. Where females, but not males trained with background context fear conditioning show reduced remote context fear. We further demonstrate the importance of training protocol when assessing behavior at both remote and recent time points. Where background but not foreground fear conditioning results in reduced remote retrieval in females, but no apparent sex differences in behavior or generalization when tested at recent time points. Sex differences in immediate early gene activation further suggest sex-specific underlying neural mechanisms in retrieval of remote context fear memories. Determining sex differences in the factors mediating retrieval of remote context fear memory has lasting implications for understanding the increased susceptibility of women to fear-related memory disorders such as PTSD.

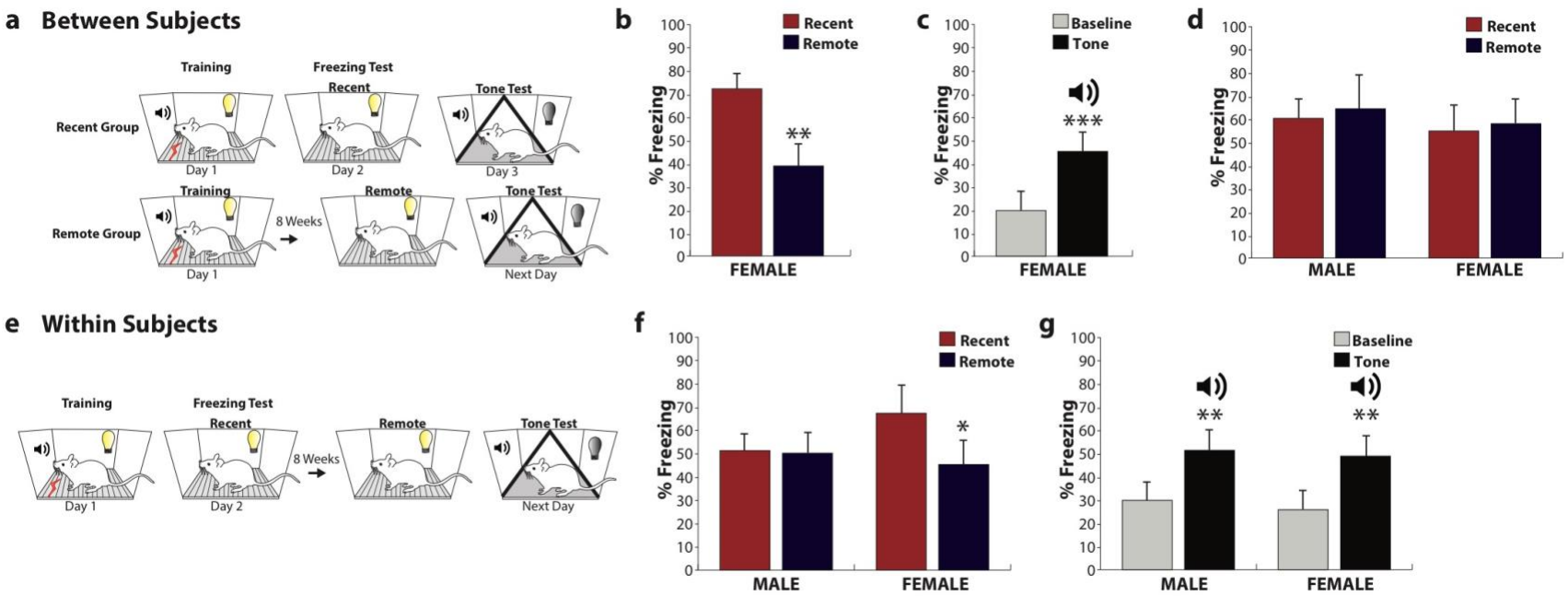

Figure 3.1. ***Females trained with background context fear conditioning show a decrease of remote context fear memory.*** (a) Between subjects experimental design. Female mice were trained with background context fear conditioning on day 1 and were either tested for freezing at a recent or remote time point and for freezing to the tone. (b) Females had lower levels of freezing when tested at the remote time point, 8 weeks after training compared with mice tested the day after training. (c) Memory for the tone is intact at a remote time point in both groups as observed by significantly higher freezing than baseline to the tone. (d) Freezing did not differ in mice tested at a recent or remote time point, 8 weeks after training. This group of mice was used for recent vs remote immunohistochemistry experiments. (e) Within subjects experimental design. On day 1, male and female mice underwent background context fear conditioning and were tested at a recent and remote time point and for freezing to the tone. (f) Females, but not males had lower levels of freezing when tested at the remote time point, 8 weeks after training compared with their recent test the day after training. This group of mice was also used for remote immunohistochemistry experiments. (g) Memory for the tone is intact at a remote time point in both sexes as observed by significantly higher freezing than baseline to the tone. Error bars represent SEM. *P<0.05, **P<0.01, ***P<0.001 cf recent test or baseline.

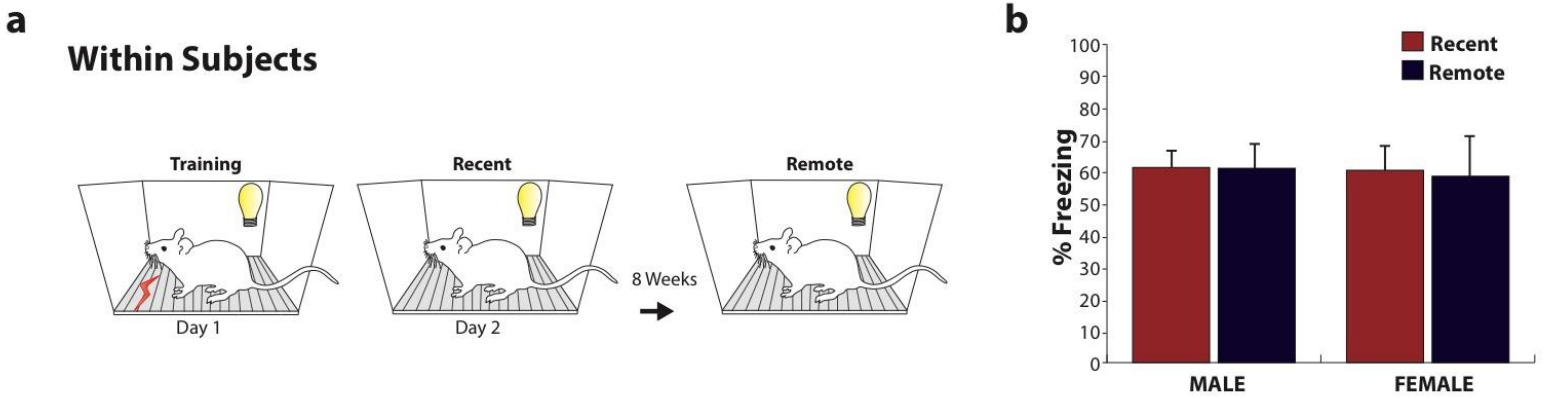

Figure 3.2. ***Females trained with foreground context fear conditioning do not show a decrease of remote context fear memory.*** (a) Within subjects experimental design. On day 1, male and female mice underwent foreground context fear conditioning and were tested at a recent and remote time point. (b) Females, nor males had different levels of freezing when tested at the remote time point, 8 weeks after training compared with their recent test the day after training. This group of mice was also used for immunohistochemistry experiments.

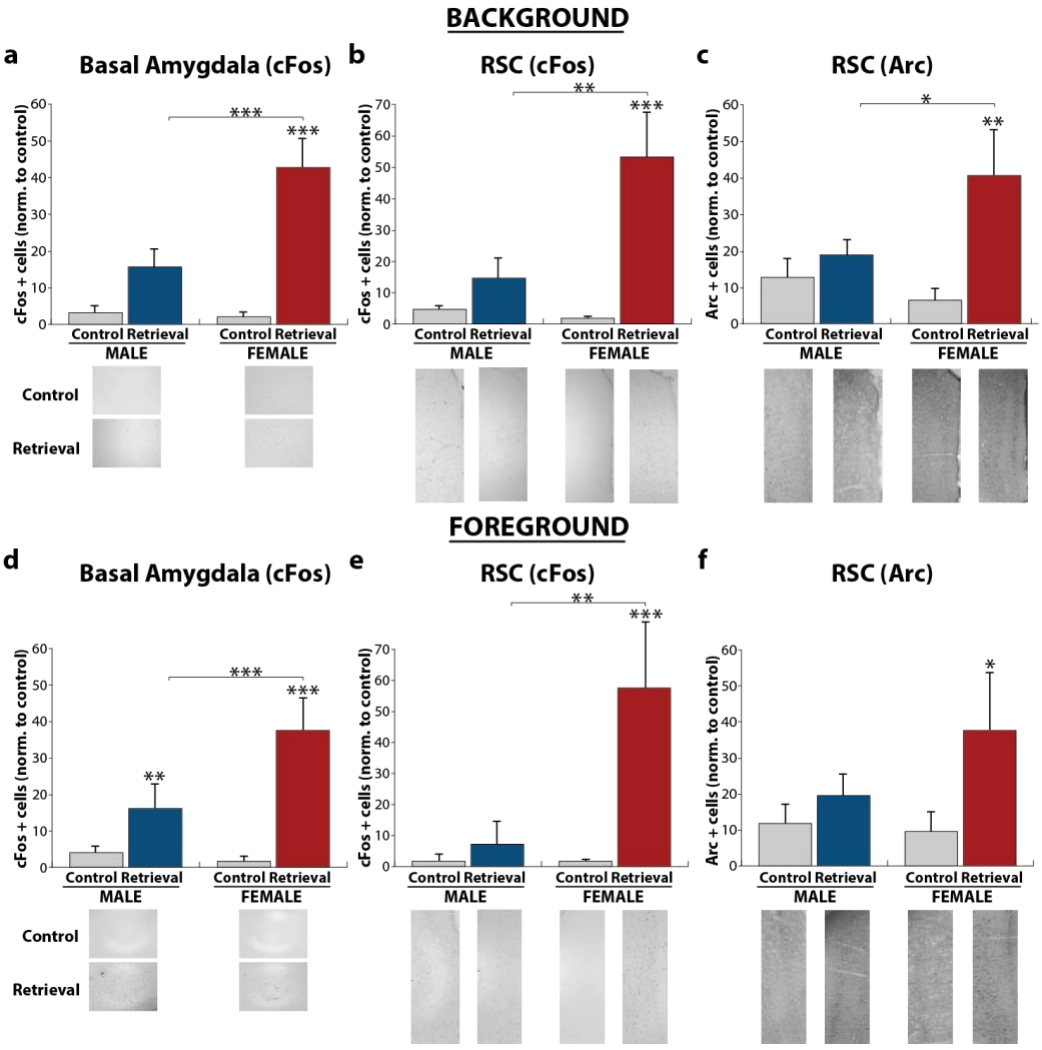

Figure 3.3. ***Sex differences in cFos and Arc activation during remote context fear memory retrieval.*** (a) Only females trained in background context fear conditioning show increases in cFos+ cells in basal amygdala following remote context fear memory retrieval compared with control. Number of cFos+ cells in basal amygdala is greater in females compared with males following remote context fear memory retrieval. (b) Only females trained in background context fear conditioning show increases in cFos+ cells in retrosplenial cortex following remote context fear memory retrieval compared with control. Number of cFos+ cells in retrosplenial cortex is greater in females compared with males following remote context fear memory retrieval. (c) Only females trained in background context fear conditioning show increases in Arc+ cells in retrosplenial cortex following remote context fear memory retrieval compared with control. Number of Arc+ cells in retrosplenial cortex is greater in females compared with males following remote context fear memory retrieval. (d) Females and males trained in foreground context fear conditioning show increases in cFos+ cells in basal amygdala following remote context fear memory retrieval compared with control. Number of cFos+ cells in basal amygdala is greater in females compared with males following remote context fear memory retrieval. (e) Only females trained in foreground context fear conditioning show increases in cFos+ cells in retrosplenial cortex following remote context fear memory retrieval compared with control. Number of cFos+ cells in retrosplenial cortex is greater in females compared with males following remote context fear memory retrieval. (f) Planned comparisons show that only females trained in foreground context fear conditioning display increases in Arc+ cells in retrosplenial cortex following remote context fear memory retrieval compared with control. Error bars represent SEM. *P<0.05, **P<0.01, ***P<0.001 cf control or retrieval males.

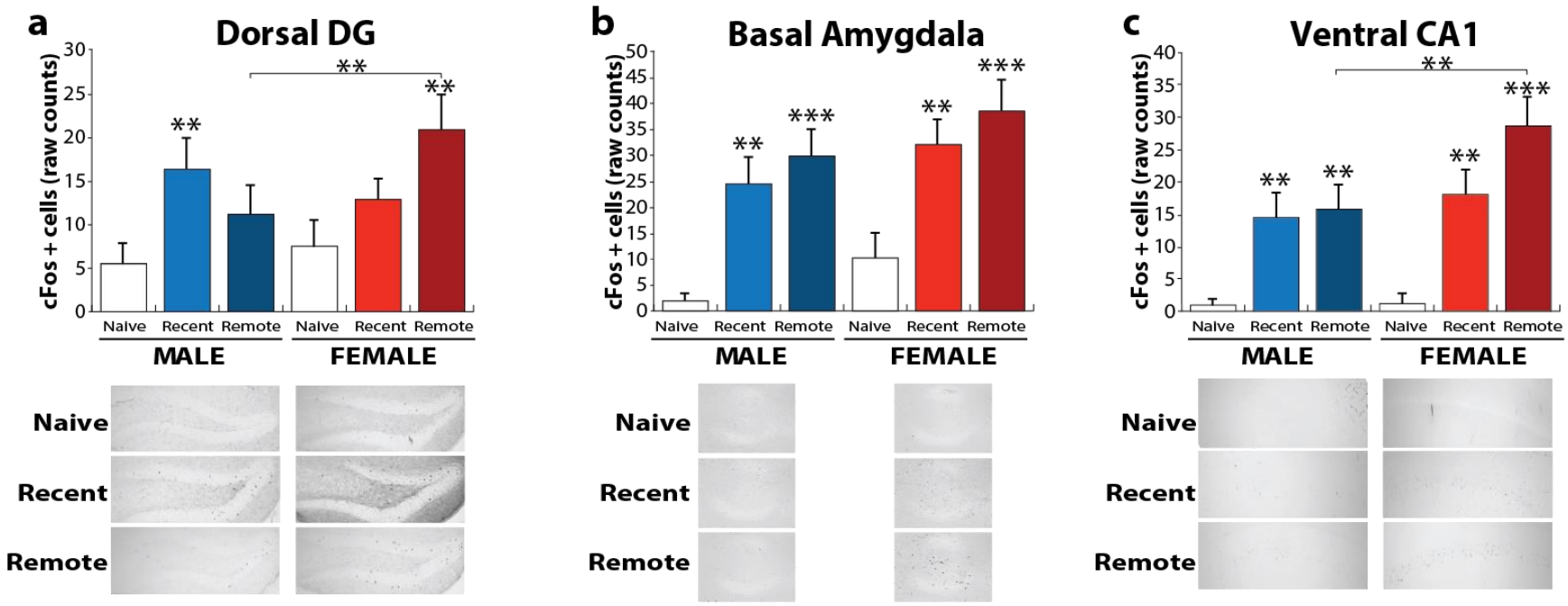

Figure 3.4. ***Sex differences in cFos activation during recent vs remote context fear memory retrieval.*** (a) Only females show increases in cFos+ cells in dorsal dentate gyrus following remote context fear memory retrieval compared with control. Number of cFos+ cells in dorsal dentate gyrus is greater in females compared with males following remote context fear memory retrieval. Males, but not females show increases in cFos+ cells in dorsal dentate gyrus following recent context fear memory retrieval compared with control. (b) Both males and females show increases in cFos+ cells in basal amygdala following recent and remote context fear memory retrieval compared with control. Overall, number of cFos+ cells in basal amygdala is greater in females compared with males. (c) Both males and females show increases in cFos+ cells in ventral CA1 following recent and remote context fear memory retrieval compared with control. Number of cFos+ cells in ventral CA1 is greater in females compared with males following remote context fear memory retrieval. Error bars represent SEM. **P<0.01, ***P<0.001 cf control or retrieval males.

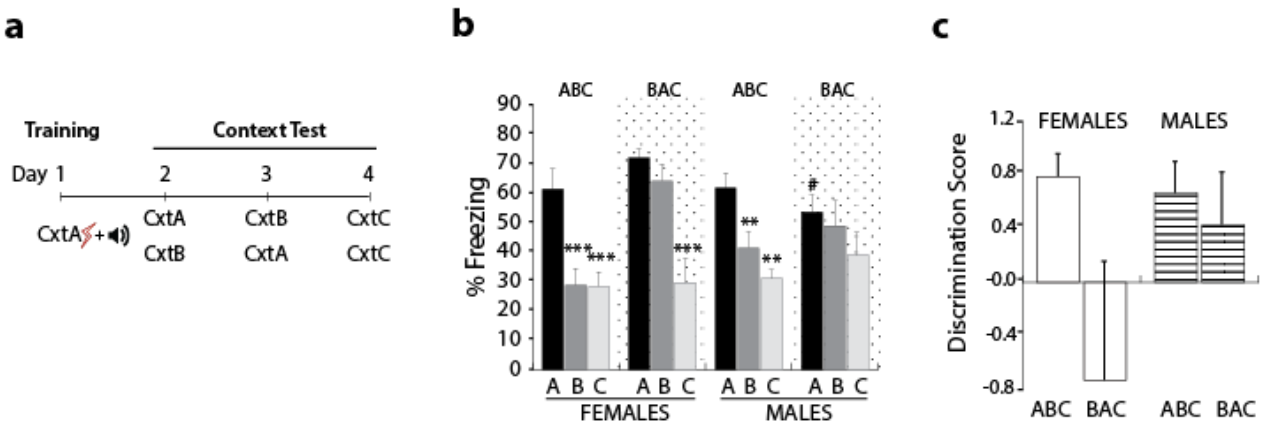

Figure 3.5. ***Males and females trained with background context fear conditioning generalize similarly to novel contexts.*** (a) Experimental design. (b) Males and females showed significantly greater freezing in the training context (A) compared with the similar context (B) and distinct context (C), only with ABC test order (ABC, white background; BAC, dotted background). c) Both males (striped bars) and females (white bars) had similar discrimination scores between the contexts. Error bars represent SEM. **P<0.01, ***P<0.001 cf training context. #P<0.05, cf females.

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Chapter IV

Sex Differences in Cognitive Strategy: Blocking and Extinction of Context fear

Abstract

Disorders of fear and anxiety such as post-traumatic stress disorder (PTSD) are more prevalent in women than in men, possibly due to sex differences in behavioral strategies used when confronted with trauma. In this project we utilized two distinct approaches to examine sex differences in behavioral strategies adapted by males and females following context fear conditioning. In experiments 1-3 we utilized a blocking task to examine whether a context-shock association is similarly retrieved in males and females and how retrieval has an effect on more complex processes such as learning a new association. In experiment 4 we assessed new learning, in the form of extinction and how this may differ behaviorally in males and females. During retrieval of context fear in experiments 1-2, females learned about a new predictor of the shock (a tone), whereas males did not. In experiment 4, females increased their freezing throughout extinction sessions while males showed a decrease in freezing. Taken together, our data demonstrate sex differences in cognitive strategies in blocking of tone and extinction of context fear. The tendency for females to learn new predictors of shock when confronted with fear and increase their freezing within an extinction trial fall in line with the possibility of a more risk averse phenotype in females when confronted with fear and a more risk-prone phenotype in males. Our findings further emphasize the need for validating behavioral tasks in females for the purpose of understanding *how* females learn and remember information. How context information is used and retrieved in males and females is likely to influence more complex

behaviors and biological processes, all of which may play a critical role in the greater susceptibility of females to fear-related memory disorders such as PTSD.

Introduction

Disorders of fear and anxiety such as post-traumatic stress disorder (PTSD) are more prevalent in women than in men, possibly due to sex differences in behavioral strategies used when confronted with trauma. While great strides have been made in assessing the ways by which males and females differentially use information to navigate a spatial environment, less is understood about strategies engaged when forced to deal with a dangerous context. Therefore, in this project we utilized distinct approaches to examine sex differences in behavioral strategies adapted by males and females following context fear conditioning.

In the non-disordered population males and females often perform equally well across a wide variety of memory tasks, but use different strategies to reach the same behavioral outcome. For example, in spatial tasks such as the Morris water maze, males largely outperform females, as indicated by their ability to more quickly find the hidden platform compared with females. However, males rely predominantly on distal cues, whereas females rely on landmarks or proximal cues to navigate a spatial environment (Rodríguez et al., 2011; Bettis and Jacobs, 2013; Keeley et al., 2013; Shah et al., 2013); thus, when a landmark such as a visual wall cue is added, sex differences in performance are abolished. Sex-specific use of spatial information has also been observed when mice were only given the ability to use either a landmark or geometric-based strategy to navigate in a Morris water maze task. Pre-training with either type of spatial information blocked learning about the other in females, whereas in males only the geometric learning blocked learning of the landmark (Rodríguez et al., 2011). Blocking is thought to ensue when there is a mismatch between what is expected and what actually does occur (Schultz, 2006;

Kamin, 1969). Therefore, in the example above, if a cue is trained to be predictive of solving a spatial task then a new geometric cue may not be learned. Kamin (1969) argued from data in males that a surprise must be present to learn in blocking and this idea was formulated into the Rescorla-Wagner model which states that that the level of association between a conditioned stimulus (CS) and an unconditioned stimulus (US) depends on a *prediction error*, also known as the difference between whether a US is expected and whether a US occurs (Rescorla and Wagner, 1972). However, blocking can also be accounted for by other theories such as the attentional theory (Mackintosh, 1975; Pearce and Hall, 1980), which suggests that blocking occurs due to inattention of a stimulus or the comparator hypothesis (Miller et al., 1988) which suggests that blocking may be due to competition between cues during memory retrieval. If males and females use different spatial or behavioral strategies to begin with, one must also question if the interpretation or what accounts for blocking in males and females may differ as well.

Sex differences in strategies use is also observed in a risky forging environment where one side of a home cage that contained food and water was paired with footshocks. While eating decreased in both sexes with the onset of shock, males increased amount ate in a single session, whereas females ate less at one time than before (Pellman et al., 2017), strategies both of which could be differentially beneficial depending on the situation and goals of each sex. Therefore, males and females differ in strategy use when presented with danger, where females may be more geared towards safety and males more willing to take risks to meet metabolic needs (For an excellent review on sex differences in behavioral strategies see Shansky, 2018).

Use of different strategies by males and females may serve to meet needs that are more likely to be beneficial for that specific sex. For an example, male and female mice live in large

communal groups involving a dominant male, several females that will breed with the male and many subordinate males (Reimer and Petras, 1967; Bronson, 1979; for review see Lonstein and De Vries, 2000). Therefore, daily functions are also likely to differ in terms of care of offspring and specific dangers may be of more heightened importance to a particular sex. For an example, it may be more advantageous for females to pay attention to specific cues signaling danger to care for her pups.

In fact, sex-specific behavioral responses to danger-paired cues that predict footshock have been observed, with a subset of females exhibiting more active, darting responses with cue onset whereas males present with a freezing response (Gruene et al., 2015). We have also recently reported sex differences in behavioral expression of fear following context fear conditioning, where females are more biased to show fear in safe contexts which resemble the shock-paired context. However, what information is retrieved by males and females and how they use this information to express fear remains an open question.

In this study we utilized two distinct approaches to examine sex differences in behavioral strategies adapted by males and females following context fear conditioning. In experiments 1-3 we utilized a blocking task to examine whether a context-shock association is similarly retrieved in males and females and how retrieval has an effect on more complex processes such as learning a new association. In experiment 4 we assessed new learning, in the form of extinction and how this may differ behaviorally in males and females. Results from these studies imply a difference in cognitive processing or strategy by males and females following context fear conditioning.

Methods

ANIMALS

9-week-old C57BL/6 mice (32 males, 64 females) from Envigo (Indianapolis, IN) were used in these experiments. Mice were individually housed throughout experiments with standard diet and water ad libitum. Individual housing in males is necessary to reduce fighting-induced stress (Meakin et al., 2013) and does not increase variance in either sex (Prendergast et al., 2014); Individual housing is consistent with University of Michigan Institutional Care and use Committee policies on management of fighting in mice. Due to independent social structures of both male and female mice (Becker and Koob, 2016), individual housing is ecologically appropriate for both sexes. The colony room was adjacent to behavioral testing rooms and was maintained at 20 ± 2 ℃ with a 12 h 0700: 1900 h light/dark cycle (lights on at 0700 h). Mice were acclimated to the colony room for at least 7 days before experiments began. Experimenters in this study were all women (Sorge et al., 2014). The University of Michigan Committee on the Use and Care of Animals approved all experimental methods performed in this research.

APPARATUS

Mice were trained and tested in conditioning chambers (9 3/4″ × 12 3/4″ × 9 3/4″; MedAssociates, VT), enclosed in sound-attenuating cubicles, equipped with a NIR camera (VID-CAM-MONO-2A). Grid floor rods were connected to a shock generator. Male and female mice were tested in separate chambers which were cleaned between each animal with either 70% ethanol, 1% acetic acid or soapy water. Freezing and locomotor activity was automatically scored with video freeze software (MedAssociates). Two experimenters, blind to experimental conditions also hand scored freezing to validate automatic scoring.

Three different contexts were used: (1) Context A: consisted of a rectangular box with white walls, lights on, an evenly sized grid floor (36 stainless steel rods, 1/8″ diameter, spaced 1/4″ apart), and 70% ethanol odor. (2) Context B: consisted of white curved wall insert and white

flat floors, with lights off and soap odor. (3) Context C: consisted of black angled walls, house lights off, staggered grid floors with alternating 1/8″ and 3/16″ grid rods, and 1% acetic acid odor.

EXPERIMENTAL DESIGN: TONE BLOCKING PROCEDURE (EXPERIMENT 1)

Phase I (days 1-6): On days 1-6 male (n = 8) and female (n = 8) mice experienced phase I foreground (unsignaled) training; a separate group of male (n = 8) and female (n = 8) mice remained in their home cage on days 1-6 (no phase I controls). In the group undergoing foreground fear conditioning, mice were placed in context A for 3 minutes, followed by delivery of a 2s, 0.5 mA footshock. Each day following fear conditioning mice were replaced in their home cage and returned to the colony room.

Phase II (day 7): On day 7 both groups of male and female mice: experimental (phase I) and control (no phase I), underwent the exact same training procedure in phase II. Mice were placed in context A in which they were allowed to explore for 2.5 minutes followed by the onset of a 30 second tone; upon termination of the tone mice received a 2s, 0.5 mA foot shock. The tone served as the new addition. Following phase II fear conditioning mice were replaced in their home cage and returned to the colony room.

Blocking Test (day 8): On day 8 male and female mice were tested for blocking of the tone (foreground trained mice). Mice were placed in a novel context (context B) to test for blocking of the tone (presented in phase II). After 2.5 minutes in context B, a 30 second tone was presented followed by a 1-minute inter-trial interval (ITI), 30 second tone, 1 minute ITI and a final 30 second tone. Freezing was measured as noted in “apparatus”.

EXPERIMENTAL DESIGN: TONE BLOCKING PROCEDURE + PROPRANOLOL PRIOR TO PHASE II TRAINING (EXPERIMENT 2)

Phase I (days 1-6): On days 1-6 female (n = 16) mice experienced phase I foreground (unsignaled) training (replication of experiment 1); a separate group of female (n = 16) mice did not receive foot shock on days 1-6 (no-shock controls). In the group undergoing foreground fear conditioning, mice were placed in context A for 3 minutes, followed by delivery of a 2s, 0.5 mA footshock; the no-shock controls received the same amount of context exposure but no footshock. Each day following fear conditioning mice were replaced in their home cage and returned to the colony room.

Phase II (day 7): 15 minutes prior to phase II training on day 7 both groups of female mice: experimental (shocked) and control (non-shocked), received an intraperitoneal injection of 10 mg/kg Propranolol or vehicle (Saline). Both shocked and non-shocked groups from phase I underwent the exact same training procedure in phase II. Mice were placed in context A in which they were allowed to explore for 2.5 minutes followed by the onset of a 30 second tone; upon termination of the tone mice received a 2s, 0.5 mA foot shock. The tone served as the new addition. Following phase II fear conditioning mice were replaced in their home cage and returned to the colony room.

Blocking Test (day 8): On day 8 female mice were tested for blocking of the tone (foreground trained mice). Mice were placed in a novel context (context B) to test for blocking of the tone (presented in phase II). After 2.5 minutes in context B, a 30 second tone was presented followed by a 1-minute inter-trial interval (ITI), 30 second tone, 1 minute ITI and a final 30 second tone. Freezing was measured as noted in "apparatus".

EXPERIMENTAL DESIGN: CONTEXT BLOCKING PROCEDURE (EXPERIMENT 3)

Phase I (days 1-6): On days 1-6 male (n =8) and female (n =8) mice experienced phase I background (signaled) training; a separate group of male (n =8) and female (n =8) mice remained

in their home cage on days 1-6 (no phase I controls). In the group undergoing background fear conditioning, mice were placed in context C for 2.5 minutes, followed by the onset of a 30 second tone; upon termination of the tone mice received a 2s, 0.5 mA foot shock. Each day following fear conditioning mice were replaced in their home cage and returned to the colony room.

Phase II (day 7): On day 7 both groups of male and female mice: experimental (phase I) and control (no phase I), underwent the exact same training procedure in phase II. Mice were placed in the new context A in which they were allowed to explore for 2.5 minutes followed by the onset of a 30 second tone; upon termination of the tone mice received a 2s, 0.5 mA foot shock. Context A served as the new addition. Following phase II fear conditioning mice were replaced in their home cage and returned to the colony room.

Blocking Test (day 8): On day 8 male and female mice were tested for blocking of the context (background trained mice). Mice were placed back in context A for 3 minutes to test for blocking of this context (presented in phase II). The control group that underwent only phase II training was placed in a novel context (context B) to test for freezing to the tone and the following day, to test for freezing of the context (context A). Freezing was measured as noted in "apparatus".

EXPERIMENTAL DESIGN: EXTINCTION (EXPERIMENT 4)

Foreground context fear conditioning was conducted as previously described (Keiser et al., 2017). Briefly, male (n =8) and female (n =8) mice were placed in the fear conditioning chambers for 3 minutes, followed by delivery of a 2s, 0.8 mA footshock. Mice were then replaced in their home cage and returned to the colony room. Each day following training, for a period of 13 days, mice were put through extinction sessions in which mice were placed in the

same context for 3 minutes and freezing was measured in 1 minute bins.

DRUGS

Propranolol (DL-Propranolol hydrochloride, 99%; Acros Organics, NJ, USA) was dissolved in saline. In experiment 2, Propranolol (10mg/kg) or saline was administered intraperitoneally (i.p.) 15 minutes prior to phase II.

STATISTICAL ANALYSIS

All statistical analyses were conducted using SPSS v23. Two-way repeated measures ANOVA (Test Bin x Group) were used to determine blocking of tone in mice that underwent foreground fear conditioning. For those that underwent background fear conditioning, blocking to the context was assessed with a one-way repeated measures ANOVA (Group). Sex differences in extinction levels were assessed using two-way repeated measures ANOVA (Sex x Day). Separate pre-planned t-tests were used to assess within-session freezing patterns for each test day in males and females. Post hoc tests with Bonferroni corrections for multiple tests were used to further examine significant effects in each experiment. The 95% confidence intervals are reported for post hoc tests. Partial η^2 (η^2_p) is reported as effect size estimate (0.01, 0.06, and 0.14 as small, medium, and large, respectively), as calculated by SPSS.

RESULTS

MALES, BUT NOT FEMALES, SHOW BLOCKING TO TONE AFTER FOREGROUND CONTEXT FEAR CONDITIONING

Males trained with foreground context fear conditioning show blocking to the tone (Figure 4.1b); (Test Bin: $F(1,14) = 11.26$, $P < 0.01$, $\eta^2_p = 0.45$, (95% CI: 5.60–25.44)). Male control mice only receiving phase II showed an increase in freezing to the tone compared with baseline $P < 0.01$ (95% CI: 9.93–37.99); whereas male mice that received phase I foreground

context fear conditioning did not freeze more to the tone compared with baseline P = 0.297. Females that have underwent foreground context fear conditioning did not show blocking to the tone (Figure 4.1c); (Test Bin: F (1,14) = 11.26, P < 0.01, η^2_p = 0.39, (95% CI: 4.91–29.12)). Female control mice only receiving phase II did not show an increase in freezing to the tone compared with baseline P = 0.225; whereas female mice that received phase I foreground context fear conditioning did freeze more to the tone compared with baseline P < 0.05, (95% CI: 6.77–41.01), indicating that females did not show blocking to the tone.

MALES AND FEMALES ACQUIRE CONTEXT FEAR CONDITIONING

Both males (P < 0.01) and females (P < 0.01) exhibited increased reactivity to the shock compared with baseline on day 1 (Figure 4.1d) (Test Bin: F (1,14) = 30.36, P < 0.001, η^2_p = 0.39, (95% CI: 161.08–366.41)), indicating that intensity of the shock did not differ between the sexes. During phase 1 freezing levels to the context differed between males and females on days 1-7 (Figure 4.1e) Sex: F (1,14) = 11.26, P < 0.001, η^2_p = 0.45, (95% CI: 7.13–32.17)), Sex x Day: F (6,84) = 5.28, P < 0.001, η^2_p = 0.27; freezing to the context was higher in females compared with males on days 2 (P < 0.05), 3 (P < 0.01), 5 (P < 0.05), 6 (P < 0.01) and 7 (P < 0.05). Both males and females increased their freezing to the context with more training trials (Figure 4.1e) Day: F (6, 84) = 85.12, P < 0.001, η^2_p = 0.86. Compared to the first context test which occurred on day 2 females showed increased freezing on days 3 (P < 0.001), 5 (P < 0.001), 6 (P < 0.001), and 7 (P < 0.001); compared to the first context test which occurred on day 2 males showed increased freezing on days 3 (P < 0.01), 4 (P < 0.01), 5 (P < 0.01), and 7 (P < 0.01). Overall, these results suggest that both males and females adequately learn context fear and increase freezing during phase 1 training with successive trials.

PROPRANOLOL ADMINISTERED PRIOR TO PHASE II TRAINING DID NOT PREVENT LEARNING ABOUT TONE IN FEMALES

Propranolol did not prevent learning about tone on phase II (Figure 4.2b) (Drug: F (1, 28) = 0.036, P = 0.851). As previously observed, females trained with foreground context fear conditioning during phase I (with shock) did not show blocking to the tone and females that underwent the same context exposure during phase I (without shock) were still able to learn about the tone (Figure 4.2b); (Test Bin: F (1,28) = 102.81, P < 0.001, η^2_p = 0.79, (95% CI: 25.00–37.66)). Females receiving shock during phase I training did not differ in freezing to the tone from females not receiving shock during phase I (Group: F (1,28) = 1.37, P = 0.252). Female mice that received shock during phase I foreground context fear conditioning did freeze more to the tone compared with baseline whether they were given Propranolol P < 0.01, (95% CI: 6.20–31.52) or vehicle P < 0.001, (95% CI: 14.88–40.19). Female mice that did not receive shock during phase I foreground context fear conditioning did freeze more to the tone compared with baseline whether they were given Propranolol P < 0.001, (95% CI: 23.70–49.02) or vehicle P < 0.001, (95% CI: 29.91–55.23), indicating that Propranolol did not prevent learning about tone on phase II and that females were still able to learn about tone with prior context exposure.

MALES, NOR FEMALES SHOW BLOCKING TO THE CONTEXT AFTER BACKGROUND CONTEXT FEAR CONDITIONING

Males and females that underwent background context fear conditioning did not show blocking to the context (Figure 4.33b and c); (Males: Group: F < 1; Females: Group: F < 1).

SEX DIFFERENCES IN FREEZING PATTERNS WITHIN EXTINCTION SESSIONS

Sex differences were not observed in levels of freezing. A significant main effect was observed for day (Day: F (12,168) = 14.06, P < 0.001, η^2_p = 0.50) but no main effect of Sex or

interaction of Sex x Day was observed. Average percent freezing to the context was significantly lower from Day 1 Test in females on Test Day 8 $P < 0.05$, Test Day 9 $P < 0.05$, Test Day 10 $P < 0.05$, Test Day 11 $P < 0.05$, Test Day 12 $P < 0.01$ and Test Day 13 $P < 0.01$. Average percent freezing to the context was significantly lower from Day 1 Test in males on Test Day 11 $P < 0.05$, Test Day 12 $P < 0.05$ and Test Day 13 $P < 0.01$. Therefore, females required 8 trials to show extinction and males required 11 trials (Figure 4.4b).

Within each session males and females showed different patterns of freezing (Figure 4.4c). Males tended to decrease freezing within a session, with a significant decrease between the first and last minute of the session observed on day 2 ($P < 0.01$). Females on the other hand, tended to increase freezing within a session, with a significant increase between the first and last minute of the session observed on day: 8 ($P < 0.001$), day 9 ($P < 0.05$), and day 13 ($P < 0.05$). When assessing differences in freezing for the first minute bin of each trial, males did not decrease their freezing from day 1 test, further suggesting that freezing decreases throughout sessions in males. Females on the other hand did show a decrease in freezing compared with the first minute bin of each trial on Test Day 8 $P < 0.05$, Test Day 9 $P < 0.05$, Test Day 11 $P < 0.05$, Test Day 12 $P < 0.01$ and Test Day 13 $P < 0.01$, further suggesting that females increase freezing throughout sessions. When assessing differences in freezing for the last minute bin of each trial, males and females decreased their freezing from day 1 test on Test days 8-13, Males: Test Day 8 $P < 0.05$, Test Day 9 $P = 0.050$, Test Day 10 $P < 0.05$, Test Day 11 $P < 0.05$, Test Day 12 $P < 0.05$, Test Day 13 $P < 0.05$; Females: Test Day 8 $P < 0.05$, Test Day 9 $P < 0.05$, Test Day 10 $P < 0.05$, Test Day 11 $P < 0.05$, Test Day 12 $P < 0.01$, Test Day 13 $P < 0.05$; suggesting that males and females extinguish fear but differ in patterns of within session freezing. Therefore, while

males and females did not differ in overall freezing levels, they did differ in patterns of freezing within test sessions and in number of test sessions required for extinction.

Discussion

Here we demonstrated sex differences in cognitive strategies following retrieval of context fear. During retrieval of context fear in experiments 1-2, females learned about a new predictor of the shock, whereas males did not. In experiment 4, females tended to increase their freezing throughout extinction sessions while males showed a decrease in freezing. Together, these findings imply a tendency of females to play it safe when confronted with fear and for males to become less risk-averse. Understanding the distinct ways in which males and females deal with a fearful environment will have strong implications for determining factors mediating the onset of fear-related memory disorders such as PTSD.

Sex differences in blocking after context fear conditioning (Experiments 1-3)

Our observation that males did not learn about a cue after context fear conditioning is consistent with earlier research in males also showing that previous context fear conditioning prevents learning of a tone or discrete cue (e.g. Ayres et al., 1985; Iordanova, 2006) and studies in males demonstrating that previous training with a cue, prevents learning about an additional compound stimulus (Kamin et al., 1969). That males with previous fear conditioning show blocking to the cue can be interpreted in line with the Rescorla-Wagner model which states that that the level of association between a conditioned stimulus (CS) and an unconditioned stimulus (US) depends on a *prediction error*, also known as the difference between whether a US is expected and whether a US occurs (Rescorla and Wagner, 1972). Therefore, the presence of a stimulus that predicts the onset of shock, which in this case is context, should result in low prediction error and therefore, result in blocking to a new cue presented before shock onset.

We observed in two separate experiments (experiment 1 and 2) that females did not show blocking to the tone after context fear conditioning. This finding could be interpreted as failure of females to acquire a context-shock association, which would fit in line with the aforementioned Rescorla-Wagner model, in which prediction error would be high and therefore the tone would be learned. However, the failure of females to acquire a strong context-shock association is unlikely given high levels of freezing to the context before shock onset during phase II background context fear conditioning (Figure 4.1e). Further, freezing to the context before shock onset was greater in females than in males and higher levels of freezing in females compared with males were observed on most trials during phase I training, suggesting that if anything, females may have acquired a stronger context-shock representation than males (Figure 4.1e). That females show greater freezing to the context after foreground context fear conditioning is consistent with previous studies in our lab (Keiser et al., 2017). Although females that underwent phase I foreground fear conditioning increased freezing to tone, home cage control females that did not receive phase I did not (Figure 4.1c), suggesting that if anything, a strong context-shock association in females *enhanced* their ability to learn about tone during phase II. In support of this idea, control females that underwent context exposures but did not receive shock during phase I increased freezing to the tone even though they too only received one tone-shock pairing (Figure 4.2b). These findings suggest that instead of females failing to develop a strong context-shock association, a strong context-shock association in females may *aid* females in learning about tone on phase II. Therefore, blocking in females may be explained by theories other than prediction error and may instead be due to increased attention to the stimulus in a dangerous situation (Mackintosh, 1975; Pearce and Hall, 1980). Therefore, it is possible that what accounts for blocking in males differs from females, where blocking in males

may be due to a strong context-shock association that prevents learning about tone (Rescorla and Wagner, 1972; Kamin, 1969) and in females a strong context-shock association may help set the stage for attention towards new cues (Mackintosh, 1975; Pearce and Hall, 1980). Lack of blocking in females may be explained by use of a cognitive strategy that differs from males. Sex differences could also exist in the type of information that is being retrieved and/ or how this retrieved information is used to reduce risks most salient for each sex which may result in a more risk-averse phenotype in females.

The interpretation of a more cautious strategy in females is supported by previous findings from our lab showing a stronger tendency for females to show a generalized fear response to a novel context after fear conditioning compared with males (Keiser et al., 2017). Stronger generalized fear to a context that resembles the shock-paired environment could be indicative of a more cautious strategy in females when confronted with fear. A more risk averse phenotype in females compared with males has been observed in studies assessing sex differences in a risky decision making task (Orsini et al., 2016). When males and females were given the option to choose between safe, small food reward and a risky large food reward (paired with footshock), females were more averse to risk as they chose the large, risky reward less than males, a finding that was not mediated by estrous phase. The authors go on to say that the differences in risk-taking between males and females may be explained by their ability to ensure reproductive success for each sex. Where a larger risk may be a necessity that allows males to mate and a more cautious, risk averse phenotype in females may allow for females to avoid harm and protect their offspring. The idea that differential behavior in males and females may serve to meet the same purpose such as reproductive success, call into question the interpretation of many behavioral experiments. In which assessment of the same behavior in males and females may

reflect a different meaning and in which different behavior, may reflect a similar meaning. Therefore, the ability to learn new potential predictors of the aversive shock may serve to be more strategic in females compared with males.

Other potential explanations for sex differences in blocking may be due to differences in stress during retrieval and how this stress response influences new learning. Stress has been shown to differentially impact spatial learning in males and females. Although similar levels of corticosterone are observed in males and females following acute restraint stress, spatial memory in a Y-maze was found to be impaired in males, but enhanced in females, a finding independent of estrous phase (Conrad et al., 2004). Similar effects have also been reported following repeated restraint stress where males showed impairments in object recognition and females showed enhanced performance (Bowman et al., 2009). However, sex differences in stress response are unlikely to be mediating our observation of sex differences in blocking after context fear conditioning as administration of the noradrenergic beta-blocker propranolol which has been shown to lower blood pressure and heart rate (Leenen et al., 1983), decrease post-traumatic stress symptoms (Brunet et al., 2011), and blunt the effect of stress in consolidation of latent inhibition (Parfitt et al., 2012), did not allow for blocking in females.

Interestingly, 6 days of background (signaled) fear conditioning did not prevent males or females on day 7 from associating the new context with foot shock. One reason for this may be due to the strong salience of a new environment, leading to an inability to solely focus on the tone and therefore, an inability to block out a novel environment that later becomes paired with footshock. For a context or environment to exhibit strong salience is not surprising as unlike a tone, which is presented before the footshock, a context must be stable, constant and unchanged (Rudy, 2009). Choice of everyday behaviors and actions largely depend of our environment and

thus, knowledge of environment serves as important for decision making and survival. While many others have examined blocking after context fear conditioning to a discrete cue (Ayres et al., 1985; Iordanova, 2006) or blocking after background fear conditioning to a compound stimulus (Kamin., 1969); our study is the first to examine sex differences in blocking in both conditions. While the underlying factors that mediate sex differences in blocking remain to be identified, our findings call into question how the lack of an effect of blocking should be interpreted in females. It remains possible that instead of females failing to recall a previously shock-paired context, that proper recall of this context results in learning about new cues and predictors of shock in females but not males. Learning new predictors of danger and attention to new cues in an already predictable environment may be more advantageous for females and may not reflect the inability to retrieve previously learned information. Future research is needed to identify the differential factors that mediate sex differences in blocking following context fear conditioning.

Sex differences in within-session extinction of context fear (Experiment 4)

In this study, males and females did not differ in overall levels of freezing each day of extinction, but did differ in within-session patterns of freezing where females tended to increase their freezing throughout a session and males tended to decrease their freezing. That males decrease freezing to the context within a session is thought to reflect proper extinction of context fear, findings that are in agreement with previous reports assessing males during extinction. Increased freezing in females throughout extinction sessions however, does not appear to reflect a failure to properly extinguish context fear. This is due to several reasons 1) both males and females extinguish context fear as observed by a decrease in average percent freezing with more extinction sessions, 2) females required less extinction trials to decrease freezing from the first

test, with females requiring 8, whereas males required 11 trials and 3) increased within-session freezing by females becomes more pronounced as overall levels of freezing drop and extinction occurs. Therefore, is likely that sex differences in within-session freezing reflect a different process, such as a different use of cognitive strategy during retrieval of extinction.

That males and females have different strategies for expressing fear during extinction is in line with previous work demonstrating sex differences in behavioral fear responses when tested for recall of a shock-paired cue. There, authors reported a stronger tendency for females to exhibit a more active, escape-like darting response when tested for fear recall of the tone, whereas males tended to exhibit a freezing response (Gruene et al., 2015). Our findings add to this body of work to show that during retrieval of *context* fear, sex-specific patterns of freezing occur within session. How such differences in freezing behavior within session seek to benefit each sex warrants further investigation.

Our findings of sex-specific patterns of freezing within extinction sessions is also in line with previous work also demonstrating that females, but not males increase freezing within an extinction session, a finding shown to be independent of gonadal hormones (Matsuda et al., 2015). We add to these findings to show that sex-specific patterns in within session extinction occur with a less robust extinction paradigm, where extinction sessions are 3 minutes in length each day, compared with those in Matsuda et al. which consisted of 20 minute sessions each day. The tendency for females to increase freezing within extinction sessions, as the number of sessions increase may be reflective of a safety strategy by females to err on the side of caution and "play it safe". As is discussed above, the tendency for females to be more risk averse may lead to benefits that result in optimal reproductive success in females, whereas in males, a riskier strategy may be more advantageous.

Conclusion

Taken together, our data demonstrate sex differences in cognitive strategies in blocking of tone and extinction of context fear. Such strategies fall in line with the possibility of a more risk averse phenotype in females when confronted with a fearful environment and a more risk-prone phenotype in males. While we cannot definitively state which strategies males and females are using during retrieval of context fear, we can state that such strategies differ between the sexes. Our findings further emphasize the need for validating behavioral tasks in females for the purpose of understanding *how* females learn and remember information. How context information is used and retrieved in males and females is likely to influence more complex behaviors and biological processes, all of which may play a critical role in the greater susceptibility of females to fear-related memory disorders such as PTSD.

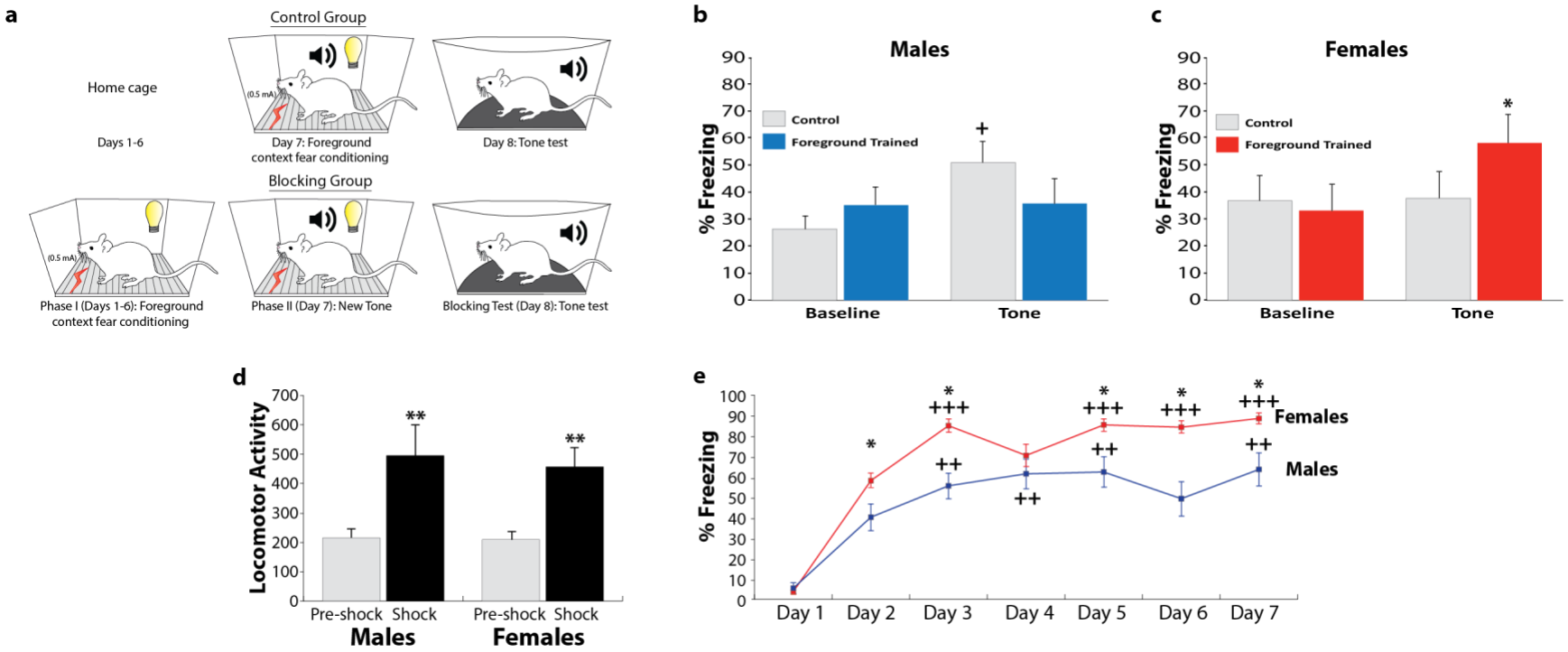

Figure 4.1. ***Males, but not females, show blocking to tone after foreground context fear conditioning.*** (a) Experimental design. The control group of male and female mice (above) remained in their home cage on days 1-6. On day 7 they underwent phase II background (signaled) fear conditioning and on day 8 were tested for freezing to the tone. The experimental group (below) underwent foreground (un signaled) context fear conditioning once a day, each day on days 1-6 (phase I training). On day 7 they underwent phase II background (signaled) fear conditioning where the tone is the new information that is added and on day 8 were tested for freezing to the tone. (b) Control males (grey bars) froze higher than baseline when tested for memory of the tone on day 8. Males from the experimental group that underwent phase I training did not freeze more to the tone compared with baseline, showing that phase I training resulted in blocking. (c) Control females (grey bars) did not freeze higher than baseline when tested for memory of the tone on day 8. Females from the experimental group that underwent phase I training however, did freeze more to the tone compared with baseline, showing that phase I did not prevent learning about the tone. (d) During day 1 of foreground context fear conditioning, both males and females show enhanced locomotor activity during shock onset compared with baseline. (e) Freezing to the context was higher in females compared with males on days 2, 3, 5, 6 and 7 and compared to the first context test which occurred on day 2 females showed increased freezing on days 3, 5, 6 and 7 and males showed increased freezing on days 3, 4, 5 and 7. Error bars represent SEM. *P<0.05, **P<0.01, cf control group baseline/pre-shock baseline/males +P<0.05, ++P<0.01, +++P<0.01, cf foreground trained baseline/ same sex freezing on day 2.

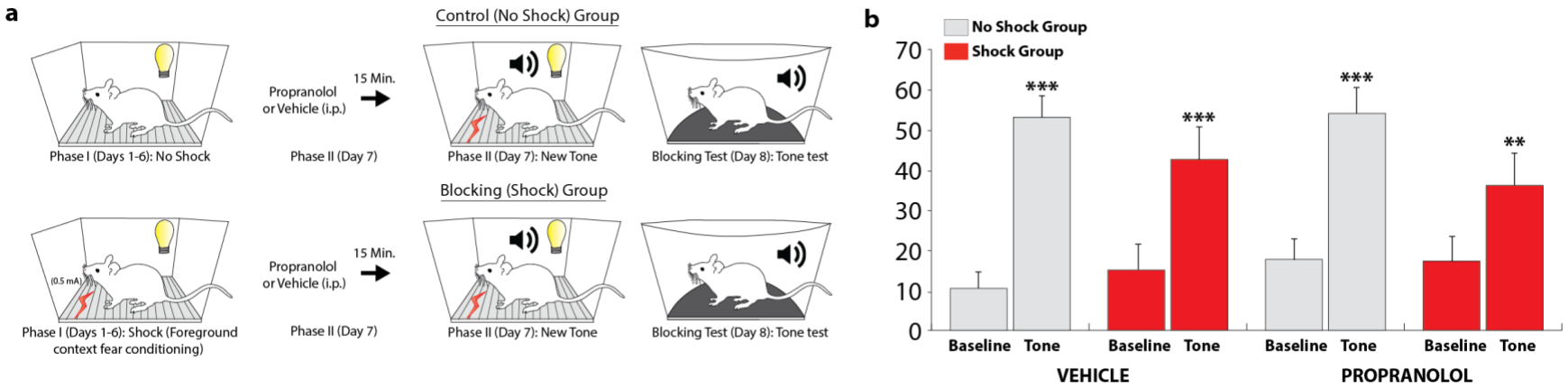

Figure 4.2. ***Propranolol had no effect on tone blocking in females*** (a) Experimental design. The control group of female mice (above) were placed in the context once a day, each day on days 1-6 (phase I training) and did not receive footshock. On day 7 they underwent phase II background (signaled) fear conditioning where they received a footshock for the first time immediately after presentation of a 30 second tone and on day 8 were tested for freezing to the tone. The experimental group (below) underwent foreground (unsignaled) context fear conditioning once a day, each day on days 1-6 (phase I training). On day 7 they underwent phase II background (signaled) fear conditioning where the tone is the new information that is added and on day 8 were tested for freezing to the tone. (b) Propranolol did not prevent learning about tone on phase II. Females from the experimental group (red) that underwent phase I training with shock froze more to the tone compared with baseline, showing that phase I did not prevent learning about the tone. Females from the control group (grey) that underwent phase I training without shock froze more to the tone compared with baseline, showing that females were still able to learn about tone with prior context exposure. Error bars represent SEM. **P<0.01, ***P<0.001, cf baseline.

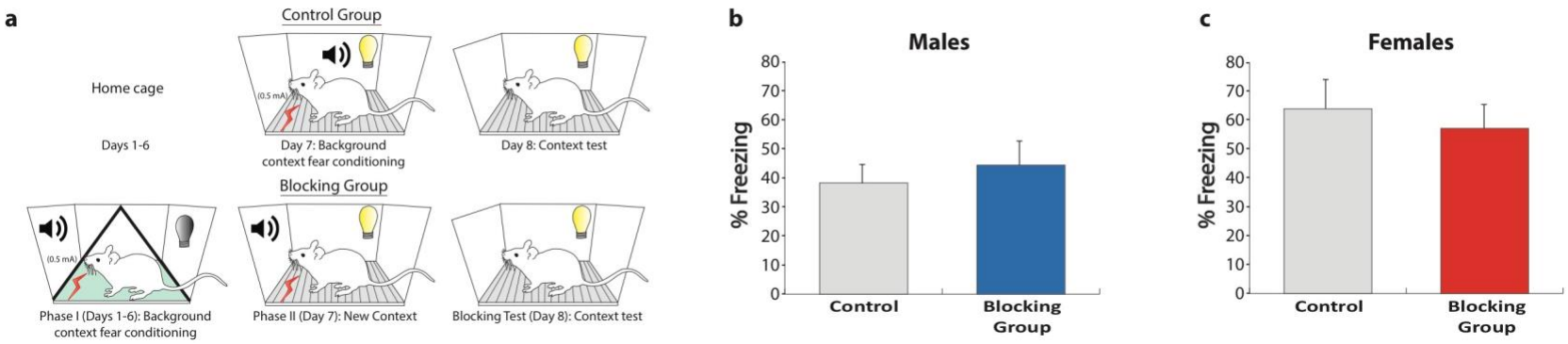

Figure 4.3. ***Neither males, nor females, show blocking to the context after background context fear conditioning.*** (a) Experimental design. The control group of male and female mice (above) remained in their home cage on days 1-6. On day 7 they underwent phase II background (signaled) fear conditioning and on day 8 were tested for freezing to the context. The experimental group (below) underwent background (signaled) context fear conditioning once a day, each day on days 1-6 (phase I training). On day 7 they underwent phase II background (signaled) fear conditioning where the context is the new information that is added and on day 8 were tested for freezing to the context. (b) Males nor (c) females that underwent phase I training significantly differed in freezing to the context compared with the control group, showing that phase I did not prevent learning about the context in either sex.

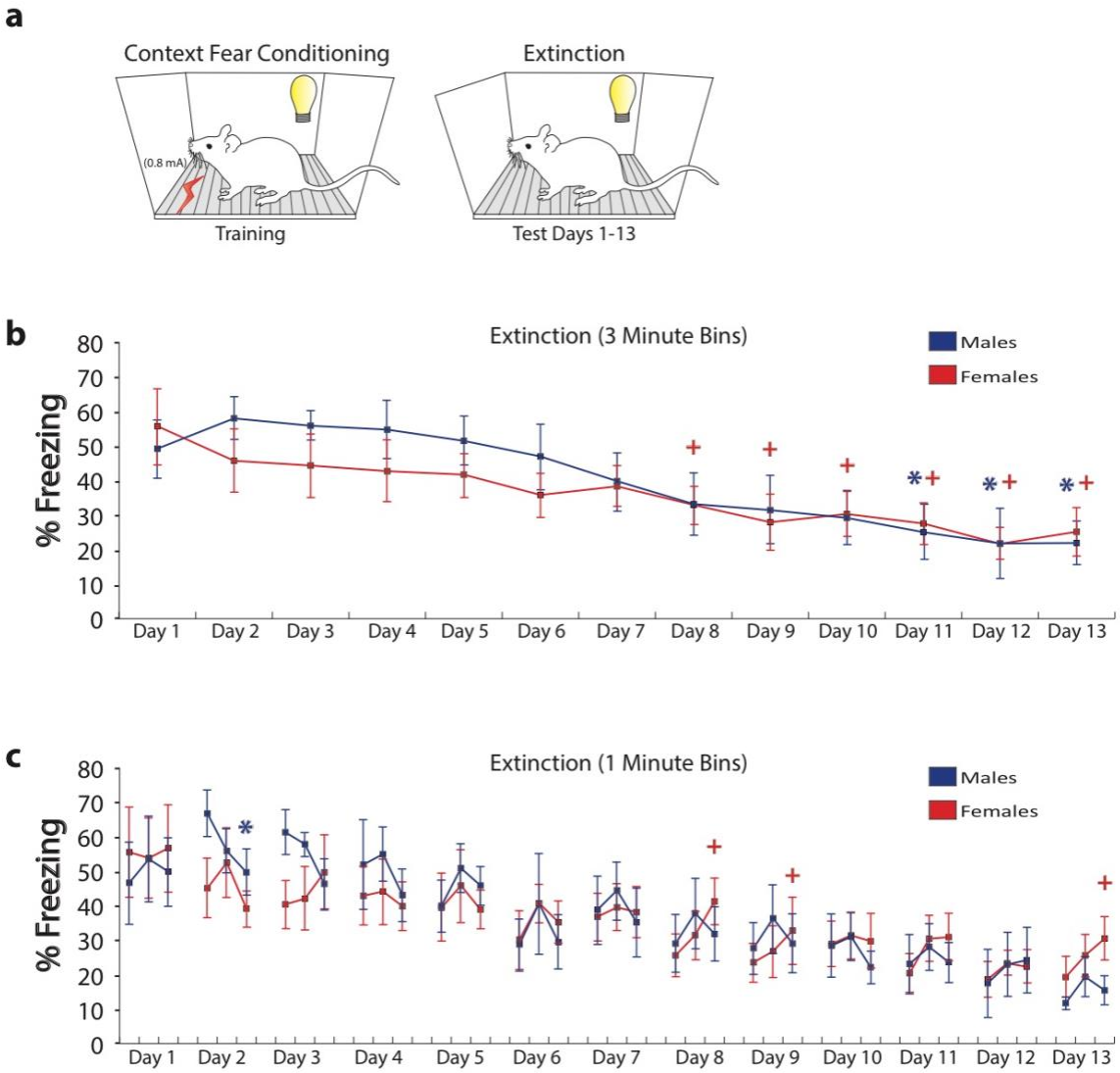

Figure 4.4. ***Sex differences in freezing patterns within extinction sessions.*** (a) Experimental design. Male and female mice underwent foreground (un signaled) context fear conditioning and underwent extinction each day following for 13 days. (b) In females (red), freezing decreased from day 1 test on days 8-13. In males (blue), freezing decreased from day 1 test on days 11-13. (c) In males (blue) freezing levels tended to decrease within sessions as in day 2, in females (red) freezing levels tended to increase within sessions as in days 8, 9 and 13. Error bars represent SEM. *P<0.05, cf day 1 males; +P<0.05, cf day 1 females.

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Chapter V

Sex Differences in Hippocampal Molecular Mechanisms Activated by Retrieval of Context Fear

Abstract

Post-traumatic stress disorder (PTSD) affects 8 million people each year, with women being more than twice as likely to be afflicted. Despite this skew in prevalence of PTSD between males and females, molecular mechanisms of fear-related memory have been primarily identified in male animals. Here we use RNA-sequencing as an unbiased measure to determine and identify differentially expressed genes in ventral hippocampus of males and females one hour following consolidation and retrieval of context fear and at baseline. We demonstrated that following consolidation or retrieval of context fear and at baseline, males and females show differential expression of many genes, suggesting distinct transcriptional regulation of ventral hippocampus by males and females, despite similar levels of context exploration, reactivity to the shock and freezing to the context. Such findings suggest that males and females may rely on differential hippocampal molecular mechanisms to reach similar behavioral outcomes. Identification of novel molecular mechanisms that mediate retrieval and consolidation of context fear memory in females will aid our understanding of *how* and by *which means* females consolidate and retrieve context fear.

Introduction

The prevalence of post-traumatic stress disorder (PTSD) in women is at a rate that is almost 3 times that of men (Kessler et al., 1995, 2012). Despite this skew in prevalence between

males and females in the occurrence of stress and memory-related disorders, basic research including females in studies of fear-related memory is sparse (Beery and Zucker, 2011; Lebron-Milad and Milad, 2012). When females have been included in the study of fear memory, sex differences have been observed in behavioral responses to shock-paired cues (Gruene et al., 2015a), generalization of context fear (Lynch et al., 2013; Keiser et al., 2017), rate of learning context (Wiltgen et al., 2001) and extinction of fear (Lebron-Milad and Milad, 2012; Milad and Quirk, 2012; Matsuda et al., 2015; Voulo and Parsons, 2017). Such differences in fear-related memory have also been observed in brain circuits involved (e.g. Gasbarri et al., 2007; Baran et al., 2010; Cahill, 2011; Bellace et al., 2013; Gruene et al., 2015b) and molecular mechanisms activated (e.g. Kudo et al., 2004; Mizuno et al., 2006, 2007; Gresack et al., 2009; Moore et al., 2010; Dachtler et al., 2011; Ter Horst et al., 2012) during acquisition or consolidation of fear-related memories. Together, these studies raise the possibility that transcriptional regulation differs in males and females during consolidation and retrieval of fear-related memories. Given that the majority of studies that have used both sexes in the study of fear-related memory have focused on the acquisition or consolidation phase, very little is known about retrieval.

Much of the work contributing to our understanding of how fear-related memories are expressed and the underlying neural circuitry involved have largely utilized a male animal model. As studies including females progress, it is becoming quite clear that females differ from males in many critical ways. However, there remains a strong need for developing the foundational work that contributes to our understanding of how fear-related memories are expressed and the underlying mechanisms that are used by females. In order to effectively accomplish this goal, an unbiased approach that steps away from what is known to be true and already occurring in males must be adapted. RNA-sequencing fits this need by not requiring pre-

selection of gene targets that we wish to assess, and thus will help to identify genes that are differentially expressed in females following consolidation or retrieval of fear-related memory.

The hippocampus is critically involved in the study of context fear memory. The dorsal hippocampus plays an important role in compiling distinct features of a fear conditioning chamber (floor, lights, sounds, colors, odor) into a single representation defined as (Rudy and O'Reilly, 1999; Reilly and Rudy, 2001; Matus-amat et al., 2004). While dorsal hippocampus is critical in formation of a representation of context, amygdala is likely storing the context-shock association (Huff and Rudy, 2004; Huff et al., 2005). Unlike dorsal hippocampus, ventral hippocampus receives reciprocal projections to and from the amygdala (Pitkanen et al., 2000; Kishi et al., 2006) and ventral hippocampus is necessary for defensive responses such as freezing (Zhang et al., 2001). There is growing evidence for sex differences in ventral hippocampus. Following context fear conditioning, males but not females show significant phosphorylation of extracellular regulated kinase (Erk), (a kinase known to be important in memory consolidation) in ventral hippocampus; whereas similar levels of Erk phosphorylation were observed in dorsal hippocampus of males and females (Gresack et al., 2009).

Sex differences in ventral hippocampus are also present after retrieval of context fear. Recent work from our lab has demonstrated greater generalization of fear to safe contexts in females compared with males and identified neural correlates following retrieval of context fear by examining immediate early gene activation. Following retrieval, females showed stronger recruitment of basal amygdala compared with males (Keiser et al., 2017) as well as stronger engagement of ventral hippocampus (Chapter 2). Given sex differences in context generalization (Lynch et al., 2013; Keiser et al., 2017) and the critical role of ventral hippocampus in retrieval of a generalized memory (Cullen et al., 2015), sex differences may also be present in the

molecular mechanisms activated by retrieval of context fear within ventral hippocampus. Less is known about the molecular basis of sex differences in in retrieval of context fear memories and to date the majority of studies examining sex differences in fear memory have focused on identifying consolidation-related molecular correlates. Further, much less is known about the role of ventral, compared with dorsal hippocampus in males and females during consolidation or retrieval of context fear memory. How males and females compare with respect to the molecular cascades in ventral hippocampus altered by consolidation and retrieval of context fear memory also remain unidentified.

Our primary objective of the present study was to identify ventral hippocampal molecular mechanisms mediating retrieval and consolidation of context fear in males and females. In order to step away from a strictly male-comparative approach, we employed RNA-sequencing as an unbiased measure to examine regulation of hippocampal gene expression after memory retrieval in females and males. Previous studies in males have determined that consolidation and retrieval of context fear downregulate different genes in hippocampus which play different functions (Peixoto et al., 2015a; Poplawski et al., 2016). We show a striking difference in number of genes differentially expressed between males and females during fear memory recall in ventral hippocampus; of particular interest, were differential expression of genes during retrieval in females that code for proteins in signaling pathways such at the PI3-Akt signaling pathway known to play a key role in spatial learning and cellular stress response. Additionally, many genes such as *growth hormone* (*gh*) and many immediate early genes were similarly regulated in ventral hippocampus of males and females during consolidation and following retrieval of context fear. Collectively, these data demonstrate sex-specific mechanisms associated with retrieval of a context fear memory. Alterations in hippocampal gene expression will inform

molecular pathways that differ in males and females during consolidation and retrieval of context fear. This knowledge may contribute to our understanding of sex-specific prevalence of disorders such as PTSD.

Methods

ANIMALS

The 9-week-old C57BL/6 mice (27 males, 27 females) from Envigo (Indianapolis, IN) were individually housed throughout experiments with standard diet and water ad libitum. Individual housing in males is required to reduce fighting- induced stress (Meakin et al., 2013), and is consistent with both previous fear conditioning studies (see e.g. Radulovic et al., 1998; Tronson et al., 2009; Tanaka et al., 2014; Van Craenendonck and Ver Donck, 2014) and University of Michigan Institutional Care and use Committee policies on management of fighting in mice, and does not increase variance in either sex (Prendergast et al., 2014). Because of independent social structures of both male and female mice (Becker and Koob, 2016), individual housing is ecologically appropriate for both sexes. The colony room was adjacent to behavioral testing rooms and maintained at 20 ± 2 °C with a 12 h 0700 : 1900 h light/dark cycle (lights on at 0700 h). All mice were acclimated to the colony room for at least 7 days before experiments began. All experimenters in this study were women (Sorge et al., 2014). The University of Michigan Committee on the Use and Care of Animals approved all experimental methods performed in this research.

APPARATUS

Training and testing conditions were performed in conditioning chambers (9 3/4″ × 12 3/4″ × 9 3/4″; MedAssociates, VT), enclosed in sound-attenuating cubicles, equipped with a NIR camera (VID-CAM-MONO-2A). Grid floor rods were connected to a shock generator. Male and

female mice were tested in separate chambers and chambers were cleaned between each animal with 70% ethanol. Video Freeze software (MedAssociates) automatically scored freezing and locomotor activity. Two experimenters, blind to experimental conditions, hand scored freezing to verify automatic scoring.

CONTEXT FEAR CONDITIONING AND RETRIEVAL

Foreground context fear conditioning was conducted as previously described (Keiser et al., 2017). Briefly, mice (18 males, 18 females) were placed in the conditioning chambers for 3 minutes, followed by delivery of a 2s, 0.8 mA foot shock. Mice were then replaced in their home cage and returned to the colony room. 24 hours after training, mice (9 males, 9 females) were placed into the same context as training for 3 minutes and freezing was measured. Following the retrieval test, mice were immediately returned to the colony room.

Behavioral Statistical Analysis:

Statistical analyses regarding freezing levels were conducted using SPSS v23. One-way ANOVA was used to determine sex differences in freezing during retrieval test.

TISSUE COLLECTION AND RNA EXTRACTION

All mice of the same sex were sacrificed on the same day. Animals were sacrificed either one hour after training (consolidation n = 9 per sex) or one hour after testing (retrieval n = 9 per sex). A separate group of home cage controls were sacrificed (n = 9 per sex) alongside the other two groups and dissections of all 3 groups were dispersed throughout the day to account for circadian effects. Ventral hippocampus was rapidly dissected on ice and immediately placed into RNA-later (Ambion, Cat#AM7020) and then in the -20 ºC until processing within the week. The dissections took place under the flow hood and the area and dissection tools were thoroughly

wiped down with RNAse away spray (Molecular Bioproducts, Cat#7002) using RNAseZap wipes (Ambion, Cat#AM9786, AM9788) to prevent degradation of RNA.

RNA was isolated using the PureLink RNA Mini Kit (Life Technologies, Cat # 12183018A,12183025. Tissue was homogenized using lysis buffer from the PureLink RNA Mini Kit and homogenized sample was placed into RNAse-free Eppendorf tubes and then on ice. RNA was purified, resulting in a total of 50 µl of RNA. RNA was analyzed for integrity with NanoDrop (Thermo Scientific). RNA was then stored in the -80 ºC until sequencing.

RNA-SEQUENCING

RNA was assessed for quality using the TapeStation (Agilent, Santa Clara, CA) using manufacturer's recommended protocols. Samples with RINs (RNA Integrity Numbers) of 8 or greater were prepped using the Illumina TruSeq Stranded mRNA Library Prep kit (Catalog #s RS-122-2101, RS-122-2102) (Illumina, San Diego, CA) using manufacturer's recommended protocols. Where 0.1-3ug of total RNA was converted to mRNA using a polyA purification. The mRNA is then fragmented and copied into first strand cDNA using reverse transcriptase and random primers. The 3 prime ends of the cDNA were then adenylated and adapters are ligated. One of the adapters that is ligated has a 6 nucleotide barcode that will be unique for each sample which allowed us to sequence more than one sample in each lane of a HiSeq flow cell (Illumina). The products are purified and enriched by PCR to create the final cDNA library. Final libraries were checked for quality and quantity by TapeStation (Agilent) and qPCR using Kapa's library quantification kit for Illumina Sequencing platforms (catalog # KK4835) (Kapa Biosystems,Wilmington MA) using manufacturer's recommended protocols. The samples were pooled, clustered on the cBot (Illumina) and sequenced on the HiSeq 4000, single-read 50 nt according to manufacturer's recommended protocols. 23,337 genes were detected as being

expressed in the ventral hippocampus with all groups of males and females combined (naïve, consolidation and retrieval groups) using the Illumina Hi-Seq platform.

SEQUENCING AND DIFFERENTIAL ANALYSIS AND STATISTICS

Sequencing was performed by the UM DNA Sequencing Core, using the Illumina Hi-Seq platform. Two different, popular techniques for differential expression analysis were used: Cufflinks/CuffDiff and HTSeq/DESeq2 (Zhang et al., 2014), using UCSC mm10.fa as the reference genome sequence. Genes and transcripts were identified as being differentially expressed based on three criteria: test status = "OK", false discovery rate (FDR) ≤ 0.05, and fold change $\geq \pm 1.5$. Genes and isoforms were annotated with NCBI Entrez GeneIDs and text descriptions.

Reads files were downloaded from the Sequencing Core's storage, and concatenated those into a single .fastq file for each sample. The quality of the raw reads data were checked for each sample using FastQC (http://www.bioinformatics.bbsrc.ac.uk/projects/fastqc/) (version v0.11.3) to identify features of the data that may indicate quality problems (e.g. low quality scores, over-represented sequences, inappropriate GC content).

The Tuxedo Suite software package was used for alignment, differential expression analysis, and post-analysis diagnostics (Langmead et al., 2009; Trapnell et al., 2009, 2013). Briefly, reads were aligned to the reference genome including both mRNAs (UCSC mm10) (ttp://genome.ucsc.edu/) using TopHat (version 2.0.13) and Bowtie2 (version 2.2.1.). Default parameter settings were used for alignment, with the exception of: "--b2-very-sensitive" telling the software to spend extra time searching for valid alignments. FastQC was used for a second round of quality control (post-alignment), to ensure that only high quality data would be input to expression quantitation and differential expression analysis.

Cufflinks/Cuffdiff (version 2.1.1):

For expression quantitation, normalization, and differential expression analysis, parameter settings: "--multi-read-correct" were used to adjust expression calculations for reads that map in more than one locus, as well as "--compatible-hits-norm" and "--upper-quartile–norm" for normalization of expression values. Diagnostic plots were generated using the CummeRbund R package.

HTSeq (version 0.6.1) /DESeq2 (version 1.14.1):

Expression quantitation was performed with HTSeq, to count non-ambiguously mapped reads only. Data were pre-filtered to remove genes with 0 counts in all samples. Normalization and differential expression was performed with DESeq2, using a negative binomial generalized linear model (https://www.bioconductor.org/packages/release/bioc/vignettes/DESeq2/inst/doc/DESeq2.pdf) . Plots were generated using variations or alternative representations of native DESeq2 plotting functions, ggplot2, plotly, and other packages within the R environment.

QUATITATIVE GENE OVERLAP

Genes that were found to be differentially expressed within sex (e.g. naïve vs retrieval) were divided into two groups: differentially expressed genes in males for that comparison and differentially expressed genes (DEGs) in females for that comparison. Using the publicly available GeneVenn program (http://genevenn.sourceforge.net), the two lists of differentially expressed genes for males and females were input to determine overlap of genes in either consolidation, retrieval or baseline groups. Genes that were found to be differentially expressed in males and females were divided into up and down-regulated groups.

PATHWAY ANALYSIS

Pathway and network analysis were performed on differentially expressed genes using Advaita Bio's iPathwayGuide (http://www.advaitabio.com/ipathwayguide). Top molecular pathways included up or down regulated differentially expressed genes. The underlying pathway topologies, comprised of genes and their directional interactions, are obtained from the KEGG database (Kanehisa et al., 2000; Kanehisa et al., 2010; Kanehisa et al., 2012; Kanehisa et al., 2014).

VOLCANO PLOTS

Volcano plots were generating using Advaita Bio's iPathwayGuide (http://www.advaitabio.com/ipathwayguide). All 84 significantly differentially expressed (DE) genes were represented in terms of their measured expression change (x-axis) and the significance of the change (y-axis). The significance was represented in terms of the negative log (base 10) of the p-value, so that more significant genes are plotted higher on the y-axis. The dotted lines represent the thresholds used to select the DE genes: 0.585 for expression change and 0.05 for significance.

HEATMAPS

Heatmaps were generated using Morpheus program from the Broad Institute (https://software.broadinstitute.org/GENE-E/). Genes included in the heatmaps were sorted from most differentially expressed (top) to least differentially expressed, including the top 20 up or down regulated genes for each particular comparison. The values included in the heatmap were Log2 of depth normalized with counts per million (CPM).

STATISTICAL ANALYSIS

Statistical analyses regarding freezing levels were conducted using SPSS v23. One-way ANOVA was used to determine sex differences in freezing during retrieval test.

Results

NO SEX DIFFERENCES IN CONTEXT FEAR CONDITIONING, LOCOMOTOR ACTIVIVITY OR SHOCK REACTIVITY

Males and females did not differ in locomotor activity prior to shock onset (Sex: $F_{(1, 13)} = 0.50$, $P = 0.495$) or in locomotor activity when the shock occurred (shock reactivity) (Sex: $F_{(1, 13)} = 0.37$, $P = 0.554$), suggesting that males and females similarly explored the context and felt the foot shock. On test day, males and females froze at similar levels (Sex: $F_{(1, 17)} = 0.003$, $P = 0.96$), suggesting that context fear was acquired to the same degree in both sexes.

DIFFERENT TRANSCRIPTIONAL PROFILES IN MALE AND FEMALE MICE AT BASELINE

We used RNA-Sequencing as an unbiased approach to identify biological correlates of sex differences at baseline. Differential expression of genes in ventral hippocampus were measured in naïve home cage control mice. A total of 84 genes were found to be differentially expressed between males and females at baseline. 86% (72) of these genes were upregulated in males compared with females (e.g. *Eif2s3y, Kdm5d, Shox2*) and the remaining 14% (12 genes) were upregulated in females (e.g. *Xist, Eif2s3x, Hmgcs, Fos, Fosb*) compared with males (Figure 5.2a). Examination of gene ontology of regulated transcripts reveal that 4 molecular pathways were differentially regulated by males and females at baseline (Figure 5.2b). These pathways included: vascular smooth muscle contraction, neuroactive ligand-receptor interaction, arrhythmogenic right ventricular cardiomyopathy, and prolactin signaling pathway.

DIFFERENT TRANSCRIPTIONAL PROFILES IN MALE AND FEMALE MICE FOLLOWING TRAINING (CONSOLIDATION) OF CONTEXT FEAR

During consolidation vs same sex naïve, a total of 68 genes were found to be differentially expressed in ventral hippocampus of males and females combined. Of the genes differentially expressed following consolidation, 51% (43 genes) were differentially expressed in

females compared with same sex naïve, leaving 49% (42 genes) in males, suggesting that consolidation of context fear resulted in a similar level of activation of transcriptional processes in males and females (Figure 5.3b). In males, 31% (13) of differentially expressed genes were upregulated following consolidation compared with naïve (e.g. *Arc*) (Figure 5.3d). In females, only 16% (7 genes) were upregulated (e.g. *Plekhf1*), whereas the remainder (84%) (36 genes) were downregulated compared with naïve (Figure 5.3d). 40% (17) of genes differentially expressed following consolidation in females overlapped with the same genes in males and in males 40% (17) of genes differentially expressed following consolidation also overlapped with the same genes in females (Figure 5.2b). Of the genes that were both differentially expressed by males and females none were regulated in opposite directions (e.g. upregulated: *Fos, Fosb, Egr1, Egr2, Egr4;* downregulated: *Gh, Aqp1, Folr1, Col8a1*) (Figure 5.2d). These data suggest that the same genes in males and females are not similarly or even oppositely expressed following consolidation, but that different molecular cascades are sex-specifically affected by consolidation of context fear. Examination of gene ontology of regulated transcripts reveal 4 enriched molecular pathways in males including: vascular smooth muscle contraction, osteoclast differentiation, amphetamine addiction, IL-17 signaling pathway and top 9 in females including: PI3-Akt signaling pathway, Jak-STAT signaling pathway, HTLV-infection, neuroactive ligand-receptor interaction, prolactin signaling pathway, amphetamine addiction, oxytocin signaling pathway, renin secretion, and IL-17 signaling pathway. Of these enriched molecular pathways in males and females, half (50%) of the top molecular pathways in males were also shared with females, and in females, 22% were shared with males; however, for one molecular pathway, different sets of genes were involved within the similar network (Figure 5.2e, 5f).

RETRIEVAL OF CONTEXT FEAR INDUCES SEX-SPECIFIC CHANGES IN VENTRAL HIPPOCAMPAL GENE EXPRESSION

Following retrieval vs same sex naïve, a total of 68 genes were found to be differentially expressed in ventral hippocampus of males and females combined. Of the genes differentially expressed following retrieval, 96% (135 genes) were differentially expressed in males compared with same sex naïve, leaving 4% (6 genes) in females, suggesting that retrieval of context fear resulted in a more active transcriptional process in males compared with females (Figure 2.4b). In females, 67% (4) of differentially expressed genes were upregulated following retrieval compared with naïve (e.g. *Sgk1*) (Figure 5.4d). In males, 9% (12 genes) were upregulated (e.g. *Egr1, Egr2, Egr4, Fosb*), whereas the remainder (91%) (123 genes) were downregulated compared with naïve (Figure 5.4d). 33% (2) of genes differentially expressed following retrieval in females overlapped with the same genes in males, whereas in males only 1% (2) of genes differentially expressed following retrieval overlapped with the same genes in females (Figure 5.4b). Of the genes that were both differentially expressed by males and females none were regulated in opposite directions (e.g. upregulated: *Fos;* downregulated: *Gh*) (Figure 5.4d). These data suggest that different molecular cascades may be sex-specifically affected by retrieval of context fear. Examination of gene ontology of regulated transcripts reveal 4 enriched molecular pathways in males including: vascular smooth muscle contraction, renin secretion, vascular smooth muscle contraction, and mineral absorption and top 4 in females including: PI3-Akt signaling pathway, Jak-STAT signaling pathway, neuroactive ligand-receptor interaction, prolactin signaling pathway. Of these enriched molecular pathways in males and females, none were regulated in both males and females (Figure 5.4e, 5f).

Discussion

Here we demonstrated that following consolidation or retrieval of context fear, males and females show different transcriptional regulation of ventral hippocampus, despite similar levels

of context exploration, reactivity to the shock and freezing to the context. Further, we demonstrate that transcriptional regulation of ventral hippocampus, a key brain region that is involved in mediating defensive and emotional responses associated with fear, differs in males and females at baseline. Together, these data highlight genes differentially expressed in ventral hippocampus of males and female following retrieval of context fear; such data provide novel insight into the molecular basis of retrieval and consolidation of context fear in males *and* females. Such findings may be used to inform sex-specific molecular pathways activated by retrieval and consolidation, information that would likely be useful for treatment of fear-related memory disorders such as PTSD.

SEX DIFFERENCES IN TRANSCRIPTIONAL PATHWAYS AT BASELINE

In addition to distinct transcriptional changes in males and females following retrieval and consolidation of context fear, we have determined sex differences in the transcriptional profile of ventral hippocampus at baseline in naïve control animals. A greater number of genes were observed to be more highly expressed in the ventral hippocampus of males compared with females and a smaller proportion of genes were found to be more highly expressed in females compared with males at baseline. Many of the genes that were differentially expressed between males and females at baseline were genes coding for sex chromosomes. These genes included X-specific transcripts such as *Xist* and *Eif2s3x* and Y-specific transcripts such as *Eif2s3y* and *Kdm5d*. The *Xist* and *Eif2s3x* genes were more highly expressed in the ventral hippocampus of females compared with males. The *Xist* gene is critical for transcriptional inactivation of one of two X-chromosomes in females. This process serves to provide dosage equivalence of the X chromosome between the sexes (Nguyen and Disteche, 2006). Inactivation of the X chromosome in females is critical and failure to inactivate it could lead to gonadal dysfunction and

abnormalities of physical features as in Turners Syndrome (Hong et al., 2014). Since the *Xist* gene is normally expressed in female and not male tissue, our observation of greater expression of this gene by females is expected and in line with previous research (Shinozaki et al., 1999; Dewing et al., 2003). Higher expression of the *Xist* gene has also been observed in the developing hippocampus of female mice vs male mice (Armoskus, 2014). Given the critical and sex-specific function of this gene, it is not surprising that we observe higher expression of the *Xist* gene in the ventral hippocampus of females that are ~9 weeks of age. The other X-linked gene more highly expressed in females is the *Eif2s3x* gene which encodes subunit three of eukaryotic translation initiation factor two which plays an important role in regulating the rate of protein translation. While the majority of genes on the inactive X-chromosome are silenced, around 3% of X-linked genes have been shown to escape X inactivation (Yang et al., 2010). *Eif2s3x* has been shown to escape X-inactivation (Wang et al., 2010; Xu et al., 2006). In agreement with our finding of higher expression of the *Eif2s3x* gene in females compared with males, other studies have also noted greater expression in female hippocampus (Armoskus et al., 2014; Xu et al., 2006). The Y-specific genes *Eif2s3y* and *Kdm5d* were more highly expressed in the ventral hippocampus of males compared with females. Similar to the X-linked *Eif2s3x*, *Eif2s3y* is a Y chromosomal homolog of *Eif2s3x* which encodes the subunit 3 of the translation elongation and initiation factor 2 (eIF2) and also plays a role in protein translation (Xu et al., 2006). Given that the *Eif2s3y* gene is Y-linked and plays a role in spermatogenesis (Ma et al., 2000; Yamauchi et al., 2009), our observation of greater expression of this gene in ventral hippocampus of males compared with females is expected. In line with our findings, many others have noted higher expression of the *Eif2s3y* gene in hippocampus of males compared with females (Xu et al., 2002, 2006; Armoskus, 2014). The other Y-specific gene *Kdm5d* codes for a

histone demethylase which demethylates di- and tri-methylated lysine 4 of histone H3 (H3K4). As with the other chromosome-linked genes, our finding of higher expression of the *Kdm5* gene in males compared with females is in agreement with other studies that note greater expression in male hippocampus (Xu et al., 2002; Armoskus, 2014). While our findings strongly replicate other studies indicating sex differences in transcription of genes located on sex chromosomes, we have also identified many other genes in ventral hippocampus that are differentially expressed between males and females at baseline.

One of the genes found to be more highly expressed in the ventral hippocampus of males compared with females was *short stature homeobox 2 (Shox2*). This gene codes for proteins which contain a 60-amino acid residue motif that represents a DNA-binding domain and these proteins serve as transcriptional regulators. One study has observed higher levels of the *Shox2* gene in the adipose tissue of men compared with women (Servera et al., 2014) but to our knowledge, the present study is the first to identify the *Shox2* gene as being differentially expressed in the ventral hippocampus of males and females. In males *Shox2* is critical for exploratory behavior and motor activity, as mutants of this gene exhibit deficits in these areas (Rosin et al., 2015); whether this gene serves a similar function in females is unknown. One of the genes found to be more highly expressed in the ventral hippocampus of females compared with males was *Hydroxymethylglutaryl-CoA synthase (Hmgcs*). This gene codes for an enzyme which serves as an intermediate in ketogenesis and cholesterol synthesis. One study has observed higher levels of the *Hmgcs* gene in the heart of female rats compared to males (Vijay et al., 2015) but to our knowledge, the present study is the first to identify the *Hmgcs* gene as being differentially expressed in the ventral hippocampus of males and females. Such diverse levels of expression of critical genes such as *Hmgcs* and *Shox2* in males and females likely result in

differential biological functions between the sexes, which may differentially affect behavior and learning and memory processes. Network analysis with differentially expressed genes in males and females at baseline demonstrated sex-specific molecular pathways such as enrichment of genes coding for members of the prolactin signaling pathway in females. An example of an enriched gene in this pathway more highly expressed in females was the gene that codes for prolactin. Prolactin is released from the pituitary and plays a critical role in many biological processes such as milk secretion (Horseman et al., 1997). In the hippocampus of OVX females prolactin has been shown to play a role in prevention of neuron loss as pre-treatment of Prl was protective against neuron loss induced by Kainic acid administration in CA1, CA3, and CA4 regions of the hippocampus (Tejadilla et al., 2010; Morales et al., 2014). In females post-treatment of subcutaneous prolactin following ICV administration of Kainic acid has also been shown to be protective against neuron loss induced by Kainic acid administration in CA1 region of the dorsal hippocampus and prevent cognitive deficits in novel object recognition (Reyes-mendoza and Morales, 2016). Given the critical role of prolactin in female maternal and biological functions, greater expression in ventral hippocampus of females compared with males is not surprising. However, its differential abundance and role in hippocampus of males and females likely results in sex-specific biological functions which may thereby affect memory processes. Surprisingly, we also observed sex differences in baseline expression of genes coding for immediate early genes *cFos* and *Fosb* in ventral hippocampus, with higher expression in females. Immediate early genes *cFos* and *Fosb* serve as markers of neuronal activity. If greater expression of *Fos* and *Fosb* results in greater expression of the cFos/Fosb protein in females, this may suggest greater overall recruitment of ventral hippocampus at baseline compared with males. Higher levels of cFos in control females would require an even greater number of cFos

positive cells in females compared with males after an experimental condition such as consolidation or retrieval of context fear to observe an increase from control. Our findings underscore the importance of normalization to control animals of the same sex, given strong differences in baseline levels of expression of key immediate early genes in ventral hippocampus. Here, we identified differential expression of genes in the ventral hippocampus of males and females at baseline previously observed to be differentially expressed between the sexes (sex chromosome-related genes), identified genes previously found to be differentially expressed in males and females in other tissues (i.e. *Shox2, Hmgcs*) and to our knowledge, are the first to observe sex differences in expression of genes coding for immediate early genes in ventral hippocampus. These findings are the first to characterize a distinct transcriptional profile in the ventral hippocampus of males and females at baseline.

TRANSCRIPTIONAL PATHWAYS IN MALES AND FEMALES DIFFER FOLLOWING CONSOLIDATION OF CONTEXT FEAR

Males and females exhibit distinct transcriptional changes after training or consolidation of context fear. Unlike following retrieval where a greater number of genes were differentially expressed in males compared with females, here, both males and females showed similar numbers of differentially expressed genes following consolidation of context fear, suggesting an equally active transcriptional response following consolidation of context fear by males and females. Many of the genes that were differentially expressed in both males and females following consolidation were immediate early genes such as: *cFos, early growth response protein 1, 2, and 4 (Egr1, Egr2, Egr4)* and *Fosb* which were more highly expressed compared with naïve, suggesting activation of ventral hippocampus by males and females following consolidation. Many of these genes have been previously shown to be upregulated following

memory acquisition or consolidation in males (Keeley, 2006; Barnes et al., 2012; Peixoto et al., 2015b). Here, our findings point to the ventral hippocampus as a region that is engaged in both males and females following consolidation of context fear. Another gene that was differentially expressed in ventral hippocampus of both males and females following consolidation was *growth hormone (gh)*, which resulted in lower expression compared with same sex naïve. Downregulation of growth hormone has been observed following chronic stress in male rats (Vander Weele et al., 2013). Given that footshock during fear conditioning is also likely to evoke a stress response, such a response may also trigger a reduction of genes encoding for *Gh*.

Examples of other genes that were differentially expressed in ventral hippocampus of both males and females following consolidation are *Aqp1, Folr1 and Col8a1*which resulted in lower expression compared with same sex naïve. These genes have been shown be regulated by the immune system (Rabolli et al., 2014; Schreiner et al., 2016). Additionally, *Aquaporin 1 (Aqp1)* is a member of the aquaporin family of water channels that are important for rapid transport of water across the plasma membrane; specifically, *Aqp1* aids in production of cerebrospinal fluid and is expressed at the apical and basolateral surfaces of the choroid plexus. Expression of the *Aqp1* gene has been shown to be decreased in the hippocampus of males following footshock (Cho et al., 2015; Mathew et al., 2016). The gene coding for *collagen type VIII alpha 1 chain (Col8a1)* is part of the Clq complement family and has been shown to be downregulated in the striatum of "losers" but not "winners" in a social defeat stress paradigm (Smagin et al., 2016), which may suggest that decreased expression of this gene by males and females in our paradigm may reflect an inherit stress response as a result of footshock, but additional research is needed to make this claim. Our findings highlight that following consolidation of context fear males and females show similar enhanced or reduced expression of

many genes in ventral hippocampus, suggesting that the transcriptional profiles are largely similar between the sexes.

Following consolidation of context fear, genes coding for the immediate early gene: *activity-regulated cytoskeleton-associated protein (Arc)* were found to be more highly expressed compared with naïve in males but not differentially expressed in females. *Arc* gene expression is responsive to broad neuronal activity such as hippocampal LTP (Link et al., 1995; Lyford et al., 1995). *Arc* differs from other immediate early genes such as the aforementioned *cFos* and *Egr's* because it's mRNA rapidly distributes throughout the dendrites soon after it is induced to reach regions that have received synaptic stimulation (Link et al., 1995; Lyford et al., 1995; Steward et al., 1998). In males, Arc in CA1 neurons has been shown to play an important role in spatial exploration (Guzowski et al., 1999) and was later shown to be critical for maintenance of LTP and consolidation of long term memory (Guzowski et al., 2000). In ventral and dorsal hippocampus, chronic restraint stress results in an increase in Arc in males (Pacheco et al., 2017), suggesting that *Arc* may be increased as a result of learning or stress exposure, both of which were likely to occur following footshock in our study. Our result of increased expression of the gene that codes for *Arc* during consolidation of context fear is in line with work depicting higher expression of Arc mRNA in the hippocampus of male mice during consolidation of context fear compared with controls (Wiltgen et al., 2010). Many studies have assessed the role of Arc in dorsal hippocampus during learning and memory processes using predominately male animals. Our data adds on to this work to show enhanced levels of *Arc* gene expression in the ventral hippocampus of males during consolidation of context fear. Further, our work reveals that *Arc* gene expression in ventral hippocampus is not similarly regulated in by males and females during consolidation.

During consolidation of context fear a number of genes were found to be differentially expressed in the ventral hippocampus of females but not males. One of the genes found to be more highly expressed in the ventral hippocampus of females following consolidation but not males was *Pleckstrin homology and FYVE domain containing 1 (Plekhf1)*. The *Plekhf1*gene was also found to be more highly expressed in the ventral hippocampus of females, but not males following retrieval. Information on the role of this gene in consolidation of memory is sparse; however, our findings of a female-specific increase in expression of this gene are line with previous reports showing greater expression of the *Plekhf1* gene in hippocampus and amygdala of females but not males following exposure to predator scent stress (Daskalakis et al., 2014). Expression of the *Plekhf1* gene has also been shown to enhance in the hippocampus following acute cort injection (Pulga et al., 2016). Given that *Plekhf1* has been shown to be regulated by stress, it is important to note that our observations of increased expression of the *Plekhf1* gene in females during consolidation may result from the consolidation process, a stress response following fear conditioning or a combination of the two. Nevertheless, our findings reveal a novel and sex-specific role of the *Plekhf1* in ventral hippocampus.

RETRIEVAL OF CONTEXT FEAR RESULTS IN DIFFERENT TRANSCRIPTIONAL PATHWAYS IN MALES AND FEMALES

A greater number of genes in males than in females were differentially expressed following retrieval compared with same sex naïve, suggesting a more active transcriptional response following retrieval of context fear in males compared with females. When comparing within sex, we noted that in males a smaller number of differentially expressed genes occurred following consolidation compared with naïve vs. retrieval compared with naive, whereas in females, a smaller number of differentially expressed genes were noted following retrieval,

suggesting that in males, a more active transcriptional response occurs following retrieval, whereas in females, a more active transcriptional response occurs in following consolidation. Unlike with consolidation, a small number of differentially expressed genes following retrieval in males overlapped with the same genes differentially expressed following retrieval in females.

Two genes were differentially expressed by males and females, one of which was *Gh (growth hormone)*, which resulted in lower expression compared with same sex naïve. Expression of this gene was also found to be reduced in both sexes following consolidation. This finding is in line with studies reporting similar levels of growth hormone-responsive cells in many brain regions in males and females, even those known to be sexually dimorphic (Furigo et al., 2017) as well as similar levels of growth hormone receptors in hippocampus (Bennett et al., 1997). Further, Gh-responsive cells are widely found to be distributed in many brain regions involved in regulation of emotional and cognitive function, including hippocampus (Furigo et al., 2017); while this study did not assess dorsal vs ventral hippocampus, another study noted a much greater density of growth hormone receptors in ventral, compared with dorsal hippocampus (Bennett et al., 1997). Given a critical role of growth hormone in memory and cognitive function (Carroll et al., 1998; Blackmore et al., 2012; Nyberg and Hallberg, 2013), and research showing that increasing Gh through a special "Gh-releasing diet" results in less impairments in context fear (Shin et al., 2008), is interesting that both sexes show a reduction, rather than an increase in expression following retrieval. However, a downregulation in growth hormone has been observed following chronic stress in male rats (Vander Weele et al., 2013). Given that retrieval of a fear memory is likely stressful, such a response may also trigger a reduction of genes encoding for *Gh*. Additionally, *Gh* gene transcription has been shown to be regulated by glucocorticoid stress hormones (Treacy et al., 1991). Recently, growth hormone in the amygdala

has been shown to increase the number of neurons activated during memory encoding and bias the allocation of neuronal activation, a potential mechanism of over-encoding (Gisabella et al., 2016). Therefore, it is possible that a reduction in Gh may be important in prevention of over-encoding. Such over-encoding can be maladaptive and related to persistently strong memories as in PTSD. Given the strong interconnection of amygdala with ventral hippocampus (Pitkanen et al., 2000; Kishi et al., 2006), it also remains possible that ventral hippocampus plays a role in the ability of Gh to influence allocation of neurons in amygdala during memory encoding. In human studies, a reduction of Gh levels have been observed in people with PTSD (van Liempt, 2012), further suggesting a role of Gh in fear-related memory. Our findings add on to the aforementioned studies to suggest that following retrieval of an emotionally-arousing context fear memory, genes coding for growth hormone in ventral hippocampus are similarly decreased in expression compared with naïve in males and females. Further, our studies point to the consistent decrease in expression of the gene that codes for growth hormone by males and females following both consolidation and retrieval of context fear.

The second gene that was common to both sexes was *Fos*, an immediate early gene which was more highly expressed following retrieval. Since *Fos*, a gene coding for cFos serves as a marker of neural activity; this finding is not at all surprising given the critical role of ventral hippocampus in defensive responses such as freezing (Zhang et al., 2001). Therefore, an increase in cFos would be expected if this region was active following retrieval in males and females. Using immunohistochemistry, we have recently reported an increase in the level of cFos+ cells in the dorsal hippocampus of males but not females following retrieval of context fear (Keiser et al., 2017) and in recent unpublished studies have demonstrated the opposite finding for ventral hippocampus, where an increase was observed in females but not males following retrieval.

Therefore, if females show higher levels of cFos+ cells at the protein level, regulation of translation or differences in time course of cFos activation between males and females may help to explain increased expression of the gene which codes for cFos in both sexes. Our findings point to the ventral hippocampus as a region that is engaged in both males and females following retrieval of context fear.

Network analysis with differentially expressed genes following retrieval in males and females demonstrated sex-specific molecular pathways such as enrichment of genes coding for members of the PI3-Akt signaling pathway in females. This pathway is known to play a key role in spatial learning (Mizuno et al., 2003) as well as fear memory (Lin et al., 2001; Chen et al., 2005). Akt, which is downstream of PI3K, plays a role in memory consolidation and LTP (Horwood et al., 2006). Here, we show that in ventral hippocampus this pathway may play an important role in females following retrieval as well. To our knowledge, no studies have assessed the role of this pathway in ventral hippocampus following retrieval of context fear in males and females. One of the genes found to be more highly expressed only in females following retrieval and enriched in the PI3-Akt signaling pathway was *serum and glucocorticoid-regulated kinase-1 (Sgk1)*. This gene encodes a serine/threonine protein kinase that plays a critical role in cellular stress response (Webster et al., 1993). While Akt is a key mediator of PI3K-dependent signaling, *Sgk1* also serves as a downstream effector of PI3K (Di Cristofano, 2017). *Sgk1* is phosphorylated by extracellular-signal regulated kinase (Erk), a kinase required for various memory processes (Feld et al., 2005; Schafe et al., 2008), and *Sgk1* directly phosphorylates cAMP responsive element binding protein (Creb), a critical transcription factor in learning and memory (Guzowski and McGaugh, 1997; Josselyn et al., 2001; Kida et al., 2002). Although the precise role of Sgk1 has not been established in retrieval of context fear memory, there is a sizeable body of evidence

implicating involvement of Sgk1 in spatial memory. For example, in male rats Sgk mRNA levels were approximately 4-fold higher in the CA1, CA3 and DG hippocampal sub regions of fast learners compared with slow learners in a water maze task (Tsai et al., 2002). Further, the authors go on to show that transfection of the *Sgk* mutant DNA to the CA1 region of the hippocampus results in impairments in in water maze performance (Tsai et al., 2002), suggesting a required role of hippocampal *Sgk* for spatial memory in male rats. Using a similar manipulation where *Sgk* mutant DNA was transfected to the CA1 region of the hippocampus of male rats, Lee et al., (2003) showed impairments in learning hippocampal-dependent tasks including Morris water maze, novel object recognition and context fear conditioning, suggesting that *Sgk* is critically involved in learning (Lee and Chao, 2003).

While these studies on *Sgk* inform a role for spatial learning in males, it's role following retrieval and how it's involvement may differ between males and females remains unknown. Our present studies add on to this data to show that the gene that codes for Sgk1 is more highly expressed in ventral hippocampus of female, but not male mice following retrieval of context fear compared with naive. Importantly, *Sgk1* was also found to be retrieval-specific, as it was not increased or decreased in either sex following learning of context fear.

Our findings of a female-specific increase in expression of the *Sgk1* gene is in line with previous reports showing greater expression of the *Sgk1* gene in hippocampus and amygdala of females but not males following exposure to predator scent stress (Daskalakis et al., 2014). Specifically, the authors reported only female rats that exhibited an *extreme* behavioral response to odor from a predator showed increased expression of the gene that codes for Sgk1 compared with control in hippocampus and amygdala (Daskalakis et al., 2014).

A main characteristic of PTSD is increased arousal or extreme behavioral responses when presented with threat (van der Kolk, 2000). Given the higher prevalence of PTSD in women compared with men (Kessler et al., 1995, 2012) and higher expression of the *Sgk1* gene in only females whom exhibited an extreme behavioral response (Daskalakis et al., 2014), *Sgk1* may serve as a female-specific mechanism that plays a role in fear memory. Given such findings, it is important to note that it is unclear if our finding of the female-specific increase in expression of the *Sgk1* gene is mediated by the act of retrieving the context fear memory or is the result of a stress response, serving as a by-product of retrieval. Nevertheless, our findings reveal a sex and retrieval-specific role of *Sgk1* in ventral hippocampus. SGK1 signaling pathway may serve as a female- specific mechanism activated by context fear memory retrieval.

Following retrieval of context fear, genes coding for immediate early genes: early growth response protein 1, 2, and 4 (*egr1, egr2, egr4*) and *fosb* were found to be more highly expressed compared with naïve in males but not differentially expressed in females. It is interesting to note that here, only males show increased expression of the Egr's as well as *Fosb*, whereas both males and females exhibited this increase following consolidation. These findings may suggest that activation of ventral hippocampus involves activation of similar immediate early genes following consolidation in males and females, but that retrieval involves sex-specific activation of molecular pathways, which include different immediate early genes. In line with this finding, recent work from our lab has shown sex differences in hippocampal immediate early gene activation following retrieval, but similar levels of immediate early gene activation between males and females following consolidation of context fear (Keiser et al., 2017). Our findings imply that ventral hippocampus is likely active in both sexes following retrieval of context fear, but in females this mechanism is likely independent of *Egr* and *Fosb* activation.

Other studies have noted regional and temporally distinct patterns within the Fos family which includes cFos and Fosb in striatal sub regions during and after chronic intravenous cocaine administration (Larson et al., 2010). In regards to Egr1, expression has been shown to correlate with the induction of hippocampal long-term potentiation (LTP) (Cole et al., 1989; Wisden et al., 1990; Worley et al., 1993) and has also been shown to increase following retrieval of context fear (Hall et al., 2001). However, all of these studies that have characterized a critical role of Egr1 in memory have been done in solely male animals. Therefore, it is likely in males, that retrieval of context fear involves engagement of early growth response proteins, whereas in females, retrieval may not result in activation of early growth response proteins. Females on the other hand are likely to engage Sgk1 following retrieval of context fear, whereas in males, retrieval may not result in activation of Sgk1. Our findings highlight novel relevance of *Sgk1* and PI3K/Akt signaling pathways as being differentially expressed in ventral hippocampus following retrieval of context fear in males and females. Such findings, may serve as a foundation for identification of female- specific and male- specific mechanisms by which context fear memories are retrieved.

CONCLUSION

Collectively, these data demonstrate a sex-specific transcriptional profile in ventral hippocampus following retrieval, during consolidation of context fear and at baseline. Our results indicate that a diverse transcriptional profile in males and females can still result in similar behavior between the sexes. Such findings highlight the idea that males and females may rely on differential hippocampal molecular mechanisms to reach similar behavioral outcomes. By adapting an unbiased approach that steps away from comparing females to established already established mechanisms in males, these results aid in identification of novel molecular

mechanisms that mediate retrieval and consolidation of context fear memory in females. This knowledge will help to understand *how* and by *which means* females consolidate and retrieve context fear. Understanding how gene expression is altered in males and females by retrieval and consolidation may be used to inform sex-specific molecular pathways which would likely be useful for treatment of fear-related memory disorders such as PTSD.

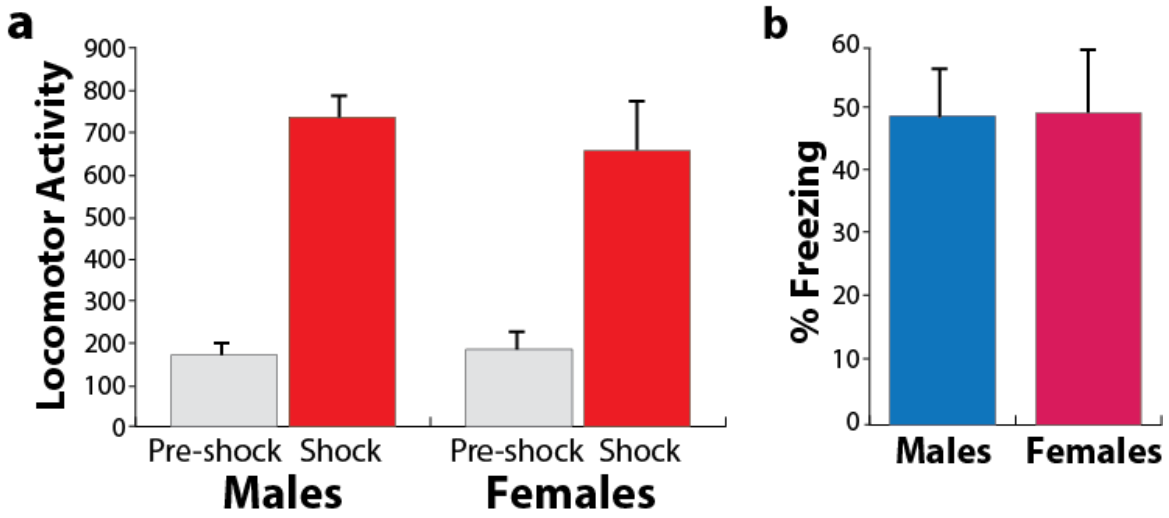

Figure 5.1. ***Males and females do not differ in pre-shock locomotor activity, shock reactivity or freezing on test day.*** (a) Males and females have similar levels of locomotor activity prior to shock onset (grey bars) and similarly increased levels of locomotor activity during shock onset (red bars). (b) Males and females show similar levels of freezing to the context during the retrieval test.

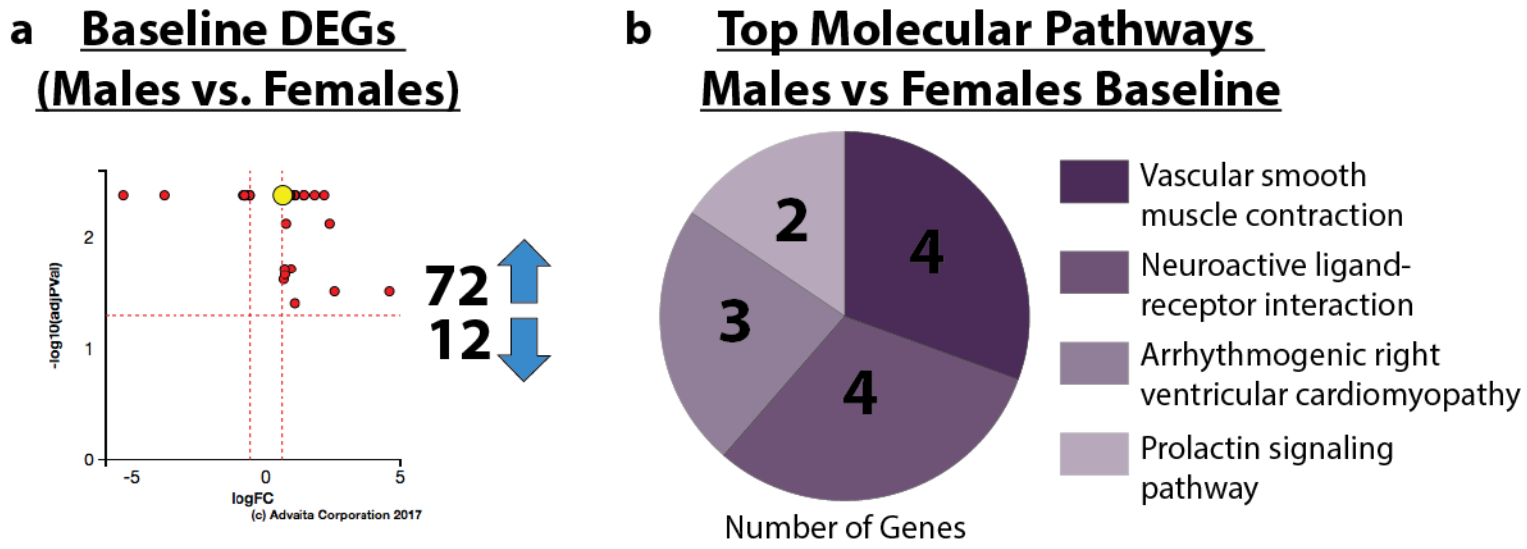

Figure 5.2. ***Sex differences in transcriptional pathways at baseline.*** (a) Volcano plot displaying the number of differentially expressed genes in ventral hippocampus in male naïve vs female naïve (−log10P-value versus log fold change) of normalized values. Upper arrow reflects genes more greatly expressed in males and downward arrow reflects genes more greatly expressed in females at baseline. (b) Top molecular pathways of differentially expressed genes in ventral hippocampus at baseline. Pathways displayed are made up of genes found to be differentially expressed by males and females at baseline (up or down regulated). Numbers in the pie chart reflect numbers of differentially expressed genes in that pathway.

Table 5.1. Top 20 genes with greater levels of expression at baseline in males vs females.

Gene name	Log fold change	*p* value	Gene name	Log fold change	*p* value
Female higher			Male higher		
Xist	- 5.312	0.004	*Eif2s3y*	4.602	0.031
Prl	- 3.806	0.004	*Kdm5d*	2.549	0.031
Adam8	- 0.840	0.004	*Uty*	2.370	0.008
Fos	- 0.799	0.004	*Tnnt1*	2.170	0.004
Gm7120	- 0.791	0.004	*Prkcd*	1.819	0.004
Dio3	- 0.788	0.004	*Ramp3*	1.438	0.004
Angptl4	- 0.766	0.004	*Tcf7l2*	1.421	0.004
Fosb	- 0.735	0.004	*Inadl*	1.072	0.004
Serpina3g	- 0.669	0.004	*Shox2*	1.053	0.039
Hmgcs2	- 0.630	0.004	*1500015ORik*	1.038	0.004
Eif2s3x	- 0.602	0.004	*Wfdc2*	0.922	0.004
Slc6a3	- 0.602	0.004	*Ninj2*	0.914	0.019
			Tnni1	0.904	0.004
			Plekhg1	0.895	0.004
			Tmem72	0.883	0.004
			Rgs16	0.881	0.004
			Opalin	0.880	0.004
			Serpinb1a	0.879	0.004
			Slc17a6	0.869	0.004
			Kcne2	0.843	0.004

Table 5.1. ***Differentially expressed genes between males and females at baseline.*** Table displaying the top 20 differentially expressed genes between males and females at baseline. To the left are genes with higher expression in females compared with males and to the right are genes with greater expression in males compared with females.

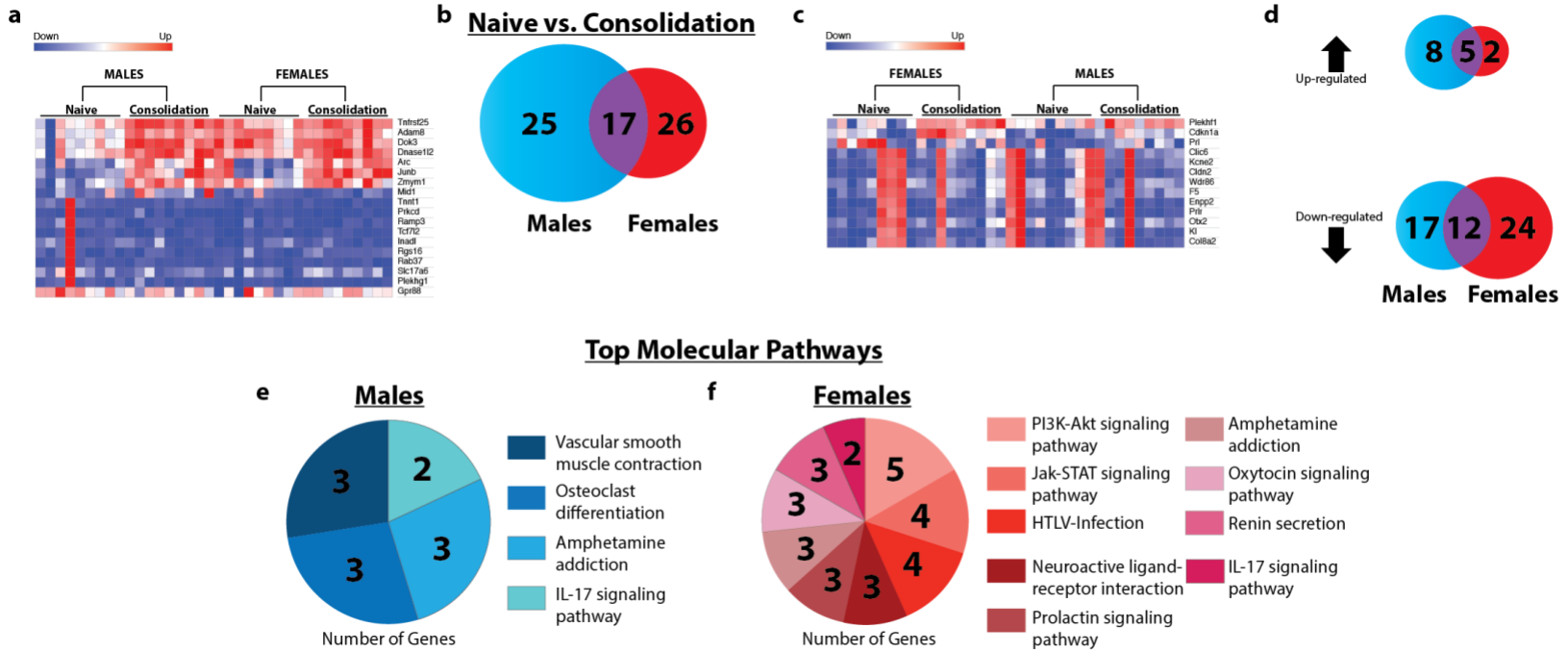

Figure 5.3. ***Transcriptional pathways in males and females differ following consolidation of context fear.*** (a) Heatmap of the top 20 genes differentially expressed (up or down) only in males during consolidation of context fear vs. male naïve compared with the same genes in females. (b) Venn diagram showing the number of differentially expressed genes in ventral hippocampus during consolidation vs same sex naïve in males and females. (c) Heatmap of the top 20 genes differentially expressed (up or down) only in females during consolidation of context fear vs. female naïve compared with the same genes in males. (d) Venn diagrams are broken down into genes with increased expression and decreased expression. (e-f) Top molecular pathways in males (e) and females (f) of differentially expressed genes in ventral hippocampus during consolidation vs. same sex naïve. Pathways displayed are made up of genes found to be differentially expressed in males and females during consolidation vs same sex naïve (up or down regulated). Numbers in the pie chart reflect numbers of differentially expressed genes in that pathway.

Table 5.2. Top 20 genes with greater or lower levels of expression following consolidation of context fear memory compared with same sex naive.

Gene name	Log fold change	*p* value	Gene name	Log fold change	*p* value
Female lower			Male lower		
Prl	- 2.976	0.004	*Tnnt1*	- 2.128	0.004
Gh	- 2.713	0.004	*Prkcd*	- 1.665	0.004
Aqp1	- 1.416	0.004	*Ramp3*	- 1.496	0.004
Wfdc2	- 1.318	0.004	*Tcf7l2*	- 1.279	0.004
Folr1	- 1.309	0.004	*Inadl*	- 1.023	0.004
1110059M19Rik	- 1.098	0.004	***Steap1***	- 0.993	0.004
Col8a1	- 1.087	0.004	*Rgs16*	- 0.966	0.004
Clic6	- 1.070	0.004	***Tmem72***	- 0.879	0.004
Kcne2	- 1.068	0.004	***Wfdc2***	- 0.869	0.024
Cldn2	- 1.043	0.004	***1110059M19Rik***	- 0.850	0.004
Wdr86	- 1.010	0.004	*Rab37*	- 0.788	0.004
1500015O10Rik	- 0.991	0.004	***Aqp1***	- 0.786	0.004
F5	- 0.990	0.004	*Slc17a6*	- 0.782	0.004
Enpp2	- 0.989	0.004	***1500015O10Rik***	- 0.752	0.004
Steap1	- 0.987	0.004	*Plekhg1*	- 0.744	0.004
Sostdc1	- 0.895	0.004	***Gh***	- 0.726	0.004
Prlr	- 0.825	0.004	***Slc4a5***	- 0.719	0.004
Otx2	- 0.815	0.004	*Gpr88*	- 0.710	0.004
Kl	- 0.799	0.004	***Folr1***	- 0.702	0.004
Col8a2	- 0.794	0.004	***Col8a1***	- 0.669	0.004
Female higher			Male higher		
Fos	1.360	0.004	***Fos***	1.896	0.004
Fosb	1.202	0.004	***Fosb***	1.723	0.004
Egr2	0.984	0.004	***Egr2***	1.133	0.004
Egr4	0.867	0.004	***Egr4***	1.016	0.004
Egr1	0.727	0.004	*Tnfrsf25*	0.896	0.004
Plekhf1	0.669	0.004	***Egr1***	0.823	0.004
Ccdc135	0.618	0.004	*Adam8*	0.783	0.004
			Dok3	0.716	0.004
			Dnase1l2	0.669	0.008
			Arc	0.651	0.004
			Junb	0.600	0.004
			Zmym1	0.590	0.004
			Mid1	0.586	0.004

Table 5.2. ***Differentially expressed genes during consolidation of context fear.*** Table displaying top 20 differentially expressed genes (up or down regulated) in ventral hippocampus of males and females during consolidation compared with same sex naïve. Bolded genes are differentially expressed in both males and females.

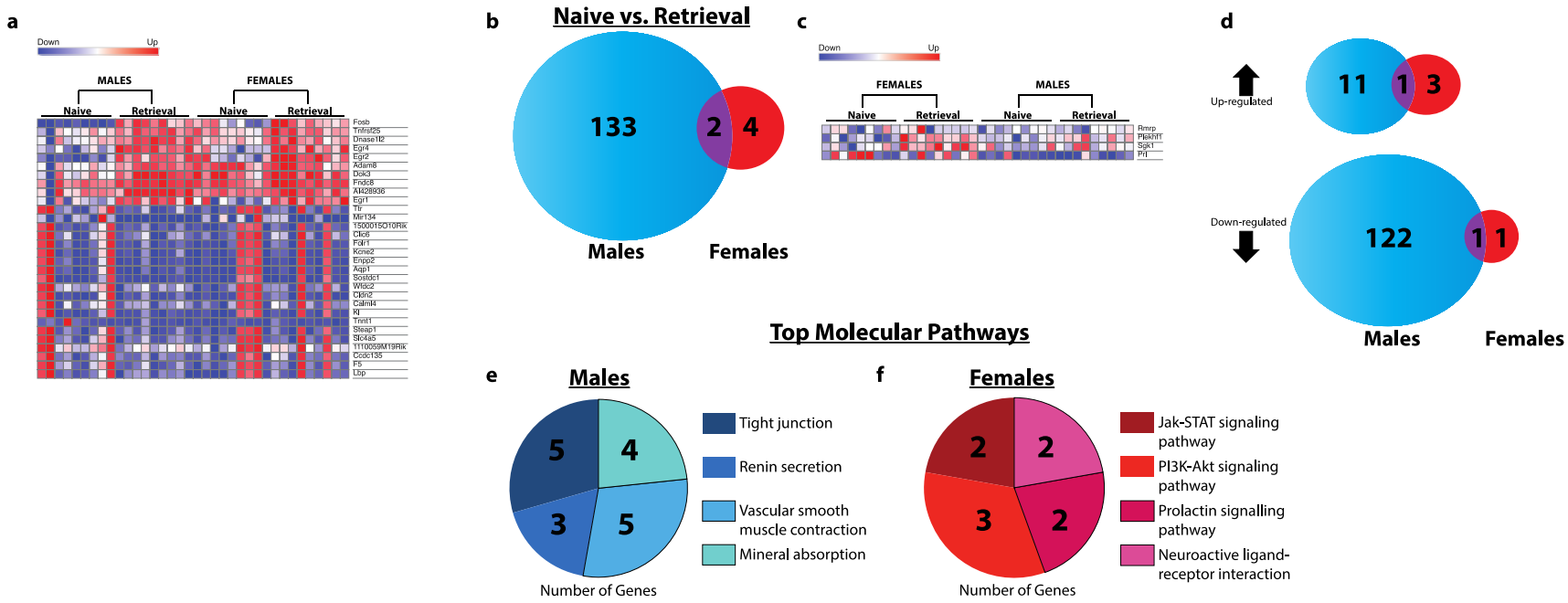

Figure 5.4. ***Retrieval of context fear results in different transcriptional responses in males and females.*** (a) Heatmap of the top 20 genes differentially expressed (up or down) only in males following retrieval of context fear vs. male naïve compared with the same genes in females. (b) Venn diagram showing the number of differentially expressed genes in ventral hippocampus following retrieval vs same sex naïve in males and females. (c) Heatmap of the top 20 genes differentially expressed (up or down) only in females following retrieval of context fear vs. female naïve compared with the same genes in males. (d) Venn diagrams are broken down into genes with increased expression and decreased expression. (e-f) Top molecular pathways in males (e) and females (f) of differentially expressed genes in ventral hippocampus following retrieval vs. same sex naïve. Pathways displayed are made up of genes found to be differentially expressed in males and females following retrieval vs same sex naïve (up or down regulated). Numbers in the pie chart reflect numbers of differentially expressed genes in that pathway.

Table 5.3. Top 20 genes with greater or lower levels of expression follwoing context fear memory retrieval compared with same sex naive.

Gene name	Log fold change	*p* value	Gene name	Log fold change	*p* value
Female lower			Male lower		
Prl	- 1.241	0.004	*Ttr*	- 4.992	0.004
Gh	- 0.912	0.004	*Mir134*	- 4.879	0.045
Female higher			*1500015O10Rik*	- 3.810	0.004
Rmrp	1.394	0.004	*Clic6*	- 3.183	0.004
Plekhf1	0.900	0.004	*Folr1*	- 3.134	0.004
Fos	0.862	0.004	*Kcne2*	- 2.886	0.004
Sgk1	0.617	0.004	*Enpp2*	- 2.747	0.004
			Aqp1	- 2.521	0.004
			Sostdc1	- 2.440	0.004
			Wfdc2	- 2.295	0.004
			Cldn2	- 2.203	0.004
			Calml4	- 2.144	0.004
			Kl	- 2.111	0.004
			Tnnt1	- 2.071	0.004
			Steap1	- 2.033	0.004
			Slc4a5	- 2.021	0.019
			1110059M19Rik	- 2.009	0.004
			Ccdc135	- 2.008	0.004
			F5	- 1.977	0.004
			Lbp	- 1.972	0.004
			Male higher		
			Fos	1.391	0.004
			Fosb	1.276	0.045
			Tnfrsf25	0.854	0.004
			Dnase1l2	0.768	0.004
			Egr4	0.746	0.004
			Egr2	0.736	0.004
			Adam8	0.724	0.004
			Dok3	0.707	0.004
			Fndc8	0.654	0.014
			AI428936	0.633	0.024
			Egr1	0.589	0.004

Table 5.3. ***Differentially expressed genes following retrieval of context fear.*** Table displaying top 20 differentially expressed genes (up or down regulated) in ventral hippocampus of males and females following retrieval compared with same sex naïve. Bolded genes are differentially expressed in both males and females.

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Chapter VI

Discussion

The studies presented in this dissertation identified sex differences in retrieval of context fear and examined the ways by which males and females differentially engage hippocampus. Throughout this dissertation I presented data illustrating a distinct behavioral phenotype in females compared with males when assessing retrieval of context fear, generalization, blocking and patterns of freezing during extinction. Whereas females differed from males both behaviorally and in patterns of immediate early gene activation, the specific mechanisms and intracellular signaling pathways by which they engaged to retrieve context fear still remain to be identified. In an effort to identify the pathways activated by retrieval of context fear in females and to step away from comparing female data to previously established male mechanisms, I use an unbiased approach with RNA-sequencing in chapter 5 to aid our understanding of the hippocampal molecular mechanisms activated by females and males following retrieval. Collectively, these data identified sex-specific molecular mechanisms associated with retrieval of a context fear memory and move the field closer from pointing out where males and females differ in learning and memory to understanding and defining *how* and *why*.

Synopsis

In chapter 2 I assess whether males and females differ in foreground context fear conditioning, generalization to a similar, safe context and determined the neural correlates of retrieval in males and females (Keiser et al., 2017). Despite strong fear conditioning in both sexes, females but not males exhibited generalized fear to the similar context. Generalization in

females was due to the similarity of the two contexts, as exposure to a non-similar context resulted in very low levels of freezing in females. Furthermore, we showed that generalization in females can be eliminated with pre-exposure to the context before it is paired with shock but not with pre-exposure to the similar context. Following retrieval of context-fear, female mice showed higher levels of cFos in basal amygdala and ventral hippocampus, whereas males showed greater levels in dorsal hippocampus (Keiser et al., 2017). In contrast, Arc levels were higher in dorsal hippocampus in females compared with males. These data demonstrate sex-specific molecular mechanisms as a consequence of context fear memory retrieval. Our data demonstrating differential activation of cFos across brain regions in males and females (Chapter 2) open the possibility that females use an amygdalar-dependent strategy in retrieval of context fear. Given competition between hippocampus and basal amygdala in context fear conditioning (Biedenkapp and Rudy, 2009) and the importance of basal amygdala in context fear conditioning (Matus-Amat et al., 2007; Amano et al., 2012; Jin and Maren, 2015), females might shift towards basal amygdala activation during memory retrieval. Sex-biased patterns of hippocampal and amygdalar mechanisms during retrieval may thereby result in greater generalization of context fear in females compared with males.

I show that males exhibit strong discrimination between a shock-paired and non-shock paired context the day following training, but in males, discrimination between these contexts diminishes as memories age and become remote, but freezing to the shock-paired context is not reduced and remains consistent (Wiltgen and Silva, 2007; Wiltgen et al., 2010). If males and females use different strategies to retrieve context fear memories during recent time points (chapter 2), there may also be differences in retrieval of remote context fear. In chapter 3 I examine sex differences in retrieval of remote context fear and determine neural correlates of

remote retrieval. Only females trained with background fear conditioning (tone), show reduced freezing to the context at remote time points; suggesting that the strategy used by females to learn background fear conditioning does not sustain them into remote retrieval of context fear. Females showed higher levels of cFos activation in ventral hippocampus, basal amygdala, and retrosplenial cortex after retrieval of background context fear memory compared with males. This pattern of activation is unlikely to explain sex differences in remote context memory retrieval, however, as this pattern was also observed after foreground context retrieval. Furthermore, we observed greater cFos activation in dorsal hippocampus during retrieval of foreground compared with background context fear conditioning in both sexes. It remains unclear whether the observed cFos activity is due to retrieval of context memory, or due to other context-exposure related memory processing. Given that the requirement of hippocampus and amygdala differs depending on whether fear conditioning is background or foreground (Kim and Fanselow, 1992; Phillips and Ledoux, 1994), and that we observe a sex-specific behavioral effect that is dependent on training protocol used, our findings may allude to the notion of sex-specific engagement or requirement of these structures during retrieval of context fear. Together, chapter 2 and 3 suggest that retrieval and its neural correlates differ between males and females at both recent and remote time points. Therefore, it is likely that males and females engage in distinct cognitive strategies to retrieve context fear. Importantly, sex differences observed in behavior do not appear to be driven by estrous phase in females during learning given similar levels of freezing in all phases. However, an effect of estrous is still possible if sample size was increased or females were tested in specific phases to enhance statistical power for examining estrous effects on freezing behavior.

One way to assess sex differences in strategy of retrieval is to examine whether the

context-shock association is similarly retrieved in both sexes. In chapter 4 I examined this using a blocking task and extinction paradigm. Despite strong context fear memory in both sexes, only males showed blocking to the tone. Data presented in chapter 4 showcase that males and females retrieve a strong context-shock association but may use a different strategy during retrieval in that males with context fear conditioning are prevented from further learning about tone, whereas fear conditioning in females may enhance new learning about the tone. Interestingly, neither sex showed blocking to the context when mice were trained with tone but a new context was presented. In extinction, sex differences in patterns of freezing were observed within session where females start off lower in freezing at the beginning of each session and show an increase in freezing throughout the session. These findings may imply a difference in strategy use during retrieval of context fear. Together, these data suggest that males and females may be retrieving qualitatively different context information or using the same information in different ways. Whereas chapters 2-4 provide data to suggest that females differ in hippocampal mechanisms normally activated by retrieval in males, it still remains unclear as to which mechanisms females engage to retrieve context fear memories that may be separate from males.

In chapter 5 I use RNA-sequencing as an unbiased approach to identify differential expression of genes in ventral hippocampus of males and females following retrieval and during consolidation of context fear compared with naïve. Despite similar behavior between the sexes during learning and retrieval, I identify a diverse transcriptional profile in ventral hippocampus of males and females following retrieval, during consolidation and at baseline. Given the lack of research assessing how females learn and retrieve fear memories, these data serve as an important first step in developing a foundational understanding of the mechanisms females engage.

Sex Differences in Behavioral Strategy During Retrieval of Context Fear

Despite the large number of studies on learning and memory, a very small percentage have included females (Milad and Quirk, 2012) and even fewer have used females to build up the framework for which theories on learning and memory have been established. Studies throughout this dissertation showcase that females do not fit with the male model, further speaking to a need for similar rigor that was used to develop an understanding of learning and memory in male animals, in females. I show that females are more prone to enhanced freezing in both a shock-paired context and a context that resembles this environment, while still showing low freezing to a distinct context (chapter 2). If discussed in line with previous studies in males, this finding would be interpreted to suggest that females are failing to develop a strong context-shock association or to accurately retrieve a representation of context. Although this possibility cannot be ruled out completely, our findings may instead indicate a more cautious-prone behavioral phenotype by females, where freezing in similar environments when presence of the shock is less certain may be evolutionarily beneficial for females but not males. In support of this idea, we found that females froze at higher levels to the context following context fear conditioning than did males suggesting that a context-shock association was adequately developed in both sexes, if anything, even more so in females. Second, pre-exposure of males and females to the context prior to context fear conditioning aided both sexes in different ways, decreasing freezing to the similar context in females but increasing freezing to the shock-paired context in males. That context pre-exposure aided males and females in some way suggests that instead of failing to retrieve or learn a context-shock association, males and females may instead be using different behavioral strategies to showcase this memory. Sex differences in fear expression have recently been outlined in a study by Gruene et al. (2015), where females but not

males show fear memory recall by presenting with an active, escape-like darting response following onset of a shock-paired tone, whereas males show a "typical" freezing response during tone onset (Gruene et al., 2015a). In the Gruene et al. (2015) study if only freezing in females were assessed, the authors may instead have concluded a failure of females to retrieve memory for the tone, when really, the *expression* of this memory took different forms in males and females, with females choosing a more active fear response and males a more passive "freezing" behavior. Therefore, failure to entertain the possibility of a similarly strong memory by males and females that results in different behavioral expression, may lead us to conclude that memory is somehow impaired, rather than indicating that it is simply *different*.

We report sex differences in remote context fear memory where females, but not males reduce their freezing to the shock-paired context when tested at a later, remote time (chapter 3). Again, if interpreted in line with previously established male findings, this may mean that memory for the context has diminished in females, while remaining consistently strong in males (Wiltgen and Silva, 2007; Wiltgen et al., 2010). Although in males, freezing to a shock-paired context is maintained when tested at a remote time (Wiltgen and Silva, 2007; Wiltgen et al., 2010), a reduction in freezing or ability to "forget" may be beneficial. As previously discussed, forgetting has been associated with an increase in hippocampal neurogenesis (Ishikawa et al., 2016), such an increase may be helpful if new learning is needed and an environment, or threats or cues within the environmental have changed, for an example. Therefore, in our studies, the ability to reduce freezing or "forget" may be differentially beneficial for females compared with males, where a reduction in freezing may allow for flexibility in behavior when environmental changes may have occurred whereas males may be better suited to maintain freezing. Although we cannot confirm these ideas with our present experiments, we cannot rule out the possibility of

sex-specific behavioral strategies and therefore should avoid comparing females to males when "typical" female behavior is not well defined or understood. Stepping away from asking where females differ from males to identifying how females learn and retrieve fear memories will help to address this issue. By consistently comparing females to males, we run risk to making the assumption that anything females display behaviorally is divergent from the norm (being males). Instead, research should seek to build a foundation that aims to define normal behavior in females in regards to context fear memory. Only with an established understanding of a behavioral phenotype in females, can we assess what is "impaired" or deficient.

I aim to examine sex differences in cognitive strategy by testing whether a context-shock association is similarly retrieved by males and females (chapter 4). First, we used a blocking task where we test how males and females use previously learned context-shock information when presented with a new predictor of the shock (tone). We find that behavior in males is consistent with the male blocking literature, where males block learning about the new cue (tone) (Kamin., 1969). Females, on the other hand, do not show blocking, a finding that again if interpreted in line with previous male literature on blocking would suggest that a context-shock association has not been adequately acquired in females and therefore prevented blocking, allowing for new learning to take place. Although this remains a possible explanation, female animals were not used in the studies that have aided in developing an interpretation of blocking (Kamin, 1969; Ayres et al., 1985; Iordanova, 2006), suggesting that in females, blocking learning context information may indicate something different from males showing blocking. In fact, there are many instances in the wild where a similar behavior in males and females is either performed for a different purpose and/or illicit different meanings. For an example a defensive behavior by females may be performed to ward off danger to her pups and a similar aggressive behavior by

males may be prompted to gain access to a female. Therefore, in our studies depending on the situation at hand, blocking in males may serve them better for their specific needs, whereas learning new information may better suit females. In our studies I argue that failure to show blocking to the tone cue in females is not the result of failure to adequately acquire a context-shock association. In support of this claim I show that one tone-shock pairing in control females (not receiving phase I training) was not enough to cause high freezing to the tone. Suggesting that if anything, prior context fear conditioning set the stage for *enhanced* learning of tone in females, implying that the interpretation of blocking in females likely differs from males. Additionally, results from chapter 2 (Keiser et al., 2017) show that a short pre-exposure time of 10 minutes' results in a strong context-shock association in females as evidenced by robust freezing to the shock-paired context and discrimination between the training context and a similar environment. Therefore, it is likely that retrieval of a *strong* context-shock association elicits different behavioral or cognitive strategies by males and females where females attend to new environmental cues and males focus on those that already predict danger. As with studies of spatial navigation where different navigational strategies are adapted by males and females to equally reach the same goal (Rodríguez et al., 2011; Bettis and Jacobs, 2013; Keeley et al., 2013; Shah et al., 2013), here it is likely that males and females use the same information differently to either block out or enhance learning a new predictor of shock.

Throughout my various assessments of male and female behavior during retrieval of fear-related memory, a similar pattern has emerged: females do not fit the established male-based paradigms. These data provide information that females trained with foreground (unsignaled) fear conditioning are more prone to higher freezing in shock-paired contexts, more likely to generalize fear to other environments, to decrease freezing to the context as more time passes,

are more likely to learn new cues when retrieving a context-shock association and to express freezing at different levels throughout extinction sessions compared with males. These behaviors in females may reflect a more cautious and hypervigilant strategy, where freezing in similar contexts, learning new predictors of shock in a fear-centered environment, and increasing freezing throughout an extinction session may prove to err on the side of caution and avoid certain risks. That males and females would be prone to different strategies when learning, retrieving and expressing memory would make sense given different types of threats and needs for each individual sex.

Although it is clear that these behavioral differences provide us with a large breadth of knowledge on where males and females differ, they fail to address *how* females engage in these different behaviors. The roadblock in preventing such an interpretation of our female-specific findings is the lack of knowledge on baseline information in females. Therefore, it will be critical to avoid assumptions that normal, baseline behavior in females will be equivalent to what is "normally" observed in males. Failure to include females in earlier studies that have laid the foundation for theories of classical conditioning are at large part to blame. To understand female baseline behavior requires a paradigm shift from asking "how are females different from males" to adoption of a female-focused approach which utilizes similar rigor to earlier studies in males.

It is critical to step towards developing a baseline understanding of learning and memory in females and to step away from a strictly male-comparative approach. Without a baseline understanding of female behavior and mechanism, we have nothing to compare and risk labeling anything that differs from males as abnormal. To step toward a foundational understanding requires determining appropriate assessments of behavior, applying a similar rigor used to develop context fear conditioning in males and taking a step back to ask what a certain behavior

means rather than coming in with expectation of that behavior, will be helpful in such assessments. Adapting this mindset will also be important when assessing behaviors in females that are similar to males as the reasons or strategies used to adapt similar behaviors may differ between the sexes. In order to step away from a male-comparative method when studying memory, we need to implement exploratory approaches that will identify female-specific mechanisms activated by memory processes. I take this approach using RNA-sequencing as an unbiased measure to examine differential expression of genes in the hippocampus of females and males following retrieval of context fear, during consolidation of context fear and at baseline (chapter 5). Importantly, use of this screening tool has pointed us towards molecular pathways previously unknown to play a role in retrieval and consolidation of context fear in both sexes.

Sex Differences in the Role of Hippocampus Following Retrieval of Context Fear

The role of hippocampus in regards to context fear conditioning has been well defined in males over many years. Specifically, the hippocampus has been shown to play a role in compiling distinct features of a fear conditioning chamber into a single representation of context (Rudy and O'Reilly, 1999; Reilly and Rudy, 2001; Matus-Amat et al., 2004). Context information from the hippocampus is then projected to the amygdala, which plays a role in regulating the emotional aspect in fear-related memories and is an important anatomical site of CS-US convergence (Phillips and Ledoux, 1992; Kim and Davis, 1993; Wilensky et al., 1999; Ledoux, 2000; Fanselow and Dale, 2003; Zelikowsky et al., 2014). The majority of research identifying the role of these structures in context fear conditioning has been done in male animals and has largely focused on learning or consolidation of fear conditioning. Throughout this dissertation I provide data showing that engagement and mechanism within hippocampus and amygdala differs to large degree during retrieval of context fear conditioning.

Sex Differences in Hippocampal Circuitry

Results from chapter 2 now show that dorsal hippocampus is more strongly recruited in males compared with females during retrieval of context fear at both recent and in chapter 3, remote time points. Given strong fear memory in females in both of these conditions, these results pave the way for many questions such as which brain regions females do engage and require and further, which neural circuits they rely on for retrieval of context fear memory. To address the first point, a consistent finding in our studies looking at retrieval of recent and remote context fear memory was that females show strong recruitment of basal amygdala and ventral hippocampus. These findings open the exciting possibility that females use an amygdalar-dependent strategy in retrieval of context fear, whereas males use a prominently hippocampal-based strategy.

Previous work states that hippocampus and basal amygdala compete in context fear conditioning (Biedenkapp and Rudy, 2009), given the necessity of basal amygdala in context fear conditioning (Matus-Amat et al., 2007; Amano et al., 2012; Jin and Maren, 2015), females may shift towards this process to retrieve context fear. Specifically, Biedenkapp and Rudy (2009) point to the basolateral amygdala as a hub to where both hippocampal and extrahippocampal systems compete for control over contextual fear in males. Further, they show that output provided by the ventral subiculum is required for the hippocampal system to win the competition with the extrahippocampal system. Although it is currently unknown whether males receive stronger output from ventral subiculm compared with females, this may be a mechanism by which males and females differ in retrieval of context fear. This mechanism may therfore allow females to circumvent strong activation of hippocamapal systems to support retrieval of context fear. Females, but not males may preferentially enagage or require the extrahippocampal

over the hippocampal system for amygdala output. Of course this idea still begs the question of which extrahippocampal brain regions females preferentially engage. Although there are few studies that have examined differntial requirement of brain regions in retrieval of context fear in males and females, medial prefrontal cortex (mPFC) may be a likely candidate. mPFC sends robust projections to the amygdala (Mcdonald et al., 1996). Sex differences in mPFC activation have been observed during extinction of context fear (Gruene et al., 2015b) and mPFC has been shown to modulate both hippocampus (Jin and Maren, 2015) and amygdala (Quirk et al., 2003; Sotres-Bayon et al., 2012) in context fear conditioning. Therefore, it is possible that instead of relying predominantly on dorsal hippocampus to retrieve context fear memories, females in our studies may instead preferentially engage mPFC-amygdalar circuitry during retrieval of context fear. If this is the case, failure to recruit dorsal hippocampus in females may not equate to impaired retrieval, as is normally the case in males.

This possibility of extrahippocampal regions playing a preferential role in retrieval of context fear in females may also be in line with our consistent finding of enhanced recruitment of both ventral hippocampus and basal amygdala in females during recent (chapter 2) and remote (chapter 3) time points. This is because projections from ventral hippocampus to mPFC have been shown to play a key role in long term storage of memories that are hippocampal-independent (Taylor et al., 2016). Whether this pathway is equally important for the transition from hippocampal-dependent to hippocampal-independent memories in females is unknown, but it is possible that in females, preferential engagement of ventral hippocampus during recent retrieval may aid them in systems consolidation. That females in our studies showed increased levels of cFos+ cells in ventral hippocampal sub regions and basal amygdala during recent and remote retrieval may suggest that females more strongly engage this pathway to retrieve context

fear memories. Our findings of strong recruitment of ventral hippocampus in females are also in line with studies showing that inactivation of hippocampus results in reduced expression of a generalized fear memory (Cullen et al., 2015); enhanced engagement of ventral hippocampal circuity by females may therefore associate with our observation of high generalization of context fear in females.

Our findings of enhanced recruitment of basal amygdala in females is also in line with many human studies that have observed sex differences in engagement of this region following exposure to emotionally arousing material (Cahill et al., 2001; Andreano et al., 2014). One such study observed that amygdala response was sustained in women but not men when exposure to negative material was familiar (Andreano et al., 2014). Further, women with more persistent amygdalar activation reported greater levels of negative effect. Given the critical role of amygdala in anxiety (Ressler, 2010) and fear (Dębiec and LeDoux, 2006), we cannot determine if enhanced amygdalar activation in females following retrieval of context fear is primarily due to the retrieval of the context-shock association or a by-product of retrieval such as a strong anxiety or fear response. Nevertheless, our findings suggest that in females either process may more strongly incorporate ventral hippocampal-amygdalar circuitry following retrieval of recent and remote context fear memories.

Sex Differences in the Role of Hippocampus in Remote Context Fear

During retrieval of context fear at a remote time point I observed enhanced recruitment of hippocampus in males and females compared with naïve. The idea that hippocampus is recruited during retrieval at a remote time point may come as a surprise given that as time passes and memories age, they are known to go through a process termed systems consolidation, where these memories are supported by more cortical regions, independent of the hippocampus (Kim

and Fanselow, 1992; Alvarez and Squire, 1994; Squire and Alvarez, 1995; Anagnostaras et al., 1999; Frankland et al., 2004; Squire et al., 2004; Wiltgen et al., 2004; Frankland and Bontempi, 2005; Sutherland and Lehmann, 2011; Wiltgen and Tanaka, 2013). However, while not required in retrieval of remote memory in males, studies have pointed to hippocampus as still being actively involved in this remote recall (Goshen et al., 2011). Goshen and colleagues (2011) used optogenetics to ensure temporally precise inhibition of CA1 region of hippocampus and observed impairments in remote context fear only initially, but with more time activity of cortical regions enhanced and memory was accurately recalled. These findings suggest that remote context fear memory retrieval naturally involves hippocampus in males, but that given the necessity and enough time, can switch to more cortical structures (Goshen et al., 2011). Given the lack of inclusion of females in studies aimed at assessing remote context fear memory, it is hard to gauge by which mechanisms or circuits females preferentially engage in this process. From our studies we can infer that given the strong recruitment of both basal amygdala and ventral CA1 region of the hippocampus, that females may preferentially engage or rely on the VHPC-BA circuit for retrieval of remote context fear memories. If as in males, hippocampus is involved, but not required for retrieval of remote context fear memories, then activation of this circuit may serve as a by-product of such retrieval driven by an anxiety or fear response. Nevertheless, it is clear from our studies that whether females are retrieving memories at a recent or remote time point, ventral hippocampus and amygdala are strongly engaged.

Role of Hippocampus in Background vs Foreground Fear Conditioning

In females, the degree of hippocampal engagement in context fear conditioning may differ depending on the type of fear conditioning (background or foreground) and result in differences in behavior. We demonstrate that females but not males trained with foreground

context fear conditioning generalize fear to a similar context and show less hippocampal recruitment compared with males (Keiser et al., 2017). Context fear conditioning is impaired following damage to hippocampus but can still be acquired with sufficient over-training given compensation by other cortical regions. However, earlier studies have suggested that over-training may not be sufficient to support learning about context fear in conditions where a tone was present in the context during training (background context fear conditioning) (Phillips and Ledoux, 1994); findings from this study suggest that background fear conditioning may more strongly recruit dorsal hippocampus.

An interesting observation from our studies in chapter 3 was that generalization of context fear was prevented in females that underwent background context fear conditioning (tone present), but not foreground context fear conditioning (tone absent). If dorsal hippocampus also plays a critical role in context discrimination in females as it does in males (Lynch et al., 2013) and if background context fear conditioning more strongly engages dorsal hippocampus in females (Phillips and Ledoux, 1994), this mechanism may explain why females in our studies discriminate between contexts if trained with background but not foreground fear conditioning. I also report that training with background but not foreground context fear conditioning often leads to reduced freezing at a remote compared with a recent time point in females but not males (chapter 3). Given that different neural circuity is involved in foreground vs background fear conditioning in males (Kim and Fanselow, 1992), it is possible that for females, differential underlying neural circuitry between the two types of training have lasting changes in behavior when tested for memory of context at a remote time point.

Sex Differences in Hippocampal Intracellular Signaling Pathways Activated Following Retrieval

Sex differences in hippocampal signaling mechanisms may also play a role in retrieval of

context fear. Although some studies have described sex differences in signaling mechanisms of dorsal hippocampus (for review see Mizuno and Giese, 2010; Keiser and Tronson, 2015), much less is understood about intracellular signaling mechanisms within ventral hippocampus in males or females. As described in chapters 2 and 3, ventral hippocampus is a region that appears to be required to retrieve context fear memories in males and females but that may be more strongly recruited in females at both recent and remote time points. Using RNA-sequencing of ventral hippocampal tissue, we aimed to get closer at understanding how intracellular signaling mechanisms may differ between the sexes by assessing differential expression of genes following retrieval. One of the genes found to be more highly expressed only in females following retrieval and enriched in the PI3-AKT signaling pathway was serum and glucocorticoid-regulated kinase-1 (*Sgk1*). Sgk1 receives upstream signals from important kinases involved in learning and memory such as extracellular-signal regulated kinase (ERK) and phosphoinositide-dependent kinase (PDK) and directly phosphorylates cAMP Responsive Element Binding Protein (CREB), a critical transcription factor in learning and memory (Guzowski and McGaugh, 1997; Josselyn et al., 2001; Kida et al., 2002). Studies have shown that following context fear conditioning, males but not females show significant phosphorylation of ERK in ventral hippocampus (Gresack et al., 2009). If activation of the ERK pathway is similar during retrieval of context fear, these findings may suggest that within ventral hippocampus females activate Sgk1 via PDK which may lead to phosphorylation of CREB. Future studies are needed to examine the necessity of this kinase and determine the specific signaling pathway involved but our studies make way for many candidate signaling pathways that could be driving activation of *Sgk1* in ventral hippocampus and be critical for retrieval of context fear in females.

Overall, studies in this dissertation suggest sex differences in retrieval of context fear and show that dorsal and ventral hippocampus are differentially recruited following retrieval but not consolidation. Ventral hippocampal sequencing data provides us with information on a multitude of signaling pathways likely to differ between males and females following retrieval that are worthy of further investigation.

When interpreting sex differences in the retrieval of context fear and the role of hippocampus it is important to consider from an ecological perspective, how these differences may be more beneficial to a specific sex. Engagement of a similar behavior by males and females may not indicate that the ethological *reason* for this behavior is also similar. Also vice versa, males and females may choose to engage in differential behavioral methods or strategies to reach a similar goal (Rodríguez et al., 2011; Bettis and Jacobs, 2013; Keeley et al., 2013; Shah et al., 2013). Throughout all of the studies presented here it is clear that both males and females are indeed retrieving a strong context fear memory but appear to be using different strategies in this process. Overall, females tend to err on the side of caution, freezing at high levels to contexts that resemble where they were shocked (Chapter 2), learning new predictors of shock when the context is already a strong predictor (Chapter 4) and increasing freezing within an extinction session even though overall freezing levels do decrease each day (Chapter 4). Use of different strategies by males and females may serve to meet needs that are more likely to be beneficial for that specific sex. Male and female mice live in large communal groups involving a dominant male, several females that will breed with the male and many subordinate males (Reimer and Petras, 1967; Bronson, 1979; for review see Lonstein and De Vries, 2000). Females therefore may provide more care for the offspring given the high percentage of subordinate males in a group and as a result, may be more likely to err on the side of caution and pay attention to

specific cues such as those that allow for protection of her pups. Although studies in a more naturalistic setting are needed to properly test this hypothesis, caution should be made against over-simplifying sex differences in behavior.

Future Directions

Studies throughout this dissertation suggest that males and females may rely on distinct hippocampal molecular mechanisms to retrieve context fear. Although I have identified ventral hippocampus as a region that appears to differ in level of recruitment following retrieval of context fear and demonstrate a sex-specific transcriptional profile in ventral hippocampus following retrieval, the precise neural circuitry and intracellular mechanisms that males and females require to retrieve these memories remain unidentified.

To test the hypothesis that the ventral hippocampal-amygdalar circuit is required for retrieval of context fear memories in females, DREADD technology could be coupled with a novel, retrogradely acting AAV viral vector (Tervo et al., 2016) to restrict viral expression to the ventral hippocampal-amygdalar circuit and inhibit this pathway to determine whether it is required for retrieval of context fear. A study using males classify the ventral hippocampal-BA pathway as being critical for retrieval of context fear while the ventral hippocampal-CEA pathway is critical for context-dependent cued fear memory retrieval (Xu et al., 2016). Given our observed sex differences in behavioral strategies of context fear retrieval, if females use a cue-based strategy, it is possible that females but not males rely on the ventral hippocampal-CEA pathway for retrieval of foreground context fear. Additionally, estradiol has been shown in a recent study to increase cFos expression in the CEA in females and was found to be correlated with enhanced extinction recall (Maeng et al., 2017).

To determine which intracellular signaling pathways are required for retrieval of context

fear I would aim to test the hypothesis that Sgk1 signaling pathway serves as a female- specific mechanism activated by context fear memory retrieval. Pharmacological or transgenic approaches could be used to assess whether inactivation of Sgk1 results in impaired retrieval of context fear in females or males. Through the use of immunohistochemistry and Western blotting we could identify signaling pathways up and downstream of Sgk1 that are activated following retrieval in female but not male ventral hippocampus. Upon identification of these pathways, cell-type specific DREADD approaches could be used to determine the intracellular signaling pathways females engage and require to retrieve context fear memories.

In addition to suggested approaches mentioned above, future research should also aim to move beyond assessing how males and females engage specific brain regions following retrieval of fear memory to examining how regions work *together* in retrieval. Examining patterns of activation of brain regions relative to one another could serve as a first step to this approach. Adaption of tracing studies, specifically technologies that allows for examining patterns of activation throughout the entire brain such as CLARITY (Chung and Deisseroth, 2013) will not only further our understanding of the dynamic interactions between structures in males and females, but will serve as innovative tools to discover novel mechanisms of learning and memory.

Conclusion

In conclusion, studies throughout this dissertation showcase that females do not fit with the male model; females are more prone to higher freezing in shock-paired contexts, more likely to generalize fear to other environments, to decrease freezing to the context as more time passes, are more likely to learn new cues when retrieving a context-shock association and to express freezing at different levels throughout extinction sessions compared with males. Females also

recruited different brain regions following retrieval of recent and remote context fear, particularly ventral hippocampus and amygdala, whereas males engaged dorsal hippocampus. Observation of differential expression of genes in ventral hippocampus in males and females provides novel insight into the molecular pathways each sex may engage following retrieval. These data further speak to a need for similar rigor that was used to develop an understanding of learning and memory in male animals, in females. Collectively, these data determine sex-specific mechanisms associated with retrieval of a context fear memory and move the field closer from pointing out where males and females differ in learning and memory to understanding and defining *how* and *why*.

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