August 2017

Sex Differences in Differential Fear Conditioning During the Acquisition and Consolidation of Learned Safety

David Sylvan Reis
University of Wisconsin-Milwaukee

SEX DIFFERENCES IN DIFFERENTIAL FEAR CONDITIONING DURING THE ACQUISITION AND CONSOLIDATION OF LEARNED SAFETY

by

David S. Reis

A Dissertation Submitted in

Partial Fulfillment of the

Requirements for the Degree of

Doctor of Philosophy

in Psychology

at

The University of Wisconsin-Milwaukee

August 2017

ABSTRACT

SEX DIFFERENCES IN DIFFERENTIAL FEAR CONDITIONING DURING THE ACQUISITION AND CONSOLIDATION OF LEARNED SAFETY

by

David S. Reis

The University of Wisconsin-Milwaukee, 2017
Under the Supervision of Professor Fred J. Helmstetter

The ability to distinguish between threatening and non-threatening situations requires careful regulation of behavioral and physiological responses to stress and fear. Deficits in fear regulation are maladaptive and can lead to the development of anxiety disorders such as PTSD. Women are nearly twice as likely to develop PTSD as are men and laboratory animal studies have shown facilitated fear acquisition, resistance to fear extinction, deficits in extinction retention and impaired discrimination between danger and safety cues in females. Taken together this suggests a propensity for reduced inhibitory control over fear responding in females. Here we investigate the mechanisms underlying fear discrimination deficits in females using an auditory differential fear conditioning procedure. Our results suggest that fear discrimination depends on successful memory consolidation of the excitatory fear signal as well as the inhibitory safety signal. Female but not male rats showed indiscriminate fear responding to both the fear and safety cue and this may be due to impairments in learned safety by female rats. Moreover, CS- retrieval in males but not females was sufficient to destabilize synapses encoding the CS+ memory trace. Together these data suggest that sex differences in the discrimination of fear and safety may be the result of deficits in the consolidation of learned safety in females and further supports the idea that deficits in fear regulation underlie the increased risk of PTSD in female.

DEDICATION

I dedicate my dissertation to my parents, Merlin and Ann Reis. I will never be able to thank you enough for the tremendous support you have given me throughout the years. The sacrifices you have made, your words of encouragement and your unrelenting optimism have helped guide me through some of the most difficult moments of my life and for that, I am eternally grateful. This is a great accomplishment as much for me as it is for you both. I would also like to recognize my brother, Jake, and my sister, Miranda for always having my back and for never failing to provide comedic relief during some of the most stressful times. Lastly, I would like to thank my fellow lab members Nicole Ferrara (Clark) and Shane Pullins for their words of wisdom, technical advice and incredible friendship during this project and throughout my graduate school career.

TABLE OF CONTENTS

LIST OF ABBREVIATIONS

ADFC – auditory differential fear conditioning

AMPA(R) – α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (receptor)

ANI – anisomycin

BLA – basolateral region of the amygdala

CaMKII – calcium calmodulin dependent protein kinase II

CBP – CREB binding protein

CeA – central nucleus of the amygdala

CREB – cAMP response element-binding protein

CS – conditioned stimulus

ERα – estrogen receptor α

ERβ – estrogen receptor β

GABA – γ-Aminobutyric acid

GAD – generalized anxiety disorder

GDX – gonadectomized

GKAP – guanylate kinase-associated protein

Grin1 – Glutamate [NMDA] receptor subunit zeta-1

IL – infralimbic region of mPFC

ITC – intercalated cells of the amygdala

K48 – lysine residue 48 linked

LA – lateral nucleus of the amygdala

LTD – long-term depression

LTP – long-term potentiation

MAPK – mitogen-activated protein kinase

mPFC – medial prefrontal cortex

mTOR – mammalian target of rapamycin

NMDA – N-methyl-D-aspartate

OVX – ovariectomized

PKA – protein kinase A

PKC – protein kinase C

PL – prelimbic region of mPFC

PreX – tone preexposure control rats

PTSD – post traumatic stress disorder

SFP – stimulus free period

UCS – unconditioned stimulus

UPS – ubiquitin proteasome system

β-LAC – clasto-Lactacystin β-lactone

SEX DIFFERENCES IN DIFFERENTIAL FEAR CONDITIONING DURING THE ACQUISITION AND CONSOLIDATION OF LEARNED SAFETY

Threat assessment is an important process that allows for successful responses to changing and unpredictable situations. This process depends on the accurate prediction of aversive outcomes, using information from familiar stimulus-outcome relationships and available environmental cues, to direct behavioral responses to novel and potentially threatening stimuli. Appropriate fear responding to situations of safety and danger depends on the carefully regulated balance between fear discrimination and generalization. Over-generalization of fear to neutral stimuli or the inability to inhibit fear responding in situations of safety can be physically and psychologically debilitating and are typifying symptoms of disorders like generalized anxiety disorder (GAD) and post-traumatic stress disorder (PTSD), respectively (Reinecke *et al.*, 2010; Mahan & Ressler, 2012; Rauch et al., 2006; Lissek et al., 2010; Jovanovic et al., 2010, 2012; Levy-Gigi et al., 2012).

Pavlovian Fear Conditioning

Our understanding of the neurobiological mechanisms underlying some anxiety disorders, and specifically deficits in fear inhibition, have benefited greatly from the use of Pavlovian fear conditioning procedures. In classical fear conditioning, a neutral conditioned stimulus (CS) is paired with an aversive unconditioned stimulus (UCS) such as a footshock. Repeated CS-UCS pairings produce an associative fear memory controlled by the CS, such that exposure to the CS alone can elicit various behavioral and physiological defensive responses including analgesia, freezing behavior and other autonomic responses (MacLennan, Jackson, & Maier, 1980; LeDoux, Cicchetti, & Reis, 1988; Fanselow, 1990, Helmstetter, 1992). Notably,

conditioned fear can be suppressed or inhibited through subsequent non-reinforced presentations of the CS in a process called fear extinction. Moreover, a CS associated with the absence of an aversive outcome can also inhibit fear responses, as in auditory differential fear conditioning (ADFC) in which one stimulus (CS+) is paired with an aversive footshock while another stimulus (CS-) is associated with the absence of footshock delivery. Several studies have demonstrated that fear extinction represents a novel, active learning experience (Bouton et al., 2006; Myers & Davis, 2007; Milad & Quirk, 2012; Maren, 2015). As a result, inhibition of conditioned fear by extinction results from competition for behavioral expression of fear between coexisting extinction and fear memories (Bouton, 2004). Like fear extinction, retrieval of safety memory has anxiolytic effects on behavior (Gewirtz *et al.*, 1997; Gillion and Ameli, 2001).

Conditioned safety signals are generally considered to be examples of conditioned inhibitors; a term that describes the ability of the safety signal to inhibit conditioned responding to a CS+ (Rescorla, 1969; Rogan *et al*., 2005). The effectiveness of a CS- as a conditioned inhibitor can be determined through tests of retardation and summation (Rescorla, 1971). In a summation test, the CS- is presented in compound with the CS+ and should result in the suppression of conditioned responses elicited by the CS+. Here the sum of the excitatory properties of the CS+ and the inhibitory properties of the CS- result in this suppression of fear. On the other hand, a retardation test assesses the degree to which the rate of excitatory fear conditioning is acquired if a previously conditioned inhibitor or safety signal is now paired with an aversive stimulus. Previously learned safety signals will be significantly delayed, or retarded, in the rate of acquisition of excitatory fear conditioning (Rescorla, 1971). Together, these tests can assess whether a prior negative relationship between a CS- and UCS retards subsequent acquisition of the conditioned behavioral response to the same CS and whether the summation of

CS- inhibitory properties with CS+ excitatory properties reduces conditioned responding (Rescorla, 1971). In support, anxiolytic properties of conditioned safety signals have been demonstrated in an anxiogenic environment such as an elevated plus maze where onset of a conditioned safety signal increased exploration in the open-arms and decreased closed-arm entries (Pollack *et al.,* 2008; Walf & Frye, 2013).

Deficits in the ability to appropriately inhibit fear is a primary characteristic of anxiety disorders. Specifically, patients with PTSD show enhanced acquisition of fear, increased resistance to extinction, and deficits in the ability to learn safety (Mahan & Ressler, 2012; Jovanovic et al., 2010, 2012). Interestingly, accumulating evidence suggests that females are twice as likely to develop PTSD as males and that this may be related to sex-specific deficits in the ability to appropriately inhibit fear responses. For example, in several studies females show enhanced acquisition of cued fear, increased resistance to fear extinction (Baran *et al.*, 2009; Gresack *et al.*, 2009; Baker-Andresen *et al.*, 2013; Matsuda *et al.*, 2015), overgeneralization of fear, as well as deficits in safety learning compared to males (Day, Reed, and Stevenson, 2016). It is possible that the pattern of sex differences in fear learning may be dependent on the stimulus modality. For example, some studies have found enhanced freezing to an auditory cue in females compared to males while at the same time demonstrating deficits in the opposite direction in contextual fear (Gresack *et al.*, 2009; Ribieiro *et al.*, 2010). Collectively these studies point to potentially large sex differences in the regulation of fear learning and expression. Whether sexual dimorphism in the neural circuitry or differing molecular mechanisms supporting fear learning in males and females is responsible for the sex differences in aversive learning remain unclear.

Neural Circuitry of Fear Conditioning

In males and females, associative fear learning is supported by an evolutionarily conserved neural circuit, consisting of several cortico-limbic brain regions including the amygdala, hippocampus and prefrontal cortex (Izquierdo, Furini, and Myskiw, 2016; Beyeler, Eckhardt, and Tye, 2014; Zelikowsky *et al.* 2014; Moustafa *et al.,* 2013). Potential sex differences fear and safety learning may be related to the degree of involvement of given brain regions during a given variation of fear conditioning.

A large body of evidence points to the amygdala as the primary site in regulation of fear learning (Fendt and Fanselow; 1999, Lavond et al., 1993; McGaugh, 2004). Anatomically, the amygdala receives sensory inputs from diverse brain regions, like the thalamus, cerebral cortex, and hippocampus. The amygdala also sends projections to various structures that mediate a variety of different fear responses (LeDoux, 1996; LaLumiere, 2014). In general, sensory inputs converge in the basal and lateral nuclei of the amygdala (BLA; Aggleton, 2000; LeDoux, 1996; Pape & Pare, 2010). In fear conditioning, the BLA plays a critical role in the formation of the CS–UCS association and is critically involved in fear memory storage. The BLA is also interconnected with the central nucleus of the amygdala (CeA), which represents the main output region of the amygdala. Specifically, the CeA sends projections to various autonomic regions that contribute to the expression of specific fear responses.

Indeed, amygdala activity and synaptic plasticity is necessary for both the formation and retrieval of fear memories (Fanselow and LeDoux, 1999; Wilensky *et al.,* 2006; Helmstetter *et al.,* 2008; Pape and Pare, 2010). Several early studies showed that neurotoxic lesions of the amygdala severely impair the formation of auditory and contextual fear memories (Helmstetter, 1992; Maren, 1999). Moreover, transient inactivation of the amygdala with the γ-Aminobutyric

acid (GABA) receptor agonist, muscimol, before auditory fear conditioning significantly impairs the formation of long-term fear memory (Helmstetter & Bellgowan, 1994; Wilensky *et al.*, 1999)

Like fear memories, memories of conditioned safety depend on appropriate levels of amygdala activity though with contrasting patterns (LeDoux *et al.*, 2000; Rogan *et al.*, 2005; Belova *et al.*, 2007; Shabel and Janak, 2009; Ostroff *et al.*, 2010; Sangha, Chadick and Janak, 2013; Thomas *et al.*, 2013). The attenuating effect of conditioned safety on amygdala activity and fear expression suggests some degree of overlap with the neural circuitry underlying fear conditioning; specifically the amygdala (Davis & Whalen, 2001; LeDoux, 2000; Heldt and Falls, 2006). While fear responses are associated with activation of the LA, other studies have shown that CS-evoked responses in the LA of the mouse significantly decreased after safety conditioning (LeDoux, 2000; Rogan *et al.*, 2005). Others have reported decreased amygdala responses to an auditory CS- following discriminative training in the cat (Collins & Pare, 2000). Moreover, *in vitro* studies have associated amygdala long-term depression (LTD) with reductions of conditioned fear responses (Wang & Gean, 1999). In fact, *in vivo* conditioned fear can be reduced with the same low-frequency stimulation used to induce amygdala LTD *in vitro* (Lin *et al.*, 2003).

The dependence of accurate threat assessment on previous stimulus-outcome associations indicates the necessity for successful formation and storage of fear and safety memories. However, with competitive and contrasting influence on amygdala activation, the specific involvement of the amygdala in the simultaneous consolidation of cued fear and safety memories remains unknown. Much of what we know about memory consolidation comes from studies of fear conditioning as opposed to other forms associative learning.

Memory Consolidation and Reconsolidation

Fear memory consolidation, or the transfer of memories from short-term (STM) to long-term (LTM), relies on the formation and stabilization of synaptic connections within the amygdala during and immediately following fear conditioning (Jarome & Helmstetter, 2013). In general, consolidation is initiated through an NMDA receptor dependent increase in intracellular calcium in the amygdala (Rodrigues, Schafe & LeDoux, 2001). This influx of calcium into excitatory glutamatergic neurons causes subsequent activation of several intracellular signaling pathways involved in the consolidation process. For example, activity-driven calcium influx results in the autophosphorylation of calcium calmodulin dependent protein kinase II (CaMKII) which significantly contributes to memory stabilization during consolidation (Rodrigues *et al.*, 2004, Johansen *et al.*, 2011; Jarome *et al.*, 2013; Jarome *et al.*, 2016).

In addition, protein kinase A (PKA), mitogen-activated protein kinase (MAPK) and protein kinase C (PKC) are also involved in the memory consolidation process (Abel *et al.*, 1997; Schafe & LeDoux, 2000; Adams & Sweatt, 2002). Intra-amygdala blockade of any of these molecules significantly impairs phosphorylation of the transcription factor CREB consequently disrupting long term fear memory formation (Dash *et al.*, 1990; Bourtchuladze *et al.*, 1994; Yin *et al.*, 1994; Josselyn *et al.*, 2001; Kida *et al.*, 2002; Pittenger *et al.*, 2002). Other intracellular signaling pathways, including protein degradation through the ubiquitin proteasome system (UPS), *de novo* protein synthesis, and mRNA transcription are necessary for memory consolidation following a number of different behavioral paradigms indicating that these may represent general mechanisms of memory consolidation including conditioned safety (Lopez-Salon *et al.*, 2001; Jarome *et al.*, 2011; Reis et al., 2013; For further review see Jarome & Helmstetter, 2014 or Rosenberg *et al.*, 2014).

Recent work has suggested that protein degradation through the UPS is a primary contributor to the dynamic nature of synaptic stability. In the UPS, proteins are targeted for degradation through the covalent attachment of a degradation specific polyubiquitin chain consisting of at least 3 ubiquitin moieties bound together at the Lys-48(K48) residue of each ubiquitin protein. Proteins covalently modified with K48-linked polyubiquitin chains are subsequently targeted by the 26S proteasomal complex for degradation. Previous work from our lab identified learning specific NMDA-dependent increases in protein degradation as well as increased degradation-specific targeting of synaptic scaffolding proteins (Jarome *et al.*, 2011).

The 26s proteasome consists of a catalytic core (20S) and two regulatory particles (19S). Proteolytic activity is mediated, in part, through the phosphorylation of Rpt6, one of 6 ATPase subunits contained in each of the 19S regulatory caps (Bedford, Paine, Sheppard, Mayer & Roelofs, 2010). Phosphorylation of Rpt6 occurs in a CAMKII-dependent manner and blockade of NMDA receptor activity attenuates activity-dependent increases in proteasome activity, degradation specific polyubiquitination and synaptic proteasome abundance (Bingol & Schuman, 2006, Jarome *et al.*, 2011).

During initial memory consolidation, UPS-mediated proteolysis primes synapses for activity-induced *de novo* protein synthesis, which allows for the stabilization of new synaptic connections (Banarjee, Neveu & Kosik, 2009; Dong *et al.*, 2014; Reis *et al.*, in prep). Inhibition of protein synthesis in the amygdala with anisomycin, or specific inhibition of the mammalian target of rapamycin (mTOR) translation control pathway, significantly impairs conditioned fear responding to an auditory cue or training context 24 hrs after training (Schafe & LeDoux, 2000, Parsons, Gafford & Helmstetter, 2006b; Kwapis *et al.*, 2011; Jarome *et al.*, 2011).

Dong and colleagues (2014) found that proteasome inhibition prior to early-phase long-term potentiation (E-LTP) in hippocampal slices enhanced LTP induction and resulted in the accumulation of translation promoting proteins. During the late-phase of LTP, proteasome inhibition prevents L-LTP maintenance, results in the accumulation of translation repressor proteins (Dong *et al.*, 2014) and disrupts reorganization of the post-synaptic density (PSD; Ehlers, 2003). Moreover, *in vivo* blockade of UPS-mediated proteolysis in the amygdala prevents activity-dependent degradation of translation repressor proteins, attenuates *de novo* protein synthesis and impairs auditory fear memory consolidation (Jarome *et al.*, 2011; Reis *et al.*, 2017). Interestingly, simultaneous inhibition of UPS-mediated proteolysis and protein synthesis during L-LTP rescues the deficit of LTP that would normally occur by blocking either one of these mechanisms individually (Fonseca et al., 2006).

Retrieval of fear memory results in the transient destabilization of amygdala synapses that were initially stabilized during memory consolidation. This period of memory destabilization after retrieval is sensitive to several pharmacological manipulations that disrupt subsequent protein-synthesis dependent synaptic restabilization, a process known as reconsolidation (Nader, Schafe & LeDoux, 2000; Parsons *et al.*, 2006a). In support of this idea, pre-retrieval infusions of the protein synthesis inhibitor anisomycin into the amygdala impair reconsolidation of auditory fear memory shown by a significant reduction in freezing to the CS 24 hrs after retrieval (Nader, Schafe & LeDoux, 2000). Memory retrieval is a key requirement for this destabilization process. Retrieval of fear memory results in increased degradation specific, NMDA-dependent polyubiquitination, proteasome activity, and AMPA receptor trafficking in the amygdala and hippocampus (Jarome *et al*., 2011; Lopez *et al.*, 2015). Moreover, this is accompanied by increased proteasomal targeting of synaptic scaffolding

proteins like GKAP and Shank, which form receptor complexes in the post synaptic density to hold receptors in place, as well as AMPA receptor endocytosis (Lee *et al.*, 2008; Jarome *et al.*, 2011). Indeed, inhibition of UPS-mediated proteolysis in conjunction with protein synthesis inhibition, prevents anisomycin induced impairments in reconsolidation of fear memory at amygdala synapses (Jarome *et al.*, 2011). Together, these data support a role for the UPS in mediating the dynamic nature of synaptic stability during learning and memory retrieval. Mechanisms supporting the consolidation and retrieval of fear memories in the amygdala may also play a role in the formation and storage of memory for safety-related cues (Genud-Gabai *et a., 2013;* Sangha et al., 2013; Senn et al., 2014; Likhtik et al., 2014; Orsini *et al.*, 2013).

Amygdala Function During Fear and Safety Learning

Discrimination training with an aversive CS+ and a safe CS- results in increased responsiveness of BLA neurons independent of CS valence (Genud-Gabai *et al., 2013;* Sangha et al., 2013; Sierra-Mercado et al., 2011). Importantly, this responsiveness seems to persist even after initial acquisition (Sangha et al., 2013; Likhtik et al., 2014). Given the role of the amygdala in attributing emotional valence to memories, these data highlight the importance of identifying mechanisms by which safety memories might be consolidated in the amygdala.

The involvement of the amygdala in the formation and storage of memories with varying emotional valence underscores the importance of precise regulatory control of amygdala activity during consolidation. Several studies have shown that a bias develops towards representing aversive rather than pleasant information when both aversive and rewarding stimuli are learned simultaneously. This is supported by evidence from Ostroff and colleagues (2010) showing bidirectional changes in LA synapse size in a stimulus-valence dependent manner. Specifically, fear conditioning was associated with large dendritic LA spines whereas as safety conditioning

resulted in smaller spines (Ostroff *et al.*, 2010). Moreover, the neural mechanisms and circuitry underlying reward and safety learning have shown some degree of overlap (Sangha *et al.*, 2013; Salzman et al., 2007; Shabel and Janak, 2009). This suggests that during fear discrimination learning, insufficient gating of amygdala activity could result in the over-shadowing of conditioned safety consolidation by fear-related plasticity (Livneh & Paz, 2012). Behaviorally, other work has shown that competition between fear and inhibitory extinction memory is transient and context dependent, implying a natural bias to drift back towards excitatory rather than inhibitory neural mechanisms (Myers and Davis, 2007). The existence of a natural bias towards fear learning presents an even greater problem for individuals predisposed to developing anxiety-related disorders and, perhaps, reflects a greater liability for females.

Much of the aberrant fear responses associated with PTSD and other anxiety disorders can be attributed to aberrant amygdala activity during the consolidation or retrieval conditioned fear, fear extinction or conditioned safety (Bremner *et al.*, 1997; Coffey et al., 1993; Lebron-Milad et al., 2012; Machado-de-Sousa et al., 2014; Shin et al., 2006). Recent work suggests that reduced inhibitory control over amygdala activity is a primary contributing factor to these disorders and may be an underlying cause of impaired safety learning during differential fear conditioning (Keiser *et al.* 2016). In one study, male and female rats were trained with context fear conditioning and were tested 24 hrs later for freezing behavior or amygdala cFOS expression following exposure to the training context or a similar but novel context. Here females but not males showed indiscriminate freezing during exposure to the training or novel context as well as indiscriminate cFOS expression in the amygdala 90 minutes after retrieval (Keiser *et al.*, 2016). Over-activation of the amygdala during a traumatic event, like fear conditioning, or fear retrieval may effectively block inhibitory control that would normally

attenuate amygdala activity during non-threatening situations, such as being placed into a context that has never been paired with footshock. Such deficits in inhibitory control of fear responses may significantly contribute to the development of PTSD.

One promising direction is the investigation into potential dysfunction of top-down control of amygdala activity during fear and safety learning. Recently, the medial prefrontal cortex has emerged as a primary candidate for top-down control of amygdala activity and consequently has been implicated in the appropriate gating of fear expression. For example, disrupted inhibitory learning, like fear extinction or conditioned safety in females, may be due to dysfunction in amygdala efferents to specific subregions of the mPFC or vice versa. Specifically, during differential fear conditioning over-excitation of amygdala efferents may cause the CS- representation to become incorporated into the memory trace of the CS+, thereby impairing safety learning. Given the opposing nature of fear and safety on synapses in the amygdala (Ostroff *et al.*, 2010), maintaining excitatory and inhibitory control of the amygdala is essential for modulating the balance between discrimination and generalization of fear.

Medial Prefrontal Cortex and Top-Down Control of Fear Expression

Like the amygdala, stimulation or inactivation of mPFC has confirmed the involvement of mPFC in fear conditioning and extinction (Burgos-Robles et al., 2007; Corcoran & Quirk, 2007; Laurent & Westbrook, 2008; Sotres-Bayon & Quirk, 2010). Interestingly, prelimbic (PL) and infralimbic (IL) subregions of the mPFC have distinct contributions to the regulation of fear (Burgos-Robles et al., 2007). For example, PL inactivation with tetrodotoxin after fear conditioning reduces fear responses whereas IL inactivation impairs the consolidation of extinction memory (Corcoran & Quirk, 2007; Laurent & Westbrook, 2009). There is increasing evidence that the opposing functional influence of PL and IL on fear behaviors during discrimination learning may be similar to their respective roles during extinction learning. For

example, extinction learning and retrieval induces expression of the immediate early gene cFOS, which is indicative of activation of IL neurons while the renewal of fear was found to selectively decrease cFOS expression in IL and ITC (Hefner et al., 2008; Herry and Mons, 2004; Knapska and Maren, 2009) and electrical stimulation of the IL facilitates extinction (Milad et al., 2004; Vidal-Gonzalez et al., 2006). In addition, pharmacological manipulation of the IL influences the consolidation of extinction memory and in some cases can induce fear extinction even in the absence of non-reinforced CS presentations (Hugues et al., 2006; Laurent and Westbrook, 2008, 2009; Mueller et al., 2010; Sierra-Mercado et al., 2011; Peters *et al.*, 2010). Injection of tracer labels into IL revealed extensive labeling in LA, the intermediate capsule and to a network of inhibitory interneurons situated between the LA and CeA, known as the intercalated cell masses (ITC).

Neurons in the ITC have vast inhibitory projections to CeA and therefore are involved in limiting excitatory input from the BLA and reducing fear responses via CeA output projections (Paré and Smith, 1993). Selective lesions of ITC neurons following extinction training resulted in significant extinction impairments and in fact increased conditioned freezing (Likhtik *et al.*, 2008). Other work has further shown that amygdala neurons involved in fear learning are under inhibitory control of local GABAergic interneurons in addition to those of the ITC and IL (Ehrlich, 2009; Amano *et al.*, 2010; Milad & Quirk, 2002). These studies suggest a potential role for IL and ITC in extinction consolidation and retention. Moreover, IL is thought to interact with amygdala during fear suppression and may therefore have a significant role in reducing conditioned fear responses during fear discrimination paradigms.

Unlike the IL, the PL is thought to play an important role in the more volitional aspects of fear expression and tracer labeling of PL was relatively limited to BLA neurons (McDonald *et*

al., 1996; 1998). Further, fear conditioning and fear expression are accompanied by an increase in PL activity, whereas the IL is active during expression of non-aversive associations or safety. Likewise, amygdala neurons that are active in response to the CS+ preferentially project to the PL (Senn et al., 2014; Sotres-Bayon et al., 2012). As a result, interactions of PL and BLA are typically thought to support fear-related behavior (Sierra-Mercado et al., 2011; Klavir et al., 2013; Klavir et al., 2012; Livneh & Pax, 2012; Knapska et al., 2012). Together, data on IL and PL interactions with amygdala and their opposing influence on fear expression suggests that amygdala activity during memory acquisition and consolidation may determine which mPFC subdivision is active during recall, and thus impact behavioral expression of fear accordingly (Livneh & Pax, 2012; Knapska et al., 2012).

In addition to sending projections to the amygdala, the mPFC also receives amygdala afferents as well as afferents from a number of other cortical and subcortical regions (Conde *et al.*, 1995; Hoover & Vertes, 2007). As previously mentioned, reciprocal connections between the mPFC subregions and the amygdala are specifically important for mediating interactions between these regions during fear conditioning and particularly for discriminating between fear and safety.

During differential fear conditioning, reciprocal connectivity between the mPFC and BLA supports the transfer of information between the two structures. One study using simultaneous dual site cell recordings found that amygdala neurons involved in valence encoding for either the CS+ or CS- fired before neurons in the mPFC (Klavir et al., 2013). Later into training, these BLA neurons begin firing after cells in the mPFC. These data indicate that early during discrimination training, BLA neurons may contribute an attentional processing component that signals information about novel association to the mPFC and seems to be

independent of the stimulus valence (Likhtik & Paz, 2015). However, during the later parts of discrimination training, these BLA neurons are valence-specific, meaning that they fire in response to either the aversive CS+ or the safe CS-. These data support the idea that during discriminative learning, the mPFC entrains amygdala activity to appropriately gate fear expression. Moreover, this finding supports the role of the mPFC in assigning valence to novel stimuli and suggests that the mPFC may play a major role in the consolidation of CS+ and CS- associations during differential fear conditioning.

In fact, deficits in neuron firing synchrony between the BLA and mPFC have been observed in non-human primates that fail to successfully discriminate (Klavir et al., 2013). In keeping with this idea, higher synchronization has been associated with successful CS+/CS- discrimination (Likhtik et al., 2014). Together, these data indicate that efficient communication between the BLA and regions of the mPFC are essential for successful discrimination and that deficits in this communication can result in discrimination deficits commonly seen in the pathology of PTSD.

Interactions between the mPFC and amygdala are essential for successful fear discrimination learning. Recent work has suggested that the mPFC relies on bidirectional communication with the amygdala to shape activity in the BLA during fear discrimination, resulting in inhibition or disinhibition of amygdala output. During successful fear discrimination, theta-frequency synchrony between mPFC and the BLA was enhanced in response to both CS+ and CS- but only in animals that successfully discriminated between the two (Rogan *et al.*, 2005).

Together, it seems that the PL and IL modulate the expression of conditioned fear and inhibition of fear, respectively. Thus, the mPFC is not required for the acquisition of fear but is

essential for regulating the expression of conditioned fear and the consolidation of inhibitory extinction and safety memories. Further evidence points to the involvement of memory consolidation mechanisms in the PFC suggesting that more complex forms of fear conditioning, such as trace or differential fear conditioning, rely on mPFC during initial memory consolidation (Morgan and LeDoux 1995; Quirk et al. 2000; Corcoran and Quirk 2007; Gilmartin and Helmstetter 2010; Reis *et al*., 2013; Gilmartin *et al.*, 2013; Gilmartin *et al.*, 2014).

Interestingly, neither conditioned inhibition nor safety conditioning appear to be sensitive to lesions of the mPFC (Gewirtz et al., 1997; Schiller and Weiner, 2004). Along with the fact that fear extinction has been compellingly coupled to the inhibitory effect of CS related information transmitted from mPFC to the amygdala suggests that the mPFC may be specifically involved in regulating fear expression during situations in which there is competition between excitatory and inhibitory mechanisms (Milad and Quirk, 2002 and Milad et al., 2004). In support of this, localized genetic knock-down of *CBP* or *Grin1* in PL had no effect on the acquisition or expression of conditioned fear but significantly impaired subsequent acquisition of CS+/CS- discrimination. Knockdown of *CBP* and *Grin1* disrupts the functions of CREB and NMDA receptors in PL excitatory neurons, respectively (Vieira *et al.*, 2014; Vieira *et al.*, 2015).

Similar findings have been observed in trace fear conditioning in which the CS is temporally separated from the UCS by a brief stimulus-free-period (SFP). In this case the prelimbic cortex is involved in maintaining a representation of tone-shock pairings across the SFP, thus allowing the CS-UCS association necessary for fear memory (Gilmartin *et al.*, 2013). NR2A containing NMDA receptors in the PL were found to mediate multiple forms of fear conditioning while NR2B-containing NMDA receptors were only necessary for trace fear conditioning (Gilmartin *et al.*, 2013). In-line with this, post-training PL infusions of the

proteasome inhibitor, β-LAC, impaired consolidation of trace but not standard delay fear conditioning (Reis *et al.*, 2013). Together these data suggest that, for more complex forms of fear conditioning, consolidation of more abstract information (i.e. temporal and spatial, or contextual details) relating to the CS-UCS association may require the mPFC.

Sex differences in Fear Discrimination

The inability to inhibit fear responding when appropriate is a major symptom of PTSD (Glover, Jovanovic, and Norrholm, 2015). Converging evidence from studies using a variety of aversive training paradigms points towards a propensity in females for enhanced fear acquisition, increased resistance to fear extinction, and deficits in safety learning (Milad *et al.*, 2009; Baker-Andresen *et al.*, 2013; Baran *et al.*, 2009; 2010; Ribiero *et al.*, 2010; Day, Reed and Stevenson, 2016). Several studies have provided strong evidence that reduced inhibitory control of fear can impair the acquisition of a conditioned safety cue resulting in indiscriminate fear responding to cues associated with danger or safety (Lissek *et al.*, 2009; Jovanovic *et al.*, 2012; Day, Reed, & Stevenson, 2016).

Recent work investigating sex differences in fear discrimination has demonstrated increased amygdala activity in females but not males following retrieval of neutral context memory (Keiser *et al.*, 2016). Moreover, impaired safety learning has recently been used to explain sex-specific deficits in cued fear discrimination following extended discriminative fear conditioning. Consistent with previous work, this study found that females but not males showed impaired discrimination between CS+ and CS- following 3 consecutive days of auditory discrimination training of 5 CS+ and 5 CS- trials per day (Day, Reed & Stevenson, 2016). A retardation test, in which animals are trained with DFC with the previously conditioned CS- serving as the new CS, revealed greater responding to the auditory cue in females than in males

and females that were only pre-exposed to the tones (Day, Reed, & Stevenson, 2016). Together these findings support the idea that impaired discrimination between stimuli predictive of safety or danger is due to impaired consolidation of the inhibitory safety memory in females.

The Role of Estrogen in Fear Inhibition

One mechanism that may contribute to sex differences in fear inhibition is the action of naturally cycling steroid hormones, specifically estrogens. In rodents, the estrous cycle is 4-5 days in length and consists of 4 distinct phases: proestrus, estrous, metestrus and diestrus. Notably, changes in estrous phase are correlated with fluctuating levels of several steroid hormones including progesterone and estrogen. A high level of estrogen is characteristic of proestrus while low levels are associated with the estrus and metestrus phases.

The role of estrogens in modulating fear extinction has received increasing attention and evidence suggests that estrogens can have a faciliatory effect on fear conditioning and fear extinction (Jasnow, Schulkin & Pfaff, 2006; Zeidan *et al.*, 2011; Lynch *et al.*, 2013; Cover *et al.*, 2014). In human and rodent studies, impaired fear extinction is associated with low estrogen levels and fluctuations in estrogen related to the menstrual cycle have been shown to modulate activity in several brain regions involved in fear and safety learning including the amygdala, hippocampus, insular and cingulate cortices, and the hypothalamus (Saleh, Connell, & Crib, 2005; Hwang *et al.*, 2015). Females that undergo extinction training during proestrus demonstrate enhanced extinction learning and administration of exogenous 17β-estradiol to ovariectomized female rats enhances extinction acquisition (Milad *et al.*, 2009; Graham and Dahler, 2016). The enhancing effect of estrogens on extinction learning is thought be mediated in part through the action of the estrogen receptor α (ERα) and β (ERβ), though with seemingly opposite roles. For example, agonists of ERβ but not ERα have been shown to facilitate context

fear extinction and enhance fear inhibition when administered locally to the dorsal hippocampus or delivered systemically (Chang *et al.*, 2009; Toufexis *et al.*, 2007). Compared to fear extinction, much less work has been done to assess the role of estrogen in aversive discrimination learning despite increasing evidence of elevated fear generalization in females (Keiser *et al.*, 2016; Lynch *et al.*, 2016; Day, Reed & Stevenson, 2016).

A recent study found that estradiol-induced enhancements in fear generalization were mediated in part by activation of cytosolic/nuclear ERβ in the dorsal hippocampus (Lynch *et al.*, 2016). In another study, gonadectomized (GDX) and estrogen-implanted male and female rats were trained in a conditional discrimination procedure. The results of this study found that GDX males and females and estrogen-implanted males were able to generalize inhibitory learning, an effect that was not observed in estrogen-implanted females. Interestingly, this study also found that estrogen did not enhance fear responding in either sex during discrimination or single-fear conditioning which suggests that estrogen may be disrupting the ability to inhibit fear responding (Toufexis *et al.*, 2007). Moreover, OVX female rats trained in a latent inhibition paradigm during proestrus exhibited attenuated latent inhibition and administration of estrogen benzoate, a synthetic steroidal estrogen, to OVX female rats abolished latent inhibition (Quinlan *et al.*, 2010; Nofrey, Ben-Shahar & Brake, 2008). Together these studies suggest that estrogen is a major contributor to sex differences in fear inhibition and that the effects of estrogen may differ between fear extinction and other forms of conditioned inhibition like safety learning.

Despite increasing work on these questions, there remains a paucity of data regarding the sex-specific effects on fear and safety learning. Recently, we have shown that females but not males would show indiscriminate fear to CS+ or CS- presentations 24 hrs after auditory differential fear conditioning (ADFC). In this study, male and naturally cycling female rats were

trained with ADFC and tested for fear discrimination the following day. Results indicate a sex difference in baseline freezing on day 1 (Figure 1), however there were no significant sex differences in freezing behavior during any other part of training (Figure 1).

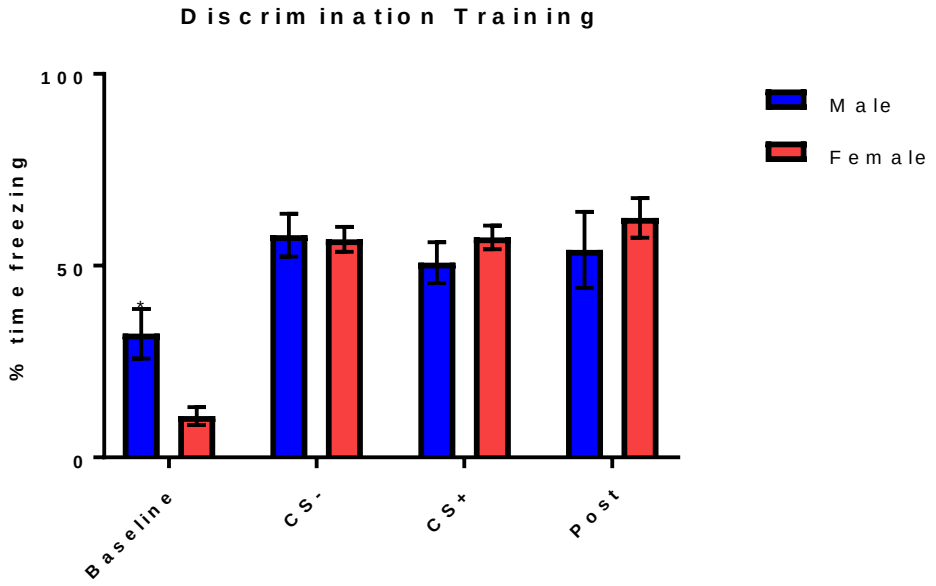


Figure 1: Average percent time spent freezing during the baseline period, CS- presentations, CS+ presentations and post period of ADFC for naturally cycle female and male rats. * p<.05.

In comparison, a sex-specific impairment in discrimination between the CS+ and CS- was observed during the test phase, 24 hrs after discrimination training. Specifically, females showed significantly greater freezing to the CS- than males (Figure 2) which resulted in significantly higher generalization index ($t(9)=4.071$, $p=0.0028$).

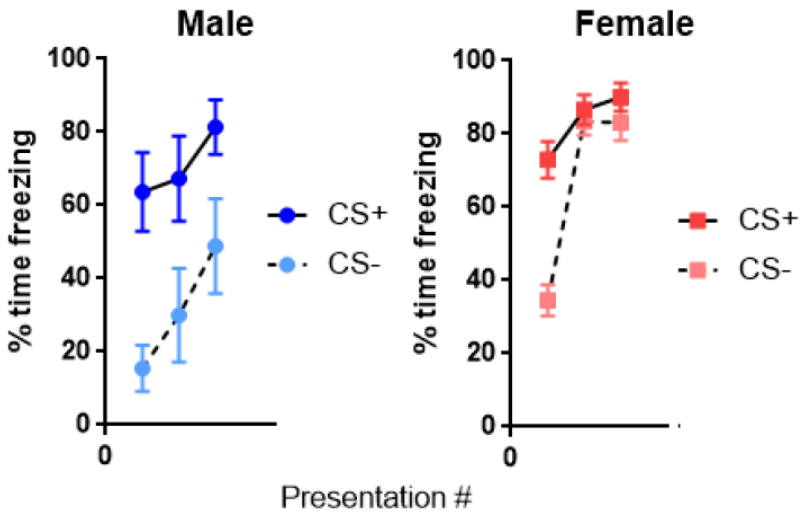


Figure 2: Average percent time spent freezing during the first 3 presentations of the CS+ and CS- for males and naturally cycling females 24 hrs after ADFC.

Notably, we did not observe an effect of estrous cycle phase during training or discrimination retrieval as seen in figures 3 and 4, respectively.

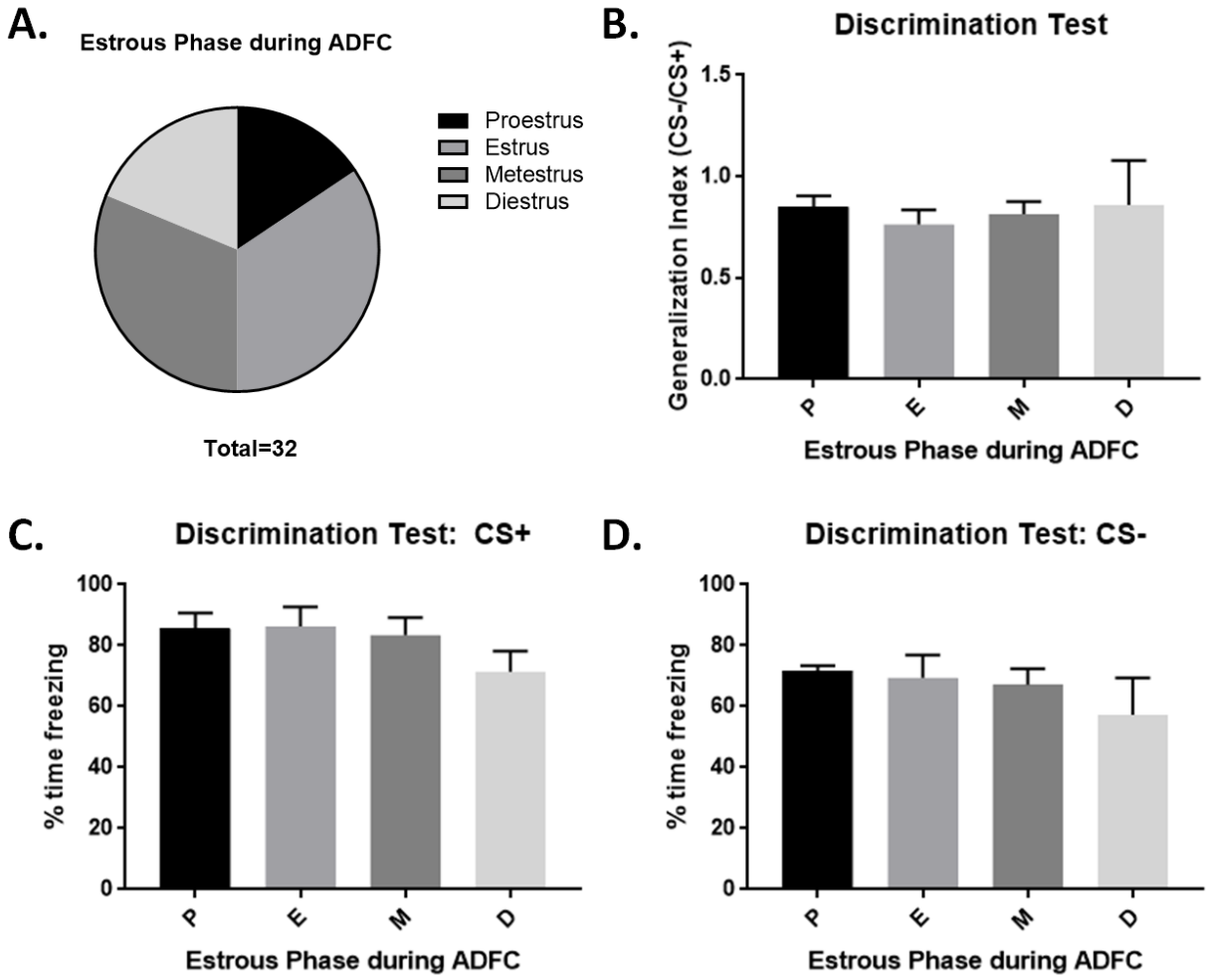


Figure 3: Generalization index and CS freezing for females grouped by estrous phase during training. A) Distribution of rats across the estrous phase on the day of ADFC. B) Generalization index (CS- freezing/CS+ freezing) as a function of estrous phase during training. C) CS+ freezing during the discrimination test in females as a function of estrous phase during training. D) CS- freezing during the discrimination test in females as a function of estrous phase during training.

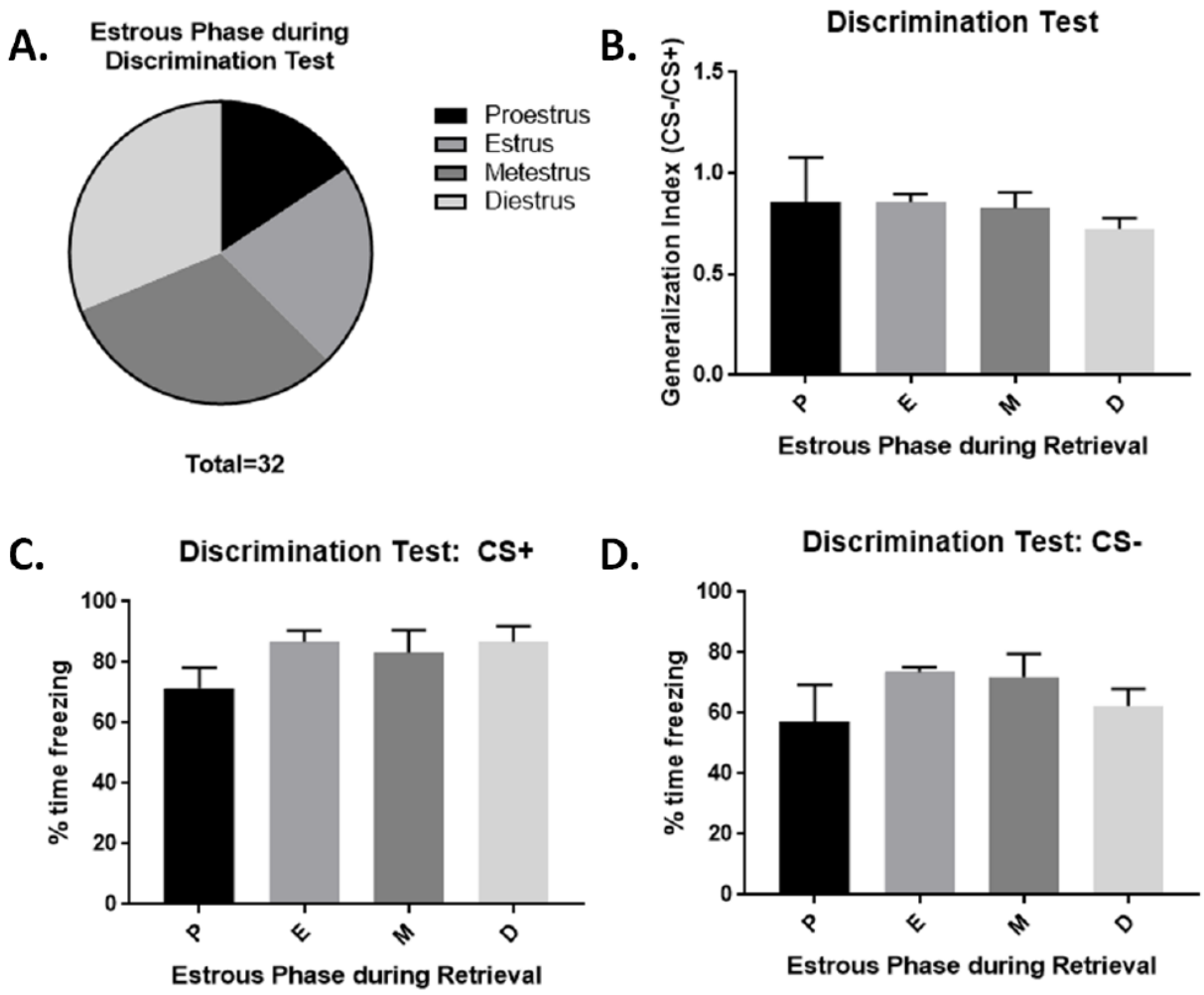


Figure 4: Generalization index and CS freezing for females grouped by estrous phase during discrimination test. A) Distribution of rats across the estrous phase on the day of retrieval. B) Generalization index (CS- freezing/CS+ freezing) as a function of estrous phase during retrieval. C) CS+ freezing during the discrimination test in females as a function of estrous phase during retrieval. D) CS- freezing during the discrimination test in females as a function of estrous phase during retrieval.

These results are consistent with previous reports of elevated fear generalization in females (Toufexis *et al.*, 2007; Keiser *et al.*, 2016). While emerging evidence from our lab and others suggests fear generalization in females is the result of impaired safety learning, many questions regarding the nature of this impairment remain.

The purpose of the present series of experiments was to further evaluate the deficits in fear discrimination seen in females and to investigate sex differences in retrieval-induced synaptic destabilization following retrieval of learned safety or learned fear memory. The results indicate that 1) males were better at discriminating between fear and safety cues following differential fear conditioning and that 2) this is due to impaired safety learning in females. Moreover, the retrieval of conditioned safety or conditioned fear induces synaptic destabilization in the amygdala of male but not female rats. 3) Lastly, CS- retrieval is sufficient to induce reconsolidation mechanisms in the amygdala of male but not female rats.

Method

Subjects

Male and female Long Evans rats weighing ~250-275 grams were obtained from Envigo (Madison, WI). All animals were individually housed and given *ad libitum* access to food and water. For all experiments, male and female rats were housed in the same colony room on opposite sides of the room. The colony room was maintained on a 14:10 hr light/dark cycle with all experiments occurring during the light period. All procedures were approved by the University of Wisconsin-Milwaukee Institutional Animal Care and Use Committee and complied with the ethical guidelines of the National Institutes of Health (NIH).

Surgery

All animals that received drug infusions were implanted with bilateral, stainless steel guide cannulae (26 ga; Plastics One Inc) aimed at the basolateral region of the amygdala (A.P. -

2.9; M.L. ±5.0; D.V. -7.0 from bregma) or the prelimbic region of the medial prefrontal cortex at a 15° angle to vertical (AP +2.9; ML ± 1.6; DV -3.2 from bregma). Coordinates are based on a rat brain atlas and have been previously used in our lab (Paxinos & Watson, 2007; Jarome *et al.*, 2011; Reis *et al.*, 2013). Prior to surgery, each rat was anesthetized with isoflurane in 100% O_2 (4% induction, 2% maintenance). Cannulae were secured to the skull with a stainless-steel screw, ethyl cyanoacrylate, and acrylic cement. Following surgery, rats were returned to their homecage and given a 7 day recovery period before any subsequent behavioral test.

Conditioning apparatus

All conditioning sessions occurred in a set of four identical Plexiglas and stainless-steel chambers each housed inside a separate sound-attenuating box (context A). Each outer box is illuminated with a 7.5 watt house light and was ventilated with a small fan. The background noise level in each of these outer boxes ranged from 46-50 dB. The floors of the Plexiglas chambers in context A were made of evenly spaced stainless steel rods through which the footshock (UCS) was delivered. Between each set of rats, each chamber was cleaned and the inside wiped down with 5% ammonium hydroxide.

All behavioral tests were conducted in a shifted context (context B). The chamber floors in context B were composed of an opaque, black piece of plastic. The chambers of context B was wiped with 5% acetic acid before each test session. For retardation training, animals were placed into context C. The floors in context C are composed of an opaque, white piece of plastic and the chamber was wiped down with lemon-scented cleaning solution before each group of animals.

Auditory Differential Fear Conditioning (ADFC)

All animals trained with ADFC underwent training in context A. Training consisted of a 6 min stimulus-free baseline period followed by randomized presentations of a CS+ or CS-

auditory tone stimulus with 10 total trials of each. A 1 or 7 kHz pure tone served as the CS+ or CS- in a counterbalanced fashion. CS+ presentations were paired with a 0.5 mA footshock, to be delivered through the floor bars of context A coincident with termination of the CS+.

Discrimination Testing

Discrimination testing occurred in context B. Testing consisted of a 60s baseline period before 3 consecutive, 30s CS- presentations separated by a 60s inter-trial interval. The final CS- was followed by 3 consecutive, 30s, non-reinforced CS+ presentations separated by a 60s inter-trial interval. Importantly, CS- trials and CS+ during discrimination testing are separated by a 180s stimulus-free period.

Retardation Test

To assess the inhibitory properties of a conditioned CS- rats were first habituated to both tones and context A. The next day half of the rats were trained with ADFC in context A. The remaining rats were trained with the same AFDC procedure but the footshocks were omitted and therefore serve as tone preexposure control group. The following day rats underwent a discrimination test in context B. On day 4, all rats were trained with a 5-trial delay fear conditioning procedure in context C, using the previously conditioned CS- as the conditioned stimulus. Conditioned freezing to CS- presentations was assessed on day 5 in context B.

Conditioned Fear Responses

The activity of each rat was recorded on digital video and the amount of movement was determined by frame-by-frame changes in pixels using FreezeScan 1.0 software (CleverSys, Reston, VA). The automatic scoring parameters are chosen such that the scored activity matches hand-scoring methods previously used in our lab to measure freezing. Analyses used percent

time freezing in response to presentations of auditory stimuli as the dependent variable for all behavioral experiments.

20S proteasome activity assay

Samples were diluted in DDH_2O and mixed with reaction buffer (250mM HEPES, pH 7.5, 5mM EDTA, 0.5% NP-40 and 0.01% SDS). Fluorogenic peptide Suc-LLVY-AMC (Millipore Sigma), and Bz-VGR-AMC (Enzo Life Sciences) was added to the samples according to the manufactures instructions. The reaction was incubated at 37^{o}C for 2-hrs and fluorescence monitored every 5-min at 360 (excitation)/ 460 (emission) on a monochromatic plate reader (Synergy H1; Biotek). Protein free blanks were used and an AMC standard curve was produced.

Drugs and Infusions

For experiments requiring intracranial infusions (experiment 3 and experiment 4) rats received bilateral infusions into the amygdala or prelimbic prefrontal cortex. For experiment 3, the protein synthesis inhibitor, anisomycin (ANI; 125 μg/μl; Tocris) will be dissolved in 2% DMSO in 1M HCl diluted in ACSF. For experiment 4, ifenprodil (Sigma Chemical; 2μg/μl) was be dissolved in 0.1 M PBS, 0.1% tartaric acid. The total volume of the infusion of ANI, βlac, Ifenprodil, or vehicle was 0.5μl per injection site, delivered at a rate of 0.5μl/min.

Western blots

Samples (10μg) were loaded on 7.5% TGX gels, ran through SDS-PAGE and transferred using a Turbo Transfer System (Biorad). Membranes were then incubated in 3% blocking buffer for 1-hr at room temperature, followed by overnight incubation in the appropriate primary antibody diluted in 3% BSA in tris buffered saline. Membranes were washed and incubated in secondary antibody (1:20,000) for 60-min. Following a final wash, membranes were incubated in enhanced chemiluminescence substrate (ECL, BioRad) for 5-min. Images were developed

using a CCD-based camera system (GBOX Chemi XT-4; Syngene) and analyzed using GeneTools software. Primary antibodies used include GluR1 (1:1000; Cell Signaling), GluR2 (1:500; Santa Cruz), GluR3 (1:1000; Cell Signaling), K48 polyubiquitin (1:500; Cell Signaling), phosphorylated TrkB (1:500; Cell Signaling) and actin (1:1000; Cell Signaling).

Estrous Phase Tracking

Naturally cycling female, Long-Evans rats were subject to at least 3 days of handling in preparation of vaginal swab collection. Cotton swabs with tips no wider than 2mm and no longer than 5mm tips were autoclaved prior to use. To collect vaginal cytological samples, autoclaved cotton swabs were first soaked in sterile dH_2O. Soaked swabs were then gently inserted into the vagina, vaginal wall swabbed, and swab gently removed. The cotton tip is then lightly rolled on to a prelabeled slide. Once dry, estrous phase was identified via light microscopy. To determine if females were naturally cycling, estrous phase was tracked through at least 3 complete cycles (12-15 days). Importantly, collection of vaginal epithelial samples occurred at the same time each day.

Statistical analyses

For quantitative protein assays, the mean pixel density was calculated for each sample and taken as a percentage of the no retrieval control group. For proteasome activity assays, each raw fluorescence reading was standardized to the AMC standard curve for that plate and taken as a percentage of the no retrieval control group. For all behavioral experiments, the average percent time spent freezing was calculated for each group. Data was analyzed using Analysis of Variance (ANOVA) and Dunnett's multiple comparisons test where appropriate.

Hypotheses

Hypothesis 1: Female but not male rats will show fear generalization following auditory differential fear conditioning due to impaired acquisition of learned safety as indicated by a test of retardation.

Hypothesis 2: If female but not male rats fail to acquire the safety signal, then synaptic destabilization associated with memory retrieval will differ between female and male rats in the amygdala, prelimbic mPFC, infralimbic mPFC and dorsal hippocampus.

Hypothesis 3: Synaptic destabilization in the amygdala, prelimbic mPFC and infralimbic mPFC will differ between CS+ and CS- retrieval, possibly in a sex specific manner.

Hypothesis 4: CS- retrieval is not sufficient to destabilize CS+ memory in male rats trained with ADFC (separate traces).

Hypothesis 5: CS- retrieval is sufficient to destabilize CS+ memory trace in female rats trained with ADFC (joint trace).

Hypothesis 6: Pretraining blockade of NR2B-containing NMDA receptors in prelimbic mPFC will impair stimulus discrimination in male rats but have no effect on female rats.

Results

Impaired fear discrimination in females is the result of a deficit in learned safety following auditory differential fear conditioning.

Previous work has shown that female but not male rats demonstrate discrimination deficits between safety and aversive cues following extended fear discrimination training. Moreover, this discrimination deficit was found to be the result of impaired safety learning during discrimination training (Day, Reed, and Stevenson, 2016). To further validate this idea, we trained male and female rats in an auditory differential fear conditioning procedure 24 hrs following tone habituation (Figure 5A). Freezing behavior of male and female rats did not differ during discrimination training (Figure 5B; $F(1,18)=0.5168$; $p=0.4814$). Consistent with our preliminary data, female rats froze significantly more in response to CS- presentations compared to baseline freezing during a discrimination test than male rats (Figure 5C; $F(1,12)=12$; $p=0.0047$). The following day, discrimination trained rats and tone pre-exposed rats (PreX) were trained using delay fear conditioning, with the previous CS- serving as the auditory stimulus paired with shock (CS- reversal). There were no differences in freezing behavior between male and female rats or between ADFC and PreX rats during reversal training (Figure 5D; $F(1,26)=0.281$; $p=0.6006$). Following CS- reversal, rats were tested for fear to the original CS-. Male but not female ADFC rats froze significantly less than PreX control rats (Figure 5E; $F(3,23)=3.41$; $p=0.0345$). Together, these data support previous work indicating that deficits in fear and safety discrimination in females following auditory differential fear conditioning is due to impairments in inhibitory safety learning.

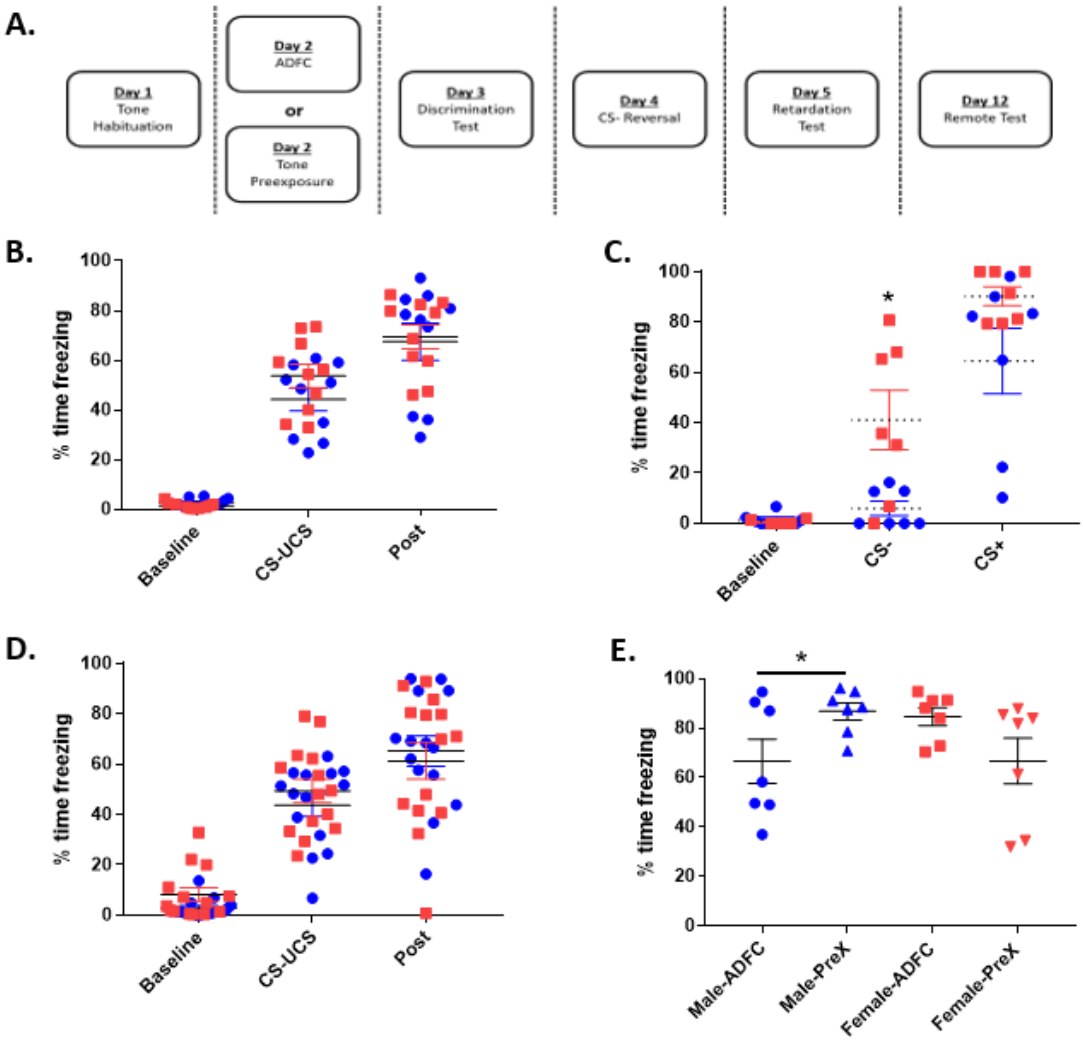
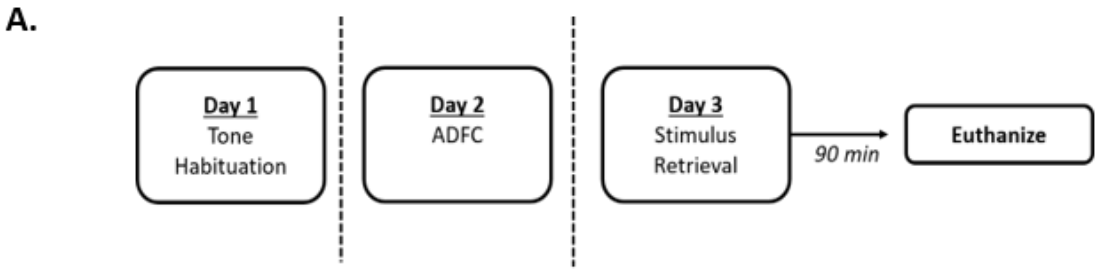


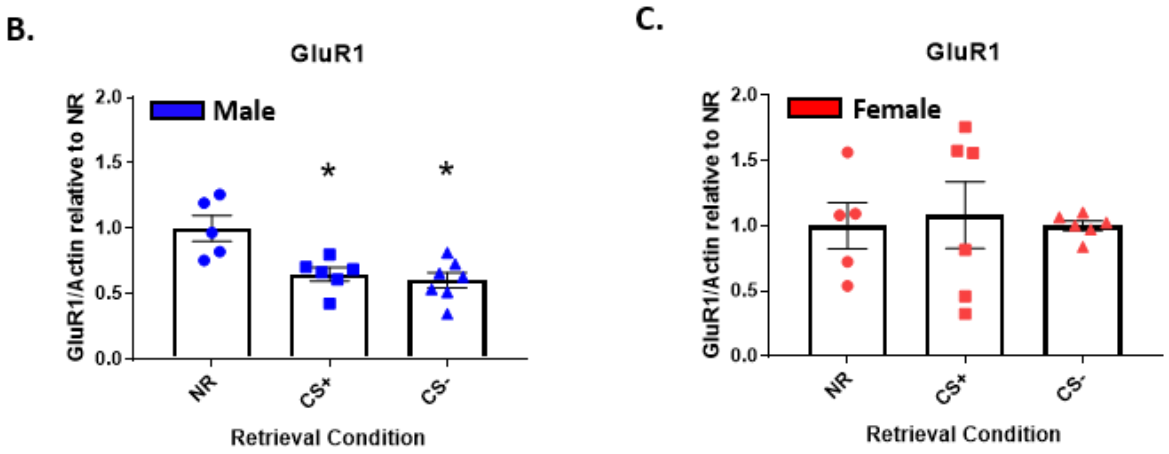
Figure 5: Females but not males show fear generalization due to impaired safety learning following differential fear conditioning. A) Behavioral procedure. B) Freezing behavior in male and female rats did not differ during discrimination training ($F(1,18)=0.5168$; $p=0.4814$). C) Female ($p=0.027$) but not male ($p=0.91848$) rats exhibited significantly more conditioned freezing to CS- presentations compared to baseline freezing during the discrimination test ($F(1,12)=12$; $p=0.0047$). D) Freezing behavior between male and female rats did not differ during any period of reversal training ($F(1,26)=0.281$; $p=0.6006$). E) Male ($p=0.035$) but not female ($p=0.1297$) rats trained with ADFC show significantly less freezing following CS- reversal compared to respective tone preexposure controls ($F(3,23)=3.41$; $p=0.0345$). *$p<.05$

Sex-specific patterns of synaptic AMPAR subunit expression and proteasome activity in amygdala following retrieval of fear or safety.

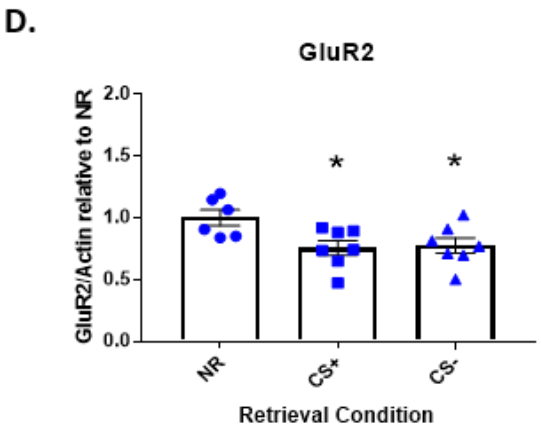
Fear memory retrieval results in and is required for synaptic destabilization in the amygdala characterized by CaMKII-dependent trafficking of AMPAR subunits and increased proteasome activity (Lee *et al.*, 2008; Jarome *et al.*, 2011; Jarome *et al.*, 2015). It is thought that this period of synaptic instability is a necessary component of memory updating through reconsolidation. Less, however, is known about similar processes in learned safety. To this end, we trained male and female rats with ADFC beginning 24 hrs after a tone habituation session. On day 3 rats received a single 30s non-reinforced presentation of the CS+ or CS- and were killed 90 min later for tissue processing. Subsets of male and female rats were killed directly from the homecage to serve as no retrieval control groups (NR). Comparisons for western blot data and activity assays were made between the NR group and each CS group for each sex.

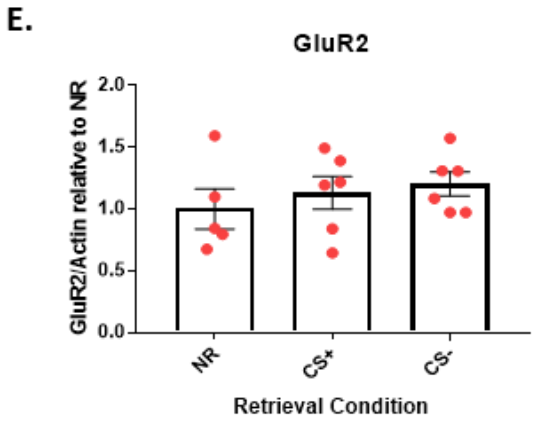
Western blot analysis of amygdala synaptosomal fractions revealed a significant reduction of GluR1 protein (Figure 6B) in males ($F(2,15)=9.247$; $p=0.0024$) but not females (Figure 6C; $F(2,14)=0.06856$; $p=0.9341$) following retrieval of CS+ ($p=0.0089$) or CS- ($p=0.0028$). Synaptic GluR2 (Figure6D) was also reduced in male ($F(2,17)=4.491$) but not female (Figure 6E; $F(2,14)=0.586$; $p=0.5696$) rats following CS+ ($p=0.0263$) and CS- ($p=0.0409$) retrieval. No significant differences in GluR3 (Figure 6F; 6G) were found between retrieval conditions for male ($F(2,16)=0.3085$; $p=0.7388$) or female rats ($F(2,16)=0.3996$; $p=0.6771$).

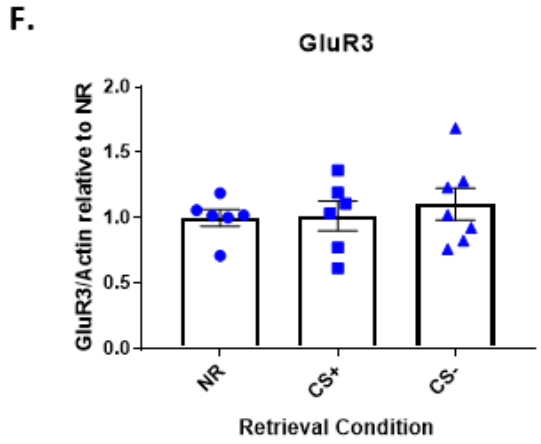

A.
Day 1
Tone
Habituation
Day 2
ADFC
Day 3
Stimulus
Retrieval
90 min
Euthanize


B.
GluR1
Male
GluR1/Actin relative to NR
2.0
1.5
1.0
0.5
0.0
*
*
NR
CS+
CS-
Retrieval Condition

C.
GluR1
Female
GluR1/Actin relative to NR
2.0
1.5
1.0
0.5
0.0
NR
CS+
CS-
Retrieval Condition


D.
GluR2
GluR2/Actin relative to NR
2.0
1.5
1.0
0.5
0.0
*
*
NR
CS+
CS-
Retrieval Condition


E.
GluR2
GluR2/Actin relative to NR
2.0
1.5
1.0
0.5
0.0
NR
CS+
CS-
Retrieval Condition


F.
GluR3
GluR3/Actin relative to NR
2.0
1.5
1.0
0.5
0.0
NR
CS+
CS-
Retrieval Condition

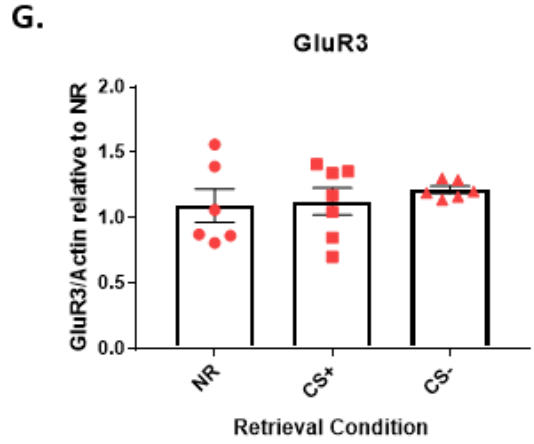

G.
GluR3
GluR3/Actin relative to NR
2.0
1.5
1.0
0.5
0.0
NR
CS+
CS-
Retrieval Condition

Figure 6: **Sex-specific differences in retrieval induced synaptic destabilization in the amygdala following ADFC.** A) Experimental design diagram. B) Male rats had significantly lower synaptic GluR1 and D) GluR 2 in the amygdala. Female rats show significantly reduced levels of synaptic GluR1 in the amygdala relative to no retrieval controls following CS+ ($p=0.0421$; $p=0.9468$) or CS- ($p=0.0264$; $p=0.99$) retrieval. C) GluR2 levels in males ($F(2,17)=0.2513$; $p=0.7806$) or females ($F(2,16)=01.124$; $p=0.3493$) did not differ between respective stimulus retrieval groups and no retrieval controls. D) No significant group differences in synaptic GluR3 were observed in male ($F(2,14)=0.08324$; $p=0.9206$) or female ($F(2,14)=2.151$; $p=0.1532$) rats.

Consistent with a reduction in synaptic GluR1, male rats showed greater chymotrypsin-like (Figure 7A; $F(2,16)=3.887$; $p=0.0421$) proteasome activity following CS+ ($p=0.0319$) but not CS- ($p=0.0903$) retrieval compared to NR controls. In females (Figure 7B), there was no difference in chymotrypsin-like activity across retrieval conditions ($F(2,16)=2.986$; $p=0.0791$). Likewise, trypsin-like activity did not differ significantly across retrieval conditions in male (Figure 7C; $F(2,15)=3.268$; $p=0.0664$) or female cohorts (Figure 7D; $F(2,17)=1.254$; $p=0.3105$). Together these data indicate that retrieval of a memory for safety (CS-), like for fear (CS+), results in GluR1 and GluR2 endocytosis at amygdala synapses in male rats and further support the idea that safety learning may be disrupted in females during ADFC.

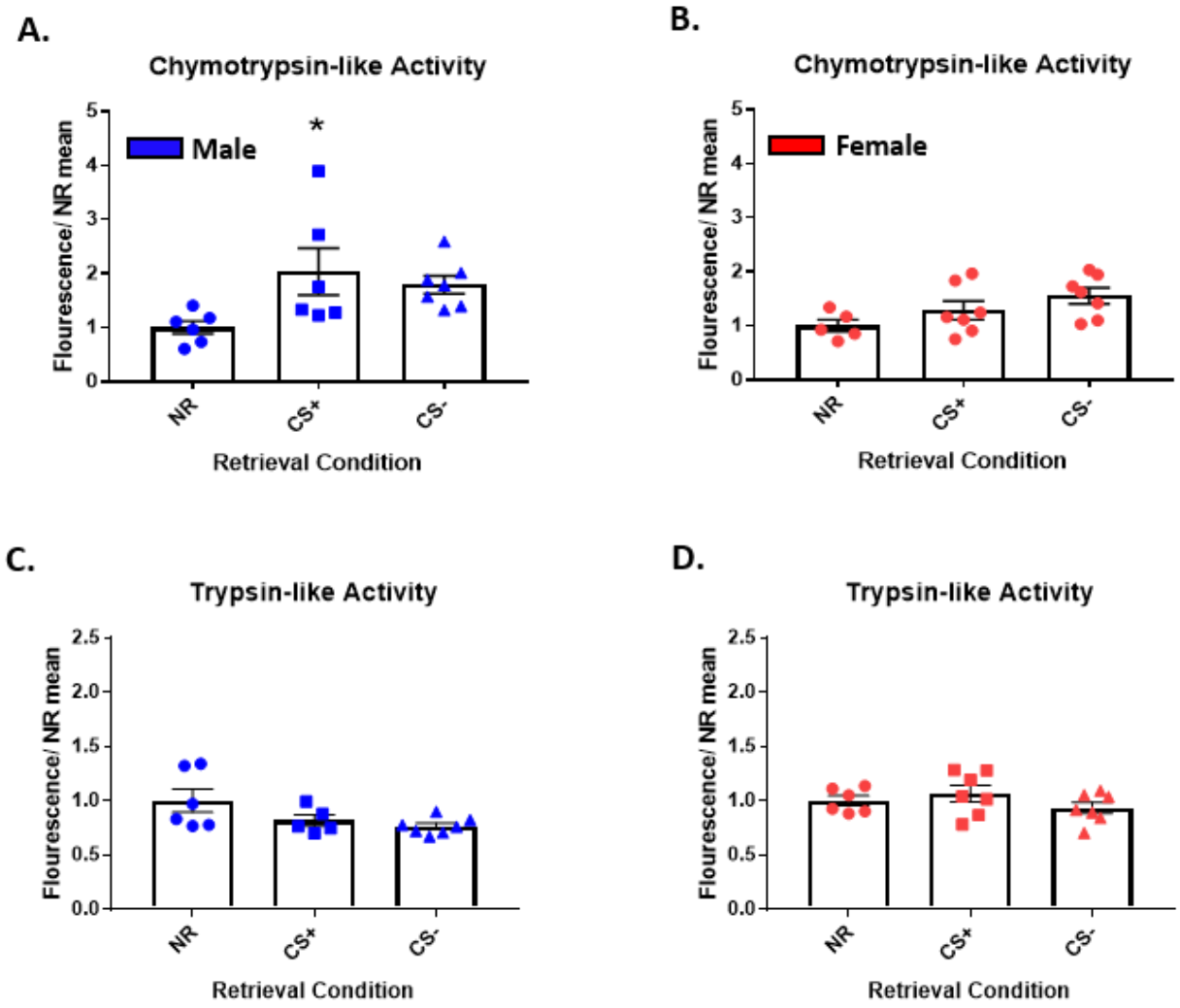


Figure 7: Sex-specific differences in retrieval induced proteasome activity in the amygdala following ADFC. A) Chymotrypsin-like activity in the amygdala is increased following CS+ retrieval. B) No significant differences in chymotrypsin-like activity across retrieval conditions in the amygdala of females. C) Trypsin-like activity did not differ across retrieval conditions in males. D) Similarly, stimulus retrieval did not change trypsin-like activity in the amygdala of females.

Unlike the amygdala, western blots of prelimbic medial prefrontal cortex synaptosomal fractions revealed no significant differences between retrieval conditions in levels of synaptic GluR1 for males (Figure 8A; $F(2,16)=0.02834$; $p=0.9721$) or females (Figure 8B; $F(2,16)=0.3466$; $p=0.7123$). Similar results were found for prelimbic GluR2 in males (Figure 8C;

F(2,17)=0.06199; p=0.9401) and females (Figure 8D; F(2,16)=1.251; p=0.3127) as well as GluR3 (Figure 8E; F(2,16)=0.3675; p=0.6982; and Figure 8F; F(2,16)=0.2812; p=0.2812) Similarly, chymotrypsin-like (Figure 9A; F(2,16)=1.901; p=0.1816; and Figure 9B; F(2,15)=0.7505; p=0.4891) and trypsin-like (Figure 9C; F(2,17)=0.3048; p=0.7412; and Figure 9D; F(2,16)=0.5443; p=0.5906) proteasome activity did not differ across retrieval groups for male or female rats in prelimbic cortex.

In the dorsal hippocampus, GluR3 (F,16)=4.907; p=0.0218) was significantly greater in females following CS+ retrieval compared to CS- retrieval (Figure 10F; p=0.0167). This was not seen in male rats (Figure 10E; F(2,16)=0.05366; p=0.9479). Similarly, GluR1 (Figure 10A; F(2,16)=0.7295; p=0.4975; and Figure 10B; F(2,16)=0.5904; p=0.5904) and GluR2 (Figure 10C; F(2,16)=0.6572; p=0.5317; and Figure 10D; F(2,16)=1.721; p=0.2104) did not differ significantly across retrieval conditions in male or female rats.

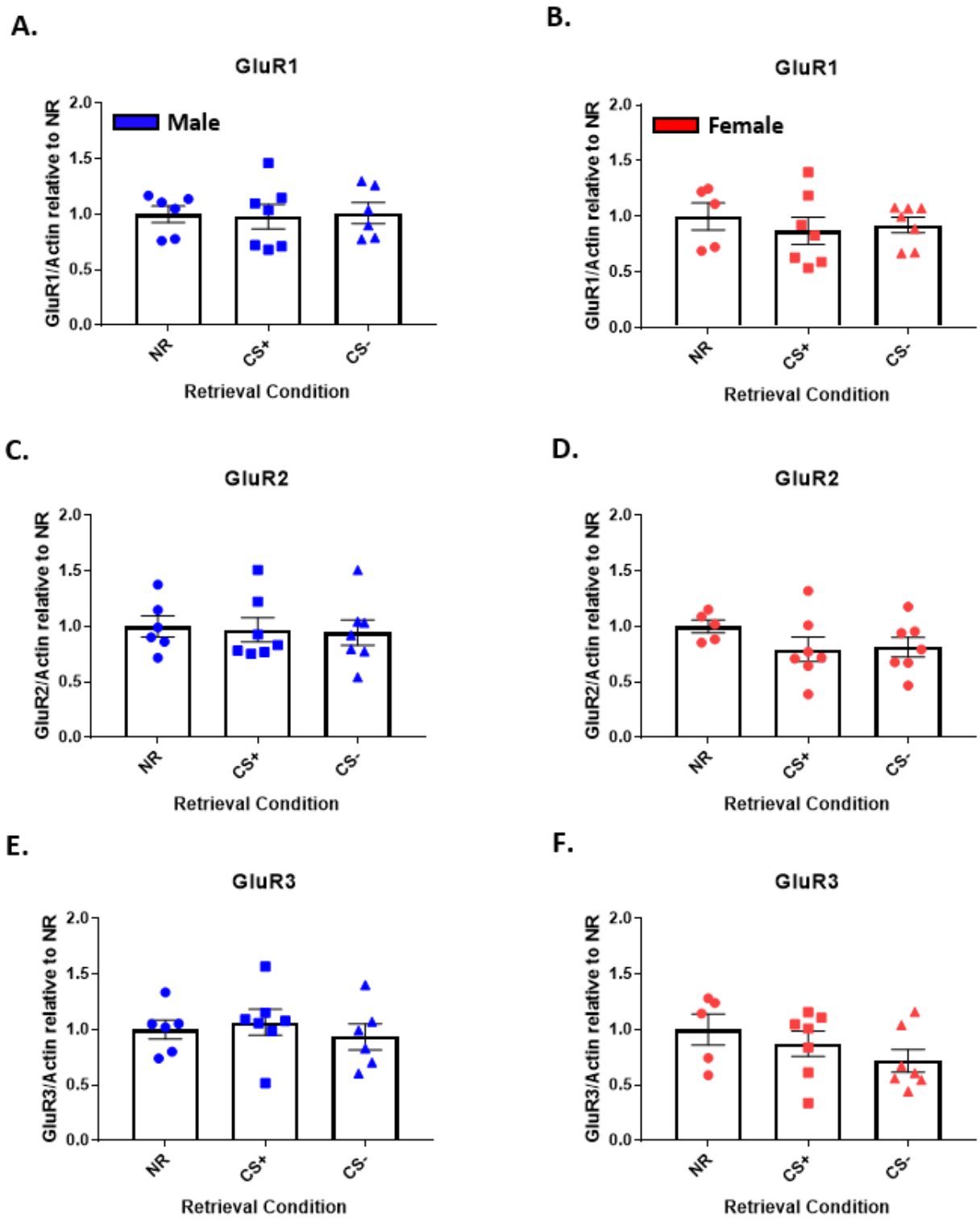


Figure 8: Synaptic AMPAR levels in the prelimbic cortex were not altered following retrieval of fear or safety. A-B) Synaptic Glur1, C-D) GluR2 and E-F) GluR3 in prelimbic medial prefrontal cortex did not differ across retrieval conditions for male or female rats.

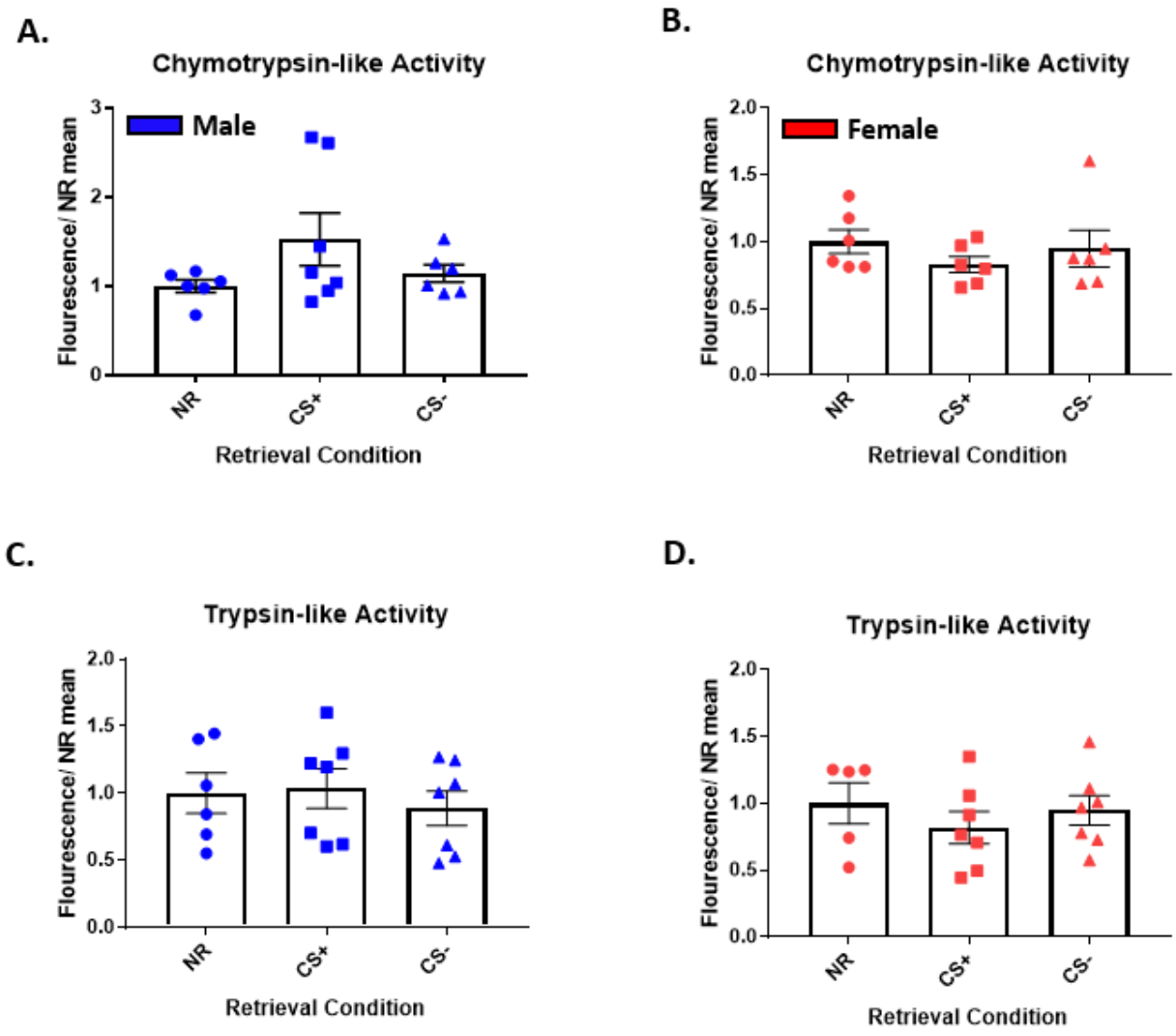


Figure 9: No differences in proteasome activity in the prelimbic cortex following ADFC. A-B) Chymotrypsin-like and C-D) trypsin-like proteasome activity did not differ significantly across retrieval conditions in male or female rats.

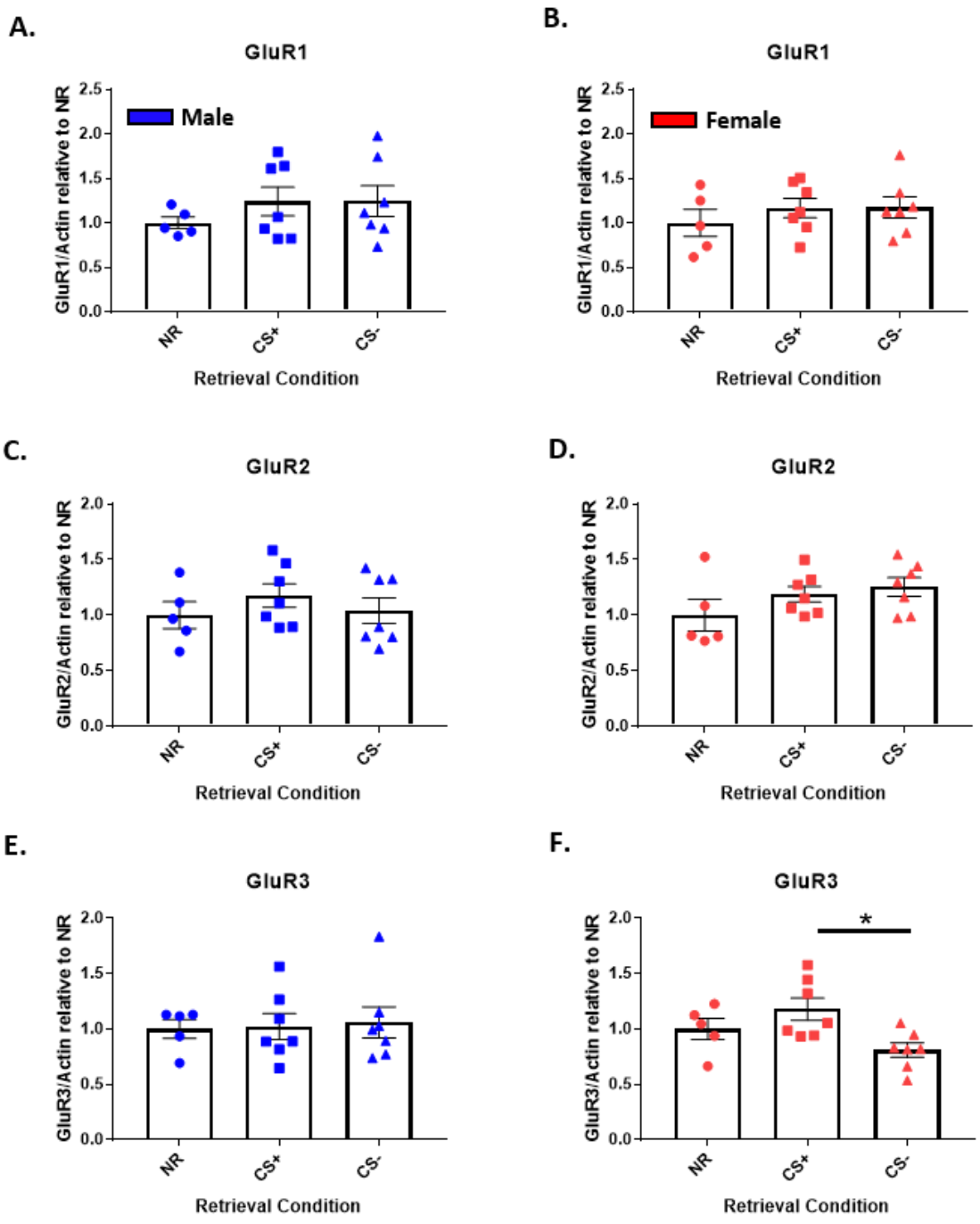


Figure 10: Sex-specific difference in GluR3 expression in the dorsal hippocampus between CS+ and CS- retrieval conditions. A-B) Synaptic Glur1and C-D) GluR2 in the dorsal hippocampus did not differ across retrieval conditions for male or female rats. E) GluR3 in the dorsal hippocampus did not differ across retrieval conditions but F) was significantly lower in females following CS- retrieval compared to CS+ retrieval.

Retrieval of conditioned safety induces fear memory reconsolidation in the amygdala in male but not female rats.

Evidence from our western blot experiment suggests that 1) retrieval of conditioned fear or conditioned safety has a similar effect on synaptic destabilization in the amygdala and 2) that retrieval induced synaptic destabilization differs between males and females. To further test these interpretations, we next examined the effects of post-retrieval protein synthesis inhibition in the amygdala on fear memory in male and female rats trained with ADFC. If retrieval of the CS- or CS+ induce similar patterns of synaptic destabilization, then protein synthesis inhibition should impair fear memory per reconsolidation theory. Following discrimination training rats received either a single CS- or CS+ retrieval followed by intra-amygdala infusions of the protein synthesis inhibitor, anisomycin (Figure 11A). The next day, rats were tested for conditioned fear to presentations of the CS+. Consistent with our previous experiments, male and female rats did not differ during ADFC training (Figure 11B; $F(1,84)=2.416$; $p=0.1239$). Interestingly during a test for CS+ memory, 2-way ANOVA revealed a significant main effect of drug infusion ($F(1,26)=14.87$; $p=0.0007$) and a significant interaction of sex and drug infusion ($F(1,26)=6.551$; $p=0.0166$). Specifically, anisomycin infusion into the amygdala following CS- retrieval was sufficient to impair conditioned fear to the CS+ in male rats ($p=0.0010$) suggesting that learned safety and learned fear may rely on overlapping cell populations. Moreover, anisomycin infusions in female rats following CS- retrieval had no effect on conditioned freezing (Figure 11D; $p=0.9254$), further supporting the idea that conditioned safety is impaired in female rats. Anisomycin infusion following CS+ retrieval had no effect on conditioned freezing in male or female rats (Figure 11C; $F(1,21)=0.2989$; $p=0.5251$). These data further support the idea that deficits in learned safety are responsible for increased generalization of fear in females and

suggest that instead of being represented by separate cell populations, the excitatory and inhibitory influences of fear and safety learning are acting on overlapping populations of cells within the amygdala (Figure 10C; F(1,21)=0.2989; p=0.5903).

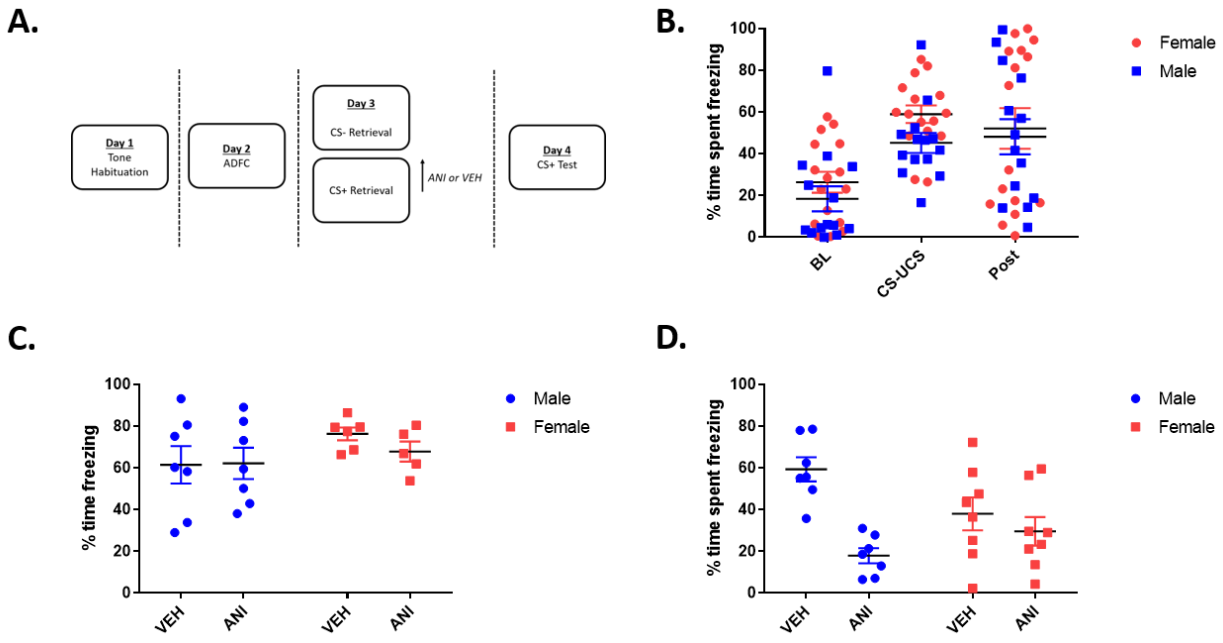


Figure 11: CS- retrieval results in synaptic destabilization of CS+ encoding synapses in the amygdala of male but not female rats. A) Experimental design B) Freezing behavior did not differ significantly between male and female rats during any phase of discrimination training (F(1,84)=2.416; p=0.1239). C) Infusion of anisomycin into the amygdala following CS+ retrieval had no effect on conditioned freezing 24 hrs later (F(1,21)=0.2989; p=0.5251). D) Following CS- retrieval, intra-amygdala infusions of ANI significantly disrupted conditioned freezing to CS+ presentations 24 hrs later in male (p=0.0003) but not female (p=0.5790) rats. INTERACTION: 2-way ANOVA F(1,26)=6.551; p=0.0166. ANI vs VEH F(1,26)=14.87; p=0.0007).

Pretraining inhibition of NR2B-containing NMDA receptors in the prelimbic mPFC facilitated freezing behavior during ADFC in males but not females.

The medial prefrontal cortex is thought to play a significant role in regulating the appropriate expression of conditioned fear. Dysregulation of mPFC-BLA synapses can impair the expression and retention of inhibitory extinction memories and facilitate fear acquisition. Interestingly, in more complex forms of fear conditioning, the mPFC relies on information from the amygdala and hippocampus to gate fear expression in an appropriate manner. While NMDA receptor activity in the PL appears to mediate several variations of fear conditioning, more complex forms specifically rely on NR2B-containingNMDA receptors in the prelimbic region of mPFC (Gilmartin *et al.*, 2013). Moreover, NMDA function is critical for activation of the ubiquitin-proteasome system and blockade of proteasome activity in the PL, likewise had no effect on a simple form of fear conditioning.

To more closely address the role of NR2B-containing NMDA receptors in the prelimbic medial prefrontal cortex, we trained male and female rats in ADFC 24 hr after tone habituation and 30 min after prelimbic infusions of ifenprodil (Figure 12A). Freezing behavior did not differ significantly between male and female rats during tone habituation (Figure 12B). A 2-way ANOVA of freezing behavior during ADFC (Figure 12C and 12D) revealed a significant main effect of drug infusion ($F(1,13)=9.214$; $p=0.0096$) and a significant drug x time interaction

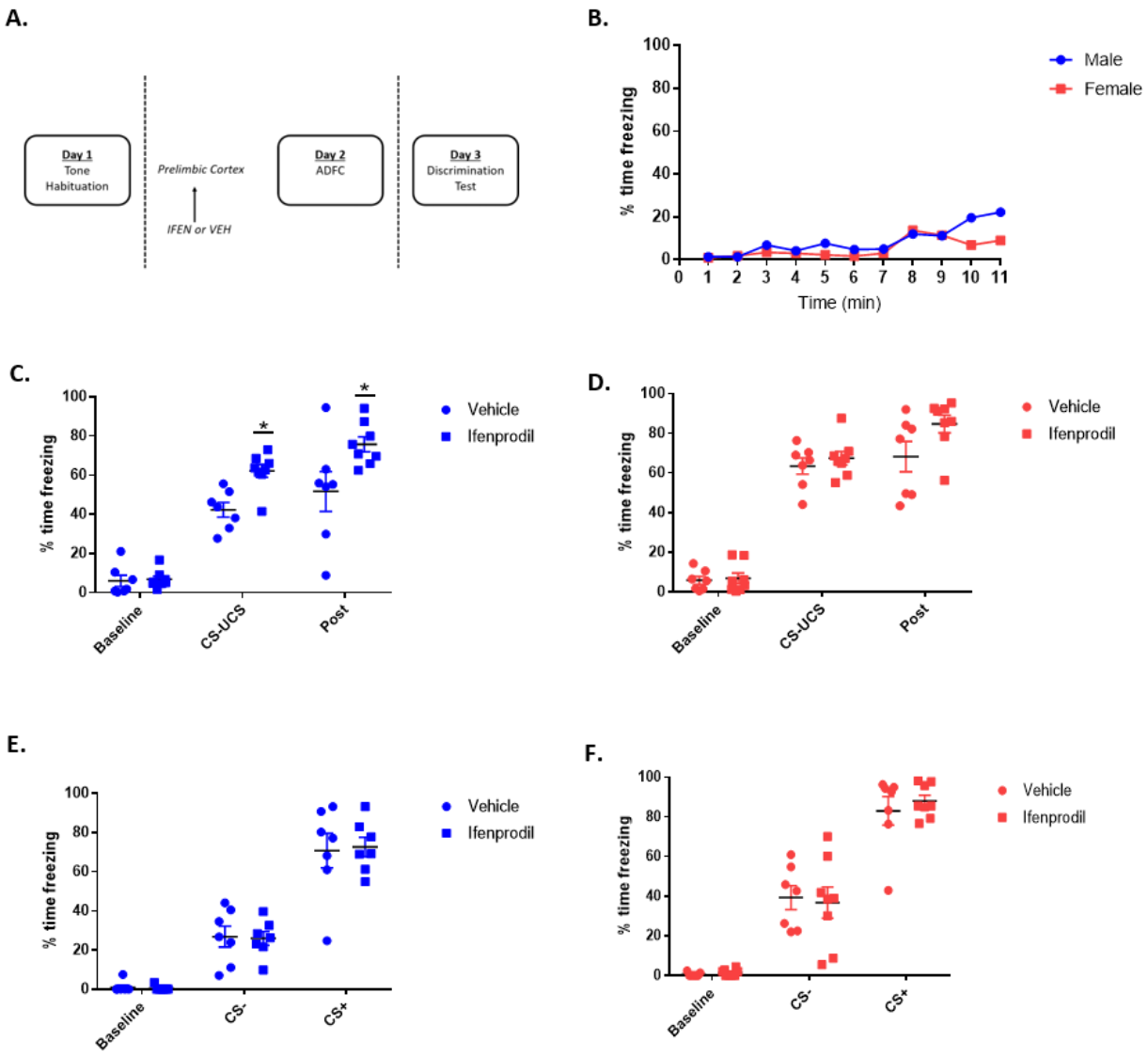


Figure 12: Pretraining infusions of ifenprodil into prelimbic cortex increases freezing during auditory differential fear conditioning in male but not female rats. B) Freezing behavior in male and female rats did not differ across the 11 min tone habituation session. C) Ifenprodil infused males froze significantly more ($F(2,13)=9.214$; $p=0.0096$) during the CS-UCS ($p=0.0181$) and post periods ($p=0.0034$) of discrimination training than vehicle infused males. D) Ifenprodil infused females did not differ from vehicle infused females during any phase of training ($F(1,13)=2.515$; $p=0.1368$). E) and F) Ifenprodil infusions had no effect on stimulus specific conditioned freezing or stimulus discrimination for male ($F(1,13)=0.05729$; $p=0.8152$) or female rats ($F(1,12)=0.01256$; $p=0.9126$) during a discrimination test.

Specifically, freezing in ifenprodil infused male rats was higher during the CS-UCS ($p=0.0181$) and post ($p=0.0034$) periods of ADFC compared to vehicle infused males. Freezing

behavior in females during ADFC did not differ between vehicle and ifenprodil infused rats ($F(1,13)=2.515$; $p=0.1368$). During a discrimination test (Figure 11E and 11F) 24 hr later, there was no difference in freezing to the CS- or CS+ between ifenprodil and vehicle infused rats in male ($F(1,13)=0.05729$; $p=0.8152$) or female cohorts ($F(1,12)=0.01256$; $p=0.9126$).

Discussion

The present set of experiments support previous literature suggesting that fear generalization in females is the result of impaired safety learning. Moreover, we extend this work by identifying, for the first time, sex-specific differences in retrieval induced synaptic destabilization in the amygdala of male and naturally cycling female rats trained in ADFC. Specifically, we found 1) slowed acquisition of fear to a previously conditioned safety signal following CS- reversal training in male but not female rats 2) decreased synaptic GluR1 and GluR2 in the amygdala of male but not female rats following retrieval of CS+ or CS- 3) increased chymotrypsin-like proteasome activity in the amygdala of male rats following CS+ retrieval 4) an anisomycin-induced impairment of CS+ reconsolidation in the amygdala following CS- retrieval and 5) a sex-specific facilitation of freezing behavior during ADFC in male rats following pretraining blockade of NR2B-containing NMDA receptors in the prelimbic mPFC. Together, these findings strongly support disrupted safety learning in females as a primary contributor to increased fear generalization and further suggest that consolidation of fear and safety conditioning may involve a similar population of cells within the amygdala.

Sex differences in Fear Inhibition

Mounting evidence points to a lack of inhibitory control as a major contributor to anxiety disorders and PTSD (Mahan & Ressler, 2012; Jovanovic et al., 2010, 2012). Given the increased

prevalence of these disorders in women, recent work has begun to focus on elucidating some of the underlying causes of this discrepancy. Multiple studies have found enhanced fear acquisition, increased resistance to fear extinction, increased fear generalization and deficits in safety learning in females but not males; all suggesting reduced inhibitory control of fear (Baran *et al.*, 2009; Gresack *et al.*, 2009; Baker-Andresen *et al.*, 2013; Matsuda *et al.*, 2015; Day, Reed, & Stevenson, 2016). Consistent with these studies, we found that reversal learning was slowed in male but not female rats trained in differential fear conditioning compared to tone preexposure controls. Our findings are consistent with recent work demonstrating an identical effect and suggests that, in females, the safety signal did not acquire the inhibitory characteristics of learned safety, specifically retarded acquisition of fear to the CS- following reversal training (Day, Reed, & Stevenson, 2016). Together with previous work, these findings suggest that fear generalization in females is the result of impaired safety learning during differential fear conditioning.

Sex Difference in Retrieval- Induced Synaptic Destabilization

It is generally accepted that memory retrieval results in the transient destabilization of synapses modified during memory consolidation (Nader, Schafe & Le Doux, 2000; Jarome *et al.*, 2011). Retrieval induced synaptic destabilization is associated with increased proteolytic activity and trafficking of AMPA receptor subunits, notably the endocytosis of GluR1 and GluR2 (Lee *et al.*, 2008; Hong *et al.*, 2013; Jarome *et al.*, 2015). While the mechanisms underlying fear retrieval and reconsolidation have been extensively studied, less is known about similar mechanisms in safety learning despite their reliance on a similar network of brain structures; including the amygdala and medial prefrontal cortex (Sangha *et al.*, 2013; Salzman et al., 2007; Shabel and Janak, 2009; Rogan *et al.*, 2005; Likhtik et al., 2014).

In the amygdala, increased responsiveness of neurons in the BLA has been associated with retrieval of aversive and safe stimuli while synapse size in LA is dependent upon stimulus valence (Ostroff *et al.*, 2010; Genud-Gabai *et al., 2013;* Sangha et al., 2013; Sierra-Mercado et al., 2011). Considering these findings, we found that retrieval of learned safety, like fear, results in the endocytosis of GluR1 and GluR2 in the amygdala of male rats, indicating that safety and fear learning may rely on a similar population of cells within the amygdala for memory consolidation or retrieval. In support of this, we also found that CS- retrieval was sufficient to induce reconsolidation of the CS+ memory in the amygdala, rendering it sensitive to disruption with anisomycin. To our knowledge this is first study to examine reconsolidation and the interaction between fear and safety memories and our results indicate that differences between CS+ and CS- elicited behavior may be due to differences in modulation of amygdala activity by other brain regions, rather than differences the consolidation of fear and safety in the amygdala.

Unlike males, GluR1, GluR2, and GluR3 in the amygdala of female rats did not differ between retrieval conditions. The absence of AMPAR endocytosis following CS- retrieval in females is consistent with our previous experiment and further supports the idea that safety learning is impaired in females. Unexpectedly, there is also an absence of AMPAR endocytosis in the amygdala of females following CS+ retrieval. In contrast with males, pharmacological blockade of reconsolidation in females following CS- retrieval had no effect on fear memory, most likely because the memory trace for the CS- was never consolidated in the first place. A primary requirement for retrieval induced synaptic destabilization is active recall of the memory. Without retrieval, memories cannot be pharmacologically disrupted (Nader, Schafe, & Le Doux, 2000; Jarome *et al.*, 2016). This suggests that in male rats, retrieval of the CS- or CS+ may be destabilizing synapses encoding memory of discrimination training more generally, rather than

discrete fear and safety memories. Currently, it is thought that reconsolidation and memory updating of auditory fear is driven by contextual novelty rather than prediction error, which is defined as a change in the relationship between previously acquired aversive cues and the ability of these cues to predict the occurrence of aversive event. Though some cases have found in both rodent and human subjects, that prediction error or alterations in the relationship between learned cues and their associated outcomes can govern the ability of a retrieved memory to undergo reconsolidation (Diaz-Mataix *et al.*, 2013; Sevenster *et al.*, 2013; Jarome *et al.*, 2015). In the present study, it is unlikely that the amygdala plays a passive role during fear memory consolidation or retrieval in females, despite our null findings in AMPAR endocytosis. In view of recent work showing elevated contextual fear generalization in females compared to males, it is possible that the retrieval session used in the present study did not provide sufficient novelty or prediction error, regarding information related to the stimuli, to induce synaptic destabilization in females. In the present study, stimulus retrieval sessions were conducted in a shifted context (different scent, brightness, and floor color) but it is possible that females generalized some part of the handling procedure despite significant adaptation to the procedures (Keiser *et al.*, 2016; Lynch *et al.*, 2016). In line with this, it possible that the conditions required to induce reconsolidation-like mechanisms differ between males and females but more work is necessary to identify possible sex differences in reconsolidation boundary conditions (Flint, Valentine, & Papandrea, 2007).

In comparison to the amygdala, memory retrieval of fear or safety did not significantly affect levels of synaptic GluR1 or GluR2 in the dorsal hippocampus in either sex, although GluR3 was significantly greater in females following CS+ retrieval compared to CS- retrieval. Interestingly, this effect was not observed in males. Work on the role of GluR3 in memory is

limited in comparison to GluR1 and GluR2. Expression of GluR3 is higher in the mPFC than in the hippocampus and amygdala and activation of these receptors through systemic injections of PEPA, an AMPAR potentiator, immediately before extinction training significantly reduced freezing behavior during extinction training and retention test (Zushida *et al.*, 2007). Given deficits in contextual fear conditioning and spatial learning in females, it is possible that the sex specific difference in GluR3 expression in the hippocampus following fear or safety retrieval may be indicative of sex differences in consolidation of differential fear conditioning.

Like the amygdala, the mPFC is thought to play a significant role in fear extinction and discrimination (Burgos-Robles et al., 2007; Corcoran & Quirk, 2007; Laurent & Westbrook, 2008; Sotres-Bayon & Quirk, 2010). Specifically, activity in the prelimbic region of the mPFC increases during fear expression in response to CS+ presentations compared to the infralimbic region which is active during safety expression and extinction retention tests (Senn et al., 2014; Sotres-Bayon et al., 2012; Hefner et al., 2008; Herry and Mons, 2004; Knapska and Maren, 2009). In the present study, the level of synaptic GluR1, GluR2, or GluR3 in the prelimbic mPFC did not differ significantly across retrieval conditions for male or female rats. Further, chymotrypsin-like and trypsin-like proteasome activity did not differ across retrieval conditions for either sex. Moreover, pretraining blockade of NR2B-containing NMDA receptors in the prelimbic cortex had no effect on fear memory or discrimination but did facilitate conditioned freezing in male rats during the training session. The enhancement of freezing behavior in male rats may indicate possible sex differences in NMDA receptor function within the prelimbic area but given that this manipulation did not affect fear memory or discrimination, it is likely that other brain regions may play a more prominent role in mediating the sex difference in learned safety such as the IL, insular cortex and ventral hippocampus.

Influence of Estrogens on Differential Fear Conditioning

An increasing body of evidence points to a role of estrogens in mediating sex differences in fear conditioning and extinction (Milad *et al.*, 2009; Baker-Andresen *et al.*, 2013; Baran *et al.*, 2009; 2010; Ribiero *et al.*, 2010). In general, exogenous estrogen treatment or naturally cycling endogenous estrogens have been found to facilitate fear conditioning, fear extinction and contribute generalization of contextual fear in human and rodent studies (Jasnow, Schulkin & Pfaff, 2006; Zeidan *et al.*, 2011; Lynch *et al.*, 2013; Cover *et al.*, 2014; Milad *et al.*, 2009; Graham and Dahler, 2016). Less has been done exploring the potential role of estrous phase in discrimination tasks using discrete auditory stimuli. A few studies have reported stimulus discrimination impairments in females, as measured by freezing behavior or fear-potentiated startle (Day, Reed, & Stevenson, 2016; Toufexis *et al.*, 2007). The former study conducted discrimination training over 3 consecutive days and were therefore unable to account for estrous phase in their analysis of conditioned freezing (Day, Reed, & Stevenson, 2016). The latter study found that estradiol treatment increased generalization. Their manipulation was in females given ovariectomies, a procedure which may disrupt the distribution of estrogen receptors (Toufexis *et al.*, 2007; Mohamed & Abdel-Rahman, 2000).

Currently, our data indicate that estrous phase during discrimination training or retrieval did not affect fear memory or discrimination between fear and safety. Despite this null result, it is possible that sex differences in estrogen receptor distribution may significantly contribute to differences in fear discrimination. For example, a virally-mediated increase in hippocampal ERα enhanced activation of plasticity related signaling and spatial memory in OVX females in the absence of exogenous estrogen (Witty *et al.*, 2012). Furthermore, ERβ expression is sexually

dimorphic in the rat brain. Specifically, females show greater expression in the anteroventral periventricular nucleus (AVPV) and the ventromedial hypothalamus and hippocampus (VMH; Ikeda et al., 2003; Orikasa et al., 2002; Zuloaga *et al.*, 2016; Zhang *et al.*, 2002). Sexual dimorphisms in estrogen receptor expression may have a significant impact on fear-related behavior, especially given the opposing functions of ERα and ERβ (Toufexis *et al.*, 2007). More work is needed in this area and specific exploration of estrogen receptor distribution in brain regions involved in regulating fear would have immediate impact on our understanding the role of estrogens in fear behavior.

Conclusion

Recent work has begun to elucidate some of the mechanisms underlying sex differences in aversive and emotional learning. Here we confirm previous reports by showing that discrimination deficits in female rats were the result of impaired safety learning. Moreover, we extend this work by demonstrating a deficit in retrieval induced synaptic destabilization in female but not male rats, evidenced by the absence of retrieval induced AMPA receptor endocytosis and increased proteasome activity. To further confirm the absence of safety memory in the amygdala of females, CS- retrieval was not sufficient to allow for anisomycin blockade of CS+ reconsolidation in females. Collectively these results point to an impairment in safety learning in females that may lead to generalized fear responses.

References

Abel, T. Nguyen, P.V., Barad, M., Deuel, T.A., Kandel, E.R. & Bourtchoulazde, R. (1997). Genetic demonstration of a role for PKA in the late phase of LTP and in hippocampus-based long-term memory. *Cell, 88,* 615-622.

Adams, J.P. & Sweatt, J.D. (2002). Molecular psychology: roles for the ERK MAPK kinase cascade in memory. *Annual Review of Pharmacology and Toxicology, 42,* 135-163.

Amano, T. et al. (2010). Synaptic correlates of fear extinction in the amygdala. Nat. Neurosci. 13, 489–494

Aspergis-Schoute, A.M., Gillan, C.M., Fineberg, N.A., Fernandez-Egea, E., Sahakian, B.J., and Robbins, T.W. (2017). Neural basis of impaired safety signaling in obsessive compulsive disorder. *PNAS. 114*(12). 3216-3221.

Bailey DJ, Kim JJ, Sun W, Thompson RF, Helmstetter FJ (1999) Acquisition of fear conditioning in rats requires the synthesis of mRNA in the amygdala. *Behav Neurosci*113. 276–282.

Baker-Andresen, D., Flavell, C.R., Li, X. and Bredy, T.W. (2013). Activation of BDNF signaling prevents the return of fear in female mice. *Learning & Memory.* 20. 237-240.

Banarjee, S., Neveu, P. and Kosik, K.S. (2009). A coordinated local translational control point at the synapse involving relief from silencing and MOV10 degradation. *Neuron*. 64. 871-884.

Baran, S.E., Armstrong, C.E., Niren, D.C., Hanna, J.J. and Conrad, C.D. (2009). Chronic stress and sex differences on the recall of fear conditioning and extinction. *Neurobiology of Learning and Memory. 91*(3). 323-332.

Baran, S.E., Armstrong, C.E., Niren, D.C., and Conrad, C.D. (2010). Prefrontal cortex lesions and sex differences in fear extinction and perseveration. *Learning & Memory.*17. 267-278.

Bedford, L., Paine, S., Sheppard, P.W., Mayer, R.J. & Roelofs, J. (2010). Assembly, structure and function of the 26S proteasome. *Trends in Cell Biology, 20,* 391-401.

Belova, M.A., Paton, J.J., Morrison, S.E., and Salzman, C.D. (2007). Expectation modulates neural responses to pleasant and aversive stimuli in primate amygdala. *Neuron. 55*(6). 970-984.

Beyeler, A., Eckhardt, C.A., Tye, K.M. (2014). Deciphering memory function with optogenetics. *Progress in molecular biology and translational science.* 122. 341-390.

Bingol, B. & Schuman, E.M. (2006). Activity-dependent dynamics and sequestration of proteasomes in dendritic spines. *Nature, 441*, 1144-1148.

Bouton, M. E., Westbrook, R. F., Corcoran, K. A., and Maren, S. (2006). Contextual and temporal modulation of extinction: behavioral and biological mechanisms. *Biol. Psychiatry* 60, 352–360. doi: 10.1016/j.biopsych.2005.12.015

Bouton, M.E. (2004). Context and behavioral processes in extinction. *Learning & Memory. 11*. 485-494.

Bremner JD, Licinio J, Darnell A, Krystal JH, Owens M, Southwick SM, Nemeroff CB, Charney DS. (1997). Elevated CSF corticotropin-releasing factor concentrations in posttraumatic stress disorder. *Am. J. Psychiatry*. 154:624–629.

Burgos-Robles, A., Vidal-Gonzalez, I., Santini, E., Quirk, G.J. (2007). Consolidation of fear extinction requires NMDA receptor-dependent bursting in the ventromedial prefrontal cortex. *Neuron.* 53. 871–880.

Coffey, C.E., Wilkinson, W.E., Weiner, R.D., Parashos, I.A., Djang, W.T., Webb, M.C., Figiel, G.S., and Spritzer, C.E. (1993). Quantitative cerebral anatomy in depression. A controlled magnetic resonance imaging study. *Arch Gen Psychiatry. 50*(1). 7-16.

Collins, D.R. and Pare, D. (2000). Differential fear conditioning induces reciprocal changes in the sensory responses of lateral amygdala neurons to the CS+ and CS-. *Learning & Memory. 7*(2). 97-103.

Conde, F., Maire-Lepoivre, E., Audinat, E. and Crepel, F. (1995). Afferent connections of the medial frontal cortex of the rat. II. cortical and subcortical afferents. *Journal of Comparative Neurology*. *352* (4). 567-593.

Corcoran, K.A. and Quirk, G.J. (2007). Recalling safety: cooperative functions of the ventromedial prefrontal cortex and the hippocampus in extinction. *CNS Spectrums. 12* (3). 200-206.

Cover, K.K., Maeng, L.Y., Lebron-Milad, K., and Milad, M.R. (2014). Mechanisms of estradiol in fear circuitry: implication for sex differences in psychopathology. *Translational Psychiatry.* 4. 1-9.

Dash, P.K., Hochner, B. and Kandel, E.R. (1990). Injection of the cAMP-responsive element into the nucleus of Aplysia sensory neurons blocks long-term faciliation. *Nature*. *345*(62777). 718-721.

Davis, M. and Whalen, P.J. (2001). The amygdala: vigilance and emotion. *Molecular Psychiatry.* 6. 13-34.

Djakovic, S.N., Schwarz, L.A., Barylko, B., DeMartino, G.N., and Patrick, G.N. (2009). Regulation of the proteasome by neuronal activity and calcium/calmodulin-dependent protein kinase II. *Journal of Biological Chemistry.* 284. 26655-26665.

Dong, C., Bach, S.V., Haynes, K.A. and Hegde, A.N. (2014). Proteasome modulates positive and negative translational regulators in long-term synaptic plasticity. *Journal of Neuroscience. 34*(9). 3171-3182.

Ehlers, M.D. (2003). Activity levels control postsynaptic composition and signaling via the ubiquitin-proteasome system. *Nature Neuroscience, 6*, 231-242.

Ehrlich, I. et al. (2009) Amygdala inhibitory circuits and the control of fear memory. Neuron 62, 757–771

Fanselow, M.S. (1990). Factors governing one-trial contextual conditioning. *Animal Learning & Behavior*. *18*(3). 264-270.

Fanselow, M.S. and LeDoux, J.E. (1999). Why we think plasticity underlying Pavlovian fear conditioning occurs in the basolateral amygdala. *Neuron. 23*(2). 229-232.

Fendt, M. and Fanselow, M.S. (1999). The neuroanatomical and neurochemical basis of conditioned fear. *Neuroscience & Biobehavioral Reviews. 23*(5). 743-760.

Fonseca, R., Vabulas, R.M., Hartl, F.U., Bonhoeffer, T. & Nagerl, U.V. (2006). A balance of protein synthesis and proteasome-dependent degradation determines the maintenance of LTP. *Neuron, 52*, 239-245.

Genud-Gabai, R. et al. (2013) Safety signals in the primate amygdala. J. Neurosci. 33, 17986–17994

Gewirtz, J.C., Falls, W.A., and Davis, M. (1997). Normal conditioned inhibition and extinction of freezing and fear-potentiated startle following electrolytic lesions of medial prefrontal cortex in rats. *Behavioral Neuroscience.* 111. 712-726.

Gilmartin, M.R. and Helmstetter, F.J. (2010). Trace and contextual fear conditioning require neural activity and NMDA receptor-dependent transmission in the medial prefrontal cortex. *Learning & Memory.* 289-296.

Gilmartin, M.R., Balderston, N.L. and Helmstetter, F.J. (2014). Prefrontal cortical regulation of fear learning. *Trends in Neuroscience. 37*(8). 455-464.

Gilmartin, M.R., Kwapis, J.L., and Helmstetter, F.J. (2013). NR2A- and NR2B-containing NMDA receptors in the prelimbic medial prefrontal cortex differentially mediate trace, delay, and contextual fear conditioning. *Learning & Memory. 20*(6). 290-294.

Glover, E.M., Jovanovic, T., and Norrholm, S.D. (2015). Estrogen and extinction of fear memories: implications for posttraumatic stress disorder. *Biological Psychiatry. 78*(3). 178-185.

Graham, B.M. and Daher, M. (2016). Estradiol and progesterone have opposing role in the regulation of fear extinction in female rats. *Neuropsychopharmacology. 41*(3). 774-780.

Gresack, J.E., Schafe, G.E., Orr, P.T., and Frick, K.M. (2009). Sex differences in contextual fear conditioning are associated with differential ventral hippocampal extracellular signal-regulated kinase activation. *Neuroscience*. 159. 451-467.

Grillon, C., and Ameli, R. (2001). Conditioned inhibition of fear potentiated startle and skin conductance in humans. *Psychophysiology.* 38. 807-815.

Heldt, S.A. and Falls, W.A. (2006). Posttraining lesions of the auditory thalamus, but not cortex, disrupt the inhibition of fear conditioning to an auditory stimulus. *European Journal of Neuroscience. 23*(3). 765-779.

Helmstetter FJ, Bellgowan PS (1994) Effects of muscimol applied to the basolateral amygdala on acquisition and expression of contextual fear conditioning in rats. *Behav Neurosci* 108. 1005–1009

Helmstetter, F.J. (1992). Contribution of the amygdala to learning and performance of conditional fear. *Physiological Behavior. 51*(6). 1271-1276.

Helmstetter, F.J., Parsons, R.G., and Gafford, G.M. (2008). Macromolecular synthesis, distributed synaptic plasticity, and fear conditioning. *Neurobiology of Learning and Memory.* 89. 324-337.

Herry, C. and Mons, N. (2004). Resistance to extinction is associated with impaired immediate early gene induction in medial prefrontal cortex and amygdala. *European Journal of Neuroscience. 20*(3). 781-790.

Hoover, W.B. and Vertes, R.P. (2007). Anatomical analysis of afferent projections to the medial prefrontal cortex in the rat. *Brain Structure and Function. 212*(2). 149-179.

Hong, I., Kim, J., Kim, J., Lee, S., Ko, H.G., Nader, K., Kaang, B.K., Tsien, R.W., and Choi, S. (2013). AMPA receptor exchange underlies transient memory destabilization on retrieval. *PNAS. 110*(20). 8218-8223.

Hugues S., Chessel A., Léna I., Marsault R., Garcia R. (2006). Prefrontal infusion of PD098059 immediately after fear extinction training blocks extinction-associated prefrontal synaptic plasticity and decreases prefrontal ERK2 phosphorylation. *Synapse*.60. 280–287.

Hwang, M.J., Zsido, R.G., Song, H., Pace-Schott, E.F., Klahr Miller, K., Lebron-Milad, K., Marin, M.F., and Milad, M.R. (2015). Contribution of estradiol levels and hormonal contraceptices to sex differences with the fear network during fear conditioning and extinction. *BMC Psychiatry. 15*(295). DOI 10.1186/s12888-015-0673-9

Izquierdo, I., Furini, C.R.G., and Myskiw, J.C. (2016). Fear memory. *Physiological Reviews. 96*(2). 695-750.

Johansen, J.P., Cain, C.K., Ostroff, L.E., and LeDoux, J.E. (2011). Molecular mechanisms of fear learning and memory. *Cell. 147*(3). 509-524.

Jarome, T.J. and Helmstetter, F.J. (2014). Protein degradation and protein synthesis in long-term memory formation. *Frontiers in Molecular Neuroscience. 7*(61).

Jarome, T.J., Ferrara, N.C., Kwapis, J.L., and Helmstetter, F.J. (2015). Contextual information drives the reconsolidation-dependent updating of retrieved fear memories. *Neuropsychopharmacology. 40*(13). 3044-3052.

Jarome, T.J., Ferrara, N.C., Kwapis, J.L., and Helmstetter, F.J. (2016). CaMKII regulates proteasome phosphorylation and activity and promotes memory destabilization following retrieval. *Neurobiology of Learning and Memory.* 128. 103-109.

Jarome, T.J., Kwapis, J.L., Ruenzel, W.L., Helmstetter, F.J. (2013). CaMKII, but not protein kinase A regulates Rpt6 phosphorylation and proteasome activity during the formation of long-term memories. *Frontiers in Behavioral Neuroscience.* 7. 115.

Jarome, T.J., Werner, C.T., Kwapis, J.L. and Helmstetter, F.J. (2011). Activity dependent protein degradation is critical for the formation and stability of fear memory in the amygdala. *PLoS ONE. 16*(9). e24349.

Jovanovic, T. et al. (2009) Posttraumatic stress disorder may be associated with impaired fear inhibition: relation to symptom severity. Psychiatry Res. 167, 151–160

Jovanovic, T., Kazama, A.M., Bachevalier, J., Davis, M. (2012). Impaired safety signal learning may be a biomarker of PTSD. *Neuropharmacology*. 62:695–704

Jovanovic, T., Norrholm, S.D., Blanding, N.Q., Davis, M., Duncan, E., Bradley, B., and Ressler, K.J. (2010). Impaired fear inhibition is a biomarker of PTSD but not depression. Depression and Anxiety.2010;27:244–251

Korzus, E. (2015) Prefrontal Cortex in Learning to Overcome Generalized Fear. *Journal of Experimental Neuroscience.* 53–56 doi:10.4137/JEN.S26227.

Keiser, A.A., Turnbull, L.M., Darian, M.A., Feldman, D.E., Song, I., and Tronson, N.C. (2016). Sex differences in context fear generalization and recruitment of hippocampus and amygdala during retrieval. *Neuropsychopharmacology.* doi: 10.1038/npp.2016.174

Kida, S., Josselyn, S.A., Pena de Ortiz, S., Kogan, J.H., Chevere, I., Masushiqe, S. & Silva, A.J. (2002). CREB required for the stability of new and reactivated fear memories. *Nature Neuroscience, 5*, 348-355.

Klavir, O. et al. (2012) Low-frequency stimulation depresses the primate anterior-cingulate-cortex and prevents spontaneous recovery of aversive memories. *Journal of Neuroscience.* 32. 8589–8597.

Klavir, O., Genud-Gabai, R., and Paz, R. (2013). Functional connectivity between amygdala and cingulate cortex for adaptive aversive learning. *Neuron. 80*(5). 1290-1300.

Knapska, E. et al. (2012) Functional anatomy of neural circuits regulating fear and extinction. Proc. Natl. Acad. Sci. U.S.A. 109, 17093–17098

Knapska, E., and Maren, S. (2009). Reciprocal patterns of c-Fos expression in the medial prefrontal cortex and amygdala after extinction and renewal of conditioned fear. *Learning & Memory.* 16. 486–49.

Kwapis, J.L., Jarome, T.J., Lonergan, M.E. & Helmstetter, F.J. (2009). Protein kinase Mzeta maintains fear memory in the amygdala but not in the hippocampus. *Behavioral Neuroscience, 123,* 844-850.

LaLumiere, R.T. (2014). Optogenetic dissection of amygdala functioning. *Frontiers in Behavioral Neuroscience.* 8. doi.10.3389.

Laurent V. and Westbrook RF (2008). Distinct contributions of the basolateral amygdala and the medial prefrontal cortex to learning and relearning extinction of context conditioned fear. *Learning & Memory.*15. 657–666.

Laurent, V. and Westbrook RF (2009). Inactivation of the infralimbic but not the prelimbic cortex impairs consolidation and retrieval of fear extinction. *Learning & Memory*. 16. 520–529.

Lavond, D.G., Kim, J.J., and Thompson, R.F. (1993). Mammalian brain substrates of aversive classical conditioning. *Annual Reviews in Psychology*. 44: 317-342

Lebron-Milad, K., Abbs, B., Milad, M.R., Linnman, C., Rougemount-Bucking, A., Zeidan, M.A., Holt, D.J., and Goldstein, J.M. (2012). Sex differences in the neurobiology of fear conditioning and extinction: a preliminary fMRI study of shared sex differences with stress-arousal circuitry. *Biology of mood & anxiety disorders. 2*(7). 2-7.

LeDoux, J.E. (2000). Emotion circuits in the brain. *Annual Reviews in Neuroscience.* 23. 155-184.

LeDoux, J.E., Iwata, J., P., Cichetti, P., and D.J., Reis (1988). Different projections of the central amygdaloid nucleus mediate autonomic and behavioral correlates of conditioned fear. *Journal of Neuroscience. 8*(7). 2517-2529.

Lee, J.L. (2008). Memory reconsolidation mediates the strengthening of memories by additional learning. *Nature Neuroscience, 11*, 1264-1266.

Likhtik, E. and Paz, R. (2015). Amygdala-prefrontal interactions in (mal)adaptive learning. *Trends in Neuroscience. 238*(3).158-166.

Likhtik, E. et al. (2014) Prefrontal entrainment of amygdala activity signals safety in learned fear and innate anxiety. *Nature Neuroscience*. 17. 106–113.

Likhtik, E., Popa, D., Apergis-Schoute, J., Fidacaro, G.A., Pare, D. (2008). Amygdala intercalated neurons are required for expression of fear extinction. *Nature. 45(*4). 642–64.

Lin, C.H., Lee, C.C., and Gean, P.W. (2003). Involvement of a calcineurin cascade in amygdala depotentiation and quenching of fear memory. *Molecular Pharmacology.* 63. 44-52.

Lissek, S., Rabin, S.J., McDowell, D.J., Dvir, S., Bradford, D.E., Geraci, M., Pine, P.S., and Grillon, C. (2010). Impaired discriminative fear-conditioning resulting from elevated fear responding to learned safety cues among individuals with panic disorder. *Behaviour Research and Therapy. 47*(2). 111-118.

Livneh, U. and Paz, R. (2012) Amygdala-prefrontal synchronization underlies resistance to extinction of aversive memories. *Neuron*. 75.133–142

Livneh, U. and Paz, R. (2012) Aversive-bias and stage-selectivity in neurons of the primate amygdala during acquisition, extinction, and overnight retention. J. Neurosci. 32, 8598–8610

Lopez-Salon, M., Alonson, M., Vianna, M.R., Mello e Souza, T., Izquierdo, I., Pasquini, J.M., Medina, J.H. (2001). The ubiquitin-proteasome cascade is required mammalian long-term memory formation. *European Journal of Neuroscience, 14*, 1820-1826.

Lynch, J.F., Winiecki, P., Vanderhoof, T., Riccio, D.C., Jasnow, A.M. (2016). Hippocampal cytosolic estrogen receptors regulate fear generalization in females. *Neurobiology of Learning and Memory.* 130. 83-92.

MacLennan, A.J., Jackson, R.L., and Maier, S.F. (1980). Conditioned analgesia in the rat. *Bulletin of the Psychonomic Society. 15*(6). 387-390.

Mahan, A.L. & Ressler, K.J. (2012). Fear conditioning, synaptic plasticity and the amygdala: implications for posttraumatic stress disorder. *Trends in Neuroscience. 35*(1). 24-35.

Maren, S. (1999). Neurotoxic basolateral amygdala lesions impair learning and memory but not the performance of conditional fear in rats. *Journal of Neuroscience.* 19. 8696–8703.

Maren, S. (2015). Out with the old and in with the new: Synaptic mechanisms of extinction in the amygdala. *Brain Research.* 1621. 231-238.

Matsuda, S., Matsuzawa, D., Ishii, D., Tomizawa, H., Sutoh, C., and Shimizu, E. (2015). Sex differences in fear extinction and involvements of extracellular signal-regulated kinase (ERK). *Neurobiology of Learning and Memory.* 123. 117-124.

McDonald, A.J. (1998) Cortical pathways to the mammalian amygdala. Prog. Neurobiol. 55, 257–332.

McGaugh, J.L. (2004). The amygdala modulates the consolidation of memories of emotionally arousing experiences. *Annual Review in Neuroscience*. 27. 1-28.

Milad, M.R., Pitman, R.K., Ellis, C.B., Gold, A.L., Shin, L.M., Lasko, N.B., Zeidan, M.A., Handwerger, K., Orr, S.P., Rauch, S.L. (2009). Neurobiological basis of failure to recall extinction memory in posttraumatic stress disorder. Biol Psychiatry. 66:1075–1082.

Milad, M.R., and Quirk, G.J. (2002). Neurons in medial prefrontal cortex signal memory for fear extinction. *Nature*. 420. 70–74.

Milad, M.R. and Quirk, G.J. (2012). Fear extinction as a model for translational neuroscience: ten years of progress. *Annual Reviews in Psychology.* 63. 129-151.

Milad, M.R., Vidal-Gonzalez, I., Quirk, G.J.(2004) Electrical stimulation of medial prefrontal cortex reduces conditioned fear in a temporally specific manner. *Behavioral Neuroscience*. 118. 389-394.

Mohamed, M.K. and Abdel-Rahman, A.A (2000). Effect of long-term ovariectomy and estrogen replacement on the expression of estrogen receptor gene in female rats. *European Journal of Endocrinology. 142*(3). 301-314.

Morgan, M.A., LeDoux, J.E.(1995) Differential contribution of dorsal and ventral medial prefrontal cortex to the acquisition and extinction of conditioned fear in rats. *Behavioral Neuroscience*. 109:681–688.

Moustafa, A.A., Gilbertson, M.W., Orr, S.P., Herzallah, M.M., Servatius, R.J., and Myers, C.E. (2013). A model of amygdala-hippocampal-prefrontal interaction in fear conditioning and extinction in animals. *Brain Cognition*. *81*(1).

Myers, K.M. and Davis, M. (2007). Mechanisms of fear extinction. *Molecular Psychiatry. 12*(2). 120-150.

Myers, K.M., Davis, M. (2007) Mechanisms of fear extinction. *Molecular Psychiatry.* 12. 120–150.

Nader, K., Schafe, G.E., and Le Doux, J.E. (2000). Fear memories require protein synthesis in the amygdala for reconsolidation after retrieval. *Nature. 406*(6797). 722-726.

Nofrey, B.S., Ben-Shahar, O.M., and Brake, W.G. (2008). Estrogen abolishes latent inhibition in ovariectomized female rats. *Brain and Cognition*. *66*(2). 156-160.

Orsini, C.A. et al. (2013) Ensemble coding of context-dependent fear memory in the amygdala. *Frontiers in Behavioral Neuroscience*. 7, 199.

Ostroff, L.E., Cain, C.K., Bedont, J., Monfils, M.H. and LeDoux, J.E. (2010). Fear and safety learning differentially affect synapse size and dendritic translation in the lateral amygdala. *Proceedings of the National Academy of Sciences of the USA. 107*(20). 9418-9423.

Pape, H.C. and Pare, D. (2010). Plastic synaptic networks of the amygdala for the acquisition, expression, and extinction of conditioned fear. *Physiological Reviews.* 90. 419-463.

Parsons, R.G., Gafford, G.M. & Helmstetter, F.J. (2006b). Translational control via the mammalian target of rapamycin pathway is critical for the formation and stability of long-term fear memories in amygdala neurons. *Journal of Neuroscience, 26*, 12977-12983.

Parsons, R.G., Gafford, G.M., Baruch, D.E., Riedner, B.A. & Helmstetter, F.J. (2006a). Long-term stability of fear memory depends on the synthesis of protein but not mRNA in the amygdala. *European Journal of Neuroscience, 23*, 1853-1859.

Pollak, D.D., Monje, F.J., Zuckerman, L., Denny, C.A., Drew, M.R. and Kandel, E.R. (2008). An animal model of a behavioral intervention for depression. *Neuron.60*(1). 149-161.

Quinlan, M.G., Duncan, A., Loiselle, C., Graffe, N., and Brake, W.G. (2010). Latent inhibition is affected by phase of the estrous cycle in female rats. *Brain and Cognition*. *74*(3). 244-248.

Rauch, S.L., Shin, L.M., Phelps, E.A. (2006). Neurocircuitry models of posttraumatic stress disorder and extinction: human neuroimaging research-past, present, and future. *Biological Psychiatry.*60:376–382.

Reinecke, A., Becker, E.S., Hoyer, J., and Rinck, M. (2010). Generalized implicit fear associations in generalized anxiety disorder. *Depress. Anxiety*. 27, 252–259

Reis, D.S., Jarome, T.J. and Helmstetter, F.J. (2013). Memory formation for trace fear conditioning requires ubiquitin-proteasome mediated protein degradation in the prefrontal cortex. *Frontiers in Behavioral Neuroscience. 7*(150). 107-116.

Reis, D.S., Rotondo, E.K., Sehgal, M., and Helmstetter, F.J. (In Prep). Regulation of activity-dependent protein synthesis through proteolytic modulation of PP2A in the amygdala during auditory fear memory consolidation.

Rescorla, R.A. (1969). Pavlovian conditioned inhibition. *Psychology Bulletin.* 72. 77-94.

Rescorla, R.A. (1971). Summation and retardation tests of latent inhibition. *Journal of Comparative Physiological Psychology.* 75. 77-81.

Ribeiro, A.M., Barbosa, F.F., Godinho, M.R., Fernandes, V.S., Munguba, H., Melo, T.G., Barbosa, M.T., Eufrasio, R.A., Cabral, A., Izidio, G.S., and Silva, R.H. (2010). Sex differences in aversive memory in rats: Possible role of extinction and reactive emotional factors. *Brain and Cognition*. *74*(1). 145-151.

Rodrigues, S.M., Farb, C.R., Bauer, E.P., LeDoux, J.E. & Schafe, G.E. (2004). Pavlovian fear conditioning regulates Thr286 autophosphorylation Ca2+/calmodulin dependent protein kinase II at amygdala synapses. *Journal of Neuroscience, 24,* 3281-3288.

Rodrigues, S.M., Schafe, G.E. & LeDoux, J.E. (2001). Intra-amygala blockade of the NR2B subunit of the NMDA receptor disrupts the acquisition but not expression of fear conditioning. *Journal of Neuroscience, 21,* 6889-6896.

Rogan, M.T., Leon, K.S., Perez, D.L., and Kandel, E.R. (2005). Distinct neural signatures for safety and danger in the amygdala and striatum of the mouse. *Neuron. 46*(2). 309-320.

Rosenberg, T., Gal-Ben-Ari, S., Dieterich, D.C., Kreutz, M.R., Ziv, N.E., Gundelfinger, E.D., and Rosenblum, K. (2014). The roles of protein expression in synaptic plasticity and memory consolidation. *Frontiers in Molecular Neuroscience.* 7. doi:10.3389/fnmol.2014.00086.

Salzman et al., 2007;

Sangha, S., Chadick, J. Z. & Janak, P. H. (2013). Safety encoding in the basal amygdala. *J. Neurosci.* 33, 3744–3751.

Schafe, G.E. & LeDoux, J.E. (2000). Memory consolidation of auditory Pavlovian fear conditioning requires protein synthesis and protein kinase A in the amygdala. *Journal of Neuroscience, 20,* RC96.

Schiller, D. and Weiner, I. (2005). Basolateral amygdala lesions in the rat produce an abnormally persistent latent inhibition with weak preexposure but not with context shift. *Behavioural Brain Research.* 163. 115-121.

Senn, V., Wolff, S.B.E., Herry, C., Herry, C., Grenier, F., Ehrlich, I., Grundemann, J., Fadok, J.P., Muller, C., Letzkus, J.J., and Luthi, A. (2014). Long-range connectivity defines behavioral specificity of amygdala neurons. *Neuron. 81*(2). 428-437.

Shabel, S.J., and Janak, P.H. (2009). Substantial similarity in amygdala neuronal activity during conditioning emotional arousal. *Proceedings of the National Academy of Science. 106*(35). 15031-15036.

Shin, L.M., Rauch, S.L. and Pitman, R.K. (2006). Amygdala, medial prefrontal cortex, and hippocampal function in PTSD. *Ann N Y Acad Sci* 1071. 67–79

Sierra-Mercado D, Padilla-Coreano N, Quirk GJ.(2011). Dissociable roles of prelimbic and infralimbic cortices, ventral hippocampus, and basolateral amygdala in the expression and extinction of conditioned fear. *Neuropsychopharmacology*. 2011;36:529–538. Sotres-Bayon et al., 2012

Sotres-Bayon F, Quirk GJ. (2010). Prefrontal control of fear: more than just extinction. *Curr. Opin. Neurobiol.*20:231–235

Thomas, E., Burock, D., Knudsen, K., Deterding, E., and Yadin, E. (2013). Single unit activity in the lateral septum and central nucleus of the amygdala in the elevated plus-maze: a model of exposure therapy?. *Neuroscience Letters.* 548. 269-274.

Vidal-Gonzalez, I., Vidal-Gonzalez, B., Rauch, S.L., Quirk, G.J. (2006). Microstimulation reveals opposing influences of prelimbic and infralimbic cortex on the expression of conditioned fear. *Learning and Memory.* 13. 728–733

Vieira, P.A., Corches, A., Lovelace, J.W., Westbrook, K.B., Mendoza, M. and Korzus, E. (2015). Prefrontal NMDA receptors expressed in excitatory neurons control fear discrimination and fear extinction. *Neurobiology of Learning and Memory.* 119. 52-62.

Vieira, P.A., Lovelace, J.W., Corches, A., Rashid, A.J., Josselyn, S.A., and Korzus, E. (2014). Prefrontal consolidation supports the attainment of fear memory accuracy. *Learning & Memory.*21. 394-405.

Walf, A.A. and Frye, C.A. (2007). The use of the elevated plus maze as an assay of anxiety-related behavior in rodents. *Nature Protocols*. *2*(2). 322-328.

Wang, S.J. and Gean, P.W. (1999). Long-term depression of excitatory synaptic transmission in the rat amygdala. *Journal of Neuroscience.* 19:10656–10663.

Wilensky, A.E., Schafe, G.E., Kristensen, M.P., and LeDoux, J.E. (2006). Rethinking the fear circuit: the central nucleus of the amygdala is required for the acquisition, consolidation, and expression of Pavlovian fear conditioning. *The Journal of Neuroscience. 26*(48). 12387-12396.

Wilensky, A.E., Schafe, G.E., LeDoux, J.E., (1999). Functional inactivation of the amygdala before but not after auditory fear conditioning prevents memory formation. *J. Neurosci*. 19, RC48.

Zelikowsky, M., Hersman, S., Chawla, M.K., Barnes, C.A. and Fanselow, M.S. (2014). Neuronal ensembles in amygdala, hippocampus, and prefrontal cortex track differential components of contextual fear. *The Journal of Neuroscience.* 8462-8466.

Zhang, J., Cai, W.Q., Zhou, D.S., and Su, B.Y. (2002). Distribution differences of estrogen receptor beta immunoreactivity in the brain of adult male and female rats. *Brain Research. 935*(1-2). 73-80.

Zuloaga, D.G., Zuloaga, K.L., Hinds, L.R., Carbone, D.L., and Handa, R.J. (2014). Estrogen receptor beta expression in the mouse forebrain: age and sex differences. *Journal of Comparative Neurology*. *522*(2). 358-371.

Zushida, K., Sakurai, M., Wada, K., and Sekiguchi, M. (2007). Facilitation of extinction learning for contextual fear memory by PEPA: a potentiator of AMPA receptors. *Journal of Neuroscience. 27*(1). 158-166.

David S. Reis

EDUCATION

2015- 2017 **University of Wisconsin-Milwaukee** **Milwaukee, WI**

- Doctor of Philosophy in Experimental Psychology awarded in *August 2017*
- Advisor: Fred Helmstetter, Ph.D.
- Departmental Major: Neuroscience
- Minor: Neurobiology, Behavioral Analysis
- Dissertation: Sex differences in differential fear conditioning during the acquisition and consolidation of learned safety

2011- 2015 **University of Wisconsin-Milwaukee** **Milwaukee, WI**

- Master of Science in Experimental Psychology awarded in *May 2015*
- Advisor: Fred Helmstetter, Ph.D.
- Departmental Major: Neuroscience
- Minor: Neurobiology, Behavioral Analysis
- Thesis: Regulation of mTOR and ERK Signaling in the Amygdala Through Proteolytic Modulation of PP2A Activity Following Auditory Fear Learning

2007- 2011 **Carthage College** **Kenosha, WI**

- Bachelor of Arts in neuroscience awarded in *May 2011*
- Advisor: Daniel Miller, Ph.D.
- Major: Neuroscience
- Minor: Biology, Psychology
- Thesis: Selective amygdala lesions facilitate acquisition of signaled lever-press avoidance in Wistar-Kyoto and Sprague Dawley rats.
- Graduated *Cum Laude*

Publications

• **Reis, D.S.**, Rotondo, E.K., Sehgal, M., Helmstetter, F.J. (2017) Regulation of protein synthesis through proteolytic modulation of protein phosphatase 2A during fear memory consolidation. *In preparation.*

• **Reis, D.S.,** Jarome, T.J. and Helmstetter, F.J. (2013). Memory for trace fear conditioning requires ubiquitin-proteasome mediated protein degradation in the prefrontal cortex. *Frontiers in Behavioral Neuroscience. 7* (150).

Abstracts

• Trace fear conditioning can affect previously acquired context memories. *The Society for Neuroscience annual meeting, San Diego, CA -Fall 2016*

• Regulation of protein synthesis through proteolytic modulation of protein phosphatase 2A during fear memory consolidation. *The Society for Neuroscience annual meeting, Molecular and Cellular Cognition Society meeting, Chicago, IL - Fall 2015*

• Activity dependent proteolysis in the amygdala modulates protein synthesis in the amygdala and dorsal hippocampus during consolidation of fear conditioning. *The Society for Neuroscience annual meeting, Washington D.C. -Fall 2014; Pavlovian Society annual meeting, Seattle, Washington - Fall 2014.*

• Ubiquitin-proteasome mediated proteolysis regulates protein translation that is critical to the formation and consolidation of auditory delay fear memory in the amygdala. *The Society for Neuroscience annual meeting, San Diego, California- Fall 2013;*

• Degradation specific polyubiquitination is increased in the amygdala and prefrontal cortex following the acquisition of auditory delay or trace fear conditioning. *The Society for Neuroscience annual meeting, New Orleans, Louisiana- Fall 2012; Milwaukee SFN Chapter meeting, Spring 2013, Milwaukee, WI*

• Selective amygdala lesions facilitate acquisition of signaled lever-press avoidance in Wistar Kyoto and Sprague Dawley rats. *The Society for Neuroscience annual meeting, San Diego, California- Fall 2010*

Talks

• University of Milwaukee Neuroscience Seminar (Fall 2015)
Title: Degrade to Create: Regulation of protein synthesis by the ubiquitin-proteasome system during memory consolidation

• University of Milwaukee Neuroscience Seminar (Fall 2014)
Title: The ubiquitin-proteasome system regulates *de novo* protein synthesis in the amygdala during fear memory consolidation.

• University of Milwaukee Neuroscience Seminar (Spring 2014)
Title: Regulation of de novo protein synthesis by the ubiquitin-proteasome system in the consolidation of fear memory.

• Association of Graduate Students in Psychology Symposium (2014)
Title: The regulation of activity-dependent protein synthesis by the ubiquitin-proteasome system

• Carthage College Social Sciences Department Colloquium (*2010)*
Title: Anxiety vulnerability and conditioned avoidance behavior

AWARDS AND HONORS

• Department of Psychology Summer Research Fellowship *2014*
• UWM Student Travel Award *2012, 2013, 2014*
• Chancellor's Graduate Student Award *2011,2012*
• Carthage College Neuroscience Student of the Year Award *2011*
• 2010 Wisconsin Laboratory Association Grant recipient *2010*
• Summer research grant from the Stress and Motivated Behavior Institute *2009, 2010*
• Carthage College Travel Award. *2009, 2010*

RESEARCH EXPERIENCE

Helmstetter Behavioral Neuroscience Laboratory
• Graduate student 2011-*present*
• University of Wisconsin-Milwaukee

Carthage College Neuroscience Laboratory
• Laboratory/Research Assistant *2008-2011*

TEACHING EXPERIENCE

Teaching Assistant - University of Wisconsin-Milwaukee
• Research Methods in Psychology (Psych 325) *2011*
2015-2016

• Advanced Physiological Psychology (Psych 654) *2016-2017*

RESEARCH ASSISTANTSHIPS

Gradaute Research Assistant - University of Wisconsin-Milwaukee
• Helmstetter Behavioral Neuroscience Lab - *Dr. Fred Helmstetter*
2012- 2015

RELEVANT TECHNIQUES

• Cannulae implantation surgeries
• Microinjections
• Western blotting
• GST- pulldown assays
• Optogenetic fiber implantation
• Virus surgeries
• RNA isolation
• rt-qPCR

- Surface Sensing of Translation Assay (SUnSET Assay)
- Immunohistochemistry
- Enzymatic activity assays
- Animal Behavior (fear conditioning, elevated plus maze, open field)

CONFERENCES ATTENDED

- Pavlovian Society meeting — Jersey City, New Jersey - *2016*
- Society for Neuroscience annual meeting — San Diego, California - *2016*
- Society for Neuroscience annual meeting — Chicago, Illinois - *2015*
- Society for Neuroscience annual meeting — Washington D.C. - *2014*
- Pavlovian Society meeting — Seattle, Washington - *2014*
- Milwaukee SFN Chapter meeting — Milwaukee, Wisconsin - *2014*
- Society for Neuroscience annual meeting — San Diego, California - *2013*
- Milwaukee SFN Chapter meeting — Milwaukee, Wisconsin - *2013*
- Society for Neuroscience annual meeting — New Orleans, Louisiana - *2012*
- Pavlovian Society meeting — Milwaukee, Wisconsin - *2011*
- Society for Neuroscience annual meeting — San Diego, California - *2010*
- Pavlovian Society meeting — Baltimore, Maryland - *2010*
- Chicago Chapter of the Society for Neuroscience — Chicago, Illinois - *2010*
- Pavlovian Society meeting — Burlington, Vermont - *2009*

PROFESSIONAL MEMBERSHIPS

- Society for Neuroscience student member — *2012-present*
- Sigma Xi — *2011-present*
- Pavlovian Society student member — *2009-present*
- Tri-Beta National Biological Honor Society — *2008-present*