Vulnerability and Resilience to Stressful and Traumatic Events: Stressor Controllability, Sex Differences and Individual Differences

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Abstract of Dissertation

Post-traumatic stress disorder (PTSD) is a debilitating disorder that affects about 10% of the US population. But, few people develop PTSD after experience of a traumatic event, so studying factors that contribute to vulnerability and resilience is important for development of treatments and preventative measures. Rodent models are useful to study the underlying neurobiology contributing to vulnerability, and several behavioral paradigms exist to study various aspects of responses to stressful experiences. For example, fear conditioning and extinction paradigms are commonly used to study fear memory processes in rodents using ‘freezing’ behavior to measure fear levels. However, most studies have used only male rodents, despite women being more likely to develop PTSD. Female rodents are generally more active in many behavioral paradigms, so it is unclear whether measuring freezing is a complete measure of fear in female rodents, or if females display active fear behaviors.

In study 1, cohorts of male and female rats were tested on the fear conditioning and extinction paradigms to investigate active fear behavior. We identify, characterize, and quantify a novel, active fear behavior “darting” and show that females are much more likely to engage in this behavior. Additionally, only a subset of females displayed darting behavior, and being a darter was associated with improved outcomes at extinction recall. Future research is necessary to investigate the neural mechanisms involved in darting and what makes some females darters. It is also unclear how darting contributes to improved outcomes at extinction recall. One possibility is that darting represents attempted escape behavior. But, escape is not actually possible within the fear conditioning paradigm, so research using other paradigms is need to investigate effects of escape from stress in females.
The aim of study 2 was to investigate dendritic spine plasticity after escapable (ES) and inescapable stress (IS) in male and female rats. As past studies have shown in males, ES reliably protects from the adverse behavior effects of stress alone. These protective effects depend on activation of prelimbic cortex (PL) neurons that project to the dorsal raphe nucleus (DRN). Dendritic spines are the major site of excitatory input on principal neurons in the brain and can change shape and size in response to synaptic activation, and spine enlargement is associated with synaptic strengthening. Thus, dendritic spine investigation focused on PL-DRN neurons and randomly selected PL neurons and it was hypothesized that ES would induce circuit specific dendritic spine changes in PL-DRN neurons. The results show that in males, ES results in dendritic mushroom spine enlargement in PL-DRN neurons only, in line with the hypothesis. In IS males, results show that thin and mushroom spines were enlarged on PL-DRN neurons and randomly sampled neurons, indicating global stress effects on dendritic spines in PL.

Very recent research found that stressor controllability (ES) does not have the same protective effects on behavior in females and does not activate the PL-DRN circuit. But, it is unclear if the lack of PL-DRN neuron activation is due to a lack of control signaling inputs to PL in females, or because of other factors that would prevent control signaling inputs from being translated into PL-DRN neuron activation during ES in females. The results from study 2 show that ES and IS affect PL dendritic morphology differently, which suggests that control is detected in females on some level, without inducing PL-DRN neuron activation that is necessary for the protective effects to occur.

The results from study 1 set the stage for future research into active fear responses in females and how active fear responses can lead to improved outcomes. Study 2 adds to the sparse research on stressor controllability effects in female rodents and the results indicate that
stressor control is at least detected in female PL on some level, even though it is not translated into protective effects behaviorally. Overall, the results of both studies highlight the need to include female rodents in preclinical research on stress responses, and to perform both between and within sex analyses when studying vulnerability and resilience to stressful events.
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Table 1. Summary of sample sizes for each experimental group, for PL-DRN and unlabeled neurons.
IS = Inescapable Stress

Kde-plot = Kernel Density Plot

KS-test = Kolmogorov-Smirnov Test

LTP = Long Term Potentiation

mPFC = Medial Prefrontal Cortex

NMDA = N-Methyl-D-aspartate

PL = Prelimbic Cortex

PTSD = Post-traumatic Stress Disorder

SSRI = Selective Serotonin Reuptake Inhibitor

US = Unconditioned Stimulus

vIPAG = Ventrolateral Periaqueductal Gray
Chapter 1

Introduction

1.1 Factors of vulnerability to stress related psychopathology

Post-traumatic stress disorder (PTSD) is a debilitating disorder that can develop after experience of a traumatic event. While the majority of the U.S. population will experience a trauma at some point, less than 10% will go on to develop PTSD (Breslau, 2009). Thus, intervention methods should be targeted to high risk individuals in order to be successful and cost-effective. Meta analyses and analysis of large data sets using machine learning have helped identify several factors that robustly predict increased risk of developing PTSD after trauma (Brewin, Andrews, Valentine, & Holloway, 2000; Kessler et al., 2014). Risk factors include lack of social support, life stress and being female (Brewin et al., 2000). Women are more likely to develop PTSD even when controlling for type of trauma (i.e. excluding sexual abuse) (Tolin & Foa, 2006), thus it is possible that there are neurobiological factors that lead to increased vulnerability in women. Identification of neurobiological factors that contribute to vulnerability and resilience in men and women differently could improve targeted treatments and preventions.

1.2 Animal models of stress related psychopathology: Fear conditioning

Fear conditioning overview

Animal models are extremely useful for studying the neurobiological underpinnings of vulnerability and resilience to stressful experiences. There is currently no animal model that can completely model PTSD, however, several rodent models exist that can probe different aspects of the disorder. A major symptom of PTSD is experience of intrusive and persistent memories of
the traumatic event that can be triggered by cues that surrounded the event. Fear conditioning is a learning paradigm that can be used in rodents to study cue associated fear memories. In Pavlovian fear conditioning a neutral cue, such as a tone, is paired with a naturally aversive stimulus, such as a foot shock. Typically, the tone is presented for 30 seconds and is terminated by the delivery of the foot shock. The repeated pairing of these stimuli leads to formation of a fear memory that can be triggered by the tone alone (Yehuda & LeDoux, 2007). In a process called extinction learning, the tone is repeatedly presented without the shock and fear (traditionally measured through freezing responses) gradually declines (Milad, Rauch, Pitman, & Quirk, 2006). Extinction learning is relevant to PTSD, as patients suffering from this disorder are impaired in extinguishing fear memories (Milad et al., 2009). In rodents, the brain circuitry involved in fear conditioning and extinction is well characterized. One key brain area is the medial prefrontal cortex (mPFC), mediates conditioned fear expression and extinction mainly through two of its subdivisions: prelimbic (PL) and infralimbic (IL) cortex. PL mediates fear expression via its projections to the basolateral amygdala (BLA), which is critical for driving fear responses (Duvarci & Pare, 2014; Giustino & Maren, 2015; Maren, 2001). IL, on the other hand, is involved in fear extinction by inhibiting BLA and fear expression (Milad & Quirk, 2002; Quirk & Mueller, 2008). In PTSD patients, there is evidence for disrupted mPFC-amygdala circuit function (Milad et al., 2009; Stevens et al., 2013). In sum, fear conditioning and extinction in rodents is a useful model to test factors that contribute to poor extinction outcomes to identify possible factors that contribute to PTSD.

Sex-differences in fear conditioning

Women are more vulnerable to developing PTSD after experiencing trauma. Yet, most previous studies using the fear conditioning and extinction paradigm have used only male
rodents (Lebron-Milad et al., 2012; Shansky, 2015). Recently, females have been included in more studies, but results regarding sex-differences in fear conditioning and extinction have been mixed. For example, different studies found that females show either impaired extinction (Baran, Armstrong, Niren, Hanna, & Conrad, 2009) or enhanced extinction (Milad, Igoe, Lebron-Milad, & Novales, 2009) after auditory fear conditioning. These studies used relatively small sample sizes of 8-10 animals per group. This is common for behavioral neuroscience research, but may not be adequate to test for sex differences in vulnerability. The development of PTSD occurs in only a small subset of people exposed to trauma and therefore represents an extreme response. Testing small groups of rodents is likely to produce results that represent normal responses. To effectively study vulnerability and resilience, a better approach would be to test large cohorts of animals and identify behavioral outliers.

In a recent study, we did precisely that. We tested large cohorts of male and female rats (n=60 each sex) on fear conditioning and extinction (Gruene, Roberts, Thomas, Ronzio, & Shansky, 2015). As discussed above, success of extinction learning is measured by levels of freezing during an extinction retrieval test. High freezing at extinction retrieval is interpreted as poor extinction while low levels of freezing represent successful extinction. In rats, levels of extinction are naturally variable and are approximately normally distributed (Bush, Sotres-Bayon, & LeDoux, 2007; Galatzer-Levy, Bonanno, Bush, & Ledoux, 2013). Testing a large number of animals allows for identification of animals who are on both ends of the extinction learning spectrum, so that factors that contribute to these individual differences can be investigated. Looking at the whole cohort, we did not find overall sex-differences in freezing levels. Looking at behavioral outliers, we found that in males, poor extinction retrieval was associated with poor performance during extinction learning (Gruene et al., 2015). In females,
however, animals with poor or successful extinction showed different levels of freezing to the CS already at initial fear conditioning. One interpretation of these findings is that resilient females show less fear during fear conditioning, while resilient males perform better during fear extinction.

Another possibility that could account for these findings is that measuring freezing in the fear conditioning paradigm is not sufficient to measure fear levels in females. In other behavioral paradigms such as elevated plus maze or open field test, females show consistently more locomotor activity (Archer, 1975; Fernandes, González, Wilson, & File, 1999; Seney, Walsh, Stolakis, & Sibille, 2012), which could make them more likely to show active fear behaviors instead of or in addition to freezing. In that case, freezing would be a good measure of fear in males, but an incomplete measure in females. Study 1 of this dissertation set out to investigate this possibility by re-analyzing behavior videos from Gruene et al (2015). Upon examination of video recordings of fear conditioning, it became apparent that a subset of females exhibited an escape like “darting” response to tone presentations as opposed to the typical freezing response. Quantification and analysis of this behavior forms the basis of study 1 of this dissertation.

It is tempting to speculate that the darting behavior exhibited by females represents an attempt to escape. In the fear conditioning paradigm, however, escape is not possible and rats cannot be trained to show escape behavior. Other rodent models exist, such as the wheel turn stressor controllability paradigm, where the effects of escaping a stressor can directly be tested. This paradigm was used in study 2 of this dissertation and is discussed below.
1.3 Animal models of stress related psychopathology: Stressor controllability paradigm

Behavioral effects of controllable and uncontrollable stress

Another predictor of resilience to stressful life events is how individuals appraise the event and employ coping strategies. One important active coping mechanism that leads to improved mental health outcomes is having either perceived or actual control over a stressor (Charney, 2004; Shapiro, Schwartz, & Astin, 1996; Southwick & Charney, 2012). In animal models, lack of behavioral control over stress can lead to “learned helplessness”. This phenomenon was first characterized in dogs. Dogs that were repeatedly shocked without being able to escape later failed to escape from shocks where escape was possible, whereas dogs who were given the power to stop the initial shocks did not display failure to escape in the later task (Maier & Seligman, 1976; Seligman & Maier, 1967).

In rodents, stressor controllability has been extensively studied over the past decades. The typical experimental design includes 3 experimental groups. In the first group, rats are exposed to a series of tail shocks each of which can be terminated when the rat turns a wheel. Thus, this group of rats has behavioral control over the stressful tail shocks. In the second group, rats are also exposed to tail shocks, but do not have the option to terminate the shock by turning a wheel. Each rat in the second group is paired to a rat in the first group and will receive the same amount and duration of shocks as the rat in the first group, resulting in a yoked control design. This way, rats in the first group (escapable stress, ES) and the second group (inescapable stress, IS) receive the same amount of physical shocks with the only difference being controllability of the stressor. A third group of rats serves as a control and remains undisturbed in their home cage (home cage, HC) (Maier, 2015; Maier & Watkins, 2005).
IS leads to various detrimental outcomes that can be physical like weight loss or development of gastric lesions (Weiss, 1968). There are also several behavioral consequences that can generally be described as anxiety-like or depression-like behaviors. The classic effect of learned helplessness can be seen in rodents in the shuttle-box test. In this test animals are placed in a box with two chambers where they will receive foot shocks that the animal can escape from by running to the other chamber. Unstressed animals and ES animals will readily learn this task while IS animals will fail to escape, thus demonstrating learned helplessness (Amat et al., 2001; Maier & Seligman, 1976). Additionally, IS leads to decreased social exploration (Christianson et al., 2008) and enhanced fear conditioning (Baratta et al., 2007; Maier, Grahn, & Watkins, 1995) while ES protects animals from all of these outcomes (Maier & Watkins, 2005). Experience with ES also protects from the adverse effects of future IS (Amat, Paul, Watkins, & Maier, 2008; Amat, Paul, Zarza, Watkins, & Maier, 2006) and even different stressors such as social defeat (Amat, Aleksejev, Paul, Watkins, & Maier, 2010). Thus, experience with controllable stress has a long-lasting “immunizing” effect against future stressors.

While it seems that the behavioral effects of IS and ES have been thoroughly characterized, it has to be acknowledged that the experiments reviewed above were conducted with male animals only. Few studies have looked into the effects of IS and ES in females. Three studies reported less severe learned helplessness in females after IS compared to males, and ES only slightly reduced escape latencies in the shuttle box task in females (Dalla, Edgecomb, Whetstone, & Shors, 2008; Kirk & Blampied, 1985; Shors et al., 2007). These studies used shuttle box training itself as the ES condition and inescapable shocks within the shuttle box as IS condition, making it a slightly different paradigm than the one discussed above. For example, freezing induced by context conditioning be a confounding factor. In a very recent study (M.
Baratta & S. Maier, personal communication, June 23, 2017), females were tested in the wheel-turn paradigm and ES did not have protective effects in females. Instead, ES and IS females showed comparable impairments in the shuttle-box test and social interaction test.

**Neurobiology of controllable and uncontrollable stress**

The underlying neuronal mechanisms of IS and ES have been extensively studied in male rats. In order to understand how ES protects from negative effects of stress, it first had to be determined how IS leads to detrimental outcomes. The key area involved is the dorsal raphe nucleus (DRN), which sends serotonergic (5HT) projections throughout the brain and specifically to structures involved in anxiety behaviors such as the amygdala (Graeff, Viana, & Mora, 1997). IS produces over-activity in the DRN as measured by increased c-fos expression (Grahn et al., 1999), and increased extracellular 5HT in the DRN itself (Maswood, Barter, Watkins, & Maier, 1998) and in the amygdala (Amat, Matus-Amat, Watkins, & Maier, 1998). Moreover, blocking DRN activity during IS blocks negative behavioral consequences such as the shuttle box escape deficit (Maier et al., 1995). To summarize, there is abundant evidence that DRN activation during IS leads to the negative behavioral outcomes of IS.

ES on the other hand, does not lead to increased activity in DRN and years of research identified the mPFC, specifically the PL region, as the key mediator of this effect. PL sends glutamatergic projections to DRN that synapse on γ-aminobutyric acid (GABA) -ergic interneurons (Jankowski & Sesack, 2004) and activation of PL leads to inhibition of DRN (Hajós, Richards, Székely, & Sharp, 1998). The first piece of evidence for PL mediation of ES effects came from a study by Amat et al. (2005). With a series of experiments the authors showed that inhibition of mPFC during ES resulted in DRN activity and extracellular 5-HT at the
same level of IS. Moreover, mPFC inhibition during ES blocks the protective effect of control on behavioral outcomes and leads to impairments in escape learning just like IS. Additionally, further research has found that experimental activation of mPFC during IS is sufficient to produce the protective effects of ES (Amat, Paul, Watkins, & Maier, 2008). By combining retrograde labeling and c-fos staining Baratta et al. (2009) showed that ES preferentially recruits DRN-projecting neurons in PL, suggesting that PL inhibits DRN directly rather than through indirect activation of other brain regions. Further, the immunizing effects of ES also depend on mPFC activation and plasticity. Inactivation of mPFC either during initial ES or at later IS exposure blocks the immunization effects of ES (Amat et al., 2006). Additionally, animals who went through ES previously, show PL activation in DRN-projecting neurons during subsequent IS as measured by c-fos activation (Baratta et al., 2009). Importantly, inhibiting protein synthesis in mPFC by infusion of anisomycin immediately before initial ES also blocks the immunization effect of ES (Amat et al., 2006). Thus, PL activation during ES likely induces plastic changes in PL that lead to PL recruitment during future stressors. There is evidence that escapable stress increased intrinsic excitability of PL neurons shortly (2 hours) after stress exposure (Varela, Wang, Christianson, Maier, & Cooper, 2012) but it is unclear if there are longer-lasting structural changes that could account for the enduring protective effects of ES.

1.4 Dendritic Morphology and Stress

Dendritic morphology is highly plastic and can change in response to stress and learning. Changes in dendritic branching and length usually take several days to develop while dendritic spines can change very rapidly, within minutes and over several hours (Farrell, Gruene, & Shansky, 2015; Kasai, Fukuda, Watanabe, Hayashi-Takagi, & Noguchi, 2010; Lai, Franke, &
Gan, 2012). Effects of acute stress on dendritic spines have been thoroughly studied in the hippocampus. Acute stress induces spine loss in hippocampal area CA3 (Chen et al., 2010; Chen, Dubé, Rice, & Baram, 2008), but increases spine density in CA1 (Leuner & Shors, 2013; Shors, Chua, & Falduto, 2001). In the barrel cortex, glucocorticoid administration for three days induces increased spine turn-over rates without affecting overall spine density (Liston & Gan, 2011). Perhaps surprisingly, acute stress effects on mPFC dendritic spines are not well studied.

In PL, one study reports increased spine density in layer II/III neurons after acute foot shock stress (Nava et al., 2015). Most studies investigating spine changes after stress in mPFC focus on chronic stress. Chronic restraint stress, as well as chronic glucocorticoid administration, results in spine loss and spine shrinkage in layer II/III PL neurons (Anderson et al., 2016; Hains et al., 2009; Radley et al., 2006, 2008) and in apical tufts of layer V PL neurons (Liu & Aghajanian, 2008).

Moreover, stress effects on mPFC dendritic morphology can be circuit- and sex-specific. For example, chronic restraint stress induces dendritic retraction in infralimbic (a sub region of mPFC) neurons in males, but neurons projecting to the BLA are protected from this effect (Shansky, Hamo, Hof, McEwen, & Morrison, 2009). In females on the other hand, stress induces increases in dendritic length in BLA projecting neurons and increases spine density (Shansky et al., 2010). In the same circuit, we found differences in dendritic length and spine density between animals exhibiting poor compared to successful extinction retrieval in males but not females (Gruene et al., 2015). Based on these findings, it is possible that ES and IS alter dendritic morphology in mPFC in circuit- and sex-specific ways. Study 2 of this dissertation will investigate whether ES and IS have unique effects on dendritic spine morphology in PL-DRN projecting neurons compared to randomly selected PL neurons and whether these effects are
different in males and females. Since ES likely induces learning related spine changes, effects of learning on dendritic spines will be discussed next.

**1.5 Dendritic Morphology in Learning and Memory**

Synaptic plasticity, specifically long term potentiation (LTP) and long term depression have been identified as underlying mechanisms of learning and memory (Bear & Malenka, 1994). The majority of excitatory synapses in hippocampus and cortex are formed on dendritic spines, which are themselves plastic and can change in shape and number (Bourne & Harris, 2007). Dendritic spines are thought to be critically involved in learning related plasticity and may represent the structural basis of memory.

Several studies have investigated how dendritic spine changes relate to LTP in vitro. Maletic-Savatic et al. (1999) used two-photon imaging of CA1 dendritic spines in developing organotypic hippocampal slices. Tetanic stimulation induced filopodia growth on dendritic segments close to the stimulation electrode. A similar study was conducted by Engert and Bonhoeffer (1999), where the authors were able to improve location specificity by using a local superfusion technique to limit synaptic activity to small areas on CA1 dendrites. The authors report increased spine formation approximately one hour after LTP induction specifically on parts of dendrites where superfusion permitted synaptic activity. In the second study the newly formed spines appeared to be thin spines rather than filopodia observed in Maletic-Savatic et al (1999).

In addition to spine formation and elimination of whole spines, individual spines can also change their shape and volume in response to stimulation. Stimulation of individual spines rapidly increases spine volume and potentiated α-amino-3-hydroxy-5-methyl-4-
isoxazolepropionic acid (AMPA) currents close to stimulated spines (Matsuzaki, Honkura, Ellis-Davies, & Kasai, 2004). The volume increase was blocked by a N-Methyl-D-aspartate (NMDA) receptor antagonist as well as by a calmodulin inhibitor or actin inhibitor. No new spine formation was observed suggesting that stimulation of multiple synapses may be necessary to induce spine formation (Matsuzaki et al., 2004).

Together, the studies discussed so far convincingly show a connection between dendritic spine changes and LTP in vitro. Transcranial two-photon imaging is a popular technique for studying learning related spine changes in living animals and has the advantage that discrete dendritic segments can be imaged multiple times over the course of an experiment. However, due to the limitations of the technique it is not yet possible to image dendritic spines in structures far away from the cortical surface. The motor cortex, being so close to the cortical surface, is an ideal target for this method and thus many studies have investigated spine changes that go along with motor skill learning. Training on the rotarod motor task, or a seed reaching task both result in increased spine formation in the motor cortex and spine changes are correlated with performance (Xu et al., 2009; Yang, Pan, & Gan, 2009). In addition to spine formation, spine elimination is also increased by motor training, with a slight delay (Xu et al., 2009; Yang et al., 2009). Additionally, most newly formed spine emerge in clusters of two or three spines (Fu, Yu, Lu, & Zuo, 2012). In this study, clustered spines also had a higher survival rate over the course of training and even four months after the end of training. Moreover, newly formed spines that emerged in clusters showed increases in head size over the course of learning while single new spines did not (Fu et al., 2012). Overall, the results suggest that clustering of newly formed spines contributes to strengthening and stabilization of new synapses after learning.
The in vivo studies summarized so far show that there is a correlation between spine changes and learning. Looking at causal relationships between spine changes and learning has been difficult so far, but Hayashi-Takagi et al. (2015) developed a new approach to directly manipulate newly formed spines. The authors developed an “optoprobe” that will tag spines potentiated by synaptic activity and upon illumination with light selectively induce shrinkage in these newly formed spines. After training on the rotarod task, newly formed spines were eliminated through activation of the optoprobe and subsequent behavioral testing revealed that this manipulation effectively decreased rotarod performance to pre-training levels, thus erasing the motor skill memory. This paper demonstrated for the first time a causal relationship between dendritic spine changes and learning.

As discussed in the previous section, stress per se can have effects on dendritic spines, so it is possible that learning related to a stressful experience. One study has used two-photon imaging in the frontal association cortex (FrA) of mice after fear conditioning and extinction to investigate spine changes corresponding to this learning paradigm. Interestingly, Lai et al. (2012) reported increased spine elimination after fear conditioning without changes in spine formation in FrA. Thus, fear conditioning seems to lead to opposite spine changes than motor training, at least in FrA.

Although it has been demonstrated that the FrA is involved in fear conditioning and extinction, this area not typically studied in relation to this learning paradigm. The hippocampus and the basolateral amygdala (BLA) are more commonly associated with fear conditioning and their role in fear conditioning has been widely studied (Duvarci & Pare, 2014; Maren, 2001), but due to their location in the brain spine changes in response to learning have only been studied through post mortem analyses. Sanders et al. (2012) used the TetTag method to label neurons
that were activated by contextual fear conditioning and analyzed dendritic spine density on hippocampal neurons after learning on tagged (activated by fear conditioning) dendritic segments compared to untagged (not activated by fear conditioning) segments. The authors report decreased spine densities on activated segments compared to non-activated segments. Additionally, another study found similar effects in BLA after fear conditioning, with activated neurons having lower spine densities compared to non-activated neurons (Gruene et al., 2016). The techniques used in the last two studies are clearly limited by not being able to image spines over the course of learning. Consequently, spine analysis is limited to looking at overall spine densities without being able to know if the effects are due to pre-existing spine differences or if they are truly due to changes in spine formation, spine elimination or both. Nonetheless, the three studies on spine changes after fear conditioning indicate that fear conditioning may have distinct effects on spine plasticity that differ from motor skill learning. This effect could be due to the stress component in fear conditioning.

To summarize, results from in vitro studies demonstrate a clear relationship between spine changes and LTP in hippocampus. LTP induction generally results in potentiation of head volume as well as growth of new spines. When it comes to relating spine changes in living animals, motor skill learning has been widely studied and generally leads to increases in spine formation and spine enlargement. However, delayed increases in spine elimination generally tend to result in no net change in spine density. Fear conditioning, on the other hand, leads to spine elimination and reduced spine densities.
1.6 Objectives and Hypotheses

The objective of this dissertation was to investigate factors contributing to vulnerability and resilience to stressful event, with a focus on sex-differences, individual differences, and controllability of stress. For study 1, we thoroughly analyzed fear behavior displayed during auditory fear conditioning and extinction. Based on previous research using different behavioral paradigms that showed increased locomotor activity in females (Shansky, 2015), and based on personal observations from watching fear conditioning behavior videos, we hypothesized that females would display more active fear behaviors. We used movement tracking software to get objective measures of active, “darting” responses and compared darting between and within sexes. We also performed exploratory analysis to investigate whether darting during fear conditioning was predictive of fear levels during extinction recall.

Results from study 1 showed that females engaged in more active fear behavior. These darting responses could represent attempts to escape the shock, even though escape is not actually possible in the paradigm we used. Study 1 also showed that female darters have improved outcomes at extinction retrieval, indicating that darting during fear conditioning is related to resilience. To directly test how escaping a stressor affects females differently, study 2 employed the stressor controllability paradigm (Maier, 2015). This paradigm has been thoroughly studied in male rats, showing that controllability leads to improved behavioral outcomes and depends on activity in the PL-DRN pathway (Maier & Watkins, 2005). The initial aim of study 2 was to examine effects of stressor controllability on dendritic plasticity in the PL-DRN pathway. Originally, it was hypothesized that females would be more susceptible to the effects of control and that would be reflected in enhanced plasticity in the PL-DRN pathway. As recent results show, however, females do not benefit from behavioral control in this pathway and
do not engage the PL-DRN pathway as a result of control (M. Baratta & S. Maier, personal communication, June 23, 2017). Based on these results, we hypothesized that only males would show circuit specific dendritic plasticity after experiencing controllable stress, while other groups would show general stress-related changes. Additionally, exploratory analysis was performed to investigate baseline sex-differences in dendritic spine morphology in the PL-DRN pathway that could contribute to functional differences during escapable stress.
1.7 Compendium of Manuscripts

Chapters 3 and 4 were taken in part from the following manuscripts:

Chapter 3


Chapter 4

Chapter 2
Materials and Methods

2.1 Study 1

Animals

Young adult (8–10 weeks) male (n=56) and female (n=58) Sprague Dawley rats were individually housed in the Nightingale Animal Facility at Northeastern University on a 12:12 light:dark cycle with access to food and water ad libitum. All procedures were conducted in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and were approved by the Northeastern University Institutional Animal Care And Use Committee. All experimenters were female.

Estrous Cycle Monitoring

Females were vaginally swabbed daily for two weeks to ensure normal estrous cycling. Collected cells were smeared on a microscope slide, stained with Nissl, and examined with a light microscope for cytology.

Apparatus and Stimuli

Rats underwent habituation, fear conditioning and fear extinction as in (Gruene et al, 2015) in one of four identical chambers constructed of aluminum and Plexiglas walls (Rat Test Cage, Coulbourn Instruments, Allentown, PA), with metal stainless steel rod flooring that was attached to a shock generator (Model H13–15; Coulbourn Instruments). The chambers were lit with a single house light, and each chamber was enclosed within a sound-isolation cubicle (Model H10–24A; Coulbourn Instruments). An infrared digital camera allowed videotaping during behavioral procedures. Chamber grid floors, trays, and walls were thoroughly cleaned.
with water and dried between sessions. Rats were allowed to freely explore the chamber for 4 min before tone presentation on each day began.

**Fear Conditioning**

After a 4-minute acclimation period, all rats were exposed to five tone (CS) presentations (habituation), followed by seven conditioning trials (CS–US pairings) on day 1. The CS was a 30-s, 5 kHz, 80 dB SPL sine wave tone, which co-terminated with a 0.5-s, 0.7 mA footshock US during fear conditioning. Mean inter-trial interval was 4 min (2–6 min range) throughout habituation and fear conditioning. Freezing was continuously recorded during the conditioning session and analyzed using FreezeFrame Software. Minimum bout was set at 2sec. After conditioning, rats were returned to their home cages.

**Extinction**

Freezing was recorded continuously during the extinction training (20 CS presentations, day 2) and test sessions (3 CS presentations, day 3). Both extinction training and testing took place in the same chamber as fear conditioning, but with different contextual cues (floor, light, and odor). Mean inter-trial interval was 4 min (2–6 min range).

**Locomotor Activity Analysis**

Video files from FreezeFrame were extracted as QuickTime File Format (.mov) and then converted to MPEG-2 files using AVS Video Converter 9.1 (Online Media Technologies LTD. 2014). The MPEG-2 files were then run through EthoVision software (Noldus), with a center point tracking with a velocity sampling rate of 3.75. Velocity data were computed by Noldus software at 3.75 Hz sampling rate and exported to Matlab (Mathworks). Darts were detected in
the exported trace using the findpeaks function with a minimum velocity of 23.5 cm/s and a minimum interpeak interval of 0.8 s. The 23.5 cm/s threshold for darts was determined by cross-referencing velocity data with experimenter scoring of darting behavior. 23.5 cm/s was the velocity at which all movements at that rate or higher were consistently scored as darts. These discrete events were registered to each trial and analyzed using a custom Matlab script.

2.2 Study 2

Animals

Adult female and male Sprague Dawley Rats were house in pairs at the University of Colorado Boulder animal facility on a 12:12 light:dark cycle with access to food and water ad libitum. All procedures were conducted in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and were approved by the Colorado University Institutional Animal Care and Use Committee.

Surgeries

Adult male and female Sprague Dawley Rats received stereotaxic surgery to infuse RetroBeads into the DRN (AP -8.3mm, DV -5mm). After surgery, animals were allowed to recover for 10 days before stress exposure. Injection sites were verified by visually inspecting DRN containing, 50µm thick slices on a Zeiss Axio Examiner A.1 microscope (Zeiss Microscopy, Thornwood, New York). Figure 1 shows an example of a successful injection site. Only animals with successful DRN injections were included in the study.
Stress Exposure and Perfusion

Males and Females were randomly assigned to ES, IS, or HC groups. Stress was administered as described in Baratta et al (2007). Briefly, test chambers were Plexiglas boxes that have a wheel mounted on one end and a rod on the other. The rat’s tail was attached to the rod with tape and copper electrodes attached to deliver the shock. ES and IS animals were run in yoked pairs for one stress session. In the stress session, animals were exposed to 100 trials of tail shocks, which were terminated for both animals once the ES rat completed a correct wheel turning response. 24 hours after stress exposure all animals were sacrificed via transcardial perfusion with 1% paraformaldehyde for one minute, followed by 4% paraformaldehyde for ten minutes. Brains were extracted and after a 6-hour post-fix time transferred to 0.1% sodium azide.

Iontophoretic Microinjections

Fixed brains were sectioned at 250µm on a vibrating microtome (Leica Microsystems, Inc, Buffalo Grove, Illinois), and mPFC-containing sections selected for microinjections. Retro-labeled PL neurons will be visualized on a Zeiss Axio Examiner A.1 microscope (Zeiss Microscopy, Thornwood, New York). Iontophoretic microinjections of fluorescent dye Lucifer Yellow were targeted to PL layer V pyramidal neurons using a DC current of 5-10nA for 10-15 minutes, followed by 2 minutes at 15nA until distal processes are filled and no further loading was observed. In addition to labeled (PL-DRN projecting) neurons, unlabeled neighboring neurons were filled. Sections were mounted on microscope slides with added seal spacers to prevent morphological distortions due to the weight of the cover glass. Then, sections were coverslipped using Vectashield (Vector Laboratories) mounting medium.
Imaging and Dendritic Spine Segment Analysis

Five to seven mPFC-DRN projecting neurons and five to seven unlabeled neurons were included in the analysis. From each neuron 2 proximal (less than 100 µm from the cell body) and 2 distal (more than 100 µm from the cell body) segments were sampled from basal dendrites. To be included in analysis, dendritic segments needed to be 1) within 80µm from the cover glass due to the working range of the microscope lens, 2) show no overlap with other dendritic segments, 3) be mostly parallel to the surface of the tissue. All images were acquired using an Olympus FV1000 confocal microscope (Optical Analysis Corporation, Nashua, New Hampshire). Once selected, segments were imaged using a 100x oil lens, 1.4 NA, zoom of 3.7 and 0.33µm step size. Using a 1024x1024 raster these settings resulted in a resolution of 0.033µm X 0.033µm X 0.33µm per pixel. Z-stacks were acquired at 2µs/pixel, with a Kalmann filter of 4, using a 458 argon laser at 30% power, and between 620-750 HV. Raw Z-stacks were deconvolved with AutoQuant (Media Cybernetics, Rockville, Maryland) and analyzed for spine number and shape (thin, stubby, or mushroom) using NeuronStudio software (Computational Neurobiology and Imaging Center, New York, New York) (classification criteria described in detail by Rodriguez et al. (2008)).

Data Processing and Statistical Analysis

Spine density analyses were performed using GraphPad Prism (GraphPad Software, Inc, La Jolla, California). Spine densities for labeled and unlabeled neurons were first averaged by neuron and then by animal. Mixed-design ANOVA was performed to test for effects of circuit and stress on spine densities. Spine head diameter and clustering analyses and plotting were performed using Python 3.5 and its relevant packages (numpy, pandas, scikit learn, scipy,
matplotlib, seaborn). Example code can be found at https://github.com/TinaGruene/spine-analysis. For head diameter analysis, neuronstudio output files (.txt) were combined for each experimental group and head diameters for thin and mushroom spines were extracted. Table 1 shows sample sizes broken down by groups and spine type.

Spine head diameters naturally vary in size and are related to spine function and synapse strength. Thus, comparing distributions of head diameters is more meaningful than comparing group means. Additionally, the large sample sizes obtained give a good estimation of the population distribution and D’Agostino & Pearson omnibus normality test showed that head diameter sizes are not normally distributed (p<0.0001 for each group). The Kolmogorov-Smirnov test (KS-test) was used to statistically test for differences in distributions and together with the cumulative distribution and kernel density estimate plots formed the basis for data interpretation. Additionally, differences in distributions were plotted for groups that showed statistical differences. To analyze potential clustering of spines, Euclidian distances were first calculated for each spine per dendritic segment (Pereira et al., 2014). Then, distances of each spine to its closest neighbor was normalized to “expected” average distance based on the spine density of each segment. Lastly, normalized minimum distances were combined for PL-DRN and unlabeled neurons of each experimental group. Kde-plots together with KS-tests were used to identify changes in spine clustering.
Figure 1. Micrograph showing an example of successful retrobead injection into DRN
Figure 1. Micrograph showing a coronal section at -7.80 Bregma with a successful retrobead injection into DRN. White dashed lines outline DRN subdivisions dorsal raphe lateral (DRL), dorsal raphe dorsal (DRD), and dorsal raphe ventral (DRV). Blue = DAPI background stain, turquoise = Retrobead injection site.
Table 1. Summary of sample sizes for each experimental group, for PL-DRN and unlabeled neurons.

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Chapter 3

Sexually divergent expression of active and passive conditioned fear responses in rats

3.1 Introduction

In the laboratory, auditory or “cued” fear conditioning and extinction in rodents are the predominant tools for studying the neural mechanisms of learning and memory for aversive stimuli (Blanchard & Blanchard, 1969; Ledoux, 2000; Maren, 2001). In these assays, the strength of a tone-shock association is traditionally measured by the fraction of time during the conditioned stimulus (CS) that subjects exhibit freezing, defined as the cessation of all movement not required for respiration (Fanselow, 1980). Accordingly, low freezing is generally interpreted as reflecting a weak association and thus poor learning. Likewise, low freezing after extinction is taken to indicate successful suppression of the conditioned response, a new memory (Quirk & Mueller, 2008). However, by their construction, these traditional assays are insensitive to alternative expressions of fear, such as escape.

Most studies of fear conditioning and extinction in rodents use exclusively male subjects (Lebron-Milad et al., 2012). The few studies that directly compare conditioned freezing responses in males and females produced mixed results (Shansky, 2015), but most frequently reported lower freezing in females (Gupta, Sen, Diepenhorst, Rudick, & Maren, 2001; Stephen Maren, De Oca, & Fanselow, 1994; Pryce, Lehmann, & Feldon, 1999). Whether this effect reflects genuine learning deficits in females, or is related to sex differences in fear response strategies is unknown. For example, females reliably exhibit heightened ambulation in a wide variety of common behavioral tests (Archer, 1975; Fernandes et al., 1999; Seney et al., 2012), which may influence their selection of responses to threatening stimuli.
To identify possible alternative fear response strategies, we evaluated locomotor activity in gonadally intact adult male (n=56) and female (n=58) Sprague Dawley rats as they were trained and tested in auditory fear conditioning (5 habituation conditioned stimuli (CS) followed by 7 CS-unconditioned stimulus (US) pairs), extinction (20 CS), and extinction retention (3 CS) tests across 3 days (Gruene et al., 2015) (Figure 2a). In many animals, we qualitatively observed a rapid ‘darting’ behavior during fear conditioning tone presentation—a rapid, forward movement across the chamber that resembled an escape-like response (illustrated in Figure 2b, and Video available at https://elifesciences.org/articles/11352#media1). We quantified these responses by identifying and counting them as discrete events in traces of each animal’s velocity for all sessions using Noldus Ethovision software and custom Matlab code (code available at https://elifesciences.org/articles/11352/figures#SD1-data, Figure 2c). We calculated darting rate (darts/min) during four non-overlapping trial epochs: 1) 60 s pre-CS period, 2) 30 s CS presentation, 3) 5 s “shock response” period, and 4) 30 s post-shock period (Figure 3a). This approach allowed us first to determine if darting reflected an alternate conditioned response, and second, whether the expression of conditioned darting predicted distinct behavioral patterns across fear conditioning and extinction.
Figure 2. Darting is an active learned response to the CS that occurs primarily in female rats.
**Figure 2.** a) Experimental timeline. (b) Darts were characterized by a brief, high velocity movement across the test chamber. (c) Velocity traces from a representative animal, demonstrating increase in conditioned darting events across fear conditioning trials. Asterisks denote events that reached criterion for darting during the CS. Time 0 denotes CS onset. (d) Temporal organization of darting in all female rats. On the left is a two dimensional histogram of dart timing relative to the CS averaged over all females for 5 habituation trials and 7 conditioning trials on day 1. Trial time is on the x-axis and colored bars denote the trial epochs we defined as CS (green), shock response (orange), and post shock response (blue). Each row represents a CS trial (habituation 1–5, and conditioning 6–12), and depicts average dart rate by the color in each 4-second bin according to the color bar. On the right are histograms of the temporal organization of darts averaged over the five habituation trials (top) and the last three conditioning trials (bottom). Darts were detected and counted as described in Materials and methods. (e) Temporal organization of darting in all male rats. Panels are organized as in (d). During habituation trials, darts occurred at low rates throughout the trial in both sexes. In contrast, after conditioning only females exhibited increased darting triggered by tone onset (‘CS’) and sustained darting after shock delivery (‘Post-shock’). Both sexes darted in response to the shock itself (Shock response). In both sexes, the first bin after the shock exceeds the limits of the y-axis.
3.2 Results

Prior to the initiation of shocks, darts were not temporally structured with respect to the CS. However, we found that females, but not males, exhibited increased dart frequency in response to CS onset during late trials (Figure 2c–e), suggesting that darting is a learned response. Figure 2d,e represent dart frequency amongst entire female and male cohorts, respectively.

We next compared darting in males and females across all test sessions. Females exhibited higher CS dart rates than males on all 3 days (Figure 3c,i,j; conditioning: ns p=0.07, Mann Whitney test. p<0.001 2-way ANOVA main effect of sex, F1,112=12.1; sex x trial interaction F11,1232=2.12, p=0.02 Extinction: p=0.01, Mann Whitney test. p<0.05, 2-way ANOVA main effect of sex, F1,112=4.05. Extinction test: p=0.008, Mann Whitney test (Pre-CS); p<0.001, 2-way ANOVA main effect of sex, F1,112=14.58 (CS)). Notably, CS dart rate in females increased as CS-US presentations progressed (Figure 3c) and decreased during extinction (Figure 3i), again suggesting that darting may reflect an alternate expression of associative learning. During fear conditioning, although both males and females reliably darted during the shock response period (Figure 3d; p<0.01 2-way ANOVA main effect of sex, F1,112=8.5), shock-evoked darts in females reached higher velocities than darts in males (Figure 3e; p<0.0001 2-way ANOVA main effect of sex, F1,112=20.35). Additionally, females were more likely to dart during the 30s post-shock period than males (Figure 2f; p<0.0001 2-way ANOVA main effect of sex, F1,112=23.27). To determine whether an animal’s shock response velocity was related to its overall propensity to dart, we correlated the mean velocity reached across all 7 US presentations with total detected darts during fear conditioning. These measures were significantly correlated in females (Figure 3g) but not males (Figure 3h), suggesting that in
females only, an animal’s immediate reaction to an aversive stimulus may influence its future response strategies.

We did not observe darting in all females, however, and so to identify possible behavioral markers and outcomes of darting, we divided animals into ‘Darter’ and ‘Non-darter’ subgroups. An animal qualified as a “Darter” if it exhibited at least one dart during fear conditioning tones (CS) 8–12. CS 8 is the 3rd CS-US pairing, and the point at which we usually observe a robust increase in freezing in males. Therefore, only darts that occurred during this same time period were considered to reflect conditioned darting. Over 40% of females qualified as Darters (Figure 4a), whereas approximately 10% of males qualified (Figure 4f; chi-square = 13.8, p=0.0002). There was no effect of the estrous cycle on darting (Figure 5). Compared to Non-darters, female Darters exhibited greater shock response velocities (Figure 4b; p=0.001 2-way ANOVA main effect of group F1,56=11.49), as well as higher dart rates in the post-shock period (Figure 4c; p<0.0001 2-way ANOVA main effect of group, F1,56=25.42.), suggesting that female Darters have a more robust and protracted response to the shock. Importantly, female Darters did not exhibit higher dart rates during pre-CS periods or during CS-only habituation trials (Figure 4d; p=0.65, Mann Whitney test), suggesting that Darters are not simply more active overall, and were not pre-disposed to dart in response to the CS. During the CS, Darters exhibited increased darting as CS-US pairs progressed (p<0.001 2-way ANOVA group x trial interaction, F11,616=8.8; main effect of group F1,56=26.35, p<0.0001). During the Extinction Pre-CS period, Darters did not dart more than Non-darters (p=0.38 Mann Whitney), but Darters exhibited increased darting during the first Extinction CS, suggesting that darting is a conserved conditioned response (2-way ANOVA interaction, F19,1064=1.584, p=0.05; *p<0.05 Sidak’s post-hoc test).
Figure 3. Sex differences in darting responses during fear conditioning and extinction.
Figure 3. (a) The 4 fear conditioning epochs in which velocity was recorded. Graphs in c-f and i-j are color coded to match, and represent mean +/- SEM. (b) In graphs c-f and i-j, females are represented by filled circle, males by an open square. (c) Pre-CS (final 60 sec before 1st CS presentation) and CS dart rate (darts/min) during conditioning. (d) number of darts observed during 5s shock (US) response periods. (e) maximum velocity reached during 5s shock (US) response periods. (f) mean dart rate observed during 30s post-shock period. (g) and (h) Pearson’s correlations of mean shock response velocity and total session dart count [note that visible male outlier was removed from analysis for being 6 SDs above mean total dart count. When included, r=0.34, p<0.05]. (i) Pre-CS and CS dart rate (darts/min) during Extinction. (j) Pre-CS and CS dart rate (darts/min) during Extinction testing. *p<0.05; **p<0.01; *** p<0.001; ****p<0.0001 males vs. females.
Figure 4. Darting subpopulations are greater in females and exhibit distinct behavioral patterns.
Figure 4. (a) and (f) proportion of females and males that qualified as Darters. (b) max velocity reached during shock response period (c) mean dart rate (darts/min) observed during 30s post-shock period. (d) Pre-CS and CS dart rate (darts/min) during conditioning, extinction, and extinction test. (e) CS freezing in female Darters vs. Non-darters. (g) Shock response velocity did not differ between male Darters and Non-darters. (h) mean dart rate (darts/min) observed during 30s post-shock period. (i) Pre-CS and CS dart rate (darts/min) during conditioning, extinction, and extinction test. (j) CS freezing in male Darters vs. Non-darters. *p<0.05; **p<0.01; *** p<0.001; ****p<0.0001 Darters vs. Non-darters.
Figure 5. Distribution of estrous cycle phase in Darters and Non-darters.
Figure 5. Distribution of animals in each estrous cycle phase did not differ between Darters and Non-darters. Chi square = 2.785, p=0.42.
We next asked whether CS darting during fear conditioning related to CS freezing behavior (Figure 4e). In females, Darters and Non-darters did not differ in pre-CS or CS-only (habituation) freezing during fear conditioning. However, as CS-US pairings progressed, Darters froze less than Non-darters, suggesting that increased darting may prevent or compete with freezing responses \( p=0.02 \) 2-way ANOVA group x trial interaction \( F_{11,616}=2.16 \). main effect of darting \( F_{1,56}=4.18, p<0.05 \). Darters and Non-darters did not significantly differ in freezing during Extinction. However, Darters also froze less during the extinction test (day 3; \( p<0.02 \), 2-way ANOVA main effect of group \( F_{1,56}=5.76 \)) despite not exhibiting increased darting at that time, suggesting that darting during fear conditioning does not simply compete with an animal’s freezing response, but may also reflect an adaptive response that predicts positive outcomes after extinction learning.

However, there are several notable distinctions between male and female Darters. First, CS dart rate in darting males was characterized by a steady low rate of darting across trials (Figure 4i), instead of the increase across trials observed in females (Figure 4d), suggesting that darting in males may not reflect a learned fear response, but general hyperactivity that results in less freezing. Second, unlike our observations in females, male Darters did not exhibit heightened shock response velocities (Figure 4g) or robust post-shock dart rates (Figure 4h; \( p=0.01 \) 2-way ANOVA group x trial interaction, \( F_{6,324}=2.8 \), no main effects) compared to Non-darters. Third, male Darters did not exhibit lower freezing during extinction testing, suggesting that the potential long-term behavioral implications of darting during fear conditioning are stronger in females than in males. Together with the large observed sex difference in darting prevalence (Figure 3a,f), these discrepancies suggest that there may be qualitative differences in the potential causes and effects of darting in males versus females. Further research will be
necessary to determine whether the neurobiological basis of darting is comparable in males and females.

3.3 Discussion

In summary, our data show that during auditory fear conditioning, a substantial subpopulation of predominantly female rats exhibit an active conditioned response associated with reduced conditioned freezing throughout fear conditioning and extinction tests. To our knowledge, this is the first formal characterization of conditioned escape-like responses during classical fear conditioning, in which the shock cannot be avoided. In contrast, learned escape behavior has been well studied in Active Avoidance (AA) paradigms (Galatzer-Levy et al., 2013; Martinez et al., 2013), and although research into potential sex differences in AA is rare, females are reported to learn AA faster than males (Dalla & Shors, 2009), which is consistent with females preferring active fear responses over freezing.

One potentially provocative finding here is that female Darters exhibited comparable freezing to Non-darters at the start of extinction, but enhanced extinction retention the following day. Importantly, lower freezing during extinction retention could not be explained by increased darting during this phase. This suggests that darting during fear conditioning does not interfere with the formation or memory of the tone-shock association, but may confer a long-term protective or adaptive state that promotes increased cognitive flexibility and thus enhanced extinction maintenance (Maren, Phan, & Liberzon, 2013). This effect is reminiscent of reports from Maier and colleagues, who have convincingly demonstrated that “escapability” in a shock stress paradigm leads to reduced escape latencies in the shuttle-box (reviewed in Maier, 2015). In a similar vein, increases in “active coping” behavior (digging, rearing, wall-sniffing) during a cued fear memory test are positively correlated with AA success (Metna-Laurent et al., 2012).
Recruitment of these active coping fear responses instead of freezing has been shown to involve neural transmission in the central amygdala (Gozzi et al., 2010) and depend on cannabinoid signaling (Metna-Laurent et al., 2012), but to date have not been studied in female rodents. Importantly, these responses have not been demonstrated during fear conditioning learning, the stage at which darting appears to be most critical. Clearly, a great deal of work remains to dissect the neurobiological mechanisms that mediate darting, and to determine its relevance to other indices of active coping, especially in female model organisms.

The finding that conditioned darting occurs primarily in females holds major implications for the interpretation of fear conditioning and extinction studies that use both male and female rats, suggesting that freezing alone may not be a complete measure of learned fear in female subjects. Specifically, female rats that exhibit low freezing levels during fear conditioning could be erroneously described as expressing low fear and/or poor learning, when in fact they have engaged darting responses. This phenomenon may also be clinically relevant, pointing to a sex-specific threat response that predicts enhanced extinction maintenance. Because the learning processes that underlie extinction form the basis for exposure therapy (a common treatment for PTSD), a better understanding of the mechanisms that drive darting could lead to improved exposure therapy success. Women are at a twofold risk for PTSD compared to men, and thus identification of the neurobiological factors that determine darting in females may provide insight into sex differences in coping strategies, as well as in stress susceptibility and resilience.
4.1 Introduction

Most people experience stressful events, but many are resilient and can recover from stress without developing a disorder. Studying factors that contribute to resilience may improve prevention and treatment outcomes for stress related disorders such as depression and PTSD. It has been widely demonstrated that having control over a stressor promotes resilience to stress. In rodents, the stressor controllability paradigm has been extensively studied over the past decades. The typical experimental design includes 3 experimental groups, where the first group of rats is exposed to a series of tail shocks each of which can be terminated when the rat turns a wheel. Thus, this group of rats has behavioral control over the stressful tail shocks. In the second group, rats are yoked to rats from the first group and receive the same amount of tail shocks, but do not have the option to terminate the shock by turning a wheel. Each rat in the second group is paired to a rat in the first group and will receive the same amount and duration of shocks as the rat in the first group. This way, rats in the first group (escapable stress, ES) and the second group (inescapable stress, IS) receive the same pattern of physical shocks with the only difference being controllability of the stressor. A third group of rats serves as a home cage control (HC) (Maier, 2015; Maier & Watkins, 2005).
IS has several behavioral consequences that can generally be described as anxiety-like or depression-like behaviors, such as deficits in the shuttle-box escape task, decreased social exploration and enhanced fear conditioning (Amat et al., 2001; Baratta et al., 2007; Christianson et al., 2008; Maier et al., 1995; Maier & Seligman, 1976), while ES protects animals from all of these outcomes (Maier & Watkins, 2005). Experience with ES also protects from the adverse effects of future IS (Amat et al., 2008; Amat et al., 2006) and even different stressors such as social defeat (Amat et al., 2010). Thus, experience with controllable stress has a long-lasting “immunizing” effect against future stressors.

While it seems that the behavioral effects of IS and ES have been thoroughly characterized, it should be noted that the experiments reviewed above were conducted with male animals only. Two studies compared the effects of ES and IS in females and males, but used escapable and inescapable foot shocks administered within the shuttle-box as their paradigm. These studies showed less severe learned helplessness in females after IS compared to males, with only a slight reduction in escape latencies in ES females (Dalla et al., 2008; Shors et al., 2007). Without strong effects of IS it is difficult to assess the potential effects of ES in females. The wheel turn paradigm used in this study eliminates confounding effects of context conditioning. Moreover, its increased shock severity and frequency is better suited to tease apart IS and ES effects by potentially producing more robust IS effects. Indeed, females who undergo IS in the wheel-turn paradigm show the classic behavioral effects seen in males, but ES does not provide protection against these effects (M. Baratta & S. Maier, personal communication, June 23, 2017).
The underlying neuronal mechanisms of IS and ES have been extensively studied in male rats. The key area involved in producing the detrimental effects of IS is the DRN, which sends 5HT projections throughout the brain and specifically to structures involved in anxiety behaviors such as the amygdala (Graeff et al., 1997). IS produces over-activity in the DRN as measured by increased c-fos expression (Grahn et al., 1999), and increased extracellular 5HT in the DRN itself (Maswood et al., 1998) and in the amygdala (Amat et al., 1998). Moreover, blocking DRN activity during IS blocks negative behavioral consequences such as the shuttle box escape deficit (Maier et al., 1995). ES on the other hand, does not lead to increased activity in DRN; years of research has identified the mPFC, specifically the PL region, as the key mediator of this effect. PL sends glutamatergic projections to DRN that synapse on GABAergic interneurons (Jankowski & Sesack, 2004) and activation of PL leads to inhibition of DRN (Hajós et al., 1998). The first piece of evidence for PL mediation of ES effects came from a study by Amat et al. (2005). With a series of experiments the authors showed that inhibition of mPFC during ES resulted in DRN activity and extracellular 5HT at the same level of IS. Moreover, mPFC inhibition during ES blocked the protective effect of control on behavioral outcomes and lead to impairments in escape learning just like IS. Additionally, further research has found that experimental activation of mPFC during IS is sufficient to produce the protective effects of ES (Amat, Paul, Watkins, & Maier, 2008). By combining retro-grade labeling and c-fos staining Baratta et al. (2009) showed that ES preferentially recruits DRN-projecting neurons in PL, suggesting that PL inhibits DRN directly rather than through indirect activation of other brain regions. In females on the other hand, PL-DRN neurons are not activated during either IS or ES, which is in line with the lack of protective effects in females (M. Baratta & S. Maier, personal communication, June 23, 2017). In males, the immunizing effects of ES also depend on mPFC
activation and plasticity. Inactivation of mPFC with the GABA agonist muscimol either during initial ES or at later IS exposure blocks the immunization effects of ES (Amat et al., 2006). Additionally, animals who went through ES previously, show PL activation in DRN-projecting neurons during subsequent IS as measured by c-fos activation (Baratta et al., 2009). Importantly, inhibiting protein synthesis in mPFC by infusion of anisomycin immediately before initial ES also blocks the immunization effect of ES (Amat et al., 2006). Thus, PL activation during ES likely induces plastic changes in PL that lead to PL-DRN recruitment during future stressors. There is evidence that escapable stress increased intrinsic excitability of PL neurons shortly (2 hours) after stress exposure (Varela, Wang, Christianson, Maier, & Cooper, 2012b), but it is unclear if there are longer-lasting structural changes that could account for the enduring protective effects of ES. It is also unclear whether there are any circuit specific plasticity effects on PL-DRN neurons induced by ES.

Dendritic spines are highly plastic and can change in response to stress, synaptic activity, and learning (Bourne & Harris, 2007; Duman & Duman, 2015; Hofer & Bonhoeffer, 2010; Moench & Wellman, 2015). Measurable changes include increased spine formation and elimination (Lai et al., 2012; Yang et al., 2014), spine clustering (Fu et al., 2012), and changes in shape and size (Matsuzaki et al., 2004), which can occur rapidly within minutes and over several hours to days.

Here, we investigate dendritic spines changes 24 hours after ES and IS in layer V PL DRN-projecting, and randomly selected neurons in male and female rats and hypothesize that ES, but not IS, would induce circuit-specific plasticity in males. Since ES in females does not recruit the PL-DRN pathway, circuit specific changes were not expected in this group. General
differences between IS and ES females, however, and would indicate that control is still detected in PL without resulting in protective effects through PL-DRN recruitment.

4.2 Results

ES and IS do not affect spine densities in either sex

We first looked for effects of IS and ES on thin and mushroom spine densities. Mixed-design ANOVA with stress condition as between animal factor and PL-DRN and unlabeled neurons as within animal factor revealed no significant effects on thin spine densities (Figure 6a, stress: F(2,23)=1.016, p=0.3777; circuit: F(1,23) = 0.8866, p=0.3564; interaction: F(2,23)=0.7828, p=0.4689), but a main effect of circuit in mushroom spine densities without interaction in males (stress: F(2,23)=2.133, p=0.1413; circuit: F(1,23)=5.737, p=0.0251, interaction: F(2,23)=0.98328, p=0.4078). In females, there was a main effect of circuit in thin spine densities without interaction (Figure 6b, stress: F(2,19)=0.08943, p=0.9148; circuit: F(1,19)=10.73, p=0.004; interaction: F(2,19)=0.04566, p=0.9555), and a marginally significant main effect of circuit on mushroom spine densities (stress: F(2,19)=0.4753, p=0.6289; circuit: F(1,19)=3.735, p=0.0683; interaction: F(2,19)=0.5645, p=0.5779). These effects are small and without stress interactions this result is likely not meaningful for the research questions at hand. Overall, neither IS nor ES seem to affect spine densities on basal dendrites of layer V pyramidal neurons.
Figure 6. Mushroom and thin spine densities after ES or IS in males and females.
Figure 6. a) Mushroom and thin spine densities in unlabeled and PL-DRN neurons of HC, ES, and IS males. The different stress conditions had no effect on thin or mushroom spine densities, but for mushroom spines there was a main effect of circuit ($p=0.0251$). b) Mushroom and thin spine densities in unlabeled and PL-DRN neurons of HC, ES, and IS females. Like in males, the different stress conditions had no effect on thin or mushroom spine densities. There was a main effect of circuit in thin spine densities ($p=0.004$), and a marginally significant main effect of circuit in mushroom spines ($p=0.0683$).
Figure 7. ES males: Mushroom spine head diameter distributions.
Figure 7. a) Cumulative frequency distribution graphs of mushroom spine head diameter sizes in PL-DRN and unlabeled neurons of ES males compared to HC males. In PL-DRN neurons, rightward shift of ES curve indicates larger mushroom spine head diameters (KS-test p=0.0471).

b) Kde-plot (left) shows the estimated population distribution of mushroom spine head diameters in PL-DRN neurons of ES males compare to HC males. The plotted difference (right) illustrates that ES males have a smaller proportion of mushroom spines with head diameters between ~0.2µm and 0.4µm, but greater proportion between 0.4µm and 0.6µm. c) Kde-plot and difference plot of mushroom spine head diameter distribution in unlabeled neurons. There is no significant difference between ES and HC distributions.
Figure 8. ES males: Thin spine head diameter distributions.
Figure 8. a) Cumulative frequency distribution graphs of thin spine head diameter sizes in PL-DRN and unlabeled neurons of ES males compared to HC males. Distributions are not significantly different in either PL-DRN or unlabeled neurons b) and c) Kde-plot (left) and difference plot (right) of thin spine head diameter distribution in PL-DRN and unlabeled neurons. There is no significant difference between distributions from ES and HC males.
Figure 9. ES males: Clustering of thin spines is increased in PL-DRN neurons but not in unlabeled neurons.
Figure 9. a) Kde-plot (left) of the normalized distance of thin spines to their closest neighboring spine in PL-DRN neurons of ES and HC males. Difference plot (right) illustrates that ES males have a greater proportion of very small distances between thin spines in PL-DRN neurons, which indicates increased clustering of thin spines (KS-test p=0.0007). b) Kde-plot and difference plots of normalized distances in unlabeled neurons. The distributions do not differ significantly.
ES males: Circuit specific enlargement in mushroom spines and clustering of thin spines

IS and ES could affect spine elimination and formation without having net effects on spine density. For example, glucocorticoids increase spine turnover but affect spine elimination and formation in adult rat barrel cortex at comparable levels (Liston & Gan, 2011) resulting in stable spine densities. With the methods used in the current study, we are unable to measure spine turnover. But, to detect other subtle changes in spines, we next analyzed spine head diameters. Spines naturally vary in size and undergo fluctuations, but synaptic activity can enlarge or shrink spines (Matsuzaki et al., 2004; Zhou, Homma, & Poo, 2004), and spine size is related to number of AMPA receptors (Asrican, Lisman, & Otmakhov, 2007; Matsuzaki et al., 2001, 2004). Thus, looking at population differences of head diameter sizes between stress groups and control animals can give insight into postsynaptic plasticity in response to IS and ES. In ES males, a rightward shift of the cumulative distribution indicates increased mushroom spine head diameters in PL-DRN neurons (Figure 7a, KS-test p=0.0471). Examination of the Kde-plots and the difference plots shows that the ES animals had fewer small mushroom spines, but an increase in the proportion of larger mushroom spines (Figure 7b). No differences between ES and HC were found in head diameter size of mushroom spines from unlabeled neurons, or thin spines from labeled or unlabeled neurons (Figure 7c, 8a-c).

Spine clustering is another form of dendritic spine plasticity that can contribute to synaptic strengthening. Learning related increases in spine clustering have been demonstrated in other brain regions, for example after LTP induction in the hippocampus (De Roo, Klauser, Muller, Muller, & Murphy, 2008) and in the motor cortex after motor learning (Fu et al., 2012). Here we analyzed distances of thin spines to their closest neighboring spine as a measure of clustering. ES males have a higher proportion of smaller distances between thin spines in PL-
DRN neurons (Figure 9a, KS-test p=0.0007) but not in unlabeled neurons (Figure 9b, KS-test p=0.209), indicating circuit specific increases in spine clustering.

**IS males: Enlarged thin and mushroom spines, clustering of thin spines independent of circuit**

In IS males, we found shifts towards larger spine head diameters in thin and mushroom spines, irrespective of circuit (Figure 10a-c and 11a-c, mushroom PL-DRN: KS-test p=0.0177, mushroom unlabeled: KS-test p=0.0578, thin PL-DRN: KS-test p<0.0001, thin unlabeled: KS-test p<0.0001). IS also resulted in increased thin spine clustering in unlabeled (Figure 12b, KS-test p<0.0001) and, to a lesser extent, PL-DRN (Figure 12a, KS-test p=0.0389) neurons.
Figure 10. IS males: Mushroom spine head diameter distributions.
**Figure 10.** a) Cumulative frequency distribution graphs of mushroom spine head diameter sizes in PL-DRN and unlabeled neurons of IS males compared to HC males. In PL-DRN and unlabeled neurons, rightward shift of IS curve indicates larger mushroom spine head diameters (PL-DRN: KS-test $p=0.0177$, unlabeled: $p=0.0578$). b) and c) Kde-plot (left) shows the estimated population distribution of mushroom spine head diameters in PL-DRN and unlabeled neurons of IS males compare to HC males. The plotted difference (right) illustrates that IS males have a smaller proportion of mushroom spines with smaller head diameters and a larger proportion of mushroom spines with larger head diameters in both PL-DRN and unlabeled neurons.
Figure 11. IS males: Thin spine head diameter distributions.
Figure 11. a) Cumulative frequency distribution graphs of thin spine head diameter sizes in PL-DRN and unlabeled neurons of IS males compared to HC males. In PL-DRN and unlabeled neurons, rightward shift of IS curve indicates larger thin spine head diameters (KS-test p<0.0001 for both). b) and c) Kde-plot (left) shows the estimated population distribution of thin spine head diameters in PL-DRN and unlabeled neurons of IS males compare to HC males. The plotted difference (right) illustrates that IS males have a smaller proportion of thin spines with smaller head diameters and a larger proportion of thin spines with larger head diameters in both PL-DRN and unlabeled neurons.
Figure 12. IS males: Clustering of thin spines increased in PL-DRN and unlabeled neurons.
**Figure 12.** a) Kde-plot (left) of the normalized distance of thin spines to their closest neighboring spine in PL-DRN neurons of IS and HC males. Difference plot (right) illustrates that IS males have a greater proportion of very small distances between thin spines in PL-DRN neurons, which indicates increased clustering of thin spines (KS-test p=0.0389). b) Kde-plot (left) of the normalized distance of thin spines to their closest neighboring spine in unlabeled neurons of IS and HC males. Difference plot (right) illustrates that IS males have a greater proportion of very small distances between thin spines in unlabeled neurons, which indicates increased clustering of thin spines (KS-test p<0.0001).
ES females: Enlarged thin and mushroom spines, clustering of thin spines independent of circuit

In ES females, we also saw shifts towards larger spine head diameters in thin and mushroom spines, irrespective of circuit (Figure 13a-c, figure 14a-c, mushroom PL-DRN: KS-test p<0.0001, mushroom unlabeled: KS-test p=0.0424, thin PL-DRN: KS-test p=0.0026, thin unlabeled: KS-test p=0.0005). ES females also showed increased thin spine clustering in PL-DRN (Figure 15a, KS-test p=0.0003) and unlabeled neurons (Figure 15b, KS-test p<0.0001).

IS females: Enlarged mushroom spines, less clustering of thin spines independent of circuit

In IS females the shift towards larger spine head diameters was limited to mushroom spines (mushroom PL-DRN: KS-test p=0.0837, mushroom unlabeled: KS-test p=0.0002, thin PL-DRN: KS-test p=0.6774, thin unlabeled: KS-test p=0.1017). Unlike ES females, IS females showed a shift towards larger distances between neighboring spines in PL-DRN (KS-test p=0.0104) and unlabeled neurons (KS-test p=0.0204), indicating decreased clustering.

Circuit specific sex-differences in thin spine size

Finally, we analyzed whether there were basic sex differences in dendritic spine morphology of PL-DRN neurons that could contribute to the lack of recruitment during ES in females. While there were no differences in thin or mushroom spine densities, we found that males have an increased proportion of small thin spines in PL-DRN neurons but not in unlabeled neurons (Figure 20 a-c, PL-DRN: KS-test p<0.0001, unlabeled: KS-test p=0.74). Males also had larger mushroom spine head diameters compared to females in unlabeled neurons but not in PL-DRN neurons (Figure 19a-c, PL-DRN: KS-test p=0.181, unlabeled: KS-test p=0.001).
Figure 13. ES females: Mushroom spine head diameter distributions.
**Figure 13.** a) Cumulative frequency distribution graphs of mushroom spine head diameter sizes in PL-DRN and unlabeled neurons of ES females compared to HC females. In PL-DRN and unlabeled neurons, rightward shift of ES curve indicates larger mushroom spine head diameters (PL-DRN: KS-test p<0.0001, unlabeled: p=0.0424). b) and c) Kde-plot (left) shows the estimated population distribution of mushroom spine head diameters in PL-DRN and unlabeled neurons of ES females compare to HC females. The plotted difference (right) illustrates that ES females have a smaller proportion of mushroom spines with smaller head diameters and a larger proportion of mushroom spines with larger head diameters in both PL-DRN and unlabeled neurons.
Figure 14. ES females: Thin spine head diameter distributions.
**Figure 14.** a) Cumulative frequency distribution graphs of thin spine head diameter sizes in PL-DRN and unlabeled neurons of ES females compared to HC females. In PL-DRN and unlabeled neurons, rightward shift of ES curve indicates larger thin spine head diameters (PL-DRN: KS-test p=0.0026, unlabeled: p=0.0005). b) and c) Kde-plot (left) shows the estimated population distribution of thin spine head diameters in PL-DRN and unlabeled neurons of ES females compare to HC females. The plotted difference (right) illustrates that ES females have a smaller proportion of thin spines with smaller head diameters and a larger proportion of thin spines with larger head diameters in both PL-DRN and unlabeled neurons.
Figure 15. ES females: Thin spine clustering increased in PL-DRN and unlabeled neurons.
**Figure 15.** a) Kde-plot (left) of the normalized distance of thin spines to their closest neighboring spine in PL-DRN neurons of ES and HC females. Difference plot (right) illustrates that ES females have a greater proportion of very small distances between thin spines in PL-DRN neurons, which indicates increased clustering of thin spines (KS-test p=0.0003). b) Kde-plot (left) of the normalized distance of thin spines to their closest neighboring spine in unlabeled neurons of ES and HC females. Difference plot (right) illustrates that ES females have a greater proportion of very small distances between thin spines in unlabeled neurons, which indicates increased clustering of thin spines (KS-test p<0.0001).
Figure 16. IS females: Mushroom spine head diameter distribution.

(a) PL-DRN

(b) PL-DRN

(c) Unlabeled
Figure 16. a) Cumulative frequency distribution graphs of mushroom spine head diameter sizes in PL-DRN and unlabeled neurons of IS females compared to HC females. In PL-DRN and unlabeled neurons, rightward shift of IS curve indicates larger mushroom spine head diameters (PL-DRN: KS-test p=0.0837, unlabeled: p=0.0002). b) and c) Kde-plot (left) shows the estimated population distribution of mushroom spine head diameters in PL-DRN and unlabeled neurons of IS females compare to HC females. The plotted difference (right) illustrates that IS females have a smaller proportion of mushroom spines with smaller head diameters and a larger proportion of mushroom spines with larger head diameters in both PL-DRN and unlabeled neurons.
Figure 17. IS females: Thin spine head diameter distributions.
Figure 17. a) Cumulative frequency distribution graphs of thin spine head diameter sizes in PL-DRN and unlabeled neurons of IS females compared to HC females. Distributions are not significantly different in either PL-DRN or unlabeled neurons b) and c) Kde-plot (left) and difference plot (right) of thin spine head diameter distribution in PL-DRN and unlabeled neurons. There is no significant difference between distributions from ES and HC females.
Figure 18. IS females: Thin spine clustering decreased in PL-DRN and unlabeled neurons.
**Figure 18.** a) Kde-plot (left) of the normalized of the normalized distance of thin spines to their closest neighboring spine in PL-DRN neurons of IS and HC females. Difference plot (right) illustrates that IS females have a greater proportion of larger distances between thin spines in PL-DRN neurons, which indicates decreased clustering of thin spines (KS-test p=0.0104). b) Kde-plot (left) of the normalized of the normalized distance of thin spines to their closest neighboring spine in unlabeled neurons of IS and HC females. Difference plot (right) illustrates that IS females have a greater proportion of larger distances between thin spines in unlabeled neurons, which indicates decreased clustering of thin spines (KS-test p=0.0204).
Figure 19. HC males vs HC females: Mushroom spine head diameter distributions.
**Figure 19.** a) Cumulative frequency distribution graphs of mushroom spine head diameter sizes in PL-DRN and unlabeled neurons of HC males compared to HC females. In unlabeled neurons, rightward shift of HC male curve indicates larger mushroom spine head diameters (KS-test $p=0.001$). b) Kde-plot(left) and difference plot(right) of mushroom spine head diameters on PL-DRN neurons of HC males and HC females. The distributions do not differ significantly (KS-test $p=0.181$). c) Kde-plot(left) and difference plot(right) of mushroom spine head diameters on unlabeled neurons show that HC males have a smaller proportion of small mushroom spines and a greater proportion of larger mushroom spines compared to HC females.
Figure 20. HC males vs HC females: Thin spine head diameter distributions.
**Figure 20.** a) Cumulative frequency distribution graphs of thin spine head diameter sizes in PL-DRN and unlabeled neurons of HC males compared to HC females. In PL-DRN neurons, rightward shift of HC female curve indicates larger thin spines in females (KS-test p<0.0001).

b) Kde-plot (left) and difference plot(right) show that males have a greater proportion of smaller thin spines and smaller proportion of larger thin spines on PL-DRN neurons. c) Kde-plot (left) and difference plot(right) of thin spine head diameters on unlabeled neurons in HC males compared to HC females. The distributions do not differ significantly.
4.3 Discussion

Chronic stress reduces and acute stress increases spine densities in PL layer II/III neurons (Moench & Wellman, 2015; Nava et al., 2015; Radley et al., 2006). In this study, we did not find changes in spine densities on layer 5 basal dendrites after ES or IS in either sex. But, plastic spine changes can be reflected in increased spine turnover, affecting spine elimination and formation at the same level and resulting in stable spine densities (De Roo et al., 2008; Liston & Gan, 2011; Xu et al., 2009). Because the technique used in the current study does not enable us to track spine turnover, we analyzed changes in head diameter size and spine clustering as additional measures of plasticity.

We found that IS males had increases in both thin and mushroom spine head diameters in unlabeled and PL-DRN neurons. Seeing plasticity in PL of IS males was somewhat surprising, since a previous study by Varela et al. (2012) showed increased excitability of PL neurons 2 hours after ES but not after IS. But, the longer (24 hours) time point after stress chosen in this study could allow for slower synaptic changes to occur. Acute foot shock stress increases glutamate release in PFC via glucocorticoid signaling (Musazzi et al., 2010; Popoli, Yan, McEwen, & Sanacora, 2011), and corticosterone treatment of cultured neurons increases NMDA and AMPA receptor surface expression (Yuen et al., 2011). Moreover, glutamatergic stimulation of spines has been shown to increase spine volume and enhance AMPA currents (Matsuzaki et al., 2004; Tanaka et al., 2008). Thus, the increased head diameters of thin and mushroom spine in IS males could be a consequence of stress induced, general enhancement of glutamate activity. This global potentiation of synapses could represent poor signal to noise ratio that may not contribute to the classic behavioral effects of IS, but could simply be a consequence of stress.
In ES males, only mushroom spines on PL-DRN neurons were enlarged, indicating a selective strengthening of synapses within the circuit. Increased activity in the PL-DRN circuit is key for protective and immunizing effects of ES (Maier, 2015), thus enlarged mushroom spines in the circuit could be both a result of enhanced activity, and contribute to the long-term effects of ES. The 24-hour time point after stress chosen in this study is long enough for acute spine changes to occur and stabilize, but future research will investigate whether the spine changes after different stress conditions are transient, remain stable, or form the basis for different plastic changes to occur.

Unlike IS males, ES males were protected from enlargement of thin spines on PL-DRN neurons and thin and mushroom spines on unlabeled neurons. Foot shock induced increases in glutamate transmission are mediated by glucocorticoid signaling (Musazzi et al., 2010), but ES does not reduce plasma corticosterone compared to IS (Maier, Ryan, Barksdale, & Kalin, 1986), so other factors are likely involved in limiting synaptic potentiation after ES to PL-DRN neurons. For example, Worley et al. (2017) hypothesized that endocannabinoid signaling could be involved in reducing stress signaling inputs while potentiating control signaling inputs, but more research is needed to support this hypothesis. It is also possible that the suppression of DRN over-activity during ES indirectly reduces stress related excitatory inputs, for example from the BLA. DRN over-activity during IS increases 5HT release in the BLA (Amat et al., 1998a), and 5HT2C receptor activation within the BLA results in increased mPFC activity (Campbell & Merchant, 2003). Additionally, IS but not ES increases 5HT and DA efflux in PL (Bland et al., 2003), which could also modulate activity and activity-related plasticity in PL during stress. To summarize, the results show that ES leads to circuit specific plasticity while protecting from global stress induced potentiation of spines outside the circuit. More research is
needed to determine the underlying mechanisms and whether these spine changes are necessary for the immunizing effects of ES to occur.

We also analyzed distances to nearest neighbors in thin spines as a measure for spine clustering and found that ES males showed increased spine clustering within the PL-DRN circuit only while IS males showed increased spine clustering in all neurons. Spine clustering has been observed after learning and LTP induction and tends to increase synaptic strength and promotes survival of newly formed spines (Kasai et al., 2010; Rogerson et al., 2014). For example, LTP induction (De Roo et al., 2008), or motor learning both induce formation of new spines in neighboring pairs, and spines that are part of a cluster have enhanced survival probabilities (Fu et al., 2012). Our findings regarding spine clustering are in line with head diameter results, showing that ES induces selective synaptic strengthening in PL-DRN neurons while IS affects clustering in all neurons.

Stressor controllability effects in females are just beginning to be explored, but results so far show that ES does not protect females from adverse behavioral outcomes and that the PL-DRN pathway is not recruited during ES (M. Baratta & S. Maier, personal communication, June 23, 2017). In line with these findings, we did not find circuit specific effects on spine morphology after ES in females. Instead, ES females showed global spine enlargement in thin and mushroom spines of PL-DRN and unlabeled neurons alike. We also found increased clustering of thin spines in PL-DRN and unlabeled neurons. Together, these results indicate that ES induces global potentiation of synapses in females that likely represent stress effects similar to those seen in IS males.

Interestingly, spine changes in IS females were slightly different than in ES females. IS females had enlarged mushroom spines on PL-DRN neurons and unlabeled neurons like ES
females, but thin spine head diameters were unaffected. IS females also had reduced clustering of thin spines on PL-DRN and unlabeled neurons. It is still unclear why the PL-DRN pathway is not activated during ES in females. One possibility is that control does not get detected in ES females, so PL would not receive control signaling inputs to activate the PL-DRN pathway. Alternatively, control is detected but fails to recruit the PL-DRN pathway, perhaps due to PL dysfunction resulting from intense stress. This seems plausible as females are generally more susceptible to stress induced mPFC dysfunction (Arnsten, 2009; Shansky & Lipps, 2013). The current results suggest that PL is affected differently by ES than IS, but future studies are needed to investigate the mechanism of this effect.

To see whether there are basic sex differences in spine morphology in the PL-DRN circuit, we compared control animals and found that males have a higher proportion of smaller thin spines. Smaller spines are generally more plastic (Bourne & Harris, 2007; Kasai et al., 2010), having more NMDA and fewer AMPA receptors (Matsuzaki et al., 2001, 2004; Noguchi, Matsuzaki, Ellis-Davies, & Kasai, 2005). Very small spines can also be “silent synapses” having only NMDA receptors but no AMPA receptors (Kasai et al., 2010; Matsuzaki et al., 2004; Zito, Scheuss, Knott, Hill, & Svoboda, 2009), which makes them more likely to be sites of plasticity. Thus, having a higher proportion of small thin spines on PL-DRN neurons could make the circuit more plastic in males, which could contribute to the sex differences in the effects of ES.

To our knowledge, this study is the first to investigate circuit specific structural plasticity after ES and IS. We found that in males, ES resulted in dendritic spine changes specifically in the PL-DRN circuit, while IS lead to global spine changes not limited to the circuit. In females, circuit specific plasticity was not expected because ES does not recruit the PL-DRN pathway in females. Indeed, ES females showed global spine changes like IS males. Surprisingly, IS in
females resulted in slightly different spine changes compared to ES, which suggests that PL is affected differently by IS and ES, despite a lack of difference in behavioral outcomes. More research is needed to test the mechanism and functional implications of structural plasticity after IS and ES, and if these changes are long-lasting enough in males to account for immunization effects of ES. Nonetheless, the results add to the vast research on IS and ES effects on male PL, while also giving insight into what may contribute to sex differences in ES effects.
Chapter 5

Discussion

Darting – Overview and possible mechanisms

To improve preventative measures and treatment outcomes of PTSD and other stress related disorders, it is important to understand behavioral and neurobiological factors that contribute to vulnerability and resilience to intensely stressful events. Using rodent models to study resilience and vulnerability to stress has unique advantages, such as being able to control experimental variables and investigate underlying brain circuitry at the synaptic level. Fear conditioning and extinction paradigms are commonly used to study fear and fear extinction processes (Yehuda & LeDoux, 2007). This behavioral paradigm, like many others, has been developed and studied using male rodents, and female rodents have only recently been included in research studies (Bangasser & Wicks, 2017; Kokras & Dalla, 2014; Shansky, 2015). Freezing behavior has been traditionally used as measure of fear levels (Duvarci & Pare, 2014), but this measure may not adequately capture fear levels in female rodents. In study 1 of this dissertation, a novel active fear response (‘darting’) was identified and characterized. The results demonstrate that females are much more likely to show darting behavior compared to males, and that darting during fear conditioning is predictive of improved extinction recall performance. One implication of these results is that future studies using fear conditioning and extinction should include analysis of darting behavior in addition to measuring freezing behavior.

Importantly, only a subset (~40%) of females showed consistent darting responses, and it is unclear what promotes darting in females. It is also not clear whether the relationship between darting and improved extinction recall is causal or merely correlational. One possibility is that
darting represents an escape-like response and that females employing this strategy have the perception of control, or perhaps even actual control, by limiting the time their paws touch the shock grid. The neurobiology and behavioral consequences of stressor controllability has been thoroughly studied in male rodents. ES compared to IS has a variety of beneficial effects on behavioral outcome measures, including reduced conditioned fear, accelerated extinction, and reduced spontaneous recovery (Baratta et al., 2007). These behavioral effects depend on activation of the PL-DRN pathway during ES, which results in suppression of stress induced DRN over-activity (Maier & Watkins, 2010). DRN over-activity during IS is thought to induce sensitization of DRN 5HT neurons so that even mild stressors would activate these neurons (Maier, 2015). For example, animals who received IS will show increased 5HT efflux in the BLA when exposed to a juvenile conspecific one day after stress (Christianson et al., 2010) ES prevents this sensitization and recruitment of the PL-DRN pathway during ES will also prime this pathway to be activated in future IS sessions (Baratta et al., 2009; Christianson et al., 2010). If darting engages the PL-DRN pathway in females it could also reduce 5HT levels in the BLA during fear conditioning and extinction.

It has also been shown that infusing corticotropin-releasing factor (CRF) into DRN increases 5HT in the central nucleus of the amygdala (CeA) and induces freezing (Forster et al., 2006). Additionally, acute selective serotonin reuptake inhibitor (SSRI) treatment (injected systemically) before fear conditioning increases freezing during fear conditioning and at recall, and SSRI treatment before recall also increases fear expression (Burghardt, Bush, McEwen, & LeDoux, 2007). The bed nucleus of the stria terminalis (BNST) is a likely target region for this effect, because direct infusion of SSRIs into the BNST is sufficient to produce the fear enhancing effects of systemic injection of SSRIs (Ravinder, Burghardt, Brodsky, Bauer, & Chattarji, 2013).
To summarize, there is evidence that increased 5HT levels in target structures such as BLA, CeA, and BNST are related to increased freezing behavior and anxiety levels. If darting is an escape-like response that engages the PL-DRN pathway, it would result in reduced 5HT levels in structures that modulate freezing and anxiety levels. Under this hypothesis, suppression of 5HT activity would reduce anxiety and arousal levels during extinction, thus improving extinction learning and resulting in improved outcomes at extinction recall.

Perhaps surprisingly, ES leads to increased 5HT levels in the dPAG during and immediately after ES exposure (Amat, Matus-Amat, Watkins, & Maier, 1998). Administration of two foot shocks the next day also resulted in increased 5HT levels in dPAG of ES animals, and 5HT levels were inversely correlated with freezing behavior after shock presentation (Jose Amat et al., 1998b). The dPAG is generally thought to mediate active fear responses (Bandler, Keay, Floyd, & Price, 2000), so differential 5HT activity in dPAG could be another potential mechanism of how darting contributes to reduced freezing during fear conditioning and possibly during extinction recall. PL also sends projections to dPAG and specifically dLPAG (Floyd, Price, Ferry, Keay, & Bandler, 2000), thus PL-dLPAG could be a potential pathway involved in promoting darting.

Several recent studies have investigated the neurobiology underlying active and passive fear responses in male rodents. Tovote et al. (2016) demonstrated that optogenetically activating glutamate neurons in vlPAG induces freezing, while activation of glutamate neurons in dLPAG induces flight. Further, the authors demonstrate that conditioned freezing depends on CeA mediated disinhibition of vlPAG neurons, while flight inducing dLPAG activation increases inhibition in vlPAG (Tovote et al., 2016). To investigate possible involvement of CeA in also mediating active fear responses, the same research group adapted the traditional fear
conditioning paradigm to promote active fear behaviors (by switching from tone CS to white noise CS). The authors found that CRF neurons in the CeA mediate conditioned flight responses while somatostatin neurons mediate conditioned freezing responses (Fadok et al., 2017). Thus, the CeA is another site of interest for potentially mediating darting responses in females.

**IS-ES dendritic spine morphology – Sex-differences**

Based on limited previous research on sex differences in the response to inescapable stress and effects of control, the finding that females respond to IS and ES using the wheel turn paradigm with identical outcomes is surprising. Kirk and Blampied (1985) studied sex-differences in the effects of inescapable tail shocks on subsequent learned helplessness measured by shuttle box escape latencies. In this paradigm, IS males showed learned helplessness as expected, while females were only minimally impaired by IS. The authors also report that females showed increased and prolonged struggling behavior during IS compared to males, which suggests that females were more resistant to the stressor (Kirk and Blampied, 1985). Reduced IS effects in females were also demonstrated using inescapable shock in the same context subsequently used to test shuttle box escape learning. IS females showed reduced learned helplessness behavior compared to IS males, and stressor controllability only slightly reduced escape latencies in females (Shors et al., 2007; Dalla et al., 2008). However, this paradigm has the caveat that IS and ES are administered in the same context (shuttle box) as the test for learned helplessness. Sex-differences in escape latencies could be due to differences in context conditioning, as a strong conditioned fear response (freezing) would interfere with escape responses. The wheel-turn paradigm circumvents this issue by administering ES and IS within an entirely different context. Another key difference between the paradigms discussed above and the wheel turn paradigm used in study 2 of this dissertation is the severity of stress administered.
Shock duration, intensity and trial frequency are all greater in the wheel turn paradigm. Females tend to be more susceptible to stress induced impairment of PFC function (Arnsten, 2009; Shansky & Lipps, 2013). It is possible that increased sensitivity to stress in females could result in PFC impairment that would prevent recruitment of the PL-DRN in ES females.

The fact that ES in females does not lead to improved behavioral outcomes and does not activate the PL-DRN pathway could mean that control is not detected in female PL to begin with. On the other hand, it could mean that control is detected, but other factors (e.g. increased sensitivity to stress) interfere with PL-DRN recruitment. The results of study 2 support the latter. ES and IS in females induced different changes in dendritic spines of PL. Consistent with the finding that ES does not activate PL-DRN neurons, the changes were not specific to PL-DRN neurons. Nonetheless, the results suggest that ES affects PL in females differently than IS. The results of study II further show that there are basic sex differences in dendritic spine morphology of PL-DRN neurons. Males have a greater proportion of smaller thin spines, which could mean that males have greater potential for plasticity. But, more research is needed to determine why the PL-DRN pathway does not get activated by ES in females.

**IS-ES dendritic spine morphology – Males**

Only a few studies have investigated plasticity in PL after IS and ES in males. Since ES has “immunizing” effects, meaning previous ES exposure protects from the detrimental effects of IS days later (Amat et al., 2006), it would be expected that PL undergoes plastic changes. This idea was broadly tested by that blocking protein synthesis in mPFC through anisomycin infusion either before or immediately after ES. Amat et al (2006) showed that blocking protein synthesis during ES blocks the immunization effect completely, while blocking it after ES reduces immunization but does not block it completely. Additionally, it has been demonstrated that PL
neurons show increased excitability 2 hours after ES but not after IS, and that his effect was due to increased sodium and calcium channel conductance (Varela et al., 2012). The immunizing effects of ES also depend on NMDA receptor activation in PL at the time of ES (Christianson et al., 2014). These studies provide evidence that plastic changes occur in PL during and after ES that are necessary for the immunizing effects. However, these studies modulated and investigated plasticity in PL as a whole without looking at pathway specific modulation of PL-DRN neurons. The results from study 2 demonstrate for the first time that ES induces selective changes in dendritic spine morphology in PL-DRN neurons.

It was somewhat surprising to see dendritic spine changes in IS males, because the studies discussed above did not show effects of blocking plasticity in PL during IS, and did not show changes in excitability of PL neurons 2 hours after IS (Christianson et al., 2014; Varela et al., 2012). But, in study 2 spine changes were measured 24 hours after stress, so it is possible that the 2 hour window used in Christianson et al (2014) was not long enough to measure stress induced plasticity. It is also possible that the global spine changes seen in IS males are simply a product of stress without having effects on behavior changes seen after IS. The very specific effects after ES, however, could indicate selective strengthening of controllability signaling inputs. Despite the vast literature on ES effects on the brain, it is still not known where exactly these inputs would come from. It has been shown that the dorsal medial striatum (DMS) is essential for the manifestation of protective effects after ES. Injecting and NMDA receptor antagonist into DMS immediately before ES does not interfere with learning the wheel turn task, but prevents the protective effects of ES (Amat et al., 2014). Thus, it is hypothesized that DMS signals controllability to PL via direct or indirect projections (e.g. through the thalamus) (Maier, 2015).
The results of study 2 cannot speak to whether spine changes are necessary or sufficient for the immunizing effects of ES. Another limitation of this study is that we can only assess spine head diameter as a population and compare experimental conditions to control conditions. For example, it is unclear whether increased proportion of larger mushroom spines in ES are a result of potentiating existing mushroom spines, thin spines, or formation of new, large mushroom spines. Similarly, it is possible that increased proportion of large thin spines in IS males is a result of potentiation of existing small thin spines, or formation of new, larger thin spines alongside elimination of small thin spines. To date, in vivo two photon imaging still lacks the necessary resolution to reliably track changes in spine size, so technical advances are needed to investigate spine size dynamics in vivo. Additionally, many of the possible interpretations of study 2 results rely on research investigating spine dynamics in hippocampal slices, so future research is needed to test whether effects in PL follow a similar pattern.

Future directions

Study 1 of this dissertation identified and characterized darting as a novel, active fear behavior that is predominantly displayed by female rats. Future research should address what makes females in general more likely to engage in darting behavior compared to males. Additionally, because only some females are ‘darters’, it will be important to study differences between darters and non-darters within sex. As discussed above, the PAG is a possible site for selection of darting over freezing behavior, with the dPAG thought to be involved in active defensive responses while the vlPAG is implicated in modulating freezing (Bandler et al., 2000; Vianna, Graeff, Brandão, & Landeira-Fernandez, 2001). PL sends projections to both dPAG and vlPAG so darting could be due to PL modulation of PAG activity. Once identified, the circuitry that elicits darting behavior can be modulated via chemogenic or optogenetic techniques to test
whether darting directly contributes to improved outcomes at extinction recall. Additionally, more research is needed to identify how darting contributes to improved outcomes at extinction recall, with PL-DRN engagement being a potential mechanism.

Dendritic spine morphology results from study 2 indicate that ES affects female PL differently, which indicates that control is detected at least on some levels in ES females. As suggested earlier, the failure of PL-DRN recruitment by ES in females could be due to increased stress sensitivity that leads to PL dysfunction. Future studies should test whether reducing shock severity or session duration during ES would result in behavioral protective effects and PL-DRN activation in females. To increase clinical relevance, it should also be tested whether modulating stress levels pharmacologically in females before and/or during ES would result in emergence of ES protective effects. It will also be important to test which inputs to PL signal controllability of stress in males to ultimately activate PL-DRN neurons and whether females do not receive similar input signals of controllability.

It is difficult to test functional relevance of spine changes in vivo, but recent technological advances allow for manipulation of dendritic spines. Hayashi-Takagi et al. (2015) developed a new approach to directly manipulate newly formed spines. The authors developed an “optoprobe” that will tag spines potentiated by synaptic activity and upon illumination with light selectively induce shrinkage in these newly potentiated spines. A similar approach could be used in future studies to investigate if the enlargement of mushroom spines in PL-DRN neurons of ES males is necessary for the protective effects of ES.
Conclusion

The findings of this dissertation have several important implications for future research on individual and sex differences in vulnerability and resilience to stress and trauma. As study 1 demonstrated, females can display active fear behaviors in the fear conditioning and extinction paradigm. Recently, improving the validity of animal models in both sexes has received more attention (Kokras & Dalla, 2014; Shansky, 2015), and study 1 contributes to the growing literature in this field. Study 2 adds to the sparse research on stressor controllability effects in female rodents and the results indicate that stressor control is at least detected in female PL on some level, even though it is not translated into protective effects behaviorally. The results set the stage for future research on why stressor controllability in the wheel turn paradigm does not have the same effect in females as it does in males. The findings in study 1 and study 2 also underscore the importance of studying between, as well as within sex differences in the response to stressful events.
References


