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Sex differences in PTSD resilience and susceptibility: Challenges for animal models of fear learning

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Abstract

PTSD occurs in only a small fraction of trauma-exposed individuals, but risk is twice as high in women as in men. The neurobiological basis for this discrepancy is not known, but the identification of biological determinants of resilience and susceptibility in each sex could lead to more targeted preventions and treatments. Animal models are a useful tool for dissecting the circuits and mechanisms that underlie the brain's response to stress, but the vast majority of this work has been developed and conducted in males. The limited work that does incorporate female animals is often inconsistent across labs and does not broadly reflect human populations in terms of female susceptibility to PTSD-like behaviors. In this review, we suggest that interpreting male vs. female comparisons in these models be approached carefully, since common behavioral outcome measures may in fact reflect distinct neural processes. Moreover, since the factors that determine resilience and susceptibility are likely at least in part distinct in men and women, models that take a within-sex approach to response variability may be more useful in identifying critical mechanisms for manipulation.

Keywords

Sex differences; Fear conditioning; Extinction; PTSD; Estrousanxiety

A person exposed to a traumatic event or stressful experience risks developing Post-Traumatic Stress Disorder (PTSD) as a result (Breslau and Kessler, 2001). These mental illnesses can be deeply debilitating and have detrimental effects on patients' physical well-being, cognitive abilities, interpersonal relationships, and general functioning in society, and thus present a major public health issue. One of the primary challenges to the biomedical research community has been that of identifying the neurobiological factors that confer susceptibility and resilience in response to stress exposure: although a majority of the population will experience a severe trauma at some point in their lifetime, the fraction of those people who develop PTSD is in fact relatively small (Yehuda and LeDoux, 2007). A better understanding of the neurobiological mechanisms that underlie individual differences

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in the consequences of stress is thus critical to progress in both treatment and prevention of this disorder.

One of the most consistently reported risk factors for PTSD is being female. Women are twice as likely as men to develop PTSD after a trauma (Haskell et al., 2010), but the reasons for this discrepancy are poorly understood. This is a particularly topical problem in the context of our recent wars in the Middle East, which have been fought by a greater percentage of women than have any international conflicts before them (D. of Defense, 2008)). Women are the fastest growing population in US Veterans Affairs (VA) hospitals, and the current percentage of female patients at VA hospitals is expected to double in the next twenty-five years (Yano et al., 2010). Women who suffer from PTSD undoubtedly will be best served by treatments that take into consideration not only the unique experiences of a woman in combat (e.g. the disproportionately high incidence of Military Sexual Trauma in women (Himmelfarb et al., 2006)), but also the distinct neurobiological background against which those experiences take place. It is thus all the more imperative that the biological ramifications of stress in women are better understood, and that sex-specific markers of susceptibility and resilience to stress-related mental health problems are identified.

For decades, the use of animal models in preclinical research has provided great insight into the neural circuits and mechanisms that mediate the effects of stress. However, despite the twofold increase in PTSD prevalence in women, the vast majority of relevant basic science work has been conducted in male animals (Lebron-Milad and Milad, 2012). We are thus left with a poor picture of stress effects that are specific to the female brain, knowledge of which could aid in the development of better treatments. Perhaps even more concerning is the lack of a behavioral model that convincingly produces sex differences that mirror those observed in humans—i.e., one in which females reliably exhibit PTSD-like symptoms more robustly and frequently than males do (Kokras and Dalla, 2014). This fundamental lack of agreement between animal and human populations may be due to the fact that the common paradigms used to measure fear and anxiety were developed using male animals. Inconsistencies observed when females are evaluated using these tools may indicate that the traditional outcome measures associated with each test in fact tap into distinct processes in females, and do not accurately reflect the emotional states assumed based on data collected in males. In this review, we will examine evidence from studies of sex differences in stress effects on classic behavioral fear learning paradigms. Ultimately, our goal is to identify measures that may require reinterpretation or adjustments in design, so that sex-specific markers of resilience and susceptibility to stress may be more accurately determined.

1. Learned fear responses

PTSD is characterized by a strong and persistent association between the memory of the trauma and its associated cues, such that the cues alone can trigger a fear response (Rothbaum and Davis, 2003). In behavioral paradigms, a similar association is created when an animal undergoes fear conditioning (Schafe et al., 2001). In this task, an animal learns to associate a previously neutral cue, like an auditory tone, with an aversive stimulus, usually a brief foot shock. When learning is successful, the animal will later express fear (measured by freezing behavior) when it hears the tone alone, even in a new context. If the tone is then

repeatedly presented without a subsequent shock, the animal's freezing will subside as it learns the tone no longer predicts the painful stimulus. This process is called extinction (Quirk and Mueller, 2008). Behaviorally, PTSD patients appear unable to extinguish the trauma-related associations they have formed (Milad et al., 2009a), and in laboratory settings PTSD patients are impaired at extinction of conditioned fear compared to healthy controls (Milad et al., 2009a). Extinction is mediated in both humans and animals by neural circuitry that is often implicated in imaging studies of PTSD—specifically, connections between the prefrontal cortex and the amygdala (Gilboa et al., 2004; Quirk et al., 2003; Knapska et al., 2012). A more comprehensive understanding of the neurobiological processes that govern extinction in animal models could thus provide critical insight into the causes of the disorder.

There is an extensive literature on extinction and its underlying mechanisms, but less than 2% of this work has been done in females (Lebron-Milad and Milad, 2012). An even smaller fraction directly compares extinction in males and females, and the limited reports that do exist are inconsistent. One might expect that since women are more likely to develop PTSD, female animals would exhibit poorer extinction than males. But while at least one group has reported that females are impaired in extinction learning compared to males (Baran et al., 2009), others report enhanced extinction in females (Milad et al., 2009b). In studies that examined contextual fear responses (freezing in response to the conditioning environment), males appear to freeze more than females during both fear conditioning and extinction (Chang et al., 2009), an effect that may be due to sex differences in hippocampal neurotransmission (Maren et al., 1994). Further complicating the issue is the potential influence of ovarian hormones; estradiol (either circulating or administered) has been reported to potentiate extinction (Milad et al., 2009b; Milad et al., 2010; Graham and Milad, 2013; Rey et al., 2014), attenuate it (Toufexis et al., 2007), or have no effect (Hoffman et al., 2010). These discrepancies may be a product of variations in protocol amongst laboratories, animal strain, or general differences in behavioral variability between the sexes, but evaluating any of these possibilities in a post-hoc fashion is not feasible.

Another consideration is that many of these studies may simply not be broad enough in sample size to confidently identify resilient and vulnerable populations, especially in females. Most animal behavior studies traditionally use experimental groups that are between 8 and 12 in number, basing analyses on group means. This approach, while useful for assessing average or "normal" behavior, is not sufficiently powered to detect inter-group differences in intra-group variability patterns. Indeed – like humans after a trauma, not all animals that undergo fear conditioning will extinguish the conditioned fear response to the same extent. Bush et al. (2007) recently demonstrated that when a large cohort of male rats undergoes extinction, the degree to which any particular animal later freezes to the tone will fall along a standard Gaussian distribution. Animals that fall on the tails of the curve represent the extremes of the population—those that are the most and least capable of suppressing freezing to the tone. Respectively, these groups may be a useful model of resilience and vulnerability, and can be exploited to probe for biological markers of variation in behavior in a traumatic context (Holmes and Singewald, 2013). Importantly, large cohorts of both sexes could provide insight into sex-specific determinants of failed and successful extinction.

Recently, we conducted a large-scale analysis of auditory cued fear conditioning and extinction in large cohorts of gonadally intact male and female rats (Gruene et al., submitted for publication). The goals of this study were 1) to evaluate a large enough sample of animals that we could be sure of observing any baseline sex differences in behavior; 2) identify “susceptible” and “resilient” sub-populations of both sexes, as characterized by poor and successful extinction retrieval; and 3) determine sex-dependent patterns of behavior in these subpopulations. Surprisingly, we found that there were no overall sex differences in freezing to the tone at any point over the course of fear conditioning, fear extinction, and extinction retrieval. Importantly, we also found similar ranges of variability in freezing between males and females. Together, these findings could be interpreted to mean that as populations, males and females do not differ in this classic learning and memory task. However, the fact that average freezing levels were comparable does not necessarily mean that the mechanisms that drive freezing are identical in males and females. To further probe the source of behavioral variability in each sex we separated animals based on freezing during extinction retrieval as susceptible (high freezing) and resilient (low freezing) subpopulations. A retrospective analysis of freezing during fear conditioning and extinction learning revealed distinct, sex-specific trajectories in susceptible vs. resilient groups. Most notably, these phenotypes emerged earlier in females – during fear conditioning – than in males, who did not distinguish as susceptible or resilient until extinction. Importantly, freezing levels during extinction retrieval did not differ between susceptible males and females, demonstrating that even identical freezing levels in males and females may in fact be indicative of distinct neural processes.

If women are more likely to develop PTSD, why don’t female rats freeze more than males in fear conditioning and extinction paradigms? One explanation could be that females express fear differently than males do. Since the introduction of the paradigm, freezing during a conditioned tone presentation has overwhelmingly been the singular measure of fear in cued fear conditioning and extinction experiments. Freezing is traditionally defined as “the complete cessation of movement with the exception of that required for respiration,” (McAllister et al., 1971) and the amount of time spent freezing is considered to be a measure of the degree to which the animal has learned the tone-shock association (Pare et al., 2004). This practice necessitates that all movement is then treated equally as non-fearful behavior. However, a number of different behaviors can be observed in response to a conditioned tone that would not be counted as freezing, but could still indicate not only recognition that the tone is meaningful (and therefore successful learning and memory), but also a fearful emotional state. These include darting and rearing, which could reflect escape-like behavior, and scanning, an expression of hypervigilance characterized by a side-to-side head motion (Choy et al., 2012). If females are more likely than males to express these non-freezing behaviors in response to the tone—either in place of or in addition to freezing—then an examination of freezing alone may not accurately reflect sex differences in fear learning, memory, and expression.

The possibility of sex-specific behavioral response profiles during learned fear tests is an especially important consideration given the common practice of removing animals that do not reach a freezing criterion for fear conditioning learning from analyses in extinction studies (Sotres-Bayon et al., 2007). Because these animals do not express high levels of

freezing at the very beginning of extinction, they are presumed not to have learned the tone-shock association, and are removed so that they do not artificially suggest accelerated extinction in their experimental group. In our work described above, using this criterion allowed us to distinguish between “resilient” animals that froze in response to the tone at the beginning of extinction (thus demonstrating learning), but successfully suppressed freezing after extinction, from those who might wrongly be classified as “resilient” because they simply never froze to the tone at any point in behavioral assessment. However, if their lack of freezing is due to the expression of any of these active responses to the tone (instead of an absence of fear, as is generally inferred), then this presumption is incorrect. We are currently revisiting our behavioral videos to determine whether there are sex differences in the expression of behavioral responses other than freezing. It may be that a more appropriate model of resilient vs. susceptible individuals lies in assessment of a complex system of responses, rather than along a spectrum of freezing alone. Importantly, the behavioral characteristics of a susceptible female animal may be distinct from those of a susceptible male. This scenario would be consistent with human studies of PTSD symptomatology, which have found sex differences in the most frequently experienced symptoms. For example, women report more distractibility and emotional distress, while men report more emotional numbness and hypervigilance (King et al., 2013).

Interestingly, measures of learned fear other than freezing produce different outcomes in males and females. In classical eyeblink conditioning, a white noise repeatedly paired with a brief shock to an animal’s eyelid produces an anticipatory eyeblink response to subsequent presentations of the noise. Landmark work by Tracy Shors has consistently shown that female rats acquire the conditioned response more rapidly, and maintain higher levels of responding than male rats (Wood and Shors, 1998; Dalla and Shors, 2009; Maeng and Shors, 2013). Whether eyeblink conditioning thus better taps into the circuits and mechanisms that mediate sex differences observed in human populations is not clear, but in the following section, we discuss the sex-specific manner in which stress modulates learning in this model. In another paradigm, fear-potentiated startle (FPS), an animal is trained to associate a neutral stimulus with a footshock, as in fear conditioning. When a startling noise is later presented in the presence of the conditioned stimulus, animals have exaggerated, or potentiated, startle responses (Walker and Davis, 2002). Mazor et al. (2009) found that female rats had a greater baseline startle amplitude than males, an effect that has also been observed in mice (Adamec et al., 2006). Toufexis et al. (2007) did not observe this sex difference; however, this group employed an extended conditioning paradigm which may have normalized the fear levels induced by the conditioned stimulus.

The work discussed above demonstrates the serious need for increased fear research in female animals. In many fear paradigms, consensus on the directionality of baseline sex differences has not been reached, something that can only be achieved with further efforts on the part of researchers to both replicate major findings and converge upon standard protocols. In the case of associative learning paradigms, whether the initial strength of the memory itself or the lasting persistence of that memory is a better marker for resilient and vulnerable phenotypes is still unknown. However, the possibility that these markers are different for males and females must be considered when interpreting experimental results. Importantly, more work must be done to identify sex-specific mechanisms that modulate

behavior in these tasks—not only gonadal hormones, but potential neurotransmitter and peptide signaling pathways as well. These systems are sexually dimorphic (Bangasser and Valentino, 2014), (Gillies et al., 2014), but their role in producing sex differences in fear behavior has only just begun to be studied.

2. Effects of stress on learned fear

Until the fifth edition of the Diagnostic and Statistical Manual of Mental Disorders (DSM-V) was issued in 2013, PTSD was classified as an anxiety disorder. The symptomatology profiles of anxiety disorders and PTSD overlap substantially, and comorbidity amongst patients is well-documented (Kessler et al., 1995), (Spinhoven et al., 2014). Like PTSD, anxiety disorders are twice as prevalent in women as in men (Wittchen et al., 2011), an epidemiological phenomenon whose biological basis also remains unknown. The neural mechanisms that underlie anxiety have been studied extensively using animal models like the elevated plus maze (EPM) and open field test (OFT), which are designed to probe the conflicting drives of an animal to both explore yet protect itself from potentially life-threatening situations (Walf and Frye, 2007), (Campos et al., 2013). As is the case with learned fear paradigms, the vast majority of this work has been done in males, but a relatively more substantial body of literature includes females as well. Surprisingly, a majority of studies that use both sexes in these tests find that females display less anxiety than males (Imhof et al., 1993), (Frye et al., 2000). This discrepancy between the directionality of sex differences in animal and human populations may be due to inherent problems in the outcome measures of the animal models themselves: specifically, while they may provide accurate indices of anxiety in males, they may in fact primarily measure general activity in females (File, 2001), (Fernandes et al., 1999). This possibility presents obvious obstacles to the interpretation of sex differences when using these models, and is discussed in detail in an excellent new review by Kokras and Dalla (2014).

PTSD is now classified as a “trauma and stress-related disorder,” meaning that exposure to a traumatic event is a primary diagnostic criterion. It could thus be argued that variability in measures of fear and anxiety alone may not identify PTSD resilient and susceptible subpopulations, but that behavior on these measures after exposure to a distinct stressful event may instead provide better insight. There are many models of stress exposure in rodents; classic approaches include repeated physical restraint, foot- or tail-shock, exposure to predator odor, or a combination of several different stressors (unpredictable mild stress). These stressors activate the hypothalamic-pituitary-adrenal (HPA) axis and can cause alterations in neuronal morphology (Shansky and Morrison, 2009), as well as affect a wide variety of behaviors and learning and memory tasks in both males and females (Shansky, 2009). It should be noted that many of these traditionally-used experimental stressors have been criticized for not accurately resembling the kinds of stressors that lead to PTSD in humans (Golden et al., 2011); attempts at more translationally valid models include underwater trauma (Richter-Levin, 1998), (Moore et al., 2014) and physical abuse by a conspecific (social defeat; (Golden et al., 2011), (Krishnan, 2014).

Although most stress work has been conducted in male animals, there is a growing body of evidence that stress affects fear learning and memory in a sex-specific manner. In eyeblink

conditioning studies, prior exposure to tailshock stress elicits opposing effects in males and females: while conditioned responses increase in males after stress exposure, females exhibit fewer conditioned responses, an effect that depends on circulating estradiol (Wood and Shors, 1998). In males, chronic restraint stress (Izquierdo et al., 2006) psychosocial stress (Wilson et al., 2014), and early-life stress (Stevenson et al., 2009) can disrupt fear extinction compared to control animals, consistent with the idea that impaired extinction in PTSD patients is due in part to trauma exposure. In females, however, findings are less consistent. Chronic restraint stress has been found to enhance extinction processes in females (Baran et al., 2009), but environmental stress (Gruene et al., 2014) has been found to impair extinction. Because of the limited reports currently in the literature, the role of estradiol in modulating stress effects on extinction is difficult to parse; however, since high estradiol status is frequently reported to enhance extinction in both women and female animals (Lebron-Milad et al., 2012), it follows that estradiol-stress interactions likely contribute to extinction outcomes (Antov and Stockhorst, 2014). This line of inquiry is particularly deserving of increased attention, with special consideration for stressor type and timing.

The studies described above examined the effects of stress during adulthood, but stress exposure during childhood or adolescence can also have long-term effects on fear conditioning and extinction processes, often in a sex-dependent manner. Such models are particularly relevant to PTSD because prior exposure to stress—especially in early life—is one of the greatest risk factors for PTSD after a trauma in adulthood (Heim et al., 1997). Maternal separation stress (MS) has been shown to impair extinction retrieval in males (Wilber et al., 2009) and produce robust spontaneous recovery of an extinguished context fear response in females (Xiong et al., 2014). Complicating this finding, however, are results from another group showing that neonatal stress can preferentially amplify footshock sensitivity in females (Kosten et al., 2005). In contrast to MS, peri-pubertal stress exposure (predator odor plus elevated platform) has been found to impair extinction in males, but facilitate it in females (Toledo-Rodriguez and Sandi, 2007). However, social stress during adolescence—a combination of isolation and recurrent cage-mate switching—impairs extinction in both males and females (McCormick et al., 2013). Collectively, findings from these studies do not paint a fully consistent picture, again emphasizing the specificity with which stressful events can affect the brain, and the care required in experimental design for future studies. In particular, it may be the case that certain stress models are more ethologically relevant to females vs. males—for example, social stress vs. predator exposure.

One of the primary issues of interpretation in studies that employ a “stress vs. no stress” group design, however, is whether the changes observed in the stress group as a whole accurately represent the disease state, or simply the normal adaptations the brain undergoes in response to trauma (Cohen et al., 2004). As noted in the introduction, PTSD occurs in a limited subset of trauma-exposed individuals, and approaches that instead examine individual stress responses in order to identify resilient and susceptible subpopulations are becoming a new standard for animal models of mental illness (Krishnan, 2014). One paradigm that has been especially fruitful has been the resident-intruder social defeat model, in which mice are repeatedly exposed to a dominant aggressor (Miczek, 1979). After chronic social defeat, mice reliably stratify on measures of social interaction when exposed to an

unfamiliar mouse, distinctions that can then be used to examine biological markers of susceptible (anti-social) and resilient (social) populations (Golden et al., 2011), (Gómez-Lázaro et al., 2011), (Elliott et al., 2010). The relationship of resilient vs. susceptible phenotypes to learned fear behavior has recently begun to be studied, but a clear picture has not yet emerged: Chou et al. (2014) found that susceptible mice exhibited greater freezing during fear conditioning compared to a resilient population, while Meduri et al. (2013) previously reported that resilient animals expressed higher and longer-sustained fear levels.

Potential sex differences in social defeat resilience are not known, primarily because common laboratory strains of female rodents do not typically display territorial aggression in the same way males do. There are several exceptions worth noting, however. First is the female California mouse, and Trainor and colleagues have used this model to identify a number of sex differences in the behavioral and cellular changes that social defeat elicits (Greenberg et al., 2014; Trainor et al., 2011), including an intriguing role for dopaminergic signaling (Campi et al., 2014). To date, however, this model has not been used to identify susceptible and resilient populations of females. A second model modifies the classic male resident-intruder paradigm, taking advantage of the aggression that a lactating female rat will express to an intruder female. This approach has been shown to induce depressive-like behavior (Bourke and Neigh, 2011; Bourke and Neigh, 2012; Ver Hoeve et al., 2013) social avoidance (Lukas and Neumann, 2014), and alterations in cocaine sensitivity (Shimamoto et al., 2011; Shimamoto et al., 2014) in female rats, lending it translational validity to a number of stress-related mental illnesses. Finally, Carmen Sandi and colleagues have developed an intriguing model of intimate partner violence. Although male rats will not normally attack females, Cordero et al. (2012) found that adult male rats that were exposed to stress during peripuberty will attack female cage mates when mildly agitated. In defeated females, the degree of aggression experienced predicted changes in serotonin transporter gene expression as well as learned helplessness, and varied according to pre-aggression anxiety (Poirier et al., 2013). Whether this stress model can be used to predict individual differences in fear conditioning and extinction tests has not been investigated, but it is also an attractive model from a translational standpoint. Interpersonal violence—especially when the attacker is a domestic partner—is one of the traumas most likely to lead to PTSD in women (Breslau et al., 1999; Forbes et al., 2014). This model may be especially relevant for military populations, since male-to-female sexual assault is unfortunately common in deployed troops (Haskell et al., 2010; Street et al., 2009).

3. Conclusions

Women are more likely than men to develop PTSD after a trauma, but whether the determinants of resilience or susceptibility are distinct in men and women are unclear. Most likely, a sex-specific combination of genetic (Ressler et al., 2011), hormonal (Lebron-Milad et al., 2012), and life experience (Kline et al., 2013) factors (Table 1) contribute to the long-term consequences of trauma exposure for a given individual. Preclinical work in animal models of stress and fear has great potential to identify these factors, but dissecting sex differences within these paradigms requires careful consideration when interpreting behavioral differences. For an excellent, comprehensive guide to launching a sex differences behavioral neuroscience research program, see Becker et al. (2005). Approaches that take

into account within-sex individual variability in behavior rather than performing simple male vs. female comparisons will likely be best able to identify the factors that confer resilience and susceptibility in each sex. Clearly, a great deal of work remains, and many mechanisms of stress and fear that have been accepted in males for years await validation in females. However, addressing the critical need for improved PTSD prevention and treatment in women is a challenge that we have no choice but to meet.

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Table 1

Summary of observed sex differences and stress effects in rodent learned fear behavioral paradigms. F = female; M = male; FC = fear conditioning; EX = extinction.

Behavioral Paradigm	Manipulation	Measure	Effect	Reference
Cued FC & EX	None	Freezing	F > M	Baran et al. (Mar. 2009)
Cued FC & EX	None	Freezing	M > F	Milad et al. (Dec. 2009b)
Context FC & EX	None	Freezing	M > F	Chang et al. (Nov. 2009)
Cued FC & EX	Estradiol (circulating)	Freezing	F ↓	Milad et al. (Dec. 2009b); Rey et al. (Apr. 2014)
Cued FC & EX	Estradiol (injection)	Freezing	None	Hoffman et al. (Oct. 2010)
Context FC & EX	Estradiol (injection)	Freezing	F ↓	Chang et al. (Nov. 2009)
Eyeblink conditioning	None	Eyeblink responses	F > M	Dalla and Shors (May 2009)
Fear potentiated startle	None	Startle magnitude	F > M	Mazor et al. (2009); Adamec et al. (Jun. 2006)
Eyeblink conditioning	Tailshock stress	Eyeblink responses	F ↓ M ↑	Wood and Shors (Mar. 1998); Maeng and Shors (Jan. 2013)
Cued FC & EX	Chronic restraint stress	Freezing	F ↓ M ↑	Baran et al. (Mar. 2009); Izquierdo et al. (May 2006)
Cued FC & EX	Psychosocial stress	Freezing	F ↑ M ↑	McCormick et al. (Nov. 2013); Wilson et al. (Jul. 2014)
Cued FC & EX	Environmental stress	Freezing	F ↑	Gruene et al. (May 2014)
Cued FC & EX	Maternal separation stress	Freezing	F ↑ M ↑	Wilber et al. (2009); Xiong et al. (Aug. 2014)
Cued FC & EX	Peri-pubertal stress	Freezing	F ↓ M ↑	Toledo-Rodriguez and Sandi (Jan. 2007)