BEHAVIORAL AND PHYSIOLOGICAL ASSESSMENT OF AN ANIMAL MODEL OF POST-TRAUMATIC STRESS DISORDER

By Sonal Goswami

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Written under the direction of

Professor Denis Pare

And approved by

_____

Elizabeth Abercrombie, PhD.

_____

Mohammed Milad, Ph.D

_____

Kevin Pang, Ph.D

_____

Denis Pare, Ph.D

_____

James M. Tepper, Ph.D

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ABSTRACT OF THE THESIS

Behavioral and Physiological assessment of an animal model of Post-traumatic Stress Disorder

By Sonal Goswami

Thesis director: Professor Denis Pare

Post-traumatic stress disorder (PTSD) is an anxiety disorder triggered by exposure to a traumatic event. Despite recent progress, the causes and pathophysiology of PTSD remain poorly understood, partly because of ethical limitations inherent to human studies. One approach to circumvent this obstacle is to study PTSD in a valid animal model of the human syndrome. In one such model, extreme and long-lasting behavioral manifestations of anxiety develop in a subset of Lewis rats after exposure to an intense predator threat (PT) that mimics the type of life-or-death situation known to precipitate PTSD in humans. Thus, the first half of this thesis tested whether the Lewis rat model reproduces salient features of human PTSD. The results of these studies established the model's face validity. The second half of this thesis used this model to identify alterations in the physiological properties of amygdala neurons that underlie the expression of PTSD. These studies revealed that PTSD is associated with differences in the synaptic responsiveness of central amygdala (CeA) neurons. Overall, these results suggest that the Lewis rat model of PTSD can be used to gain mechanistic insights in the pathophysiology of PTSD.

PREAMBLE

Exposure to a severe traumatic event leads to the expression of a syndrome called post-traumatic stress disorder (PTSD), which affects around 7% of the population (Nemeroff et al., 2006). The development and expression of PTSD involves predisposing factors along with physiological, behavioral, emotional, and cognitive changes that emerge after trauma (Afifi et al., 2010; Nugent et al., 2008). However, progress in identifying these factors has been hampered by ethical limitations associated with human research. For instance, humans cannot be randomly assigned to trauma, and importantly, the invasive techniques required to study the pathophysiology of PTSD can only be used in animals. Thus, a promising approach towards understanding the pathophysiology of PTSD is to study the disease in a valid animal model that reproduces salient features of the human syndrome.

Human studies have consistently revealed that PTSD is associated with abnormal regulation of conditioned fear responses (Orr et al. 2000; Peri et al., 2000; Blechert et al., 2007; Milad et al., 2008, 2009), and impaired performance on hippocampal-dependent tasks (Shin et al., 2004; Lindauer et al., 2006; Gilbertson et al., 2007; Thomaes et al., 2009; Hayes et al., 2011; reviewed in Samuelson, 2011). Interestingly, identical twin studies have revealed that impaired extinction of classically conditioned fear responses develops as a result of trauma (Milad et al., 2008), while hippocampal deficits were shown to predate trauma (Gilbertson et al., 2007). Thus, the first half of this thesis will use these criteria to test the validity of the Lewis rat model of PTSD.

Furthermore, human neuroimaging studies consistently report higher amygdala activity in individuals with PTSD. Given the limited spatial resolution of fMRI, the specific mechanisms of this increased activation remain unclear. Therefore, we performed ex vivo analyses of the physiological properties of amygdala neurons in PTSD-like and resilient rats, using the whole-cell patch technique. We found that expression of the PTSD-like phenotype was associated with alterations in the synaptic responsiveness of central amygdala neurons.

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TABLE OF CONTENTS

LIST OF ABBREVIATIONS

aCSF – artificial cerebrospinal fluid

ACTH – adrenocorticotropic hormone

AMPA – 2-amino-3-(3-hydroxy-5-methyl-isoxazol-4-yl) propanoic acid

AOR – allocentric object-place recognition

BL – basolateral nucleus of the amygdala

BLA – basolateral complex of the amygdala

BM – basomedial nucleus of the amygdala

BNST – bed nucleus of the stria terminalis

BNST-AL – anterolateral portion of the bed nucleus of the stria terminalis

BNST-AM – antermedial portion of the bed nucleus of the stria terminalis

CeA – central amygdala

CeL – lateral sector of the central amygdala

CeM – medial sector of the central amygdala

CORT – corticosterone/cortisol

CS – conditioned stimulus

CVS – chronic variable stress

DI – discrimination index

DSM – Diagnostic and Statistical Manual for Mental Disorders

EBMA – extreme behavioral manifestations of anxiety

EC – external capsule

EOR – egocentric object-place recognition

EPM – elevated plus maze

EPSP – excitatory postsynaptic potential

fMRI – functional magnetic resonance imaging

HPA – hypothalamic-pituitary-adrenal axis

IL – infralimbic region of the rodent medial prefrontal cortex

IPSP – inhibitory postsynaptic potential

ITC – intercalated cell masses

LA – lateral nucleus of the amygdala

LF – late-firing neuron

LTB – low threshold bursting neurons

NOR – novel object recognition

OF – open field test

PACAP – pituitary adenylate cyclase activing peptide

PTSD – post-traumatic stress disorder

PT – predator threat

RS – regular spiking neurons

SPS – single prolonged stress

US – unconditioned stimulus

UR – unconditioned response

vmPFC – ventromedial prefrontal cortex

LIST OF TABLES

Chapter III

Chapter VI

LIST OF ILLUSTRATIONS

Chapter II

Chapter III

Chapter IV

Chapter VI

CHAPTER I

INTRODUCTION TO PTSD AND RELATED ANIMAL MODELS

1.1 Changing views on the origin, symptoms, and treatment of PTSD

Although PTSD has gained considerable attention in recent decades, it has not always been clear what constitutes adaptive versus maladaptive responses to traumatic stress. However, PTSD-like symptoms were identified early on, as evidenced in classical literature and religious texts. Perhaps the most famous example is Part I of *King Henry IV*, written by William Shakespeare in the 1500s. Here, a soldier's wife describes combat-related PTSD-like symptoms that include nightmares, heightened physiological activity, emotional numbness, social withdrawal, and depression. This impressive description of PTSD came hundreds of years before the French Revolution, which led to a large number of soldiers exhibiting similar traumatic stress symptoms. Although traumatic stress symptoms were first recognized within the context of military combat, the Industrial Revolution brought vast increases in occupational disaster victims expressing similar traumatic stress symptoms. These events led to the conclusion that traumatic stress symptoms could occur outside the realm of combat experience.

Around the late 1800s, an intellectual rivalry was brewing between proponents of two different theories regarding the causes of traumatic stress symptoms (Crocq and Crocq, 2000). On one side was Jean-Martin Charcot, who proposed that traumatic stress symptoms were a form of hysteria resulting from emotional shock. In other words, Charcot did not believe that traumatic symptoms had biological underpinnings. This is particularly surprising, given that Charcot was a student of Duchenne (discussed in section 5.1), who advocated

the examination of emotional expression at the biological level. On the other side, Hermann Oppenheim, suggested that traumatic stress symptoms were the result of small lesions within the brain or spinal cord (Oppenheim, 1892).

Since empirical evidence regarding biological theories of PTSD were lacking, treatment of PTSD has its roots in psychoanalysis. Freud and others began to explore the role of early life experiences and unconscious processes in the expression of traumatic stress symptoms. By World War II, more questions were raised regarding the treatment of PTSD. For instance, based on experience with cohorts of war veterans, Abram Kardiner (1941) was the first to suggest that this "neurosis" should be treated immediately after trauma before it consolidated. He believed that once consolidated, these symptoms could lead to chronic forms of the syndrome that would become more resistant to treatment (Kardiner, 1941). This was a particularly insightful idea for that time, since only recently have neuroscientists began to understand the process of memory consolidation and reconsolidation, which indeed have great implications for PTSD treatment. Later, the American psychiatrists Grinker and Spiegel (1945) attempted to treat the syndrome with barbiturates, marking the first attempt at pharmacotherapy for traumatic stress symptoms. In addition, Grinker and Spiegel were the first to identify an acute and delayed form of the syndrome, which is still recognized in current diagnostic standards.

Up to the second World War, theories of PTSD emphasized vascular irregularities in response to trauma, which led to such names as "irritable heart", or "soldier's heart" syndromes, and "neurocirculatory asthenia", and other names

such as “shell shock”, “traumatic neurosis”, “combat exhaustion”, and “effort syndrome” (Jones, 2006). The first version of the *Diagnostic and Statistical Manual for Mental Disorders* (DSM) was written in 1952, when these traumatic stress syndromes were referred to as “gross stress reaction” (DSM, 1952). Interestingly, this syndrome was completely left out of the second version of the DSM, and then reintroduced in 1980 for the third version, bearing the name “post-traumatic stress disorder”.

1.2 Current clinical diagnosis, and heritability studies

This section will summarize the diagnostic features of this anxiety disorder based on the criteria provided in the current *DSM-IV* (American Psychiatric Association, 2000). According to the DSM-IV, PTSD is a type of anxiety disorder triggered by exposure to a traumatic event. Depending on the type of trauma and its intensity, the proportion of individuals who develop PTSD varies greatly. For instance, traumatic events of human design (i.e. violent crime, rape, war) lead to a much higher incidence of PTSD than natural disasters (North et al., 2012).

In order to receive a diagnosis of PTSD, the individual must have experienced a traumatic event that produced feelings of intense fear, horror or helplessness. Once this criterion is met, individuals must pass a symptom threshold for each of three symptom clusters: re-experiencing, avoidance, and hyperarousal. Re-experiencing occurs when individuals involuntary re-live the traumatic event in a variety of ways, including recurrent nightmares and flashbacks. The avoidance symptom cluster is characterized by individuals’

efforts to avoid and emotionally detach or numb themselves from people, places, things, and activities that remind them of the traumatic event. The third symptom cluster, hyperarousal, is characterized by heightened physiological reactivity as evidenced by exaggerated startle response, difficulty concentrating, and hypervigilance. Symptoms must occur for at least one month and cause significant impairment in the individual's social, occupational, or other areas of functioning.

Not all individuals who experience a traumatic event develop PTSD. Individual variation is explained, in part, by a significant genetic contribution to this anxiety disorder. For instance, children of parents who had PTSD are at a higher risk for developing the disorder. Also, the correlation of PTSD status is higher among identical than fraternal twins (Nugent et al., 2008; Afifi et al., 2010). Consistent with this, genetic studies have identified DNA variations that show a strong association with PTSD status and likely confer susceptibility/resilience to some individuals (Mahan and Ressler, 2012). Interestingly, PTSD heritability coincides with that of other psychiatric conditions such as generalized anxiety, panic disorder, and depression (Chantarujikapong et al., 2001; Fu et al., 2007), suggesting that these disorders gain expression through common biological mechanisms. While these findings point to a strong genetic contribution to PTSD susceptibility and resilience, it should be recognized that the impact of a traumatic event also depends on complex interactions with environmental factors such as social support and early life experiences (Delahanty and Nugent, 2006; Saxe et al., 2006; Teicher et al., 2006).

1.3 Mechanisms involved in the expression of PTSD

In PTSD, the fear responses triggered by the initial trauma are repetitively re-experienced, often through flashbacks and recurring nightmares, even though the threat is no longer present. Moreover, fear tends to generalize to other stimuli and situations, contributing to avoidance of fear provoking places, activities, and people. Consistent with this, numerous laboratory studies support the view that fear is regulated abnormally in PTSD (Orr et al., 2000; Peri et al., 2000; Guthrie and Bryant, 2006; Milad et al., 2008). In the following, we briefly review this evidence.

The leading experimental model to study how organisms learn to predict danger based on experience is classical or Pavlovian fear conditioning (LeDoux, 2000). In this model, a neutral conditioned stimulus (CS), such as a context or tone, is paired with a noxious unconditioned stimulus (US), typically a mild electrical shock to the hand or wrist. After a few CS-US pairings, presentation of the CS alone comes to elicit conditioned fear responses (CR; e.g. galvanic skin conductance, pupil dilation). A similar network of brain structures, particularly the amygdala and the ventromedial prefrontal cortex (vmPFC), regulate fear learning in humans and animals (Phelps and LeDoux, 2005). As in animals, human with bilateral amygdala lesions cannot form Pavlovian fear memories even though they acquire declarative memories of the training sessions (Bechara et al., 1995; LaBar et al., 1995). In addition, fMRI studies in humans indicate that Pavlovian fear conditioning leads to increases in CS-evoked BOLD signals in the amygdala

(Buchel et al., 1998; LaBar et al., 1998). Moreover, a strong correlation was found between the magnitude of this increase and the amplitude of conditioned fear responses (Phelps, 2004). However, due to the low spatial resolution of fMRI, we have no human data about the relative contribution of different amygdala nuclei to learned fear.

While it is clear that fear is abnormally regulated in PTSD, the evidence is mixed as to whether individuals with PTSD acquire and/or express stronger conditioned fear responses than controls (Grillon et al., 1998; Orr et al., 2000; Blechert et al., 2007; Norrholm and Ressler, 2009; Jovanovic et al., 2012). In contrast, there is consensus that those with PTSD display increased baseline startle responses (Morgan et al., 1995; Grillon et al., 1998; Kumari et al., 2001). It has been proposed that this form of fear dysregulation results from an inability of individuals with PTSD to differentiate safe from threatening contexts (Grillon et al., 1998). Indeed, Grillon et al., (2002) found that when individuals with PTSD were confronted with situations perceived as stressful, and presented with unpredictable adverse events, they exhibited potentiated startle responses compared to controls. These findings support the role of unrealistic danger expectations, which can contribute to a chronic state of anxiety or hyperarousal that allows fear to generalize to previously safe situations and progressively invade more aspects of an individual's life.

In addition, it was shown that subjects who suffer from PTSD are deficient at learning that stimuli previously associated with adverse outcomes no longer present a threat (Orr et al., 2000; Peri et al., 2000; Rothbaum et al., 2001;

Guthrie and Bryant, 2006; Milad et al., 2008; Norrholm and Ressler, 2009; Jovanovic et al., 2012). As a result, PTSD is often characterized as a failure to learn safety (Milad et al., 2008). In the laboratory, this form of learning is modeled by repeatedly presenting the CS in the absence of the US. This process, termed extinction, is closely related to an approach commonly used by clinicians to treat PTSD (prolonged exposure therapy; (Bisson et al., 2007; Powers et al., 2010; Rauch et al., 2012). In this approach, individuals with PTSD are presented with actual or imagined trauma reminders depicting the most feared aspects of the traumatic event. These cues, analogous the CS, are not followed by danger (or US). With sufficient repetition and when paced appropriately, extinction occurs and PTSD symptoms begin to disappear. Here, it is important to understand that extinction training does not reverse or erase the original fear memory, but leads to the formation of a new inhibitory memory (CS-no US) that competes with the original CS-US association for control of behavior (Myers and Davis, 2002).

While extinction deficits can be partially remedied with prolonged extinction training, it remained unclear whether the observed extinction deficits characteristic of PTSD precede or follow trauma. A study of monozygotic twins discordant for trauma exposure recently addressed this question. Milad et al., (2008) found that PTSD is associated with impaired extinction retrieval *after* but not before trauma. This suggests that the extinction deficit seen in PTSD is not a pre-existing condition but that it develops as a result of trauma (Milad et al., 2008).

Overall, prior research on conditioned fear demonstrates that individuals with PTSD have difficulty differentiating safe from threatening contexts and tend to generate heightened fear responses that generalize to safe cues and contexts. Moreover, they fail to extinguish fear responses without therapeutic intervention. Thus, it is clear that these fear dysregulation processes contribute to the maintenance of PTSD.

1.4 Mechanisms involved in the susceptibility to PTSD

The hippocampus plays a critical role in the formation of declarative memories and is rich in receptors for glucocorticoids, a class of steroid hormones released by the adrenal glands during stressful conditions. It is well established that high levels of circulating stress hormones lead to cellular atrophy in the hippocampus (Sapolsky, 2000; McEwen, 2007; Popoli et al., 2012), inhibition of hippocampal neurogenesis (Gould et al., 1997; Galea et al., 2006) and of activity-dependent synaptic plasticity (Pavlides et al., 2002). Moreover, stress impairs hippocampal-dependent memory retrieval in humans (de Quervain et al., 2000; Wolf et al., 2001), an effect mediated in part by direct glucocorticoid effects in the hippocampus (Roozendaal et al., 2003).

Human imaging studies indicate that individuals with PTSD have smaller hippocampal volumes (Gilbertson et al., 2002; Bremner et al., 2003; Kitayama et al., 2005; Wang et al., 2010)and exhibit impaired performance on hippocampal-dependent tasks (for instance, see Shin et al., 2004; Lindauer et al., 2006; Gilbertson et al., 2007; Thomaes et al., 2009; Hayes et al., 2011; reviewed in

Samuelson, 2011). In particular, Gilbertson et al., (2007) examined the impact of PTSD on performance in allocentric spatial tasks that rely on the identification of spatial relationships between neighboring stimuli. These tasks cannot be solved using an egocentric frame of reference and are believed to depend on hippocampal functioning (Langston and Wood, 2010), a view supported by virtual reality experiments (Suthana et al., 2009). In monozygotic twins discordant for combat exposure, Gilbertson et al., (2007) found that individuals with PTSD and their non-traumatized co-twin were impaired on allocentric tasks relative to non-PTSD control twins.

The significance of these hippocampal deficits for the etiology of PTSD comes from earlier work on the relationship between different memory systems. Indeed, it was shown that many learning tasks engage multiple memory systems in the brain ("habit", striatal-dependent vs. "cognitive", hippocampal-dependent; reviewed in (Poldrack and Packard, 2003). It was also found that these systems interact in a competitive manner such that lesions or inactivation of one system leads the subject to favor the other, an interpretation supported by functional imaging studies in humans (Poldrack et al., 1999). Particularly relevant to PTSD is the observation that in such dual-solution memory tasks, stress biases rats (Kim et al., 2001; Packard and Wingard, 2004) and humans (Schwabe et al., 2007; Schwabe et al., 2008) toward striatal-dependent learning strategies. Moreover, it was shown that the basolateral amygdala mediates these effects of stress (Wingard and Packard, 2008). Therefore, it is possible that the antecedent hippocampal deficits and amygdala hyper-responsiveness seen in PTSD lead to

a greater engagement of the striatum and automatic responding to trauma-related cues (Goodman et al., 2012). In addition, since the hippocampus is required for differentiating contexts (Rudy et al., 2004), its impaired function might interfere with the contextual regulation of fear responses, promoting fear generalization.

1.5 Extant animal models of PTSD

Animal models provide an important avenue for studying the pathophysiology of PTSD because they circumvent ethical limitations associated with human research in three important ways. First, in contrast with human studies where participants cannot be randomly assigned to trauma exposure, animal models allow researchers to manipulate all aspects of the stressor including type, timing, and intensity. Second, these manipulations allow investigators to separate preexisting factors from those that are acquired after exposure to the stressor. Finally, animal models allow the use of more invasive techniques than ethically acceptable in humans.

Fortunately, much work has already been performed to define animal models of PTSD that reproduce the salient features of the human syndrome (Adamec et al., 2006; Cohen et al., 2006a; Siegmund and Wotjak, 2006). In these models, animals are exposed to various types of stressors, leading to long-lasting changes in circulating levels of stress hormones and/or in anxiety-like behaviors, assessed with standardized tests such as the elevated plus maze (EPM), open field (OF), social interaction test, and acoustic startle. Below, the

most common rodent PTSD models are described with regard to whether they reproduce salient features of human PTSD. Attention is focused on inter-individual differences in trauma susceptibility, deficits in fear extinction, and evidence of impaired hippocampal functioning. A thorough review of this vast literature would remain outside the scope of this introduction. Therefore, an overview of key findings is provided below.

1.5.1 Models using physical stressors

The traumatic events that precipitate PTSD in humans often involve potentially life threatening bodily harm. Similarly, many animal models of PTSD use physical stressors such as inescapable footshocks, underwater/forced swim paradigms, immobilization/restraint stress, or a combination of multiple stressors.

Delivery of inescapable and unsignaled footshocks leads to the formation of robust fear associations to the context where the shocks were administered (Rudy et al., 2004). In addition, some studies report that delivery of numerous (2-10) inescapable footshocks of high intensity (~ 1.5 mA) increase anxiety-like behavior on the EPM (Armario et al., 2008) and cause persistent increases in circulating levels of adrenocorticotropin hormone (ACTH) and corticosterone (CORT) levels (Daviu et al., 2012). It is important to note that inescapable footshock stress is also widely used to model depression, as it can induce symptoms of learned helplessness (Seligman and Maier, 1967). Consistent with this, administration of antidepressants attenuates the long-term behavioral effects of inescapable footshocks (e.g. enhanced acoustic startle) (Manion et al.,

2007). This finding highlights a major confound associated with many animal models of PTSD, particularly those relying on physical stressors: it is unclear whether they model PTSD and/or depression. However, PTSD and depression often coexist in humans. Therefore, this is not necessarily a weakness.

Underwater trauma has also been used in rats to model the trauma that precipitates PTSD in humans. For example, rats subjected to underwater trauma (40 s swim and 20 s submersion) show an immediate and persistent decrease in open arm time in EPM compared to rats that swim without submersion (Moore et al., 2012).

Restraint stress, which involves placing rats in restraining tubes for 2 to 6 hours, has been used extensively in the stress literature and leads to increased manifestations of anxiety in the EPM (Vyas et al., 2002), and changes in neuronal morphology within brain regions implicated in stress, fear, and anxiety processing (Miller and McEwen, 2006). A related procedure is immobilization stress, where rodents are restrained onto a wooden platform either acutely (single session) or chronically (several sessions). Immobilization stress was reported to cause a long-term desensitization of the hypothalamic-pituitary-adrenal (HPA) axis activity to subsequent exposure to the same immobilization stressor (homotypic stressor), but sensitized HPA responsiveness (increased plasma ACTH and CORT levels) to novel (heterotypic) stressors (Armario et al., 2004; Armario et al., 2008; Belda et al., 2008; Belda et al., 2012). However, this is not always the case using other stressors (see below).

Other models use a combination of stressors typically referred to as single prolonged stress (SPS). Contrary to what this name implies, these paradigms actually involve the administration of *various* stressors (restraint stress, forced swim, and ether exposure). Although SPS does not address the unique contributions of each stressor independently or potential confounds associated with repetitive stress, they have been used widely and feature some similarities with human PTSD.

Finally, there are even more intense physical stressor models than those mentioned so far: the "variable stress" models. For example, chronic variable stress (CVS) involves exposing animals to a different stressor daily for 6 days, and this procedure is typically repeated for several weeks in a row (Molina et al., 1990; McGuire et al., 2010). CVS was reported to cause decreased time on the open arms of the EPM (Zurita et al., 2000). Admittedly, this class of models is extreme and it likely elicits a wide range of physiological and psychological disturbances. Yet, it probably comes closest to simulate the chronic stress conditions experienced by military personnel in front-line positions.

1.5.2 Models using psychosocial stressors

In another approach to model PTSD, animals are submitted to a variety of psychosocial stressors such as housing instability, social defeat, and social isolation. Recent work with victims of partner abuse supports the ecological validity of housing instability as an important risk factor for humans and animals alike. Indeed, Rollins et al., (2012) found that difficulties maintaining a stable

living environment predicted PTSD, even after level of danger was statistically controlled (Rollins et al., 2012). Researchers have attempted to reproduce this phenomenon by subjecting animals to social instability for weeks by changing home cages as well as cage mates daily (Park et al., 2001; Zoladz et al., 2008; Zoladz et al., 2012). When social instability is chronic, or combined with other stressors, animals exhibit long-lasting anxiety-like behaviors reminiscent of human PTSD symptoms (Zoladz et al., 2008; Zoladz et al., 2012; Saavedra-Rodriguez and Feig, 2013).

Social defeat has also been shown to induce long-term signs of anxiety (Huhman et al., 1992; Huhman, 2006). Most studies using this stressor have been done in Syrian hamsters, because unlike rats, hamsters are solitary animals and readily exhibit signs of territorial aggression (Huhman, 2006). Here, an “intruder” animal is placed in the territory belonging to a larger “resident” animal, prompting the resident to attack the intruder. In a representative experiment using this model, resident-intruder pairings occurred for 15 minutes a day over 4 days. On day 5, physical interactions did not occur (a mesh barrier separated the two animals), and stress hormone levels were tested. Plasma levels of ACTH and CORT were elevated among submissive animals, but not the dominant animals (Huhman et al., 1992), suggesting that the hormonal changes do not reflect physical contact, but a more psychological process akin to human victimization by interpersonal violence. Even a single exposure to social defeat can lead to conditioned defeat, whereby previously defeated animals engage in submissive behaviors, even after becoming residents themselves (Jasnow and

Huhman, 2001). Conditioned defeat persists a month after the initial social defeat event (Huhman, 2006). Finally, when social defeat was investigated in rats, it was shown to cause a persistent enhancement of the acoustic startle response (Pulliam et al., 2010), and reduced open arm exploration in the EPM (Narayanan et al., 2011).

1.5.3 Models using early life stressors

In humans, prior exposure to trauma, particularly during development can cause long-term hormonal abnormalities and increased risk of developing PTSD (Delahanty and Nugent, 2006). Pioneering studies beginning with Harlow's monkeys (Young et al., 1973) have led to a large body of research examining the impact of maternal separation and early life stressors. Collectively, these studies show that neonatal isolation enhances stress and anxiety responses upon exposure to severe stress later in life (Diehl et al., 2012). When combined with other stressors, social isolation leads to a marked enhancement of anxiety-like behaviors (Imanaka et al., 2006). Moreover, social isolation pre- or post-weaning increases plasma levels of CORT and hypothalamic levels of corticotropin-releasing hormone mRNA (Zhang et al., 2011). Studies have also demonstrated that juvenile rats exposed to a severe psychological stressor are more likely to develop extreme anxiety-like phenotypes when exposed to the same stressor again in adulthood (Cohen et al., 2006a; Cohen et al., 2007). This contrasts with studies of immobilization, where it was found that repeated exposure to the same stressor causes a desensitization of the stress response (Armario et al., 2004;

Armario et al., 2008; Belda et al., 2008; Belda et al., 2012). Nevertheless, studies of humans (Yehuda et al., 1998b; Yehuda et al., 1998a) and animals (de Kloet et al., 2005; Cohen et al., 2007; Bazak et al., 2009) generally show that exposure to high levels of stress or glucocorticoids early in life predisposes individuals to be more susceptible to subsequent stressors. In animals, this susceptibility can be transmitted through generations (Seckl and Meaney, 2006).

1.5.4 Genetic models of PTSD-like symptoms

Interesting genetic models have been developed in rodents including rat or mouse strains that exhibit high trait anxiety and/or marked fear extinction deficits (Landgraf and Wigger, 2002; Camp et al., 2009; Holmes and Singewald, 2013). Another noteworthy line of investigation focuses on variations in anxious temperaments in monkeys (Kalin, 2003; Fox et al., 2008). However, PTSD-like abnormalities associated with these models do not require exposure to trauma, so they will not be further considered in this thesis.

1.5.5 Models using psychogenic stressors

The physical stressor models reviewed above involve physical pain or discomfort. In contrast, the ones discussed in this section involve threat, but usually no pain. In these models, rodents are exposed to species-relevant predators (predator stress) or their odor (predator threat) (Blanchard and Blanchard, 1988; Dielenberg and McGregor, 2001). While live predators constitute a more intense stressor than their odor, the latter is a convenient

alternative that allows for greater repeatability and control over the intensity of the trauma. In addition, a major strength of these models is their ecological relevance. Field studies of animals in their natural habitat have shown that exposure to predators or associated cues lead to increased glucocorticoid levels, and decreased number of offspring (Clinchy et al., 2010). In addition, the rise in maternal glucocorticoid levels appeared to be transmitted through generations (Sheriff et al., 2010).

In the predator stress model, rats are exposed to a cat for one or two sessions of up to 45 min each. Typically, the rats are placed in a small protective enclosure that prevents direct physical interactions (Diamond et al., 1999). However, in some laboratories, no such measures are taken (Adamec et al., 1998). In the predator threat (PT) model, rats are presented with predator odors from natural or synthetic sources. Natural odors are usually obtained from felines (cat fur, bedding, litter). The most common synthetic odor is trimethylthiazoline (TMT), a component of fox feces that rats find aversive. While feline odors can be used as US to support contextual fear conditioning (Blanchard et al., 2001), TMT cannot (Wallace and Rosen, 2000). Consistent with this, it was reported that cat odors and TMT elicit different patterns of responses with cat odors triggering anxiety-like behaviors whereas TMT evokes avoidance responses (Dielenberg and McGregor, 2001).

Arguably, predator stress and threat models constitute a better replica of the kind of life-or-death circumstances that precipitate PTSD in humans than physical stressors. Consistent with this, rodents exposed to predators or their

odor develop long lasting (three weeks or more) manifestations of anxiety as seen in a variety of behavioral assays including the EPM, social interaction test, and acoustic startle (Adamec and Shallow, 1993; Adamec et al., 1998; Mesches et al., 1999; Hebb et al., 2003; Adamec et al., 2006; Roseboom et al., 2007; Nanda et al., 2008; Zoladz et al., 2008; Zoladz et al., 2012).

1.6 Importance of individual differences in susceptibility to trauma

A limitation of most existing animal PTSD models is that the stressful event affects all animals similarly. Individual differences are either not present or reported; all comparisons are between naive vs. trauma-exposed subjects. This is in contrast with human PTSD where only a proportion of trauma-exposed individuals develop the disorder. Animal models that can capture individual differences in vulnerability to trauma have greater ecological and face validity because they allow researchers to investigate factors conferring resilience and susceptibility to trauma.

The PT model of PTSD reproduces the individual variations in trauma vulnerability seen in humans. Indeed, previous studies from Cohen and colleagues (2006a,b) have shown that PT exposure leads to the development of extreme manifestations of anxiety (EBMAs) in a proportion of subjects. Interestingly, the incidence of EBMAs following PT was found to be much higher in the inbred Lewis rat strain (50%) than in Sprague-Dawley rats (20%), and Fisher rats (10%; (Cohen et al., 2006a; Cohen et al., 2006b). This finding, which was replicated in our studies (Goswami et al., 2010), strongly implicates a

genetic contribution towards the development of extreme anxiety-like states, facilitating investigations of gene-environment interactions.

Although the incidence of the PTSD-like phenotype (~50%) is much higher in Lewis rats than in humans, there are many reasons why this is useful. First, tests can be conducted before or after exposure of the rats to predators or their odor to determine if differences between susceptible and resilient animals predate or are a consequence of the stressor. Second, random groups of Lewis rats include a nearly equal fraction of susceptible and resilient subjects, so fewer rats have to be studied to compare the two groups on any dimension. This is particularly advantageous for labor-intensive studies such as those relying on single-unit recordings in behaving animals. Along the same lines, because PT induces a bimodal distribution of anxiety-like behaviors, there is no need to eliminate animals from the analyses in order to examine only extreme responders as is commonly done in other stress models (Nalloor et al., 2011). In one such approach, one week following a single 10-minute exposure to soiled cat litter, Lewis rats are tested on the EPM (Cohen et al., 2006a; Cohen et al., 2006b; Goswami et al., 2010; Goswami et al., 2012). Rats with extremely compromised exploratory behavior (zero time spent on open arms of the EPM) are categorized as PTSD-like, whereas rats that explore the open and closed arms of the EPM are categorized as resilient. Thus, the Lewis rat model of PTSD provides an extremely efficient and practical model that easily lends itself to a wide variety of experimental procedures.

1.7 Objectives of this thesis

This thesis pursues two objectives: (1) To test the validity of the Lewis rat model of PTSD; and (2) use this model to examine some of the neural mechanisms involved in the expression and maintenance of PTSD. Thus, this thesis will involve detailed behavioral assessments of factors that might predispose individuals to develop PTSD, along with ex vivo analyses of the mechanisms involved in the expression and maintenance of PTSD. Given the critical role played by the amygdala in generating fear and anxiety behaviors, we will compare the physiological properties and synaptic responsiveness of amygdala neurons in susceptible and resilient Lewis rats.

CHAPTER II

GENERAL METHODS

2.1 Animals

Adult male Lewis rats weighing 200-225g upon delivery were used for all experiments (Charles Rivers, New Field, NJ). Animals were housed individually with ad libitum access to food and water. Rats were maintained on a 12-hour light/dark cycle (lights on at 7am), and habituated to the animal facility and handling for one week before experiments begin.

2.2 Predator Threat (PT)

Predator threat is the laboratory model we used to induce trauma in Lewis rats. It consists of a single ten-minute exposure to soiled cat litter. The litter is in use for approximately 48 hours by two neutered male cats, and sifted for stools. For experiments in Chapter 3, two cups of litter were placed in a standard rat cage with a wire mesh top. For the remainder of the experiments in this thesis, the above protocol was slightly modified. Modifications included 2 cups of cat litter in a large square weigh boat placed in the corner of the rats' home cage. The cage was then covered with clear Plexiglas.

2.3 Elevated Plus Maze (EPM)

The EPM is a well-established test of anxiety based on the assumption that anxious rats prefer small, dark places rather than less secure, open areas. The maze is shaped like a plus and elevated 60 cm from the ground. The arms extend 60 cm long and are 10 cm wide. Two opposing arms are black, and enclosed by 30 cm tall black Plexiglas walls, while the other two arms are made

of white Plexiglas and are not enclosed by walls. EPM testing is performed under red light, and begins by placing the rat in the center of the maze facing an open arm. Rats are given 5 minutes to explore the maze, while their behavior is recorded by an overhead camera. We categorize rats that completely avoid the open arms of the maze as “PTSD-like”, and rats that explore all parts of the maze as resilient.

2.4 Auditory Classical Fear Conditioning

Fear conditioning and extinction training and testing were performed in conditioning chambers from Coulbourn Instruments. These chambers (25 x 29 x 28 cm) have aluminum and Plexiglas walls. The floor consists of 0.5-cm-diameter stainless steel bars spaced at 1.8 cm through which a mild footshock can be delivered. The chambers were located inside a sound-attenuating box, which contained a ventilation fan, and were illuminated by a single house light. Fear conditioning and extinction occurred in different contexts (contexts A and B, respectively). For fear conditioning (context A), rats were placed in the rodent conditioning chamber described above. For extinction training and testing, the appearance of the chamber was modified as follows: a black Plexiglas floor washed with peppermint-scented soap covered the original floor (context B). The rats’ behavior was recorded with a video camera and scored off-line. Behavioral freezing to the CS was measured with a stopwatch, while remaining blind to the rats’ behavior on the EPM. Behavioral freezing was defined as the arrest of all movements other than breathing.

On day 1, the rats were habituated to contexts A and B for 20 min each. On day 2, the rats were subjected to an auditory fear-conditioning protocol in which they were presented with four pairings of the CS and US. The CS was a 1-kHz tone lasting 30 sec (75 dB). The US was a 0.5-mA footshock lasting 1 sec. The CS and US co-terminated (variable inter-CS intervals of 2–5 min). On day 3, the rats underwent extinction training in context B. Here, the rats received 20 presentations of the CS alone (no US). On day 4, extinction recall was tested with 10 additional presentations of the CS alone in context B.

2.5 Open Field Test (OF)

The OF can be used to assess baseline anxiety levels and exploratory behavior. Our OF apparatus was a square box (1 x 1 m) surrounded by walls, 60 cm high. Red lines drawn on the floor divided the arena in 36 squares of equal area. Rats were placed one at a time in the center of this novel environment and allotted 5 minutes to explore the arena under dim light. Their exploratory behaviors were recorded by an overhead video camera and scored offline.

2.6 Object and Object-Place Recognition Tasks

We subjected different samples of Lewis rats to one of three tasks commonly used to assess various forms of recognition memory in rodents (e.g., Mumby, 2001; Langston and Wood, 2010). In these tasks, preferential exploration of the novel relative to the familiar items is used to assess recognition memory. In the first task, the novel object recognition task (NOR), the rats'

exploratory behavior during the test phase was driven by object identity (see below). In contrast, in the next two tasks, the egocentric (EOR) or allocentric (AOR) object recognition tasks, the rats could rely on different frames of reference to identify the novel location of the familiar object: egocentric in the EOR task and allocentric in the AOR task.

2.6.1 Aspects common to all tasks

The apparatus and habituation procedures used in the three tasks were identical. All object recognition experiments were conducted in an arena made of black Plexiglas (76 by 76 cm, with walls 60 cm high), under red light illumination. Objects were secured to the floor of the arena with Velcro, 5 cm away from the corners. Objects used in these tasks included plastic beach toys, mugs, aluminum cans, bottles, and pencil holders. Pilot tests showed that the objects used were of similar interest to Lewis rats. This was confirmed in the actual experiments where the time exploring the different objects during the sampling phase did not differ. In particular, less than ± 4 s deviation was seen between maximal and minimal exploration times of individual objects relative to the average exploration time of all objects used within each task. Active exploration of these objects included behaviors such as touching the objects with the nose or paws. In addition, three-dimensional objects were secured to the walls of the arena to provide the rats with visual spatial cues. On Day 0, the rats were habituated to the test apparatus for 20 min with no objects present. This was followed by a second 20 min period of habituation with two identical objects

secured at the northeast and northwest corners. After each trial, the testing arena was wiped clean with a 20% ethanol solution to eliminate odor cues.

2.6.2 Novel object recognition (NOR) task (Figure 1A)

In this task, the sample and test phases were carried out 24 h after habituation. *Sample phase*: two identical objects were secured near the north wall. Rats were placed in the arena facing the south wall (away from the objects) and given 5 min to explore the objects. *Inter-phase interval*: rats were placed in their home cage for 2 min. During this time, one object was replaced with a novel object. The location of the replaced object was counterbalanced across subjects. *Test Phase*: rats were placed back into the arena facing the south wall and given 5 min to explore the objects.

2.6.3 Egocentric object-place recognition (EOR) task (Figure1B)

Starting 24 h after habituation, each rat experienced one trial per day on four consecutive days. The sample and test phases were separated by a 2 min interval on each trial. *Sample Phase*: rats were placed in the arena facing the south wall and given 5 min to explore two objects that were distinct and novel. *Inter-phase Interval*: rats were placed in their home cage for 2 min. During this time, one object was replaced by a second copy (duplicate) of the remaining object. *Test Phase*: The rats were placed facing the south wall and given 5 min to explore the new object-place configuration.

2.6.4 Allocentric object-place recognition (AOR) task (Figure1B)

In this task, all aspects of the protocol were identical to the EOR task except for the positioning of the rats at the start of the test phase. Instead of starting the trials facing the south wall, rats were placed facing either the east or west wall (two trials each) and given 5 min to explore the new object-place configuration. As a result, the only difference between the EOR and AOR tasks is that in the first case the rats could use an egocentric frame of reference to identify the novel item location whereas in the second, they had to rely on an allocentric frame of reference.

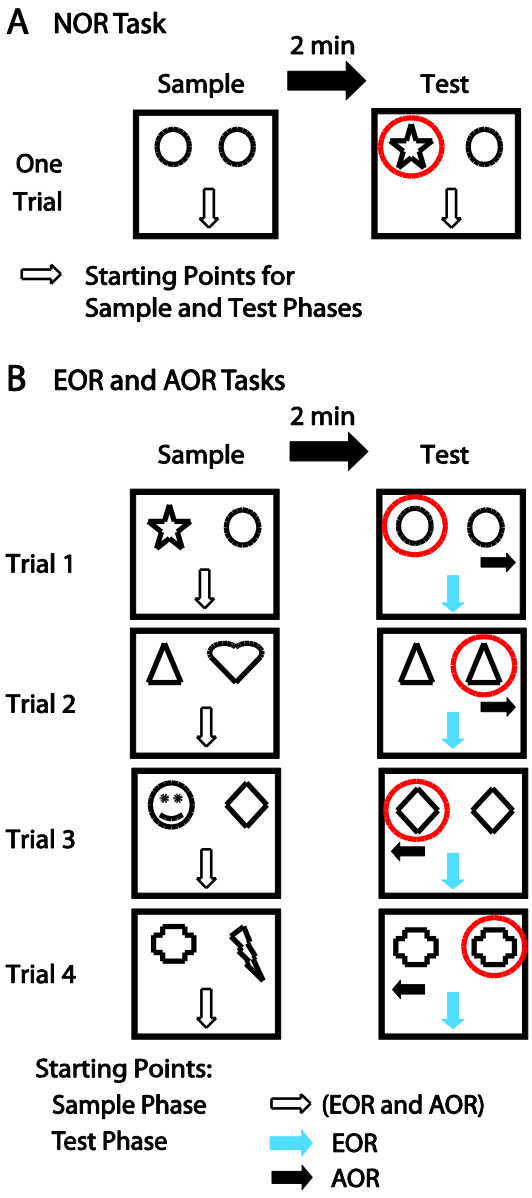

Figure 1. **Experimental paradigm.** (A) In the novel object recognition (NOR) task, rats were first presented with two identical objects (sample phase, 5 min, left), and returned to their home cage for 2 min. During this period, one of the two objects was replaced with a novel object whose location (east or west) was varied randomly across subjects. During the test phase (right), the rats were allowed to explore the two objects for 5 min. The rats' starting positions in the sample and test phases were identical: facing the south wall. (B) In the egocentric (EOR) and allocentric (AOR) object-place recognition tasks, all aspects of the sample phase were identical: the rats were presented with two novel objects. As for the NOR task, they faced the south wall at the start of this sample phase. After a 5 min exploration period, the rats were returned to their home cage for 2 min. During this period, one object was replaced with an identical copy of the other. The position of this object duplicate was counterbalanced across trials. In the EOR task, the starting position of the rat was identical to that used in the sample phase (facing the south wall, blue arrow). In the AOR task, the rats' starting position varied across trials (facing the east or west wall, black arrow). Four such trials were conducted, on four consecutive days.

2.6.5 Inclusion criteria and measured variables

In order for the rats' behavior to be considered on a given test trial, they must have explored both objects for at least 5 s during the sample phase. In the NOR task, if a subject did not meet this criterion, a second trial was conducted the next day. In the EOR and AOR tasks, if a subject did not meet this criterion, the rat's behavior on that trial was ignored. This occurred rarely (EOR, 2 trials; AOR, 3 trials). Time spent exploring the two objects in the sample and test phases was scored off-line by experienced while

remaining blind to the rat's phenotype (PTSD-like vs. resilient, as determined by the EPM).

Note that the number of trials in the NOR vs. AOR and EOR tasks differs. In the AOR task, because the rats' starting position differs in the sample and test phase, it is standard practice to run four trials, allowing counterbalancing of the difference in starting positions between the sample and test phases as well as left or right location of replaced objects. The same is done in the egocentric version of the object-place task to facilitate comparisons of the results in the EOR and AOR versions of the task. In contrast, the NOR task did not require such a design, as rats always started in the same position.

2.7 Electrophysiology

2.7.1 In vitro slice preparation

One to three days after the EPM test, rats were deeply anesthetized with Avertin (300 mg/kg, i.p.). After abolition of all reflexes, they were perfused through the heart with a cold (4°C) modified artificial cerebrospinal fluid (aCSF) that contained (in mM): 248 sucrose, 2.5 KCl, 7 $MgCl_2$, 23 $NaHCO_3$, 1.2 NaH_2PO_4, 7 glucose. Their brains were then extracted and cut in 300 µm-thick coronal slices with a vibrating microtome while submerged in the same solution as above. After cutting, slices were transferred to an incubating chamber where they were kept at 32°C for 30 minutes, then allowed to recover for at least one hour at room temperature in a control aCSF with the following composition (in mM) 124 NaCl, 2.5 KCl, 1.25 NaH_2PO_4, 26 $NaHCO_3$, 1 $MgCl_2$, 2 $CaCl_2$,

10 glucose. (pH 7.3, 300 mOsm). The slices were then transferred one at a time to a recording chamber perfused with the latter solution (5 ml/min). Before the recordings began, the temperature of the chamber was gradually increased to 32°C.

2.7.2 Electrophysiological recordings

The recordings were obtained while remaining blind to the rats' phenotype, which was revealed only after analysis. Under visual guidance with differential interference contrast and infrared video-microscopy, we obtained whole-cell patch recordings of amygdala neurons using pipettes (7-10 MΩ) pulled from borosilicate glass capillaries and filled with a solution containing (in mM): 130 K-gluconate, 10 N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid, 10 KCl, 2 $MgCl_2$, 2 ATP-Mg, and 0.2 GTP-tris (hydroxy-methyl) aminomethane (pH 7.2, 280 mOsm). The liquid junction potential was -10 mV with this solution and the membrane potential was corrected accordingly. Recordings were obtained with an Axoclamp 2B amplifier and digitized at 10 kHz with a Digidata 1200 interface (Axon Instruments, Foster City, CA).

To characterize the electroresponsive properties of recorded cells, graded series of depolarizing and hyperpolarizing current pulses (20 pA steps, 500 ms in duration) were applied from a pre-pulse potential of -80 mV. The input resistance (R_{in}) of the cells was estimated in the linear portion of current-voltage plots.

To activate synaptic inputs to the recorded cells, stimulating electrodes were positioned at one of three sites: in the external capsule (EC) and lateral

amygdala (LA) when recording basolateral amygdala (BL) neurons, or in BL when studying CeA neurons. To minimize variability between experiments, we always selected the same coronal level (2.0, 2.5, or 3.2 mm posterior to bregma for CeM, CeL, and BL recordings, respectively) and positioned the stimulating electrodes at the same site. Unless otherwise noted, electrical stimuli (100 μs) were delivered at a low frequency (0.1 Hz), in a range of intensities (100-800 μA), and from a membrane potential of -65 mV. At least three stimuli were delivered at each intensity and responses averaged.

2.7.3 Glutamate uncaging Experiments

To assess potential differences in postsynaptic responsiveness to glutamate, we used glutamate uncaging. In these experiments, the composition of the aCSF was as above except for the addition of caged glutamate (4-Methoxy-7-nitroindolinyl-caged-L-glutamate, 1.0 mM; Tocris Bioscience, Bristol, UK). Glutamate was uncaged by applying UV light pulses (5-40 ms) over the recorded cell. Responsiveness to uncaged glutamate was assessed from a membrane potential of –80 mV, as determined by intracellular current injection. The UV light stimuli were delivered at 0.1 Hz by a LED source (365 nm, 60 mW; CoolLED, Andover, UK) via a 60X immersion objective, yielding UV light spots of ≈150 μm in diameter.

CHAPTER III

PROPERTIES OF EXTINCTION IN RESILIENT AND PTSD-LIKE RATS

3.1 Rationale

One factor thought to play a critical role in the persistence of PTSD, is a compromised ability to extinguish fear memories (for review, see Quirk and Mueller 2008). Two main lines of evidence support this notion. First, in functional imaging studies, the brain structures that normally support fear expression and extinction (for review, see Pape and Pare 2010) show abnormal activity patterns in PTSD (Rauch et al., 2006; Shin et al., 2006; Bremner et al., 2008; Milad et al., 2009). Second, several studies have reported that individuals with PTSD are deficient at extinguishing classically conditioned fear responses (Orr et al., 2000; Peri et al., 2000; Blechert et al., 2007; Milad et al., 2008; Milad et al., 2009). Of particular interest, a study of identical twins discordant for trauma exposure has revealed that this extinction deficit was not a pre-existing condition but developed as a result of trauma (Milad et al., 2008). Previous studies have found that rats classified as “susceptible” are also impaired at the extinction of contextual fear conditioning, compared to “resilient” rats (Nalloor et al., 2011). Surprisingly, whether abnormalities in the regulation of conditioned fear responses predate the onset of a PTSD-like state in animals has not been investigated. Therefore, we subjected separate groups of Lewis rats to an auditory fear conditioning paradigm either before or after PT. Exploratory behavior on the EPM one week after PT was used to classify subjects as resilient or PTSD-like (see section 3.3).

3.2 Hypothesis

Based on prior findings, we hypothesize that PTSD-like Lewis rats will exhibit impaired extinction of classically conditioned fear responses, compared to resilient rats. Since human studies suggest that this extinction impairment is acquired after exposure to trauma, we expect to see extinction deficits in PTSD-like rats after, but not before exposure to PT.

3.3 Methods

The first experiment aimed to determine whether mere exposure to the fear-conditioning protocol was sufficient to cause the emergence of EBMAs in Lewis rats. To address this question, three groups of Lewis rats were tested on the EPM. In the first group (naïve), rats were not subjected to PT and remained in their home cage until tested on the EPM. A second group of Lewis rats (PT7) was tested on the EPM seven days after PT. The last group of Lewis rats (FC7) was tested on the EPM seven days after fear conditioning but without PT exposure. A delay of 7 days between PT and EPM testing was used to reproduce the conditions found in a previous study in Lewis rats (Cohen et al. 2006b). For details, see sections 2.2 and 2.3.

The next series of experiments aimed to determine whether resilient and PTSD-like rats differed in their ability to extinguish conditioned fear responses. A second objective was to assess whether such differences required prior exposure to PT or not. To this end, two groups of Lewis rats were subjected to fear conditioning and extinction (section 2.4) either after or before PT exposure.

Statistical analyses

A mixed between and within-subjects design was used to evaluate differences in extinction between resilient and PTSD-like rats. To test statistical significance, two-way (group by trial) repeated MANOVAs were conducted. Prior to conducting these MANOVAs, assumptions of sphericity and equality of covariances were evaluated with Mauchly's and Box's M tests. Sphericity assumptions were violated for every multivariate analysis. Thus, Greenhouse-Giesser ($\varepsilon < 0.75$) or Haynh Feldt ($\varepsilon \geq 0.75$) estimates for sphericity were used to test statistical significance of within-subjects effects. Significance levels with these degree of freedom adjustments were the same as the unadjusted F-tests. Thus, for the sake of brevity, only unadjusted F-tests are reported. To identify the source of significant effects, Bonferroni adjusted paired t-tests were computed to examine changes over time (first vs. last CS only). Corrected model univariate F-tests and Bonferroni adjusted t-tests were used to compare PTSD-like and resilient rats on CS trials. All values are reported as average ± SEM. Prior to these posthoc analyses, the Levene test for equality of error variances was used to evaluate differences in dispersion around the mean for each group and trial. When error variances were unequal, significance tests for unequal variances were used.

3.4 Results

3.4.1 Effect of PT or fear conditioning on behavior in the EPM

In order to study the impact of PT on extinction, it was critical that we first determine the incidence of EBMAs in naïve animals, assess to what extent PT influences this incidence, and establish whether fear conditioning, by itself, causes the emergence of the anxious phenotype in Lewis rats. To address these questions, we compared the exploratory behavior of three groups of rats in the EPM (Fig. 2A): naïve rats (n = 31) vs. rats subjected to classical auditory fear conditioning (FC7, n = 28) or PT (PT7, n = 22) 1 week prior to EPM testing. The anxious phenotype was defined as severely compromised exploratory behavior in the EPM (zero time in the open arms during a 5-min test period).

As shown in Figure 2B, the incidence of the anxious phenotype was ≤12.9% in the naïve and FC7 groups, whereas it was 45.4% in the PT7 group, replicating earlier findings (Cohen et al. 2006b). The higher proportion of Lewis rats with the extremely anxious phenotype in the PT7 group was statistically significant (Fisher exact probability test, P = 0.002). Although this pattern of results suggests that PT causes the emergence of an extremely anxious phenotype in a subset of susceptible animals, there is an alternative interpretation. Indeed, it is conceivable that PT caused an overall reduction in the time spent in the open arms of the EPM in all Lewis rats. According to this view, the drastically higher proportion of rats avoiding the open arms in the PT7 group would be a simple reflection of a floor effect: Because the distribution of time in the open arms shifts toward low values 1 week following PT, a greater proportion

of rats end up spending no time in the open arms of the EPM. However, if PT causes a general increase in anxiety in the PT7 group, then one would expect that comparisons of time spent in the open arms excluding rats that did not go in the open arms at all should yield a significantly lower average in the PT7 group compared with the other groups. At odds with this notion, however, an ANOVA on time spent in the open arms (excluding rats with no time in the open arms) revealed no main effect of group ($F = 0.939$, $P = 0.397$). In fact, when the extreme subjects were excluded, the average time spent in the open arms was higher in the PT7 group, although this difference did not reach significance (PT7 = 31.5 ± 7.6 sec; other groups, 24.6 ± 3.0 sec, t-test, unequal variance, $P = 0.39$). Similarly, comparisons of time spent in the open arms between the PT7 group vs. the naïve and FC7 groups considered together or separately also failed to reveal significant differences when all subjects were included (t-tests, unequal variance, $P \geq 0.36$).

While it appears that PT does not produce a general increase in anxiety in all subjects, another question is whether the resilient rats in the PT7 group show other behaviors suggestive of resilience. Further support for the characterization of the rats that explored the open arms of the EPM as resilient was obtained by comparing other measures of risk assessment in the naïve vs. the resilient rats of the PT7 group. In particular, we examined whether the number of stretch-attends or head dips (protected and unprotected head dips considered separately) differed significantly in the two groups. However, we failed to find significant differences along these three dimensions (Stretch-attends: naïve 3.2 ± 0.7, PT7

= 3.1 ± 1.11, t-test, P = 0.9; protected head dips: naïve 2.9 ± 0.5, PT7 = 3.5 ± 0.9, t-test, P = 0.43; unprotected head dips: naïve 1.5 ± 0.4; PT7 = 1.8 ± 0.8, t-test, P = 0.71). In contrast, compared with resilient rats of the PT7 group, rats with the anxious phenotype based on the lack of exploration of the open arms (hereafter called PTSD-like rats) displayed a clear reduction in two of the above three risk assessment behaviors (protected and unprotected head dips, t-tests, P < 0.002).

Thus, these results suggest that PT indeed causes the emergence of EBMAs in a subset of susceptible Lewis rats, and not simply a general increase in anxiety expressed by all subjects. Moreover, the absence of differences in risk assessment behaviors (head dips and stretch-attends) among naïve and resilient rats of the PT7 group validates using exploration of the open arm of the EPM as a means by which to classify rats as resilient.

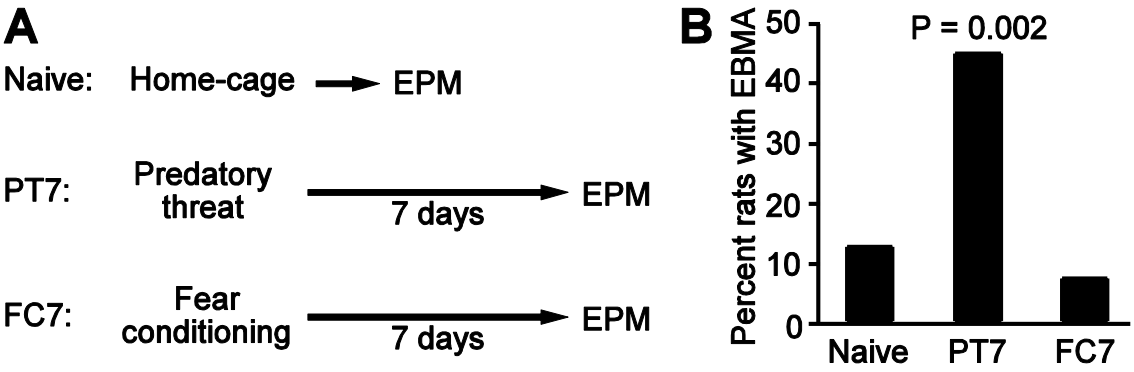

Figure 2. Impact of predator threat and fear conditioning on the incidence of extreme behavioral manifestations of anxiety (EBMA). (A) Experimental groups. (B) Graph plotting the proportion of subjects that spent zero time exploring the open arms of the EPM during a 5-min test period.

3.4.2 Fear extinction in resilient vs. PTSD-like rats after PT

Next, we examined whether PTSD-like rats are deficient at extinguishing conditioned fear responses, as was seen in humans with PTSD (Orr et al. 2000;

Peri et al. 2000; Blechert et al. 2007; Milad et al. 2008, 2009). Thus, the PT7 group was subjected to a classical fear-conditioning protocol 1 week after EPM testing (Fig. 3A1). Details regarding the fear conditioning and extinction protocols, see section 2.4.

Figure 3A2 plots the percentage of time spent freezing (y-axis) in these Lewis rats during the various phases of the fear conditioning protocol (x-axis). Rats are sorted as a function of their behavior in the EPM: resilient rats (black, n = 12) that explored the EPM's open arms vs. PTSD-like rats (red, n = 10) that spent no time exploring the open arms. A group by trial repeated measures MANOVA was used to evaluate within- and between-group effects. During fear conditioning, PTSD-like and resilient rats both significantly increased the amount of time spent freezing across conditioning trials ($F = 59.65$, $P < 0.001$). There were no significant differences in levels of freezing between the PTSD-like and resilient rats at any time point ($F < 1$) and no significant group by trial interaction ($F < 1$). During the last CS, both groups exhibited nearly identical levels of freezing behavior (t-test, $P = 0.62$, PTSD = 85.7 ± 3.6%; resilient = 82.3 ± 5.3%). During extinction training, there was no statistically significant group by trial interaction effect. Both PTSD-like and resilient rats significantly decreased in the amount of time spent freezing in response to the CS ($F = 31.48$, $P < 0.001$), with significant change from the first to the last CS (paired t-tests, resilient $P < 0.001$, PTSD-like $P < 0.001$). However, PTSD-like rats displayed a significantly higher level of

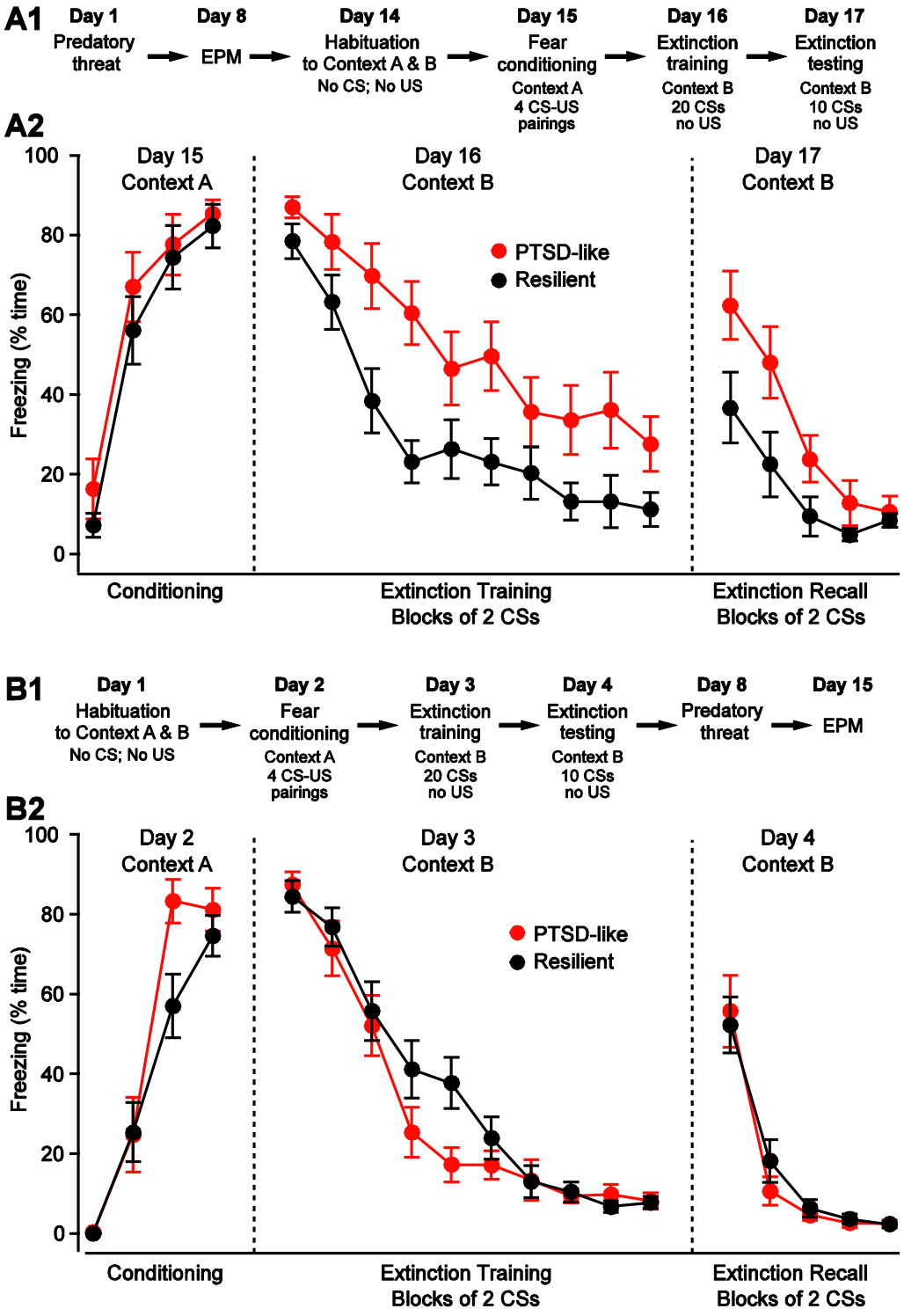

Figure 3. Impact of predator threat on the acquisition and extinction of conditioned fear responses. Lewis rats were subjected to a fear-conditioning protocol either after (A1, A2) or before (B1, B2) PT as detailed in A1 and B1, respectively. Panels A2 and B2 plot the percentage of time spent freezing (y-axis) during the various phases of the fear-conditioning protocol (x-axis). In A2 and B2, rats are sorted as a function of their behavior on the EPM with rats exploring the open arms in black (resilient) and rats that avoided the open arms entirely in red (PTSD-like).

freezing, i.e., were slower to extinguish freezing to the CS, than resilient rats, as evidenced by a multivariate group effect ($F = 10.26$, $P < 0.004$). Post-hoc analyses revealed that both groups of rats displayed a similar amount of freezing behavior to the first four CSs. However, by the fifth CS, PTSD-like rats displayed significantly higher levels of freezing than resilient rats ($F = 7.33$, $P = 0.014$). In addition, when data across CS trials 5 through 20 were pooled, PTSD rats displayed significantly higher freezing, on average, than resilient rats (t-test, $P < 0.005$). In the above, it is possible that the higher freezing levels seen in PTSD-like rats are not due to an extinction deficit but result from the fact that they acquire higher levels of conditioned fear than resilient rats to begin with. Although freezing levels did not differ significantly at the beginning of the extinction training session, there was a trend (t-test, first two CSs, $P = 0.13$) that, combined with potential ceiling effects, warranted further analysis. Thus, to address this question, we repeated the above analyses using freezing during the first two CSs as a covariate, and found identical results. We also looked at this question another way. That is, we matched levels of conditioned fear at the beginning of extinction training by dropping subjects at opposite poles of the distribution in each group (Fig. 4A1, arrow). However, even after matching freezing levels, PTSD-like rats still froze significantly more than resilient rats at the end of the extinction training session (last six CSs, t-test, $P = 0.045$).

A group by trial repeated measures MANOVA was conducted to evaluate within-and between-group effects during extinction recall (Fig. 3A2). Expectedly, there was a significant decrease in freezing across trials ($F = 13.5$, $P < 0.001$) in

both groups (paired t-tests, first vs. last CS, PTSD-like, $P < 0.001$; resilient, $P < 0.005$). In addition, PTSD-like rats froze significantly more during extinction recall than resilient rats (multivariate $F = 5.21$, $P < 0.04$). Post-hoc t-tests confirmed that PTSD-like rats froze significantly more than resilient rats from the first to the fourth CS of the extinction testing session (t-tests, $P < 0.05$).

While the higher freezing levels seen in PTSD-like rats at the end of extinction training is strongly suggestive of a short term within-session extinction deficit, the significance of the higher freezing level they display during extinction recall (Fig. 3A2) is less clear. Here, the critical issue is whether PTSD-like rats are deficient in the overnight consolidation of extinction as seen in humans with PTSD (Milad et al. 2008, 2009). One possible explanation for the higher freezing levels exhibited by PTSD-like rats during extinction recall is that because they extinguish less fear during extinction training, they show more freezing the next day. At odds with this, however, there was a negligible correlation between freezing levels at the end of extinction training and beginning of extinction testing in PTSD-like rats ($r = -0.023$). Alternatively, it could be that PTSD-like rats are deficient in the overnight consolidation of extinction. In support of this possibility, there are many instances in the literature of dissociations between within-session and between-session extinction with some lesions and pharmacological treatments leaving within-session extinction intact or marginally reduced, yet severely reducing between-session extinction (Sotres-Bayon et al., 2007; Quirk and Mueller, 2008). To directly address whether consolidation of extinction memory was affected in PTSD-like rats, we compared freezing levels in the two

groups at the beginning of the extinction test after matching their freezing levels at the end of extinction training (Fig. 4B1, arrow). To achieve this, we dropped extreme subjects in the two groups, resulting in nearly identical average freezing levels at the end of extinction training (Fig. 4B1,B2). At the beginning of the extinction recall test, PTSD-like rats still showed significantly higher levels of freezing than resilient rats (resilient = 23.3 ± 9.2%; PTSD-like = 64.7 ± 14.1%; t-test, P = 0.038), suggesting that PTSD-like rats are also deficient at consolidating extinction memory.

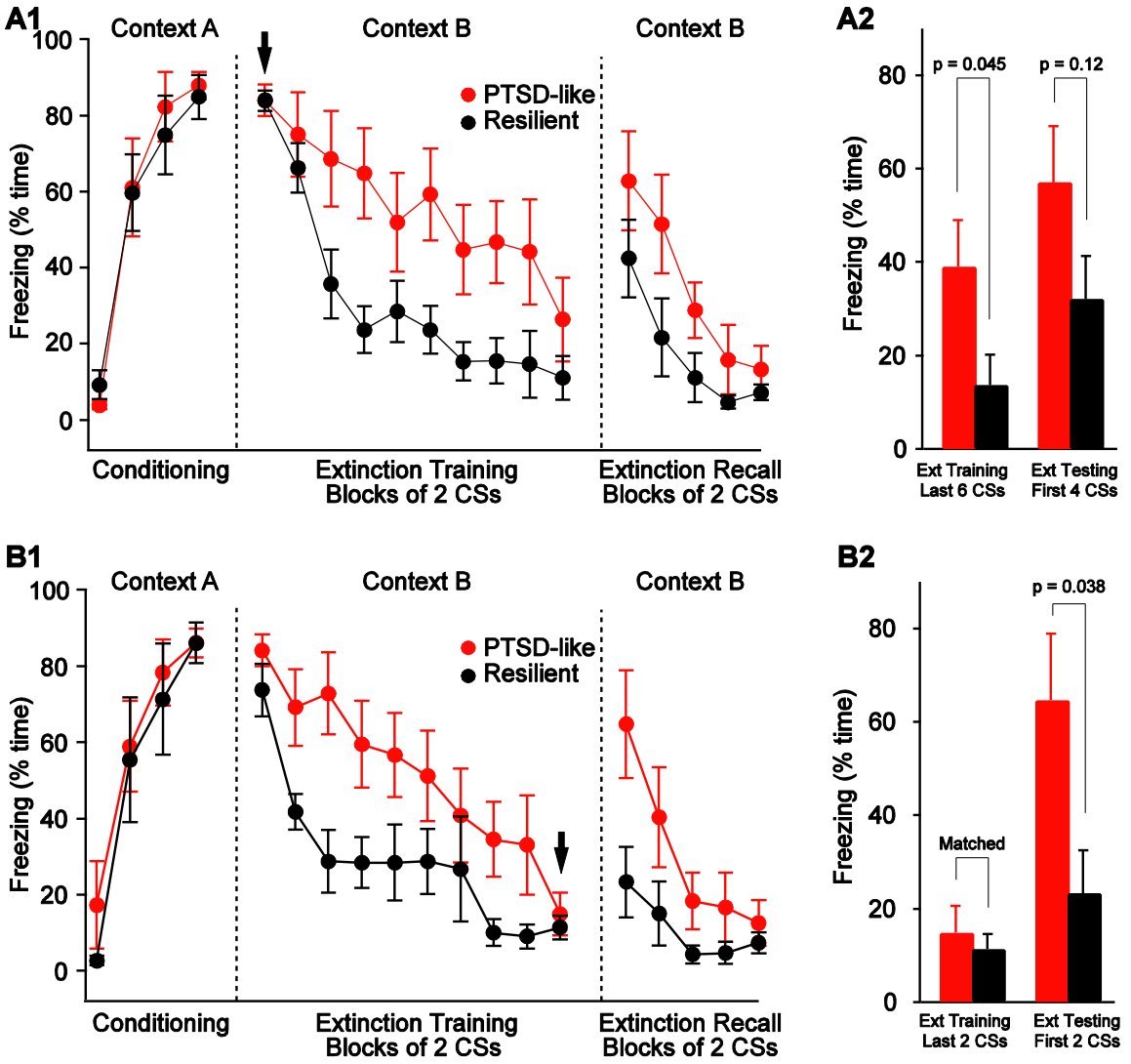

Figure 4. Matching of fear levels to analyze the mechanisms underlying the within- and between-session extinction deficit of PTSD-like rats. (A1,A2) Higher levels of conditioned fear do not explain within-session extinction deficit seen in PTSD-like rats. Freezing levels at the beginning of the extinction training session (arrow) were matched by dropping subjects at opposite poles of the distributions in the two rat groups. Two samples of six rats remained. (A1) Time spent freezing (y-axis) during the various phases of the fear-conditioning protocol (x-axis). (A2) Bar graph plots freezing levels in these subsets of PTSD-like (red) and resilient (black) rats during the last six CSs of the extinction training session (left) and first four CSs of the extinction testing session (right). (B1,B2) Higher freezing levels of PTSD-like rats during extinction recall are not due to a within-session extinction deficit. Freezing levels at the end of extinction training (last two CSs, arrow) were matched between the resilient and PTSD-like rats. To achieve this, we dropped subjects at the opposite ends of the distributions in the PTSD-like and resilient rats. We remained with samples of six PTSD-like and five resilient rats. (B1) Graph plotting percentage of time spent freezing (y-axis) during the various phases of the fear-conditioning protocol (x-axis) in these two subsets of rats. (B2) Bar graph plots freezing levels in these subsets of PTSD-like (red) and resilient (black) rats during the last two CSs of the extinction training session (left) and first two CSs of the extinction testing session (right). Even after matching freezing levels at the end of extinction training, we still observed significantly higher freezing at the beginning of the extinction test, suggesting that PTSD-like rats are deficient in the overnight consolidation of extinction.

3.4.3 Fear extinction in resilient vs. PTSD-like rats prior to PT

The above suggests that a subset of Lewis rats are prone to develop EBMAs following PT and that these rats are also deficient at extinguishing conditioned fear responses, as seen in human PTSD (Orr et al. 2000; Peri et al. 2000; Blechert et al. 2007; Milad et al. 2008, 2009). These results raise the question of whether the physiological mechanisms responsible for this extinction deficit are a consequence of PT or whether they predate it. The next set of experiments addressed this question by subjecting Lewis rats (n = 35) to the same protocol as above with the exception that fear conditioning occurred prior to PT and EPM testing, as described in Figure 3B1.

In this sample, the incidence of rats spending zero time in the open arms of the EPM was 40% (or 14 of 35), not significantly different from that seen in the PT7 group (Fisher exact probability test, P = 0.79). Figure 2B2 plots percentage of time spent freezing (y-axis) in resilient (black, n = 21) and PTSD-like (red, n = 14) Lewis rats during the various phases of the fear-conditioning protocol (x-axis). As in section 3.4.2, PTSD-like and resilient rats both displayed significantly increased freezing levels as a result of fear conditioning ($F = 152.48$, $P < 0.001$), as well as the expected decrease in freezing during extinction training ($F_{(9,25)} = 115.56$, $P < 0.001$), and extinction recall ($F = 19.16$, $P < 0.001$). There were no significant differences in levels of freezing between the PTSD-like and resilient rats in any condition (fear conditioning [$F = 2.18$, $P = 0.15$], extinction training [$F < 1$], and extinction recall [$F < 1$]) and no significant group by trial interaction (fear conditioning [$F = 2.31$, $P = 0.10$], extinction training [$F = 1.63$, $P = 0.16$], and

extinction recall [F < 1]). These results suggest that the extinction deficit seen in PTSD-like rats of section 3.4.2 is not an antecedent condition but rather a consequence of PT exposure.

Although MANOVAs failed to reveal overall group effects, there were trends for group by trial interactions during fear conditioning and extinction training (P = 0.10 and 0.15, respectively). Indeed, inspection of the data revealed that PTSD-like rats appeared to acquire the CS–US association faster than resilient rats during fear conditioning (Fig. 3B2). Specifically, PTSD-like rats displayed significantly higher freezing levels during the third CS–US pairing compared to resilient rats (t-test, P = 0.02). Also, during the extinction training session, freezing levels in PTSD-like rats reached near floor levels faster than resilient rats (t-test comparing freezing during CSs 9–10, P = 0.024). These observations may be taken as evidence that, prior to PT, PTSD-like rats are more sensitive to stimulus contingencies than resilient rats, even though they do not exhibit an extinction deficit.

Figure 5 contrasts the results obtained in PTSD-like (red) and resilient (black) rats when fear conditioning was performed before (empty bars) vs. after (filled bars) PT. While there were no group differences in freezing levels at the end of the fear-conditioning session (Fig. 5A, t-tests, P ≥ 0.18) or beginning of extinction training (Fig. 5B, t-tests, P ≥ 0.11), PTSD-like rats trained on fear conditioning after PT froze significantly more than all other groups at late stages of extinction training (Fig. 5C, t-tests, P ≤ 0.024) and early stages of extinction testing (Fig. 5D, t-tests, P ≤ 0.028), with no differences between the other groups.

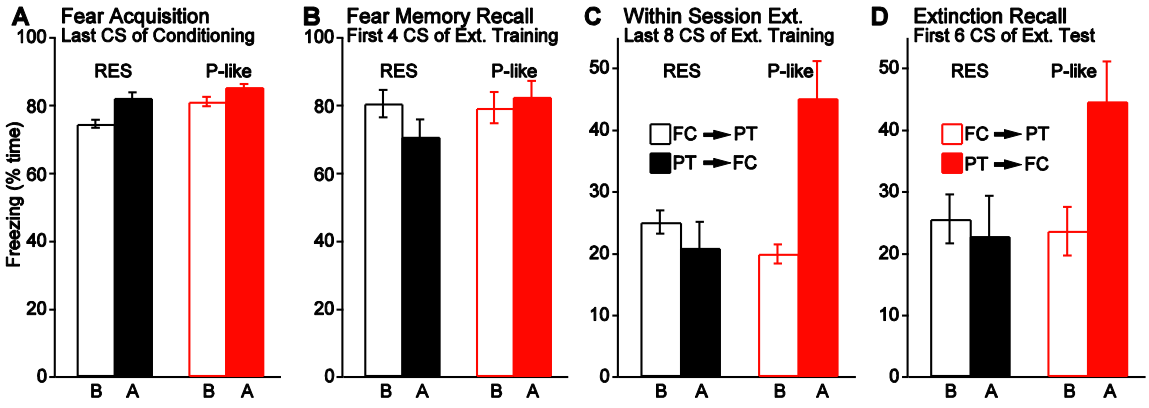

Figure 5. Behavior of PTSD-like and resilient rats depending on timing of PT. Bar graphs plot freezing levels in PTSD-like (red) and resilient (black) rats when fear conditioning was performed before (empty bars) vs. after (filled bars) PT. Four phases are considered: (**A**) end of fear-conditioning session, (**B**) beginning of extinction training session, (**C**) end of extinction training session, and (**D**) beginning of extinction testing session.

3.5 Discussion

While the role of fear-learning mechanisms in the etiology and pathophysiology of anxiety disorders is controversial (Keane et al., 1985; McNally, 2002; Mineka and Ohman, 2002; Poulton and Menzies, 2002; Mineka and Oehlberg, 2008), in our view, it is easy to see how an extinction deficit could contribute to the maintenance of PTSD. Indeed, PTSD is often characterized as a failure to forget fear associations. However, the role of fear learning mechanisms in the acquisition of the disorder is less clear. Here, it is perhaps significant that even though resilient and PTSD-like rats displayed similar levels of conditioned fear prior to PT, PTSD-like rats seemed to acquire the CS–US association faster than resilient rats (Fig. 3B2). Thus, it appears that naïve PTSD-like rats may be more sensitive to stimulus contingencies than resilient rats, possibly because they are primed to interpret threat in their environment resulting in faster learning of associations of fear-based contingencies. While it

remains unclear whether this property accounts for their greater susceptibility to show EBMAs after PT, this possibility should not be discounted. On the other hand, the slower within-session extinction seen after PT could be interpreted as the opposite, a reduced sensitivity to stimulus contingencies. Alternatively, this could reflect an additional deficit whereby PTSD-like rats are impaired at forming new, safety-based associations for previously learned danger-related cues.

In conclusion, the present findings indicate that the Lewis rat model of PTSD reproduces several aspects of the human syndrome. Indeed, the PTSD-like state of susceptible Lewis rats only develops after a life-threatening experience, and only in a proportion of subjects. Moreover, as in the human syndrome, PTSD-like rats display an extinction deficit that does not predate disease onset but develops as a result of the traumatic experience. However, while the Lewis rat model of PTSD has face validity, it should be tested further, for instance, by comparing the performance of resilient vs. PTSD-like rats on memory tasks dependent on hippocampal function, known to be impaired in humans with PTSD (Gilbertson et al. 2002, 2007; Kitayama et al. 2005).

CHAPTER IV

IMPAIRED HIPPOCAMPAL-DEPENDENT BEHAVIORS ASSOCIATED WITH PTSD-LIKE PHENOTYPE

4.1 Rationale

Many animal models of PTSD are also associated with signs of hippocampal dysfunction. For instance, immobilization stress (Andero et al., 2012), underwater trauma (Richter-Levin, 1998; Wang et al., 2000), and chronic social instability combined with predator stress impair spatial memory (Diamond et al., 1999; Park et al., 2008, reviewed in section 1.5). In addition, it was reported that following SPS (Wang et al., 2012), or chronic social instability combined with predator stress, the rats' performance on a novel object recognition task was impaired (Zoladz et al., 2008). Interestingly, one study reported that acute exposure to predator stress alone selectively impairs hippocampal-dependent working memory, while sparing hippocampal-independent reference memory in the radial arm water maze (Woodson et al., 2003).

Confidence that the hippocampal alterations seen in animal models of PTSD and in the human disorder depend on similar mechanisms would be increased if they were apparent prior to the traumatic event, as in human PTSD (Gilbertson et al., 2007). Fortunately, much work has been done in rodents to identify tasks that critically depend on hippocampal functioning. Previous studies have revealed that rats with ibotenic acid lesions of the entire hippocampus can identify novel objects normally (Langston and Wood, 2010). However, they are greatly impaired in their ability to recognize novel spatial configurations of these objects in an allocentric but not egocentric frame of reference (Langston and

Wood, 2010). We will take advantage of this knowledge for the design of the present experiments.

4.2 Hypothesis

Prior to PT, we tested separate groups of Lewis rats on three types of recognition memory tasks that varied in the types of clues the rats could use to identify item novelty; identity of the objects or their location in ego- or allo-centric coordinates. Since only the latter is known to depend on hippocampal functioning (Langston and Wood, 2010), we expect that prior to PT exposure, rats predisposed to develop a PTSD-like phenotype will show greater recognition deficits compared to resilient rats, but only when identifying item novelty based on allocentric encoding.

4.3 Methods

4.3.1 Open Field

To assess whether resilient vs. PTSD-like rats differed in their baseline anxiety levels and exploratory behavior, we tested Lewis rats on the OF prior to PT (see section 2.5 for details). A total of 81 Lewis rats were used in these experiments.

4.3.2 Object and object-place recognition tasks

Distinct samples of Lewis rats were tested in the three object or object-place recognition tasks (NOR, *n* = 31; EOR, *n* = 36; AOR; *n* = 48). Two days after

task completion, they were subjected to PT and tested on the EPM one week later. We aimed to obtain samples that included at least 8–12 PTSD-like rats for each task. Every week, 4–8 rats underwent the paradigm until we reached the target number of PTSD-like rats for each task. However, unexpected differences in the incidence of the PTSD-like phenotype in the three tasks required that different sample sizes be used to reach the target of 8–12 PTSD-like rats in the three tasks.

4.4 RESULTS

4.4.1 Behavior of resilient and PTSD-like rats in the open field

Forty-four (54%) of the rats were classified as PTSD-like, whereas 37 rats (46%) were classified as resilient. The incidence of the PTSD-like phenotype in this sample is consistent with that found in previous studies using the same paradigm (45-50%; Cohen et al. 2006a; Goswami et al. 2010), and much higher than in naïve Lewis rats (not subjected to PT; 13%; Goswami et al. 2010). Importantly, by comparing various measures of anxiety in naïve vs. resilient rats, the latter study determined that PT did not cause a general increase in anxiety expressed by all subjects, but the emergence of EBMAs in a subset of susceptible Lewis rats.

As shown in Table 1, we compared the behavior of PTSD-like rats and resilient rats in the OF prior to PT along eight different dimensions that included measure thought to assess anxiety levels (e.g. time in periphery) as well as measures of global locomotor activity (e.g. total numbers of corners or quadrants

visited). A One-way MANOVA revealed no significant group differences (Wilks' λ = 1.34, p = 0.232). These negative results suggest that if behavioral differences are detected between resilient and PTSD-like rats in the object or object-place recognition tasks, they are unlikely to reflect disparities in exploratory behaviors or anxiety levels.

Table 1 | Comparison between the behavior of resilient and PTSD-like rats in the open field.

	Resilient ($n = 35$)	PTSD-like ($n = 44$)
Time in the center	37.26 ± 7.77	41.57 ± 9.86
Time in the periphery	261.57 ± 7.82	256.57 ± 9.90
Latency to leave the center	31.51 ± 6.56	39.16 ± 9.94
Number of stretch attends	6.60 ± 0.65	6.43 ± 0.5
Number of rears	6.31 ± 0.63	6.34 ± 0.58
Number of corners visited	5.74 ± 0.86	4.98 ± 0.76
Number of quadrants visited	5.91 ± 0.81	4.98 ± 0.73
Time in the corners	223 ± 9.62	233.93 ± 9.78

Values are expressed in seconds. Note that two resilient rats had to be excluded due to technical difficulties with the camera.

4.4.2 Common patterns of exploratory behaviors in the NOR, EOR, and AOR tasks

The incidence of the PTSD-like vs. resilient phenotypes differed markedly between groups (Figure 6A, Chi-square test, p = 0.002). In the NOR sample, 39% of Lewis rats (or 12 rats of 31) exhibited the PTSD-like phenotype, consistent with the high incidence seen in earlier studies (Cohen et al. 2006a; Goswami et al. 2010) and in the sample used for the OF test. In contrast, the incidence of PTSD-like rats was 25% in the EOR (9 out of 36) and AOR (12 out

of 48) tasks (Figure 6A). Thus, it appears that some aspect of the EOR or AOR tasks, perhaps the increased handling of the rats, reduces the incidence of the PTSD-like phenotype. Consistent with this, when we compared the rat groups subjected to little (OF, NOR) or more extensive handling (EOR or AOR), a significant difference in the relative incidence of the PTSD-like and resilient phenotypes was observed (Chi-square test, $p = 0.0004$). This phenomenon is reminiscent of prior studies that describe how some stressors or early life experiences can have protective effects on subsequent susceptibility to emotional challenges (Parker and Maestripieri, 2011).

The exploratory behavior of Lewis rats on the various tasks was similar in many respects. We first describe these similarities and then highlight differences between PTSD-like and resilient rats. Figure 6B shows the time spent by resilient (blue) and PTSD- like (red) rats exploring the objects during the 5 min sampling period. A two-factor analysis of variance (ANOVA) using task identity (NOR, EOR, AOR) and behavioral phenotype (PTSD- like, resilient) as between

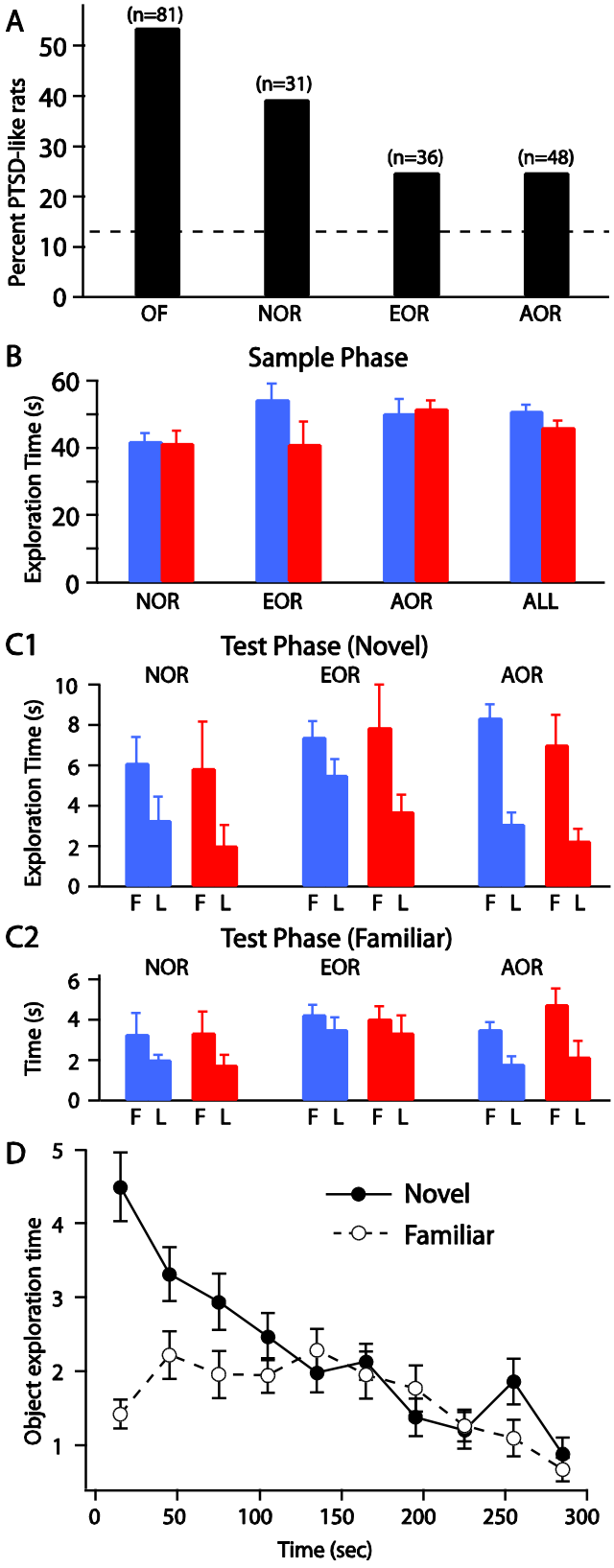

Figure 6. Incidence of PTSD-like phenotype and common patterns of exploratory behavior across the NOR, EOR, and AOR tasks. (A) Different samples of rats were tested on the EPM one week after predator threat and classified as PTSD-like if they failed to explore the open arms. Depending on the sample (n's above each bar), prior to predator threat, the rats were either subjected to the open field test (OF) or one of the object (NOR) or object-place recognition (EOR, AOR) tasks. (B) Total time exploring objects during the sample phase of the three tasks in Resilient (blue) vs. PTSD-like (red) rats. Data obtained in the three rat samples is combined on the right (ALL). (C) Time exploring novel (C1) or familiar (C2) items during the first (F) and last minute (L) of the testing phase in Resilient (blue) vs. PTSD-like (red) rats. (D) Fluctuations in time spent exploring the novel (solid circles and continuous line) or familiar (empty circles and dashed line) item during the test phase of the AOR task. The data is plotted in 30 s bins.

factors revealed no main effects of task [$F = 1.61$, $p = 0.21$] or phenotype [$F = 1.64$, $p = 0.20$] and no interactions between task and phenotype [$F = 2.45$, $p = 0.09$]. Consistent with this, we found no significant difference in total exploration of the objects between resilient and PTSD-like rats during the sample phase of all tasks were combined (*t*-test, $t = -1.54$, $p = 0.13$).

In studies using object or object-place recognition tasks, it is customary to compare exploration of the novel and familiar objects (or object-place configurations) over the entire test phase. However, this approach assumes that the pattern of exploration is consistent across the duration of the test phase. We tested this assumption by comparing exploration of the novel (Figure 6C1) and familiar items (Figure 6C2) during the first (F, left) and last (L, right) minute of the test phases in the three tasks. As shown in Figures 6C1,2, irrespective of group identity, Lewis rats spent a similar amount of time exploring the novel and familiar items during the test phase of the three tasks. Also, there was an overall tendency for the subjects to spend more time exploring the objects early than late in the 5 min testing period, irrespective of task or phenotype. However, the latter trend appeared to be differentially expressed in relation to the novel (Figure 6C1) vs. familiar (Figure 6C2) items. In particular, the difference between early and late exploration times was markedly higher for the novel than the familiar item. This was confirmed by a 3 (task) by 2 (phenotype) by 2 (object identity) by 2 (exploration period) MANOVA using task identity (NOR, EOR, AOR) and behavioral phenotype (PTSD-like, resilient), as between factors and object identity (novel, familiar) and exploration period (early, late) as within- subject

factors. This revealed a main effect of object identity [$F = 26.6$, $p < 0.001$] and exploration period [$F = 46.6$, $p < 0.001$] with a significant interaction between them [$F = 8.6$, $p = 0.004$], but no effect of phenotype [$F = 0.5$, $p = 0.51$]. There was also a main effect of task [$F = 3.8$, $p = 0.025$] such that rats showed lower exploration times of the novel and familiar items in the NOR as compared to both EOR and AOR tasks (Scheffe, p's < 0.015). However, there was no interaction with phenotype (Task-Phenotype interaction [$F = 0.05$, $p = 0.95$]).

Overall, the above suggests that irrespective of behavioral phenotype, Lewis rats spend more time exploring the items early than late in the test phase and that this effect is more pronounced for the novel items. A better appreciation of this non-uniformity can be gained by examining Figure 6D where we plot fluctuations in the exploration times of the novel (solid line) vs. familiar (dashed line) object-place configurations for all rats subjected to the AOR task. In this and other tasks, it is obvious that preferential exploration of the novel item is maximal during the early part of the test phase and that it decays later. Importantly, exploration of the novel and familiar items becomes nearly indistinguishable toward the end of the test phase. Thus, in order to enhance the sensitivity of comparisons between novel vs. familiar item explorations, it is important to target the early portion of the test phase.

4.4.3 Task-dependent differences in time exploring novel vs. familiar items

An approach frequently used to quantify differential object exploration in NOR, EOR, and AOR tasks is to compute a discrimination index (DI). The DI

normalizes differences in exploration times between novel and familiar items by the combined exploration time of both items. This minimizes the impact of inter-individual differences in locomotor activity. The DI is computed using the following equation: (Novel–Familiar)/(Novel + Familiar). We adopted this approach to compare the exploratory behavior of resilient and PTSD-like rats during the test phase of the three tasks. However, note that DIs were not normally distributed in two of the three tasks (Kolmogorov–Smirnov tests, $p < 0.05$). Thus, non-parametric statistical tests are used to assess this data.

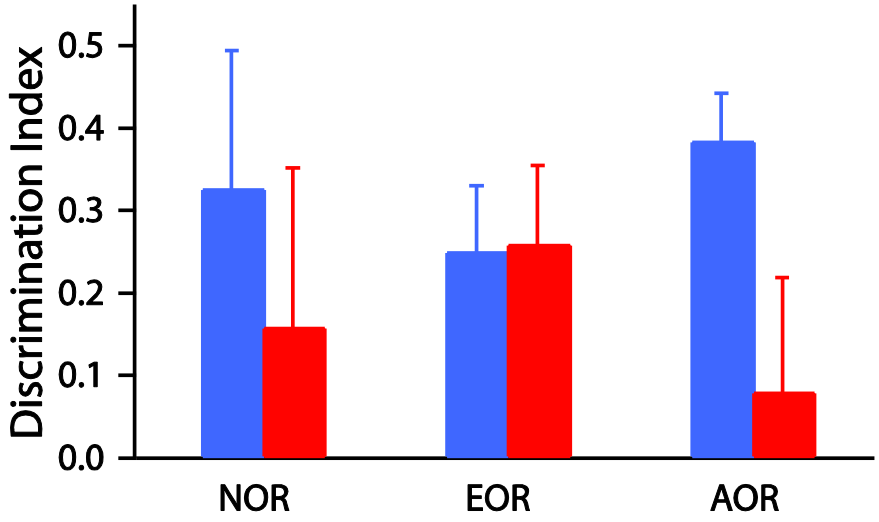

Figure 7: Differential exploration of novel vs. familiar items in the three tasks. The bar graph illustrates the discrimination index of Resilient (blue) and PTSD-like (red) rats in the three tasks (NOR, left; EOR, middle; AOR, right). To compute the discrimination indices, we used exploration times during the first 1.5 min of the test phase.

Because differential exploration of the novel and familiar items was maximal during the first 1.5 min of the task (Figure 6D), we used the data obtained during this period to compare the two groups (Figure 7: resilient, blue; PTSD-like, red). In all three tasks, Lewis rats had positive average DIs indicating preferential exploration of the novel items irrespective of the type of information

required to assess item familiarity/novelty. This was confirmed by comparing the proportion of rats with DIs > or ≤ 0 to a random (50:50) distribution (Chi-square, $p < 0.001$). However, when the proportion of rats with DIs > or ≤ 0 was compared between the two phenotypes after collapsing the data in the three tasks, a significant difference emerged (Chi-square, $p = 0.035$). Insights into the origin of this effect can be obtained by comparing the DIs of resilient and PTSD-like animals in the three tasks. As shown in Figure 7, differences in DIs relative to inter-individual variability seemed negligible in the NOR and EOR tasks, but substantial in the AOR task. Consistent with this, a Mann–Whitney U test comparing the DIs of resilient vs. PTSD-like rats on the AOR task revealed a significant difference ($p = 0.024$) with PTSD-like rats exhibiting lower discrimination of the novel items than resilient rats. Note that the trend apparent in the NOR task was driven by one extreme subject of the PTSD- like group that nearly spent all of the available time during the test phase exploring the familiar object. When this extreme subject was excluded, the trend vanished (DIs: PTSD-like = 0.25 ± 0.17; resilient = 0.32 ± 0.16). Consistent with this, when we compared the proportion of PTSD-like vs. resilient rats with DIs > or ≤ 0 in the NOR task, it did not approach significance (Fisher test, $p = 0.28$).

4.5 Discussion

To assess hippocampal functioning, we used three different recognition tasks (NOR, EOR, AOR) where subjects manifest that they have previously encountered objects or object-place configurations by preferential exploration of

novel items. It should be mentioned that there is significant debate in the literature regarding the hippocampal dependence of the three tasks (Mumby, 2001; Langston and Wood, 2010). However, we note that recognition of item familiarity using an allocentric frame of reference, as in our AOR task, seems to be particularly dependent on hippocampal functioning in rats (Langston and Wood, 2010) and humans (Suthana et al., 2009). Importantly, the exploratory behavior of resilient and PTSD-like rats was indistinguishable in a novel OF and during the sampling phase of the three tasks. This suggests that there were no disparities between two rat groups in their initial object investigations. In contrast, during the test phase, the performance of PTSD-like rats was inferior to that of resilient rats, but only on the AOR task. That is, PTSD-like rats spent less time exploring the novel relative to the familiar object-place configuration than resilient rats. In light of prior work indicating that hippocampal functioning is especially critical for detecting familiarity in an allocentric spatial frame of reference (Langston and Wood, 2010; Suthana et al., 2009), these results support the notion that PTSD-like rats reproduce the hippocampal deficit seen in human PTSD. Combined with our prior findings on fear extinction (Goswami et al., 2010), the present results suggest that the Lewis rat model of PTSD has face validity. Therefore, comparing limbic neuronal interactions in resilient vs. at risk Lewis rats might shed light on the pathophysiology of human PTSD.

CHAPTER V

INTRODUCTION TO THE ROLE OF THE AMYGDALA IN EMOTIONAL LEARNING AND MEMORIES

In the first part of this thesis, I tested the validity of a rat model of PTSD. Having found evidence that the model reproduces salient features of the human syndrome, in the second part of the thesis, I will use this model to shed light on the pathophysiology of PTSD. However, for this endeavor to be successful, the neural mechanisms that control the expression of fear and anxiety must be highly conserved across species. Therefore, the following reviews evidence that a highly conserved network of brain structures regulate the expression of fear and anxiety in mammals (Phelps and LeDoux , 2005). Since my experiments examined whether the intrinsic and synaptic responsiveness of amygdala neurons differ between resilient and PTSD-like rats, the following will focus on this structure.

5.1 Evolutionary theory of emotions

Early on, it was suggested that facial expressions provided a nonverbal form of communication before the development of language. In the 1860s, the French physician Duchenne de Boulogne, aimed to identify the specific facial muscles involved in emotional expressions. These were among the first studies that used electrophysiological techniques to study biological readouts of emotional expression. Duchenne's studies were included in Charles Darwin's book, *The expression of emotions in man and animals.* In this book, Darwin suggested that emotions are inherited, passed along to us from our ancestors. The evidence he discusses to support this notion included the following: primal emotions are similarly expressed (1) by difference species (e.g. the case of fear

responses, most species exhibit behaviors including orienting towards the stimulus, cessation of ongoing activity, cardiovascular changes, piloerection, antinociception, and defecation); (2) by infants and adults (e.g. smiling or frowning), (3) by geographically isolated cultural groups, and (4) and by individuals born blind and with normal vision. Taken together, Darwin's observations suggest that, like physical characteristics, emotions are inherited. Although Darwin never attempted to attribute emotional expression to specific regions of the brain, others were quick to fill that void.

In the 1950s, Paul McLean introduced his concept of the "Triune brain," incorporating the ideas of Darwin and other contemporaries. This concept suggested that the human brain was essentially made up of 3 different brains, each reflecting distinct stages of evolution. According to McLean's view, the most primitive region referred to as the reptilian brain or "R-complex" consisted of the basal ganglia, which mediated the execution of motor programs. The next stage of evolution involved the "limbic system", which McLean believed was responsible for emotional expression. Finally, the newest component was the neocortex, which seemed particularly pronounced in higher mammals such as nonhuman primates and humans. Interestingly, the amygdala was actually discovered and named (i.e. amygdalae latin for "almond") by Karl Friedrich Burdach in the early 1800s, but direct evidence supporting its role in emotional processing would not come until landmark studies in monkeys.

The number of studies relating animal behavior and human emotions increased steadily in the 19th and 20th centuries, and began to implicate the

amygdala in emotional expression. For instance, in 1888, scientists Brown and Schafer commented on the peculiar emotional effects of bilateral temporal lobectomy in monkeys. These experiments were repeated by Heinrich Kluver and P. Bucy during the 1930s, leading to two major observations: 1) the monkeys appeared unable to recognize familiar objects, despite having an intact visual system, and 2) they had lost normal fear and anger reactions. The resulting mixture of emotional and memory deficits came to be known as the Kluver-Bucy syndrome. Later, it was shown that specific amygdala lesions could reproduce the affective components of the Kluver-Bucy syndrome (Weiskrantz, 1956). These studies led to the current concept that the amygdala is involved in adding emotional salience to the sensory representations of stimuli.

5.2 Phylogenetic conservation of the amygdala complex

Recent evidence continues to support the notion that the amygdala, or homologous structures found in lower vertebrates are evolutionarily conserved across phylogeny. For instance, conditioned avoidance learning is impaired in goldfish after lesions of the medial portions of the dorsal telencephalic pallium (Dm; Portavella et al. 2004). Detailed tract-tracing studies in goldfish have shown that Dm receives auditory, chemosensory, and audiovisual inputs, while exhibiting hypothalamic projection patterns similar to the amygdala of tetrapods (Northcutt, 2006, 2011). In fact, recent studies suggest that Dm is analogous to the basolateral amygdala (BLA), whereas peri-commissural regions within the subpallium may be homologous to CeA and bed nucleus of the stria terminalis

(BNST) regions of the mammalian brain (Maximino et al. 2013). Therefore, it is believed that the brain of teleosts, which date back more than 400 million years ago (Volff, 2005), contain regions homologous to mammalian amygdala nuclei.

Consistent with this view, developmental gene expression studies have led to the identification of mammalian amygdala homologues of the CeA and LA in urudele amphibians (salamanders), and anuran amphibians (frogs and toads; Moreno and Gonzalez, 2007a,b). Similarly in reptiles, portions of the dorsal ventricular ridge (DVR) contain homologues of mammalian amygdala nuclei (Guirado et al. 2000), based on connectivity with sensory cortical and hypothalamic regions (Novejarque et al. 2004). In particular, the posterior DVR (PDVR) is an sensory associative structure, and tract-tracing studies have revealed that the reptilian PDVR projects to the ventral striatum, suggesting that PDVR is homologous to the mammalian basolateral complex of the amygdala (BLA; Novejarque et al. 2004).

Volumetric studies have examined the relative sizes of the various amygdala nuclei across vertebrate evolution. For instance, an expansion of the cortical-like BLA is seen when comparing monkeys to rats, whereas CeA maintain similar volumes across species (Chareyron et al. 2011). These findings suggest that the relative expansion of the BLA may be related to the increase in neocortical mass. Indeed, the size of the BLA is positively correlated with the size of the neocortex, whereas CeA represents a more primitive structure heavily involved in autonomic processing which mammals have in common with earlier amniotic ancestors (Barton et al., 2003). Further evidence of this comes from the

developmental trajectories of the various amygdala nuclei during the lifespan of rats. Indeed, the BLA increases in volume by 113% within the first 3 weeks of life, whereas the volume of CeA increases by only 30% during this period (Chareyron et al. 2012a). Taken together, evolutionary perspectives continue to gain support by studies indicating that regions homologous to the mammalian amygdala are present in phylogenetically older species. Furthermore, the volumes of various amygdala nuclei change across phylogenesis in a way that is consistent with that of the structures providing the major inputs.

5.3 Amygdala development in mammals

The mammalian telencephalon is derived from multiple embryonic origins from the pallium or subpallium, which give rise to excitatory and inhibitory neurons, respectively. Most amygdala neurons are derived from the border where pallial and subpallial progenitor pools meet. This is consistent with the heterogeneity of cell types found in the amygdala. Pallial origins are subdivided into dorsal, medial, lateral, and ventral regions. Subpallial origins include the lateral (LGE), medial (MGE), and caudal ganglionic eminences (CGE). Various studies have indicated that BLA neurons are derived mostly from lateral and ventral pallial origins (Tang et al., 2012). On the other hand, CeA appears to originate from subpallial regions particularly the LGE and possibly the CGE (Tang et al., 2012). This is consistent with the notion that pallial progenitors give rise to excitatory neurons, while subpallial regions give rise to inhibitory neurons (Hirata et al., 2009).

5.4 Neuronal characteristics of the mammalian amygdala

Although the amygdala has been divided into as many as a dozen nuclei (Swanson and Petrovich, 1998), the present discussion will focus on the main components involved in generating fear and anxiety-like behaviors: First is the basolateral amygdala (BLA) complex, which consists of LA, basolateral amygdala (BL), and basomedial (BM; also known as the accessory basal nucleus, AB) amygdala nuclei; second, is the CeA. The morphology and physiology of these principal BLA neurons is similar to that of cortical pyramidal cells. For instance, approximately 80% of BLA neurons are excitatory, using glutamate as the main neurotransmitter (Pape and Pare, 2010). They contain multipolar dendritic arbors with spiny dendrites, and highly collateralized axons (McDonald, 1992). However, given these extensive excitatory collaterals, the baseline firing rates of BLA neurons are surprisingly low (Pape and Pare, 2010). These low firing rates are partially explained by the existence of a large network of inhibitory interneurons.

The diversity of GABAergic interneurons in the BLA is similar to that found in most cortical regions, as determined by the expression of various calcium-binding proteins, neuropeptides, and firing properties. It was reported that up to 50% of BLA interneurons express parvalbumin, forming either perisomatic nets (basket cells) or synaptic contacts with the axon initial segments of principal neurons (axo-axonic/chandelier cells; McDonald and Betette, 2001; Rainnie et al., 2006). Thus, these interneurons are strategically placed to exert powerful

inhibitory influences over BLA output. In addition, BLA contains many other physiological subtypes of interneurons. However, reviewing this diversity is beyond the scope of this thesis.

A major intra-amygdala recipient of BLA inputs is the CeA. This region has a distinct embryonic origin, (section 5.3) and the morphology of its neurons closely resemble striatal medium spiny neurons (MSNs). Although GABA is the major neurotransmitter used by CeA cells, this region is also rich in peptides (Cassell et al., 1986; Cassell and Gray, 1989). Detailed investigations have revealed two distinct regions within CeA: a lateral sector (CeL), and a medial sector (CeM). While CeM contributes most amygdala outputs to brainstem and hypothalamic structures (Hopkins and Holstege, 1978), it is thought to be under tonic inhibitory control of CeL (Ciocchi et al. 2010).

Thus, the amygdala is composed of a heterogeneous group of nuclei with different embryonic origins, as well as unique morphological, physiological, and neurochemical characteristics. Together, these components of the amygdala are critically involved in regulating conditioned fear responses.

5.5 Amygdala circuits underlying the acquisition and extinction of conditioned fear

The paradigm typically used to study fear learning is Pavlovian fear conditioning (LeDoux, 2000), where an initially neutral stimulus or context CS acquires the ability to elicit conditioned fear responses after pairing with a noxious US. The LA is a key site of synaptic plasticity for fear learning (Blair et al.

2001; LeDoux, 2000; Maren, 2001). Indeed, convergence of CS and US inputs during fear conditioning increases the efficacy of synapses conveying information about the CS to LA neurons (McKernan and Shinnick-Gallagher, 1997; Rumpel et al., 2005). As a result, subsequent presentations of the CS alone evoke larger responses in LA (Quirk et al., 1995; Collins & Paré, 2000; Repa et al., 2001). In turn, LA evokes fear responses via CeM (Kapp et al., 1979; Davis, 2000; Ciocchi et al., 2010), the main source of amygdala projections to brainstem and hypothalamic fear effector neurons (Hopkins & Holstege, 1978).

However, transfer of CS information from LA to CeM is indirect (Pare et al., 2004). Indeed, LA lacks direct projections to CeM. Instead, it projects to CeL, to intercalated cell masses (ITC), and to the basal amygdala nuclei (BA) (Krettek and Price, 1978a; Smith and Pare, 1994; Pare et al., 1995; Pitkanen et al., 1997), all of which project to CeM. In fact, recent data indicate that transfer of CS information from LA to CeM involves two mechanisms: *excitation* of CeM cells via glutamatergic BA neurons (BA; Amano et al,. 2011) and *disinhibition* of CeM neurons from GABAergic inputs arising in CeL (Ciocchi et al., 2010; Haubensak et al., 2010; Duvarci et al., 2011; Li et al., 2013) and ITC cells at the BA-CeM border (Amir et al., 2011). In addition, BL is involved in the acquisition of predator odor conditioning (Takahashi et al. 2008), and behavioral stress was shown to increase dendritic branching and spine density in BL neurons (Vyas et al., 2002; Mitra et al., 2005). Finally, application of exogenous glucocorticoids was shown to enhance the excitability of BL neurons *in vitro* (Duvarci and Pare, 2007).

Although the exact mechanisms remain unclear, it is now well established that the intrinsic amygdala pathways described above are regulated by the mPFC. Indeed, PL inactivation blocks the expression of conditioned fear (Sierra-Mercado et al., 2011), an effect thought to depend on PL projections to the BLA. In addition, the infralimbic (IL) region regulates the extinction of conditioned fear. At the level of the amygdala, extinction learning depends on the reinforcement of an active inhibitory process. In part, this effect involves an increased recruitment of ITC neurons by CS-related inputs from the BLA and the consequent inhibition of CeM output cells (Likhtik et al., 2008; Jungling et al., 2008). Importantly, this process is under the facilitatory control of IL (Quirk et al., 2003; Royer & Pare, 2002). Indeed, IL neurons project to ITC cells (McDonald et al., 1996), they acquire CS responsiveness as a result of extinction training (Milad et al., 2002) and their activation by electrical microstimulation accelerates extinction (Milad et al., 2004).

Although far less data is available in humans, the available evidence is entirely consistent with animal findings. As in animals, human subjects with bilateral amygdala lesions cannot form Pavlovian fear memories even though they acquire declarative memories of the training sessions (Bechera et al., 1995; LaBar et al., 1995). In addition, fMRI studies in humans indicate that fear conditioning leads to increases in CS-evoked BOLD signals in the amygdala (Buchel et al., 1998; LaBar et al., 1998). Moreover, a strong correlation was found between the magnitude of this increase and the amplitude of conditioned fear responses (Phelps, 2004). The substrates of extinction learning also appear

to be the same in humans and animals. For instance, extinction training in humans causes a reduction in the CS-related BOLD signal in the amygdala (LaBar et al., 1998; Knight et al., 2004; Phelps, 2004) and a delayed increased in CS-evoked BOLD signal in the human homologue of the IL region of rats and monkeys (Phelps, 2004). However, due to the low spatial resolution of fMRI, we have no human data about the relative contribution of different amygdala nuclei to learned fear. All that fMRI studies can reveal is whether the BOLD signal in the amygdala as a whole increases or decreases.

5.6 The networks mediating the acquisition and extinction of conditioned fear show abnormal activity patterns in humans with PTSD.

Functional imaging studies in humans, typically combat veterans, have revealed that the amygdala and the human homologue of IL (vmPFC) show different patterns of activation in traumatized individuals with vs. without PTSD. Indeed, when those with PTSD are presented with reminders of traumatic events, the amygdala was generally found to be hyper-responsive (reviewed in Shin et al. 2006). In contrast, vmPFC was found to be hypo-responsive and smaller in volume (Bremner et al. 2008). Given the critical role played by IL in extinction, these observations suggest that hypo-activity in IL may be responsible for the extinction deficits of PTSD subjects (Milad et al. 2008). More evidence indicating that the amygdala is critically involved in the etiology of PTSD comes from a retrospective study of Vietnam war veterans who had suffered localized brain injuries and emotionally traumatic memories: subjects with amygdala damage

had a dramatically lower incidence of PTSD than subjects with lesions to other parts of their brain (Koenigs et al. 2008). Further strengthening the link between aberrant amygdala activation in PTSD, a prospective study found that higher activation of the amygdala in response emotional images before trauma predicted reports of higher posttraumatic stress symptom severity (Admon et al. 2009).

So far I have mainly discussed amygdala circuitry as it relates to the generation of phasic fear responses – a process that appears to be abnormally regulated in patients suffering from PTSD. The remaining discussion will address anxiety-like states that are reminiscent of more long-term PTSD symptoms.

5.7 Fear versus anxiety: the role of the bed nucleus of the stria terminalis

As mentioned earlier, PTSD is triggered by a severely traumatic experience, which elicits feelings of extreme fear or terror. Nevertheless, long-term behavioral changes induced by this experience are more akin to anxiety. In other words, PTSD is caused by overwhelming activation of the fear circuit, which can lead to changes in closely related brain regions. It has been proposed that the amygdala mediates fear, while the BNST mediates anxiety (Walker et al. 2003; for review, see Davis et al. 2010). These authors suggest that fear responses are evoked by discrete stimuli, and typically subside upon the removal of the stimulus, whereas anxiety is produced by more complex stimuli generating a longer-duration response (anticipatory fear or apprehension). If this theory is

true, then it is conceivable that trauma may induce long-term changes within the amygdala that may alter activity within the BNST to produce chronic anxiety-like behaviors.

5.7.1 Behavioral dissociations between fear and anxiety

Behavioral studies have dissociated the contributions of the amygdala and BNST to fear and anxiety mainly by manipulating properties of the CS. For instance, one study compared cued and contextual fear conditioning, after lesions of either the CeA or BNST (Sullivan et al. 2004). The authors found that electrolytic lesions of the CeA significantly decreased cued and contextual freezing, and decreased fear-induced CORT release. On the other hand, BNST lesions decreased contextual freezing and CORT release, while leaving cued conditioning intact. Although both structures can influence HPA axis output, these findings suggest that the amygdala mediates multiple aspects of fear, while the BNST preferentially mediates fear associations with the context. Other studies using inactivation or lesion techniques sparing fibers of passage have confirmed the role of the BNST in contextual fear (Resstel et al. 2008; Duvarci et al. 2009, Zimmerman and Maren, 2011).

Further evidence that the BNST mediates anxiety comes from a study comparing the effects of BNST-lesions on fear conditioning using either a long- or short-duration CS (Waddell et al. 2006). The authors suggest that a long-duration CS evokes a form of anticipatory fear more akin to anxiety. In this series of experiments, BNST lesions did not disrupt conditioned fear responses, a short-

duration CS, presumably because this type of CS evokes an immediate fear response that predominately depends on the amygdala. However, BNST lesions reduced conditioned fear responses to a long-duration CS, indicating that the BNST is recruited upon conditions of anticipatory fear.

The generalization of fear responses to innocuous stimuli is a common symptom of PTSD that often results in maladaptive avoidance behavior in humans. Strong evidence implicates the BNST in fear generalization, as measured by discriminative fear conditioning, where one CS is paired with a foot shock (CS+) and one is not (CS-; Duvarci et al 2009). In this study, rats that received ibotenic acid lesions of the entire BNST before training had better discriminative abilities, and reduced contextual fear compared to sham-lesioned controls. These authors also showed that BNST lesions increased the amount of time rats spent on the open arm of the EPM. Similar results were obtained with the elevated zero maze (Waddell et al. 2006). Thus, behavioral studies indicate that BNST activity contributes to anxiety, since inactivating or lesions of this structure tend to reduce anxiety-like behaviors.

5.7.2. The amygdala and BNST share a close anatomical relationship

The BNST comprises over a dozen nuclei that reside above and below the anterior commissure. The unique projection patterns and behavioral roles of each nucleus has not yet been systematically investigated; however, anatomical studies suggest that most of the nuclei within the anterolateral portion receive very strong inputs from CeA and BLA (Dong and Swanson, 2004; Dong et al.

2001; see Davis et al. 2010 for review). In addition, the CeA and BNST contribute overlapping projections to brainstem and hypothalamic structures mediating fear responses. Interestingly, a recent study suggests CeA can project either directly to the brainstem, or indirectly via the stria terminalis (Nagy and Pare, 2008). Thus, the amygdala is in a convenient position to regulate anxiety by modulating the activity patterns of BNST neurons.

5.7.3 Stress affects the morphology of structures regulating fear and anxiety

The close anatomical relationship between the amygdala and BNST is complemented by studies of stress-induced morphological differences within neurons of these structures. Studies in rats have revealed that acute immobilization stress increases dendritic branching and spine formation within the BLA (Mitra et al. 2005), while shorter BLA dendrites have been linked to resilience (Mitra et al. 2009). Furthermore, dendritic hypertrophy in response to immobilization stress was also found within the BNST (Vyas et al. 2003).

CHAPTER VI

INTRINSIC PROPERTIES OF AMYGDALA NEURONS IN PTSD-LIKE AND RESILIENT RATS

6.1 Rationale

Overall, the first half of this thesis establishes the face validity of the Lewis rat model of PTSD, suggesting it could be used to shed light on the pathophysiology of PTSD. Since prior functional imaging studies suggest that a major regulator of fear expression, the amygdala, is hyperactive in human PTSD (Rauch et al. 2006; Shin et al. 2006; Bremner et al. 2008; Milad et al. 2009), the present study uses the Lewis rat model of PTSD to compare the synaptic and intrinsic excitability of amygdala neurons in resilient vs. susceptible rats.

6.2 Hypothesis

We anticipate that the expression of the PTSD-like phenotype is associated with alterations in the physiological properties of amygdala neurons.

6.3 Methods

Lewis rats (n = 83) were subjected to PT and tested on the EPM one week later. Rats with extremely compromised exploratory behavior (zero time in the EPM's open arms) were classified as PTSD-like (50.6% or 42 of 83) whereas the other rats were classified as resilient (49.4% or 41 of 83). One to three days later, the rats were used for in vitro electrophysiological experiments. Here, visually-guided patch clamp recordings were performed in coronal slices of the amygdala to test whether the physiological properties or synaptic responsiveness of recorded cells varied as a function of the rats' phenotype. Importantly, the

recordings were obtained while remaining blind to rats' phenotype (PTSD-like vs. resilient).

6.4 Results

6.4.1 Incidence and passive properties of amygdala cell types in PTSD-like vs. resilient rats

The following is based on samples of 82 BL (resilient, n = 38; PTSD-like, n = 44), 138 central lateral (CeL; resilient, n = 69; PTSD-like, n = 69) and 71 central medial (CeM, resilient, n = 26; PTSD-like, n = 45) neurons. CeL and CeM neurons are considered separately because they form contrasting connections with fear output networks. Indeed, CeM contributes most brainstem projections of the amygdala to brainstem fear effector structures such as the periaqueductal gray, nucleus tract solitaris, and dorsal motor nucleus of vagus (Hopkins and Holstege, 1978; Veening et al., 1984). In contrast, the brainstem projections of CeL are largely limited to parabrachial nucleus (Petrovich and Swanson, 1997). However, CeL is thought to regulate fear expression via GABAergic projections to CeM (Lopez de Armentia and Sah, 2004; Ciocchi et al., 2010; Haubensak et al., 2010; Duvarci and Pare, 2011) and BNST (Dong et al., 2001). Below, we first compare passive neuronal properties and incidence of physiological cell types between resilient and PTSD-like rats at the three sites and then examine their synaptic responsiveness.

BL neurons

Using criteria derived from studies that correlated the physiological and morphological properties of BL neurons (Washburn and Moises 1992a; Rainnie et al. 1993; Pare et al. 1995a; Faber and Sah 2002), we classified BL neurons as putative projection cells (Fig. 8A1,2) or interneurons (Fig. 8A3) based on their contrasting electroresponsive properties (reviewed in Sah et al., 2003; Pape and Pare 2010). In particular, BL neurons were classified as principal cells when they displayed spike frequency adaptation during depolarizing current pulses and generated action potentials of comparatively long duration (≥0.8 ms at half amplitude). Given the heterogeneous firing patterns of BL interneurons reported in previous studies (Sosulina et al. 2006; Woodruff and Sah 2007; Jasnow et al. 2009), we relied primarily on spike duration to identify these cells (≤ 0.6 ms at half amplitude). Because a very low proportion of recorded cells met this criterion, they will not be considered further below.

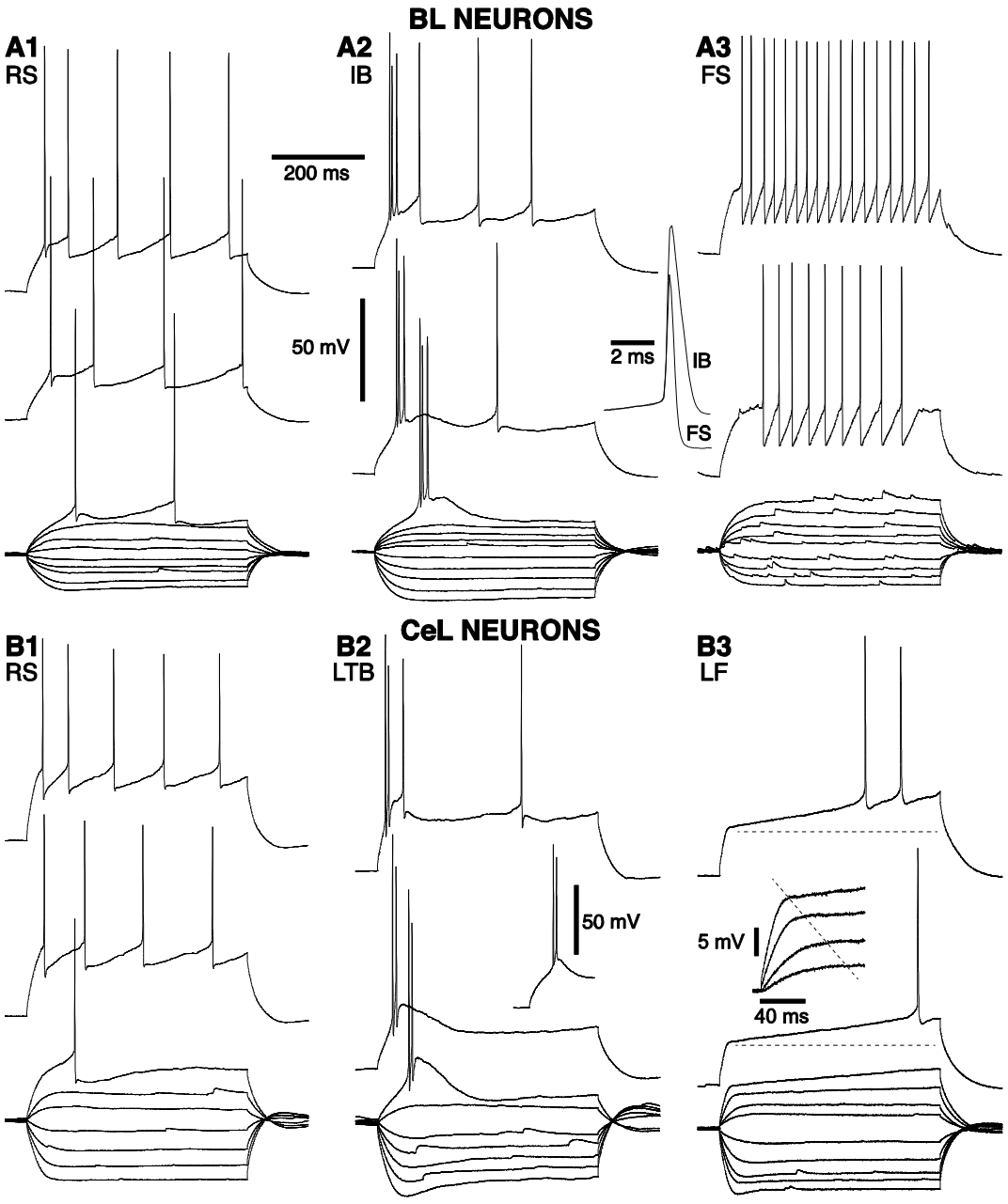

Figure 8 Physiological classes of amygdala neurons. Voltage responses of six different cells to negative and positive current pulses of progressively increasing amplitude (current steps of -0.04 nA for negative and sub-threshold positive pulses; current steps 0.02 nA for supra-threshold pulses). Unless otherwise noted, stimuli were applied from a membrane potential of -80 mV, as determined by steady intracellular current injection. (A) In BL, three types of neurons could be distinguished: regular spiking (RS; A1), intrinsically bursting (IB; A2) and fast spiking (FS; A3). Inset between A2 and A3 overlays action potentials generated by FS and IB cells. (B) In CeL, three types of neurons could be distinguished: RS (B1), low-threshold bursting (LTB; B2), and late firing (LF, B3). Inset below top trace of B2 shows rebound spike doublet generated at the break of a -0.2 nA hyperpolarizing pulse applied from -65 mV. Inset in B3 shows change in the time course of voltage responses to sub-threshold depolarizing current pulses. Voltage and time calibrations between A1 and A2 apply to all panels with the exception of insets.

Consistent with previous findings (Pare et al., 1995a), two types of BL projection cells could be distinguished based on their responses to depolarizing current pulses: cells generating only single spikes (Fig. 8A1), hereafter termed regular spiking (RS) cells, and neurons generating spike doublets or bursts (Fig. 8A2), hereafter termed intrinsically bursting (IB). As shown in figure 9A, the incidence of RS and IB neurons did not vary between resilient and PTSD-like rats (Fisher's exact test, P = 0.8230). Similarly, spike duration, amplitude, and threshold did not vary significantly as a function of the rats' phenotypes, nor did their resting potential, time constant, or input resistance (t-tests, P's>0.1; Table 2). The same negative result pattern was obtained when we separately compared the properties of the two cell types in resilient and PTSD-like rats.

Table 2. Physiological Properties of BL neurons

			Action Potential			
	Rest, mV	R_{in}, MΩ	Threshold, mV	Amplitude, mV	Duration, ms	Time Constant, ms
Resilient N = 52	-67.9 ± 0.6	128.3 ± 8.1	-40.9 ± 0.6	90.7 ± 1.5	0.85 ± .02	40.8 ± 2.2
PTSD-like N = 50	-67.5 ± 0.6	135.3 ± 6.9	-40.9 ± 0.5	91.8 ± 1.7	0.87 ± .02	38.4 ± 2.9

Values are means ± SE. BL, basolateral nucleus of the amygdala; Rest, resting potential; R_{in}, input resistance.

CeL neurons

Consistent with prior studies in rats (Dumont et al. 2002; Lopez de Armentia and Sah 2004; Amano et al., 2012), we identified three main cell types in CeL, based on variations in the temporal dynamics of current-evoked spiking:

regular spiking (RS; Fig. 8B1), low-threshold bursting (LTB, Fig. 8B2), and late-firing (LF, Fig. 8B3). However, as shown in figure 9B, their incidence did not vary significantly between resilient vs. PTSD-like rats (chi-square test, P = 0.211). Moreover, as seen with BL neurons, the two behavioral phenotypes were not associated with differences in spike or passive properties, whether we considered the three cell types together (Table 3) or separately.

Table 3. Physiological Properties of CeL neurons

			Action Potential			
	Rest, mV	R_{in}, MΩ	Threshold, mV	Amplitude, mV	Duration, ms	Time Constant, ms
Resilient N = 69	-62.8 ± 0.7	414.7 ± 22.8	-39.5 ± 0.8	84.2 ± 1.3	1.00 ± .03	42.8 ± 2.8
PTSD-like N = 69	-63.9 ± 0.8	373.4 ± 17.7	-38.3 ± 1.4	84.1 ± 1.2	1.05 ± .03	43.2 ± 2.5

Values are means ± SE. CeL, central lateral amygdala; Rest, resting potential; R_{in}, input resistance.

CeM neurons

In prior studies (Dumont et al. 2002; Martina et al. 1999), the same physiological classes of neurons identified in CeL were found in CeM, albeit with differences in their relative incidence. Our results in CeM matched these earlier findings with the exception that we encountered no LF cells. In contrast with BL and CeL neurons, marked differences in the incidence of CeM cell types were observed as a function of the rats' behavioral phenotype (Fig. 9C). In particular, RS cells prevailed in PTSD-like rats whereas the incidence of LTB neurons was higher in resilient rats (Fisher test, p = 0.017).

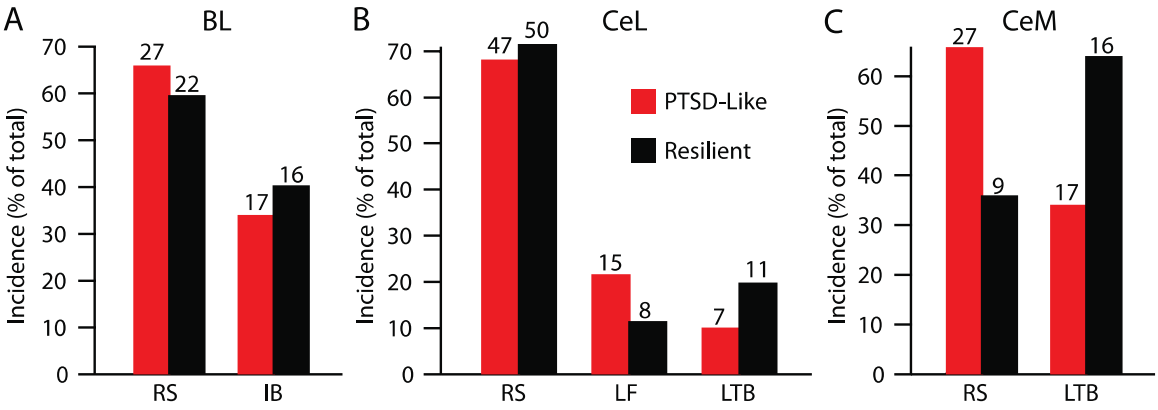

Figure 9 Incidence of different physiological classes of amygdala neurons. (**A-C**) Amygdala neurons recorded in BL (**A**), CeL (**B**), and CeM (**C**). Sample sizes: (**A**) BL neurons from resilient (n= 52) and PTSD-like rats (n = 50); (**B**) CeL neurons from resilient (n = 69) and PTSD-like rats (n = 69). (**C**) CeM neurons from resilient (n = 25) and PTSD-like rats (n = 41). Numbers above bars indicate sample sizes for respective cell type.

To assess whether this contrast resulted in an overall difference in the intrinsic excitability of CeM between PTSD-like vs. resilient rats, we next compared the spike and passive properties of LTB and RS cells combined. However, no differences could be detected (Table 4A). When the two cell types were considered separately, the properties of LTB cells did not vary with behavioral phenotype (Table 4B), whereas that of RS did along a few dimensions (Table 4C). However, the overall significance of these differences is unclear because the direction of the changes was inconsistent. For instance, in PTSD-like rats, RS cells had a more depolarized resting potential than in resilient rats, yet a more negative spike threshold. Moreover, these differences were detected with an uncorrected significance level of $\alpha = 0.05$; none of them approached significance with Bonferroni correction ($p = 0.006$ in this case).

Table 4A. Physiological Properties of CeM neurons

			Action Potential			
	Rest, mV	R_{in}, MΩ	Threshold, mV	Amplitude, mV	Duration, ms	Time Constant, ms
Resilient N = 26	-63.8 ± 1.3	450.9 ± 62.1	-42.1 ± 1.5	79.5 ± 2.1	0.84 ± .05	31.8 ± 2.6
PTSD-like N = 45	-62.1 ± 1.0	472.9 ± 29.8	-44.7 ± 0.9	84.5 ± 1.8	0.75 ± .03	31.6 ± 2.3

Values are means ± SE. CeM, central medial amygdala; Rest, resting potential; R_{in}, input resistance.

Table 4B. Physiological Properties of LTB neurons

			Action Potential			
	Rest, mV	R_{in}, MΩ	Threshold, mV	Amplitude, mV	Duration, ms	Time Constant, ms
Resilient N = 16	-61.9 ± 1.5	454.5 ± 51.2	-46.0 ± 0.9	79.2 ± 2.6	0.91 ± .08	31.3 ± 3.6
PTSD-like N = 14	-64.5 ± 1.7	490.0 ± 42.0	-45.5 ± 1.1	80.5 ± 3.1	0.84 ± .06	29.4 ± 2.2

Values are means ± SE. LTB, low-threshold bursting neurons in CeM; Rest, resting potential; R_{in}, input resistance.

Table 4C. Physiological Properties of RS neurons

			Action Potential			
	Rest, mV	R_{in}, MΩ	Threshold, mV	Amplitude, mV	Duration, ms	Time Constant, ms
Resilient N = 9	-66.8 ± 2.4	575.1 ± 108	-37.4 ± 3.0	80.7 ± 3.9	0.76 ± .05	32.6 ± 4.4
PTSD-like N = 27	-60.6 ± 1.3	504.6 ± 39.6	-43.7 ± 1.1	84.7 ± 2.2	0.73 ± .02	35.2 ± 3.4

Values are means ± SE. RS, Regular spiking neurons in CeM; Rest, resting potential; R_{in}, input resistance.

6.4.2 Synaptic responsiveness of amygdala neurons in resilient vs. PTSD-like rats

BL neurons

Prior tracing studies have revealed that the BL nucleus receives strong excitatory inputs from a variety of cortical fields (McDonald et al., 1999) and from LA (Pare et al., 1995). To test whether the responsiveness of BL neurons to these inputs differed between the two phenotypes, we positioned stimulating

electrodes in the EC, which carries most cortical axons ending in BL, and in the ventral part of LA. We then compared the responses elicited by electrical stimuli (100 µs; 0.1-0.8 mA) delivered at these two sites in BL neurons (resilient, n = 34; PTSD-like, n = 24). As shown in figure 10, whether the stimuli were delivered in the EC or LA, no difference were seen between the two phenotypes in the proportion of stimuli eliciting spikes ($F = 0.002$, $p = 0.965$; Fig. 10A1), in the amplitude of evoked excitatory (EPSPs) or inhibitory (IPSPs) postsynaptic potentials (EPSPs, $F = 1.515$, $P = 0.236$; IPSPs, $F = 1.012$, $P = 0.329$; Fig. 10A2) or in the slope of EPSPs ($F = 0.260$, $P = 0.615$; Fig. 10A3).

CeL neurons

BL neurons constitute a major source of glutamatergic inputs to CeL and CeM (Krettek and Price, 1978; Pare et al., 1995b; Pitkanen et al., 1997; Royer et al., 1999). Therefore, we positioned stimulating electrodes at this site and compared the properties of BL-evoked synaptic responses in the two phenotypes. In contrast with BL neurons, CeL cells (resilient, n = 28; PTSD-like, n = 34) displayed marked differences in synaptic responsiveness as a function of behavioral phenotype (Fig. 10A2). First, the proportion of BL stimuli eliciting spikes was significantly higher in PTSD-like than resilient rats ($F = 8.693$, $p = 0.005$; Fig. 10B1) and this effect was seen in both RS and LF neurons. Consistent with this, EPSP (but not IPSP) amplitudes (Fig. 10B2; $F = 6.248$, $p = 0.018$) and slopes (Fig. 10B3; $F = 5.284$, $P = 0.026$) where higher in CeL neurons

from PTSD-like than resilient rats, particularly in an intermediate range of stimulation intensities (0.2-0.5 mA).

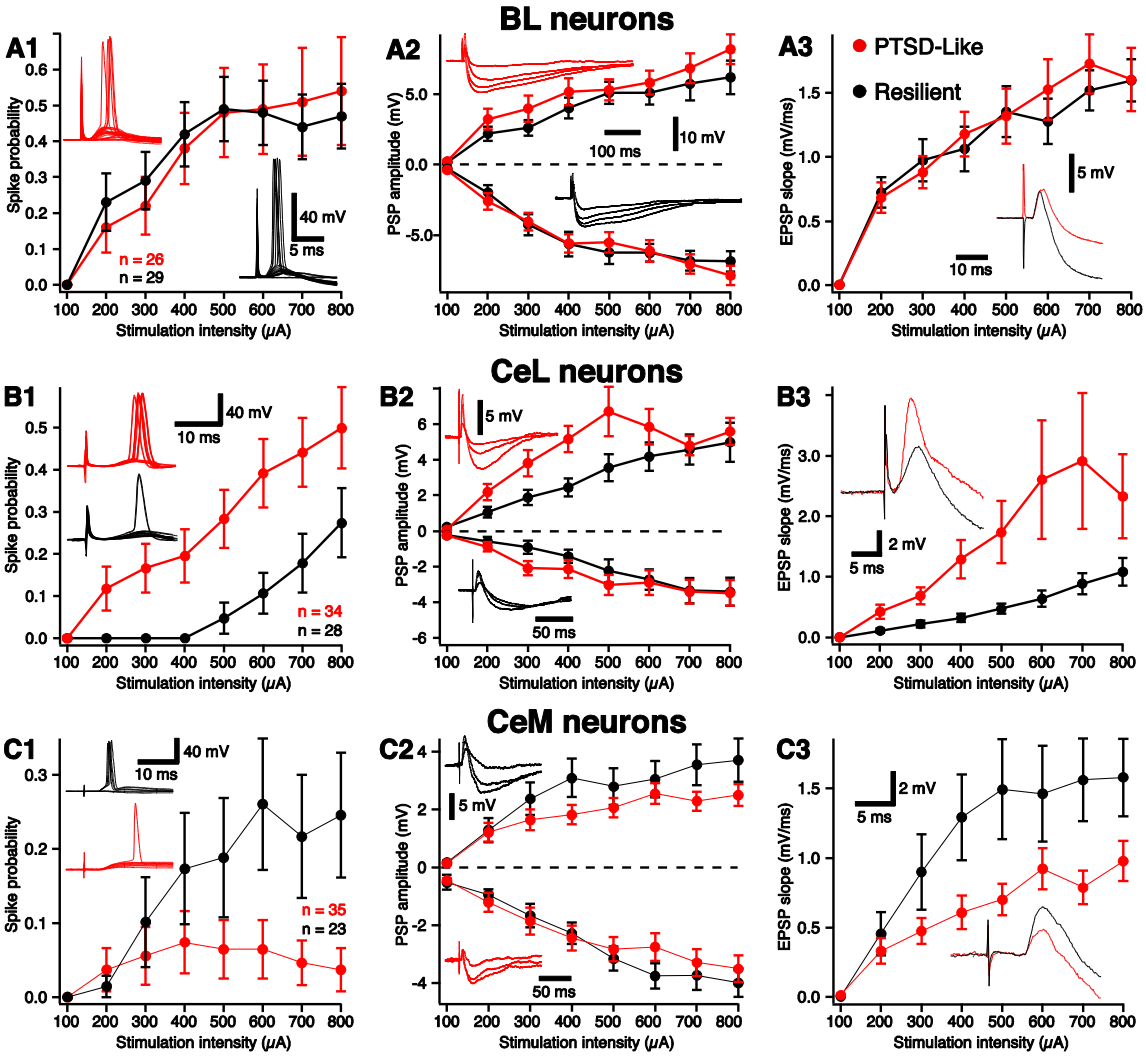

Figure 10. Synaptic responsiveness of BL (A), CeL (B), and CeM (C) neurons in resilient (black) and PTSD-like (red) rats. In all panels, the x-axis represents stimulation intensity whereas the y-axis shows (1) proportion of trials eliciting orthodromic spikes, (2) the amplitude of evoked EPSP and IPSP (positive and negative values, respectively), as well as (3) EPSP slopes (measured in the first 2 ms). Stimulation sites were LA (A1), EC (A2-3), BL (B,C). Insets show representative examples of evoked responses for neurons recorded in resilient (black) and PTSD-like rats (red).

To determine whether the increased synaptic responsiveness of CeL neurons in PTSD-like rats was due to pre- or post-synaptic factors, we compared properties of paired-pulse facilitation (PPF) in the two groups (Fig. 11A). In this

analysis (Katz and Miledi, 1968), two identical stimuli are applied in rapid succession. When the interval between the two stimuli is sufficiently brief, the second one typically elicits a larger response (reviewed in Zucker and Regher, 2002). The magnitude of PPF is believed to be inversely proportional to the probability of transmitter release because manipulations that increase release probability decrease PPF and conversely (Creager et al., 1980; Manabe et al., 1993). Thus, in voltage-clamp mode and in the presence of picrotoxin (100 µM), we applied two BL stimuli separated by 50 ms and computed the ratio of the EPSC amplitude elicited by the two stimuli (EPSC1/EPSC2) in CeL neurons from PTSD-like (n = 5) and resilient (n = 6) rats. However, the paired pulse ratio did not differ significantly in the two groups (Fig. 11A; t-test, resilient = 1.524; PTSD-like = 1.552; $p > 0.769$).

Therefore, to test whether a difference in the postsynaptic sensitivity of CeL neurons to glutamate mediates the group differences in BL-evoked responses, we used photic uncaging of glutamate. In this approach, slices are bathed in an aCSF solution containing 4-Methoxy-7-nitroindolinyl-caged-L-glutamate (1.0 mM). Ultraviolet light stimuli (diameter, ≈150 µm; 5-30 ms), centered over the soma of the recorded cell, are applied at a low frequency (0.1 Hz) to uncage glutamate.

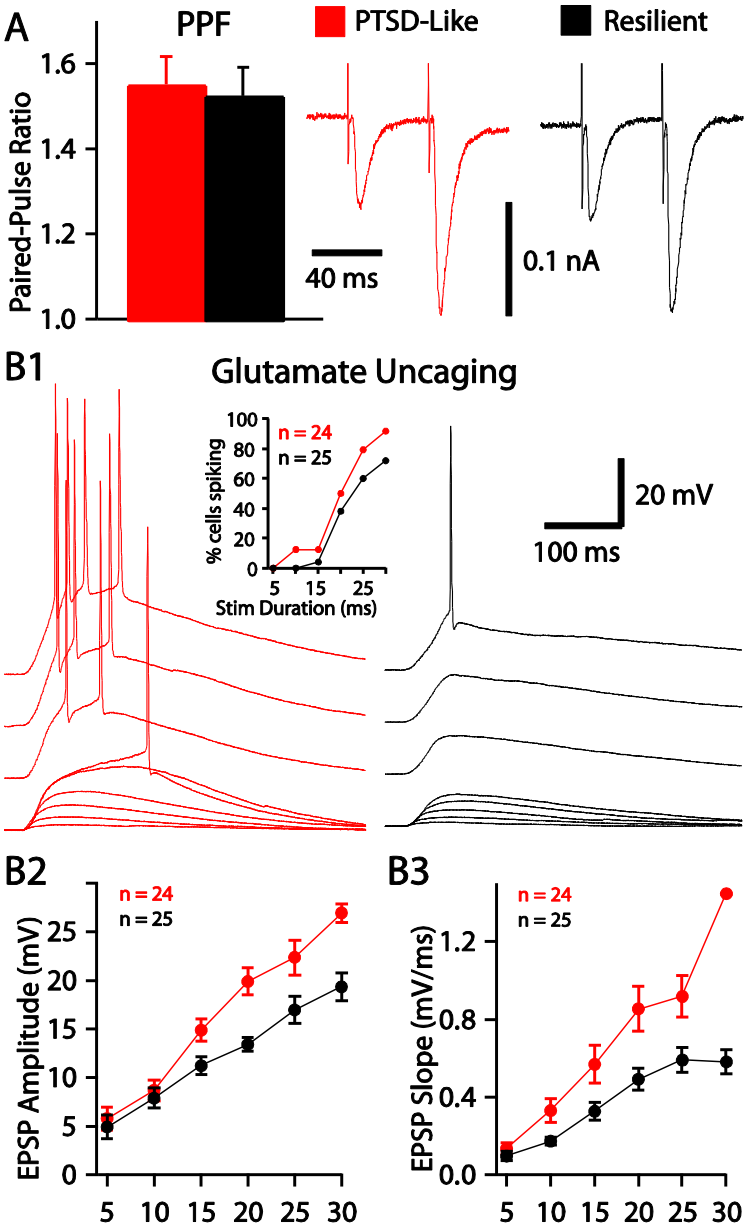

Figure 11 Mechanisms underlying increased responsiveness of CeL neurons to BL inputs in PTSD-like (red) relative to resilient (black) rats. (A) Properties of paired-pulse facilitation at BL inputs to CeL neurons did not vary as a function of the rats' phenotype. Left: ratio of second to first EPSC amplitudes. Right: representative examples of responses evoked by two BL stimuli separated by 50 ms in CeL neurons. (B) Responses of CeL neurons to uncaged glutamate vary as a function of the rats' phenotype. (B1) Representative examples of responses elicited by UV light pulses of gradually increasing duration (5 to 45 ms). Inset: proportion of cells spiking (y-axis) as a function of UV stimulus duration (x-axis). Beyond 30 ms, all cells fired. (B2) Peak amplitude of EPSPs elicited by glutamate uncaging (y-axis) as a function of UV stimulus duration (x-axis). In this and the next panel, all supra-threshold responses were excluded, resulting in progressively diminishing n's with UV stimuli of increasing durations (see inset of B1). (B3) Slope of EPSPs elicited by glutamate uncaging (y-axis) as a function of UV stimulus duration (x-axis).

Figure 11B1 illustrates representative examples of responses to UV stimuli of progressively increasing duration (bottom to top) in CeL neurons from PSTD-like (red, left) and resilient (right, black) rats. As in these representative examples, the amplitude and slope of EPSPs elicited by uncaged glutamate was significantly higher in samples of CeL cells recorded from PTSD-like compared to resilient rats (PTSD-like, n=24; resilient, n=25; amplitude, F = 16.076, P = 0.005; slope, F = 17.206, P = 0.004). Note that these differences were detected despite the fact that the analyses of the data represented in Figure 11B1 and Figure 11B2 excluded trials where cells fired in response to uncaged glutamate. At all stimulation intensities, a higher proportion of supra-threshold trials were seen in the PTSD-like rats (Fig. 11B1, inset). Thus, excluding trials with supra-threshold responses likely minimized group differences.

CeM neurons

Opposite to the results obtained in CeL, CeM neurons from resilient rats had a higher synaptic excitability. First, the proportion of BL stimuli eliciting spikes (Fig. 10C1) was significantly higher in CeM cells from resilient than PTSD-like rats (intensities 0.4 – 0.8 mA, F = 4.571, P = 0.037). Similarly, EPSP slopes (Fig. 10C2) were significantly higher in the resilient group (F = 6.373, P = 0.015). EPSP amplitudes (Fig. 10C3) displayed a parallel trend but group differences did not reach significance (F = 2.047, P = 0.159). We are currently analyzing whether the opposite group differences in synaptic excitability seen between

CeM and CeL neurons are due to the fact that they are targeted by distinct types of BL axons.

6.5 Discussion

Several lines of evidence suggest that PTSD is associated with aberrant amygdala activity. However, due to the limited spatial and temporal resolution of fMRI, and the ambiguous significance of changes in BOLD, the location and nature of the alterations in amygdala activity remained unclear. The present study was undertaken to shed light on these questions using a rat model of PTSD. We performed patch recordings of amygdala neurons in susceptible and resilient rats. By comparing neuronal properties in the two behavioral phenotypes, we tested whether the intrinsic excitability and/or synaptic responsiveness of neurons in different parts of the amygdala is altered in the PTSD-like state. Our results suggest that the PTSD-like state is associated with an increased synaptic responsiveness of CeL neurons and the opposite in CeM cells. No difference in the excitability of BL neurons was detected between the two phenotypes.

In contrast with BL neurons where no differences in intrinsic or synaptic excitability could be detected as a function of the rats' phenotypes, robust differences were observed in CeA. However, they had an opposite polarity in CeL and CeM. In CeL, the amplitude and slope of BL-evoked EPSPs was higher in PTSD-like rats with no difference in IPSP amplitudes. In CeM, BL-evoked EPSPs were higher in resilient rats. For CeL neurons, analyses of PPF (no

group differences) and of the responses elicited by glutamate uncaging (higher responses in PTSD-like rats) support the view that the group differences in synaptic responsiveness are dependent on postsynaptic factors, possibly a change in the number and/or biophysical properties of 2-amino-3-(3-hydroxy-5-methyl-isoxazol-4-yl) propanoic acid (AMPA) receptors. At present, the mechanisms underlying the group differences in the responsiveness of CeM neurons to BL inputs are unknown. Ongoing experiments in our laboratory are pursuing this question.

CHAPTER 7

GENERAL DISCUSSION

7.1 Validity of the Lewis rat model of PTSD

Ethical limitations inherent to human studies hinder progress in understanding the pathophysiology of PTSD. One approach to circumvent this problem is to study this disorder in a valid animal model of the human syndrome, allowing the use of more invasive techniques than possible in humans. One such approach focuses on the impact of species-relevant threatening stimuli that mimic the type of life-or-death experiences known to precipitate PTSD in humans. For instance, rodents exposed to predators or their odor develop enduring (≥ 3 weeks) signs of anxiety as determined with several behavioral assays such as the EPM, acoustic startle, and social interaction test (Adamec and Shallow, 1993; Blanchard et al., 2003; Adamec et al., 2006). As in human PTSD, differential susceptibility to PT was seen in rodents. For instance, Cohen et al. (2006b) reported that following PT, the incidence of extreme behavioral manifestations of anxiety varied markedly in different rat strains: 50% in Lewis rats compared to 20% of Sprague-Dawley and 10% of Fisher rats. The equal prevalence of susceptible and resilient subjects among Lewis rats led us to focus on this strain. We reasoned that because random groups of Lewis rats include a roughly equal proportion of susceptible and resilient subjects, fewer rats would have to be studied to compare the two groups on any dimension. This would be particularly advantageous for labor-intensive studies such as those relying on single-unit recordings in behaving animals. Accordingly, the objectives of this thesis were two-fold: 1) assess the validity of the Lewis rat model of PTSD, focusing on whether it reproduces salient features of human PTSD; and 2) use this model of

identify some of the physiological correlates of PTSD expression.

We began assessing the validity of the Lewis rat model of PTSD by testing whether this model replicates the compromised ability of human PTSD subjects to extinguish conditioned fear responses, a trait thought to play a critical role in the persistence of PTSD (reviewed in Quirk and Mueller, 2008). Indeed, humans with PTSD exhibit an extinction deficit (Orr et al., 2000; Peri et al., 2000; Blechert et al., 2007; Milad et al., 2008, 2009). Importantly, a study of identical twins discordant for trauma exposure revealed that this deficit develops as a result of trauma and does not predate it (Milad et al., 2008). Consistent with this, we observed that PTSD-like Lewis rats showed a clear extinction deficit if PT occurred before, but not after, fear conditioning (Goswami et al., 2010). In contrast, the relative timing of PT and fear conditioning did not change the properties of extinction in resilient rats (Goswami et al., 2010). This pattern of results suggested that the extinction deficit manifested by PTSD-like rats is not an antecedent condition but is acquired as a result of PT, paralleling human findings.

A vast literature indicates that individuals with PTSD are impaired on hippocampal-dependent tasks (for instance, see Shin et al., 2004; Lindauer et al., 2006; Gilbertson et al., 2007; Thomaes et al., 2009; Hayes et al., 2011; reviewed in Samuelson, 2011). Moreover, studies of monozygotic twins discordant for combat exposure have revealed that hippocampal abnormalities *predate* onset of the disorder (Gilbertson et al., 2002, 2007), in contrast with the fear extinction deficit (Milad et al., 2008). Therefore, PTSD susceptibility was assessed by

testing whether the Lewis rat model of PTSD reproduces the hippocampal abnormalities seen in humans. Separate groups of Lewis rats were subjected to one of 3 novel object recognition paradigms that vary in the degree of hippocampal dependence (NOR, EOR, AOR; Langston and Wood, 2010) prior to PT. We observed that PTSD-like rats showed a selective impairment in their ability to recognize novel object configurations in the hippocampal-dependent AOR task, paralleling human findings. Notably, this impairment was detected in the absence of baseline differences in locomotor and/or exploratory behaviors measured during the OF test, and the sample phase of the object recognition tasks.

Further support for the hippocampal-dependent nature of the AOR task can be found in the literature that explores the effects of stress on striatal-and hippocampal-dependent learning strategies (e.g., Packard and Wingard, 2004; Schwabe et al., 2007; Schwabe and Wolf, 2009). There is converging evidence that, under conditions of stress, animals and humans show preference for striatal-dependent (or response learning), as opposed to hippocampal-dependent (or place learning). The mechanisms for this switch remain unclear but it has been suggested that it could be related to the release of stress hormones (Schwabe et al., 2010). While task completion in the present study preceded stress, these data support the idea that rats predisposed toward PTSD may already evidence a hippocampal deficit, which creates a preference toward striatal-dependent learning. Future studies should relate these behavioral findings to hippocampal volumes or other deficits to validate these findings.

Admittedly, additional tests will be required to ascertain whether the model we used faithfully reproduces the human syndrome. However, given the limited options currently available, we submit that the evidence reviewed above amply justifies the use of the PT models to gain mechanistic insights in the pathophysiology of PTSD.

7.2 Limitations of the ex vivo approach

While studying the physiology of amygdala neurons in brain slices has great analytical power, this approach has significant limitations. Indeed, many connections, particularly those involving distant structures, are lost in brain slices. This results in abnormally hyperpolarized membrane potentials and drastically reduced spontaneous activity (Pare et al., 1998). Consequently, network phenomena that likely play an important role in PTSD cannot be studied with this approach.

This limitation may account for some of our negative findings. As mentioned above, prior functional imaging studies in humans revealed that the amygdala is hyperactive in PTSD. Given the limited spatial resolution of fMRI and since the basolateral amygdala occupies a much larger volume than other amygdala nuclei in humans, it is natural to assume that an increase in the activity/excitability at this site accounts for the fMRI results. However, all the properties we examined in BL neurons were identical in the two phenotypes, including incidence of physiological cell types, their passive properties, and synaptic responsiveness. While this negative data suggests that BL activity does

not differ between the two phenotypes, it is also possible that due to dissimilarities in the activity of afferent neurons, BL cells are more active in PTSD. However, such effects cannot be detected in brain slices because the inputs to BL are cut. Furthermore, the BLA is known to facilitate the formation of long-term memories that are stored elsewhere, such as cortex (Chavez et al. 2013), medial temporal lobe (Pitkanen, 2000), and striatum (Kita and Kitai, 1990; Paz and Pare, 2013). An additional challenge is the temporal specificity required for immediate early gene detection used as neuronal activity markers. Therefore, we cannot rule out the possibility that BL activity transiently increases during and/or shortly after predator stress to facilitate plasticity elsewhere in the brain, while returning to baseline levels at the time of our physiological experiments (> 1 week after PT). Unit recordings in behaving animals will be required to settle this question.

7.3 Opposite alterations in the synaptic responsiveness of CeL and CeM neurons in PTSD-like vs. resilient rats

Irrespective of the underlying mechanisms, the differences in synaptic responsiveness seen in CeL and CeM as a function of the rats' phenotypes likely have significant implications for the pathophysiology of PTSD. Indeed, CeL contributes GABAergic projections to CeM and the anterolateral part of the BNST. These two structures are part of an anatomical entity termed the extended amygdala (Alheid and Heimer, 1988; de Olmos and Heimer, 1999), a concept based on similarities in the morphology and neurotransmitter content of BNST

and CeA neurons (McDonald, 2003), shared inputs from the basolateral amygdala (Krettek and Prince, 1978ab; Pare et al., 1995; Savender et al., 1995; Dong et al., 2001a), as well as common projections to brainstem nuclei that generate various aspects of fear/anxiety responses (Hopkins and Holstege, 1978; Sofroniew, 1983; Veening et al., 1984; Holstege et al., 1985; Dong et al., 2000, 2001b; Dong and Swanson, 2004, 2006a-c).

Despite these similarities however, BNST and CeA play different roles. Indeed, lesion (Hitchcock and Davis 1987, 1991; LeDoux et al., 1988; Campeau and Davis, 1995; Jimenez and Maren, 2009), local drug infusion (Kim et al. 1993; Wilensky et al. 2006), optogenetic (Ciocchi et al., 2010) and unit recording studies (Duvarci et al., 2011) suggest that CeA is required for the rapid expression of conditioned fear responses to discrete sensory cues, functions that are unaffected by BNST lesions (Walker and Davis 1997; Gewirtz et al. 1998; Sullivan et al. 2004). Rather, lesions of BNST interfere with the genesis of longer "anxiety-like" states in response to more diffuse environmental contingencies (reviewed in Walker et al. 2003; Sullivan et al. 2004; Duvarci et al., 2009). For instance, BNST lesions disrupt CORT release and freezing responses to contextual stimuli previously paired with aversive outcomes (Sullivan et al., 2004).

However, other lines of evidence suggest that BNST exerts a dual influence over fear expression. For instance, a recent unit recording study from our lab revealed that neurons in the anterolateral sector of BNST (BNST-AL) acquire inhibitory responses to conditioned stimuli (CS) predicting adverse

outcomes whereas cells in its anteromedial sector (BNST-AM) exhibit the opposite behavior (Hauffler et al., 2012, SfN). Importantly, BNST-AL and AM neurons displayed the same opposite behavior in relation to the expression of contextual fear with the exception that BNST-AM cells were recruited more strongly during contextual fear than in responses to discrete CSs (Hauffler et al., 2013, SfN). Overall, these findings suggest that BNST-AM and AL exert opposite influences over the expression of fear/anxiety, with the former exerting anxiogenic and the latter anxiolytic influences.

Consistent with this, it was recently reported that infusions of calcitonin gene-related peptide (CGRP) in BNST increase fear-potentiated startle and Fos expression in CeA (Sink et al., 2011). Yet, patch-clamp studies from our lab revealed that CGRP enhances GABAergic inhibition in neurons of BNST-AL (Gungor and Pare, 2012, SfN), the BNST region receiving CGRP inputs from the parabrachial nucleus (Gustafson and Greengard, 1990; Dobolyi et al., 2005). Since most BNST neurons are GABAergic (Esclapez et al. 1993; Poulin et al. 2009), these results suggest the startle enhancement produced by intra-BNST infusions of CGRP is due to the inhibition of BNST-AL neurons, further reinforcing the notion that BNST-AL exerts anxiolytic influences.

When interpreted in this context, the opposite changes in CeL and CeM responsiveness seen in PTSD-like vs. resilient rats suggest that the following network alterations are involved in maintaining the PTSD-like state. The increase responsiveness of CeL neurons in PTSD-like rats would alter the balance between CeM and BNST control of fear/anxiety expression, in favor of

the latter. Since BNST-AL neurons contribute strong GABAergic projections to BNST-AM (Turesson et al., 2013), the increased inhibition of BNST-AL by CeL in PTSD-like rats would cause a disinhibition of BNST-AM, leading to increased expression of anxiety. An ongoing ex vivo study comparing the synaptic responsiveness of BNST neurons in PTSD-like and resilient rats supports the above model (Rodriguez-Sierra et al., 2011, SfN). Indeed, these studies reveal that extrinsic GABAergic inputs to BNST-AL are potentiated in PTSD-like relative to resilient rats and that BNST-AM neurons show the opposite trend.

7.4 Role of peptides in the expression of a PTSD-like state

As our understanding of the contribution of various peptides to the expression of anxiety improves, opportunities for novel therapeutic interventions can be unveiled. Recent studies in humans have shown increased levels of pituitary adenylate cyclase activating peptide (PACAP) in individuals with PTSD (Ressler et al. 2011). This peptide binds to one of 3 receptors. Two of these receptors bind PACAP, as well as vasointenstinal peptide (VIP). However, the PAC1 receptor selectively binds PACAP, and is expressed heavily in the amygdala and BNST (Ressler et al. 2011). In addition, PAC1R mRNA levels were shown to increase in mice subjected to Pavlovian fear conditioning, and strong positive correlations were found between freezing levels and levels of PAC1R mRNA expression within the amygdala (Ressler et al. 2011). Detailed investigations suggest that CeL contains the most PACAP-positive presynaptic terminals than other amygdala nuclei (Cho et al. 2012). Furthermore, exogenous

application of PACAP increases the synaptic excitability of CeL neurons via postsynaptic mechanisms (Cho et al. 2012), closely paralleling our findings. Therefore, these results combined with our recordings of CeL neurons in PTSD-like rats vs. resilient rats suggest that the increased excitability of CeL neurons in PTSD-like rats may involve altered PACAP signaling after trauma. However, it should be noted that it is currently unclear whether differences in PACAP levels in humans with PTSD are acquired after trauma, or predate onset of the syndrome.

A critical challenge for future studies will be to determine whether these inferences are correct by comparing the activity of BNST, BL, and CeA neurons in PTSD-like vs. resilient rats during the presentation of unconditioned as well as conditioned fear inducing stimuli. It will also be important to compare activity profiles in this network before and during PT. Such comparisons might reveal pre-existing differences between the two rat groups, thereby shedding light on the factors that predispose some subjects to trauma susceptibility.

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VITA

Sonal Goswami

1983	Born April 5th, in Flin Flon, Manitoba, Canada.
2001	Graduated from Plano East Senior High, Plano, TX.
2001 – 2005	Attended the University of Texas at Dallas, Richardson, TX.
2005	B.A. Psychology, University of Texas at Dallas, Richardson, TX.
2005	B.S. Neuroscience, University of Texas at Dallas, Richardson, TX. School of Behavioral and Brain Sciences Honors.
2003 – 2005	Student assistant at University of Texas at Dallas, Richardson, TX.
2007 – 2013	Graduate work at Rutgers University, Newark, NJ.
2008, 2011, 2012	Teaching assistant at Rutgers University, Newark, NJ.
2010	Article: Impact of predatory threat on fear extinction in Lewis rats. Learn Mem. vol. 17, p. 494-501.
2012	Article: A rat model of post-traumatic stress disorder reproduces the hippocampal deficits seen in the human syndrome. Front Behav Neurosci. 6:26
2012	Article: Morphology, PKCd expression, and synaptic responsiveness of different types of rat central lateral amygdala neurons. J Neurophysiol. vol. 108, p. 3196-205.
2013	Article: Animal models of post-traumatic stress disorder: face validity. Front Neuroscience. In press.
2013	PhD in Behavioral and Neural Sciences, Rutgers University, Newark, NJ.