INDIVIDUAL DIFFERENCES IN VULNERABILITY TO MALADAPTIVE FEAR IN RATS AND HUMANS: BEHAVIORAL AND NEURAL CORRELATES

by

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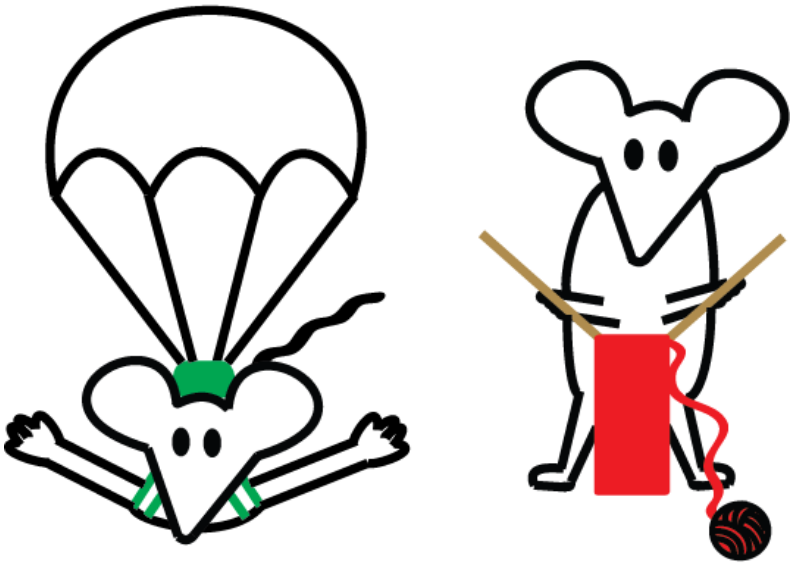
Differences in Temperament



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DEDICATION

This dissertation is dedicated to my family, for their love and support of me throughout my life and my years in graduate school. For my grandfathers, who shared their passion for the natural world and the sciences with me, and because of whom I continue to look to the stars and the sea for inspiration. For my grandmothers, who were and are some of the smartest and kindest women I know. For my parents, whose life-long caring and support has been instrumental in helping me achieve my dreams. You shared with me your love of science and science fiction, and I am forever grateful for all of the opportunities you've given me and for all of the wonderful times we've shared. For my little sister who isn't so little anymore, your knowledge of and enthusiasm for your own field of work is inspiring and I'm so glad we've grown closer over the years. For my wonderful husband, Sandor, who believed in me even when I didn't, and who continues to make me laugh. All of your constant support and encouragement has enabled me to complete so much more than I thought was possible. Thank you.

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ABSTRACT

Certain individuals are vulnerable to developing post-traumatic stress disorder (PTSD), but mechanisms underlying this selective vulnerability are unknown. We hypothesize that genetic predisposition, together with environmental modulation, influences this selective vulnerability. One potentially useful model are rat lines selectively bred for high (bHR) or low (bLR) locomotor response to novelty, a model of differential vulnerability to affective pathology. Using a standard fear conditioning and extinction paradigm, we determined that bLRs demonstrated decreased extinction learning and retention, while bHRs displayed faster extinction and greater retention. bLRs, like PTSD patients, may be genetically vulnerable to maladaptive fear behaviors while bHRs remain resilient. We next asked whether this genetic predisposition was modifiable by administering fibroblast growth factor 2 (FGF2), a treatment previously shown to facilitate extinction learning. Perinatal FGF2 administration facilitated extinction retention in bHRs, but not bLRs, demonstrating that individual variation is an important determinant of FGF2's ability to modulate fear behavior. Additionally, we examined how social environment might alter vulnerability in the genetic model. The company of in-group phenotype animals facilitated extinction for bHR and bLR rats. Outbred rats characterized as HRs and LRs responded similarly. To understand how this gene-by-environment interaction might influence neural circuitry, we examined brain region coactivation using cFos after extinction learning. Both bHR and bLR animals displayed different patterns of coactivation from outbred rats. We observed potential

compensatory coactivations in areas of the prefrontal cortex in bLRs when they displayed extinction behavior. To extend our findings to humans, we performed a similar analysis using functional magnetic resonance imaging during extinction learning. We observed increased coactivation between the anterior cingulate cortex and the amygdala in individuals with high trait anxiety. Together, these coactivation studies implicate prefrontal cortex – amygdala circuit dysfunction in individuals with high anxiety across species. We conclude that individual differences in emotional responsiveness significantly impact fear extinction learning. Moreover, these differences interact with the efficacy of pharmacological agents and environmental factors to reduce vulnerability to maladaptive fear responses. Importantly, these differences in fear extinction are reflected at the neural level, and the altered circuit function in vulnerable individuals is present in both our rodent model and human subjects.

Chapter 1.

Introduction

Maladaptive Fear and the Development of Post-Traumatic Stress Disorder

Fear is an emotion that almost all living organisms exhibit (Panksepp, 2011). Avoidance of individuals or situations that can cause harm increases the odds that an individual will survive, making fear an adaptive response to environmental stimuli (Bolles, 1970; Fanselow & Lester, 1988; Maren, 2011; Ohman & Mineka, 2001). Since fear is evolutionarily important, fear responses such as increased sympathetic tone (e.g. high heart rate, breathing, sweating) and release of glucocorticoids (e.g. corticosterone in rodents and cortisol in humans) are well-conserved across species (Kozlowska, Walker, McLean, & Carrive, 2015; Ohman & Mineka, 2001). In most potentially threatening situations, responses are reflexive and behaviors are innate and often species-specific, allowing individuals to quickly respond to potentially threatening stimuli (Bolles, 1970; Mineka & Ohman, 2002; Ohman & Mineka, 2001).

While defensive and fear responses often have short durations (present while the threatening stimulus is near) memories of events or stimuli that triggered fear responses can be long-lasting (Maren, 2001, 2011; Ohman & Mineka, 2001). Fear memories are made up of a number of different components including the unconditioned stimulus (US), conditioned stimulus (CS) and context elements (Maren, Phan, & Liberzon, 2013). The US is a stimulus innately feared by the individual such as pain, height, a predator, or

other indicator of imminent injury or death. The CS is a cue that occurred in close proximity to an interaction with the US but does not ordinarily elicit fear. Contextual information is information about the surroundings of an individual, or the environment, in which a fear-inducing event is experienced. The elements of the fear memory are usually tightly linked, with the presence of the CS eliciting defensive behaviors even in the absence of the US or in a different context, allowing the transfer of defensive responses to unknown but potentially threatening situations (LeDoux, 2000; Maren, 2001).

Due to the longevity and transferable nature of fear memories, anxiety disorders can result when aspects of a fear memory become altered in a way that the memory loses contextual information and/or becomes generalized to other similar stimuli (Liberzon & Sripada, 2008; Maren et al., 2013). Fear is differentiated from anxiety by the length of the emotion; fear is usually short-lived, while anxiety is a persistent state of heightened arousal and attention to potentially threatening stimuli (Grupe & Nitschke, 2013). Maladaptive versions of fear memories that elicit defensive responses to non-threatening stimuli or in non-stressful circumstances may lead to anxiety and stress disorders such as phobias, panic disorder, and post-traumatic stress disorder (PTSD; Bouton, Mineka, & Barlow, 2001; Grupe & Nitschke, 2013; Mineka & Oehlberg, 2008; Mineka & Ohman, 2002; Ohman & Mineka, 2001).

PTSD in particular is considered a disorder of fear learning and regulation (Dejean et al., 2015; Desmedt, Marighetto, & Piazza, 2015; Likhtik & Paz, 2015; Milad, Rosenbaum, & Simon, 2014; Mineka & Oehlberg, 2008). PTSD is a debilitating disorder that stems from the experience of a traumatic event that elicits extreme fear in an individual (Friedman, Resick, Bryant, Strain, et al., 2011). The majority of individuals

will experience many of the symptoms used to diagnose PTSD after a traumatic event, but symptoms must continue for over a month after trauma to confer a diagnosis of PTSD. Individuals who suffer from PTSD often experience physiological fear responses to cues in their environment that (sometimes only vaguely) resemble stimuli that might have been present during their traumatic event (see Table 1.1; Friedman, Resick, Bryant, & Brewin, 2011).

Table 1.1 The symptoms of PTSD according to DSM-5, modified from (Friedman, Resick, Bryant, & Brewin, 2011).

Diagnostic Category	Symptoms
Re-experiencing	• Flashbacks • Nightmares • Large physiological fear responses to reminders of the trauma
Avoidance	• Staying away from place, person, or object reminders of trauma • Avoiding thoughts or conversations regarding the trauma
Negative cognitions and mood	• Difficulty remembering details of the traumatic event • Persistent negative bias about the world and others • Decreased interest in significant activities • Persistent inability to feel positive emotions
Alterations in arousal	• Irritable, angry, or aggressive behaviors • Reckless or self-destructive behavior • Exaggerated startle response • Hypervigilance • Insomnia

PTSD patients also re-live their trauma, without the ability to realize that it is a past memory, indicating that their fear memory may have lost the contextual element of autobiographical timing. The combination of flashbacks and increased physiological fear responses often leads to avoidance of situations or cues that might remind an individual of their trauma (Friedman, Resick, Bryant, & Brewin, 2011). This heightened state of anxiety, as well as negative bias in emotional and cognitive responses and increased aggression and arousal are characteristic of PTSD patients (Friedman, Resick, Bryant, & Brewin, 2011). This potentially debilitating disorder is costly, with an estimated 3.3 billion dollars spent on treatment of PTSD symptoms in a given year (IoM, 2014). The cost to both individual lives and in productivity lost makes it imperative that we understand the factors that lead to maladaptive fear memories and responses in disorders such as PTSD with the ultimate goal of providing better treatment options or preventing PTSD altogether.

Individual Differences Play a Major Role in the Etiology of PTSD

While PTSD affects many individuals, not all who experience a traumatic event will develop this disorder. Surveys indicate that over 50% of the US population has experienced a traumatic event (such as rape, physical attack or combat experience), with some estimates as high as 92.2% of men and 87.1% of women having experienced at least one traumatic event in their lifetime (Breslau & Kessler, 2001; Kessler, Sonnega, Bromet, Hughes, & Nelson, 1995). Despite the high rates of trauma, the prevalence of PTSD in the US population remains at approximately 6.8% (Kessler et al., 2005). In certain populations, such as veterans of the Iraq and Afghanistan wars (Operation Iraqi

Freedom and Operation Enduring Freedom), the prevalence of PTSD is estimated to be approximately 16%, one of the higher rates of PTSD prevalence in a given population (Dursa, Reinhard, Barth, & Schneiderman, 2014). The disparity in numbers between the extremely high rates of trauma exposure and the respectively low rates of PTSD diagnosis indicate that there is large individual variation in the etiology of PTSD. Despite this, we have limited insight into why certain individuals develop PTSD while others remain unaffected.

A focus on individual differences in response to trauma could not only lead to discoveries about what vulnerability factors might better predict PTSD in humans, but resilient individuals that do not go on to develop maladaptive responses may provide additional treatments and therapy options to assist vulnerable individuals. As a construct, we believe that resilience is not necessarily the absence of vulnerability. Instead, resilience may involve counter-regulatory mechanisms that could be exploited to decrease vulnerability in individuals. Thus, it is critical that in both human studies and animal models the field develop ways to assess individual differences in response to trauma that lead to vulnerability or resilience to PTSD.

Aberrant Brain Activity in PTSD Patients

There are identifiable patterns of brain activity that differentiate PTSD patients from trauma-exposed or non-combat controls, which may allow identification of the biological underpinnings of the development of maladaptive fear memory in specific individuals. Certain aspects of PTSD-related brain activity, such as hyperactivity of the amygdala to aversive stimuli, are similar to other anxiety disorders such as panic

disorder, social phobia and generalized anxiety disorder (Etkin & Wager, 2007; Killgore et al., 2014; Phan, Fitzgerald, Nathan, & Tancer, 2006). However, a recent study has indicated that this hyperactivity of the amygdala may be more specific to trauma or fear-related imagery than previously thought (van Rooij, Rademaker, et al., 2015). Hyperactivity of the amygdala is hypothesized to be due to lowered inhibition from the prefrontal cortex (Dejean et al., 2015). Indeed, PTSD patients often exhibit reduced prefrontal cortical activity in both ventromedial prefrontal cortex (vmPFC), and anterior cingulate cortex (ACC) on a variety of tasks (Dejean et al., 2015; Milad et al., 2009; Shin et al., 2005; Werner et al., 2009). PTSD patients also have reduced amygdala to vmPFC connectivity during emotion processing, further suggesting dysfunction in the limbic system (Stevens et al., 2013). Connectivity analyses also show region-specific amygdala-PFC dysfunction across genders in PTSD, with connectivity between the amygdala and ACC being reduced in male PTSD patients, and connectivity between the amygdala and vmPFC being reduced in female PTSD patients (Koch et al., 2016). An additional study demonstrated reduced connectivity within the default mode, sensory, and salience networks in PTSD patients compared to controls (Zhang et al., 2015). However, some of the changes observed in these studies may not be specific to PTSD, but instead result from trauma exposure (DiGangi et al., 2016; Reuveni et al., 2016).

The hippocampus also often shows hyperactivity in studies of PTSD patients (Dejean et al., 2015; Werner et al., 2009). However, at least one study has found hypoactivation of the hippocampus, indicating that hippocampal activity may be task-dependent, or inconsistent, in studies of PTSD (Astur et al., 2006). PTSD patients have higher levels of white matter in their hippocampus (Chao, Tosun, Woodward, Kaufer, &

Neylan, 2015), and additional studies have consistently identified reduced hippocampal volume in PTSD patients (Bremner et al., 1995; Bremner et al., 1997; Kitayama, Vaccarino, Kutner, Weiss, & Bremner, 2005; van Rooij, Kennis, et al., 2015). These findings indicate that structural changes may underlie differences in hippocampal activity in PTSD patients. However, one of the drawbacks of these studies of amygdala, PFC and hippocampal dysfunction is that they were all completed on patients who currently carried a diagnosis of PTSD, and therefore, it is impossible to disentangle differences that were present prior to the onset of the disorder from those that are part of the disorder itself.

Several prospective studies of brain activity demonstrated differences present prior to the onset of PTSD symptoms. For instance, increased amygdala activity to negative stimuli was predictive of PTSD onset after a terrorist attack, indicating that increased amygdala activity may be present prior to the onset of PTSD (McLaughlin et al., 2014). Increased resting metabolic rate and hyperactivity of the dorsal ACC was a familial risk factor, and correlated with symptoms, of PTSD suggesting that increased metabolism and activity in dorsal ACC may be predictive of vulnerability to PTSD (Shin, 2011; Shin et al., 2009). One study identified smaller hippocampal volume as a risk factor for PTSD using monozygotic twins (Gilbertson et al., 2002). Together, these studies provide evidence that some brain activity and metabolic dysfunction as well as structural changes may be present prior to trauma and the onset of PTSD; however, further prospective studies are needed to fully test these results. Collectively, dysfunctional activity in medial prefrontal cortices, amygdala and hippocampus may be neural predictors of vulnerability to PTSD (Milad et al., 2009; Shin et al., 2005; Werner

et al., 2009). The development of dysfunction in these regions, especially as it relates to individual predisposition or experience, remains unclear despite the potential for improving biomarkers of the disease.

PTSD is Moderately Heritable But Genetic Candidates Are Often Non-Specific

Although there are some consistent neural correlates of PTSD, a better prediction method may rely on genetic and/or biological markers other than neural dysfunction. Studies in multi-generational families and twins provide evidence that PTSD is moderately heritable, with heritability ranging from 30 to 70%, depending on the study population (Goenjian et al., 2008; Sartor et al., 2012; Sartor et al., 2011; Stein, Jang, Taylor, Vernon, & Livesley, 2002; True et al., 1993; Yehuda, Halligan, & Bierer, 2001). Most consistently, PTSD is estimated to be 30-40% heritable (Sartor et al., 2012; Stein et al., 2002; True et al., 1993). Collectively, these epidemiological studies indicate that PTSD may have a genetic component, as there are likely biological underpinnings of a moderately heritable disorder.

More recently, genome-wide association studies (GWAS) and candidate gene studies have identified a variety of potential candidate genes that may underlie the heritable aspects of PTSD (Ashley-Koch et al., 2015; Bomyea, Risbrough, & Lang, 2012; Nievergelt et al., 2015; Skelton, Ressler, Norrholm, Jovanovic, & Bradley-Davino, 2012; Smoller, 2016). However, replication across GWAS studies has been low, perhaps due to the variable sample size, gender, and trauma types studied (Ashley-Koch et al., 2015; Digangi, Guffanti, McLaughlin, & Koenen, 2013; Nievergelt et al., 2015). Genetic polymorphisms associated with the serotonin and dopamine neurotransmitter systems

were linked to the development of PTSD (Bomyea et al., 2012; Cheung & Bryant, 2015; Smoller, 2016). However, the results of many studies are mixed, and some of these polymorphisms may better predict gene-by-environment interactions (Bomyea et al., 2012; Telch et al., 2015).

The most well studied genetic polymorphism is that of the FK506 binding protein 5 (FKBP5). A number of candidate gene studies (Bomyea et al., 2012; Cheung & Bryant, 2015; Smoller, 2016) demonstrated that the short allele of FKBP5 is associated with vulnerability to PTSD and negative emotional states as well as intrusive thoughts (characteristic of PTSD symptoms). While the genetics of FKBP5 seem promising, polymorphisms of FKBP5 have also been associated with attention deficit hyperactivity disorder, autism spectrum disorders, and major depressive disorder, all of which imply that genetic variance in FKBP5 may have broader effects rather than conferring specific vulnerability to PTSD (Isaksson, Allen, Nilsson, & Lindblad, 2015; Patel, Crider, Pandya, Ahmed, & Pillai, 2015; Szczepankiewicz et al., 2014). Instead, the short allele of FKBP5 may confer vulnerability to mental illness in general, and interact with other vulnerability factors that result in more specific clinical outcomes.

While identifying the specific genetic underpinnings of PTSD is an ongoing process, the GWAS studies along with twin studies have identified significant overlap between the heritability of PTSD and other mental disorders, similar to the findings in FKBP5. Overlap between the heritable components of PTSD has been seen with bipolar disorder (Nievergelt et al., 2015; Solovieff et al., 2014; Sumner, Duncan, Ratanatharathorn, Roberts, & Koenen, 2016) and schizophrenia (Sumner et al., 2016) as well as other anxiety disorders (Goenjian et al., 2008). The most consistently seen

overlap is major depressive disorder (MDD) with PTSD. Overlap between PTSD and MDD ranges from 20 to 100% overlap (Fu et al., 2007; Goenjian et al., 2008; Sartor et al., 2012; Solovieff et al., 2014). These studies imply that there may be a common biological substrate (possibly genetic) that underlies susceptibility to multiple mental health disorders rather than a specific genetic cause for PTSD alone.

The moderate heritability of PTSD is lower than heritability rates of bipolar disorder and schizophrenia, which are 70-80% heritable (Lawrie, O'Donovan, Saks, Burns, & Lieberman, 2016). Furthermore, the variability and lack of specificity of candidate genes makes it challenging to identify risk genes for PTSD. These issues are compounded by high degrees of overlap between risk genes and different mental health disorders (Lee et al., 2013). These issues suggest that the currently known genetic markers are not reliable predictors of PTSD.

Temperament May Provide Better Prediction For PTSD Than Individual Genes

While not necessarily linked to specific genetic causes, there are additional biological and personality factors that may influence vulnerability to PTSD. These include decreased heart rate variability (Minassian et al., 2015) and traits such as lower intelligence, higher antisocial personality, and higher neuroticism, that are each predictive of developing PTSD, as is a previous history of mental health disorders (Brewin, Andrews, & Valentine, 2000; Orr et al., 2012; Ozer, Best, Lipsey, & Weiss, 2003; Perrin et al., 2014). While these traits are often seen in those who have developed PTSD, it is often unclear whether personality characteristics and biological changes are a predisposing factor or a result of the disorder.

Personality traits are part of a larger construct of temperament, or a stable way of responding to environmental stimuli. A number of temperaments have been found to be highly heritable (Heath, Cloninger, & Martin, 1994). Internalizing and externalizing are two commonly applied temperament categories that encompass various mental health disorders (Krueger, McGue, & Iacono, 2001). MDD, anxiety, and other mood disorders are categorized as internalizing disorders, while substance use disorders, and antisocial personality disorders are examples of externalizing disorders (Krueger et al., 2001; Wolf et al., 2010). A series of studies in twins has demonstrated that internalizing and externalizing disorders are heritable (Amstadter, Maes, Sheerin, Myers, & Kendler, 2015; Kendler & Myers, 2014; Kendler, Myers, Maes, & Keyes, 2011; Khan, Jacobson, Gardner, Prescott, & Kendler, 2005; Wolf et al., 2010). At least one study has determined that using the externalizing dimension better identifies candidate genes for mental health disorders than studying individual disorders alone (Dick et al., 2008). Based on these findings, it is likely that temperament is a heritable construct that may allow better prediction of candidate genes for certain disorders.

PTSD is traditionally thought to fall into the category of internalizing disorders; however, one study found that heritability of disorders was better modeled when PTSD was allowed to load onto both externalizing and internalizing dimensions, indicating that PTSD in particular may blur the lines between these categories (Wolf et al., 2010). Whichever category each disorder falls in, it appears that broader descriptions of the heritability of temperament are useful for deciphering the complex genetic correlates of mental health disorders (Wolf et al., 2010). Based on the research above, it may be that

this construct of temperament, especially as it relates to internalizing or externalizing tendencies, is a better predictor of heritable mental health disorders such as PTSD.

Genetic and Temperament Vulnerability Factors Interact With the Environment

In addition to genetics, environmental resources such as income, social support, and previous as well as current life stressors can significantly influence an individuals' vulnerability to PTSD (Bonanno, Galea, Bucciarelli, & Vlahov, 2007). Given that a diagnosis of PTSD requires an external trauma or event, it is likely that gene-by-environment interactions give rise to the disorder. Despite the need for further examination of the gene-by-environment interactions between candidate genes and trauma or other environmental factors, very few studies of the genetics underlying PTSD have assessed these interactions (Digangi et al., 2013). The studies that have identified gene-by-environment interactions have found early life stress interactions with glucocorticoids and the HPA axis (Yehuda et al., 2010), the transmission of PTSD to successive generations influenced by parental stress (Rodgers & Bale, 2015), and potential immune dysregulation in response to stressors (Zannas, Provencal, & Binder, 2015). However, none of these gene-by-environment interactions have led to full prediction of an individual's susceptibility to developing PTSD, indicating that further studies of the ways in which environment interacts with genetics and other potential vulnerability factors are needed.

Despite significant research into the genetic, temperament, and environmental factors, as well as gene-by-environment interactions, so far no factor in any of these categories, and no combination of them, has allowed us to reliably predict vulnerability to

PTSD. Since temperament constructs may be better predictors of the heritability of mental health disorders than candidate risk genes, it is possible that interactions between temperament and the environment could better predict vulnerability to PTSD. Indeed, several studies have found that environmental influences predict the specific disorder that an individual develops (Amstadter et al., 2015; Kendler et al., 2011; Wolf et al., 2010). Further research is needed to clarify the interactions between temperament and the environmental conditions that lead to specific mental health disorders such as PTSD.

Significant Questions Remain Unanswered to Predict an Individual's Propensity to Develop PTSD

The sections above have reviewed a significant amount of research in humans that has identified maladaptive fear memories as the underlying cause of PTSD. Despite this, we have limited insight into why only a fraction of the individuals exposed to trauma develop PTSD. Here, we summarize the conclusions of the previous sections and outline the outstanding questions that remain.

While there are neural correlates, both in activity and structure that are characteristic of PTSD, these biological markers are often studied after PTSD is present, making it difficult to determine the predisposing factors that an individual might have had prior to trauma and PTSD onset (Dejean et al., 2015; Shin et al., 2005). PTSD is known to be moderately heritable, and there are several candidate genes that have been identified (Bomyea et al., 2012; Smoller, 2016). However, PTSD is not as heritable as other disorders such as schizophrenia and bipolar disorder, and the heritability and gene candidates often display overlap with other mental health disorders (Goenjian et al.,

2008; Lawrie et al., 2016; Solovieff et al., 2014). Temperament may be a better predictor of heritability for mental health disorders, but the interactions between temperament and environmental influences that lead to PTSD remain unclear (Digangi et al., 2013; Wolf et al., 2010). ***We still do not understand when or how the neural correlates of PTSD become dysfunctional or the role that temperament plays in determining vulnerability to PTSD, especially in gene-by-environment interactions. Developing a better understanding of these potential vulnerability factors may lead to identification of temperament, biological, genetic, or environmental factors along with their interactions that trigger PTSD, allowing for the development of better treatments or prevention options.*** In order to develop a better understanding of these factors, it is important to identify animal models that allow us to better control specific genetic, environmental and biological variables to tease apart the role they play in the development of PTSD. These models then allow us to compare the results of tightly controlled animal studies with studies in humans to develop a greater understanding of the influences that cause specific individuals to be vulnerable to developing PTSD.

Animal Models of Stressor-Induced PTSD

A distinct advantage for research investigating PTSD is that fear can be studied in other species, allowing for better control over genetic and environmental influences that may affect vulnerability to PTSD-like behaviors. A number of animal models display characteristics similar to those of PTSD patients after trauma (Borghans & Homberg, 2015). Several of these animal models use one or a series of physical stressors to mimic trauma encoding in humans. In the single prolonged stress model, rats are given restraint

stress, forced to swim in a group, and then given ether until unconscious (Knox et al., 2012). This model displays some behavioral characteristics of PTSD including reduced extinction learning (see section on fear conditioning below), increased glucocorticoid response, and changes in sleep after the stressors (Knox et al., 2012; Vanderheyden et al., 2015). Restraint stress by itself increases spontaneous anxiety in rodents, along with changes in glucocorticoid response similar to alterations seen in PTSD patients (Gameiro et al., 2006; Valles, Marti, & Armario, 2006). Subjecting rats to underwater trauma (forced swim followed by holding the rodent underwater for thirty seconds) also increases spontaneous anxiety for at least three weeks after the stressor (Richter-Levin, 1998). Several models also expose rodents to predator odors such as cat collars to elicit increases in fear behavior (Corley, Caruso, & Takahashi, 2012). While all of these models exhibit some behaviors similar to that of PTSD patients, in many cases they are used in combination with fear conditioning and extinction to better assess the model's propensity for PTSD.

Fear Conditioning and Extinction as an Animal Model of PTSD

Pavlovian fear conditioning and extinction is one particularly prevalent model of PTSD since it can be applied across multiple species in the laboratory, including in humans and rodents (Bowers & Ressler, 2015; Mahan & Ressler, 2012; Milad et al., 2014). Pavlov originally discovered that stimuli can become connected with behavioral responses through enduring memories (Pavlov & Anrep, 1927). During fear conditioning, a neutral cue (such as a tone, light, or odor) is presented along with an aversive unconditioned stimulus (US), such as a mild footshock. After repeated pairings, the

neutral cue becomes a conditioned stimulus (CS), with the individual displaying species-specific fear behaviors and increased endocrine response to the CS (Figure 1.1A; LeDoux, 2000). The conditioned fear responses can also be elicited by pairing an US with a context, which is known as contextual fear conditioning. In rodents, the species-specific response to fearful stimuli is freezing, or the absence of all movement with the exception of that required for respiration (Blanchard & Blanchard, 1969a, 1969b). Fear conditioning, as described here, can be likened to the traumatic events experienced by individuals that develop PTSD as the fear memories generated are long-lasting and the fear responses are difficult to reduce (Milad et al., 2014; Yehuda & LeDoux, 2007).

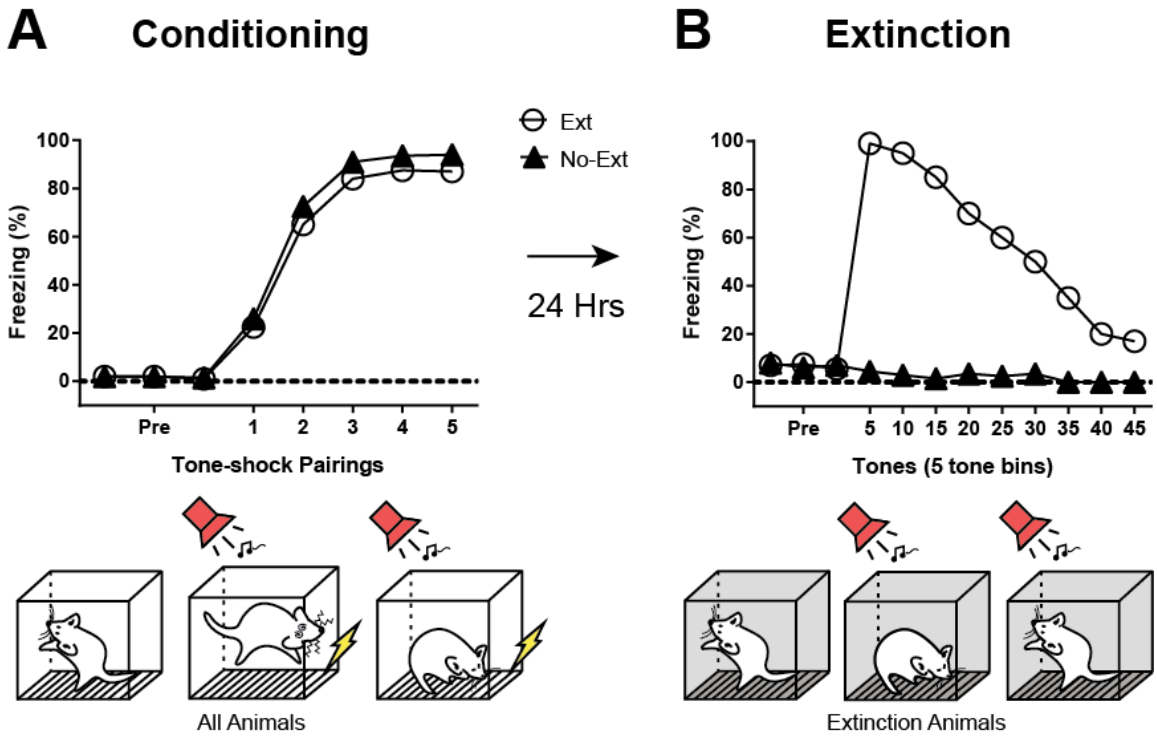

Figure 1.1 Examples of fear conditioning and extinction in rodents. *When a rat is placed in a novel environment, typically they explore the environment, which produces low levels of freezing (seen during the Pre-Period of Conditioning and Extinction). During conditioning (A), repeated pairings of a neutral tone with a mild footshock leads to a characteristic increase in freezing behavior. Traditionally, extinction learning (B) occurs 24 hours after conditioning in a novel environment (grey box vs. white box context). Rats are divided into two groups, those that receive extinction, and those that*

simply enter the novel environment and receive no presentations of the CS (No-Extinction). No-Ext rats therefore display low levels of freezing throughout the Extinction session. Ext rats display very high levels of freezing upon initial tone presentations that decrease over repeated presentations of the CS in the absence of the US. This decrease in freezing over time is the behavioral marker of extinction learning.

One of the most common behavioral therapies for PTSD is exposure therapy, which relies on the learning process of extinction (Foa, 2006; Norton & Price, 2007). Extinction is the process of presenting the CS (or conditioned context) repeatedly without the presence of the fear-inducing US. Over time, these repeated presentations of the CS lead to decreased fear expression and a return to normal, active, behavior in most individuals (Figure 1.1B; Chang et al., 2009; Maren, 2011; Milad et al., 2014). After extinction (usually 24 hours later), individuals return to the extinction context and are presented with the CS to test the retention of extinction memories. This allows researchers to understand the ability of individuals to learn and retain extinction, and to test various environmental and pharmacological manipulations that might improve extinction learning. It is important to note that extinction does not occur due to unlearning the original fear, but consists of new context-dependent learning of "safety". The context-dependence of extinction learning can lead to relapse when an individual is in a new environment, or over time (Bouton, Westbrook, Corcoran, & Maren, 2006). Previous studies have directly linked extinction to PTSD, with PTSD patients showing reduced extinction learning and retention in the laboratory (Milad et al., 2009; Orr et al., 2000), and individuals with reduced extinction showing a greater likelihood of developing PTSD (Lommen, Engelhard, Sijbrandij, van den Hout, & Hermans, 2013). Extinction and retention of extinction learning can both be tested effectively in rodent models and the ease of using the fear conditioning and extinction paradigm has made it a

particularly popular way to model the development of maladaptive fear memories and PTSD in both rodents and humans (Bowers & Ressler, 2015; Dejean et al., 2015; Desmedt et al., 2015; Dias, Banerjee, Goodman, & Ressler, 2013; Maren, 2011; Maren et al., 2013). Despite the large literature that has developed around fear conditioning in animals as a model of PTSD, little is known about how individual differences in temperament or genetics influence the dynamics of maladaptive fear memory, especially in animal models.

Neural Correlates of Fear and Extinction

Research on fear conditioning and extinction in rodents has led to the understanding that the brain regions underlying fear and extinction responses are similar across species (Bowers & Ressler, 2015; Dejean et al., 2015; Quirk & Beer, 2006). In rodents, similar to humans, fear conditioning and extinction activate the amygdala, hippocampus, and areas of the prefrontal cortex (Figure 1.2). While these brain regions are also involved in other forms of emotion and emotion regulation, the focus here will be on the role specific brain regions play in fear conditioning and extinction. The sections below provide a brief overview of the neural circuits involved in fear conditioning and extinction with a focus on the anatomy of the rodent (for further information, the reader is referred to these excellent reviews: Dejean et al., 2015; Duvarci & Pare, 2014; Giustino & Maren, 2015; Grillon, 2002; Grundemann & Luthi, 2015; Herry et al., 2010; Johansen, Cain, Ostroff, & LeDoux, 2011; LeDoux, 2000; Maren, 2001; Maren & Fanselow, 1996; Orsini & Maren, 2012; Pape & Pare, 2010; Quirk, Garcia, & Gonzalez-Lima, 2006; Sotres-Bayon & Quirk, 2010; Tovote, Fadok, & Luthi, 2015).

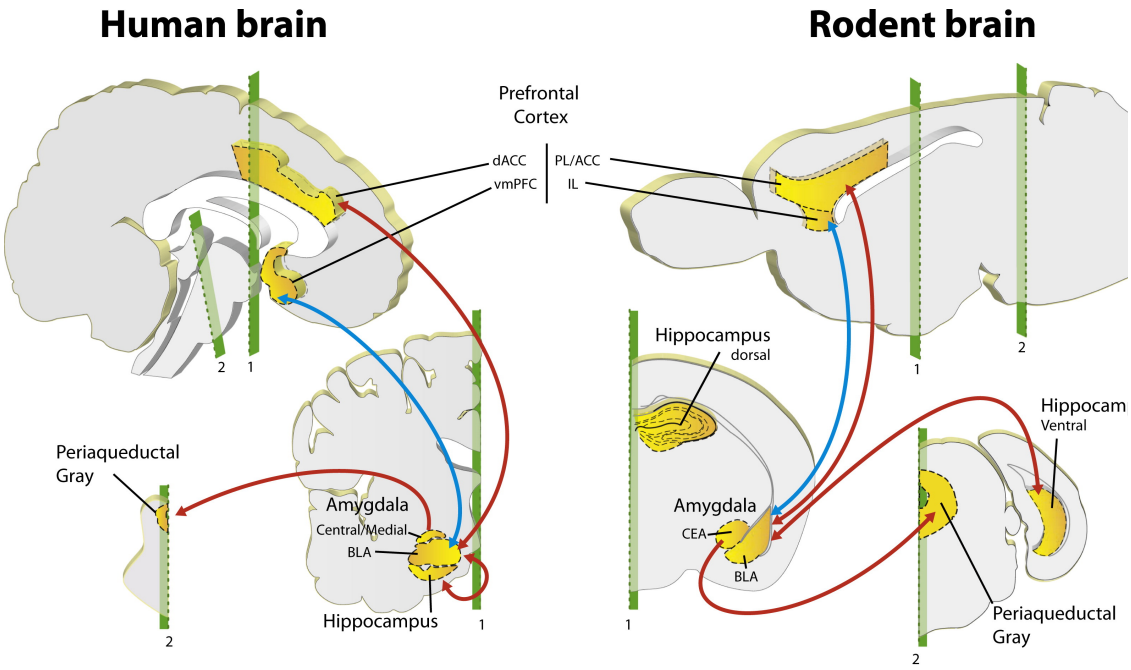

Figure 1.2 Neural circuitry underlying fear conditioning and extinction in humans and rodents. *On the left, brain regions important for fear conditioning and extinction in humans are highlighted. On the right, the complementary regions of the rodent brain important for fear conditioning and extinction are highlighted. The blue lines are connections particularly important for fear extinction. Reprinted from Biological Psychiatry, 78/5, DeJean, Courtin, Rozeske, Bonnet, Dousset, Michelet, and Herry, Neuronal Circuits for Fear Expression and Recovery: Recent Advances and Potential Therapeutic Strategies, 298-306, 2015 with permission from Elsevier. http://www.sciencedirect.com/science/journal/00063223*

Neural correlates of fear conditioning

During fear conditioning, the brain must integrate a variety of sensory information regarding the cues and unconditioned stimuli, place it in context with the location and previous knowledge of that location, and determine whether the animal should express defensive behaviors. This integration of sensory, contextual and emotional information requires a large network of regions primarily defined by the amygdala, hippocampus and medial prefrontal cortex (Dejean et al., 2015; Pape & Pare, 2010).

Amygdala. The amygdala is composed of several subnuclei located in the temporal lobe that are involved in fear learning and fear expression. One of these amygdala subnuclei, the basolateral amygdala (BLA; composed of the basal and lateral amygdala nuclei) is uniquely positioned to receive information about both the CS and the US, and previous work has shown it to be involved in the acquisition and storage of fear memories (Herry et al., 2008; Maren, 1999a; Maren & Fanselow, 1996). The BLA receives input from the hippocampus as well as sensory thalamic nuclei (both auditory and somatosensory) that allows the integration of sensory, cued and contextual information into a single fear memory (Hubner, Bosch, Gall, Luthi, & Ehrlich, 2014; LeDoux, Farb, & Ruggiero, 1990; Ledoux, Ruggiero, Forest, Stornetta, & Reis, 1987). Inactivation of the BLA prevents learning during fear conditioning, and abolishes the fear memory after learning has occurred (Cousens & Otto, 1998; Maren, 1999a; Maren, Aharonov, & Fanselow, 1996); strongly implicating the BLA as the site of fear memory storage in the brain (Maren, 2001). Additional research in rodents has implicated specific neurons in the learning and storage of fear memories, with certain "fear" neurons increasing their responding during conditioning and while an animal expresses fear (Herry et al., 2008; Repa et al., 2001). It has recently been shown that these fear neurons project directly to the prelimbic region of the prefrontal cortex, further enhancing our understanding of the circuitry underlying fear learning and expression (Senn et al., 2014).

After integration in the BLA, information about whether or not the individual should express fear is routed to the central nucleus of the amygdala (CEA), which has connections with fear expression regions of the brainstem (such as the periaqueductal grey) to promote fear responses such as freezing and autonomic arousal (Ciocchi et al.,

2010). More recently, it was shown that the central nucleus of the amygdala has two subdivisions; the medial subdivision which underlies conditioned and unconditioned freezing responses and is under tonic control of the other, lateral subdivision, which plays a role in fear learning (Ciocchi et al., 2010; Haubensak et al., 2010). These complicated microcircuits within the central amygdala hold specific control over outputs to brainstem nuclei and other regions that control the response to fearful stimuli.

Hippocampus. Information from the hippocampus is integrated into fear memories in the BLA, implicating the hippocampus in fear learning. In particular, the hippocampus is involved in learning the context of the fearful event, including spatial, temporal, internal state, and other cues (Maren, 1999b, 2001; Maren & Fanselow, 1997). The role of the hippocampus in fear learning has primarily been identified by the effects of temporary inactivation or lesions on the ability of an animal to recall the location in which it should be afraid (Kim, Rison, & Fanselow, 1993; Maren & Fanselow, 1997). The dorsal hippocampus is the primary location of place cells, or neurons that encode specific locations in the environment (Moser, Rowland, & Moser, 2015), and these cells change their firing in response to fear conditioning, indicating that they may encode long term memory of the location in which an animal encountered a fearful stimulus (Wang et al., 2012).

Prefrontal Cortex. Certain areas of the prefrontal cortex of the rat have direct and reciprocal anatomical connections with both the hippocampus and amygdala (Hoover & Vertes, 2007; McDonald, 1991; McDonald, Mascagni, & Guo, 1996), and indeed, they too are involved in fear learning. Specifically, the prelimbic cortex (PL) plays a role in fear acquisition and expression. Activation of the PL is necessary for the expression

specifically of learned fear (Corcoran & Quirk, 2007), and activity in PL increases freezing behavior (Sierra-Mercado, Padilla-Coreano, & Quirk, 2011; Vidal-Gonzalez, Vidal-Gonzalez, Rauch, & Quirk, 2006). Electrophysiology of PL neurons after fear conditioning has shown that they display sustained firing correlating strongly with freezing behavior, and that their firing and bursting rates increase with conditioning (Burgos-Robles, Vidal-Gonzalez, & Quirk, 2009). Together, these findings strongly implicate the PL area of the prefrontal cortex as a primary structure involved in the acquisition and expression of fear conditioning.

Taken together, the data above provide strong evidence for a set of functional and anatomical neural pathways for fear conditioning. Fear stimuli activate areas of the visual, auditory, and somatosensory pathways as part of normal sensory experience. Some of these signals are integrated into a contextual representation in the hippocampus, while cue-related signals are sent directly to the BLA (LeDoux et al., 1990; Maren, 2001). Reciprocal connections between the amygdala and prefrontal cortex integrate signals between the BLA and PL (Pape & Pare, 2010; Senn et al., 2014). The PL and BLA then signal the central amygdala, which sends output to brainstem areas such as the periaqueductal grey to induce freezing responses in the animal (Ciocchi et al., 2010; Dejean et al., 2015). These networks between amygdala, hippocampus, and prefrontal cortex provide evidence that fear learning is similar across species, as the same regions are implicated in studies of human fear conditioning (Dejean et al., 2015).

Neural Correlates of Extinction Learning

Similar to fear conditioning, the amygdala, hippocampus and prefrontal cortex are implicated in the extinction of fear (Figure 1.2). Interestingly, different neuronal populations in the BLA and different areas of the prefrontal cortex are involved in fear conditioning versus fear extinction (Dejean et al., 2015; Pape & Pare, 2010).

Amygdala. Similar to fear conditioning, the BLA is thought to play a central role in the reduction of fear during fear extinction and the storage of the extinction memory (Dejean et al., 2015; Orsini & Maren, 2012). Temporary inactivation of the BLA during extinction results in impaired memory of fear extinction during the retention test, indicating that the BLA is important for extinction learning and retention (Sierra-Mercado et al., 2011). Additional studies have implicated both molecular pathways associated with memory (ERK/MAPK) and specific receptors involved in synaptic plasticity (NMDA receptors containing NR2B) in the BLA as necessary for extinction learning (Herry, Trifilieff, Micheau, Luthi, & Mons, 2006; Sotres-Bayon, Bush, & LeDoux, 2007). As described previously, the BLA contains a population of fear neurons that are more active during fear conditioning and when an animal expresses fear (Herry et al., 2008). During fear extinction, a second population of "extinction" neurons becomes active, and fear neurons suppress their firing over the course of extinction learning (Herry et al., 2008). These extinction neurons have direct connections with the infralimbic area of the prefrontal cortex, implicating this connection in the reduction of fear responses during extinction (Senn et al., 2014).

Hippocampus. Extinction learning is context-specific, meaning that expression of extinction only occurs in the context in which extinction learning originally occurred,

directly implicating the hippocampus in extinction learning (Bouton et al., 2006; Orsini, Kim, Knapska, & Maren, 2011; Orsini & Maren, 2012). Inactivation of the ventral or dorsal hippocampus impaired both freezing during extinction and the retention of extinction (Corcoran, Desmond, Frey, & Maren, 2005; Sierra-Mercado et al., 2011). Additionally, inactivation of the dorsal hippocampus removed the context-specificity of the extinction memory behaviorally and in BLA neuronal firing (Maren & Hobin, 2007). A recent study has also implicated hippocampal place cell remapping during extinction as well as conditioning, indicating that place cell activity may help to differentiate fear and extinction memories (Wang, Yuan, Keinath, Ramos Alvarez, & Muzzio, 2015). The ventral hippocampus has been associated with emotion and anxiety-like behavior in rodents (Bannerman et al., 2003; Bannerman et al., 2004), and this region has a distinct molecular makeup and different anatomical targets than its dorsal hippocampal counterpart (Fanselow & Dong, 2010). Ventral hippocampus in particular plays a role in fear extinction, with inactivation inhibiting the contextual recall of extinction (Orsini et al., 2011). Thus, both dorsal and ventral hippocampus appear to be involved in the appropriate contextualization of fear extinction memories.

Prefrontal Cortex. The infralimbic prefrontal cortex (IL) is strongly implicated in the process of fear extinction (Dejean et al., 2015; Orsini & Maren, 2012). Inactivation of IL reduces the acquisition and retention of extinction learning (Sangha, Robinson, Greba, Davies, & Howland, 2014; Sierra-Mercado, Corcoran, Lebron-Milad, & Quirk, 2006; Sierra-Mercado et al., 2011). Additionally, neurons in IL increase their activity during extinction, demonstrating both greater excitability and burst firing, and stimulation of IL can mimic extinction learning by reducing freezing (Milad & Quirk, 2002; Santini,

Quirk, & Porter, 2008; Vidal-Gonzalez et al., 2006). A recent study demonstrated that IL neurons that project to the BLA have direct control over expression of fear behavior, with increased IL projection firing causing reductions in freezing while inhibition of IL projection firing increased freezing behavior (Senn et al., 2014). Together, these data point to the need for IL, and IL-BLA circuitry in particular, for extinction learning and retention.

The data summarized above indicate that a network of brain regions, including the amygdala, hippocampus and prefrontal cortex, is involved in fear extinction. Similar to fear conditioning, the BLA integrates contextual and sensory information by encoding the extinction memory (Orsini & Maren, 2012; Tovote et al., 2015). BLA influences, and is influenced by, the IL in the prefrontal cortex, which contributes to expression of the extinction over the fear memory (Senn et al., 2014; Tovote et al., 2015). Involvement of the hippocampus, potentially through interactions with IL, BLA, or both, leads to the context-specificity of the extinction memory (Maren & Hobin, 2007; Orsini & Maren, 2012; Sierra-Mercado et al., 2011). Together these long-range circuits lead to a reduction in fear behavior and subsequent retrieval of extinction memories in appropriate locations.

It is clear that great strides have been made in understanding the underlying neurobiology of fear and extinction processes in rodents. It is important to note that many of the brain regions underlying fear conditioning and extinction in rodents are also seen to be active during fear conditioning and extinction in humans (Dejean et al., 2015; Delgado, Nearing, Ledoux, & Phelps, 2008; LaBar, Gatenby, Gore, LeDoux, & Phelps, 1998; Milad et al., 2009; Milad et al., 2014; Phelps, Delgado, Nearing, & LeDoux, 2004). Additionally, these same brain regions that underlie fear conditioning and extinction are

those often seen to be dysregulated in PTSD patients (Dejean et al., 2015; Milad et al., 2009; Milad et al., 2014; Shin et al., 2004; Shin et al., 2005). Together, this lends further credence to the idea that PTSD is a disorder of maladaptive fear and extinction. While we understand a great deal about the neural circuitry underlying fear learning, it is still unclear how dysfunction in this circuitry allows fear learning to become a maladaptive memory that is challenging to extinguish and how individual differences might mediate that dysfunction.

Animal Models of Individual Differences

Despite the clear need for an individual understanding of maladaptive fear behavior, most studies using fear conditioning collapse data across individuals to determine the group effect (Bush, Sotres-Bayon, & LeDoux, 2007). This use of average behavior across all animals eliminates the ability of researchers to identify and manipulate individual responses to fear and extinction learning. To address this need, a small but growing body of work is focused on identifying individual differences in fear and extinction behavior in rodents. One promising approach may be to use selective breeding for differences in "temperament" to assess the heritability of individual differences in fear and extinction behavior.

One study divided rats into high and low fear reactivity based on their responses to fear conditioning, and high and low fear recovery based on their responses to fear extinction (Bush et al., 2007). While this was a very important step toward identifying individual differences in rodents, the criteria described in the study can only be used to identify individuals in a *post hoc* manner after the behavior has been displayed. This

negates the ability to test protective strategies aimed at enhancing resilience, and does not allow for prospective assessment of vulnerability factors.

One way to develop a model of individual differences that does not require *post hoc* division of individuals is to investigate differences in behavior between inbred strains of rats or mice. Several studies have compared different rat or mouse strains to study differences in startle or fear behavior between individuals in those groups (Falls, Carlson, Turner, & Willott, 1997; Farook et al., 2001; Stohr et al., 2000). While this provides some information about differences between inbred strains of rodents, none of these studies have looked at individual differences in extinction learning, an important component of human maladaptive responses to fear. Additionally, these studies do not discuss the potential diversity of individual responses within a strain.

Another way to predetermine certain characteristics of individuals is selective breeding. Selective breeding can amplify a chosen behavioral or neurological characteristic, such as temperament. However, frequently other characteristics aside from those that were specifically selected are consistently differentiated in these lines. Several studies of selectively bred rats have identified differences in startle amplitude or fear behavior between individual lines (Brush, Gendron, & Isaacson, 1999; McQueen, Overstreet, Ardayfio, & Commissaris, 2001), although not all selectively bred lines differ on these characteristics (Blizard & Adams, 2002). Two studies have identified individual differences specifically in fear conditioning and extinction behavior using a selective breeding strategy to define rats with individual differences in "temperament" (Lopez-Aumatell et al., 2009; Muigg et al., 2008). Despite their ability to better assess prospective differences in individual behavior that might lead to vulnerability or

resilience to maladaptive fear, the fear behavior of these rats remains poorly characterized. These studies provide evidence that a selective breeding strategy may be useful for demonstrating individual differences in fear conditioning and extinction that mimic the human individual variability in developing PTSD after trauma. Indeed, animal models of "temperament" may provide better models of the heritability of mental health disorders, similar to humans, than specific genetic lines. This may be especially true if the animals display stable tendencies toward internalizing or externalizing disorders, which have been shown to be particularly heritable and predictive of mental health disorders in humans (Amstadter et al., 2015; Kendler & Myers, 2014; Kendler et al., 2011; Khan et al., 2005; Wolf et al., 2010).

Our lab has extensively characterized two lines of selectively bred rats, demonstrating consistent and extreme differences in "temperament" between the two lines. The "temperaments" of our selectively bred animals are similar to a collection of traits that in humans are thought to be associated with externalizing or internalizing disorders (Khan et al., 2005). Like in humans, the "temperaments", or behavioral phenotypes displayed by the rats are stable across time and are heritable, generating lines of rats that have differential emotional responses to the environment (Stead et al., 2006; Wolf et al., 2010). These lines are selectively bred for their locomotion in a novel environment, with bred high responders (bHRs) having high novelty-induced locomotion, and bred low responders (bLRs) having low novelty-induced locomotion (Stead et al., 2006). While a normal population of outbred Sprague Dawley rats displays variability in this locomotor phenotype, selective breeding has generated two lines of rats that display the extreme ends of the locomotor behavioral spectrum(Stead et al., 2006). Along with

alterations in locomotor activity, these rats also display a variety of other behavioral changes that result in distinct and contrasting phenotypes. The bLRs characteristically display high levels of spontaneous anxiety-like behavior on apparatus such as the elevated plus maze, the open field, and the light-dark box, all classic tests of spontaneous anxiety-like behavior in rodents (Clinton, Miller, Watson, & Akil, 2008; Garcia-Fuster et al., 2012; Perez, Clinton, Turner, Watson, & Akil, 2009; Stead et al., 2006; Turner, Clinton, Thompson, Watson, & Akil, 2011). In contrast, bHRs display low levels of spontaneous anxiety-like behavior on the same tests (Clinton et al., 2008; Garcia-Fuster et al., 2012; Perez et al., 2009; Stead et al., 2006; Turner et al., 2011). The bLRs also display high levels of depressive-like behavior on sucrose preference and the forced-swim test while bHRs display low levels of depressive-like behavior (Garcia-Fuster et al., 2012; Stedenfeld et al., 2011; Turner et al., 2011). These characteristic differences in "temperament" are also seen on tests of drug seeking, with bHRs showing increased propensity to seek drugs and increased levels of addictive behavior compared to bLRs (Clinton et al., 2012; Flagel et al., 2010). These studies demonstrate that the selective breeding strategy has developed two lines of rats with large individual differences in their response to stressful stimuli; however, no studies have assessed how these animals respond to learned fear using fear conditioning or extinction.

These bHR and bLR rodents provide a genetic model of "temperament" and susceptibility to a variety of mental health disorders, and they display known differences in several neural systems and molecular families. Here, we use the term "genetic model" to indicate that the behavioral phenotype and associated biological underpinnings are heritable and likely rely on genetic changes, although the specific genetic biology of the

animals is currently under study. Based on the data above, bLRs are prone to internalizing disorders (low novelty seeking, depressive- and anxiety-like behaviors are increased) while bHRs are prone to externalizing disorders (high novelty seeking, increased drug seeking and addiction; Clinton et al., 2012; Flagel, Waselus, Clinton, Watson, & Akil, 2014; Stead et al., 2006; Stedenfeld et al., 2011). These behaviors are seen early in life, and are not dependent on maternal behavior, although maternal care can alter them (Clinton et al., 2008; Clinton, Stead, Miller, Watson, & Akil, 2011; Cohen et al., 2015).

Besides stable alterations in behavior, these rats also display differences in the neural circuitry of the serotonin and dopaminergic systems of the brain (Dellu, Piazza, Mayo, Le Moal, & Simon, 1996; Flagel et al., 2014). bHRs demonstrate increased release of dopamine in the nucleus accumbens (Flagel et al., 2010). These differences in the dopaminergic system between bHR and bLR individuals may underlie the changes in drug seeking and addiction liability seen between these animals (Flagel et al., 2010; Flagel et al., 2014). bHRs also have increased and prolonged glucocorticoid secretion in response to stress, and fewer glucocorticoid receptors in the hippocampus, as well as increased presence of repressive epigenetic modifications on the GR promotor, indicating that their HPA axis function is altered (Chaudhury et al., 2014; Clinton et al., 2008; Flagel et al., 2014). The HPA axis is a major component of the stress response, and these differential responses of the glucocorticoid system in the HPA axis may lead to the decreased anxiety and high novelty seeking observed in bHRs (Flagel et al., 2014). These rat lines provide a rich context in which to further assess individual differences in

vulnerability to developing maladaptive fear behavior, given their stable phenotypes and differential activity in neural systems known to underlie emotional responses.

In addition to their differences in behavior, glucocorticoid and dopaminergic responses, our lab has determined that the bHRs and bLRs differ in the amount of fibroblast growth factor two (FGF2) in their hippocampus, which may contribute to the differences in "temperament" between the lines (Chaudhury et al., 2014; Eren-Kocak, Turner, Watson, & Akil, 2011; Perez et al., 2009). FGF2, like other growth factors, is known to be involved in cell proliferation, survival, differentiation and maintenance (Beenken & Mohammadi, 2009; Turner, Eren-Kocak, Inui, Watson, & Akil, 2015). FGF2 was initially of interest because it is downregulated in the post-mortem brains of humans with major depressive disorder (Bernard et al., 2011; Evans et al., 2004). Further studies in rodents demonstrated that FGF2 is downregulated after social defeat, a model of major depressive disorder (Turner, Calvo, Frost, Akil, & Watson, 2008), and that administration of FGF2 causes antidepressant like effects on a variety of behaviors (Elsayed et al., 2012; Turner, Gula, Taylor, Watson, & Akil, 2008). While this evidence points to FGF2 being involved in major depressive disorder and the action of antidepressants, our lab also found differences in anxiety-like behavior after FGF2 administration or knock-down, indicating that FGF2 has both anxiolytic and antidepressant actions (Chaudhury et al., 2014; Eren-Kocak et al., 2011; Perez et al., 2009; Turner et al., 2011). Interestingly, these studies also showed that FGF2 was elevated in the hippocampus of low-anxiety bHR animals compared to high anxiety bLR animals, and that early life administration of FGF2 could selectively impact the anxiety-like behavior of bLR animals (Perez et al., 2009; Turner et al., 2011). A more recent study demonstrated that knocking down FGF2

in the hippocampus eliminated phenotypic differences in anxiety-like behavior, indicating that hippocampal FGF2 may be critical to the phenotype of these selectively bred animals (Chaudhury et al., 2014). The basal differences in FGF2 seen in the hippocampus of these animals, and the ability of FGF2 to alter affective behavior, indicates that FGF2 may play a role in emotional responsiveness and may prove relevant to understanding vulnerability or resilience to a number of stress disorders, including PTSD. Yet no previous studies have examined fear conditioning and extinction behavior in these selectively bred lines to determine whether their differences in "temperament" would also influence their propensity for PTSD-like behaviors.

Specific Aims and Hypotheses of the Dissertation

Individual differences are involved in the etiology of PTSD, and their study is vital to further developing predictive tools for clinical use (Holly & Miczek, 2015; Holmes & Singewald, 2013). Individual differences could arise from genetic, temperament, or environmental factors, or gene-by-environment interactions, but the underpinnings of these individual differences are important to study in order to understand how they lead to vulnerability or resilience to PTSD (Bomyea et al., 2012; Perrin et al., 2014; Skelton et al., 2012). While animal models of individual differences have been developed, only a few have been tested using fear conditioning as a preclinical model of PTSD (Lopez-Aumatell et al., 2009; Muigg et al., 2008). No model has yet been extensively characterized for differences in vulnerability to PTSD-like behaviors, and used to test potential protective or resilience-inducing factors. ***This dissertation therefore endeavors to answer three overarching questions:***

1. ***Are PTSD-like behaviors heritable in a genetic animal model and malleable by experimental manipulation?***
2. ***Are heritable maladaptive fear behaviors influenced by environmental manipulations and are there gene-by-environment interactions?***
3. ***How well do the neural correlates of individual differences in fear behavior in rats translate to humans?***

In Chapter 2, we assess Question 1. *Can a rodent model of individual differences in temperament provide a heritable model of PTSD-like behaviors, and if so, can we change that heritable behavior by experimental manipulation?* We characterize bHR and bLR animals using a fear conditioning and extinction paradigm to examine their propensity to maladaptive fear behavior. We hypothesize that both bHRs and bLRs will show conditioned behaviors similar to standard outbred animals but will differ substantially in their behavior during extinction, with bHRs showing facilitated extinction learning and bLRs showing reduced extinction learning. We then examine whether this predicted heritable vulnerability to reduced extinction in bLRs can be modulated by the administration of FGF2 early in life. We hypothesize that early life FGF2 in bHRs will have no effect on their fear conditioning or extinction behavior while administration of FGF2 early in life will reduce vulnerability to PTSD-like behaviors in bLRs by facilitating their extinction learning, making them appear more like outbred animals.

In Chapter 3, we examine Question 2. *Can the environment manipulate the genetic model by changing extinction behavior for either bHRs or bLRs, will manipulating both the "genetic load" and the environment have an additive effect, and what are the neural correlates of the effect of environment?* We begin by manipulating

the social context surrounding animals during fear conditioning and extinction. We hypothesize that social context might facilitate fear extinction for outbred rats, but not for the more extreme selectively bred rats. We hypothesize that there might be an interaction of genes with environment where reducing the heritable anxiety phenotype of bLRs (their "genetic load") along with providing them a specific social context during extinction might facilitate extinction learning. We examine the neural correlates of this gene-by-environment interaction by examining the brain activity after extinction learning as indexed by immediate early gene activity. We hypothesize that the infralimbic prefrontal cortex and basolateral amygdala will show changes in activity based on the social context of extinction for bLR and bHR rats.

In Chapter 4, we investigate Question 3. *Do the neural correlates of heritable anxiety seen during extinction in our rodent model translate to humans?* We examine the neural correlates of individual differences in humans by analyzing a functional magnetic resonance imaging study of fear extinction learning. We hypothesize that during extinction, individuals with high trait anxiety would display reduced amygdala – subgenual cingulate cortex coactivation compared to individuals with low trait anxiety, but that we might see increased coactivation of orbitofrontal cortex with amygdala and hippocampus, similar to the rodent data.

Together, the goal of this dissertation is to better elucidate the patterns of individual differences that underlie vulnerability or resilience to developing PTSD-like behaviors and to begin to understand the brain mechanisms that might contribute to these differences in both humans and rodents.

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Chapter 2.

Selectively Bred Rats Provide a Unique Model of Vulnerability to PTSD-like Behavior and Respond Differentially to FGF2 Augmentation Early in Life

In the US alone, it is estimated that over 50% of men and women have experienced at least one trauma in their lifetime, yet incidence rates of post-traumatic stress disorder (PTSD) remain at around 8% of the population (Kessler, Sonnega, Bromet, Hughes, & Nelson, 1995). This suggests that individual differences in vulnerability to trauma play a critical role in the etiology of PTSD. While considerable effort has been directed at understanding the genetic and behavioral risk factors for PTSD (Bomyea, Risbrough, & Lang, 2012; Lommen, Engelhard, Sijbrandij, van den Hout, & Hermans, 2013; Orr et al., 2012), we do not yet have a method to accurately predict which individuals will develop PTSD. Building a reliable prediction method has the potential to advance research on therapeutics for PTSD, but is unlikely to happen based solely on human research. It is therefore unsurprising that a call was recently made for further development of animal models of individual differences in response to trauma (Holly & Miczek, 2015).

To this end, we have developed a rodent model of individual differences in which rats have been selectively bred based on their locomotion in a novel environment (for a review see Flagel, Waselus, Clinton, Watson, & Akil, 2014). While a typical population of outbred Sprague Dawley rats will display variability in this trait, the selective breeding

approach has magnified these differences, yielding two lines of rats that differ drastically in their "temperament" (Stead et al., 2006). Specifically, the selectively bred "High Responders" (bHRs) characteristically display low spontaneous anxiety- and depression-like behavior, while the "Low Responders" (bLRs) characteristically display high anxiety- and depression-like behavior (Perez, Clinton, Turner, Watson, & Akil, 2009; Stead et al., 2006). These selectively bred lines are a model for "temperament" or stable tendencies in humans that predispose to internalizing (e.g. anxiety and depression) versus externalizing (e.g. addiction and impulsivity) disorders (Khan, Jacobson, Gardner, Prescott, & Kendler, 2005). The lines offer a unique opportunity to explore how individual differences in emotional responsiveness influence the learning and memory processes involved in the etiology of PTSD, including the conditioning and extinction of fear (Maren, Phan, & Liberzon, 2013).

Fear conditioning and extinction are common models of trauma encoding and exposure therapy, respectively (Milad, Rosenbaum, & Simon, 2014). It has previously been shown that PTSD patients have reduced extinction learning and retention (Blechert, Michael, Vriends, Margraf, & Wilhelm, 2007; Milad et al., 2009; Norrholm et al., 2011; Orr et al., 2000), and that reduced extinction learning may be predictive of future PTSD (Lommen et al., 2013). Given that a history of depression is known to be a risk factor for PTSD (Orr et al., 2012) and that bLRs specifically display depressive-like behaviors, we hypothesized that our selectively bred rats might provide a model of individual differences in PTSD-like vulnerability during fear conditioning and extinction.

One advantage of our selectively bred animal model is that the phenotype of offspring can be predicted with 99% accuracy; therefore, we can test early life

interventions that may have lasting effects on behaviors such as fear extinction. It has recently been shown in adult rodents that administration of FGF2, which is known to play a role in cell survival and differentiation, reduces anxiety and depression-like behavior, including in bLRs (Eren-Kocak, Turner, Watson, & Akil, 2011; Perez et al., 2009; Turner, Clinton, Thompson, Watson, & Akil, 2011; Turner, Eren-Kocak, Inui, Watson, & Akil, 2015). Furthermore, FGF2 has been shown to be a potent enhancer of fear and extinction learning in juvenile and adult outbred rats (Graham & Richardson, 2011): a single dose of FGF2 administered before the extinction session enhanced both extinction learning and the retention of the extinction memory 24 hours later (Graham & Richardson, 2009). However, it is unknown whether FGF2 differentially affects extinction learning in individuals with differing genetic predispositions to negative affect, and whether there is a critical developmental time window for this modulation. Our lab has shown that a single dose of FGF2 the day after birth can significantly reduce anxiety- and depression-like behavior in bLR rats when tested in adulthood (Turner et al., 2011). This suggested that a single administration of FGF2 the day after birth might also be able to influence extinction learning in adulthood, including in vulnerable individuals.

Thus, the aim of the present studies was to characterize a new animal model of individual differences in fear and extinction learning and test whether early life administration of FGF2 might have differential effects on individuals of different temperaments during extinction learning in adulthood. We hypothesized that bHRs and bLRs would display fear learning similar to that of outbred animals, but that bLRs would show significantly reduced extinction learning and retention, indicating a PTSD-like phenotype. Based on our previous data (Turner et al., 2011), we hypothesized that

administration of FGF2 the day after birth would reduce spontaneous anxiety as measured by the elevated plus maze, and also facilitate extinction learning and retention specifically in bLRs.

Methods

Animals

Outbred Rats. Forty-eight male Sprague Dawley rats from Charles River Laboratories (Chicago, IL) weighing approximately 225-250 grams were compared with selectively bred rats from our laboratory colony.

Selectively Bred Rats. Selectively bred rats were originally Sprague Dawley outbred animals continuously bred with partners chosen based on their locomotion in a novel environment (Stead, et al., 2006). Rats that show high locomotion in a novel environment (see Behavior for description) are bred with rats that also display high locomotion in a novel environment (high responders; HRs); rats that show low locomotion in a novel environment are bred with rats that display low locomotion in a novel environment (low responders; LRs). Successive generations of breeding in this way has led to a stable line of complex traits that have been described in a number of publications (for a review, see Flagel, et al. 2014). 142 male selectively bred animals from generations 36, 40 and 41 of our colony were used in these experiments (72 bHRs, 70 bLRs). Additionally, 32 male bred Intermediate Responders (bIRs), a first-generation cross of bHR-bLR parents, were generated to compare with the selectively bred animals.

All animals participating in these studies were pair-housed (with non-littermates when known) on a 12/12 hour light-dark schedule with access to food and water *ad-libitum*. Animals were handled for two minutes a day for at least five days prior to beginning behavioral testing to acclimate the animals to the experimenter. The University Committee on the Use and Care of Animals (UCUCA) at the University of Michigan approved all protocols and procedures. There are eight animals per group unless otherwise noted.

Behavior

Locomotor Testing. Rats were placed in a 43 x 21.5 x 25.5 cm clear acrylic cage with a stainless steel grid floor for one hour. The cages are separated by black acrylic dividers and micro-isolator cage tops so that rats cannot see or smell each other. Horizontal (walking/running) and vertical (rearing) locomotor activity was monitored every five minutes by two panels of photocells that record beam breaks as described previously (Stead et al., 2006). Horizontal and vertical locomotor measures were added together to create a total locomotion score for each individual animal. Between animals, the cages were cleaned with 70% ethanol. The locomotion-testing equipment and software was developed in-house at the University of Michigan. All locomotor testing took place between 0800 and 1200 hours.

Elevated Plus Maze. The elevated plus maze (EPM) in our laboratory has four black Plexiglass arms arranged in a perpendicular cross that are raised off the floor (70 cm from the floor, each arm is 45 cm long and 12 cm wide). Two arms of the EPM are enclosed by 45 cm –high walls while two consist of only flooring. At the intersection of

the four arms of the maze is a 12 x 12 cm platform that allows access to all of the arms (Pellow, Chopin, File, & Briley, 1985). Animals were transported to the testing room in their home cage and tested immediately prior to or after their cage mate. Selectively bred animals were randomized and counterbalanced such that bLR and bHR cages were interleaved during testing. During the five-minute testing period, the room is dimly lit (~ 40 lux) by a single bulb hung from the ceiling above the center of the maze, and behavior is monitored using a computerized video tracking system (Noldus Ethovision, Cincinnati, OH). At the start of the five-minute test, the rat is placed on the center platform facing one of the open arms of the maze. The tracking system recorded the time spent in the open and closed arms and the center platform as well as the total distance traversed by the animal. After every animal, the EPM apparatus was cleaned with 30% ethanol. All EPM testing occurred between 0830 and 1130 hours. Animals that spend a greater percentage of time in the closed arms are considered more anxious than those that spend a greater percentage of time exploring the open arms. Animals underwent fear conditioning and extinction one week after EPM testing.

Fear Conditioning and Extinction. Fear conditioning and extinction parameters were very similar to those described in Chang et al. (2009). Animals were randomly assigned to conditions in these studies, and fear conditioning occurred when animals were approximately 67-73 days of age. Behavioral testing occurred between 0830 and 1600 hours. During testing, video was collected of each animal.

In brief, animals were transported to the testing room in individual plastic chambers that did not contain bedding except on extinction and extinction retention days when a layer of ¼ size corn cob bedding was placed in the chamber. Eight individual

chambers were placed together on a cart so that eight animals could be transported to and from testing at one time.

The testing room contained eight identical Med Associates (St. Albans, VT) testing chambers containing a house light, a speaker, and a 19 rod (4.8 mm diameter) grid floor housed inside sound-attenuating chambers (dimensions: 30.5 x 24.1 x 21.0 cm). The testing chambers were constructed of a clear Plexiglas ceiling, back wall and door, and the aluminum modular walls of the chambers were flat paneling except for the speaker and the house light, which were placed on opposite sides of the chamber. Each sound-attenuating chamber was equipped with a ventilation fan that caused background noise in the testing chamber to be approximately 65 decibels. Each chamber was cleaned before and after each animal with a solution of 1% acetic acid (Fisher Scientific Glacial Acetic Acid A491-212) or 1% ammonium hydroxide (Fisher Scientific A669-212) on acquisition and extinction/retention days respectively. The waste pan below the grid in each chamber was filled with a small amount of the cleaning liquid to scent the chamber and allow easy cleaning of the pan after an animal was present in the chamber. Additionally, a small container of the scented cleaning liquid was placed in the testing room to scent the air in the room.

During fear conditioning in context A, a three-minute habituation period was provided for the animals to familiarize themselves with the context before beginning fear conditioning. Fear conditioning consisted of five tone-shock pairings. A 2 kHz, 80 decibel tone was played for ten seconds that co-terminated with a two second long 0.6 mA scrambled shock through the grid floor of the chamber. The inter-trial interval was one minute. Before each experiment commenced, the tones and shocks for each chamber

were calibrated so that all chambers were identical. Animals were removed from the chamber one minute after the last tone-shock pairing and returned to their home cage via the same transportation chambers described above. During fear conditioning, all lights in the hallways and in the testing room were bright white fluorescent lighting, the doors to the sound-attenuating chambers were open, the house lights inside the Med Associates chambers were white, and 1% acetic acid was used for cleaning (context A).

Twenty-four hours after fear conditioning, animals were returned to the testing room for fear extinction learning (or no-extinction) in context B. Extinction retention was tested 24 hours after extinction learning with the same context and protocol as extinction and all animals receiving tone stimuli (ABB design). During fear extinction and extinction retention, a three-minute habituation period was provided for the animals to explore the novel context before beginning fear extinction. Fear extinction consisted of 45 tone-alone presentations, and no shocks were given. The tone was exactly as presented during fear conditioning, a 2 kHz, 80 decibel tone for ten seconds. The inter-trial interval was thirty seconds. A three-minute period of silence was also left at the end of extinction. No-Extinction animals were brought to the extinction context and placed into the chambers for 35 minutes and thirty seconds, the same amount of time as the extinction animals, but were not presented with tones or shocks as a control for extinction learning. Animals were removed from the chamber and transported back to their homecage using the transportation chambers described above. During fear extinction and retention, all lights in the hallways and the testing room were red, the houselights inside the Med Associates chambers were red, the grid floor was covered with a solid piece of black

acrylic, the doors of the sound attenuating chambers were closed, and 1% ammonium hydroxide was used for cleaning to differentiate the extinction context (context B).

Twenty-four hours after extinction retention testing, animals were returned to the fear conditioning context (context A) for thirty minutes to test their acquisition of fear to the context itself. No tones or shocks were presented during this time. The context was exactly as presented during fear conditioning with white lights and 1% acetic acid used to clean the boxes (context A).

A trained observer recorded the number of fecal boluses excreted by the animal during each behavioral session, as the amount of defecation has been linked to the degree of fear experienced by the animal (Antoniadis & McDonald, 1999; Sutherland & McDonald, 1990).

Behavioral Analysis. Videos were analyzed using a computerized system (CleverSys Inc., Reston, VA) that calculates the percentage of freezing during specified times. Freezing is a species-specific fear response identified by the absence of motion with the exception of that required for respiration (Blanchard & Blanchard, 1969a, 1969b). Freezing was calculated to occur if the motion of the animal was below a specified number of the pixels in the video and remained below that threshold for two seconds. As soon as motion exceeded the threshold, the animal was considered to be moving. Escape behavior was defined as climbing, jumping, darting, or biting (on parts of the chamber including the grid floor) and was hand-scored by a trained observer blind to the condition of the animal. Using a metronome, a visual observation was taken every two seconds to record what behavior the animal was expressing at that time. The freezing behavior scored by hand correlated strongly with that scored by the CleverSys software

(R = 0.700 or greater). Area under the curve was calculated by summing the area of trapezoids between adjacent data points for each animal.

Early-life FGF2 Administration

Administration protocols were based on Turner et al. (2011). On the day after birth (post-natal day [PND] 1), dams were removed from the homecage for 8 to twelve minutes, during which pups were sexed and injected. Pups and dams remained in the same room, although they were in separate cages. Pups were placed under a heat lamp to maintain their body heat during the procedure. Litters were culled to match male and female composition of the litters, leaving no more than six pups of each sex. All pups from a litter were injected with either Fibroblast Growth Factor 2 (FGF2; 20 ng/g in 50 µl 0.1 M PBS with 0.1% BSA; 98% pure BSA from Sigma-Aldrich, St. Louis, MO A9706) or vehicle (0.1 M PBS with 0.1% BSA) subcutaneously into the axillary space. The experimenters changed gloves between each litter. The FGF2, also known as basic FGF, used in these studies is Human FGF2 that has been purified after being recombinantly expressed in e-coli (Sigma-Aldrich F0291). FGF2 was dissolved in 1 ml of 0.1 M PBS with 0.1% BSA, aliquoted and stored at -20 °C until use. On the day of injection, one aliquot of FGF2 was thawed on wet ice, diluted 8.3 fold, and kept on wet ice during injections. Pups were returned to the mother immediately after all pups were injected. Rats were weaned at PND 21, and pair-housed thereafter.

Corticosterone Assay

Blood was collected 48 hours prior to fear conditioning, and, on average, 10 minutes after conditioning. Whole blood was collected from the lateral tail vein by making a small incision toward the apex of the tail for baseline and post-fear conditioning collections while the rat was restrained lightly. No rat was incised more than three times and a new incision was made each time. Blood was collected in heparinized micro-hematocrit capillary tubes (Fisher Scientific 22-362-566) and immediately spun for 10 minutes at 4000 RPMs to separate the plasma. Five µl of plasma was collected in 1.5 ml microcentrifuge tubes and immediately frozen on dry ice. Samples were stored at -80 °C until use.

Corticosterone was detected using radioimmunoassay (RIA; MP Biomedicals, 0712103, ImmuChem Corticosterone double antibody 125 Iodine Kit for Rats and Mice, Santa Ana, CA). The lot number of kits was matched when multiple kits were ordered for a single study. All reagents were brought to room temperature and rat serum samples were thawed on wet ice. Corticosterone controls were reconstituted as per kit instructions with 2.0 mls of distilled water and allowed to sit at room temperature. Serum samples were diluted 1:200 with Steroid Diluent provided in the kit. Samples, including controls, were run in duplicate. Combining small amounts of serum from between 4 and 8 rats representing different conditions, we made a "Random Mix" control sample. This "Random Mix" control was run in duplicate across every centrifuge spin to account for technical variation. The assay was run in 12 x 75 mm borosilicate glass round bottom test tubes (Fisher Scientific 14-958-10B). 200 µl of ^{125}I was added to all corticosterone controls and samples using a repeat pipette. 200 µl of anti-corticosterone was added to all

tubes excluding the non-steroid bound controls. All tubes were mixed using a vortex and incubated at room temperature (approximately 25°C) for two hours. After incubation, 500 µl of the precipitant solution was added to all tubes, which were vortexed for 30 seconds apiece. Tubes were centrifuged in batches at 2400 RPMs for 15 minutes. The supernatant was decanted into radioactive waste, and the test tubes were blotted to remove any excess liquid. A gamma counter was used to assess the amount of ^{125}I present in each tube of the assay. The sensitivity of the corticosterone assay was 12.5 ng/ml.

The assay was analyzed using the instructions provided in the kit. The coefficient of variation was calculated for the duplicates; duplicates where the coefficient of variation was above 10% were removed from the analysis and re-run. Duplicate samples that had small coefficient of variation were averaged to generate a single value for each animal and the non-steroid bound control value was subtracted. This corrected value for each control and animal was then divided by the 0 corticosterone control corrected value and multiplied by 100 to obtain the % of bound corticosterone. A corticosterone control calibrator curve was calculated from these % bound values for each centrifuge run. Sample corticosterone values (ng/ml) were then calculated using the slope and intercept of the exponential curve defined by the corticosterone control calibrator samples.

Data Analyses

Locomotion and EPM data were analyzed using a one-way ANOVA. Corticosterone data were analyzed using a one-way ANOVA with repeated measures for collection time. Freezing and escape data were analyzed using a two-way (phenotype and extinction group) or three-way (phenotype, treatment and extinction group) ANOVA

with repeated-measures across tones. All statistics were calculated using SPSS (IBM). When Mauchly's test of sphericity was found to be significant, the conservative Greenhouse-Geisser correction was applied (Jaccard & Becker, 2009). When a given main effect or interaction met criterion for significance ($p < 0.05$), Bonferoni correction was applied to all *post hoc* comparisons.

Results

Locomotion Differentiates Selectively Bred and Outbred Phenotypes

There was a significant main effect of phenotype on locomotor response to novelty (Figure 2.1) with bHRs ($M = 1692.00$, $SD = 147.83$) > bIRs ($p < 0.001$; M = 692.00, $SD = 241.06$) > outbreds ($p < 0.001$; $M = 438.69$, $SD = 159.63$) > bLRs ($p < 0.001$; $M = 91.19$, $SD = 30.04$; $F(3, 88) = 311.28$, $p < 0.001$). All *post hoc* tests demonstrated significant differences between all rat phenotypes (Figure 2.1; $p < 0.001$).

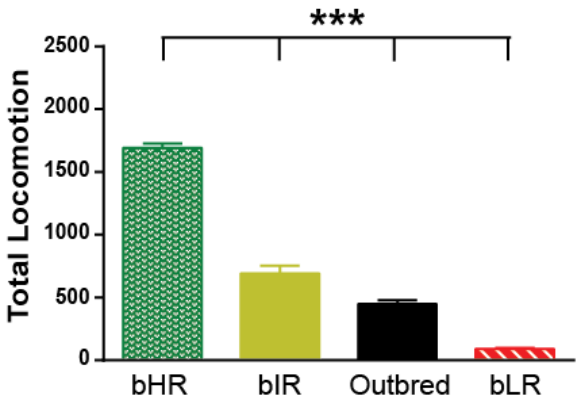

Figure 2.1 Locomotor behavior in a novel environment significantly differentiates all phenotypes. *Locomotor testing of selectively bred high responder (bHR) and low responder (bLR) rats illustrates their characteristic phenotypes, and shows how their locomotion is different from their first generation cross (bIR) or outbred Sprague Dawley rats. All phenotypes differ significantly from all other phenotypes in their locomotor response to novelty. *** = $p < 0.001$*

Experiment One.

Selectively Bred Animals Exhibit Different Defensive Behaviors During Conditioning

Outbred Sprague Dawley rats and bIR animals (the first generation cross of bHR and bLR parents) never differed in their fear behavior during conditioning, extinction, or extinction retention (Figure 2.2) and therefore were combined into a control group for all further analyses ($p > 0.05$ for all comparisons of outbreds to bIRs).

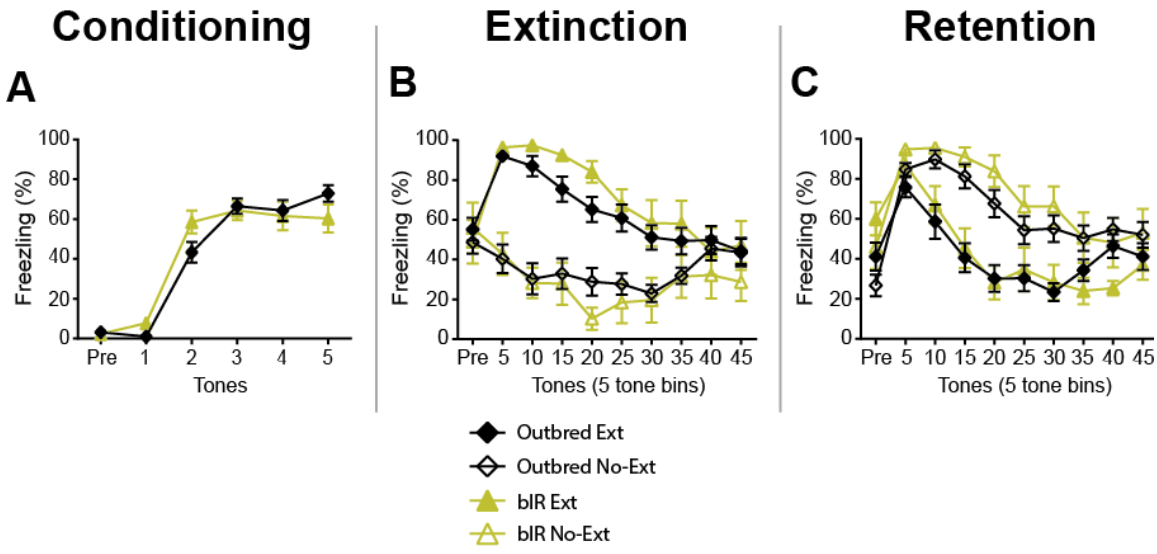

Figure 2.2 Outbred rats and a first generation cross of the selectively bred rats (bred Intermediate Responders, bIRs) demonstrate identical fear conditioning and extinction behavior. *Outbred Sprague Dawley rats and bIR rats show almost identical freezing behavior during fear conditioning (A), extinction (B), and extinction retention (C). The behavior between the two phenotypes was never significantly different, so they were combined into a single "control" group for subsequent statistical analysis and graphical display.*

During fear conditioning there was a main effect of tone where bHR, bLR and Control freezing behavior increased across the session ($F(3.54,318.56) = 69.60$, $p < 0.001$; Figure 2.3A). Surprisingly, there was a main effect of phenotype ($F(2,90) = 23.52$, $p < 0.001$; Figure 2.3A), and *post hoc* tests revealed that bHRs had lower freezing levels

than all other phenotypes ($p < 0.001$ for both bLR and control rats). There was also a tone by phenotype interaction ($F(7.08,318.56) = 4.78$, $p < 0.001$), indicating that, relative to the other groups, bHRs had lower freezing during tones 2-5 (Figure 2.3A). No other effects reached statistical significance.

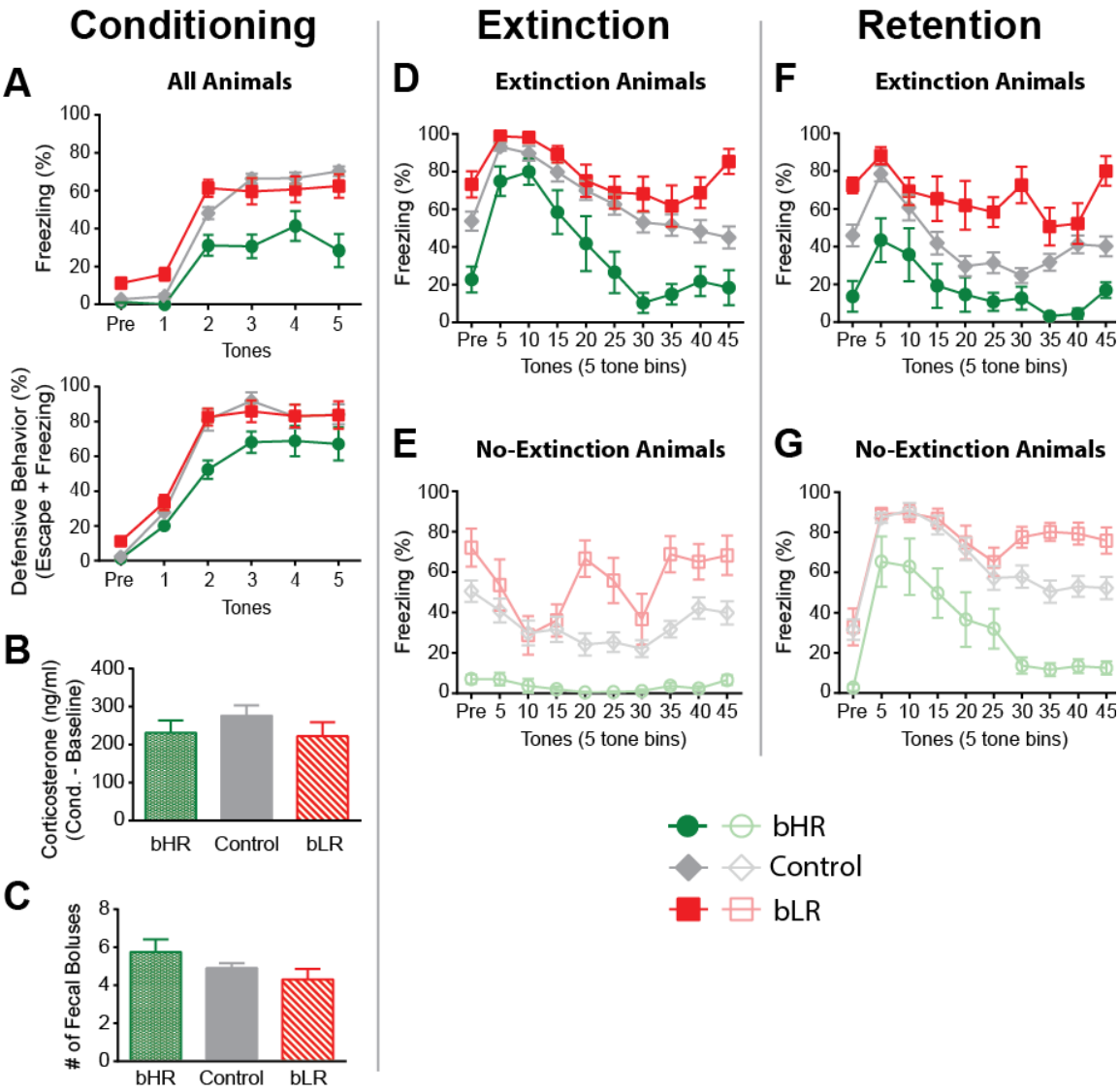

Figure 2.3 Fear conditioning in rats selectively bred for locomotion in a novel environment reveals individual differences in fear conditioning and extinction behavior. *During fear conditioning, bHR rats display a more active response to inescapable shock leading to lower levels of freezing. When escape behaviors are included with freezing as a measure of defensive behavior, bHR fear levels are more similar to those of bLR and controls (A). There are no phenotype differences in rise in corticosterone levels after conditioning (B) or number of fecal boluses excreted (C).*

During extinction learning, bHRs show facilitated within-session extinction, while bLRs show a slight reduction in extinction learning (D). No-Extinction controls show phenotype differences in freezing levels, but do not show extinction (E). bLRs show reduced extinction retention and bHRs show facilitated retention (F). The behavior of No-Extinction animals during extinction retention replicates our finding that bLRs show reduced extinction learning while bHRs show facilitated extinction learning (G). The reduced extinction learning evidenced by bLR rats indicates a more PTSD-like behavioral phenotype, while bHR rats seem to be particularly resilient.

In order to determine why bHR freezing was so low during the conditioning session, all videos were hand-scored by a trained observer for freezing and escape behaviors. Analysis of the escape behavior during conditioning showed a main effect of tones such that escape behavior increased across the session ($F(2.54, 106.74) = 8.02$, $p < 0.001$; data not shown). There was also a main effect of phenotype ($F(2,42) = 3.82$, $p = 0.03$) where bHRs had significantly higher escape behavior than bLRs (*post hoc* $p = 0.04$) but not higher than controls (*post hoc* $p = 0.12$; data not shown). Interestingly, when escape behavior is combined with freezing during fear conditioning to generate a measurement of defensive behavior, the main effect of phenotype remains ($F(2,42) = 7.22$, $p = 0.002$; Figure 2.3A).

While the lower freezing levels observed in bHRs could indicate a lower level of fear, other measures indicate that their fear level is equal to that of controls and bLRs. For graphical purposes the corticosterone data are displayed as the difference in corticosterone levels post-pre conditioning collection time (Figure 2.3B). A repeated measures ANOVA for collection time revealed that while corticosterone levels were significantly different from baseline to post-conditioning ($p < 0.001$), the main effect of phenotype was not significant, with bHRs having similarly high corticosterone levels compared to all other phenotype groups ($F(2,41) = 0.82$, $p = 0.45$). There was also no significant interaction between collection time and phenotype ($p = 0.45$). Additionally,

the main effect of phenotype on the number of fecal boluses produced during conditioning was not significant ($F(3,88) = 1.45$, $p = 0.233$; Figure 2.3C). As can be seen in Figure 2.3D, bHRs are capable of freezing to express their fear during extinction, and do so when shocks are not present in the context.

bHRs are Resilient, Demonstrating Facilitated Fear Extinction Learning, While bLRs are Vulnerable to Reduced Fear Extinction Learning and Retention

During extinction, there was a significant main effect of tones ($F(4.47, 402.62) = 11.53$, $p < 0.001$; Figure 2.3D) where freezing decreased across the session. Animals displayed higher freezing during the first two tone bins relative to subsequent tone bins (*post hoc* tests comparing tone bins 5 and 10 with all subsequent tone bins $p < 0.01$), indicating that extinction learning occurred (Figure 2.3D). There was a significant main effect of phenotype that influenced extinction learning ($F(2,90) = 23.67$, $p < 0.001$; Figure 2.3D), with bHRs displaying significantly lower freezing (*post hoc* tests $p < 0.001$) and bLRs showing significantly higher freezing (*post hoc* tests $p < 0.005$) than all other groups. There was a main effect of extinction group ($F(1,90) = 44.42$, $p < 0.001$; Figure 2.3E), where rats undergoing extinction had higher levels of freezing than No-Extinction rats (graph comparing Extinction to No-Extinction not shown). There was an interaction of extinction group with tones where the pattern of freezing for Extinction and No-Extinction rats differed throughout the session ($F(4.45, 391.83) = 19.50$, $p < 0.001$; Figure 2.3D, E). No other effects reached statistical significance.

The mildly reduced extinction learning seen in bLRs (Figure 2.3D) was confirmed during extinction retention testing, where bLRs showed very high freezing compared to

other phenotypes (Figure 2.3F). During extinction retention there was a main effect of tones ($F(3.51, 308.86) = 28.90$, $p < 0.001$; Figure 2.3F) where freezing decreased across the session. Animals demonstrated significantly higher freezing during the first two tone bins of the session relative to subsequent tone bins (*post hoc* tests comparing tone bins 5 and 10 with all subsequent tone bins $p < 0.015$). During retention there was a main effect of phenotype ($F(2,90) = 34.16$, $p < 0.001$; Figure 2.3F), where bHRs had lower freezing than controls (*post hoc* $p < 0.001$) which were significantly lower than bLRs (*post hoc* $p < 0.001$). There was a main effect of extinction group ($F(1,88) = 29.01$, $p < 0.001$; Figure 2.3F, G), where No-Extinction animals who were receiving extinction for the first time had higher freezing than Extinction animals (graph comparing Extinction to No-Extinction not shown). No other effects reached statistical significance.

Experiment Two.

Early-life FGF2 Administration Reduces Spontaneous Anxiety-like Behavior on EPM

In a second experiment, early life administration of FGF2 had no main effect on locomotor behavior ($p = 0.693$, Figure 2.4), although there remained a highly significant main effect of phenotype on locomotor behavior ($F(1,57) = 1227.26$, $p < 0.001$; Figure 2.4). *Post hoc* tests demonstrated that bHRs had significantly higher locomotor response to novelty than bLRs ($p < 0.001$).

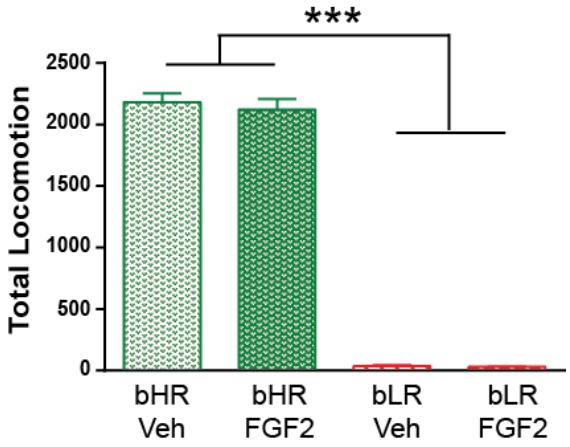

Figure 2.4 Early life FGF2 administration has no effect on locomotor behavior in a novel environment. *While large differences in phenotypic locomotor response to novelty remain, with bHRs showing greater locomotion than bLRs, there is no effect of early life FGF2 administration on either phenotype. *** = $p < 0.001$*

However, early life administration of FGF2 produced a significant main effect of treatment on the elevated plus maze (EPM; $F(1,54) = 6.88$, $p = 0.011$; Figure 2.5A), with animals that received early life FGF2 administration demonstrating significantly more time in the open arms of the EPM than rats administered vehicle. As expected, the significant main effect of phenotype remained, with bHRs spending significantly more time on the open arms than bLRs ($F(1,54) = 61.30$, $p < 0.001$; Figure 2.5A). While early

life FGF2 administration decreased spontaneous anxiety-like behavior on the EPM, it did not change the total distance traveled (main effect of treatment $p = 0.874$; Figure 2.5B).

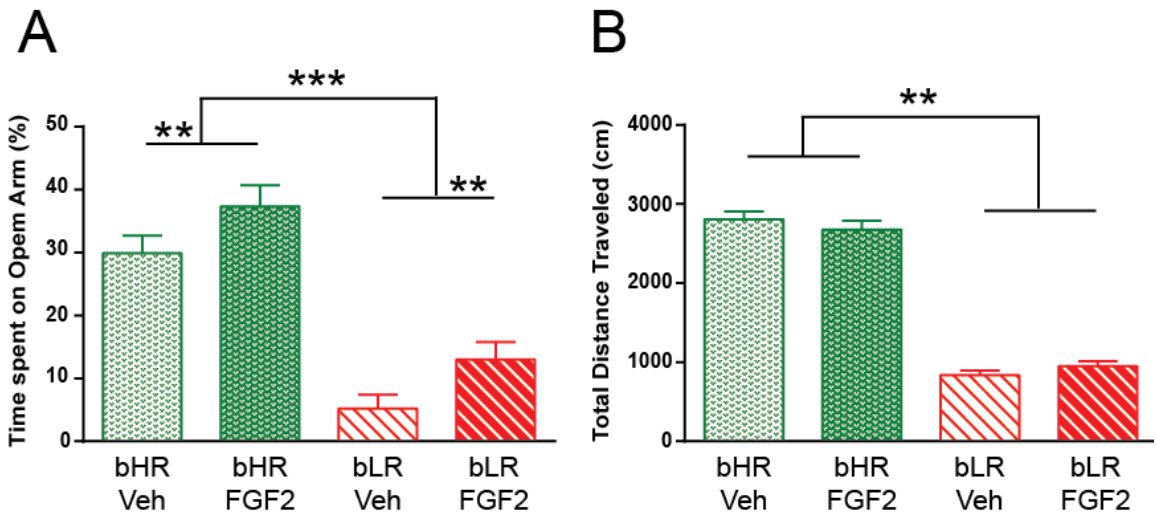

Figure 2.5 Early life administration of FGF2 reduces anxiety-like behavior but not distance traveled on the elevated plus maze. *Administration of FGF2 on the day after birth reduced anxiety-like behavior, as shown by an increase in the percentage of time spent in the open arms of the elevated plus maze for both bHR and bLR animals (A). While the phenotype difference remains clear between the two lines of animals, early life FGF2 did not change the distance traveled from vehicle levels for either bHRs or bLRs, indicating that the anxiolytic effects of FGF2 are specific and not due to a general increase in locomotor behavior (B). ** = $p < 0.01$, *** = $p < 0.001$*

Early-life FGF2 has a Subtle Effect on Fear Learning

During conditioning, there was a main effect of tones ($F(4,204) = 11.31$, $p < 0.001$; Figure 2.6A) where freezing increased across the session. Animals displayed significantly higher freezing levels to tones 2 through 5 than to tone 1 (*post hoc* $p < 0.001$), indicating that fear learning occurred. There was a significant main effect of phenotype on fear conditioning ($F(1,51) = 89.60$, $p < 0.001$), with bHRs showing lower freezing than bLRs (Figure 2.6A). There was also a significant interaction of tones by phenotype ($F(4,204) = 2.78$, $p = 0.028$), where bHRs demonstrated a different pattern of freezing than bLRs during conditioning. There was a significant interaction between

treatment and phenotype ($F(1,51) = 7.59$, $p = 0.008$) where bHRs showed an effect of FGF2 on their freezing during conditioning while bLRs did not; however, there was no main effect of treatment ($p = 0.853$; Figure 2.6A). As in Experiment One, there was a significant main effect of phenotype on escape behavior $F(1,753) = 744.32$, $p < 0.001$), with bHRs showing greater escape behavior than bLRs (data not shown). There was no effect of treatment on escape behavior, and no treatment by phenotype interaction, indicating that escape behavior did not account for the differential freezing behavior seen in early life FGF2-injected bHR animals ($p = 0.66$, and 0.38 respectively; data not shown). When freezing and escape were combined to create a measure of defensive behavior, the treatment by phenotype interaction remained mildly significant ($F(1,51) = 4.314$, $p = 0.04$; Figure 2.6B). Like Experiment One, there was no difference between phenotypes on the number of fecal boluses excreted (main effect of phenotype $p = 0.24$) indicating that by this measure, both phenotypes experienced equal levels of fear (data not shown).

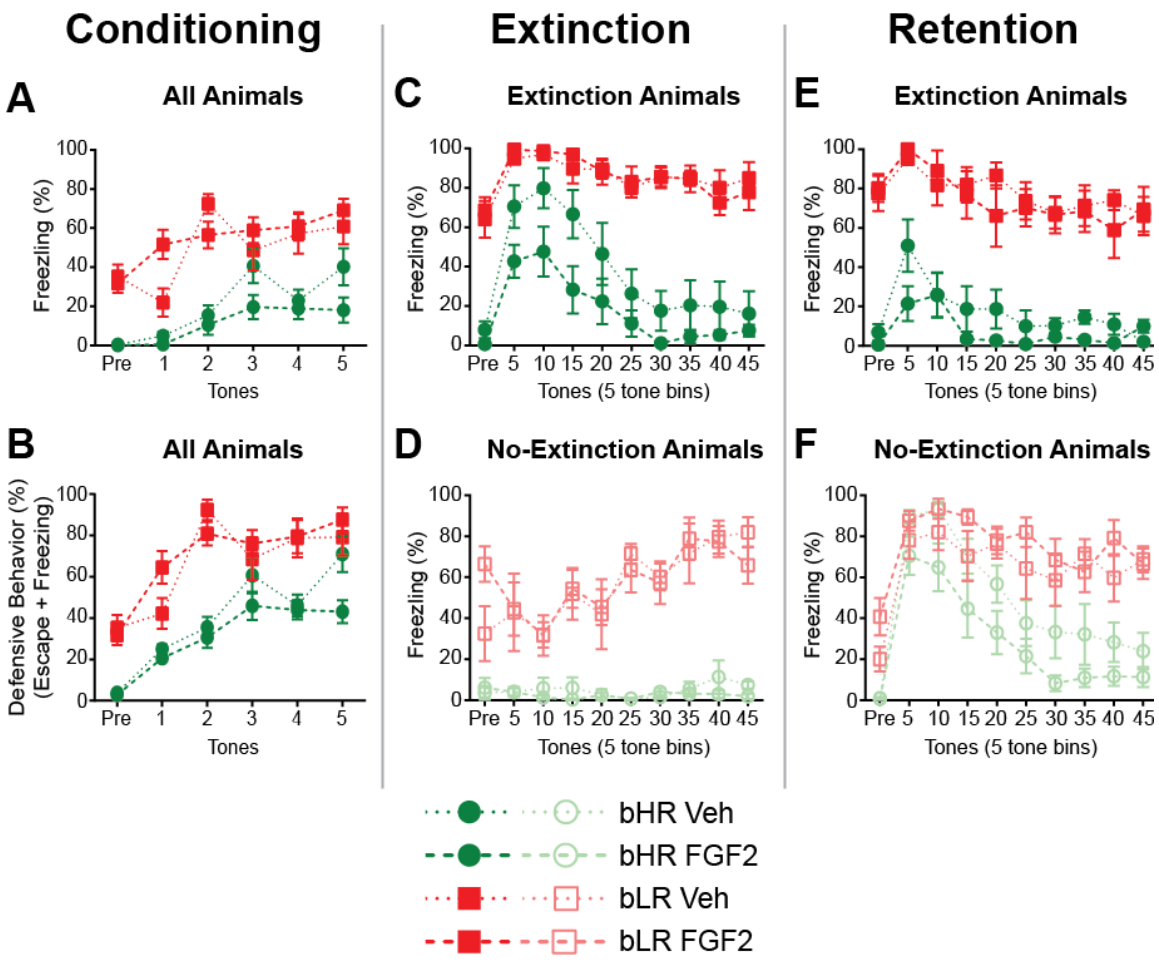

Figure 2.6 Early life administration of FGF2 facilitates extinction learning and retention only in more resilient bHR animals. *Administration of FGF2 the day after birth does not change fear learning in bLR animals; however, it does reduce freezing in bHR animals (A). Defensive behavior continues to modestly differentiate between bHR and bLR animals during conditioning, but the drug by phenotype interaction is less significant (B). During extinction, bLRs show no effect of early life FGF2, while there is a trend for bHRs given FGF2 to demonstrate facilitated extinction learning (C). There is no effect of FGF2 on No-Extinction animals during the extinction session (D). During extinction retention, bHRs given FGF2 early in life show facilitated extinction retention, while there is no effect on bLRs (E). The behavior of the No-Extinction animals during the extinction retention session replicates the finding that bHRs show facilitated extinction learning after FGF2 administration early in life, while there is no effect on bLR animals, indicating that there are individual differences in the effectiveness of early life FGF2 administration (F).*

Early-life FGF2 Administration Selectively Facilitates Fear Extinction of Resilient Animals in Adulthood

During extinction, there was a main effect of tones ($F(5.95,291.77) = 2.84$, $p = 0.011$; Figure 2.6C) where freezing decreased across the session. There was a significant tone by phenotype interaction ($F(5.95,291.77) = 10.158$, $p < 0.001$; Figure 2.6C), with bLRs maintaining a pattern of freezing throughout the session, while bHRs demonstrated extinction learning (*post hoc* tests between all tone bins by phenotype $p < 0.01$). There was a significant main effect of phenotype where bHRs displayed lower freezing than bLRs ($F(1,49) = 247.34$, $p < 0.001$; Figure 2.6C). There was a trend toward a main effect of treatment ($F(1,49) = 3.22$, $p = 0.079$) where vehicle-injected animals showed higher freezing than FGF2-injected animals, indicating that early life administration of FGF2 facilitated extinction learning in adulthood (Figure 2.6C). There was a significant main effect of extinction group where Extinction animals displayed significantly higher freezing than No-Extinction animals ($F(1,49) = 58.92$, $p < 0.001$; Figure 2.6D; graph comparing Extinction to No-Extinction not shown). There was a significant interaction between tones and extinction group where the pattern of freezing across the session differed between No-Extinction and Extinction animals ($F(5.95,291.77) = 23.007$, $p < 0.001$; graph comparing Extinction to No-Extinction not shown), indicating that extinction learning occurred in the extinction animals. While there was no significant phenotype by treatment interaction on freezing, there was a trend toward a phenotype by treatment interaction on area under the curve ($F(1,49) = 3.24$, $p = 0.078$), indicating that early life administration of FGF2 facilitated extinction learning, particularly in bHRs, over the course of the extinction session.

These effects of early life FGF2 administration were more robust during extinction retention testing because bHRs in both the Extinction and No-Extinction groups demonstrated effects of FGF2 treatment on extinction behavior. There was a significant main effect of tones ($F(4.76,233.34) = 28.00$, $p < 0.001$; Figure 2.6E), indicating that freezing once again decreased across the session. Animals demonstrated significantly higher freezing during the first two tone bins of the session relative to subsequent tone bins (*post hoc* tests comparing tone bins 5 and 10, with all subsequent tone bins $p < 0.001$). There was a significant interaction between tones and phenotype, where bHRs showed a steeper decline in freezing across the session than bLRs ($F(4.76,233.34) = 3.27$, $p = 0.008$; Figure 2.6E). There was a significant interaction between tones and extinction group, where No-Extinction animals had a higher peak and steeper curve than animals that had already undergone extinction ($F(4.76,233.34) = 2.79$, $p = 0.020$; Figure 2.6E, F; graph comparing Extinction to No-Extinction not shown). There was a significant phenotype by extinction group interaction ($F(1,49) = 12.90$, $p = 0.001$) where bHR extinction animals retained their extinction learning from the day before while bLRs showed no difference between Extinction and No-Extinction animals indicating that they did not retain extinction (Figure 2.6E, F). There was a significant tones by phenotype by extinction group interaction ($F(4.76,233.34) = 3.31$, $p = 0.008$) where the freezing pattern of the bLR Extinction and No-Extinction groups differed significantly across the session from those of the bHR Extinction and No-Extinction groups. There was a main effect of phenotype; bHRs had significantly lower freezing than bLRs overall ($F(1,49) = 134.78$, $p < 0.001$; Figure 2.6E). There was a significant main effect of extinction group; Extinction animals showed retention of their extinction

learning by having lower freezing than No-Extinction animals ($F(1,49) = 10.46$, $p = 0.002$). There was a significant phenotype by treatment interaction ($F(1,49) = 4.69$, $p = 0.035$), where early life FGF2 administration reduced freezing compared to vehicle animals for bHRs, but not bLRs, indicating that FGF2 facilitated extinction retention specifically in bHRs.

Discussion

The present work reveals that selectively bred differences in response to novelty can predict individual differences in PTSD-like behaviors. During fear conditioning, bHRs and bLRs displayed very different behavioral responses: bHRs exhibited more active escape behaviors, while bLRs exhibited higher levels of freezing behavior. This response profile has some parallels with active and passive defensive responses in humans (Blanchard, Hynd, Minke, Minemoto, & Blanchard, 2001; Harrison, Ahn, & Adolphs, 2015). Although fear conditioning in the selectively bred animals was similar to outbred animals, bLRs show reduced extinction learning and retention, a feature that is also characteristic of human PTSD patients (Blechert et al., 2007; Milad et al., 2009; Norrholm et al., 2011; Orr et al., 2000). Furthermore, the present work demonstrated that a single administration of FGF2 the day after birth is effective at facilitating extinction retention only for a subset of individuals. Early life FGF2 selectively affected bHRs by further reducing their fear and increasing their retention of extinction learning while having no effect on the more vulnerable bLRs. Collectively, these results provide a better understanding of how individual differences in temperament may influence an

individual's potential to develop PTSD, and demonstrate that a potential treatment like FGF2 may have differential effects based on temperament.

bHRs and bLRs Show Behavioral Differences During Fear Conditioning and Extinction

Our studies provide evidence that bLR rats present a unique model of vulnerability to PTSD. In humans, both a history of depressive episodes and a negative bias in cognitive tone have been linked to increased risk for developing PTSD (Bomyea et al., 2012; Orr et al., 2012). Previous studies have shown that bLRs display high levels of spontaneous anxiety- and depression-like behavior (Perez et al., 2009; Stead et al., 2006; Turner et al., 2011), and here we observe differences in their behavior during fear conditioning and extinction. bLRs showed reduced extinction learning and retention compared to outbred animals and bHRs, and in particular, bLRs show a deficit in extinction learning despite having similar levels of freezing to control animals at the start of the extinction (or retention, for No-Extinction animals) session. Reduced extinction learning has been demonstrated in human PTSD patient populations (Blechert et al., 2007; Norrholm et al., 2011; Orr et al., 2000), and in one case has been shown to be predictive of future onset of PTSD (Lommen et al., 2013). We therefore interpret the reduced extinction learning and retention in our bLR rats as PTSD-like behavior, indicating that these individuals have an increased propensity to develop PTSD-like symptoms.

In contrast, bHR animals may provide a model of resilience to developing PTSD. During conditioning, bHRs exhibited more active escape responses during the

conditioning session. Interestingly, active coping in humans correlates with reduced PTSD symptoms in a study of trauma-exposed veterans (Contractor, Armour, Shea, Mota, & Pietrzak, 2015). Similarly, we observed facilitated extinction learning in bHR animals, indicating that active coping during fear conditioning may contribute to lower levels of fear retention and PTSD-like behavior afterward. Additionally, it has recently been shown that outbred animals with higher levels of endogenous FGF2 in the hippocampus show lower levels of fear in contextual and cued fear conditioning (Graham & Richardson, 2016). The more resilient bHR animals have high endogenous expression of FGF2 in the hippocampus (Perez et al., 2009), which may at least partly mediate their lower levels of freezing and their facilitated extinction. Overall, bHRs demonstrate better extinction learning and retention than controls, indicating that they are particularly resilient to PTSD-like behaviors.

While the bHRs and bLRs represent more extreme responses to fear conditioning and extinction learning, it is interesting to note that a first generation cross (bIRs) displays normal behavior indiscriminable from that of outbred animals. This indicates that although the parents are extreme, a heterozygous cross of these selectively bred animals provides a good measure of typical rat behavior. Additionally, the bIRs provide evidence that while the bHRs and bLRs might represent extreme phenotypes of vulnerability and resilience, their temperament and genetic makeup are not extremely different from outbred animals. In other words, the behavior of bIR animals would not match that of outbred animals so closely if their parents were not simply extreme ends of the outbred temperament spectrum. Our selectively bred model therefore provides a useful way of studying individual differences in vulnerability and resilience to PTSD-like

behaviors while also providing an internal control of selectively bred animals that behave like outbred animals.

Since our animals are selectively bred according to their locomotor response to a novel environment, movement in the novel fear conditioning chambers could be interpreted as a potential confound in these studies. However, our observations suggest that this confound cannot fully explain our results. While bLRs move less in a novel environment than outbred animals, they are not usually freezing (their pre-period freezing levels prior to fear conditioning are below 20%). Post-shock freezing levels are very high in bLRs, supporting the interpretation that they use passive coping responses. However, their freezing decreases during the extinction session, indicating that their immobility is likely due to fear and not purely due to their phenotypic lack of locomotion. Likewise, facilitated fear extinction in bHRs is not solely due to their increased locomotion, as their freezing levels at the start of the extinction session are very close to those of outbred animals. While we believe that the freezing behavior described here provides strong evidence for phenotypic differences in fear behavior, future studies can further assess differences in response to fear stimuli in these selectively bred rodents by using fear-potentiated startle and measures of heart rate.

Early Life FGF2 Facilitates Fear Extinction Only in Resilient bHR Animals

As we develop better models of individual differences in PTSD-like behavior, we can investigate the role of those intrinsic differences in responsiveness to potential prevention or treatment options. Graham and Richardson (2009, 2010) have previously reported that administration of FGF2 immediately before extinction learning in rats

enhanced extinction learning and retention as well as reduced the return of fear after extinction. This provides strong evidence that FGF2 might be a promising pharmacological tool to enhance extinction learning (Graham & Richardson, 2015). We have previously shown that FGF2 can reduce anxiety-like behavior in adults (Perez et al., 2009), and also plays an organizational role in development to change emotional responsiveness and epigenetic factors that mediate lifelong changes in reactivity to the environment (Chaudhury et al., 2014). We therefore asked whether FGF2 could also have an organizational influence on fear extinction when administered at a developmental timepoint.

We assessed whether a single administration of FGF2 the day after birth might enhance extinction learning and retention in adulthood, and whether there were differing effects on individuals of different temperaments. We replicated our previous finding that early life administration of FGF2 significantly reduces spontaneous anxiety on the elevated plus-maze (Turner et al., 2011). While this effect of early life FGF2 on spontaneous anxiety was seen in both bHRs and bLRs in the current study, contrary to our hypothesis, only bHRs demonstrated any effect of FGF2 on their fear extinction behavior. bHRs showed a mild enhancement of extinction learning, and a significant enhancement of extinction retention with FGF2 treatment, indicating that early life administration of FGF2 reduced fear responses in resilient individuals. In contrast, early-life FGF2 treatment had no effect on bLR behavior during extinction or retention, indicating that early life administration of FGF2 was unable to exert significant effect on the extinction deficit of vulnerable individuals. Interestingly, Graham and Richardson (2009) previously showed that administration of FGF2 did not enhance extinction

retention when extinction learning did not occur. This may partially explain why bLRs show no effect of early life FGF2 administration: because they show little evidence of learning extinction, FGF2 could not enhance their retention. While the underlying cause remains unclear, these data demonstrate a clear difference in how early life administration of FGF2 affects fear extinction learning in adulthood, with only resilient animals demonstrating facilitated extinction learning and retention after FGF2 administration.

Although early life administration of FGF2 had no effect on extinction in the vulnerable bLR animals, there remain other possibilities for testing pharmacological enhancers of extinction such as d-cycloserine, which has often been shown to facilitate extinction in rats (Fitzgerald, Seemann, & Maren, 2014) and to be effective at reducing PTSD behaviors in humans (Difede et al., 2014). Future studies should also demonstrate whether these individual differences in fear learning and extinction learning and retention also differentiate responses to the return of fear after extinction. For example, it is unclear whether bHRs would demonstrate renewal (the return of fear in a new context) or reinstatement (the return of fear after experiencing a fear reminder) compared to outbred animals. While the genetic causes of the differences in emotional responsiveness of the bHRs and bLRs are currently under study, it is likely that a host of genes may contribute to the differences in vulnerability and resilience in the two lines. In some cases, while the intrinsic vulnerability factors may not be reversible, counter-regulatory mechanisms may be used to compensate for these vulnerability factors and induce resilience. Thus, further study of the bHR animals may lead to insights into the molecular and neural

underpinnings of their resilience that can be used to manipulate the vulnerability shown by bLRs.

Summary

We have developed an animal model that demonstrates differences in PTSD-like behavior that are markedly similar to the differences observed in humans. We also demonstrated that a single administration of FGF2 early in life facilitates extinction in the resilient animals, although it fails to do so in vulnerable animals. This was contrary to our hypothesis, but suggests that it is important to tailor the treatment or prevention strategy to the existing propensity of the individual being treated. Future work will examine how the model resembles human disease and whether other interventions may be effective at reducing the behavioral vulnerability that bLRs exhibit.

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Chapter 3.

In-group Social Context Enhances Extinction Learning for Both Outbred Rats and Rats Selectively Bred for Their Locomotor Response to a Novel Environment

While the majority of American adults will experience a traumatic event in their lifetime, only a small percentage will actually go on to develop post-traumatic stress disorder (PTSD; Kessler, Sonnega, Bromet, Hughes, & Nelson, 1995), indicating that there are likely individual differences that influence vulnerability. Individual differences may also play a role in the response to both behavioral and pharmacological therapies for PTSD. For both types of therapy, only approximately 60% of PTSD patients respond, indicating that there may be individual characteristics that promote higher or lower efficacy of the therapeutic intervention (Cusack et al., 2016; Stein, Ipser, & Seedat, 2006). It is therefore important that we not only explore the individual differences that might lead to the increased vulnerability and treatment resistance seen in some individuals, but that we fully understand how environmental influences interact with individual differences to produce these effects.

Our rodent model of individual differences provides a promising way to explore the interaction of heritable individual differences with environmental manipulations. In the preceding chapter of this dissertation, we demonstrated that our rodent model of individual differences in "temperament" (a consistent way of responding to environmental stimuli) also exhibited stable and differential fear conditioning and

extinction behavior. One line of these selectively bred animals demonstrated reduced extinction learning, a characteristic of PTSD patients (Lommen, Engelhard, Sijbrandij, van den Hout, & Hermans, 2013; Milad et al., 2008; Milad et al., 2009). We consider our animal model a "genetic model" because the temperament characteristics of the rats are heritable and stable across generations, and are likely due to biological underpinnings that remain to be identified. Since temperament may be a better predictor of heritable vulnerability to mental health disorders in humans (Kendler & Myers, 2014; Kendler, Myers, Maes, & Keyes, 2011; Wolf et al., 2010), our animal model is particularly useful for understanding the interplay between "genes" and the environment, including exposure to trauma, which is a requirement to diagnose PTSD (Friedman et al., 2011). Almost by definition then, heritable propensities, such as temperament, that promote vulnerability or resilience in certain individuals are likely to interact with environmental factors (like trauma) to predict the development of PTSD. However, little is known about these interactions in humans or in animal models.

Social support is one environmental manipulation relevant to PTSD. Observations that social support facilitates recovery from stress led to the social buffering hypothesis, which posits that social support can mediate the effects of stress on an individual (Cobb, 1976; Cohen & Wills, 1985; Eisenberger, 2013a, 2013b). Social support was also linked directly to PTSD, with the presence of social support facilitating a reduction in PTSD symptoms and lowering the probability of developing PTSD (Bomyea, Risbrough, & Lang, 2012; Ozer, Best, Lipsey, & Weiss, 2003; Wilcox, 2010). Given that individual differences likely influence the efficacy of both the behavioral and pharmacological therapeutic interventions available for PTSD, it would not be surprising to find that there

are also individual differences in response to social buffering. We do not currently understand how these individual differences might or might not interact with an environmental manipulation like social buffering.

Environmental manipulations like social support may be useful to improve the efficacy of therapy, especially for vulnerable individuals. To our knowledge, only two studies have investigated the effects of social support on therapy efficacy (Price, Gros, Strachan, Ruggiero, & Acierno, 2013; Thrasher, Power, Morant, Marks, & Dalgleish, 2010). In both studies, one a civilian sample and another a military sample, social support positively influenced the results of therapy, indicating that social support may play a role in the ability of individuals to recover after trauma (Price et al., 2013; Thrasher et al., 2010). Interestingly, some research indicated that social support declines with increasing PTSD symptoms, suggesting that the dynamics of social support interactions may be more complicated than a simple social buffering model would suggest (King, Taft, King, Hammond, & Stone, 2006; Pietrzak, Johnson, Goldstein, Malley, & Southwick, 2009). It may be that the interactions of environmental factors like social support with genetic or temperament dispositions are more important than environmental factors alone for understanding why and how PTSD symptoms change. While the majority of this research points to positive effects of social support that may influence the resilience of an individual to developing PTSD, it is still unclear how this environmental variable interacts with individual genetic predisposition to reduce PTSD symptoms and vulnerability.

Studies in animals provide more control over the genetic and environmental variables to be manipulated in a given study, and social support is a relevant

manipulation for animal studies as well. Animals, like humans, display social buffering by demonstrating reduced fear responses when in a group with at least one other individual. This effect has been seen across a variety of species including macaques, marmosets, squirrel monkeys, cats, mice, and rats (Davitz & Mason, 1955; Guzman et al., 2009; Hennessy, Chun, & Capitanio, 2016; Liu et al., 2013; Rukstalis & French, 2005; Stanton, Patterson, & Levine, 1985; Young, Majolo, Heistermann, Schulke, & Ostner, 2014). While some of these studies demonstrated social buffering of stress in the natural environment (Young et al., 2014), many of these studies use fear conditioning to manipulate stress levels of individual animals (Guzman et al., 2009; Kiyokawa, Hiroshima, Takeuchi, & Mori, 2014; Kiyokawa, Honda, Takeuchi, & Mori, 2014; Kiyokawa, Kikusui, Takeuchi, & Mori, 2004; Stanton et al., 1985). A model of trauma encoding, fear conditioning is a paradigm in which an emotionally neutral stimulus such as a tone is repeatedly paired with an aversive stimulus such as a mild footshock (LeDoux, 2000). This repeated pairing leads an individual to express fear to the tone stimulus even when the aversive stimulus is not present, and leads to enduring memories of the fearful association (LeDoux, 2000). The ability of social support to modulate fear and extinction behavior in animals suggests that it is a useful environmental manipulation to examine gene-by-environment interactions.

The majority of studies in animals have assessed the efficacy of social support during or shortly after fear conditioning. A number of studies demonstrated that having another individual (a conspecific) present when a conditioned animal is reintroduced to the feared stimulus reduces the amount of fear expressed by the conditioned animal (Kiyokawa, Honda, et al., 2014; Kiyokawa et al., 2004; Kiyokawa, Kodama, Takeuchi, &

Mori, 2013; Stanton et al., 1985; Takahashi et al., 2013). These studies also showed that physical interaction was not necessary for the social buffering effect to occur (Kiyokawa, Hiroshima, et al., 2014; Kiyokawa, Honda, et al., 2014; Kiyokawa et al., 2013; Rukstalis & French, 2005; Takahashi et al., 2013). In fact, olfactory stimuli are sufficient, and familiar conspecifics (and mates in particular) were potent stimuli to induce social buffering of the fear response after conditioning (Kiyokawa, Hiroshima, et al., 2014; Kiyokawa, Honda, et al., 2014; Kiyokawa et al., 2013; Rukstalis & French, 2005; Takahashi et al., 2013). Having a conspecific present during fear conditioning also reduced fear responses to the presentation of the fear stimulus after conditioning (Lee & Noh, 2016). Additionally, Guzmán and colleagues (2009) showed that observing other mice acting in a non-fearful manner reduced subsequent fear conditioning to a context in a long-lasting manner. Together, these studies demonstrated that interacting with, observing, or smelling other individuals, especially familiar ones, can reduce fear across paradigms and species. Despite this, we have little insight into how individuals with different temperaments or genetic predispositions might influence each other during fear conditioning.

Fewer studies have investigated the effects of social buffering on extinction learning in animals. Extinction is the process by which fear to a conditioned stimulus is reduced by repeated presentations of the tone without the footshock, or other aversive stimulus (Maren, 2011). This procedure is a good model of exposure therapy in humans (Bowers & Ressler, 2015; Milad, Rosenbaum, & Simon, 2014). Thus, understanding the effects of social support on extinction processes would carry important translational implications. Nowak and colleagues (2013) showed that observing a conspecific

demonstrate freezing significantly increased freezing in rats that had previously been extinguished, while freezing remained low in rats that had been extinguished and observed a conspecific also showing low levels of fear. While these results indicate that social interaction can influence behavior during the extinction of fear, the authors did not investigate whether a non-extinguished rat would show reduced freezing when observing a conspecific demonstrating low levels of fear. Since these results do not fully investigate the idea of social buffering, it is still unclear how the behavior of other individuals might reduce fear, or enhance extinction, during learning. Furthermore, no studies have examined the effects of individual differences in temperament on social buffering during extinction, or how the two might interact.

While social buffering may be a useful environmental manipulation to investigate vulnerability to PTSD-like behavior, investigating the neural correlates of this environmental variable is important for developing increased understanding of how this variable impacts behavior. Several studies in animals have demonstrated a role for specific brain regions in social buffering. A study that examined brain activity where animals were pair-housed after fear conditioning demonstrated increased basal amygdala and periaqueductal grey (PAG) activation as determined by the immediate early gene cFos (Kiyokawa, Takeuchi, & Mori, 2007). Another study showed greater lateral amygdala and infralimbic (IL) prefrontal cortex cFos activity when animals were both pair housed and re-exposed to the conditioned stimulus in pairs (Kiyokawa et al., 2007). Both of these behavioral procedures reduced behavioral fear responses to the conditioned stimulus, but activated slightly different brain regions. This indicates that while amygdala, PAG and prefrontal cortex may be some of the neural correlates of social

buffering, different brain regions may play a role under different experimental conditions. In contrast to these results of increased amygdala activation, another study using electrophysiology to directly measure neuronal firing observed reduced lateral amygdala activity during social buffering (Fuzzo et al., 2015). An additional study using Zif268 as a marker of neuronal activity demonstrated reduced basolateral (BLA) and medial amygdala activity when the social partner was familiar compared to an unfamiliar partner (Hodges, Green, Simone, & McCormick, 2014). These data indicate that the BLA is likely involved in social buffering, but whether the activity is suppressed or increased may depend on the nature of the social buffering paradigm. Several additional regions including the lateral septum, and the posteromedial region of the olfactory peduncle have also been implicated in the effects of social buffering, suggesting that a larger network of brain regions may underlie the behavioral effects (Guzman et al., 2014; Kiyokawa, Wakabayashi, Takeuchi, & Mori, 2012). While it appears that amygdala and prefrontal cortical areas are the primary brain regions involved in the social buffering response, it remains unclear how certain brain regions respond to different social paradigms, and whether those brain regions would also be important for social buffering of extinction learning rather than simply fear expression.

Despite evidence that social support can buffer the effects of stress and that medial prefrontal cortex and BLA are involved in social buffering in rodents, it remains unknown how heritable individual differences might influence the efficacy of social support and change brain activity accordingly. We employed selectively bred high responder (bHR) and low responder (bLR) animals, which we previously determined to be a model of differential vulnerability to PTSD-like behavior. *In the current studies, we*

assess how an environmental manipulation such as social buffering influences the genetic propensity of these selectively bred animals to be vulnerable or resilient to PTSD-like behavior and the neural correlates of this gene-by-environment interaction.

In Experiment One, we experimentally manipulated the social context of the rats to examine whether this variable would affect extinction behavior in outbred and selectively bred animals. We hypothesized that in-group phenotype social context might facilitate extinction learning within the extinction session for outbred Sprague Dawley animals. However, we expected to see no change in fear extinction behavior in the selectively bred bHR and bLR rats since their propensity to extreme behavioral responses is strong. In Experiment Two, we manipulated the locomotor phenotype of the selectively bred animals and observed the interaction of this change in "genetic load" with the social context along with the neural correlates of this interaction. We hypothesized that there might be an interaction of genes with environment where reducing the heritable anxiety of bLRs along with providing them an in-group phenotype social context during extinction might facilitate extinction learning while there would be an effect of environment but no gene-by-environment interaction for bHRs. We hypothesized that the infralimbic prefrontal cortex and basolateral amygdala would show changes in activity of the immediate early gene cFos based on the social context of extinction for bLR and bHR rats.

Methods

Experiment One.

Animals.

Outbred Rats. Seventy-two male Sprague Dawley rats from Charles River Laboratories weighing approximately 225-250 grams were compared with selectively bred rats from our colony. These outbred rats underwent locomotor testing (described below) after which we divided the population evenly into thirds and labeled them as HR, LR and Intermediate (IR) phenotypes. Forty-eight of the outbred rats identified as outbred HRs (oHR) or outbred LRs (oLR) were divided into in-group phenotype (N=8 per phenotype), mixed phenotype (N=8 per phenotype) and isolated (N=8 per phenotype) social context groups (depicted in Figure 3.1). IRs were not used in these studies.

Selectively Bred Rats. Selectively bred rats were originally Sprague Dawley outbred animals continuously bred with partners chosen based on their locomotion in a novel environment as described previously (Stead, et al., 2006). Forty selectively bred animals (20 bHRs and 20 bLRs) from generation 43 were divided into in-group phenotype (N=8 per phenotype), mixed phenotype (N=8 per phenotype) and isolated (N=4 per phenotype) social context groups (Figure 3.1).

All animals participating in this study were pair-housed (with non-littermates when known) on a 12/12 hour light-dark schedule with access to food and water *ad-libitum*. Animals were handled for two minutes a day for at least five days prior to beginning behavioral testing to acclimate the animals to the experimenter. The University Committee on the Use and Care of Animals (UCUCA) at the University of Michigan approved all protocols and procedures.

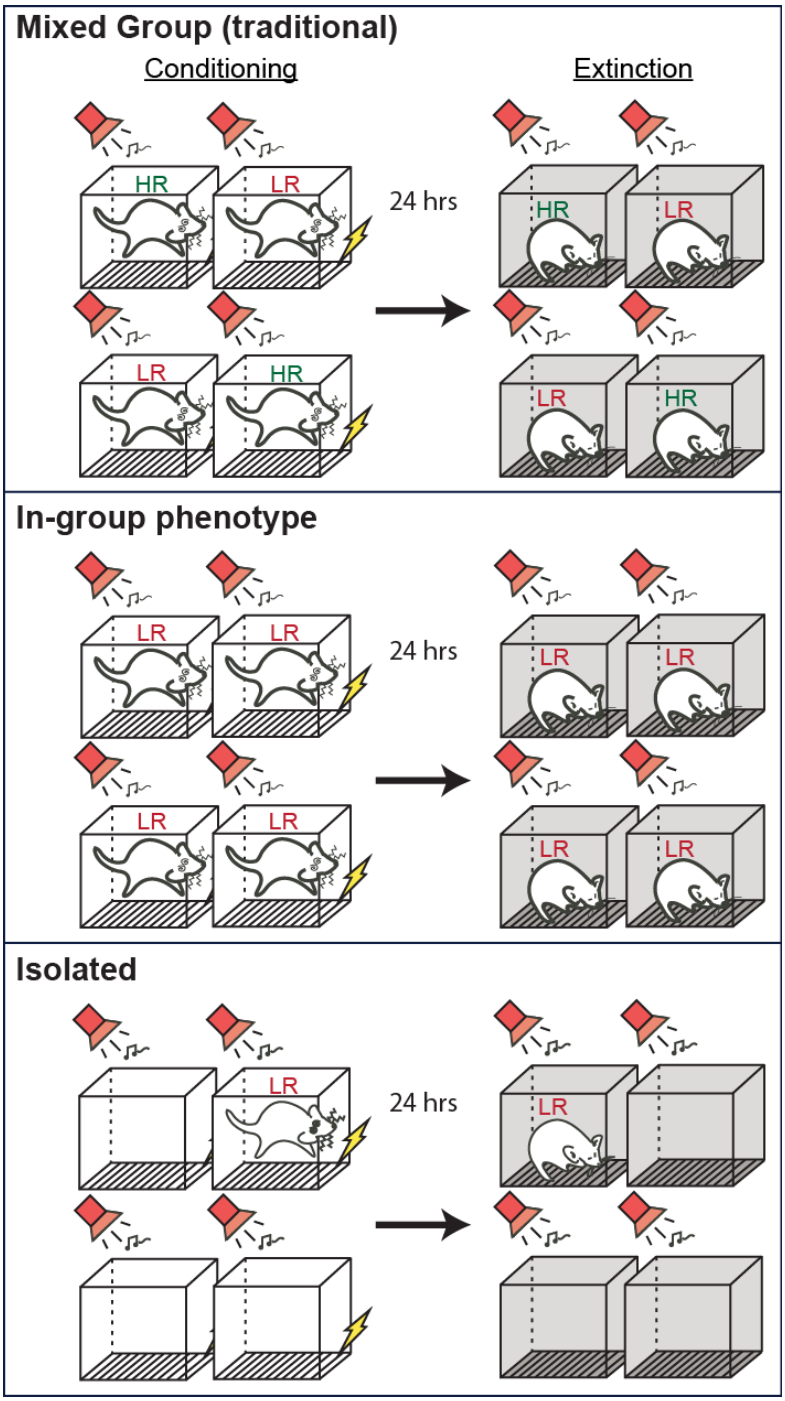

Figure 3.1 Experimental design. *These graphical representations demonstrate the different social context conditions outlined in this chapter. In the mixed group social context, all the testing chambers were filled and a mixture of phenotypes were present (equal numbers of HR and LR rats). In the in-group phenotype condition, all the chambers were filled with rats from the same phenotype. In the isolated condition, only one in four chambers contained a rat. The graphical representations demonstrate half of the chambers and rats that were present in the room during testing. These conditions were identical for both outbred and selectively bred rats.*

Behavior

Locomotor Testing. Rats were placed in a 43 x 21.5 x 25.5 cm clear acrylic cage with a stainless steel grid floor for one hour. The cages are separated by black acrylic dividers and micro-isolator cage tops so that rats cannot see or smell each other. Horizontal (walking/running) and vertical (rearing) locomotor activity was monitored continuously and recorded every five minutes by two panels of photocells that assess beam breaks. Horizontal and vertical locomotor measures were added together to create a total locomotion score for each individual animal. Between animals, the cages were cleaned with 70% ethanol. The locomotion-testing equipment and software was developed in-house at the University of Michigan. All locomotor testing took place between 0800 and 1200 hours.

Fear Conditioning and Extinction. Fear conditioning and extinction parameters were very similar to those described in Chang et al. (2009). Animals were randomly assigned to conditions in these studies, and fear conditioning began when animals were approximately 67 days of age. In brief, animals were transported to the testing room in individual plastic chambers that did not contain bedding except on extinction and extinction retention days when a layer of ¼ size corn cob bedding was placed in the chamber. Eight individual chambers were placed together on a cart so that eight animals could be transported to and from testing at one time. For the in-group and mixed phenotype conditions, each transport chamber contained a rat of the appropriate phenotype (all rats of a single phenotype in the in-group condition, mixed phenotypes for the mixed social context condition). Rats in the isolated condition were transported to the testing room individually.

The testing room contained eight identical Med Associates testing chambers containing a house light, a speaker, and a 19 rod (4.8 mm diameter) grid floor housed inside sound-attenuating chambers (dimensions: 30.5 x 24.1 x 21.0 cm; St. Albans, VT). The testing chambers were constructed of a clear Plexiglas ceiling, back wall and door, and the aluminum modular walls of the chambers were flat paneling except for the speaker and the house light, which were placed on opposite sides of the chamber. Each sound-attenuating chamber was equipped with a ventilation fan that caused background noise in the testing chamber to be approximately 65 decibels. For the in-group phenotype and mixed phenotype conditions, all eight chambers were filled with a single rat each of the appropriate phenotype group (Figure 3.1). Rats in the isolated condition were placed in chambers the greatest distance apart in the testing room to provide as little social interaction as possible (Figure 3.1).

Each chamber was cleaned before and after each animal with a solution of 1% acetic acid (Fisher Scientific Glacial Acetic Acid A491-212) or 1% ammonium hydroxide (Fisher Scientific A669-212) on conditioning or extinction days respectively. The waste pan below the grid in each chamber was filled with a small amount of the cleaning liquid to scent the chamber and allow easy cleaning of the pan after an animal was present in the chamber. Additionally, a small container of the scented cleaning liquid was placed in the testing room to scent the air in the room. Behavioral testing occurred between 0830 and 1600 hours. During testing, video was collected of each animal. The number of fecal boluses excreted by the animal during testing was counted after each animal was removed from the chamber.

Fear conditioning parameters were identical to those described in Chapter 2 of this dissertation. In brief, during fear conditioning in context A, a three minute habituation period was provided for the animals to familiarize themselves with the context before beginning fear conditioning. Fear conditioning consisted of five tone-shock pairings. A 2 kHz, 80 decibel tone was played for ten seconds that co-terminated with a two second long 0.6 mA scrambled shock through the grid floor of the chamber. The inter-trial interval was one minute. Before each experiment commenced, the tones and shocks for each chamber were calibrated so that all chambers were identical. Animals were removed from the chamber one minute after the last tone-shock pairing and returned to their home cage via the same transportation chambers described above. During fear conditioning, all lights in the hallways and in the testing room were bright white fluorescent lighting, the doors to the sound-attenuating chambers were open, the house lights inside the Med Associates chambers were white, and 1% acetic acid was used for cleaning (context A).

Twenty-four hours after undergoing fear conditioning, animals were returned to the testing room for fear extinction learning in context B (AB context design). Extinction parameters were identical to those described in Chapter 2 of this dissertation. In brief, during fear extinction, a three minute habituation period was provided for the animals to explore the novel context. Forty-five tone-alone presentations followed the habituation period, no shocks were given. The tone was exactly as presented during fear conditioning, a 2 kHz, 80 decibel tone for ten seconds. The inter-trial interval was thirty seconds. A three minute period of silence was also left at the end of extinction. Animals were removed from the chamber and transported back to their homecage using the

transportation chambers described above. During fear extinction, all lights in the hallways and the testing room were red, the houselights inside the Med Associates chambers were red, the grid floor was covered with a solid piece of black acrylic, the doors of the sound attenuating chambers were closed, and 1% ammonium hydroxide was used for cleaning (context B).

Behavioral analysis. Videos were analyzed using a computerized system (CleverSys Inc., Reston, VA) that calculates the percentage of freezing during specified times as described previously in Chapter 2 of this dissertation.

Data Analyses

Locomotion data were analyzed using a one-way ANOVA. Freezing data were analyzed using a two-way (phenotype by social context condition) ANOVA with repeated-measures across tones. Statistics were calculated using SPSS (IBM). When Mauchly's test of sphericity was found to be significant, the conservative Greenhouse-Geisser correction was applied (Jaccard & Becker, 2009). When a given main effect or interaction met criterion for significance ($p < 0.05$), Bonferoni correction was applied to post hoc comparisons.

Experiment Two.

Animals

Outbred Rats. Outbred rats were controls for the neural correlates analysis described later. Eight male Sprague Dawley rats from Charles River Laboratories weighing approximately 225-250 grams were compared with selectively bred rats from

our laboratory colony. Outbred rats underwent locomotor testing and were identified as oHRs or oLRs. These animals were run in mixed-phenotype groups (Figure 3.1) and received either Extinction (N=4) or No-Extinction (N=4) conditions.

Selectively Bred Rats. Selectively bred rats were originally Sprague Dawley outbred animals continuously bred with partners chosen based on their locomotion in a novel environment as described previously (Stead, et al., 2006). Thirty-two male selectively bred animals from generation 46 of our colony (16 bHR and 16 bLR) were divided into in-group phenotype (N=8 per phenotype) and mixed phenotype (N=8 per phenotype) social context groups (Figure 3.1). The isolated social context condition was not used in this study. Of note, the selective breeding schema for generation 46 was slightly different from that previously described by Stead and colleagues (2006) and by previous studies in this dissertation. Instead of selecting the most extreme individuals from the population produced by the colony to breed, individuals with locomotor scores closer to the average for their phenotype were bred together to produce the offspring used in this study.

All animals participating in this study were pair-housed (with non-littermates when known) on a 12/12 hour light-dark schedule with access to food and water *ad-libitum*. Animals were handled for two minutes a day for at least five days prior to beginning behavioral testing to acclimate the animals to the experimenter. The University Committee on the Use and Care of Animals (UCUCA) at the University of Michigan approved all protocols and procedures.

Behavior

Behavioral procedures were identical to those described in Experiment One of this chapter (above). The only difference is that rats in this study were sacrificed 15 minutes after the end of the extinction session. Brains were extracted, immediately flash frozen using 2-methylbutane, and stored at -80ºC until use.

Tissue Analysis

mRNA in situ hybridization (ISH). All *in situ* probes were synthesized in-house; the rat mRNA sequence used for generating the probe was complimentary to the RefSeq database number for cFos: X06769.1. All cDNA segments were extracted (Qiaquick Gel Extraction kit, Qiagen, Valencia, CA), subcloned in Bluescript SK (Stratagene, La Jolla, CA) and confirmed by nucleotide sequencing. The probes were labeled in a reaction mixture of 2µl of linearized plasmid specific to the probe of interest, 10X transcription buffer (Epicentre Technologies, Madison, WI), 125µCi of ^{35}S-labeled UTP, 125µCi of ^{35}S labeled ATP, 150µM CTP and GTP, 12.5mM dithiothreitol, 1µl of RNAse inhibitor, and 1µl of T7 RNA polymerase and incubated at 37ºC for 1.5 hours. Labeled probes were purified on Micro Bio-Spin 6 Chromatography Columns (BioRad, Berkeley, CA) according to the manufacturer's instructions. An additional 1µl of 1M dithiothreitol was added to the labeled mRNA after purification and the mixture was stored at -20ºC overnight.

Ten-micron sections were taken every 100 µm and mounted onto Superfrost Plus slides (Fisher, Waltham, MA) at -20°C using a cryostat. Slide-mounted tissue was fixed in 4% paraformaldehyde solution for 60 minutes, washed three times for five minutes

each with 2X SSC (1X SSC is 150 mM sodium chloride and 15 mM sodium citrate), and treated with 0.1M triethanolamine with 0.25% acetic anhydride for 10 minutes. Slides were rinsed for half an hour and then dehydrated in graded ethanols before air-drying. After air-drying, slides were treated with hybridization buffer containing the labeled probe (2-2.5 x 10^6 counts/75 μL buffer; 50% formamide, 10% dextran sulfate, 3X SSC, 50 mM sodium phosphate buffer (pH = 7.4), 1X Denhardt's solution, 0.1 mg/ml yeast tRNA, and 10 mM dithiothreitol). All slides were cover-slipped and stored in humidified chambers at 55°C during the approximately 16 hour hybridization period. After hybridization, sections were washed three times for five minutes each in 2X SSC and incubated in an RNase solution (100 μg/mL RNase in Tris buffer with 0.5M NaCl, pH=8) at 37ºC for one hour. Sections were then sequentially washed in 2X, 1X, and 0.5X SSC for five minutes each before being incubated in 0.1X SSC at 65ºC for 1 hour. Sections were rinsed in distilled water and dehydrated through graded ethanols. Slides were exposed to Kodak BioMax MR Scientific Imaging Film (Sigma Aldrich), and the 4 week exposure time was experimentally determined for optimal signal.

Autoradiograph Quantification Procedures. mRNA expression signals from autoradiographic films were quantified using the computer-assisted optical densitometry software ImageJ (National Institutes of Health, Bethesda, MDD).

Measurements were collected for each hemisphere of a given region. Regions of interest were the infralimbic cortex (IL), prelimbic cortex (PL), orbitofrontal cortex (OFC), piriform cortex, and basolateral amygdala (BLA). For regions of the prefrontal cortex and amygdala, a standard sized rectangle was used to take a measurement. For dorsal hippocampus (dHC) and ventral hippocampus (vHC), an outline was made of each

CA field (CA1, CA3, dentate gyrus, and CA2 only in dHC) to take a measurement. Sections with visible folding, tearing, or otherwise unusual signal were discarded from analysis. We defined approximate coordinates from Bregma for each brain region as follows: PL: +4.68mm to +2.52mm; IL: +3.72mm to +2.52mm; OFC: +5.16mm to +3.00mm; piriform: +3.24 to +2.28; BLA: -2.28mm to -3.48mm; dHC: -2.92mm to -3.48mm; and vHC: -5.16mm to -5.76mm (Paxinos & Watson, 2007). Data from >5 sections were averaged to create a mean signal measurement for each animal and group averages and standard error of the mean were calculated (background was found to correlate across brain regions, so to remove confounding effects of background correlation no background correction was applied).

Data Analyses

Behavioral Data. Locomotion data were analyzed using a one-way ANOVA. Freezing data were analyzed using a two-way (phenotype by social context condition) ANOVA with repeated-measures across tones. Statistics were calculated using SPSS (IBM). When Mauchly's test of sphericity was found to be significant, the conservative Greenhouse-Geisser correction was applied (Jaccard & Becker, 2009). When a given main effect or interaction met criterion for significance ($p < 0.05$), Bonferoni correction was applied to post hoc comparisons.

cFos Coactivation Analyses. cFos activity for all animals of Experiment Two were analyzed. This included outbred animals that underwent Extinction or No-Extinction conditions, as well as selectively bred rats that underwent extinction in the in-group phenotype and mixed-group social contexts. Following similar procedures to those

of Flagel and colleagues (2011), we calculated correlations in the cFos signal between brain regions as a measure of "coactivation" between regions. The mean signal from a given animal for two brain regions of interest was entered into a group analysis and used to calculate a correlation for signal strength between the two regions. This procedure was performed iteratively for all combinations of brain regions, generating a correlation matrix for animals in each phenotype and social context group. To correct for the large number of comparisons generated by these correlations, the p-values were corrected using false discovery rate (FDR; http://qvalue.princeton.edu/). Only correlations below FDR $q < 0.05$ were considered significant. This method of correction is not as stringent as Bonferroni correction, but it is commonly used in large datasets. To plot results, FDR corrected *q*-values were examined, and where a significant correlation existed for a given group, a line was drawn in the figures, with the thickness scaling with the correlation coefficient (R-value) for a given correlation.

We used linear regression to determine relationships between regional coactivation and phenotype. Linear regression analysis was conducted in R (R Core Team; Vienna, Austria; https://www.R-project.org). Data from the cFos analysis were centered and scaled so that the data could be compared across regions. The relationships between regional coactivation and phenotype were examined using the linear model:

$$\textbf{Equation 1: } (\text{Activity in Region A}) \approx \beta_0 + \beta_1(\text{Activity in Region B}) + \beta_2(\text{Phenotype}) + \beta_3(\text{Activity in Region B} * \text{Phenotype})$$

This means that we examined whether there was a significant relationship between activation of two regions while controlling for phenotype, and we further examined if regional coactivation was systematically altered by phenotype. We applied this model for each pair of regions included in our analyses and corrected for multiple

comparisons using the Benjamini-Hochberg procedure with α set to 0.05. We ran this analysis twice: once including the outbred animals, and once comparing just bHR and bLR animals. The reduced power from the reduction in sample size of the selectively bred phenotype model (bHR and bLR only) showed no significant effects after p-values were corrected with the Benjamini-Hochberg procedure. Uncorrected p-values are provided where noted. An additional linear model was run to assess the interaction of phenotype by social context; however we did not have enough power to effectively assess this more complicated model, so results are not shown.

Results

Experiment One.

Social Context Changes Extinction Learning for Outbred Animals. Outbred animals screened for their locomotion in a novel environment displayed a significant main effect of novelty-seeking phenotype on locomotor behavior, with outbred HRs (oHRs) having significantly higher total locomotor behavior than outbred LRs (oLRs; $F(1,47) = 1620.73$, $p < 0.001$; Figure 3.2A).

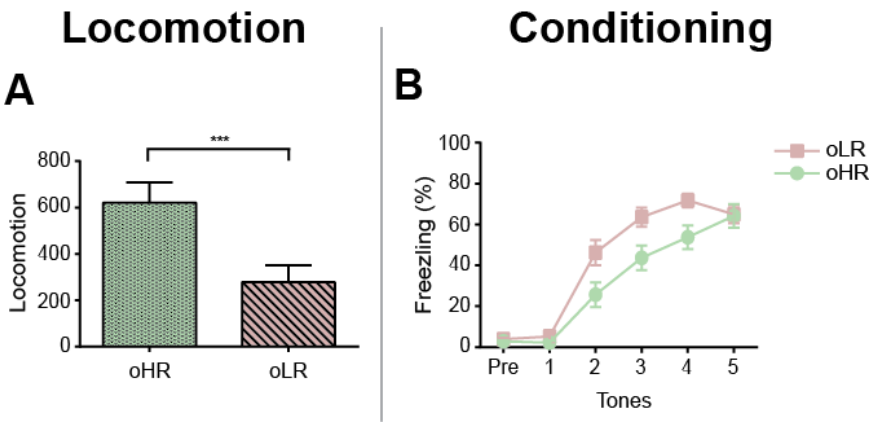

Figure 3.2 Outbred Sprague Dawley rats display differences in locomotion and fear conditioning similar to selectively bred rats. *Outbred Sprague Dawley rats screened for their locomotor response to a novel environment demonstrate significant differences in their locomotor behavior between outbred HR (oHR) and outbred LR (oLR) rats (A). During fear conditioning, oHR rats demonstrated lower freezing compared to oLR rats, although both phenotypes demonstrated the same level of freezing at the end of the conditioning session (B). *** = $p < 0.001$*

Fear Conditioning. There was a significant main effect of tones ($F(4,164) = 62.79$, $p < 0.001$) where freezing increased across the session, indicating that animals did condition (Figure 3.2B, Figure 3.3A). Animals demonstrated significantly lower freezing during the first tone of the session relative to subsequent tones (*post hoc* $p > 0.31$). The novelty-seeking phenotype in outbred rats significantly influenced fear conditioning, with a significant main effect of phenotype ($F(1,41) = 10.87$, $p = 0.002$) where oHRs displayed lower freezing levels than oLRs (Figure 3.2B). There was a main effect of social context ($F(2,41) = 4.32$, $p = 0.020$; Figure 3.3A), with rats in the in-group social context displaying lower freezing than rats in the isolated social condition (*post hoc* $p = 0.026$). Rats in the mixed social context did not differ from either of the other two groups (*post hoc* $p > 0.093$; Figure 3.3A). There was no significant interaction between social context conditions and novelty-seeking phenotype ($p = 0.861$). While there were significant differences between social context conditions on freezing behavior, there was

no difference in the number of fecal boluses excreted by animals of different phenotypes ($p = 0.056$) or in different social contexts ($p = 0.147$), suggesting that their levels of fear were equally high (data not shown). Additionally, during the last tone of the conditioning session, the main effect of social context was not significant, indicating that all groups demonstrated the same amount of freezing behavior, and that all groups conditioned equally well ($p = 0.507$; Figure 3.3A). No other main effects or interactions were significant.

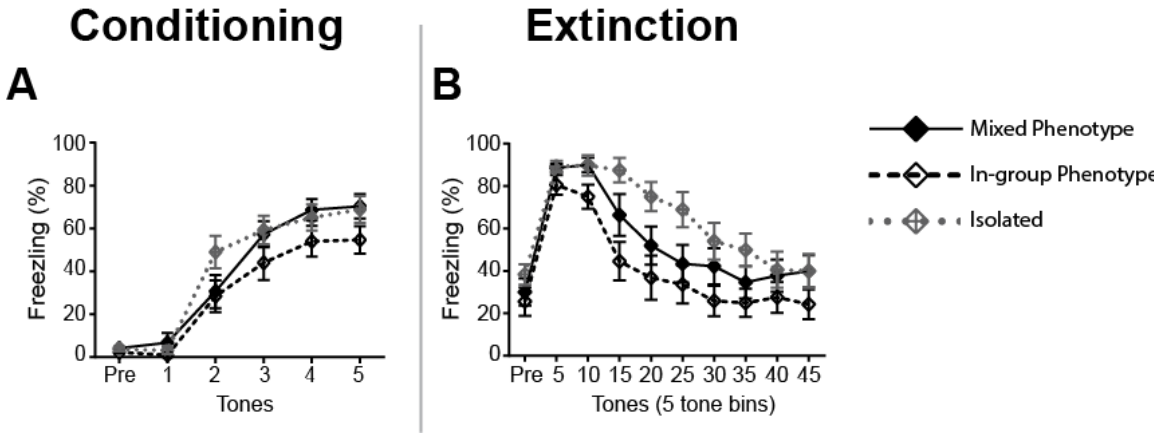

Figure 3.3 Social context changes extinction learning in outbred animals. *During fear conditioning, there were small differences between social contexts, with in-group social context rats displaying lower freezing than rats in the isolated condition (A). During fear extinction there are significant differences in extinction learning, with animals extinguished in the isolated social context demonstrating reduced extinction learning compared to animals extinguished in the in-group social context, indicating that extinction learning during isolation is worse than learning when surrounded by like-phenotype individuals (B).*

Extinction. Unlike conditioning, there was no main effect of outbred novelty-seeking phenotype on fear extinction, with both oHRs and oLRs exhibiting similar levels of freezing throughout the session ($p = 0.530$; data not shown). There was also no interaction between social context and outbred novelty-seeking phenotype ($p = 0.754$; data not shown). There was a significant main effect of tones ($F(4.38,179.38) = 47.77$, $p < 0.001$), where freezing decreased across the session indicating that animals

extinguished (Figure 3.3B). Animals demonstrated significantly higher freezing during the first two tone bins of the session relative to subsequent tone bins (*post hoc* tests comparing tone bins 5 and 10, with all subsequent tone bins $p < 0.005$). There was a significant main effect of social context on extinction ($F(2,41) = 4.55$, $p = 0.016$; Figure 3.3B), with animals in the in-group social context displaying lower levels of freezing than animals in the isolated social condition (*post hoc* $p = 0.013$), and animals in the mixed phenotype social context displaying freezing levels not different from either of the other two groups (*post hoc* $p > 0.320$). No other main effects or interactions were significant.

We cannot rule out the fact that outbred animals in the in-group social context simply do not condition as well, leading to their reduced freezing during extinction learning. However, animals in all social context conditions displayed equal freezing levels during the first five tones of the extinction session, suggesting that the level of fear was equally high for all social conditions at the beginning of the session ($p = 0.14$; Figure 3.3B).

Together, these data demonstrate that while outbred novelty-seeking phenotype has a significant influence on fear conditioning, it does not influence behavior during extinction. Social context, however, did impact extinction behavior: rats that were extinguished in the in-group phenotype social context displayed significantly facilitated extinction compared to isolated animals (Figure 3.3B). There was no interaction of outbred novelty seeking phenotype and social context during extinction.

Social Context Facilitates Extinction Learning for Selectively Bred Animals. Fear Conditioning. Social context had no effect on fear conditioning ($p = 0.973$; Figure 3.4A). Selectively bred animals maintained the significant main effect of phenotype on

fear conditioning behavior seen in Chapter 2, with bHR animals displaying lower freezing than bLR animals ($F(1,34) = 32.34$, $p < 0.001$). There was a significant main effect of tones ($F(2.92,99.40) = 17.89$, $p < 0.001$) where freezing increased across the conditioning session, indicating that animals conditioned (Figure 3.4A). Animals demonstrated significantly lower freezing during the first tone of the session than subsequent tones (*post hoc* tests comparing tone 1 and subsequent tones $p < 0.001$). No other effects reached significance.

Extinction. Social context significantly impacted extinction learning ($F(2,34) = 3.21$, $p = 0.05$; Figure 3.4B), with animals in the in-group social context displaying lower freezing than animals in the mixed social context (*post hoc* $p = 0.049$). Animals in the isolated social context did not differ from either of the other two social context conditions (*post hoc* $p > 0.81$). The main effect of phenotype seen during conditioning remained, with bHRs displaying lower freezing during extinction than bLRs ($F(1,34) = 332.32$, $p < 0.001$; Figure 3.4B). There was a main effect of tones where freezing decreased across the session, indicating that animals learned extinction ($F(4.44, 151.04) = 20.48$, $p < 0.001$; Figure 3.4B). Animals demonstrated significantly higher freezing during the first two tone bins relative to subsequent tone bins (*post hoc* tests comparing tone bins 5 and 10 to all subsequent tone bins $p < 0.001$). As seen in Chapter 2, there was a significant interaction of tones with phenotype where bHRs demonstrated facilitated extinction learning compared to bLRs ($F(4.44, 151.04) = 2.31$, $p = 0.05$; Figure 3.4B). No other effects were significant.

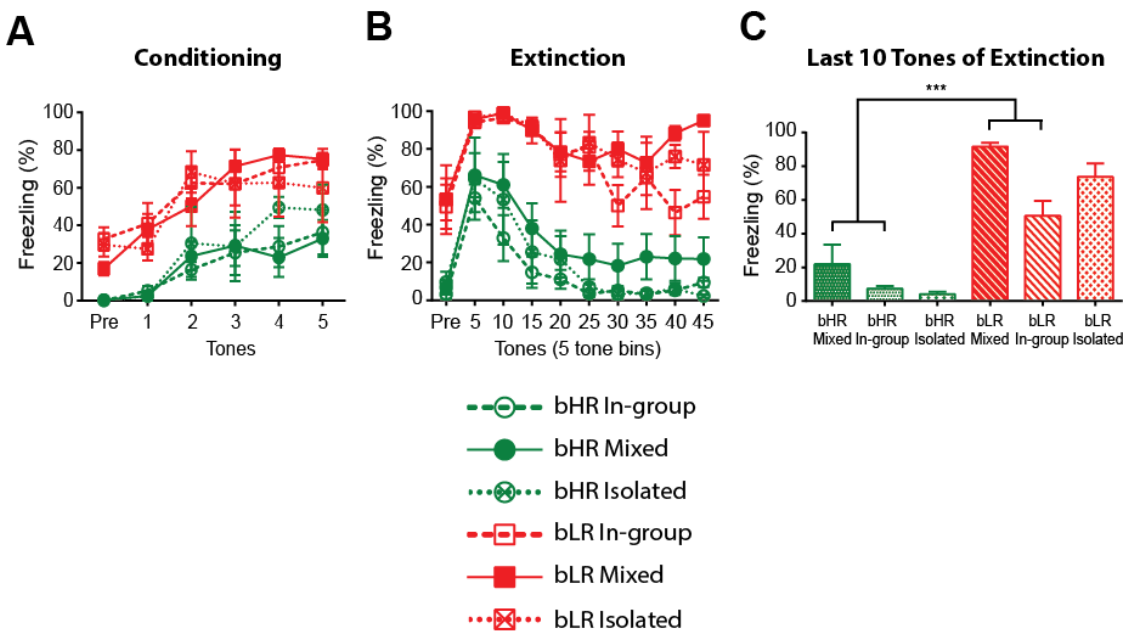

Figure 3.4 Social context significantly impacts extinction learning in rats selectively bred for their locomotor response to novelty. *There were no effects of social context on fear conditioning in selectively bred animals (A). Social context significantly impacted fear extinction, with rats in the in-group social context demonstrating significantly lower freezing than animals in the mixed group social context (B). This reduction in freezing behavior during extinction learning was particularly noticeable at the end of extinction (C). *** = $p < 0.001$*

The significant differences in behavior between phenotypes and our *a priori* interest in whether social context might facilitate extinction in bLR animals led us to do an additional analysis of the final tone bins of extinction and to include the phenotype by social context condition interaction. An analysis of the last two tone bins of the extinction session demonstrated highly significant differences between social context groups ($F(2,34) = 8.26$, $p = 0.001$; Figure 3.4C), with animals in the in-group phenotype social context demonstrating lower freezing than those in the mixed phenotype social context (*post hoc* $p = 0.001$), while isolated animals did not differ from either of the other two groups (*post hoc* $p > 0.124$).

Contrary to our hypothesis, social context has significant impacts on extinction learning in selectively bred animals. The in-group phenotype social context significantly

facilitated extinction learning for both bHR and bLR animals compared to rats in the mixed-group social context. For selectively bred rats, isolation did not reduce extinction as much as being mixed with the out-group phenotype.

Experiment Two.

Extinction Behavior is Stable Across Generations of Selectively Bred Rats.

Data from our previous studies of fear conditioning performed on selectively bred rats from generations 40, 41, and 43 were compared (Figure 3.5). Neither locomotor behavior, nor extinction behavior were significantly different between generations 40, 41, and 43. In these generations, locomotor behavior for both bHRs and bLRs was stable and extreme, and fear extinction behavior was stable, with bLRs demonstrating reduced extinction learning and bHRs demonstrating facilitated extinction learning. These data indicate that we have a reliable genetic model of individual differences in fear extinction behavior.

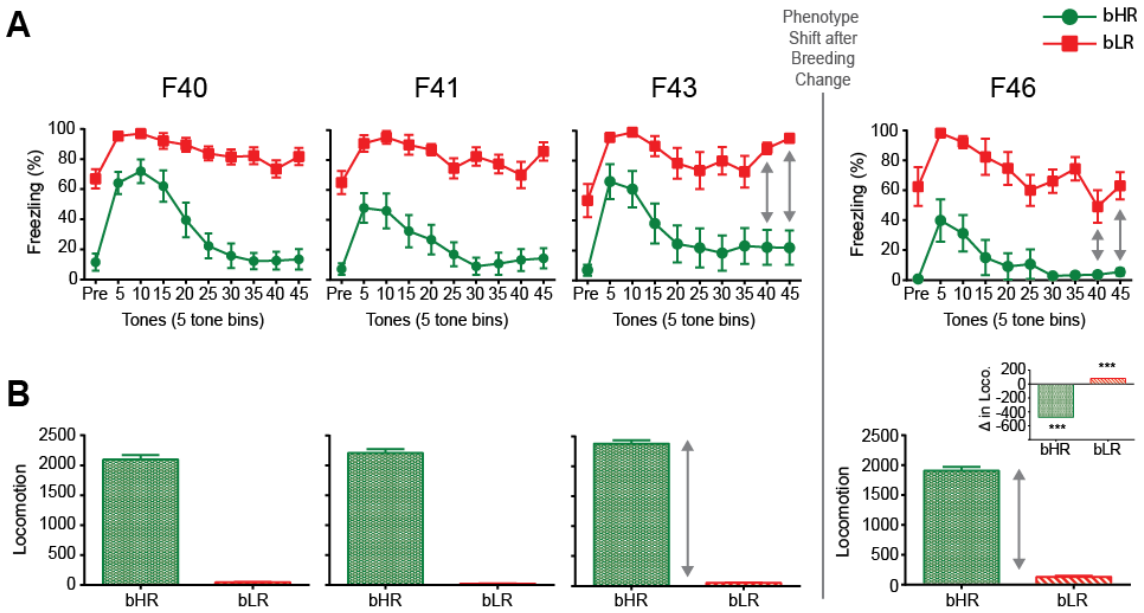

Figure 3.5 Extinction behavior and locomotor response to novelty are similar across generations until the change in selective breeding. *Extinction learning was stable and displayed distinct differences between bHRs and bLRs for generations 40 through 43, prior to the change in the selective breeding process. Generation 46, however, displayed facilitated extinction learning for bLRs compared to previous generations, indicating that the change in phenotype significantly affects extinction learning (A). Locomotion in a novel environment was consistent across generations 40 through 43, with bHRs and bLRs displaying extreme locomotor phenotypes (B). In contrast, both bHRs and bLRs displayed highly significant changes in locomotor behavior from generation 43 to generation 46 after the selective breeding schema changed (inset[1]). These changes decreased the locomotor response of bHRs and increased that of bLRs. While both selectively bred lines remain extreme, they were brought closer together in their locomotor phenotype, which also changed their extinction behavior.* [1] *The inset data were calculated by subtracting the locomotion scores of F43 from F46 for each phenotype.*

Changes in Locomotor Phenotype Significantly Alter the Extinction Behavior of Selectively Bred Rats. Comparison of the locomotor behavior from generation 46 of our colony with locomotor behavior from generation 43 demonstrated significant changes in the locomotor phenotype of both bHR and bLR animals based on a change in the selective breeding schema (Figure 3.5B). bLRs had significantly greater locomotion in a novel environment in generation 46 than generation 43 ($p < 0.001$; Figure 3.5B inset).

bHR animals had significantly less locomotion in a novel environment in generation 46 than generation 43 ($p < 0.001$; Figure 3.5B inset). In both cases the locomotor behavior of these selectively bred lines is still extreme, however, each phenotype trended toward a more moderate locomotor phenotype than seen in previous generations.

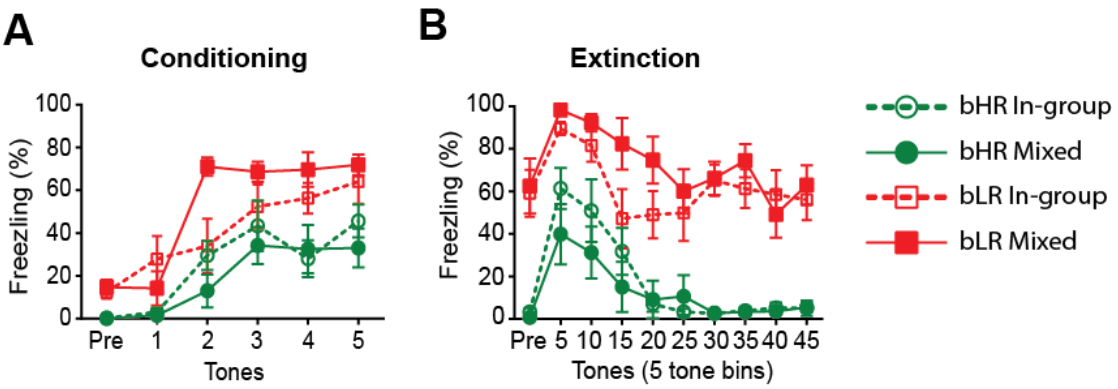

Figure 3.6 Social context trends toward facilitating extinction learning for bLRs but not bHRs after the extreme locomotor phenotype is modestly reduced. *bLRs and bHRs demonstrated no differences between social context groups during fear conditioning (A). bLRs again demonstrated facilitated extinction learning, with in-group social context animals demonstrating reduced freezing levels compared to mixed group context animals, although the effect was earlier in extinction learning than previously observed (B). bHRs showed no effect of social context during extinction (B).*

Extinction data from generation 46 of our colony confirmed that social context facilitates extinction in bLRs (Figure 3.6B). There was a main effect of tones where freezing decreased across the session, indicating that animals learned extinction ($F(3.30, 92.39) = 17.24$, $p < 0.001$; Figure 3.6B). Animals demonstrated significantly higher freezing during the first two tone bins relative to subsequent tone bins (*post hoc* tests comparing tone bins 5 and 10 to all subsequent tone bins $p < 0.003$). There was a main effect of phenotype, with bHRs having lower freezing than bLRs ($F(1,28) = 75.74$, $p < 0.001$; Figure 3.6B). There was a trend toward a tone by phenotype by social context interaction ($F(3.3, 92.39) = 2.37$, $p = 0.07$), with bLR animals in the in-group social context showing facilitated extinction learning early in the session compared to bLR

mixed phenotype social context animals while bHR animals did not differ in their extinction learning based on social context (Figure 3.6B). No other effects were significant in this dataset.

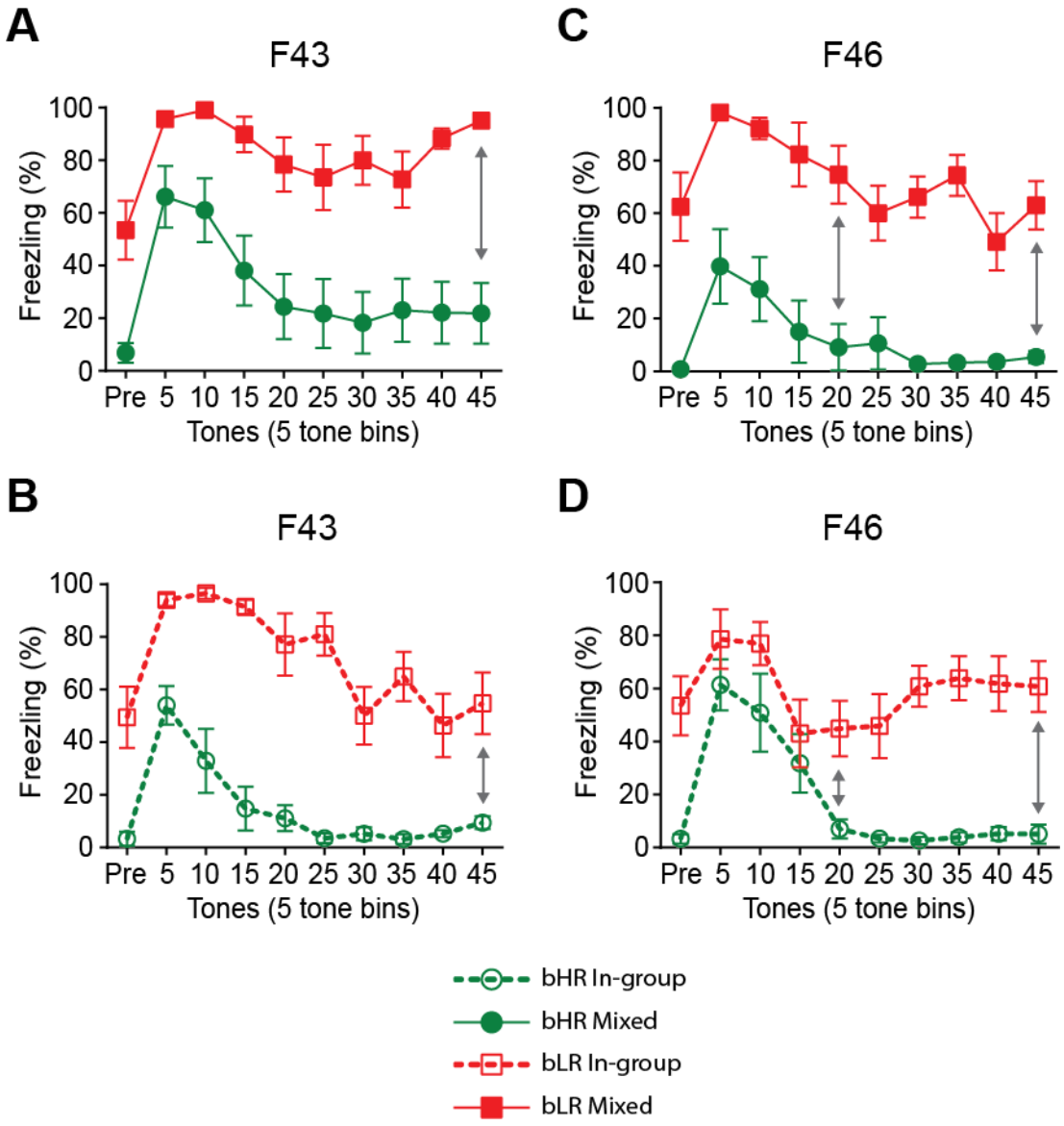

Figure 3.7 Selectively bred LR rats demonstrate a gene-by-environment interaction when social context and locomotor phenotype are manipulated. *Prior to the change in the selective breeding process (F43), bLRs demonstrated significantly reduced extinction learning compared to bHRs (A). In-group phenotype social context facilitated extinction for bLRs even when their phenotype was particularly extreme (B). In generation 46, after the change to the selective breeding process, bLRs exhibited facilitated extinction learning in the mixed group social context (C). bLRs showed a trend toward further facilitation of extinction when they underwent extinction in the in-group phenotype social*

context (D). Their freezing levels nearly matched that of bHRs for a portion of the session, indicating that manipulating both the locomotor phenotype and the environment of these rats can additively facilitate extinction learning. Together these data provide initial evidence for a gene-by-environment interaction.

The extinction behavior of our selectively bred rats is remarkably consistent across generations (Figure 3.5), only when the selective breeding schema changed locomotor behavior did extinction behavior change. This change in the "genetic load" of the selectively bred animals changed locomotor behavior for both bHR and bLR animals. As the locomotor behavior of bLRs increased, their extinction learning also improved (Figure 3.7 A, C), and as the locomotor behavior of bHRs decreased their extinction learning decreased slightly. These clear changes in extinction behavior indicate that the genetic phenotype of the animal significantly impacts fear and extinction learning. Additionally, there is a significant gene-by-environment interaction, where bLR rats from generation 46 that were placed in the in-group phenotype social context show a trend toward better extinction learning, bringing their freezing almost to the level of bHR rats, a phenomenon not seen in any previous generation (Figure 3.7D).

Outbred Rat Extinction and No-Extinction Controls. Outbred rats served as Extinction and No-Extinction controls for the neural correlates of gene-by-environment interaction in this experiment. The expected extinction group by tone interaction was present ($F(8,48) = 3.22$, $p = 0.005$; Figure 3.8), where outbred rats in the Extinction condition demonstrated standard extinction behavior with freezing that reduced across the session. Outbred rats in the No-Extinction condition did not change their freezing across the session.

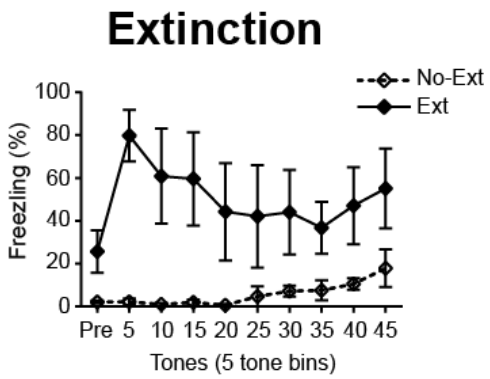

Figure 3.8 Outbred rats show standard fear extinction behavior. *Outbred Sprague Dawley rats were run in mixed-group social context and demonstrate standard fear extinction behavior with freezing in the No-Extinction group remaining very low throughout the session, while the rats undergoing extinction training demonstrate the standard high freezing to the initial tones and then a reduction in freezing across the rest of the session.*

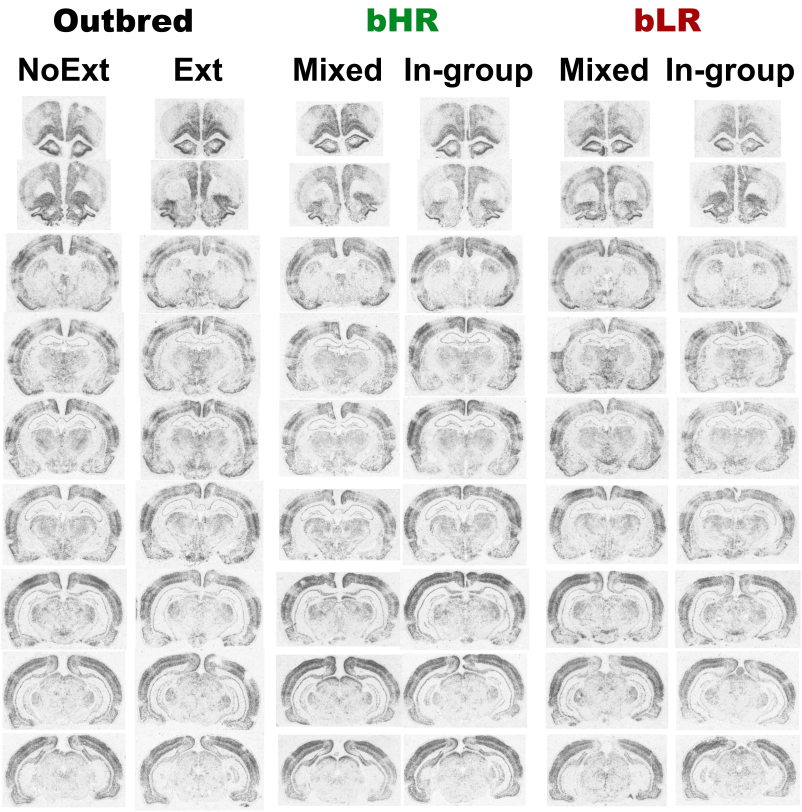

Figure 3.9 Representative sections from rats of the various phenotype and experimental groups displaying cFos mRNA as visualized by in situ hybridization. *A single representative individual was chosen based on the mean value of cFos for each phenotype. Sections through the brain of that individual are displayed through each of*

the regions quantified for the coactivation analysis. Outbred animals provided Extinction and No-Extinction controls for the selectively bred animals. bHR and bLR phenotype groups display a representative individual from the mixed and in-group phenotype social context experimental conditions. No significant differences are seen on within-region cFos expression between social context manipulations.

Both bHR and bLR Rats May Show Differential Coactivation From Outbred Rats During Extinction. Representative sections of brains labeled for cFos mRNA by *in situ* hybridization from individuals of the various phenotype and experimental groups are displayed in Figure 3.9. Surprisingly, bHR rats and bLR rats both showed different coactivation networks during extinction learning than outbred Sprague Dawley rats. While we cannot directly compare the correlation between groups, correlations between different pairs of regions were highly significant and survived FDR correction for different phenotypes (Table 3.1; Table 3.2). Outbred rats had significant coactivations between the IL and BLA and between various subfields of the dorsal and ventral hippocampus after extinction learning (Figure 3.11). Outbred rats that did not undergo extinction did not show significant correlations in these regions (Figure 3.11). Our linear regression analysis allowed us to more fully assess the interaction between phenotype and coactivation between regions. Using the interaction term of the regression analysis, we observed that phenotype significantly influenced coactivation between regions for the dorsal CA2 and IL ($p = 0.018$), PL ($p = 0.008$), OFC ($p = 0.029$), BLA ($p = 0.028$), dorsal CA1 ($p < 0.001$), dorsal CA3 ($p < 0.001$), ventral CA1 ($p = 0.007$), and ventral DG ($p = 0.007$). We also observed that phenotype significantly influenced coactivation between regions for the ventral CA3 and IL ($p = 0.047$), PL ($p = 0.038$), dorsal CA1 ($p = 0.047$), dorsal CA3 ($p = 0.047$), and ventral DG ($p < 0.001$). For the significant interactions between phenotype and various regions with dorsal CA2, bLRs consistently displayed a

greater relationship between the regions that bHRs, while outbred rats showed almost no coactivation between regions (Figure 3.10A, B, C). The significant interactions between phenotype and various regions with ventral CA3 displayed a different pattern (Figure 3.10D, E, F). For ventral CA3, bLRs still displayed the most significant relationships between regions, with outbreds often showing a relationship between regions, while bHRs showed little to no relationship between regions (Figure 3.10D, E). This was true with the exception of the relationship between ventral CA3 and ventral DG, where all three phenotypes displayed a significant relationship between regions, but the strength of that relationship differed, with bLRs greater than bHRs, greater than outbred rats (Figure 3.10F). Together, we can conclude that neither bHRs nor bLRs displayed a series of coactivations between regions that was similar to outbred rats.

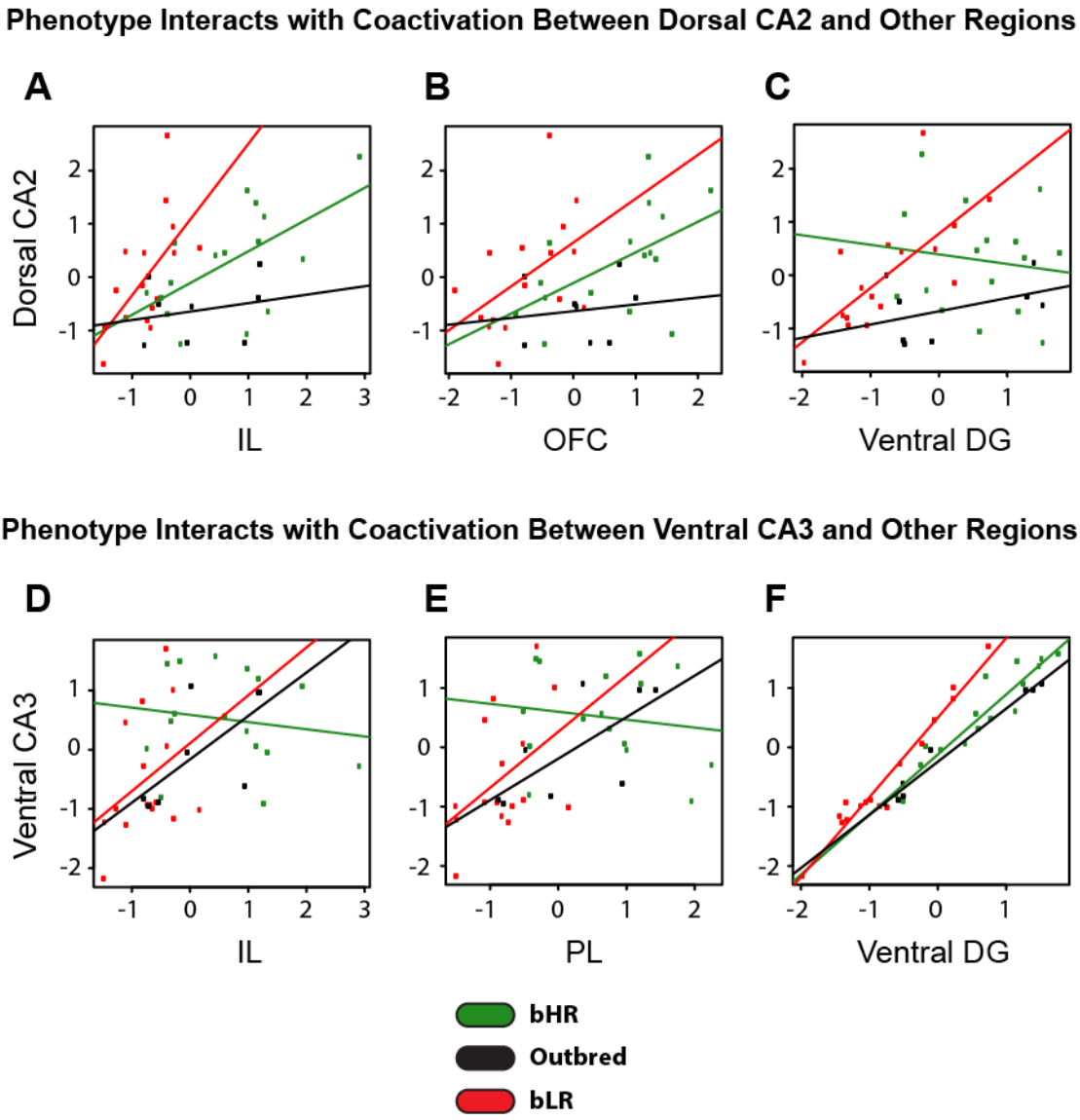

Figure 3.10 Significant Interactions Between Phenotype and Regional Coactivation. *The dorsal hippocampal subfield of CA2 displayed significant interactions between coactivation and phenotype for a number of regions including IL (A), OFC (B), and ventral DG (C). For the majority of these interactions, outbred animals had little to no relationship between regions, while selectively bred animals, and bLRs in particular, showed strong positive relationships. The ventral hippocampal subfield of CA3 also displayed significant interactions between coactivation and phenotype for a number of regions, including IL (D), PL (E), and ventral DG (F). For interactions between ventral hippocampus and prefrontal cortex (D,E), bHRs had little to no coactivation between regions, while outbred rats and bLRs in particular showed strong positive relationships between regions. In contrast, the phenotype interaction with ventral CA3 and ventral DG shows strong positive relationships with all phenotypes, however bLRs have the strongest positive relationship between regions, followed by bHRs and then by outbred animals. Together, these data suggest that coactivation between regions is significantly different depending on the novelty-seeking phenotype of the animal.*

Table 3.1 Coactivation of hippocampal regions in Outbred, bLR, and bHR extinction and social context groups.

	q-values					
Region Pair	**Outbred No-Ext**	**Outbred Ext**	**bLR Mixed**	**bLR In-group**	**bHR Mixed**	**bHR In-group**
dHC_CA1 - dHC_CA2	0.078	**0.035**	0.060	0.165	**0.038**	0.072
dHC_CA1 - dHC_CA3	0.194	**0.022**	**0.000**	**0.008**	**0.000**	**0.039**
dHC_CA1 - dHC_DG	0.200	**0.049**	0.070	**0.008**	0.055	**0.008**
dHC_CA1 - vHC_CA1	0.109	**0.007**	0.116	**0.027**	0.260	0.178
dHC_CA1 - vHC_CA3	0.224	**0.038**	0.094	0.055	0.293	0.368
dHC_CA1 - vHC_DG	0.230	**0.034**	0.073	**0.049**	0.302	0.342
dHC_CA1 - Piriform	0.249	0.112	0.113	**0.046**	0.167	0.051
dHC_CA2 - dHC_CA3	0.224	**0.022**	0.060	0.070	**0.024**	0.106
dHC_CA2 - dHC_DG	0.249	0.078	0.136	0.092	**0.029**	0.119
dHC_CA2 - vHC_CA1	0.177	**0.035**	0.058	0.119	0.297	0.361
dHC_CA2 - vHC_CA3	0.277	0.062	**0.046**	**0.045**	0.173	0.293
dHC_CA2 - vHC_DG	0.279	**0.036**	**0.029**	0.058	0.165	0.355
dHC_CA2 - Piriform	0.230	0.138	0.245	0.104	0.165	**0.030**
dHC_CA3 - dHC_DG	0.182	0.054	0.099	**0.012**	**0.027**	0.055
dHC_CA3 - vHC_CA1	0.111	**0.023**	0.109	**0.029**	0.351	0.297
dHC_CA3 - vHC_CA3	**0.046**	**0.046**	0.081	**0.030**	0.226	0.273
dHC_CA3 - vHC_DG	**0.047**	**0.022**	0.060	**0.027**	0.235	0.226
dHC CA3 - Piriform	0.078	0.131	0.111	**0.038**	0.188	**0.038**
dHC_DG - vHC_CA1	0.265	0.054	0.134	0.058	0.242	0.283
dHC_DG - vHC_CA3	0.194	**0.016**	0.221	0.058	0.055	0.273
dHC_DG - vHC_DG	0.188	**0.047**	0.173	0.058	0.058	0.246
dHC DG - Piriform	0.109	0.123	0.051	0.055	0.118	0.099
vHC_CA1 - vHC_CA3	0.114	**0.047**	**0.008**	**0.024**	0.113	0.065
vHC_CA1 - vHC_DG	0.121	**0.037**	**0.008**	**0.021**	0.165	0.172
vHC CA1 - Piriform	0.188	0.109	0.057	0.058	0.188	0.245
vHC_CA3 - vHC_DG	**0.007**	**0.036**	**0.000**	**0.000**	**0.000**	**0.012**
vHC_CA3 - Piriform	0.114	0.123	0.126	**0.021**	0.213	0.253
vHC_DG - Piriform	0.112	0.143	0.099	**0.021**	0.197	0.197

Table 3.2 Coactivation of prefrontal regions and BLA in Outbred, bLR and bHR rats.

	q-values					
Region Pair	**Outbred No-Ext**	**Outbred Ext**	**bLR Mixed**	**bLR In-group**	**bHR Mixed**	**bHR In-group**
IL - PL	**0.036**	0.088	**0.000**	**0.038**	**0.000**	**0.029**
IL - OFC	0.078	**0.049**	0.203	0.197	**0.043**	**0.046**
IL - BLA	0.120	**0.047**	0.169	0.232	0.188	**0.027**
IL - dHC_CA1	0.181	0.131	0.253	0.369	0.190	**0.039**
IL - dHC_CA2	0.177	0.143	0.077	0.094	0.063	0.094
IL - dHC_CA3	0.054	0.143	0.235	0.286	0.192	0.056
IL - dHC_DG	0.166	0.155	0.188	0.317	0.140	**0.037**
IL - vHC_CA1	0.140	0.121	0.092	0.236	0.297	0.283
IL - vHC_CA3	0.109	0.153	0.109	0.192	0.226	0.362
IL - vHC_DG	0.109	0.161	0.058	0.216	0.197	0.326
IL - Piriform	0.052	**0.042**	0.192	0.203	**0.038**	**0.027**
PL - OFC	0.104	0.140	0.142	0.104	0.051	**0.012**
PL - BLA	0.105	0.112	0.221	0.192	0.165	0.087
PL - dHC_CA1	0.174	0.150	0.245	0.368	0.116	**0.037**
PL - dHC_CA2	0.188	0.140	0.090	0.099	**0.046**	0.050
PL - dHC_CA3	**0.033**	0.153	0.213	0.266	0.131	**0.021**
PL - dHC_DG	0.188	0.200	0.197	0.297	0.122	**0.037**
PL - vHC_CA1	0.109	0.140	0.060	0.287	0.297	0.318
PL - vHC_CA3	0.075	0.190	0.090	0.113	0.225	0.324
PL - vHC_DG	0.077	0.177	**0.042**	0.131	0.207	0.361
PL - Piriform	0.075	0.131	0.134	0.104	0.055	**0.027**
OFC - BLA	0.204	0.078	0.295	**0.038**	0.119	0.081
OFC - dHC_CA1	0.279	0.112	0.131	0.064	0.173	0.051
OFC - dHC_CA2	0.251	0.140	0.185	0.060	0.173	**0.037**
OFC - dHC_CA3	0.105	0.133	0.131	**0.038**	0.221	**0.041**
OFC - dHC_DG	0.080	0.120	0.090	0.068	0.207	0.087
OFC - vHC_CA1	0.219	0.109	**0.042**	0.072	0.195	0.355
OFC - vHC_CA3	0.134	0.120	0.114	**0.023**	0.283	0.350
OFC - vHC_DG	0.130	0.143	0.068	**0.023**	0.275	0.355
OFC - Piriform	**0.026**	**0.007**	**0.027**	**0.000**	**0.016**	**0.012**
BLA - dHC_CA1	0.065	0.181	0.060	**0.024**	**0.038**	**0.024**
BLA - dHC_CA2	0.120	0.197	0.195	0.072	0.087	0.160
BLA - dHC_CA3	0.121	0.197	0.068	**0.037**	**0.037**	0.065
BLA - dHC_DG	0.273	0.202	0.106	**0.021**	**0.027**	0.050
BLA - vHC_CA1	0.052	0.176	0.233	**0.037**	0.351	0.064
BLA - vHC_CA3	0.148	0.200	0.190	**0.024**	0.170	0.188
BLA - vHC_DG	0.153	0.218	0.178	**0.024**	0.202	0.232
BLA - Piriform	0.180	0.073	0.160	**0.023**	0.072	**0.040**

Compensatory Networks May Help to Facilitate Fear Extinction in In-group Phenotype Contexts for Vulnerable Animals. Contrary to our hypotheses, no analysis of within-region activation of cFos demonstrated differences between social contexts in the bLR or bHR rats (data not shown). However, we did observe differences in the coactivation between regions that interacted with bHR and bLR phenotype prior to multiple comparison correction (Appendix 3.1).

For further analysis, we focus on the bLR rats to better understand the neural correlates of the gene-by-environment interaction that they displayed behaviorally. This analysis is preliminary, as our sample size is too low to statistically compare social context groups within or between phenotypes. The coactivation patterns of bLR brains appear quite different from outbred brains (Figure 3.10), as would be expected given their extreme phenotype (Figure 3.11). bLR animals that underwent extinction in the mixed phenotype group show coactivation patterns that appear somewhat similar to outbred no-ext animals, with limited inter-prefrontal cortex coactivation and some intra-hippocampal coactivation, but no IL – BLA coactivation (Figure 3.11A; Table 3.1; Table 3.2). In contrast, the coactivation map of bLRs that underwent fear extinction in the in-group phenotype context shows enhanced coactivation, with multiple prefrontal and hippocampal regions demonstrating coactivation with the BLA (Figure 3.11B). While there is increased prefrontal-BLA coactivation, we still did not observe coactivation of IL – BLA. Instead, the orbitofrontal cortex (OFC) and piriform cortex appear to show coactivations with both the BLA and areas of the hippocampus, which were not seen in outbred animals that underwent extinction learning (Figure 3.11B; Table 1.1; Table 1.2). BLA itself also appears to have increased coactivation with areas of both dorsal and

ventral hippocampus in bLRs extinguished in the in-group phenotype context. These additional coactivations are not seen in outbred animals or bHRs and this may indicate that there are compensatory networks brought online that serve to facilitate extinction in bLRs when they are in the in-group phenotype context.

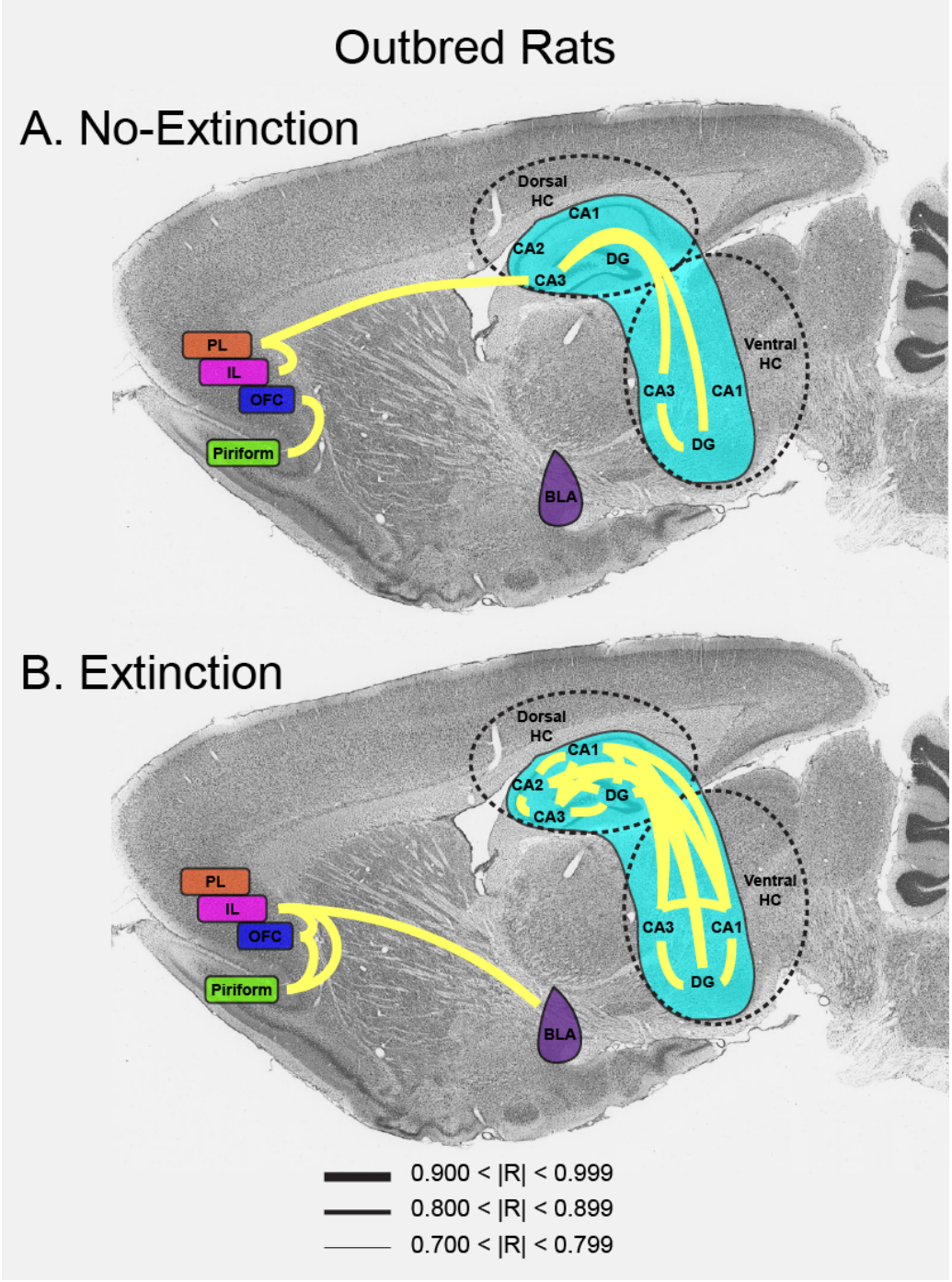

Figure 3.11 Outbred coactivation patterns in response to novel environment and extinction learning. *Outbred Sprague Dawley rats that did not undergo extinction training demonstrate few coactivations between regions (A). Outbred rats that underwent extinction learning demonstrate significant coactivations between IL and BLA, as well as within the dorsal and ventral hippocampus, indicating that extinction learning may alter the coactivation of these regions (B). (Rat brain image is derived from nissl stained tissue.)*

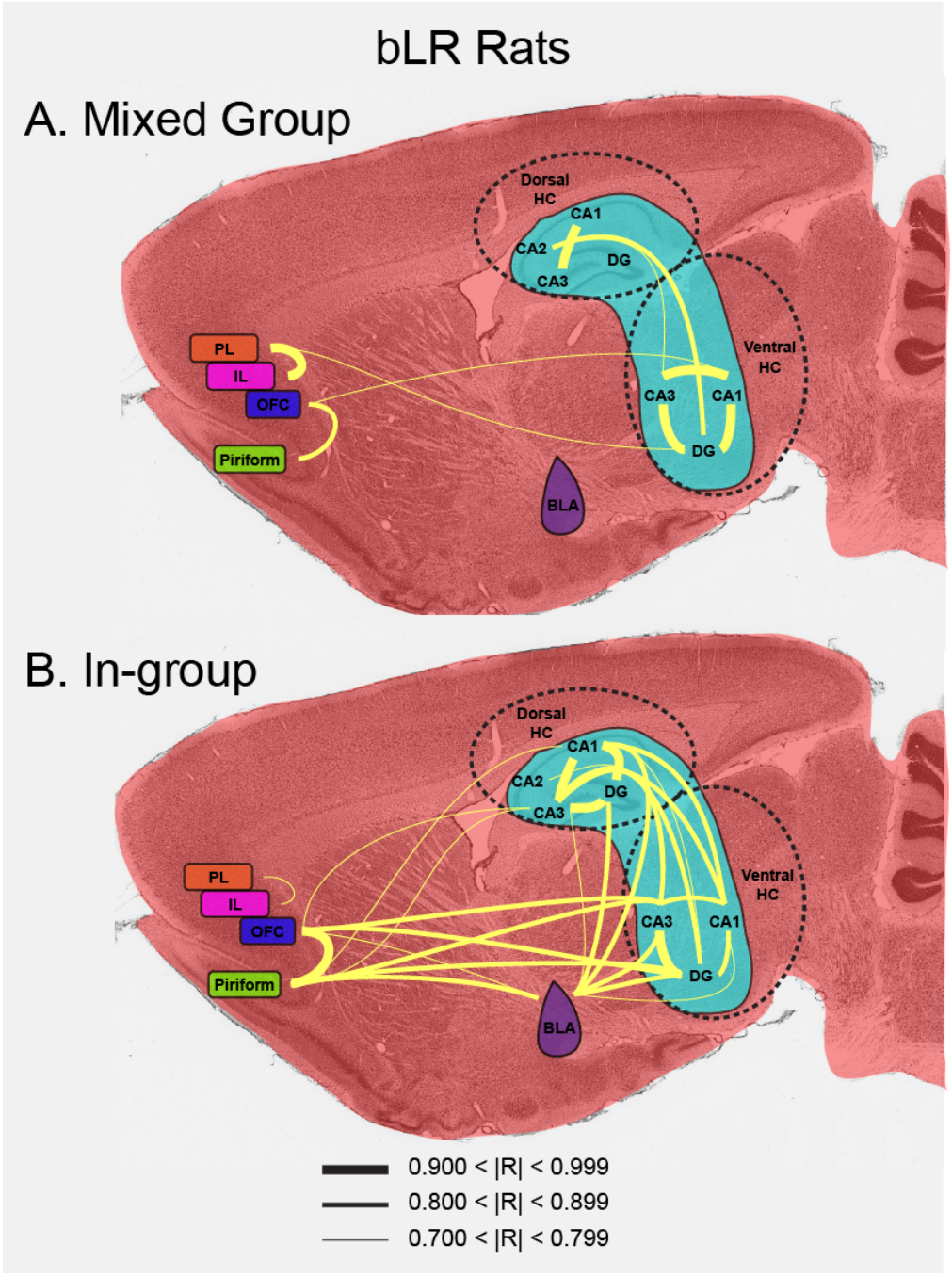

Figure 3.12 bLR coactivation patterns in response to extinction learning in mixed and in-group social contexts. *bLR rats extinguished in the mixed group social context demonstrate less coactivation than seen in outbred extinction animals, indicating that their lack of extinction behavior may be due to limited coactivation of standard extinction learning networks (A). In contrast, bLRs in the in-group social context demonstrate coactivation of OFC – BLA and piriform – BLA as well as BLA – hippocampal regions not seen in outbred extinction animals (B). Given their facilitated extinction behavior, it may be that coactivation of OFC, Piriform, BLA and hippocampus allows usually*

vulnerable animals to extinguish their fear. (Rat brain image is derived from nissl stained tissue.)

Discussion

In these studies, we have shown that the interaction of social context with individual differences in phenotype can significantly influence extinction learning in rodents. In Experiment One, we determined that the environmental manipulation of social context was powerful enough to facilitate extinction learning for both outbred and selectively bred rats. In outbred rats screened for their locomotor response to a novel environment, animals that underwent extinction training in the company of in-group phenotype individuals extinguished their fear better than animals extinguished in the isolated condition. This was also true for animals selectively bred for their locomotor response to a novel environment, with the in-group phenotype social context facilitating fear extinction compared to the mixed group condition. This was contradictory to our hypothesis that we would see no effect of social context in selectively bred animals because their genetic propensity for specific extinction behavior was too strong. Instead, we demonstrated that in-group social context is a powerful facilitator of extinction learning for both outbred and selectively bred rats.

In Experiment Two, we observed initial evidence for a gene-by-environment interaction between social context and heritable temperament and assessed the neural correlates of this interaction. We manipulated the novelty-seeking phenotype of our selectively bred animals by reducing the extremity of their phenotypic locomotor responses and demonstrated that this significantly changed extinction behavior,

particularly for bLRs. Consistent with our hypothesis, we showed that in-group social context trended toward further facilitating extinction learning specifically in bLRs, indicating that this particular environmental manipulation is useful even when the heritable propensity for reduced extinction behavior is lessened. We examined the neural correlates of this gene-by-environment interaction in bLRs, and found that under in-group phenotype conditions, areas of the prefrontal cortex, including the orbitofrontal cortex (OFC), and piriform cortex have increased coactivation with the basolateral amygdala (BLA) and areas of the hippocampus. While these results were unexpected given our hypothesis of differential activity in IL and BLA, these data suggest that these other areas of prefrontal cortex might be important for the gene-by-environment interaction that facilitated extinction learning.

Collectively, these studies indicate that the social context present during extinction significantly influences the behavior and brain activity in both outbred and selectively bred rats, and that the effects of this environmental manipulation may interact with genetic predisposition, providing a useful model of gene-by-environment interactions to study vulnerability to PTSD. Finally, these studies provide some of the first evidence of the neural correlates of social buffering during extinction learning in rodents, indicating that coactivations with the prefrontal cortex may be particularly important.

Social Buffering and Fear Extinction Are Impacted by Individual Differences in the Novelty-seeking Phenotype of Outbred Rats

To our knowledge, Experiment One described here is the first to use the novelty-seeking phenotype to facilitate social buffering in outbred animals in general, but particularly during extinction learning. The current studies assessed the novelty-seeking phenotype of outbred Sprague Dawley rats and divided these rats into outbred high responder (oHR) and outbred low responder (oLR) phenotypes based on their locomotion in a novel environment. During a standard fear conditioning and extinction paradigm, the social context of the animals significantly influenced extinction learning, with rats extinguished in the in-group phenotype condition showing facilitated extinction learning compared to rats in the isolated condition. Many studies have demonstrated significant reductions of fear in animals as a result of social buffering between pairs of individuals (Fuzzo et al., 2015; Guzman et al., 2009; Kiyokawa, Hiroshima, et al., 2014; Kiyokawa, Honda, et al., 2014; Knapska, Mikosz, Werka, & Maren, 2010; Knapska et al., 2006). However, no previous study has manipulated the social context using the novelty-seeking phenotype to assess the impact of individual differences in phenotype on behavior. These findings impact the study of fear conditioning and extinction in two ways: 1) researchers should be cognizant of how many rats they test at a given time and how that may influence their results, and 2) researchers should consider whether the phenotype of the animals may influence the results of their experiment and take it into account accordingly.

The study of fear extinction has been around for many years; however, little attention has been paid to the number of animals conditioned or extinguished at any given

time. Indeed, in their recommendations for procedure, Chang et al. (2009) state that the number of animals run at any given time will depend on the number of behavioral chambers available to the experimenter. While this is true, and studies have been run with single chambers (Burgos-Robles, Vidal-Gonzalez, & Quirk, 2009; Graham & Richardson, 2009), four chambers (Bush, Sotres-Bayon, & LeDoux, 2007), eight chambers (Orsini, Kim, Knapska, & Maren, 2011), and potentially more, our data suggest that these different conditions may influence fear extinction in rats. It is clear that in outbred rats, rats conditioned and extinguished in the presence of only one other individual learn within-session extinction less well than those conditioned and extinguished in the presence of seven other individuals, particularly when they are all of the same novelty-seeking phenotype. While our data cannot currently predict how extinction learning might vary with other numbers of individuals present, we can say with some certainty that the data from laboratories where there are fewer individuals present in the room during extinction is likely to differ from that of laboratories where there are many individual rats together during extinction learning. This may be one reason why there is variability in effects of extinction across publications.

An additional measure that investigators studying fear extinction may need to consider in future studies is the phenotype of individual animals in their studies. While most studies ignore individual differences in behavioral responses, preferring to average over a group, at least one other study has shown that individual differences in fear extinction exist in the outbred population (Bush et al., 2007). Our data show that rats in in-group phenotype social contexts learned within-session extinction better than isolated controls. Although social context will not influence the results of laboratories where rats

are tested individually, it is still important to consider phenotype in single-tested rats, as the sequential testing of different phenotypes may also influence the behavior of individuals. Our data do not demonstrate significant differences between the mixed-phenotype social context and the in-group social context, indicating that for outbred rats, investigators may continue to choose to ignore individual differences in phenotype. However, we are limited by our sample size, and there is a hint that those two groups may diverge, especially in the selectively bred animals where the phenotypic differences during fear extinction were particularly pronounced. With a greater sample size, it is possible that phenotype may influence extinction learning even when group extinction environments are used for outbred animals. Further studies are needed to assess this effect, as these subtle individual differences may influence extinction learning, and the efficacy of pharmacological (and other) interventions. Collectively, our data indicate that there are further variables to carefully consider when designing fear conditioning and extinction experiments, even in outbred rats.

In-group Phenotype Social Contexts Promote Social Buffering in Both Outbred and Selectively Bred Rats

In Experiment One, we observed that in-group phenotype social context facilitated extinction learning for both outbred and selectively bred rats. Our findings fit nicely with the literature on social buffering in animals, which consistently demonstrates that individual animals in a social context show reduced fear (Davitz & Mason, 1955; Guzman et al., 2009; Hennessy et al., 2016; Kiyokawa, Honda, et al., 2014; Kiyokawa et al., 2013; Liu et al., 2013; Rukstalis & French, 2005; Stanton et al., 1985; Young et al.,

2014). Our research extends these findings in both outbred and selectively bred rats to demonstrate that the presence of like-individuals (in-group phenotype) facilitates fear extinction.

To our knowledge, this is the first finding demonstrating in-group preferences during fear extinction in animals; however, there have been a series of studies in humans demonstrating that the in-group or out-group status of other individuals influence both the acquisition and extinction of conditioned fear. For instance, individuals condition more strongly to images of a person in their racial out-group, but this can be moderated by prior experience and interaction with members of that racial outgroup (Golkar, Bjornstjerna, & Olsson, 2015; Olsson, Ebert, Banaji, & Phelps, 2005). Additionally, individuals do not readily extinguish their fear to images of male racial out-group individuals (Navarrete et al., 2009). Interestingly, it has also been shown that humans can learn fear extinction vicariously, by watching someone else go through extinction learning, and that this mode of extinction learning is in fact more robust and is retained better than standard extinction learning (Golkar, Selbing, Flygare, Ohman, & Olsson, 2013). These studies demonstrate that the individuals present during learning influence fear and extinction, and that the social experience of extinction can be more powerful than standard extinction learning. In the study by Golkar and colleagues (2013), the participants were all male, and they observed a male receive extinction training. Our results indicate that in-group individuals may help promote extinction, similar to their results. It would therefore be interesting to understand whether the results obtained by Golkar and colleagues (2013) of vicarious extinction learning would be less robust if the observed individual were from an out-group. Our findings, together with those of the

human literature, indicate that the in-group status of individuals present during fear conditioning and extinction can have significant effects on behavior, and that this effect is common across species.

It should be noted that it is unclear how social buffering is transmitted between animals in our experimental paradigm. It has previously been shown that the odor of a familiar conspecific is better at reducing fear responses after conditioning than an unfamiliar conspecific, although both conditions show a reduction in fear compared to having no conspecific odor present (Kiyokawa, Honda, et al., 2014). The communication between animals during fear extinction in our paradigm may be olfactory, and we cannot rule out that a familiar conspecific (cagemate) was in the room, which may have further influenced extinction learning. However, cagemates were always several cages distant from each other, seemingly decreasing the possibility that facilitation of extinction is due solely to having a cagemate present in the room. The transmission of social information between individuals may well still be through olfactory information, and it has been demonstrated that olfaction, particularly the activation of certain olfactory receptors, may be important for the social buffering phenomenon (Klein et al., 2015). We use strong odors as part of our standard contextual cues, which may partially mask social odors present from other rats. However, it is currently unclear whether our chosen contextual odors would mask social odors well enough to mitigate social buffering via odor communication. It may be that rats in our paradigm are communicating through olfactory signals, although further investigation is needed.

Alternatively, rats may be communicating using ultrasonic vocalizations. Rats are known to emit ultrasonic vocalizations in a variety of situations, in particular during

aversive or rewarding circumstances (Brudzynski, 2013). In addition, it has been shown that rats find certain calls (50 kHz) rewarding and will self administer them, while calls that are emitted in aversive situations (22 kHz) are avoided (Burgdorf et al., 2008). It has also been shown that ultrasonic vocalizations can directly influence the fear behavior of other rats in a social situation (Kim, Kim, Covey, & Kim, 2010), indicating that ultrasonic vocalizations may play a role in the social response to fear extinction learning in our paradigm. While we have evidence that bHR and bLR rats emit different levels of ultrasonic vocalizations both at baseline and in response to stimuli (Perez-Sepulveda et al., 2013), we do not currently have data on their calls in response to fear learning or extinction. It would be interesting to know whether the negative (22 kHz) calls are reduced in in-group phenotype situations during fear extinction learning, and whether those calls are different based on the phenotype of the animal.

Social Context Manipulations Provide an Opportunity to Explore Gene-by-Environment Interactions

Our data also suggest that a gene-by-environment interaction alters how well bLR animals extinguish their fear. We have previously shown (Chapter 2) that bLR animals demonstrate reduced fear extinction learning and retention of that extinction. Those studies were completed solely in mixed-group social contexts, and indicate that there is a genetic disposition to impaired extinction learning. In Experiment One, we demonstrated that although bLRs in the mixed-group social context demonstrate reduced fear extinction learning, surrounding them with in-group phenotype individuals facilitates extinction learning. This effect of social context appears to depend on the magnitude of the original

phenotypic differences, because the social effect diminished slightly in Experiment Two; completed after we altered our selective breeding paradigm. In Experiment Two, the bLR individuals demonstrated a significantly higher locomotor phenotype from the previous generation (Figure 3.6), giving them a slightly more bHR-like locomotor phenotype. The bLRs in the mixed-group social context showed significantly better extinction learning during Experiment Two than seen in previous studies (Figure 3.5A, F46). This reduced phenotypic effect on fear extinction created a smaller difference seen between in-group and mixed-group social contexts during extinction learning. Currently, we cannot determine whether the facilitated extinction behavior seen in this generation was due to increased extinction learning or to increased locomotion in bLR rats. Although further studies are needed to determine if this trend is stable and due specifically to extinction learning, there may be a gene-by-environment interaction such that reducing the genetic vulnerability facilitates extinction learning, but manipulating the social context provides even better resilience and promotes extinction learning further.

OFC and Piriform Cortex Coactivation with BLA and Hippocampus May Facilitate Extinction in bLRs

When we examined the neural correlates of the gene-by-environment interaction in bLR rats, we observed increased coactivation between orbitofrontal cortex (OFC) and BLA as well as OFC coactivation with subfields of the hippocampus that differentiated bLR rats from the other phenotypes (including bHR rats; Appendix 3.1). These coactivations also appear to separate bLR rats in the in-group social context from those in the mixed group social context. We also showed that bLR rats appear never to coactivate

the infralimbic prefrontal cortex (IL) and BLA, which we observed in outbred animals after extinction learning. This suggests that the unusual coactivations seen in bLRs in the in-group social context might be compensatory mechanisms to assist in facilitating their extinction learning.

Our findings implicate coactivation of brain regions such as the OFC with the BLA and hippocampus as critical to facilitated extinction learning in in-group social contexts. OFC has reciprocal projections with the amygdala and also has connections with the ventral hippocampus, indicating that it may play a role in processing emotional information, and fear in particular (Cavada, Company, Tejedor, Cruz-Rizzolo, & Reinoso-Suarez, 2000; Hoover & Vertes, 2007, 2011). The OFC has previously been implicated in fear extinction learning, with one study demonstrating that neurotoxic lesions of the OFC prior to conditioning caused fear generalization and reduced extinction learning (Zelinski, Hong, Tyndall, Halsall, & McDonald, 2010). A more recent study demonstrated that pharmacological inactivation of the medial OFC reduced fear expression during extinction but did not affect retention of extinction (Rodriguez-Romaguera, Do-Monte, Tanimura, Quirk, & Haber, 2015). While these studies are not cohesive in their conclusions about the role of the OFC in extinction learning, both indicate that there is a role for the OFC, and our data suggest that this may be particularly true for situations in which extinction is facilitated by in-group social contexts. Studies in humans using lesion patients have also identified the OFC as important to emotion and the ways emotion can influence working memory and recognition of emotion in others (Goodkind et al., 2012; Levens, Devinsky, & Phelps, 2011). Therefore, the OFC may be coactivated specifically when an in-group social context facilitates extinction learning

because within this situation, individuals are interacting with and interpreting the emotional output of others to determine their own behavior. While the exact role of the OFC remains unclear, it is likely to be involved in the processing of emotional information and in learning, although how those two functions intersect is still a subject of current research (Stalnaker, Cooch, & Schoenbaum, 2015).

Our findings additionally provide evidence for increased coactivation between the piriform cortex and BLA and areas of the hippocampus in the bLR rats that were extinguished in the in-group social context compared to the mixed group social context. Like the OFC, the piriform cortex also has direct bilateral projections with the basolateral amygdala, implicating it in fear and extinction processing (Hoover & Vertes, 2007; Majak, Ronkko, Kemppainen, & Pitkanen, 2004). Moreover, one of the main functions of the piriform cortex is olfaction (Uchida, Poo, & Haddad, 2014). In our paradigm, one of the contextual elements that differentiate the conditioning from the extinction session is the odorant used in the room and to clean the chambers the rats are placed in (acetic acid or ammonium hydroxide). It is therefore not surprising that the piriform cortex might be activated, and coactivated with the amygdala and hippocampus to determine whether the animal should remain afraid in this context. Dorsal hippocampus in particular is involved in perception and storage of contextual information during fear conditioning and extinction learning (Maren & Fanselow, 1997; Maren & Hobin, 2007; Sutherland & McDonald, 1990; Wang et al., 2012), and we observed increased coactivation between piriform cortex and some subfields of dorsal hippocampus in bLRs during extinction learning. Interestingly, it has recently been proposed that piriform cortex itself might participate in threat perception and retention (Li, 2014), and therefore it may be that the

observed coactivation of piriform cortex with amygdala and hippocampal areas is a result of updating the threat detection memory, allowing extinction to occur. An alternative explanation is that the additional coactivation of piriform with BLA and hippocampus is a result of the social context because odor may play a significant role in social buffering (Klein et al., 2015; Takahashi et al., 2013). Additional studies in outbreds and selectively bred rodents are needed to confirm our results and tease apart these different hypotheses.

While both OFC and piriform cortex may play distinct roles in interpreting and contextualizing extinction learning, both of these prefrontal structures were coactivated with the hippocampus. We observed that the coactivations between OFC and piriform cortex with hippocampal subfields were stronger in bLRs from the in-group social context than in outbred animals. We also found evidence that coactivation between OFC and piriform cortex with ventral hippocampus in particular was increased in bLR animals compared to bHRs (Appendix 3.1). Both dorsal and ventral hippocampus are involved in extinction learning (Corcoran, Desmond, Frey, & Maren, 2005; Dejean et al., 2015; Maren, 2001; Maren & Fanselow, 1995; Maren & Hobin, 2007; Orsini et al., 2011; Sierra-Mercado, Padilla-Coreano, & Quirk, 2011; Wang, Yuan, Keinath, Ramos Alvarez, & Muzzio, 2015). The dorsal hippocampus is the primary location for place cells (Moser, Rowland, & Moser, 2015), which are involved in contextual learning, including during both fear conditioning and extinction (Wang et al., 2012; Wang et al., 2015). As previously mentioned, we see increased coactivation primarily between piriform cortex and dorsal hippocampus, indicating that these structures may be increasing coactivation to better identify, encode, and utilize contextual information to modulate freezing behavior in bLRs that are actively learning extinction. Ventral hippocampus is involved

in anxiety-like behavior (Bannerman et al., 2003; Bannerman et al., 2004), and has been implicated in both fear expression and extinction learning (Orsini et al., 2011; Sierra-Mercado et al., 2011). Coactivations between OFC and piriform cortex with ventral hippocampus may therefore influence extinction learning and the expression of anxiety behavior such as freezing. Our results suggest that coactivations between both dorsal and ventral hippocampus with areas of the prefrontal cortex are important for facilitating extinction learning of bLRs in in-group social contexts. Further studies are needed to better differentiate the roles of dorsal and ventral hippocampus in parsing social context.

Additional studies are also needed to further interpret the cFos data described in this chapter. For instance, currently it is unknown if the differential coactivations displayed by bHR and bLR animals are specific to extinction learning. Studies of basal bHR and bLR brains can determine whether cFos coactivation networks are differentially regulated in bHR and bLR animals under non-behavioral conditions. Additional studies using No-Extinction controls in selectively bred animals would also help to differentiate whether cFos coactivation between regions seen in our current study are specific to extinction learning or are non-specific coactivation in response to environmental stimuli. Finally, studies are needed to better delineate whether additional coactivation networks observed in bLR animals including OFC and piriform cortex are due to extinction learning, or potentially due to social context information. Studies in outbred animals manipulating social context can help to clarify this, as can studies in selectively bred animals that utilize No-Extinction controls in different social contexts. These studies will assist in further interpretation of the findings of increased coactivation of bLR animals in the in-group phenotype social context found here.

It should be noted that there are several limitations to our interpretation of the cFos correlations. First, our sample size for the outbred Extinction and No-Extinction groups was very low for correlation analyses. Increased sample size will provide more robust results. Additionally, our sample size was too low to be able to statistically compare coactivations between social context groups within phenotypes. Further studies with increased sample size will be necessary to fully parse the differences between coactivation networks of in-group phenotype and mixed group phenotype social contexts. We also do not know which cell types expressed cFos in our study, so it is currently unknown whether these regions may have been primarily active or inhibited, as cFos is expressed in both excitatory and inhibitory neurons as well as glia in response to stimuli (Herdegen & Leah, 1998; Pfarr et al., 2015). While we see positive correlations between all regions, and therefore identified them as significantly "coactivated" in this study, it is unclear whether that might have been an increase in the activity of inhibitory interneurons or primary projection cells. An additional caveat is that while many of these regions do have direct anatomical projections between them, it is unclear from our data what coactivation of cFos between regions indicates in terms of actual functional connectivity and communication. While these limitations prevent us from making stronger claims about the involvement of OFC and piriform cortex in fear extinction, we believe that the coactivation results we present provide preliminary evidence that these regions are important, perhaps particularly in social situations that facilitate fear extinction.

Conclusion

This set of studies provides unique insight into the way an environmental manipulation based on novelty-seeking phenotype can impact the extinction of learned fear and the neural correlates of that interaction. We have found that experimental design should be carefully determined to account for the social dynamics of the group being tested, including phenotypic individual differences, even in outbred Sprague Dawley rats. These design choices are even more important when working with selectively bred rodents, and potentially for genetic models that may be more or less vulnerable to poor fear extinction learning. We have also identified several brain regions, including orbitofrontal cortex and piriform cortex that may coactivate with amygdala and hippocampus when animals are surrounded by a context including in-group individuals that facilitate extinction learning. The ability to use social context during extinction learning to further assess gene-by-environment interactions in animal models is promising. Together, our work provides strong evidence that social context significantly impacts the extinction of learned fear in outbred and selectively bred rats, and that the neural correlates of this may involve the orbitofrontal cortex and piriform cortex.

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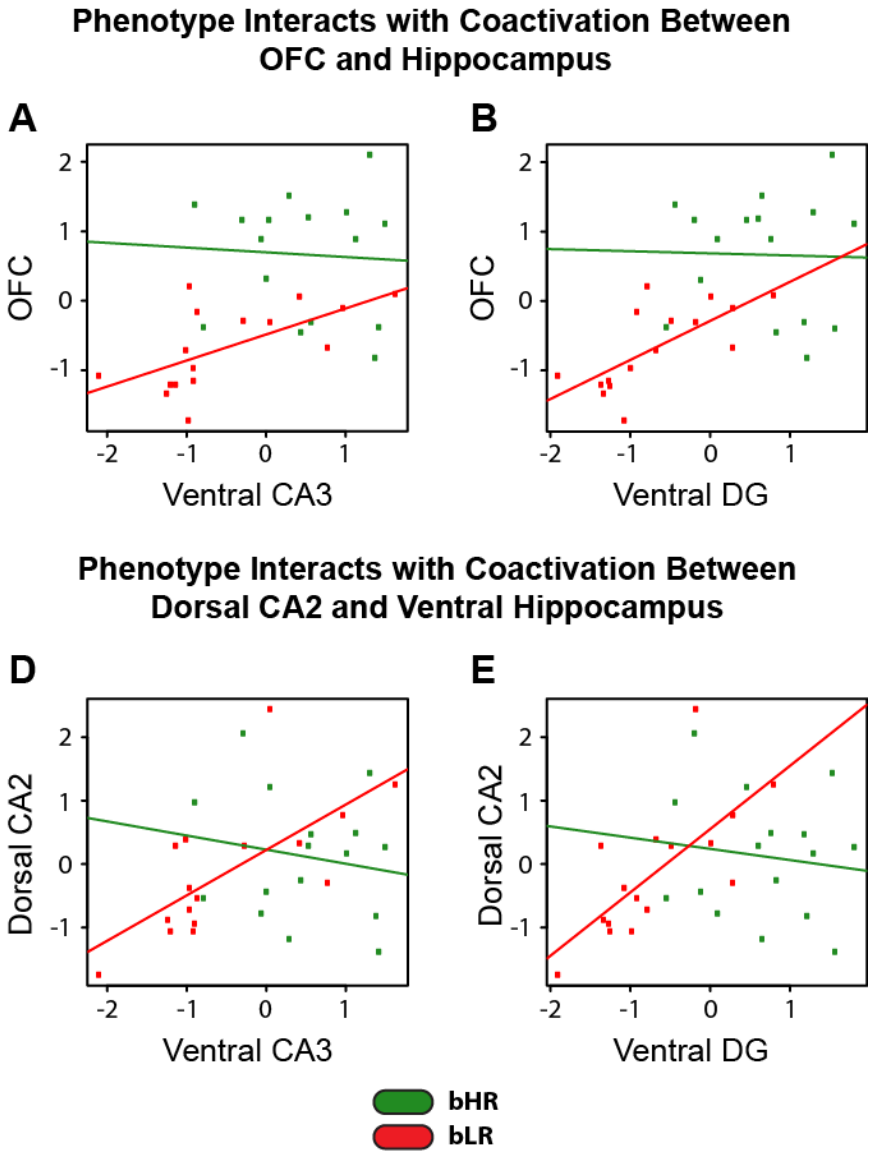
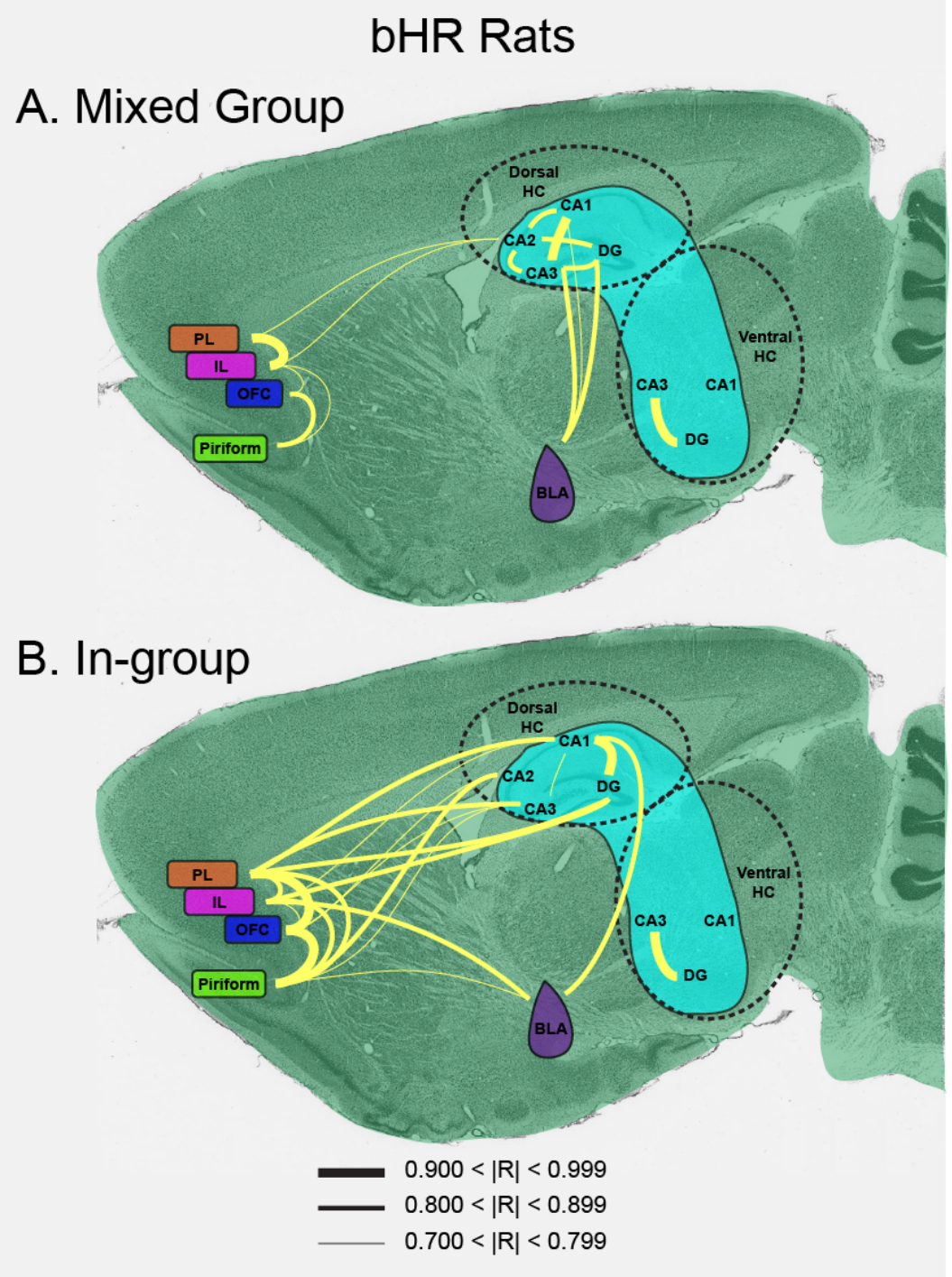
Appendix 3.1
bHR Coactivation Data and Comparison with bLR Coactivation Data

We observed differences in the coactivation between regions interacting with phenotype in our linear regression when only bHRs and bLRs were compared (uncorrected *p*-values). Since this analysis required the use of uncorrected p-values, we limit our explanation of findings to the five most significant ($p < 0.02$). At this uncorrected threshold, there was a significant interaction between phenotype and OFC with ventral CA3 ($p = 0.015$) and ventral DG ($p = 0.017$) where bHRs showed almost no relationship between regions while bLRs had a positive relationship (Supplementary Figure 3.1A, B). There was also a significant interaction between phenotype and dorsal CA2 with ventral CA3 ($p = 0.009$) and ventral DG ($p = 0.018$) where bHRs show a slight negative relationship between regions while bLRs show a stronger positive relationship (Supplementary Figure 3.1C, D). Finally, there was a significant interaction between phenotype and ventral CA3 with ventral DG ($p = 0.012$) where both bHRs and bLRs demonstrate strong positive relationships between regions but the relationship is stronger for bLRs (Figure 3.10 F). Several of these relationships were observed when outbred animals were included in the model and correction for multiple comparisons was applied, indicating that with further sample size and greater power, these relationships may reach full statistical significance.



Supplementary Figure 3.1 Interactions Between Selectively Bred Phenotype and Regional Coactivation. *The orbitofrontal cortex showed significant interactions between coactivation with subfields of the ventral hippocampus, including ventral CA3 (A) and ventral DG (B), and the phenotype of selectively bred animals. In these interactions, bHRs display little to no coactivation between regions, while bLRs demonstrate positive relationships between regions. The dorsal hippocampal subfield CA2 also showed significant interactions between coactivation with subfields of the ventral hippocampus, including ventral CA3 (D), and ventral DG (E), and the phenotype of selectively bred animals. These relationships were similar to those observed with the OFC, with bHRs showing slight negative relationships between regions and bLRs showing stronger positive relationships between regions. Together, these data suggest that although these interactions do not meet multiple comparison correction cut-offs, bHRs and bLRs also likely differ in their regional coactivation indicating that selectively bred phenotype also influences coactivation.*



Supplementary Figure 3.2 bHR coactivation patterns in response to extinction learning in mixed and in-group social context. *bHR rats extinguished in the mixed social context appear to display reduced coactivations than outbred extinction animals (A). bHRs extinguished in the in-group social context show more coactivations, including between IL and BLA (B). These results suggest that social context affects coactivation between brain regions even in animals that don't exhibit changes in behavior. (Rat brain image is derived from nissl stained tissue.)*

Chapter 4.

Amygdala and Anterior Cingulate Cortex Coactivation and Connectivity are Altered by Pathological Anxiety[1]

Since it is often challenging to differentiate potential vulnerability factors using behavioral measures, it is important to study brain function to determine whether individual differences in brain circuits may lead to vulnerability. The previous two chapters in this dissertation have demonstrated that individual differences in "temperament" (stable ways of responding to environmental stimuli) are associated with different behavioral and neural activation during fear extinction in rodents. In Chapter 3, we demonstrated that rats selectively bred for low locomotion in a novel environment also exhibit higher anxiety, a more "internalizing" temperament, reduced extinction learning, and show decreased coactivation of amydala with medial prefrontal cortex during extinction learning. These results indicate that individual differences in anxiety are predictive of reduced extinction learning in these rats, a pattern similar to that of PTSD patients (Bonanno, Galea, Bucciarelli, & Vlahov, 2007; Brewin, Andrews, & Valentine, 2000; Ozer, Best, Lipsey, & Weiss, 2003; Perrin et al., 2014). We also found evidence that individuals placed in a social context that facilitated extinction learning differentially

[1] *Portions of this chapter have previously been published in the journal of Depression and Anxiety in 2012 as "Aberrant amygdala-frontal cortex connectivity during perception of fearful faces and at rest in generalized social anxiety disorder" by Katherine E. Prater, Avinash Hosanagar, Heide Klumpp, Mike Angstadt, and K. Luan Phan and are reproduced here with permission of the publisher, John Wiley and Sons under license number 3867160443014.*

coactivated regions of the medial prefrontal cortex (including the orbitofrontal cortex, OFC) with the amygdala and hippocampus during extinction learning. This indicates that compensatory networks involving the medial prefrontal cortex, amygdala and hippocampus might be brought online to facilitate extinction in these high anxiety individuals. While these findings in rats are intriguing, it is unclear whether humans might show similar changes in coactivation with changes in anxiety level.

Fear conditioning has been used to test fear memory encoding and extinction in humans, and in particular, to develop an understanding of the ways in which the human brain responds to fear and fear memories. Using functional magnetic resonance imaging (fMRI), a number of studies have identified a network of brain regions including the ventromedial prefrontal cortex (vmPFC), the amygdala, and the hippocampus that are active during fear extinction in non-psychiatric individuals (Delgado, Nearing, Ledoux, & Phelps, 2008; Hartley, Fischl, & Phelps, 2011; LaBar, Gatenby, Gore, LeDoux, & Phelps, 1998; Linnman et al., 2012; MacNamara et al., 2015; Milad, Orr, Pitman, & Rauch, 2005; Milad, Quirk, et al., 2007; Milad, Rosenbaum, & Simon, 2014; Milad, Wright, et al., 2007; Phelps, Delgado, Nearing, & LeDoux, 2004). Unlike studies in animals, fMRI studies in humans that assess fear learning and extinction use several stimuli in their conditioning paradigms. In general, there is a conditioned stimulus that is paired with an aversive stimulus, most often a shock, (CS+), and a stimulus that is not paired with an aversive stimulus (CS-) to control for the neural activity caused by viewing stimuli during extinction. Studies of fear conditioning and extinction learning in humans have shown consistent amygdala activity when the CS+ is presented (Knight, Smith, Cheng, Stein, & Helmstetter, 2004; LaBar et al., 1998; Phelps et al., 2004).

During fear extinction in particular, areas of the prefrontal cortex are differentially active including the anterior cingulate cortex (ACC), or Brodmann's Area (BA) 32, which was more active to the CS+, while vmPFC and subgenual cingulate (BA25) were more active to the CS- (Ahs, Kragel, Zielinski, Brady, & LaBar, 2015; Phelps et al., 2004). In contrast, other studies have found consistent evidence of vmPFC activation during extinction with greater activation during the CS+ rather than the CS- (Kalisch et al., 2006; Milad, Wright, et al., 2007). Hippocampal activation is also seen during fear extinction, and studies that employ stronger contextual information in their stimuli find more robust evidence for hippocampal involvement in extinction learning (Kalisch et al., 2006; Knight et al., 2004; Lissek, Glaubitz, Uengoer, & Tegenthoff, 2013; Milad, Wright, et al., 2007). Overall, it would appear that a network of brain regions including the ACC, vmPFC, amygdala and hippocampus are active during fear extinction in humans.

One thing to note about these studies is that the term vmPFC is often used to indicate activation in a variety of different medial prefrontal cortical regions, including rostral areas of the ACC, as well as subgenual cingulate (BA25), medial prefrontal cortex, and sometimes parts of OFC, with different studies sometimes reporting one region and not the others. This use of a general regional term while the data themselves indicate possible region-specificity in response means that it is currently unclear which areas of medial prefrontal cortex are particularly important for extinction learning in humans, and in some cases the direction of activation (to CS+ or CS-) is also unclear.

Since PTSD is often considered a disorder of extinction learning (Dunsmoor & Paz, 2015; Lissek & van Meurs, 2015; Morey et al., 2015), it would be reasonable to expect that a diagnosis of PTSD would alter the neural activity seen during extinction

learning. Two studies have demonstrated reduced vmPFC activity and either increased dorsal ACC or amygdala activity during extinction in PTSD patients (Milad et al., 2009; Rougemont-Bucking et al., 2011). In contrast, several studies have demonstrated no differences in the activation of brain areas between PTSD patients and trauma-exposed controls during extinction learning (Garfinkel et al., 2014; Shvil et al., 2014; Steiger, Nees, Wicking, Lang, & Flor, 2015). Only one of these studies has compared against a non-trauma exposed control group, but there were also no differences between PTSD patients and the non-trauma exposed controls during extinction (Steiger et al., 2015). It is more common in the literature to find that PTSD patients appear to have reduced activity in vmPFC during fear extinction recall, which is tested *after* extinction learning (Garfinkel et al., 2014; Milad et al., 2009; Shvil et al., 2014), or during CS- trials during fear conditioning (Morey et al., 2015). These data indicate that although PTSD patients may have reduced activity in vmPFC, it may not be during extinction learning *per se*. Rather, alterations in activity may appear either beforehand or afterward, perhaps in situations where the CS is considered safe by non-psychiatric controls (CS- trials during conditioning and CS+ trials after extinction learning). These results stand in direct contrast to studies where deficits in the behavior of fear extinction learning have been consistently seen in PTSD patients (Blechert, Michael, Vriends, Margraf, & Wilhelm, 2007; Norrholm et al., 2011; Orr et al., 2000), and reduced extinction learning appears to be predictive of PTSD (Lommen, Engelhard, Sijbrandij, van den Hout, & Hermans, 2013). These inconclusive results of neural activation differences for PTSD patients during extinction are therefore surprising, and require further inquiry to determine

whether there are previously missed changes in neural activity that may underlie these consistent behavioral effects.

While it is currently unclear how a diagnosis of PTSD influences the neural circuitry underlying fear extinction, patients with other psychiatric disorders such as anxiety display more consistent changes in their brain activity during extinction learning. Patients who have panic disorder with agoraphobia demonstrate differential activation in ACC and amygdala during extinction learning when comparing patients who respond well to therapy and those who do not (Lueken et al., 2013). Individuals with generalized anxiety disorder or social phobia also demonstrated reduced ACC and vmPFC activity when viewing extinguished stimuli (Britton et al., 2013), and patients with obsessive compulsive disorder demonstrated reduced activity in vmPFC compared to controls during extinction and extinction recall (Milad et al., 2013). Together, these studies indicate that disrupted activation of vmPFC may be more prevalent in psychiatric disorders other than PTSD during extinction learning. The disorders discussed above, panic disorder, anxiety disorders, and obsessive compulsive disorder, are all members of the internalizing disorder transdiagnostic category (Carragher, Krueger, Eaton, & Slade, 2015; Eaton, Rodriguez-Seijas, Carragher, & Krueger, 2015; Krueger, McGue, & Iacono, 2001; Wolf et al., 2010). This categorical similarity may explain why disorders with such different symptoms might all display similar neural activity during extinction learning. PTSD is also often thought to fall in the category of internalizing disorders; however a recent study indicated that it may also share heritable traits with the externalizing disorders (Wolf et al., 2010). If PTSD does share characteristics with both the internalizing and externalizing disorders, this may explain why PTSD patients do not

show similar neural activity patterns as patients who have perhaps more strictly defined internalizing disorders. Overall, it appears that reduced vmPFC and increased amygdala responding during fear extinction learning are consistent across several of the internalizing disorders, indicating both that these regions play a crucial role in fear extinction and that propensity for internalizing alters standard brain activation patterns.

In this chapter, we assessed whether there are similarities between the rodent brain coactivation data described in Chapter 3 and human brain coactivation data during an extinction task. To understand how coactivation varies with anxiety in humans, we observed coactivation in participants with varying levels of trait anxiety during extinction learning. We hypothesized that during extinction, individuals with high trait anxiety would display reduced amygdala – subgenual cingulate cortex coactivation compared to individuals with low trait anxiety, but that we might see increased coactivation of hippocampus with orbitofrontal cortex, similar to the rodent data.

Methods

Participants

Seventy-two right-handed individuals participated in this study. Forty-seven had a primary psychiatric diagnosis while 25 were free of any prior or current diagnosis. Psychiatric diagnostic classification of participants was based on administration of the Structured Clinical Interview for DSM-IV (SCID-IV). Trained clinicians conducted all clinical assessments. All participants provided written informed consent and the study was approved by the institutional review board at the University of Illinois at Chicago.

Table 4.1 shows the participants' demographic and clinical characteristics. All participants were free of psychoactive medication and had negative urine toxicology tests at the time of scan. Female participants also tested negative for pregnancy on the scan day. Participants with a psychiatric diagnosis had a variety of primary diagnoses, with 18 participants diagnosed with generalized anxiety disorder (GAD), 11 with major depressive disorder (MDD), 9 with social anxiety disorder (SAD), 5 with PTSD, 3 with panic disorder, and one participant whose primary diagnosis was dysthymia. Additionally, these participants had a number of comorbid, or secondary diagnoses, including 10 with MDD, 10 with SAD, 8 with GAD and 5 with PTSD as well as two participants with a secondary diagnosis of specific phobia. Participants in this study are part of a study designed to assess the biological basis of negative valence systems as suggested by the Research Domain Criteria of NIMH.

Table 4.1 Participant demographics and clinical characteristics.

	Group Mean (SD)			
	Psychiatric	Non-psychiatric	*t*-value	*p*-value
Age	27.55 (8.53)	26.24 (12.35)	-0.53	0.598
Gender	13 M / 34 F	7 M / 18 F	0.001[a]	0.976
Education (years)	16.46 (3.47)	15.04 (2.91)	-1.74	0.087
STAI-T	54.91 (8.02)	27.24 (5.60)	-17.08	< 0.001
HAM-A	18.02 (7.66)	1.00 (1.66)	-14.6	< 0.001
LSAS	61.43 (23.94)	13.12 (9.77)	-12.07	< 0.001
PSWQ	63.57 (9.22)	32.48 (8.30)	-14.09	< 0.001
BDI	24.13 (8.68)	0.80 (1.41)	-17.99	< 0.001
HAM-D	12.53 (4.69)	0.40 (0.76)	-17.31	< 0.001

[a] χ^2 analysis.

STAI-T, Spielberger State-Trait Anxiety Inventory - Trait; HAM-A, Hamilton Anxiety Rating Scale; LSAS, Liebowitz Social Anxiety Scale; PSWQ, Post-traumatic Stress; BDI, Beck Depression Inventory; HAM-D, Hamilton Depression Rating Scale

Experimental Tasks

At the beginning of the experiment, all participants performed a fear conditioning task developed by me and tested in the Phan lab (MacNamara et al., 2015) that is a modified version of a task used previously (Milad et al., 2005; Milad et al., 2009; Milad, Quirk, et al., 2007). Fear conditioning was performed prior to entering the fMRI scanner. During fear conditioning, participants learned to associate the color of a streetlight (pink, blue, or yellow) with the possibility of receiving an electric shock on their left foot. One color became a conditioned stimulus that would later be extinguished during the extinction task (CS+E), one color became a conditioned stimulus that would remain un-extinguished (CS+U), and the other color served as a non-conditioned stimulus (CS-). During each trial, participants viewed photos of a streetlight that was lit in one of the colors (blue, pink, or yellow) in an outdoor scene (forest or school). The two contextual scenes (forest or school) were used to differentiate the conditioning context from the extinction context. The CS+E and CS+U trials were partially reinforced (60% of trials co-terminated with a 500ms presentation of a mild electric shock to the left foot). The other color of streetlight (blue, pink, or yellow, accordingly) was a non-conditioned stimulus (CS-) and was never reinforced. All stimuli and scenes were counterbalanced across participants. The stimulus timing was as follows: the unlit streetlight scene (context) was presented for 3 seconds, immediately following which the same streetlight appeared lit with the stimulus color for 6 seconds. These trials were interleaved by a fixation cross with an inter-trial interval varying from 12 to 25.5 seconds. The conditioning session consisted of 20 CS+E and 20 CS+U trials where 24 (12 of each conditioned stimulus) were reinforced with mild electric shock, and 20 CS- trials that

were never paired with shock. All trials were intermixed in pseudo-random order, and presented in two runs of approximately 12 minutes each. Prior to the beginning of the task, the shock level was calibrated based on participant feedback so that the electric current applied to their left foot was uncomfortable but not painful.

Immediately following fear conditioning, all participants completed the fear extinction task inside the fMRI scanner. Participants confirmed that the shock level used during the training task was uncomfortable, but not painful, prior to beginning the task. In the extinction task, the scene behind the streetlight (forest or school) was the opposite of the fear conditioning task. The CS+E and CS- colors were presented, but no shocks were delivered during either trial type. As in the fear conditioning task, the context was presented for 3 seconds before the appearance of the color in the streetlight, which remained for 6 seconds. During the presentation of the colored streetlight, participants were asked to rate their expectancy level of receiving a shock on a three-point scale. These trials were also interleaved with a fixation cross for an inter-trial interval varying between 12 and 25.5 seconds. 30 psychiatric patients and 13 non-psychiatric individuals completed the task with the parameters as described. The remaining 17 psychiatric and 12 non-psychiatric participants completed an identical task with slightly different stimulus timing parameters. In particular, the context was presented for 3 to 8 seconds prior to the appearance of the streetlight color, which remained for 4 seconds. We found no differences between activity on the two tasks in any brain region, and therefore have combined participants who completed these tasks with slightly different timing in this study. The inter-trial interval for this version of the task ranged from 4 to 9 seconds. The extinction session for both versions of the task consisted of 20 CS+E trials and 20 CS–

trials presented in two runs of approximately 8 minutes each. Participants were randomly assigned to one of two task versions that contained different pseudorandom stimulus presentation orders.

Analysis of Extinction Learning

During the extinction task, participants were asked to rate the likelihood of being shocked by pressing one of three buttons on a keypad. A response of 1 corresponded to "Yes I will be shocked", a response of 2 indicated "No I will not be shocked," and a response of 3 indicated that the participant was "Unsure" about whether they would receive a shock during that trial. Shock rating responses were recoded on a scale from 0 to 1, where 0 corresponded to "No", 0.5 corresponded to "Unsure" and 1 corresponded to "Yes" for shock likelihood (this corresponds more closely to the probability of being shocked). Based on this recoding, participant responses were averaged and compared using a one-way ANOVA with repeated measures for the trials in SPSS (IBM, Armonk, NY).

Functional Magnetic Resonance Imaging (fMRI) Parameters

Images were acquired on a 3.0T GE MR 750 scanner (General Electric Healthcare, Waukesha, WI) using an eight-channel phased-array radio frequency head coil. A gradient echo-planar imaging sequence was used (TR = 2000 ms, TE = 22.2 ms, 64 x 64 matrix, flip angle of 90°, FOV = 22 cm, 3.4 x 3.0 x 3.0 mm voxels) with 44 axial slices per volume.

fMRI Data Analyses

Data were preprocessed and analyzed using Statistical Parametric Mapping 8 (SPM8) software (http://www.fil.ion.ucl.ac.uk/spm). The first four volumes were discarded prior to analysis to allow for T1 equilibration effects. Images were realigned to correct for motion, corrected for errors in slice timing, spatially transformed to standard MNI space using the echo-planar imaging template provided with SPM8, resampled every 2mm using sinc interpolation and smoothed with an 8mm full-width-half-maximum Gaussian kernel to decrease spatial noise prior to statistical analysis. Translational movement in millimeters (x, y, z) and rotational motion in degrees (pitch, roll, yaw) were calculated based on the SPM8 parameters for motion correction. None of the participants included in this study had movement greater than 2 mm translation or 2° rotation in any direction.

We used a combination of region of interest (ROI) templates based on Brodmann's areas (BA) defined by cytoarchitectonic mapping and provided with SPM8 (Brodmann, 1909), and cytoarchitectonic mapping of post-mortem human brains (Amunts et al., 2005; Amunts & Zilles, 2015; Henssen et al., 2016). The ROIs used in this study were BA 11, BA 32, BA 25, and the basolateral (BLA) and central (CEA) subdivisions of the amygdala as well as the CA fields of the hippocampus. ROIs were split at the midline so that data from both right and left hemispheres could be extracted. The average hemodynamic response was extracted from each ROI during the CS+E or CS- trials during the extinction task for each participant using MarsBaR (http://marsbar.sourceforge.net/).

Additional Statistical Analysis

The linear regression analysis was conducted in R (R Core Team; Vienna, Austria; https://www.R-project.org). Analysis and comparison of participant demographics was conducted in SPSS (IBM, Armonk, NY).

We used linear regression to determine relationships between regional coactivation and trait anxiety. Data from the ROI analysis were centered and scaled so that the data could be compared across regions. Trait anxiety was a continuous measure across participants generated by using the value for each individual's STAI-T result (Spielberger, 1983). Note that the regression currently assumes the intercept of trait anxiety is zero. The relationships between regional coactivation and trait anxiety were examined using the linear model:

$$\textbf{Equation 1: } (\text{Activity in Region A}) \approx \beta_0 + \beta_1(\text{Activity in Region B}) + \beta_2(\text{STAI-T}) + \beta_3(\text{Activity in Region B} * \text{STAI-T})$$

This means that we examined whether there was a significant relationship between activation of two regions as it would appear in subjects with low anxiety (effect of region), and we further examined if regional coactivation was altered with changes in trait anxiety (the interaction of region by anxiety). We applied this model for each pair of regions included in our analyses and corrected for multiple comparisons using the Benjamini-Hochberg procedure with α set to 0.05. To display how the relationship between activation of two regions changed with increasing anxiety, participants were divided into thirds based on their STAI-T value and categorized as low, mid-level, or high anxiety individuals. It is important to note that our regression model contains multiple terms that will impact the way the model predicts the dependent variable, and therefore may not produce symmetrical relationships. That is, the activity in region A

might significantly predict that of region B (when the effects of other predictors are removed), however the reverse (activity from region B predicting activity in region A) may not be statistically significant, especially after multiple comparison correction.

Results

Levels of Trait Anxiety Modulate Extinction Learning

Participants displayed a main effect of trial, with shock likelihood ratings decreasing across the session, indicating that extinction occurred ($F(7.40,503.05) = 34.52$, $p < 0.001$; Figure 4.1). *Post hoc* analyses indicated that shock likelihood ratings were significantly higher during the first two CS presentations than during subsequent CS presentations (*post hoc* $p < 0.001$). Participants also displayed a main effect of CS type ($F(1,68) = 26.72$, $p < 0.001$), with higher shock likelihood ratings to the CS+ than the CS- (*post hoc* $p < 0.001$) indicating that conditioning to the CS+ occurred (Figure 4.1). There was a significant interaction between CS type and trial ($F(7.39,502.24) = 14.65$, $p < 0.001$), where the differences between CS+ and CS- were stronger at the beginning of the session, indicating that extinction occurred down to levels of CS- responding (Figure 4.1). There was no significant main effect of level of trait anxiety ($p = 0.953$). There was a significant interaction between trial and level of trait anxiety ($F(14.80,503.05) = 1.89$, $p = 0.023$) where individuals with high trait anxiety demonstrated faster extinction than individuals with moderate or low levels of trait anxiety, particularly during trials 4, 5, and 6 (Figure 4.1). No other main effects or interactions reached significance.

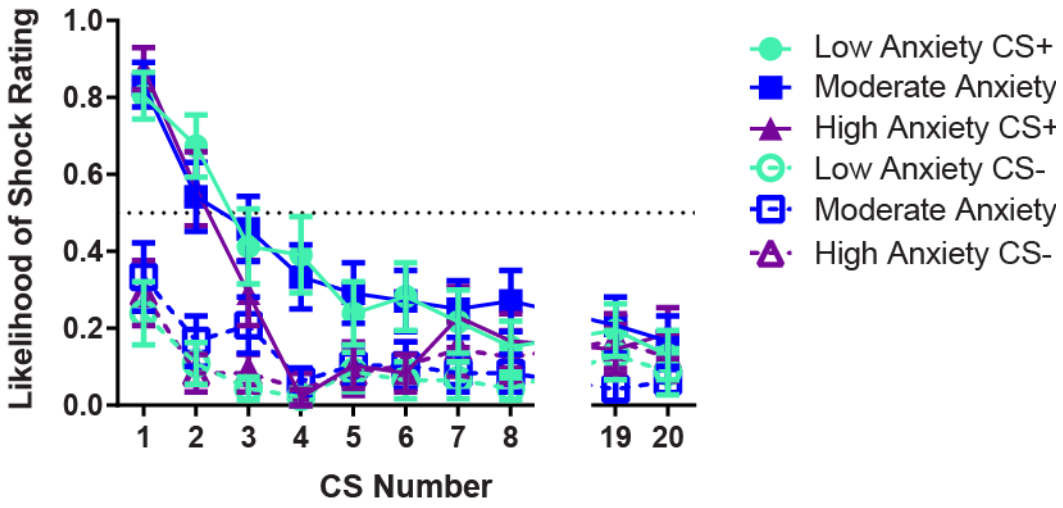


Figure 4.1 Behavioral ratings of shock expectancy demonstrate facilitated extinction learning for individuals with high trait anxiety. *The differences between the CS+ and the CS- shock likelihood ratings at the beginning of the session indicate that fear conditioning was effective for participants. Additionally, the decrease in shock likelihood ratings for CS+trials indicates that participants extinguished their fear, and then remained low throughout the session, similar to CS- shock likelihood levels. Individuals with high levels of trait anxiety demonstrate a quicker return to CS- levels of their shock expectancy ratings, while individuals with moderate and low levels of trait anxiety do not differ and extinguish more slowly. The dotted line at 0.5 denotes the level at which participants are "unsure" whether they will be shocked in a given trial.*

Levels of Trait Anxiety Modulate the Coactivation of BLA and ACC During Fear Extinction

Coactivation Between Regions During Extinction.

The relationships observed are for participants with low trait anxiety. During CS+ trials there were significant positive relationships between left hemisphere regions and their right hemisphere counterparts. There were also significant relationships between left OFC and left subgenual cingulate, left ACC and left OFC, left BLA and left hippocampus, left subgenual and right OFC, BLA, CEA and hippocampus, left ACC and right OFC, as well as left BLA and right hippocampus (Table 4.2).

Table 4.2 P-values for relationships between the left hemisphere and other brain regions during the CS+E for low-anxiety individuals.

		Left Hemisphere					
		OFC (BA11)	Subgenual Cingulate (BA25)	ACC (BA32)	BLA	CEA	Hippocampus
Left Hemisphere	OFC (BA11)		0.072	**0.020**	0.799	0.717	0.525
	Subgenual Cingulate (BA25)	**0.038**		0.236	0.707	0.639	0.208
	ACC (BA32)	0.065	0.556		0.094	0.688	0.794
	BLA	0.513	0.191	0.603		0.063	**0.000**
	CEA	0.927	0.967	0.757	0.693		0.525
	Hippocampus	0.327	0.073	0.910	**0.000**	0.247	
Right Hemisphere	OFC (BA11)	**0.000**	**0.007**	**0.031**	0.693	0.688	0.257
	Subgenual Cingulate (BA25)	0.083	**0.000**	0.603	0.671	0.688	0.212
	ACC (BA32)	**0.047**	0.080	**0.000**	0.359	0.739	0.973
	BLA	0.083	**0.002**	0.910	**0.000**	0.063	**0.000**
	CEA	0.625	**0.029**	0.910	0.591	**0.000**	0.212
	Hippocampus	0.432	**0.018**	0.910	**0.000**	0.227	**0.000**

Bold text indicates a significant relationship between regions
Colored text indicates a relationship that is present in CS+ and not CS- trials (extinction specific)

During the CS+ trials significant relationships exist between each right hemisphere region and its counterpart in the left hemisphere except for the CEA (Table 4.3). Additional relationships are significant between the right OFC and left subgenual cingulate, the right OFC and the right ACC, the right BLA and bilateral hippocampus, the right subgenual cingulate and right OFC, the right subgenual cingulate and the right BLA, CEA, and hippocampus, the right CEA and right BLA, and the right hippocampus and bilateral BLA (Table 4.3).

Table 4.3 P-values for relationships between the right hemisphere and other brain regions during the CS+E for low-anxiety individuals.

		Right Hemisphere					
		OFC (BA11)	Subgenual Cingulate (BA25)	ACC (BA32)	BLA	CEA	Hippocampus
Left Hemisphere	OFC (BA11)	**0.027**	0.128	0.143	0.901	0.932	0.841
	Subgenual Cingulate (BA25)	**0.027**	**0.000**	0.204	0.332	0.096	0.227
	ACC (BA32)	0.196	0.777	**0.005**	0.105	0.361	0.348
	BLA	0.313	0.128	0.422	**0.000**	0.178	**0.000**
	CEA	0.919	0.986	0.934	0.924	0.051	0.841
	Hippocampus	0.212	0.108	0.934	**0.002**	0.230	**0.000**
Right Hemisphere	OFC (BA11)		**0.003**	**0.046**	0.716	0.367	0.453
	Subgenual Cingulate (BA25)	**0.027**		0.379	0.332	0.113	0.225
	ACC (BA32)	**0.046**	0.128		0.332	0.932	0.841
	BLA	0.142	**0.001**	0.934		**0.048**	**0.000**
	CEA	0.313	**0.035**	0.934	0.537		0.225
	Hippocampus	0.157	**0.014**	0.934	**0.000**	0.053	

Bold text indicates a significant relationship between regions
Colored text indicates a relationship that is present in CS+ and not CS- trials (extinction specific)

All pairs of regions that demonstrated a significant relationship were positively correlated with each other during the CS+E trials of the extinction session, indicating substantial coactivation between regions. Almost all pairs of regions that displayed significant relationships also had a strong positive relationship during the CS- trials. Notable exceptions were the left hippocampus with left BLA, left OFC with right ACC, left subgenual cingulate with right CEA, the right OFC with right ACC, right subgenual cingulate with right CEA, the right CEA with right BLA, and the right hippocampus with left BLA, none of which were significantly coactivated during CS- trials. This lack of coactivation during CS- trials may indicate that coactivation of these regions is specific to extinction learning.

Coactivations Modulated by Trait Anxiety.

No brain regions had a significant relationship directly with the STAI-T, indicating that trait anxiety alone does not predict activation in these brain regions during extinction learning. None of the interactions between coactivation and the STAI-T for the stimulus being extinguished (CS+E) are present when activity during the presentation of the non-conditioned stimulus (CS-) is examined (Figure 4.3B, D). This indicates that the interactions between region coactivation and anxiety are specific to extinction learning.

Coactivation of the left BLA and left ACC is modulated by anxiety as measured by the STAI-T ($p = 0.001$; Table 4.4; Figure 4.3A). In this interaction, increasing anxiety levels increase coactivation between the regions (that is, the relationship between regions is stronger with higher anxiety scores; Figure 4.2).

Table 4.4 P-values for the interaction of trait anxiety with coactivation between left hemisphere brain regions and other regions during the CS+E.

		Left Hemisphere *p*-values					
		OFC (BA11)	Subgenual Cingulate (BA25)	ACC (BA32)	BLA	CEA	Hippo-campus
Left Hemisphere	OFC (BA11)		0.745	0.649	0.097	0.765	0.303
	Subgenual Cingulate (BA25)	0.838		0.715	0.179	0.765	0.747
	ACC (BA32)	0.838	0.509		**0.001**	0.437	0.110
	BLA	0.838	0.988	0.194		0.839	0.303
	CEA	0.838	0.355	0.194	0.108		0.303
	Hippocampus	0.838	0.988	0.336	0.888	0.905	
Right Hemisphere	OFC (BA11)	0.838	0.745	0.455	0.598	0.905	0.747
	Subgenual Cingulate (BA25)	0.838	0.988	0.649	0.437	0.765	0.790
	ACC (BA32)	0.838	0.988	0.315	0.072	0.765	0.343
	BLA	0.838	0.509	0.336	0.315	0.765	0.110
	CEA	0.838	0.895	0.336	0.211	0.765	0.747
	Hippocampus	0.838	0.745	0.336	0.442	0.974	0.343

Colored text indicates a relationship that is present in CS+ and not CS- trials (extinction specific)

Coactivation between the right BLA and the left ACC is modulated by anxiety as measured by the STAI-T (p = 0.004; Table 4.5; Figure 4.3C). In this interaction, increasing anxiety levels again increase the coactivation between regions (the relationship between regions is stronger with higher anxiety scores; Figure 4.2).

Table 4.5 P-values for the interaction of trait anxiety with coactivation between right hemisphere brain regions and other regions during the CS+E.

		Right Hemisphere *p*-values					
		OFC (BA11)	Subgenual Cingulate (BA25)	ACC (BA32)	BLA	CEA	Hippo-campus
Left Hemisphere	OFC (BA11)	0.961	0.815	0.834	0.059	0.927	0.028
	Subgenual Cingulate (BA25)	0.961	0.810	0.864	0.336	0.931	0.444
	ACC (BA32)	0.961	0.703	0.594	**0.004**	0.197	0.028
	BLA	0.961	0.822	0.447	0.743	0.931	0.444
	CEA	0.961	0.574	0.594	0.094	0.625	0.132
	Hippocampus	0.961	0.822	0.594	0.793	0.931	0.252
Right Hemisphere	OFC (BA11)		0.810	0.691	0.418	0.931	0.444
	Subgenual Cingulate (BA25)	0.961		0.704	0.418	0.931	0.592
	ACC (BA32)	0.961	0.822		0.059	0.927	0.252
	BLA	0.961	0.574	0.594		0.931	0.252
	CEA	0.961	0.810	0.632	0.195		0.521
	Hippocampus	0.961	0.718	0.632	0.908	0.931	

Colored text indicates a relationship that is present in CS+ and not CS- trials (extinction specific)

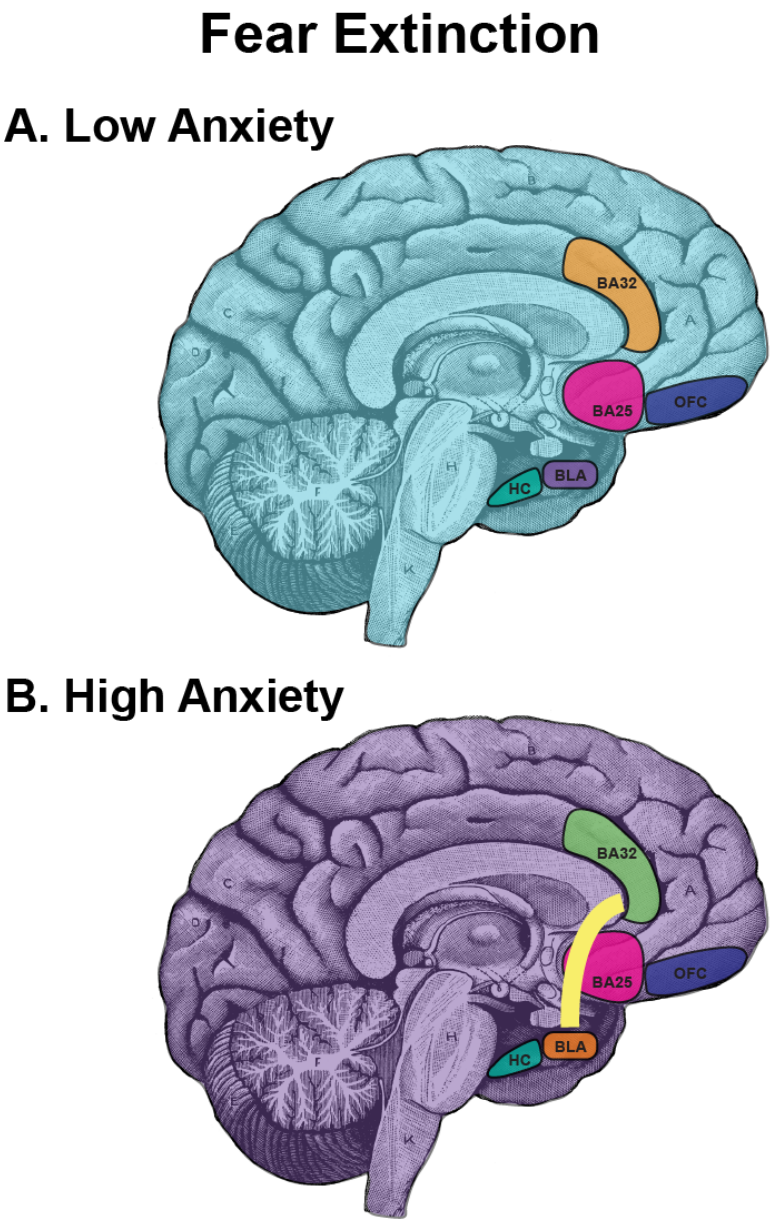


Figure 4.2 Diagram of the presence or absence of bilateral coactivation between BLA and ACC in low and high anxiety individuals. *In individuals with low levels of trait anxiety, there is little to no coactivation of BLA and ACC (A), while for individuals with the highest levels of trait anxiety there is significant coactivation between BLA and ACC (B).*

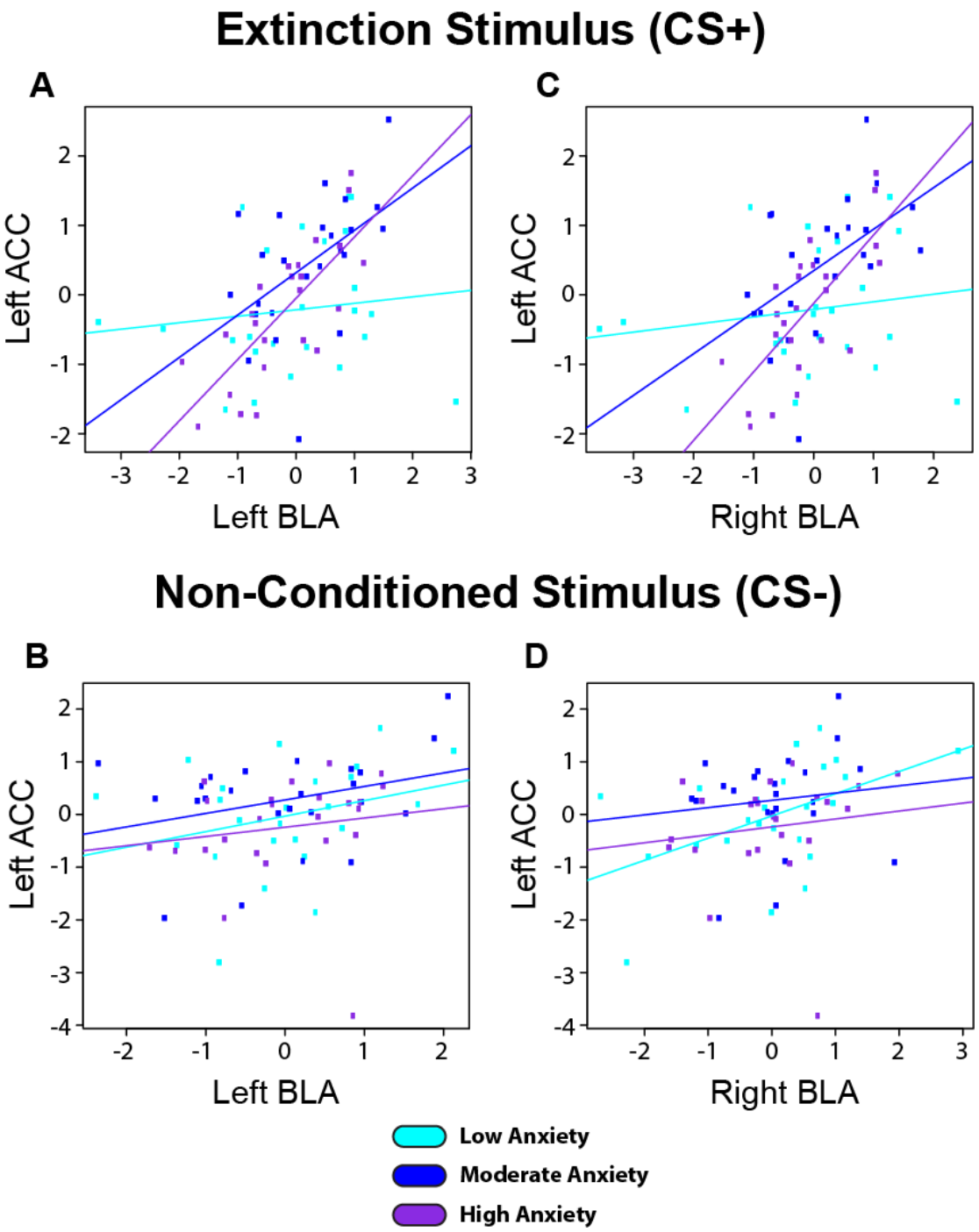


Figure 4.3 The relationship between BLA and ACC is influenced by trait anxiety levels during CS+ but not CS- trials. *The relationship between left BLA and left ACC is stronger with increasing levels of anxiety during CS+ trials (A), whereas there is little to no relationship between regions and no effect of anxiety during CS- trials (B). These same relationships hold for the right BLA and left ACC with anxiety modulating the relationship between regions during CS+ trials (C) but not during CS- trials (D).*

Discussion

The data presented here make a strong case for the dysregulation of amygdala-prefrontal cortex circuits during extinction in individuals with high levels of trait anxiety. These high trait anxiety individuals also carry a DSM diagnosis of internalizing disorders, however, we focused on trait anxiety as a continuous measure to observe its effects on neural circuit activity. Interestingly, while all individuals extinguished their fear, individuals with high trait anxiety extinguished their fear faster than individuals with moderate or low levels of trait anxiety as measured by shock likelihood ratings. In the fMRI data, we observed coactivations between hippocampus, amygdala, and areas of the medial prefrontal cortex that were specific to extinction learning in low-anxiety individuals, but most were not present in high anxiety individuals. By contrast, during extinction learning, BLA coactivation with ACC is increased in individuals with high trait anxiety, while individuals with low trait anxiety show little coactivation between these two regions. Although the pattern of coactivation observed during fear extinction learning did not support our hypothesis of reduced coactivation between BLA and subgenual ACC in high anxiety individuals, our data do indicate that there is dysregulation of the amygdala-prefrontal circuit when these more anxious individuals are extinguishing their conditioned response. These data suggest that changes in patterns of coactivation between amygdala and prefrontal cortex may be prevalent across species, and that dysfunction in the amygdala-prefrontal circuit may be a biomarker for high anxiety individuals.

Individuals with High Trait Anxiety Demonstrate Facilitated Extinction Learning

All participants in this study extinguished their fear to the CS+ quickly during the extinction session. Surprisingly, individuals with high trait anxiety extinguished their fear more quickly than those with moderate or low levels of trait anxiety. A previous study of the influences of trait anxiety on fear extinction demonstrated no behavioral differences between individuals with differing levels of trait anxiety (Sehlmeyer et al., 2011). However, this prior study was completed on a sample of purely non-psychiatric individuals, while our population includes patients with a variety of internalizing psychiatric diagnoses. Our behavioral data indicating that individuals with high trait anxiety extinguish more quickly than individuals with moderate or low trait anxiety is also in contrast to studies where PTSD patients demonstrate reduced extinction learning compared to control groups (Blechert, Michael, Vriends, Margraf, & Wilhelm, 2007; Lommen, Engelhard, Sijbrandij, van den Hout, & Hermans, 2013; Norrholm et al., 2011; Orr et al., 2000). While our participant population includes those with psychiatric diagnoses, a minority are those with PTSD, suggesting that different psychiatric diagnoses may influence the behavioral response to fear extinction.

While behavioral ratings provide us with a measure of extinction learning, it may be that participants with high levels of trait anxiety consciously respond to fear conditioning and extinction differently because of their attentional bias toward threatening stimuli in the environment. The use of unconscious measures such as galvanic skin response may provide a different measure of extinction learning, and perhaps one that is slower than that of low trait anxiety participants. Additional studies are needed to assess the potential coping skills developed by individuals with high trait

anxiety, and may provide important and interesting insight into the ways that trait anxiety affects interactions with the environment and experimental manipulations such as fear extinction in particular.

Coactivation vs. Functional Connectivity

Before we compare our results to the literature, it is important to discuss how measures of coactivation and connectivity differ. In Chapter 3 of this dissertation, we used a measure of cellular activity (cFos mRNA) to examine whether pairs of regions were coactivated or not during extinction learning. In order to match this technique as closely as possible, in this chapter we extracted the peak hemodynamic response function value from pairs of regions and used a regression analysis to assess coactivation during extinction learning (Figure 4.4A). Coactivation in both of these studies means that there is a significant positive relationship between activity measurements taken from a single point in time across pairs of regions (as activity in a region increases activity in the other region also increases). That is, activity in these regions is an average across the entire task of extinction learning and does not assess how brain regions might change their activity throughout the task. Thus, coactivation is a simple measure of whether two regions are more active in the same individuals.

In contrast to measures of coactivation, functional connectivity as assessed in many fMRI studies is a measure of how highly correlated regions are across time (Figure 4.4B). Fluctuations in the hemodynamic response function are calculated for a given region. These calculated fluctuations are called a timeseries. The timeseries is then correlated with a timeseries from another region. If the two regions have similar

timeseries (that is, as Region A increases Region B increases with approximately the same timing and as Region B decreases Region A also decreases with approximately the same timing), their correlation will be stronger. These correlations of the timeseries between regions can then be averaged across different groups of participants and compared between participant groups.

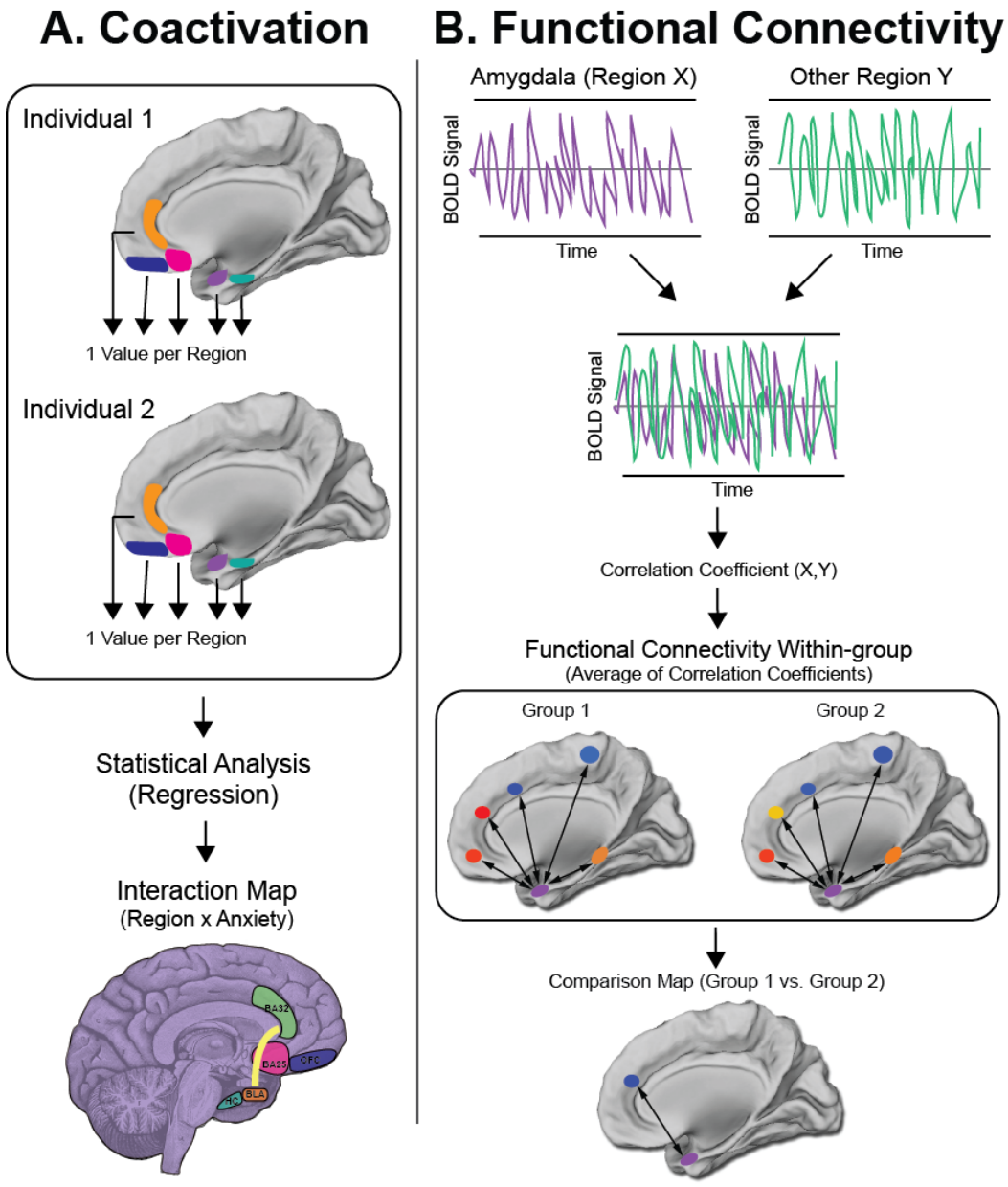


Figure 4.4 Coactivation Differs From Functional Connectivity by Comparing Results From a Single Timepoint. *When we examine coactivation in both humans and rodents*

(A), we collect a single average measure of neuronal activity from a variety of brain regions. These values are then analyzed using regression which allows us to determine which regions display significant relationships that are modulated by anxiety. In contrast, functional connectivity (B) takes the timeseries from a seed region such as the amydala and correlates that time series with the timeseries from all other brain regions. These correlation coefficients can then be viewed as connectivity maps within a group of participants, and compared between participant groups to assess where and how functional connectivity has changed between groups.

The coactivation analysis that we performed here is no longer a standard analytical approach to parse fMRI data, while functional connectivity is widely used. We chose to use coactivation in our study of extinction learning to better compare our results to the rodent data previously described. It is also important, however, to determine whether more standard analyses would lead to similar findings, which is why we compare our results to functional connectivity from our previous study (Prater, Hosanagar, Klumpp, Angstadt, & Phan, 2013). While these analyses are not directly comparable, we believe that they both offer insight into the ways in which brain regions act together to produce various behaviors during a variety of tasks.

Extinction-Specific Coactivation of Regions in the Human Brain

During fear extinction learning, several areas were coactivated in low-anxiety individuals, including areas of the prefrontal cortex, amygdala and hippocampus. Specifically, we observed coactivations specific to extinction learning for the hippocampus with BLA, the OFC with ACC, the subgenual cingulate cortex with the central amygdala, and in a more limited way, for CEA with BLA. It is well known that hippocampus, amygdala, and medial prefrontal cortex are involved in processing extinction learning (Delgado et al., 2008; Garfinkel et al., 2014; Kalisch et al., 2006; LaBar et al., 1998; Milad, Quirk, et al., 2007; Milad, Wright, et al., 2007; Phelps et al.,

2004). What is unique to our analysis here is the ability to assess coactivation between pairs of regions. Interestingly, one other study has used a region-of-interest based approach to analyze activity in a fear extinction paradigm. Ahs and colleagues (2015) extracted values from different regions and then used mediation analysis to determine that a hippocampal-vmPFC-amygdala circuit was particularly strong during recall of extinction learning. While we employed linear regression instead of mediation analysis here, our analysis of coactivation implicates similar structures in the processing of extinction learning.

While the regression analysis performed here to assess coactivation is not the same as a functional connectivity analysis as traditionally performed on fMRI data, to our knowledge there are no functional connectivity studies during extinction learning. A number of studies have examined functional connectivity after fear conditioning and have shown increased amygdala-ACC functional connectivity that predicts fear responses (Feng, Feng, Chen, & Lei, 2014). In another study, functional connectivity of amygdala with vmPFC after fear conditioning predicted the efficacy of extinction learning, indicating that connectivity between these regions might be important for extinction learning (Feng, Zheng, & Feng, 2015). Finally, resting amygdala metabolism as assessed by positron emission tomography using fluordeoxyglucose was positively correlated with activation of the vmPFC during extinction learning (Linnman et al., 2012). While each of these studies demonstrates amygdala and prefrontal cortex connectivity around tasks of fear conditioning or extinction, none of them directly analyzes connectivity during extinction learning itself. While our coactivation study does not assess how activity between regions changes over time as functional connectivity does, it does assess how

regions act within a subject and therefore provides some insight into how a circuit might be activated. Our study of coactivation is therefore the first to implicate coactivation of prefrontal, hippocampal and amygdala areas in extinction learning in low-anxiety individuals.

Anxiety-modulated Amygdala and ACC Coactivation During Extinction Learning

Coactivation between BLA and ACC was the only relationship modulated by trait anxiety, and it was modulated in a bilateral fashion. Interestingly, the coactivation of these two regions increased with trait anxiety across individuals; participants who demonstrated the highest levels of trait anxiety also showed the strongest coactivation between BLA and ACC.

In this analysis, we sought to compare coactivation of brain regions during extinction across species. In Chapter 3, we demonstrated that high anxiety bLR rats show reduced prefrontal-amygdala coactivation during extinction learning compared to standard outbred rats that have low levels of spontaneous anxiety. These bLR rats demonstrated increased coactivation of BLA with hippocampus and OFC when the animals learned extinction, indicating that these may be compensatory networks engaged to facilitate extinction learning in these animals. In our human study, we investigated how trait anxiety altered coactivation between regions. We hypothesized that trait anxiety (as measured by the STAI; Spielberger, 1983) is roughly analogous to the spontaneous anxiety exhibited by our rats. Similar to our coactivation data in the rodent model, our human results indicate that individuals with high levels of trait anxiety show increased amygdala-ACC coactivation when they learned extinction. We did not observe increased

OFC-amygdala connectivity in high anxiety humans, as we hypothesized based on the results from our animal model. While the specific prefrontal regions coactivated with the amygdala differ slightly between species, we observed that amygdala-prefrontal cortex coactivation increased when highly anxious rats and humans extinguished their fear.

There are a number of possibilities to explain why amygdala-prefrontal coactivation might differ between rats and humans. These differences may be due to the fact that the fear conditioning in humans is often a milder procedure than the standard protocols used in rats. Therefore our human participants (even those with high anxiety) fully extinguished their fear, while our bLRs demonstrate significantly reduced extinction learning. One other major difference between the two studies is that hemodynamic response is a correlative measure of cellular activity, while cFos mRNA is a more direct measure of cellular activity. Additionally, our rodent model is intended to mimic aspects of PTSD, but there were few PTSD patients in this particular human sample. Moreover, because we're examining trait anxiety, dissimilar patient populations may display different neural activity during extinction learning, which may have led to our divergent results between species. Therefore, while these experiments comparing neural coactivation in rodents and humans do not align directly, both studies indicate that there is clear dysfunction in the amygdala-prefrontal circuit in individuals with high levels of anxiety. Further studies will be needed to determine how coactivation changes across different patient populations.

Other researchers have observed that trait anxiety modulated brain activity in humans during fear conditioning and extinction tasks. Elevated trait anxiety across individuals increased the amount of activity in the somatosensory cortex during fear

conditioning, indicating that increased trait anxiety influenced brain activity, including in non-psychiatric participants (Greening, Lee, & Mather, 2016). Trait anxiety also increased amygdala response to fearful stimuli and decreased hippocampal connectivity with vmPFC during fear conditioning (Indovina, Robbins, Nunez-Elizalde, Dunn, & Bishop, 2011). Although the vmPFC regions described by Indovina and colleagues (2011) were smaller than the OFC used in the current study, the peak coordinate used to define their regions falls within BA11 (our OFC). Our data demonstrate no differences in coactivation between hippocampus and OFC for high anxiety individuals during extinction, indicating that the reduced connectivity seen in their study may be specific to fear conditioning. Another study demonstrated that high trait anxiety in non-psychiatric participants increased amygdala activity during the early parts of extinction learning and decreased dorsal ACC response during the end of the extinction task (Sehlmeyer et al., 2011). This reduced dorsal ACC activity during extinction appears to be in direct contrast to our results, which showed increased BLA-ACC coactivation in high anxiety individuals. However, our coactivation analysis is substantially different from a standard activation analysis, and therefore it is challenging to determine whether our results are actually contradictory.

While the previous studies demonstrate that high trait anxiety can modulate activity in brain regions, including the amygdala and PFC during extinction learning, all of those studies were conducted in non-psychiatric participants, while our study includes both non-psychiatric controls and individuals diagnosed with a mood or anxiety disorder. Fear conditioning and extinction are often thought to model processes particularly relevant to the development and treatment of PTSD, so it is interesting to note that there

are few studies that show differences in brain activity between PTSD patients and controls during extinction learning (Milad et al., 2009; Rougemont-Bucking et al., 2011). It is more consistent to find studies that demonstrate differential neural activity during extinction learning in other anxiety disorders, including panic disorder, generalized anxiety disorder, and obsessive compulsive disorder (Britton et al., 2013; Lueken et al., 2013; Milad et al., 2013). Given that a history of previous anxiety may be a vulnerability factor for developing PTSD (Bonanno, Galea, Bucciarelli, & Vlahov, 2007; Brewin, Andrews, & Valentine, 2000; Ozer, Best, Lipsey, & Weiss, 2003; Perrin et al., 2014), and that reduced vmPFC activity during extinction seems to be consistent across anxiety disorders other than PTSD, it is possible that the inconsistency in altered vmPFC activity across neuroimaging studies is due to comorbid anxiety diagnoses in the samples rather than to PTSD itself. However, this hypothesis requires further investigation.

Our study contains patients with primary psychiatric diagnoses that span a variety of disorders, including generalized anxiety disorder, panic disorder, and social anxiety disorder, and this may influence our results. While the studies of anxiety disorder patients discussed in the introduction generally demonstrate reduced vmPFC activity in anxiety patients compared to healthy controls, we show that BLA-ACC coactivation increases with anxiety. Previous studies demonstrated negative correlations between amygdala and vmPFC in PTSD patients (Shin et al., 2004; Shin et al., 2005). These negative correlations are not found in our study, as we saw only positive relationships (coactivation) between regions. Our patient population does not include many PTSD patients, so it may be that the differences between trait anxiety and an actual diagnosis in PTSD differentially modulate the coactivation between amygdala and regions of the

medial prefrontal cortex. Our patient population does include a large number of generalized anxiety disorder patients, and previous studies have demonstrated that these patients show positive correlations between amygdala and ventrolateral prefrontal cortex (McClure et al., 2007; Monk et al., 2008). While these previous studies did not examine correlations between orbitofrontal cortex or ACC, and we did not examine ventrolateral prefrontal cortex, it appears that generalized anxiety disorder patients may show increased coactivation between amygdala and multiple prefrontal cortex regions. Further studies will be needed to assess how consistently generalized anxiety disorder patients display increased coactivation between regions, and whether different anxiety disorders demonstrate distinct differences in the direction of relationships between amygdala and different areas of the prefrontal cortex.

Increased coactivation between amygdala and prefrontal cortex may also be a result of threat detection. Activity in the ACC is associated with both startle amplitude in response to phobic stimuli (Pissiota et al., 2003), as well as fear expression (Milad, Quirk, et al., 2007; Rougemont-Bucking et al., 2011). Dorsal ACC metabolism at rest predicted both PTSD symptoms and neural response to fear extinction in a variety of brain regions, including its own activity, vmPFC, and hippocampus in both PTSD patients and trauma-exposed controls (Linnman et al., 2012; Marin et al., 2016). Together, these findings suggest that activity in ACC may be a result of increased fear or threat detection in the environment. This heightened ability to identify threat in the environment might lead to facilitated extinction behavior as observed in our high trait anxiety individuals, as they are more aware of the contingencies during the extinction task.

Increased amygdala activity is also thought to result from increased arousal or bias toward threat in the environment (Bögels & Mansell, 2004; Etkin & Wager, 2007; Stein, Goldin, Sareen, Zorrilla, & Brown, 2002), indicating that our participants with high anxiety may coactivate ACC and amygdala because of increased threat perception. Studies in monkeys using electrophysiology have demonstrated that successful reversal learning of an aversive conditioning task requires amygdala and ACC functional connectivity, with ACC propagating signals back to the amygdala regarding whether the expected US was present or omitted (Klavir, Genud-Gabai, & Paz, 2013). These findings suggest that functional connectivity between the ACC and amygdala is necessary for adaptive responding to changing aversive contingencies. In the current study, individuals with high trait anxiety extinguished their fear faster than those with moderate or low levels of trait anxiety. It may be that the high trait anxiety participants exhibited greater BLA-ACC coactivation because they learned the contingencies of extinction better and more quickly than other participants. Interestingly, further evidence in monkeys indicates that synchrony between amygdala and ACC may predict when memories will resist extinction learning (Livneh & Paz, 2012), and that inducing lower firing rates of neurons in ACC during extinction learning reduces the spontaneous recovery of fear (Klavir, Genud-Gabai, & Paz, 2012). Collectively, these studies in monkeys indicate that communication between amygdala and ACC is vital for extinction learning that is lasting, and that increased ACC activity or synchrony between ACC and amygdala can result in decreased extinction learning and retention. Our current study does not assess whether these individuals effectively extinguished their fear across sessions, further studies are needed to assess whether the increased coactivation between ACC and amygdala

observed in our study leads to decreased between-session extinction learning. It appears that *high trait anxiety biases individuals toward increased amygdala and prefrontal cortex coactivation as a result of increased threat detection.* This increased threat detection could arise from a general attentional bias to threat, potentially facilitating the behavioral extinction of fear, and future studies can clarify this hypothesis.

Amygdala-Frontal Connectivity Abnormalities Are Also Present Across Tasks in Anxious Individuals

In a previous study of anxiety patients, we observed less amygdala connectivity to the frontal cortex that was localized in the same general ACC area during both fearful minus happy faces and at rest (Appendix 4.1; Prater, Hosanagar, Klumpp, Angstadt, & Phan, 2013). This area of ACC was very similar to the one we used to measure coactivation during extinction learning in the current study. This additional finding suggests that aberrant amygdala-ACC connectivity in anxiety patients may exist across tasks and mental states (Prater et al., 2013). ACC is thought to provide feedback to amygdala by modulating the extent to which it responds to salient emotional signals (Goldin, Manber-Ball, Werner, Heimberg, & Gross, 2009). Failure to recruit ACC in the regulation of a provoked or unprovoked anxiety state may lead to persistent amygdala activity often seen in patients with anxiety across a variety of social-emotional threat-related tasks (Etkin & Wager, 2007; Stein et al., 2002) due to decreased attentional control and an increased attentional bias to threat (Bögels & Mansell, 2004). The decreased connectivity seen across rest and task potentially illustrates the increased vigilance for threatening information seen in anxiety patients in the absence of a threat

cue (i.e. at rest; Bögels & Mansell, 2004), and their decreased emotion regulation and increased response to threat when a threatening cue is present (i.e. during viewing of fearful faces; McTeague et al., 2009).

Other studies have also demonstrated dysfunction in amygdala-ACC circuits. Hahn et al. (2011) observed reduced amygdala connectivity to ACC/ventral mPFC in anxiety patients at rest. It should be noted, however, that using different connectivity analytic approaches ('effective connectivity' as determined by Granger causality analysis, GCA, and independent component analysis, ICA), Liao et al. observed *increased* amygdala to ventral mPFC or ACC connectivity in subjects with anxiety during the resting state (Liao, Chen, et al., 2010; Liao, Qiu, et al., 2010). This finding from Liao et al. better match the findings of our human data during extinction, indicating that there may be commonalities across mental states (resting state and extinction) that appear using specific techniques and specific anxious populations.

Our previous finding indicates that the deficit in amygdala-ACC connectivity may be relevant to a brain model of anxiety involving abnormalities that both persist at the baseline state, and during signals of threat. The finding of group differences is also consistent with evidence in generalized anxiety disorder in which decreased connectivity between ACC and amygdala is associated with a failure of implicit emotion regulation (Etkin, Prater, Hoeft, Menon, & Schatzberg, 2010), and with major depressive disorder, where patients exhibit decreased amygdala to ACC connectivity in response to emotion processing (Carballedo et al., 2011). These results indicate a possible commonality across anxiety disorders, and perhaps across both anxiety and depressive disorders, implicating internalizing disorders in general. Similar aberrant connectivity patterns at rest *and*

during task indicate that this differential connectivity between fear generating (amygdala) and fear regulating (ACC) regions may underlie the pervasive attention, interpretive, and memory bias for threatening information and persistent negative self-reflection seen in patients with gSAD (Bögels & Mansell, 2004).

Coactivation and Functional Connectivity Results Implicate an Altered BLA-ACC Relationship in Individuals with High Anxiety

The coactivation and connectivity results described appear at first glance to contradict each other. In the coactivation study, individuals with high levels of trait anxiety show increased BLA-ACC coactivation. In the connectivity results described in the discussion, anxiety patients show decreased BLA-ACC connectivity across both task and rest (Prater et al., 2013). As discussed previously, coactivation is not a measure of functional connectivity. Thus, although we can say that BLA and ACC both increased their activity similarly in individuals with high trait anxiety, the current data do not allow us to determine whether or how that relationship changed over time throughout the extinction task. It may be that functional connectivity analysis in these participants would also demonstrate reduced connectivity in high trait anxious individuals despite the increased coactivation. Thus, we cannot say whether the functional connectivity data match or contradict the coactivation data. Further analyses will need to directly compare coactivation with connectivity in the same participants to determine how they relate. Additionally, it may be that the mental state of extinction learning is significantly different from threat perception or the resting state tasks used in previous studies. Thus, the coactivation between regions might alter directionality based on the specific task

being performed. Further studies should examine coactivation during threat perception and the resting state as it relates to trait anxiety to determine if there are similarities or differences in coactivation direction between the tasks. Despite the seemingly opposite direction of our effects paired with our previous data, we observed dysregulation of the same brain regions across multiple tasks, and therefore we believe these data implicate dysfunction of the BLA and ACC across tasks in high anxiety individuals.

Limitations and Future Directions

The current findings should be considered in the context of several noteworthy limitations. The coactivation analysis does not directly compare the relationship between CS+ and CS-. Thus, although we can say that there are significant relationships that may exist during one and not the other, further statistical analysis is needed to fully parse the differences in coactivation between these two stimuli. In the current analysis we used a variety of regions defined by post-mortem cytoarchitectonic mapping of the human brain, and those defined by Brodmann's areas using older cytoarchitectonic maps. It has recently come to our attention that a new set of cytoarchitectonic maps for the orbitofrontal cortex has been released (Henssen et al., 2016), and future studies should employ these to determine if a more precisely defined OFC coactivates differently with other regions. Additionally, the high anxiety participants in this analysis consist of individuals with a variety of primary diagnoses including major depressive disorder which, although often comorbid with anxiety, is not itself an anxiety disorder. Further analysis of this dataset will provide information on whether primary diagnosis is meaningful for the different relationships in coactivation of amygdala and ACC,

including whether these results are specific to anxiety. It will also be of interest to assess functional connectivity in these individuals during both extinction learning and during the resting state to understand how well our coactivation analysis matches the more often used measures of functional connectivity. This analysis will also determine whether the direction of effects (increased amygdala-pfc coactivation) in this participant population replicates or is different from the anxiety population in our previous experiment. Further studies should address these points, as they will provide important information for further interpretation of this increased coactivation between regions in high anxiety individuals.

Conclusion

The major contribution of the approach described is the use of coactivation in participants with varying levels of trait anxiety to implicate dysfunctional amygdala-prefrontal circuits across species and mental states. The current dataset combined with our previous study also implicates amygdala-prefrontal dysfunction across anxiety disorders. A recent meta-analysis of fMRI studies of anxiety and stress disorders identified dysfunctional amygdala-PFC connectivity across anxiety disorders (Duval, Javanbakht, & Liberzon, 2015). This confluence of results indicates that amygdala-prefrontal dysfunction may be very consistent across species and across specific anxiety disorders for individuals with high levels of anxiety. Our results and those of previous studies indicate that different task and rest paradigms each provide unique and important information about brain function in anxiety patients. While stronger amygdala-ACC coactivation was present in high anxiety individuals during extinction, we observed reduced amygdala-ACC connectivity during an emotional faces task and at rest in a

previous study of anxious individuals (Prater et al., 2013). While the strength of the connectivity or coactivation might change across tasks, it would appear that abnormal amygdala-PFC relationships represent a tonic (present across multiple tasks and rest) state for the anxious brain. Patients with anxiety often show a constant attention bias for threatening information (Bögels & Mansell, 2004); we believe the dysregulated amygdala connectivity with ACC seen during extinction, an emotion task that uses threat perception and at rest may be related to this altered attention state, and may apply to other anxiety disorders as well. Since the amygdala-PFC network appears to be dysregulated consistently in individuals with high anxiety, this dysfunction may be a potential biomarker for anxious individuals. These findings should prompt further studies to better delineate the functional relevance of amygdala-frontal networks and the strength of their connectivity that account for both task-dependent and task-independent patterns of brain connectivity in health and disease.

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Appendix 4.1

Methods and Results for Comparison of Resting-state and Emotion Task-related Functional Connectivity in Anxiety Patients

Methods

Participants

Twenty right-handed patients with generalized social anxiety disorder (gSAD) and 17 matched healthy controls (HC) participated in the study. Psychiatric diagnostic classification of participants was based on administration of the Structured Clinical Interview for DSM-IV (SCID-IV). gSAD participants were additionally verified to have gSAD based on the clinician-administered Liebowitz Social Anxiety Scale (LSAS). HCs were required to be free of prior or current psychiatric disorder. Trained clinicians, including a board-certified psychiatrist (K.L.P.) conducted all clinical assessments. All participants provided written informed consent and the study was approved by the institutional review boards at the University of Michigan and the University of Chicago.

Supplementary Table 4.1 shows the participants' demographic and clinical characteristics. Two gSAD patients were on a selective serotonin reuptake inhibitor with no change in medication or dosage for at least 8 weeks prior to the scan. All other participants were free of psychoactive medications at the time of scanning and urine toxicology screens were negative for all participants on scan day. No patients had a major depressive episode or substance abuse within a six-month period prior to scanning. Some

gSAD patients had psychiatric co-morbidity (n=3 with current specific phobia, one of whom also had current generalized anxiety disorder; n=1 had current obsessive-compulsive disorder; and n=1 with current panic disorder); of note, for all patients, gSAD was the primary, most clinically salient diagnosis at the time of study entry.

Supplementary Table 4.1 Experiment Two Participant Demographics and Clinical Characteristics

	Group Mean (SD)			
	gSAD	Control	*t*-value	*p*-value
Age	25.95 (5.39)	25.71 (7.15)	0.12	0.907
Gender	9 M / 11 F	7 M / 10 F	0.06[a]	0.815
LSAS	79.35 (15.41)	7.94 (7.05)	18.56	< 0.001
BDI	14.35 (8.33)	0.82 (1.07)	7.19	< 0.001
STAI-T	46.45 (11.88)	26.00 (3.04)	7.42	< 0.001

[a] χ^2 analysis.

LSAS, Liebowitz Social Anxiety Scale; BDI, Beck Depression Inventory; STAI-T, Spielberger State-Trait Anxiety Inventory – Trait

Experimental Tasks

All participants performed both the emotional face-matching task (EFMT) and the resting state scan (RS) following conventional procedures previously described in healthy and gSAD subjects (Hahn et al., 2011; Labuschagne et al., 2010; Liao, Chen, et al., 2010). The EFMT, a variant of the task originally described by Hariri et al. (2002), is designed to isolate amygdala response to social signals of threat. In brief, this task involved photographs from a validated set of face stimuli (Gur et al., 2002) presented in a block-design during which participants view a trio of faces and select one of two faces (bottom) that expressed the same emotion (happy, fearful or angry) as the target face (top). The identity of all three faces was always different, and an equal number of male and female faces were used in the task. The face-matching task was interspersed with an

identical geometric shape-matching task. There were a total of 18 blocks in the task, three of each of the three emotions, and a corresponding nine blocks of shape matching. Each block lasted 20 seconds with five presentations of either faces or shapes per block. The order of emotion blocks was counterbalanced across participants, however the task always began with face matching and alternated with shape matching.

The RS scan is designed to probe intrinsic connectivity patterns at rest; subjects were instructed to fixate on a crosshair on a blank gray screen, relax, and let their mind wander without falling asleep for 5 minutes.

fMRI Parameters

Images were acquired on two identical 3.0T GE Signa scanners using the standard radiofrequency head coil and associated software (LX 8.3, Neuro-optimized gradients, General Electric). Seven participants (4 gSAD, 3 HCs) were scanned on one scanner while the remaining participants were scanned on an identical scanner at a different institution. There was no difference between image acquisition parameters or processing steps between scanners. Whole-brain functional MRI scans were acquired using a T2-weighted reverse spiral gradient-recall echo sequence (TR = 2000 ms, TE = 25 ms, 64 x 64 matrix, flip angle of 77°, FOV = 240 mm, 3.75 mm^2 in-plane voxels) with 30 contiguous 5mm axial slices per volume.

fMRI Data Analyses

Data were preprocessed and analyzed using Statistical Parametric Mapping 5 (SPM5; http://www.fil.ion.ucl.ac.uk/spm). The first four volumes from each task run and

the first eight volumes from each resting run were discarded to allow for T1 equilibration effects. Images were realigned to correct for motion, corrected for errors in slice timing, spatially transformed to standard MNI space using the echo-planar imaging template provided with SPM5, resampled every 2mm using sinc interpolation and smoothed with an 8 mm full-width-half-maximum Gaussian kernel to decrease spatial noise prior to statistical analysis. Translational movement in millimeters (x,y,z) and rotational motion in degrees (pitch, roll, yaw) were calculated based on the SPM5 parameters for motion correction. None of the participants had movement greater than 2 mm translation or 2° rotation.

In order to extract signal from a region of interest (ROI) that is robust, yet unbiased to any one particular emotional expression (fearful face, happy face) or to any one particular group (gSAD, HC), we used a 'functional' localizer ROI approach that also takes into account anatomical constraints (Poldrack, 2007). We defined an amygdala seed derived from the functional activation of the "all faces" vs. "all shapes" task contrast of the second level GLM with a threshold of $p < 0.001$. Results from the conjunction of task activation from both groups were confined within the AAL defined anatomical amygdala (Tzourio-Mazoyer et al., 2002). The resulting right amygdala ROI 'seed' was 696 mm^3 in volume and the left amygdala ROI 'seed' was 1008 mm^3 in volume.

To examine amygdala-frontal connectivity to fearful faces, we employed conventional steps using Psycho-Physiological Interaction (PPI) analysis (Friston et al., 1997). PPI analysis allows us to isolate the context-dependent coupling ('PPI-FC') pattern during the task of perceiving social signals of threat and comparing 2 face stimuli directly allows us to de-confound non-salient features that are common to both types of

stimuli. For the PPI analysis, the interaction term of the amygdala seed timeseries with the task parameters (fearful vs. happy) was the variable of interest. The timeseries of the seed itself as well as the task covariate and the six movement parameters were all included as effects of no interest.

rsFC was implemented using conventional methods previously described (Etkin, Prater, Schatzberg, Menon, & Greicius, 2009). Importantly, we used the same right and left amygdala seeds for both of the two (PPI-FC and rsFC) connectivity analyses. In brief, the resting data were first bandpass filtered between frequencies of 0.008 to 0.1 to limit the analysis to resting state frequencies of interest (Cordes et al., 2001). The seed ROI timeseries was used as a covariate of interest in a first level model for each participant to provide whole-brain correlation values. The six motion parameters and the global signal were covariates of no interest.

All second-level analyses for between-group results consisted of random effects models. For between-group comparisons (two samples *t*-test) we set a whole-brain voxel-wise significance threshold for peak voxel significance at $p < 0.05$, cluster-level corrected for multiple comparisons across the entire brain (cluster volume > 28048mm^3, for the PPI-FC analysis; cluster volume > 25512mm^3 for the rsFC analysis); these cluster-level thresholds were calculated using Monte-Carlo simulations (AFNI AlphaSim, http://afni.nimh.nih.gov/afni/doc/manual/AlphaSim). After multiple comparison correction, the AAL toolbox for SPM was used to further identify the surviving clusters by computing the volume of each cluster overlapping the anatomical regions defined by the AAL. Within significant clusters, we searched for those PFC regions *a priori* hypothesized to exert group differences in amygdala connectivity (ACC, mPFC, DLPFC,

OFC). Results are given as peak Z-score of the cluster and volume of each *a priori* region within those significant clusters. To clarify group differences, beta (β) weights (an estimate of connectivity strength in arbitrary units) were extracted from 5mm-radius spheres surrounding the peak voxel within the *a priori* region.

Additional Statistical Analysis

We conducted *post hoc* Pearson's correlations between symptom severity measures and the connectivity beta weights from peak ROIs described above using SPSS. Beta weights were correlated with total LSAS score as well as the $LSAS_{Fear}$ and $LSAS_{Avoidance}$ subscales. Correlations were Bonferroni corrected for multiple comparisons using α set to 0.05 leading to a significance threshold of $p < 0.008$.

Results

Behavioral Results

Both groups performed the EFMT well, achieving means of greater than 90% accuracy and reaction times less than 2000ms on average per trial. Overall, there was no main effect of group or group x emotion interaction on accuracy (gSAD patients M = 94.99, SE = 0.75; HCs M = 94.88, SE = 0.64) or reaction time (gSAD patients M = 1471.84, SE = 46.50; HCs M = 1400.63, SE = 54.17; all $p_s > 0.05$).

Functional MRI Results

Task Activations. The EFMT is designed to detect between group differences in amygdala activity (Phan, Fitzgerald, Nathan, & Tancer, 2006; Stein et al., 2002), and thus we restrict our report of activations to between group results. gSAD patients showed

greater right amygdala activity than healthy controls in the fearful versus happy faces contrast ($p < 0.05$, cluster extent > 200 voxels). There were no differences in amygdala activity between gSAD patients and controls when viewing angry faces versus happy faces even at this low threshold, thus we restricted our subsequent connectivity analysis to fearful versus happy faces. There were no group differences in activation seen in the anterior cingulate; however, gSAD patients did show greater activity than healthy controls in DLPFC regions ($p < 0.05$, cluster extent > 1000; Supplementary Table 4.3).

Connectivity. We report and discuss here group differences in amygdala connectivity to discrete frontal brain areas for which we had an *a priori* hypothesis, namely within the ACC, mPFC, OFC and DLPFC (Supplementary Table 4.2). We display the findings as: 1) between-groups whole-brain voxel-wise *t*-maps; and 2) mean (S.E.M.) extracted β-weights for each group to clarify the within-group connectivity driving the group differences (Supplementary Figure 4.1). Of note, using scanner location as a covariate of no interest to remove any potential confound in the data made no change to the results described.

Supplementary Table 4.2 Between group differences in amygdala connectivity to fearful faces and at rest in* a priori *areas

Scan	Region	**Cluster Z** Volume (mm^3)	MNI Coordinate x	y	z
Right Amygdala:					
Threat Connectivity		**3.46**			
	ACC	649	14	38	22
	DLPFC	860	44	46	4
Left Amygdala:					
Threat Connectivity		**4.44**			
	ACC	82	8	20	22
	DLPFC	47	-24	-6	48
Right Amygdala:					
Rest Connectivity		**3.39**			
	ACC	772	-2	8	24
Left Amygdala:**					
Rest Connectivity		*3.78*			
	ACC	650	-4	34	-6

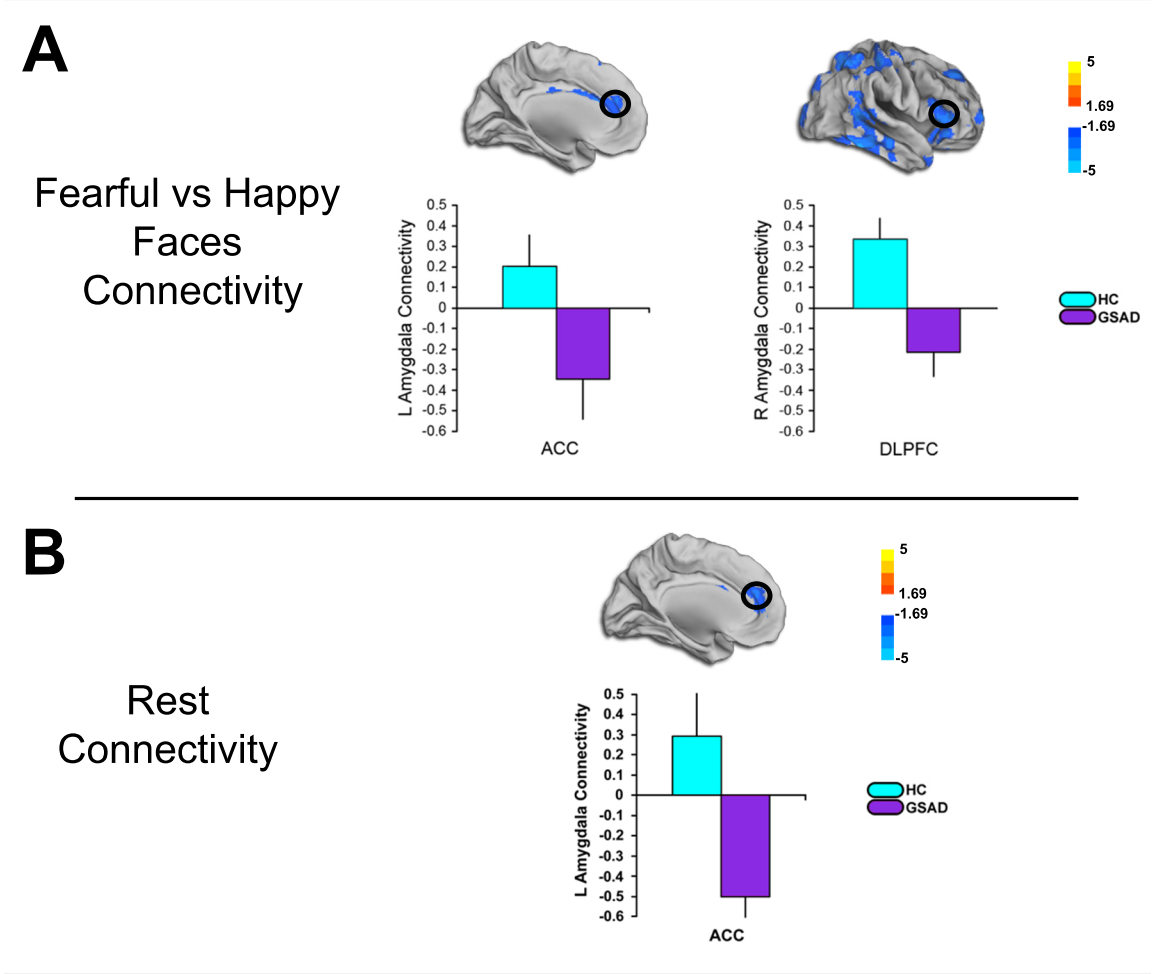
Cluster-level significance set at p< 0.05, whole brain corrected for multiple comparisons. Within each significant cluster, Z-score and associated a priori region are noted along with Montreal Neurological Institute (MNI) atlas coordinates of peaks. Of note, the volume is specifically that contained within the anatomically defined a priori region, not the total cluster volume. ACC, Anterior Cingulate Cortex; DLPFC, Dorsolateral Prefrontal Cortex.

***Although this ACC region did not fall within a cluster that survived correction for multiple comparisons, given its similarity in extent and location to the pattern observed in relation to the right amygdala, we include it here for completeness.*

Fearful vs. Happy Face Connectivity (PPI-FC). From both the left and right amygdala, within the medial frontal wall, gSAD patients exhibited *less* connectivity to rostral ACC during viewing of fearful minus happy faces than HCs (Supplementary Figure 4.1A; Supplementary Table 4.2; Supplementary Table 4.4). From both the left and right amygdala, at the lateral prefrontal wall, gSAD patients exhibited *less* connectivity to

bilateral DLPFC during viewing of fearful minus happy faces than HCs (Supplementary Figure 4.1A; Supplementary Table 4.2). In contrast, we did not observe any *a priori* areas in the medial or lateral PFC that showed greater connectivity to either left or right amygdala in the gSAD group compared to HCs. We did not observe group differences in amygdala connectivity to mPFC or OFC during perception of fearful (vs. happy) faces.

Rest Connectivity (rsFC). From both the left and right amygdala, within the medial frontal wall, gSAD patients exhibited *less* connectivity with rostral ACC than HCs (Supplementary Figure 4.1B; Supplementary Table 4.2; Supplementary Table 4.5); of note, only the ACC cluster connected to right amygdala exhibited a group difference significant for cluster-level correction for multiple comparisons. We did not observe group differences in amygdala connectivity with DLPFC, mPFC, or OFC at rest. In order to eliminate concern about the global signal regression introducing false negatives into our results (Fox, Zhang, Snyder, & Raichle, 2009), a rsFC analysis without the use of global signal regression was conducted and yielded similar results (Supplementary Table 4.6).

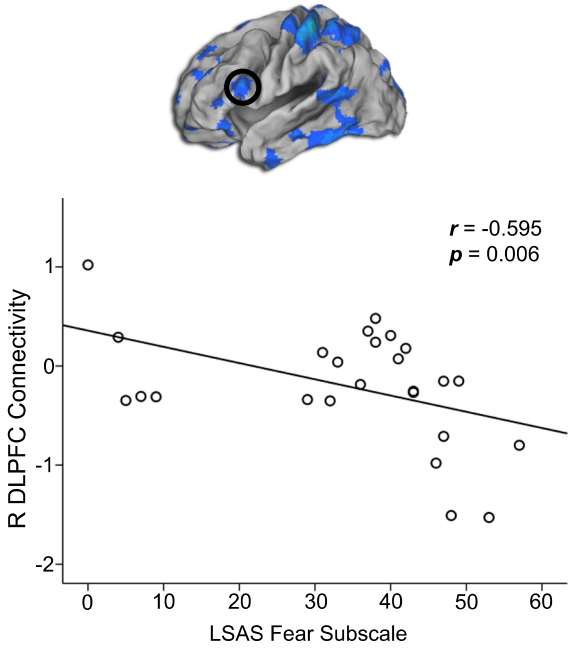


Supplementary Figure 4.1 Reduced BLA-ACC in anxiety patients' connectivity across an emotion task and the resting state. *(A) shows less amygdala connectivity to the rostral ACC and DLPFC during viewing of fearful faces minus happy faces; bar graphs show extracted measure of connectivity within each group. (B) shows less amygdala connectivity to the rostral ACC during rest; bar graphs show extracted measure of connectivity within each group. Color scale reflects t-score. GSAD, Generalized Social Anxiety Disorder; HC, Healthy Control*

Overlap between Rs-FC and PPI-FC. We explored the overlap between the ACC connectivity findings for both the PPI-FC and the rsFC using a conjunction analysis. The connectivity findings showed a shared volume of 3456 mm^3, indicating that the ACC cluster showing hypo-connectivity to amygdala during threat perception overlaps approximately 40% with the ACC cluster showing hypo-connectivity to amygdala at rest.

This indicates that the reduced connectivity seen in individuals with gSAD is consistent across both the emotion task and resting state studied here.

Post hoc Correlation Analysis. We found that right amygdala connectivity with right DLPFC in the PPI-FC analysis was significantly correlated with the $LSAS_{Fear}$ symptom measure in gSAD patients (r = -0.595, p = 0.006; Supplementary Figure 4.2). No other connectivity measures showed significant correlations with any symptom scale.



Supplementary Figure 4.2 Amygdala connectivity with DLPFC correlates with anxiety symptoms. *Symptoms of anxiety, as measured by the Leibowitz Social Anxiety Scale were significantly negatively correlated with activity in the DLPFC during viewing of fearful minus happy faces. These data indicate that as symptoms of anxiety increase, connectivity between amygdala and DLPFC decrease, potentially leading to decreased ability to regulate emotion.*

Additional Analysis. In order to account for potentially confounding effects, connectivity analyses (rsFC and PPI) were re-conducted excluding the gSAD patients on medications, the gSAD patients with comorbid psychiatric disorders, and the seven participants scanned at a different institution, all of which yielded similar results.

Supplementary Table 4.3 Task Activations for gSAD Patients Minus Healthy Controls, Fearful versus Happy Faces

gSAD - HC

Cluster	**Volume (mm^3)**					
Peak	*Name*	*Side*	*X*	*Y*	*Z*	*t-value*
Cluster 1	**31776**					
	Superior Parietal	*R*	*26*	*-76*	*56*	3.21
	Middle Occipital	*R*	*52*	*-78*	*6*	2.99
	Middle Temporal	*R*	*52*	*-76*	*4*	2.68
	Superior Occipital	*R*	*28*	*-68*	*48*	2.46
	Lingual Gyrus	*R*	*16*	*-96*	*-16*	2.44
	Calcarine Sulcus	*R*	*26*	*-102*	*-0*	2.41
	Angular Gyrus	*R*	*30*	*-68*	*48*	2.40
	Calcarine Sulcus	*L*	*4*	*-94*	*-8*	2.37
	Inferior Occipital	*R*	*26*	*-102*	*-2*	2.36
	Cuneus	*R*	*12*	*-94*	*28*	2.29
	Cerebellum	*R*	*20*	*-90*	*-20*	2.22
	Middle Temporal Pole	*R*	*60*	*18*	*-24*	2.16
	Precuneus	*R*	*14*	*-72*	*60*	2.06
	Cuneus	*L*	*2*	*-92*	*18*	1.98
	Inferior Parietal	*R*	*38*	*-70*	*58*	1.91
	Precuneus	*L*	*0*	*-66*	*58*	1.71
Cluster 2	**9928**					
	Insula	*L*	*-26*	*28*	*2*	3.54
	Inferior Frontal Triangularis	*L*	*-42*	*38*	*0*	3.41
	Inferior Orbitofrontal	*L*	*-40*	*24*	*-4*	2.73
	Inferior Frontal Operculum	*L*	*-50*	*14*	*0*	2.58
	Middle Frontal	***L***	***-38***	***42***	***-2***	**2.33**
	Superior Temporal Pole	*L*	*-52*	*14*	*-2*	2.18
	Middle Orbitofrontal	*L*	*-40*	*44*	*-2*	2.08
	Rolandic Operculum	*L*	*-54*	*10*	*2*	1.83
Cluster 3	**12128**					
	Superior Temporal Pole	*R*	*58*	*22*	*-18*	2.96
	Middle Temporal Pole	*R*	*52*	*20*	*-34*	2.80
	Middle Orbitofrontal	*R*	*46*	*46*	*-18*	2.48
	Inferior Orbitofrontal	*R*	*46*	*44*	*-18*	2.43
	Insula	*R*	*42*	*20*	*-6*	2.38
Cluster 4	**12464**					

	Thalamus	*L*	*-10*	*-24*	*16*	3.74
	Caudate	*L*	*-12*	*-4*	*22*	2.95
	Thalamus	*R*	*12*	*-16*	*18*	2.30
	Hippocampus	*L*	*-20*	*-36*	*8*	2.19
	Caudate	*R*	*16*	*-14*	*20*	2.05
	Lingual Gyrus	*L*	*-14*	*-34*	*-4*	1.82
	Globus Pallidus	*R*	*20*	*-2*	*6*	1.77
Cluster 5	**14464**					
	Middle Frontal	***R***	***32***	***38***	***26***	**3.56**
	Inferior Frontal Triangularis	*R*	*34*	*32*	*26*	2.78
	Superior Frontal	*R*	*28*	*44*	*22*	2.46
	Inferior Frontal Operculum	*R*	*52*	*20*	*26*	2.31
	Precentral Gyrus	*R*	*56*	*12*	*36*	2.17

The between groups results for gSAD patients minus healthy controls for fearful versus happy faces. Cluster volumes are given, as well as the anatomical regions and coordinates for peaks in MNI coordinates and corresponding t-values for the peak within that region. No anterior cingulate activation is found, however, gSAD patients do show greater lateral frontal activity than healthy controls.

Supplementary Table 4.4 Full Brain PPI Results for Between-Group Differences

L Amygdala Seed

Cluster	Volume (mm^3)					
Peak	*Name*	*Side*	*X*	*Y*	*Z*	*t-value*
Cluster 1	**311512**					
	Cerebellum	*L*	*-54*	*-56*	*-26*	6.71
	Postcentral Gyrus	*L*	*-40*	*-34*	*62*	4.87
	Superior Parietal	*R*	*12*	*-70*	*68*	4.48
	Inferior Temporal	*R*	*64*	*-48*	*-10*	4.43
	Middle Temporal	*R*	*64*	*-48*	*-8*	4.23
	Precentral Gyrus	*L*	*-38*	*-30*	*62*	4.17
	Postcentral Gyrus	*R*	*60*	*-16*	*18*	4.08
	Superior Parietal	*L*	*-30*	*-44*	*70*	4.08
	Rolandic Operculum	*R*	*60*	*-14*	*16*	4.06
	Superior Temporal Pole	*L*	*-24*	*24*	*-30*	3.82
	Inferior Orbitofrontal	*R*	*56*	*32*	*-10*	3.79
	Inferior Orbitofrontal	*L*	*-38*	*32*	*-20*	3.70
	Supramarginal Gyrus	*R*	*58*	*-16*	*22*	3.67
	Precuneus	*L*	*-12*	*-62*	*56*	3.66
	Superior Orbitofrontal	*R*	*12*	*18*	*-26*	3.56
	Inferior Parietal	*L*	*-48*	*-42*	*60*	3.55
	Precuneus	*R*	*10*	*-66*	*68*	3.53
	Superior Orbitofrontal	*L*	*-16*	*34*	*-26*	3.52
	Middle Temporal	*L*	*-66*	*-20*	*-22*	3.51
	Superior Occipital	*L*	*-12*	*-100*	*12*	3.51
	Precentral Gyrus	*R*	*30*	*-8*	*48*	3.43
	Middle Occipital	*L*	*-36*	*-70*	*16*	3.40
	Inferior Temporal	*L*	*-56*	*-46*	*-26*	3.37
	Superior Frontal	*R*	*28*	*-4*	*66*	3.36
	Superior Temporal	*R*	*50*	*-32*	*10*	3.29
	Middle Temporal Pole	*R*	*52*	*14*	*-24*	3.27
	Rectus	*R*	*10*	*20*	*-26*	3.27
	Superior Temporal Pole	*R*	*52*	*14*	*-22*	3.20
	Inferior Parietal	*R*	*30*	*-54*	*54*	3.19
	Calcarine Sulcus	*L*	*-24*	*-70*	*8*	3.10
	Paracentral Lobule	*L*	*-6*	*-38*	*74*	3.07
	Rectus	*L*	*2*	*34*	*-26*	3.02

R Amygdala Seed

Cluster	Volume (mm^3)					
Peak	*Name*	*Side*	*X*	*Y*	*Z*	*t-value*
Cluster 1	**169784**					
	Superior Temporal	R	68	-8	2	3.81
	Inferior Temporal	R	54	-44	-24	3.78
	Superior Occipital	R	18	-94	20	3.71
	Middle Temporal	R	64	-36	0	3.63
	Postcentral Gyrus	R	62	-20	44	3.50
	Supramarginal Gyrus	R	62	-22	44	3.41
	Cuneus	R	16	-82	30	3.40
	Superior Frontal	L	-28	58	30	3.35
	Middle Frontal	**L**	**-26**	**56**	**32**	**3.32**
	Inferior Frontal Triangularis	R	58	24	-2	3.32
	Fusiform Gyrus	R	34	-16	-36	3.27
	Inferior Orbitofrontal	R	58	24	-4	3.27
	Angular Gyrus	R	50	-60	24	3.24
	Superior Frontal	R	16	66	0	3.16
	Middle Frontal	**R**	**44**	**46**	**4**	**3.11**
	Cuneus	L	4	-92	14	3.09
	Superior Orbitofrontal	R	24	68	-4	3.07
	Rolandic Operculum	R	46	-30	20	3.07
	Calcarine Sulcus	R	6	-92	12	3.05
	Calcarine Sulcus	L	4	-92	12	3.02
	Superior Medial Frontal	R	14	64	0	3.01
	Anterior Cingulate	**R**	**14**	**38**	**22**	**2.96**
	Middle Occipital	R	42	-68	4	2.95
	Supplementary Motor	R	14	-2	50	2.87
	Superior Medial Frontal	L	-14	64	10	2.83
	Medial Orbitofrontal	R	14	62	-2	2.72
	Superior Parietal	R	28	-62	70	2.71
	Caudate	L	-4	12	8	2.66
	Heschl's Gyrus	R	68	-4	6	2.65
	Precentral Gyrus	R	38	-24	64	2.63
	Anterior Cingulate	**L**	**-4**	**12**	**24**	**2.62**
	Parahippocampal Gyrus	R	18	-10	-26	2.54

Inferior Occipital	*L*	*-50*	*-60*	*-14*	3.01		Middle Orbitofrontal	R	42	48	-2	2.54
Cuneus	*L*	*-10*	*-82*	*14*	2.93		Middle Cingulate	R	4	44	30	2.54
Inferior Frontal Triangularis	*R*	*58*	*24*	*6*	2.90		Caudate	R	8	12	6	2.52
Fusiform Gyrus	*L*	*-42*	*-60*	*-20*	2.89		Inferior Parietal	R	40	-62	58	2.49
Supplementary Motor	*L*	*-14*	*16*	*66*	2.88		Superior Occipital	L	-14	-82	28	2.43
							Inferior Frontal					
Angular Gyrus	*L*	*-40*	*-52*	*30*	2.86		Operculum	R	46	20	16	2.41
Cerebellum	*R*	*54*	*-62*	*-26*	2.84		Putamen	R	18	12	2	2.31
Cuneus	*R*	*8*	*-82*	*16*	2.83		Supplementary Motor	L	0	20	68	2.30
Middle Occipital	*R*	*38*	*-80*	*14*	2.82		Middle Cingulate	L	0	-14	32	2.26
Middle Frontal	***R***	***26***	***-4***	***52***	**2.79**		Superior Orbitofrontal	L	-20	62	-2	2.18
Superior Frontal	*L*	*-14*	*14*	*68*	2.77		Precuneus	R	14	-70	62	2.15
Calcarine Sulcus	*R*	*8*	*-80*	*16*	2.77		Inferior Occipital	R	54	-66	-16	2.07
Inferior Frontal Triangularis	*L*	*-52*	*18*	*20*	2.74		Globus Pallidus	R	16	10	-2	2.05
Inferior Frontal Operculum	*R*	*52*	*6*	*24*	2.73		Hippocampus	R	40	-32	-10	1.97
Superior Temporal	*L*	*-54*	*-46*	*12*	2.73		Cerebellum	R	26	-28	-38	1.93
Lingual Gyrus	*R*	*12*	*-78*	*-4*	2.71		Superior Temporal Pole	R	70	2	0	1.92
Middle Cingulate	*L*	*-12*	*-24*	*50*	2.71		Insula	R	40	0	16	1.91
Inferior Frontal Operculum	*L*	*-52*	*16*	*22*	2.68		Middle Occipital	L	-20	-80	18	1.88
Lingual Gyrus	*L*	*-16*	*-62*	*2*	2.66		Medial Orbitofrontal	L	-14	66	-2	1.78
Middle Orbitofrontal	*L*	*-34*	*42*	*-14*	2.55	**Cluster 2**	**35960**					
Angular Gyrus	*R*	*28*	*-58*	*52*	2.55		*Middle Temporal*	*L*	*-56*	*-36*	*4*	3.70
Middle Frontal	***L***	***-24***	***-6***	***48***	**2.47**		*Rolandic Operculum*	*L*	*-46*	*-16*	*18*	3.66
Superior Occipital	*R*	*22*	*-92*	*24*	2.46		*Postcentral Gyrus*	*L*	*-50*	*-16*	*18*	3.34
Paracentral Lobule	*R*	*6*	*-44*	*62*	2.42		*Inferior Temporal*	*L*	*-64*	*-24*	*-18*	3.32
Heschl's Gyrus	*L*	*-34*	*-28*	*6*	2.35		*Fusiform Gyrus*	*L*	*-32*	*-12*	*-42*	3.25
Inferior Occipital	*R*	*48*	*-62*	*-14*	2.28		*Superior Temporal*	*L*	*-58*	*-44*	*12*	3.05
Caudate	*R*	*10*	*12*	*0*	2.28		*Supramarginal Gyrus*	*L*	*-52*	*-22*	*16*	2.69
Superior Medial Frontal	*L*	*-12*	*24*	*60*	2.25		*Insula*	*L*	*-36*	*-22*	*20*	2.60
Caudate	*L*	*-4*	*12*	*8*	2.22		*Angular Gyrus*	*L*	*-46*	*-56*	*38*	2.24
Thalamus	*R*	*14*	*-6*	*-2*	2.21		*Inferior Parietal*	*L*	*-46*	*-54*	*38*	2.18
Fusiform Gyrus	*R*	*42*	*-44*	*-18*	2.17		*Heschl's Gyrus*	*L*	*-48*	*-16*	*10*	2.17
Anterior Cingulate	***R***	***8***	***20***	***22***	**2.17**		*Cerebellum*	*L*	*-54*	*-48*	*-30*	2.16
Superior Medial Frontal	*R*	*14*	*28*	*60*	2.16		*Parahippocampal Gyrus*	*L*	*-22*	*-14*	*-34*	1.76
Hippocampus	*R*	*14*	*-2*	*-16*	2.16		*Superior Frontal*	*R*	*18*	*66*	*18*	1.73
Supplementary Motor	*R*	*14*	*22*	*60*	2.12		*Inferior Occipital*	*L*	*-58*	*-64*	*-16*	1.72

Supramarginal Gyrus	*L*	*-48*	*-42*	*24*	2.12		*Inferior Orbitofrontal*	*R*	*50*	*42*	*-4*	1.70
Parahippocampal Gyrus	*R*	*14*	*-2*	*-18*	2.11	**Cluster 3**	**34248**					
Middle Temporal Pole	*L*	*-28*	*20*	*-34*	2.10		*Paracentral Lobule*	*L*	*-14*	*-32*	*76*	3.59
Heschl's Gyrus	*R*	*58*	*-10*	*8*	2.03		*Postcentral Gyrus*	*L*	*-30*	*-38*	*68*	3.46
Middle Orbitofrontal	*R*	*30*	*38*	*-22*	2.02		*Superior Parietal*	*L*	*-38*	*-48*	*64*	3.16
Anterior Cingulate	***L***	***-2***	***16***	***22***	**1.96**		*Inferior Parietal*	*L*	*-46*	*-48*	*60*	2.95
Putamen	*R*	*16*	*-2*	*0*	1.95		*Precuneus*	*L*	*-12*	*-42*	*74*	2.87
Insula	*R*	*30*	*24*	*-4*	1.87		*Superior Frontal*	*L*	*-10*	*-10*	*80*	2.49
Rolandic Operculum	*L*	*-36*	*-38*	*18*	1.80		*Supplementary Motor*	*L*	*-8*	*-10*	*78*	2.39
Middle Cingulate	*R*	*14*	*-8*	*48*	1.79		*Precuneus*	*R*	*8*	*-56*	*52*	2.37
Putamen	*R*	*16*	*12*	*-2*	1.78		*Precentral Gyrus*	*L*	*-48*	*-4*	*56*	2.29
							Superior Parietal	*R*	*14*	*-44*	*66*	2.26
							Postcentral Gyrus	*R*	*16*	*-42*	*66*	2.23
							Paracentral Lobule	*R*	*2*	*-32*	*62*	2.03
							Middle Cingulate	*L*	*-4*	*-44*	*54*	1.90
							Supplementary Motor	*R*	*2*	*-26*	*62*	1.70
							Middle Orbitofrontal	*R*	*44*	*50*	*-8*	1.65
							Superior Orbitofrontal	*R*	*22*	*66*	*-10*	1.48

The whole brain between group differences found for both the left and right amygdala seed during threat perception. The volume of the clusters (in mm^3) are given in **bold**. The peak coordinate (in MNI space) and t-score for each region contained within the cluster are shown in *italics*. *A priori* regions of interest discussed in the text are shown in ***bold italics***.

Supplementary Table 4.5 Supplementary Table 3: Full Brain rsFC Results for Between Group Differences

L Amygdala Seed **

Cluster	Volume (mm^3)					
Peak	*Name*	*Side*	*X*	*Y*	*Z*	*t-value*
Cluster 1	**13040**					
	Anterior Cingulate	***L***	***-4***	***34***	***-6***	**4.23**
	Olfactory	*L*	*-6*	*30*	*-2*	3.87
	Anterior Cingulate	***R***	***4***	***38***	***8***	**3.82**
	Superior Medial Frontal	*R*	*2*	*50*	*4*	3.02
	Medial Orbitofrontal	*L*	*-4*	*36*	*-10*	3.01
	Superior Medial Frontal	*L*	*0*	*54*	*4*	2.93
	Medial Orbitofrontal	*R*	*0*	*32*	*-10*	2.70
	Caudate	*L*	*-4*	*20*	*0*	1.93
	Superior Orbitofrontal	*L*	*-12*	*58*	*-6*	1.81

R Amygdala Seed

Cluster	Volume (mm^3)					
Peak	*Name*	*Side*	*X*	*Y*	*Z*	*t-value*
Cluster 1	**169784**					
	Anterior Cingulate	***L***	***-2***	***8***	***24***	**3.56**
	Globus Pallidus	*L*	*-18*	*-2*	*2*	3.56
	Superior Medial Frontal	*L*	*-14*	*40*	*22*	3.40
	Caudate	*L*	*-16*	*22*	*14*	3.22
	Putamen	*L*	*-20*	*2*	*4*	3.22
	Anterior Cingulate	***R***	***2***	***10***	***24***	**3.20**
	Caudate	*R*	*18*	*14*	*18*	3.09
	Superior Frontal	*R*	*18*	*22*	*40*	2.90
	Middle Frontal	*R*	*30*	*30*	*42*	2.80
	Superior Frontal	*L*	*-14*	*42*	*26*	2.75
	Superior Medial Frontal	*R*	*4*	*70*	*16*	2.69
	Middle Frontal	*L*	*-24*	*28*	*44*	2.61
	Middle Cingulate	*R*	*16*	*20*	*36*	2.50
	Putamen	*R*	*28*	*-4*	*14*	2.42
	Inferior Frontal Triangularis	*R*	*30*	*14*	*26*	2.15
	Inferior Frontal Operculum	*R*	*30*	*12*	*28*	1.99
	Middle Cingulate	*L*	*-8*	*28*	*32*	1.98
	Supplementary Motor	*R*	*4*	*24*	*46*	1.89
	Insula	*L*	*-30*	*12*	*16*	1.88
	Medial Orbitofrontal	*L*	*-8*	*54*	*-2*	1.85
	Supplementary Motor	*L*	*2*	*24*	*46*	1.83
	Olfactory	*L*	*-4*	*28*	*-2*	1.76
	Rolandic Operculum	*R*	*42*	*-16*	*22*	1.73

The whole brain between group differences found for both the left and right amygdala seed during the resting state. The volume of the cluster (in mm^3) is given in **bold**. The peak coordinate (in MNI space) and t-score for each region contained within the cluster are shown in *italics*. *A priori* regions of interest discussed in the text are shown in ***bold italics***. ** At a corrected threshold no results survive for the left amygdala seed. Results in the table above are shown for p=0.05, cluster threshold > 1000 voxels.

Supplementary Table 4.6 Full Brain rsFC Without Global Signal Results for Between Group Differences

R Amygdala Seed

Cluster	**Volume (mm^3)**					
Peak	*Name*	*Side*	*X*	*Y*	*Z*	*t-value*
Cluster 1	**6552**					
	Anterior Cingulate	***L***	***-2***	***8***	***24***	**2.56**
	Caudate	*L*	*-16*	*18*	*16*	1.98
	Cerebellar Vermis		*-2*	*-36*	*-6*	1.61
	Thalamus	*L*	*-6*	*-16*	*18*	1.25
	Caudate	*R*	*20*	*-4*	*20*	0.99
	Middle Frontal	*L*	*-24*	*30*	*42*	0.95
	Superior Frontal	*L*	*-18*	*34*	*30*	0.92
Cluster 2	**1400**					
	Thalamus	*R*	*8*	*-6*	*10*	1.20
	Cerebellar Vermis		*0*	*-36*	*-10*	1.11
	Hippocampus	*L*	*-34*	*-36*	*-4*	1.10
Cluster 3	**1008**					
	Hippocampus	*L*	*-34*	*-38*	*-4*	1.16
	Parahippocampal Gyrus	*L*	*-32*	*-40*	*-4*	1.09
Cluster 4	**2288**					
	Cerebellar Vermis		*-2*	*-36*	*-12*	1.30

The whole brain between group differences found for both the left and right amygdala seed during the resting state from an analysis done without using the global signal as a regressor. The volume of the clusters (in mm^3) are given in **bold**. The peak coordinate (in MNI space) and t-score for each region contained within the cluster are shown in *italics*. *A priori* regions of interest discussed in the text are shown in ***bold italics***. Note that the peak for the Anterior Cingulate exactly matches that found in the analysis above when global signal was used as a covariate.

Chapter 5.

Discussion

Summary of Findings

As discussed throughout the preceding chapters of this dissertation, fear conditioning and extinction have been used for many years to model how fear memories, which should be adaptive and helpful to an individual, become maladaptive in disorders such as post-traumatic stress disorder (PTSD) and anxiety disorders. Despite much research, it remains unclear which factors, or which combination of factors, might be most important to confer vulnerability only in certain individuals. Temperament (defined here as emotional responsiveness traits that are stable over time) may be a good predictor of the heritability and comorbidity of psychiatric disorders (Carragher, Krueger, Eaton, & Slade, 2015; Eaton, Rodriguez-Seijas, Carragher, & Krueger, 2015; Krueger, McGue, & Iacono, 2001; Wolf et al., 2010). Internalizing disorders, such as PTSD and anxiety as well as major depressive disorder, share certain commonalities in neural circuits and behavioral dysfunction; thus, internalizing temperament may predict vulnerability to PTSD. In order to better assess how these vulnerability or resilience factors intersect, we aimed to identify a rodent model of individual differences in vulnerability to PTSD. *In particular, we were interested in the question: how do individual differences in temperament influence fear conditioning and extinction? To approach this larger question, we endeavored to answer three overarching questions:*

1. *Are PTSD-like behaviors heritable in a genetic animal model and malleable by experimental manipulation?*
2. *Are heritable maladaptive fear behaviors influenced by environmental manipulations and are there gene-by-environment interactions?*
3. *How well do the neural correlates of individual differences in fear behavior in rats translate to humans?*

We investigated the first two questions using rats selectively bred based on their locomotion in a novel environment (Stead et al., 2006). While outbred Sprague Dawley rats demonstrate variability in their locomotor response to a novel environment, selective breeding has magnified these differences, generating two lines of rats that differ drastically in their emotional responsiveness (see Flagel, Waselus, Clinton, Watson, & Akil, 2014 for a review). Among other differences, bred high responders (bHRs) display low levels of anxiety- and depressive-like behavior, while bred low responders (bLRs) exhibit high levels of anxiety- and depressive-like behavior (Flagel et al., 2010; Perez, Clinton, Turner, Watson, & Akil, 2009; Stead et al., 2006). bLRs in particular, exhibit some behavioral characteristics that are similar to humans with internalizing disorders (Flagel et al., 2014; Khan, Jacobson, Gardner, Prescott, & Kendler, 2005). The phenotypes of these animals are stable and heritable, leading us to consider them a "genetic" model of internalizing temperament. Here, we use the term "genetic" to mean heritable and stable over generations. Formal analyses show this heritability to be highly significant and mostly unaltered by maternal behavior as evidenced by cross-fostering studies (Stead et al., 2006). Whole exome sequencing analyses implicate several chromosomal loci in these genetic differences, although the functional relationship

between genetic variants and behavioral differences remain to be established (unpublished data). Given that the characteristic behavioral differences displayed by these animals might mimic some of the risk factors for PTSD previously described in humans, including previous history of mental health disorders (Orr et al., 2012), we hypothesized that these selectively bred rodents might be differentially vulnerable to PTSD-like behaviors.

In humans, PTSD patients have reduced ability to extinguish their fear after conditioning (Milad et al., 2008; Milad et al., 2009; Orr et al., 2000; Rougemont-Bucking et al., 2011). Additionally, reduced extinction learning may be predictive of whether an individual develops PTSD after trauma (Lommen, Engelhard, Sijbrandij, van den Hout, & Hermans, 2013). Given that PTSD patients show reduced extinction learning and retention, animals that also show reduced extinction learning and retention could therefore be considered to have PTSD-like behavior.

We found that bHR and bLR animals demonstrate differences in both fear conditioning and extinction. Interestingly, bHR animals show reduced freezing during fear conditioning, despite evidence that their fear levels are equal to those of bLRs and controls. This active response to fear conditioning is unusual, as rodents typically display passive freezing behaviors during fear conditioning (Blanchard & Blanchard, 1969a, 1969b). bHRs also demonstrated facilitated fear extinction and retention of extinction compared to bLRs and controls. In contrast, bLRs exhibited identical levels of freezing to controls during fear conditioning, but greater freezing during extinction and extinction retention. Together, these results demonstrate that bHRs are particularly resilient to PTSD-like behavior, while bLRs show reduced extinction and extinction retention similar

to PTSD patients. These selectively bred rats therefore provide a unique model of individual differences in vulnerability to PTSD-like behavior.

Since bLRs are more vulnerable to PTSD-like behavior, we wanted to determine whether pharmacological manipulation could improve their extinction learning and retention. Fibroblast growth factor 2 (FGF2) is anxiolytic and anti-depressant when given chronically in adulthood (Perez et al., 2009; Turner, Gula, Taylor, Watson, & Akil, 2008). Additionally, when given as a single injection early in life, FGF2 alters both anxiety-like behavior and epigenetic modifications in our selectively bred rats when assessed during adulthood, indicating that it has an organizational capacity to mediate emotional responsiveness during early life (Chaudhury et al., 2014; Turner, Clinton, Thompson, Watson, & Akil, 2011). FGF2 also facilitates extinction and reduces the return of fear after extinction when administered acutely in outbred animals (Graham & Richardson, 2009a, 2010b, 2011). Given that early life FGF2 is particularly effective at reducing anxiety-like behavior in bLRs and that FGF2 facilitates extinction, we predicted that early life FGF2 would facilitate extinction specifically for bLRs.

Surprisingly, only bHR animals demonstrated facilitated extinction and extinction retention along with a small reduction in fear during conditioning after early life FGF2, indicating that early life FGF2 may decrease fear responses in bHRs in general. While bLRs did demonstrate reduced anxiety on the elevated plus maze after early life FGF2, they showed no differences in extinction or extinction retention. These data inform our interpretation of how FGF2 might influence the extinction of fear, indicating that the efficacy of FGF2 may be moderated by individual temperament, and that early life administration of FGF2 facilitates extinction solely for already resilient individuals.

We then assessed whether an environmental manipulation would display similar changes in efficacy based on individual differences. We first tested the effects of social context manipulations during extinction on outbred Sprague Dawley rats. We found that socially isolated rats (two rats present in the room as far apart as possible) had significantly reduced extinction compared to in-group phenotype social context rats (eight rats all of the same novelty-seeking phenotype in the room). To our knowledge, this is the first study to demonstrate that the number and novelty-seeking phenotype of rats present during extinction learning influences behavior. This finding was similar for the selectively bred rats: they showed reduced extinction learning when in a mixed-group social context (bHRs and bLRs in the room together) compared to the in-group phenotype social context (all bLRs or all bHRs in the room), with isolated animals having extinction levels in between the other groups. These data indicate that extreme phenotypes are detrimental to extinction learning when placed in the same room together.

We also identified evidence for a gene-by-environment interaction in our selectively bred LR rats. We manipulated the novelty-seeking phenotype of the animals to be slightly less extreme by modifying our selective breeding schema. When the novelty-seeking phenotype changed modestly and became slightly more bHR-like, bLRs exhibited facilitated extinction learning. These data suggest that manipulating the novelty-seeking phenotype of bLR animals significantly alters their characteristic behavior during extinction. In addition, social context continued to affect extinction learning in bLRs, with a trend toward facilitated extinction for the bLRs in the in-group phenotype social context. Collectively, we have demonstrated that the phenotype of these

animals significantly impacts their extinction behavior, and that this interacts with environmental manipulations to further alter behavior.

Along with these behavioral findings, we also observed differential coactivation of brain regions involved in fear between outbred, bHR, and bLR rats. We chose to examine bLRs in particular because only they displayed a gene-by-environment interaction when both novelty-seeking phenotype and social context were manipulated. We found that outbred animals show coactivation of basolateral amygdala (BLA) and infralimbic prefrontal cortex (IL) as well as some intra-hippocampal coactivation during extinction learning. The IL in particular is thought to be important for extinction learning, and its inhibition of the BLA central to the reduction in fear typically seen during fear extinction (Milad & Quirk, 2002; Quirk, Garcia, & Gonzalez-Lima, 2006; Santini, Quirk, & Porter, 2008; Sierra-Mercado, Corcoran, Lebron-Milad, & Quirk, 2006; Sierra-Mercado, Padilla-Coreano, & Quirk, 2011; Sotres-Bayon & Quirk, 2010; Vidal-Gonzalez, Vidal-Gonzalez, Rauch, & Quirk, 2006). In contrast to the coactivations seen in outbred animals, bLRs displayed no coactivation between BLA and IL, even when extinction occurred within the in-group phenotype social context. bLRs in the mixed social context that show characteristic reduced extinction learning had reduced coactivation of BLA, hippocampus and prefrontal cortex in general. In contrast, bLRs that underwent extinction in the in-group social context and showed facilitated extinction behavior (though still not to outbred levels) had coactivation between a number of regions, and many more coactivations between regions than their mixed social context counterparts. In particular, we observed coactivations between the orbitofrontal cortex (OFC) and BLA as well as the OFC and hippocampus that appear strongest in bLRs

within the in-group social context during extinction. This suggests that OFC coactivation with BLA and hippocampus may help to facilitate extinction in bLRs and may provide a potential compensatory mechanism for the lack of IL-BLA coactivation seen in bLRs.

Together, ***our studies in rodents provide a unique animal model that displays both differences in temperament and also differences in propensity to develop PTSD-like behaviors such as reduced extinction learning. We have demonstrated that individual differences in predisposition for internalizing temperament also influence the efficacy of early life FGF2 administration, facilitating extinction only in the more resilient bHR animals. We have identified a novel environmental manipulation to influence extinction learning, demonstrating in both outbred and selectively bred rats that a social context composed of in-group phenotype individuals facilitates extinction learning. We also observed a gene-by-environment interaction using social context to facilitate bLR extinction when the novelty-seeking phenotype was altered. Facilitated extinction learning in bLR animals also appears to increase coactivation between OFC and hippocampus, which may be a compensatory coactivation due to the lack of IL-BLA coactivation seen in these animals.*** Our studies are some of the first to go beyond demonstrating individual differences in extinction learning in a rodent model, assessing individual differences in pharmacological therapeutics as well as environmental manipulations, gene-by-environment interactions, and potential neural mechanisms.

While our studies in rodents provide evidence that temperament can determine vulnerability to PTSD-like symptoms, their value is increased if we can demonstrate similar changes in the human brain. Our bLR animals display high anxiety-like behavior on a variety of tests, and this general level of high anxiety may be similar to high levels

of trait anxiety in humans (Chaudhury et al., 2014; Perez et al., 2009; Spielberger, 1983; Turner et al., 2011). We therefore examined whether high trait anxiety influenced coactivation between brain regions during extinction in humans.

Humans with high trait anxiety (many of whom were also diagnosed with internalizing disorders) exhibited greater coactivation between BLA and anterior cingulate cortex (ACC), an area of the PFC during extinction learning. To our knowledge, ours is one of the first studies to examine coactivation during extinction in humans with varying levels of trait anxiety. Using a method other than coactivation, we have previously observed reduced functional connectivity between BLA and ACC in highly anxious patients. These data, along with that of other labs demonstrating reduced connectivity between amygdala and areas of the prefrontal cortex in anxiety patients (Ding et al., 2011; Hahn et al., 2011; W. Liao, Chen, et al., 2010; W. Liao, Qiu, et al., 2010; Wei Liao et al., 2011; Prater, Hosanagar, Klumpp, Angstadt, & Phan, 2013), indicate that anxiety may disrupt the function of amygdala-prefrontal circuits.

Together, these findings lead us toward a more complete understanding of individual differences in fear and extinction learning by combining data across animal models and studies of humans with varying degrees of internalizing temperament. ***Our results indicate that individual differences in temperament significantly alter behavior as well as responsiveness to pharmacological and environmental intervention. Additionally, our findings in both humans and rodents implicate dysfunction in amygdala-prefrontal circuits in high anxiety individuals.*** We believe these data allow us to further evaluate individual differences in vulnerability and resilience to PTSD and anxiety disorders, and to ask more informed questions moving forward.

In particular, it is important to further examine how animal models of PTSD might be improved, how environmental variables impact extinction learning, how temperament influences both pharmacological and environmental interventions and the neural circuits underlying individual differences in extinction learning across species. To begin to address these issues, we will discuss:

- The limitations of using within-session extinction as a measure of overall therapeutic effect.
- Whether definitions of the concepts of resilience and vulnerability fit our bHR and bLR rats.
- The need to evaluate our animal model of individual differences in temperament for other symptoms of PTSD-like behavior in addition to reduced extinction learning.
- The role of FGF2 in reducing anxiety across a variety of behaviors with differential effects on specific phenotypes.
- The potential implications of social context manipulations for human studies of group therapy.
- The comparative anatomy of brain regions across species and dysfunction in prefrontal-amygdala circuits as a potential biomarker of individuals with high anxiety.

In the long term, it will be important to identify how communication occurs in social contexts during extinction learning, the specific role of the OFC in extinction learning, and how differences in temperament displayed by the bHR and bLR rats may influence the storage and circuits underlying fear memory storage. Finally, it will be necessary for

future studies to investigate how coactivation and connectivity relate to each other both within studies and across species, especially during fear extinction, to allow us to better translate our findings to the human condition. These points are further discussed below.

Building Better Animal Models of Individual Differences in Vulnerability and Resilience to PTSD

The Limitations of Using Within-Session Extinction as a Proxy for Overall Extinction Learning

A number of studies in rats and humans have determined that the ability of an individual to extinguish during a session of fear extinction or exposure therapy is not tied to retention of that extinction learning between sessions, or the success of treatment overall (Brown, LeBeau, Yi Chat, & Craske, 2016; Plendl & Wotjak, 2010; Sripada & Rauch, 2015). In particular, individuals who extinguish within-session may show little retention of that extinction between sessions, indicating that within-session extinction does not predict between session fear levels (Plendl & Wotjak, 2010). This dissociation between within-session and between session extinction is an important limitation of the studies discussed in this dissertation and also for models of PTSD in general.

While we acknowledge that within- and between-session extinction are dissociable, we believe that the results of our studies are still pertinent for a number of reasons. First, bLRs show reduced extinction learning within-session, but also reduced retention of extinction learning between sessions. While further studies are needed to determine how long lasting this effect is, we believe the reduced extinction learning seen in these rats within-session may also predict reduced extinction between sessions. In

contrast, bHRs show facilitated fear extinction both within- and between-sessions. Together, our results suggest that selective breeding for locomotion in a novel environment may alter the behavior of individual animals in such a way that their within-session extinction behavior is predictive of between-session fear reductions, although further studies are needed to verify this effect. In addition, our studies indicate that the ability of FGF2 to facilitate within-session extinction learning is also predictive of fear behavior during retention, with bHR animals that were administered FGF2 showing reduced fear behavior during within-session extinction and during the retention session 24 hours after extinction learning. While these studies provide one line of evidence that temperament predicts PTSD-like behavior and that this behavior may be consistent both within- and between-sessions, further studies are needed to extend the number of sessions and confirm our results.

One area where we cannot provide evidence for between-session extinction learning is for the studies where we manipulated social context. Future studies should assess both whether the facilitated extinction seen within-session for rats in the in-group phenotype social context remains consistent during retention testing, and whether social context affects between-session extinction learning as well as within-session extinction learning.

Overall, many studies that identify pharmacological agents or environmental manipulations that facilitate within-session extinction learning would benefit from extending their findings to between-session extinction. By expanding beyond the confines of within-session extinction learning, we will build better models of PTSD in animals that may provide greater translational benefit to humans as well.

Development of a Definition of Resilience and Vulnerability For Use in Translational Research

Throughout this dissertation we have used the terms "vulnerability" and "resilience" to refer to rodents, yet a translational definition of these terms for both humans and animals is lacking. While animals are often referred to as vulnerable or resilient, these terms were originally defined, and redefined, in studies of humans and human psychology over a number of years. Animal studies of individual differences in response to stress, trauma, and rewarding events are key to developing a better understanding of the biology underlying a variety of mental health disorders including PTSD and major depressive disorder. Yet, if we cannot assess whether individuals in other species are more or less resilient, then we cannot easily apply our findings to provide positive benefit to human individuals. In order for these studies in animals to truly be translational in nature it is important to develop a working definition of vulnerability and resilience that will be applicable across species. This means that the definition of resilience or vulnerability needs to be made up of behaviors or traits that are similar and measurable across multiple species.

Several models of resilience have been proposed (Bonanno, Galea, Bucciarelli, & Vlahov, 2007; Campbell-Sills & Stein, 2007; Davydov, Stewart, Ritchie, & Chaudieu, 2010; DeSimone, Harms, Vanhove, & Herian, 2016; Khosravi & Nikmanesh, 2014; Kobasa, 1979; Ponce-Garcia, Madewell, & Kennison, 2015; Richardson, 2002); however most of them are challenging to assess in animal models. We focus on resiliency as described by Connor and Davidson (2003) because it is most easily translated into an

operational definition that would fit an animal model. Connor and Davidson (2003) developed a scale to rate the resiliency of an individual in humans. Their scale, the CD-RISC, assesses how well people successfully deal with stress. The CD-RISC has five factors that indicate individuals with high resilience more often (1) feel competent, have high standards for themselves and are tenacious, (2) are more tolerable of negative affect and are strengthened by stress, (3) accept change as a positive and have secure relationships, (4) feel control over their lives, and (5) often feel there are spiritual influences in their lives (Connor & Davidson, 2003). It has recently been suggested that the CD-RISC may have one factor (a one-dimensional measure of resilience) instead of 5, and that the last two factors proposed by the original description were not statistically valid (Arias Gonzalez, Crespo Sierra, Arias Martinez, Martinez-Molina, & Ponce, 2015). While the presence of five factors may not be parsimonious, the original five factors provide definitions by which we can assess whether the construct of resiliency as defined by the CD-RISC can be effectively evaluated in non-human models, therefore we will reference the five-factor model here.

The CD-RISC is a self-report measure, which means that some aspects are challenging to assess in rodents; however, others appear to translate well. Persistence as a corollary of tenacity can be assessed in rodents as a measure of their continued behavior even in the face of lack of reward or direct punishment. While tolerance of negative affect as a construct is challenging to observe in model species, one aspect of it assessed by the CD-RISC is the ability to perform under pressure, which can be assessed in other species by increasing the level of stress imposed on the animal while the individual continues to perform a task. While assessing whether a rodent actively has secure

relationships is challenging, modifications can be made to the housing arrangements of species such as rodents to provide them with social interaction or a lack-thereof to assess some aspects of the third factor of the CD-RISC in animal models. Unfortunately, the final two factors, locus of control and spirituality would appear to be next to impossible to assess in non-human individuals, but these factors may have been less valid in the original human model as well (Arias Gonzalez et al., 2015). While there are inherent challenges in modeling all aspects that the CD-RISC scale evaluates, three of the five factors may be assessed in animal models, indicating that of the definitions explored so far, this is the one most suitable to translational research. This translational definition would be that resilient individuals are more persistent or tenacious in the face of challenge, they continue to perform and focus in the face of stress, and they build relationships that enhance their performance under stressful situations.

While we have begun to build a working definition of what resilient individuals look like across species, there are additional factors that are thought to contribute to "resilience." In building a case for studying resilience in a translational manner, Yehuda and colleagues (2006) suggest that studies need to differentiate between "resistance" and "recovery." They define resistance as being unfazed or impervious to the effects of stress, while recovery is the ability to cope effectively after stress has affected an individual. This enhances our definition by adding the timing of how an individual copes with a stressor. Additionally, Davydov and colleagues (2010) propose that resilience is composed of both individual and larger societal factors that promote an individual's ability to recognize and respond to a stressor effectively. These mechanisms of effective stress response can be built, similar to innate immunity, by developing responses to

specific stressors through experience, or they can be conferred either through genetics, or social influences, as the appropriate response given a specific stressful situation. Like the ideas proposed by Yehuda et al. (2006), this idea of resilience also involves the building of protective mechanisms over time. This idea of resilience is particularly attractive because it implies that resilience is not something an individual is born with or genetically imbued with, but that resilience can be built and improved even in spite of genetic or psychological vulnerability.

Combining these ideas, ***resilience is the presence of physical, psychological or environmental influences that enhance the ability of an individual to recognize and respond to stress in ways that promote health. Resilient individuals have specific measurable characteristics such as persistence, the ability to perform under pressure, and close social networks that allow improved performance. Resilience mechanisms can be innate (genetic), but the development of resilience is a life-long process that allows experience to modify the ways an individual responds to stress. Individuals may draw on their experience to dynamically tune their behaviors, psychology, or environmental influences, allowing them to consciously modify their resilience at a given time.***

If we apply the above definition to our studies of selectively bred rodents, we can now more effectively determine whether bHRs are more resilient than bLRs. bHRs are more persistent in attempting to escape during conditioning; as persistence is one of the traits identified as being present in resilient individuals, the bHRs could be considered more resilient. If we assume that the process of extinction learning is inherently mildly stressful (uncertainty about whether shocks will occur is part of the learning process),

bHRs show facilitated extinction and retention, indicating that they are successfully learning under stressful circumstances, another measure of resilience. bHRs also develop relationships that assist them in performing extinction learning, as it is seen in Chapter 3 that in-group phenotype social groups facilitate extinction learning. bHRs demonstrate persistence, focused learning under stress and relationship building, indicating that their resilience level is high. We can also say with some confidence that in this situation it is unlikely that bHRs perceive lower levels of stress given their glucocorticoid and fecal bolus responses to fear conditioning. Therefore, the resilience is likely because of their ability to respond to stress rather than their inability to perceive the stressors provided. Thus, by our translational definition of resilience, bHRs appear to be resilient individuals. There also may be molecular families that influence the resilient bHR phenotype. For example, previous work from our lab demonstrated that bHRs have increased levels of fibroblast growth factor 2 (FGF2) in the hippocampus, and that these levels are necessary for their decreased anxiety-like (more resilient) phenotype (Chaudhury et al., 2014; Perez et al., 2009). These findings indicate that bHRs may not only display behavioral characteristics of resilience, but may also show biological markers of resilience. While our behavioral and biological measures suggest that bHRs are more resilient, further work will be needed to identify the process of resilience building in these animals, and the potential genetic and environmental mediators of resilience.

In contrast to the large literature on resilience, vulnerability has been much less debated as a construct. In some cases, vulnerability was assumed to be the opposite of resilience (Davydov et al., 2010). That is, vulnerability is simply a lack of resilience, or perhaps the inability to respond to stress effectively. While vulnerable individuals are

likely to have fewer resilient characteristics, we would argue that vulnerability itself is *not* the opposite of resilience. As discussed in Chapter 1, there are many studies that have sought to identify traits (genetic or psychological) or environmental factors that confer additional risk to the development of disorders such as PTSD and anxiety (Andrews, Brewin, & Rose, 2003; Bomyea, Risbrough, & Lang, 2012; Brewin, Andrews, & Valentine, 2000; Minassian et al., 2015; Ozer, Best, Lipsey, & Weiss, 2003; Skelton, Ressler, Norrholm, Jovanovic, & Bradley-Davino, 2012; Smith et al., 2013; Smoller, 2016; Telch et al., 2015; van Rooij et al., 2015; Yehuda et al., 2010). These studies compare individuals who have developed a disorder such as PTSD with those who have experienced similar adverse experiences such as trauma but have not developed PTSD, and therefore are assumed to be resilient. However, in performing these studies, many investigators fail to define what vulnerability actually means. Here, we define ***vulnerability as the presence of physical, psychological, or environmental influences that increase the likelihood that an individual will respond to stress or adverse events in ways that promote harm. Vulnerable individuals may have specific measurable characteristics like a past history of mental health disorders, reduced ability to perform under stress, and/or certain genetic (FKBP5 or BDNF polymorphisms) or psychological (trait anxiety) risk factors. Vulnerability mechanisms can be innate (genetic), but the development of vulnerability may be a life-long process that allows experience to modify the ways an individual responds to stress. Individuals may draw on their experiences to dynamically tune their behaviors, psychology, or environmental influences, allowing them to consciously modify their vulnerability at a given time.***

Applying this translational definition to our selectively bred animals, we can now more effectively determine whether bLRs are more vulnerable. During conditioning, bLRs quickly resort to passive responses indicating a potential lack of persistence, which may make them more vulnerable. Again assuming that the process of extinction learning is inherently mildly stressful, bLRs show reduced extinction learning and retention, indicating lower learning performance in a stressful situation. This indicates that bLRs may be less resilient. bLRs also show high anxiety- and depression-like behavior on a variety of tasks (Chaudhury et al., 2014; Perez et al., 2009; Turner et al., 2011). This may be similar to a "prior history of mental health disorders" which is thought to promote vulnerability in humans. Finally, bLRs develop relationships that assist them in performing extinction learning, as it is seen in Chapter 3 that in-group phenotype social groups facilitate extinction learning. While bLRs demonstrate this one aspect of resilience, they demonstrate more behaviors associated with vulnerability. Similar to bHRs, bLR rats also show a potential biological indicator of vulnerability. Previous findings from our lab have shown that bLRs have reduced hippocampal FGF2, and that this influences their anxiety-like (more vulnerable) phenotype (Chaudhury et al., 2014; Perez et al., 2009). Thus, by our translational definition of resilience, bLRs appear to be more vulnerable individuals. Further work will be needed to further refine the specific genetic, environmental, and other factors that promote vulnerability in bLRs.

These translational definitions of resilience and vulnerability allow us to better apply these terms to individuals across species, yet it is also important to note that these concepts are not a one-dimensional continuum. In other words, plasticity-associated reductions in resilience do not necessarily translate directly into a gain of vulnerability or

vice versa. These concepts more likely exist on separate but overlapping spectrums, with time, experience, external and internal factors, and conscious tuning of cognition and behaviors in response to stress **intersecting** to determine behavioral coping as the output of the levels of resilience and vulnerability. For example, individuals with a prior history of mental health disorders are more prone to developing PTSD (Bonanno et al., 2007; Brewin et al., 2000; Orr et al., 2012; Ozer et al., 2003; Perrin et al., 2014). If an individual with prior history of mental health disorder is aware that this history places them at higher risk, they can choose to manipulate their environment to provide less opportunity for overly stressful or traumatic events and teach themselves healthy coping mechanisms so that they can deal with future stressors effectively. These adjustments to environmental and psychological influences may have differential (but complimentary) effects on vulnerability and resilience: manipulating environmental influences may reduce the vulnerability, and the psychological adjustments involving coping strategies may promote resilience. Both changes can occur independently, but they are not changes of the same construct in a single dimension. Furthermore, we believe that resilience and vulnerability are not necessarily one-dimensional based on the way they can manifest differently in different individuals. Strategies that an individual may consciously adopt in order to develop resilience may produce a phenotype that is both similar to and qualitatively different from individuals who were more resilient as a result of biological variables, including gene expression, HPA axis responsivity, etc. For example, individuals with a genetic predisposition to vulnerability that have been environmentally and psychologically biased toward more resilient coping strategies may display some of the same behaviors in response to stress as individuals that are genetically predisposed to

resilience, but will not have an identical resilience phenotype. These two psychological constructs may influence each other (building resilience *may* reduce vulnerability along one influential line), however vulnerability and resilience are not inherently opposites of each other.

We believe that the translational definitions of resilience and vulnerability developed here will be useful as future studies pursue questions regarding individual differences and how they affect the development of various mental health disorders across species. A number of authors have suggested that the study of the biological underpinnings of resilience are important to understand in order to develop resilience-promoting factors (Charney, 2004; Yehuda et al., 2006), and we agree; however, without a translational definition of what resilience or vulnerability looks like in an individual it is challenging to assess the biological mechanisms. It is also important to note that these are preliminary, working, definitions, and that further studies will refine these definitions as a greater understanding of what makes an individual resilient or vulnerable across species is developed.

Animal Models Should Demonstrate Generalization, Sleep Disturbance, and Autobiographical Memory Amnesia as well as Sex Differences to Better Represent PTSD Symptoms

While the animal models that exist currently are providing important information about how the brain stores fear memories and how those memories might become maladaptive in PTSD, there are improvements that could be made so that animal models better mimic PTSD symptoms. While reduced extinction learning and recall is thought to

be characteristic of human PTSD patients, it is not the only symptom of this debilitating disorder (Lommen et al., 2013; Milad et al., 2008; Milad et al., 2009; Orr et al., 2000). Other major symptoms of PTSD include: re-experiencing events such as flashbacks and nightmares, hyperarousal including increased aggression and insomnia, negative cognitions including attention biases and some amnesia, and avoidance of cues and situations that might provide reminders of a traumatic event (Friedman, Resick, Bryant, & Brewin, 2011). While some aspects of these symptoms are more challenging to model in animals than others, the best animal model of PTSD will display many of these symptoms post-fear conditioning, and help to understand the individual differences that provide increased vulnerability and resilience to developing these symptoms.

Fear Generalization. One aspect of PTSD symptomatology that has recently received significant attention is fear generalization. The process of generalization is thought to lead to increased avoidance of stimuli that were not initially part of the trauma experienced by the individual, and to contribute to the heightened anxiety seen in PTSD patients (Lissek, 2012). Fear generalization is defined as the transfer of fearful responses to stimuli that are similar, but are not the same as, the original stimuli present during fear conditioning (Dunsmoor & Paz, 2015; Lissek, 2012; Lopresto, Schipper, & Homberg, 2016). Fear generalization can be tested easily in both humans and animals by using stimuli or contexts that vary slightly along one dimension (such as size) to assess a generalization gradient of how different a stimulus needs to be before fear responses decrease (Figure 5.1). Previous studies have demonstrated that patients with generalized anxiety disorder and panic disorder both show increased fear to stimuli that appear different enough to be seen as safe by non-anxious individuals (Lissek et al., 2014; Lissek

et al., 2010). It is assumed that PTSD patients will show a similar fear response to stimuli that should be safe, and ongoing studies are working to test this. Since fear generalization appears to be characteristic of some anxiety disorders, potentially including PTSD, and is thought to partially underlie the increased avoidance seen in PTSD, good animal models of PTSD symptoms should also demonstrate fear generalization after conditioning.

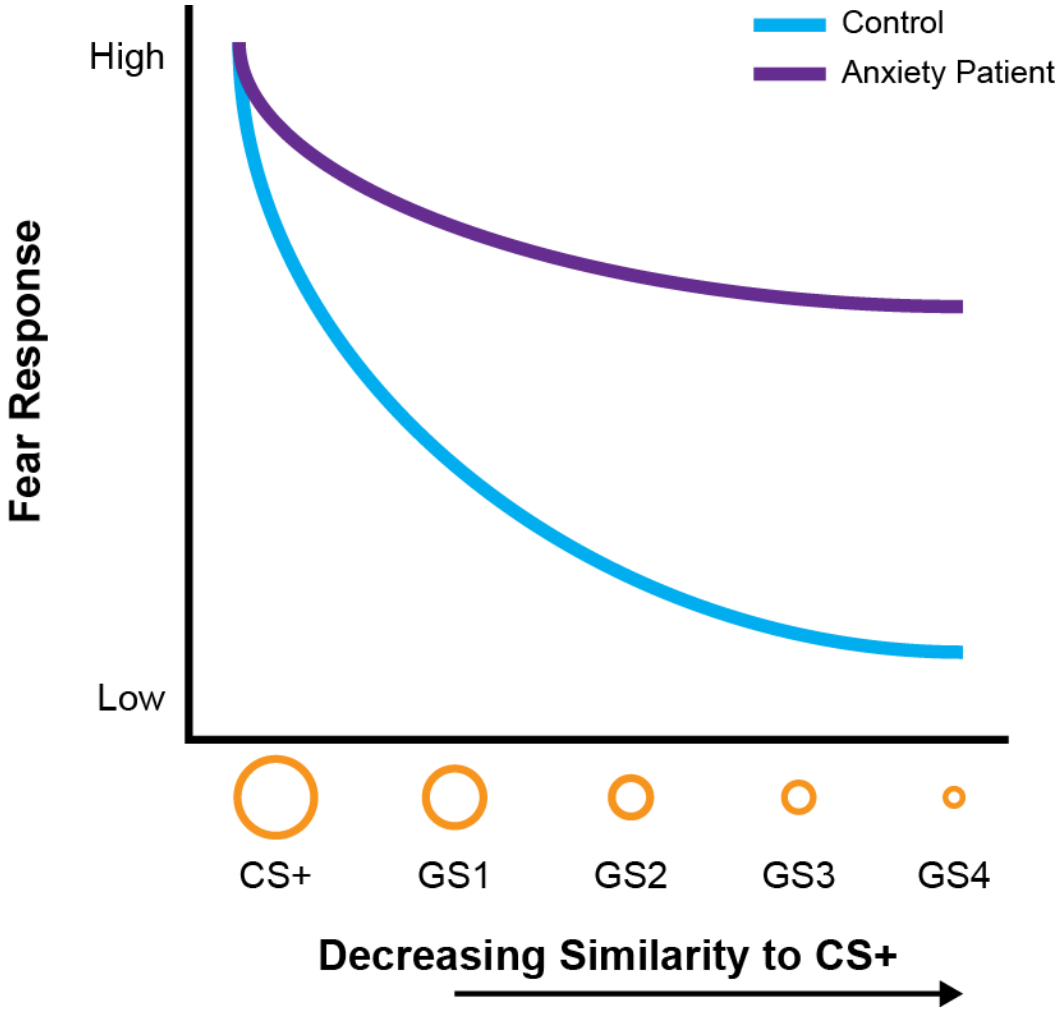


Figure 5.1 Fear generalization differs between non-psychiatric control participants and anxiety patients. *Fear generalization paradigms use a gradient of generalization stimuli (GS; varying on an aspect such as size) to assess the level of fear response to stimuli that were not directly conditioned but that are similar to the original conditioned stimulus (CS+). Non-psychiatric participants generally show a sharp decrease in fear responses as stimuli become less similar to the CS+, while patients with anxiety have less steep fear response curves, indicating increased fear to stimuli less similar to the CS+. This increased fear to GSs is fear generalization.*

While we did not assess generalization directly in our selectively bred bHR and bLR rats, there is some preliminary evidence from our experiments that bLRs may have increased generalization of context fear. When bLRs and bHRs are first introduced to the conditioning context, they both show low levels of freezing in the habituation period (Figure 5.2). When the animals are brought to the extinction context the following day, bHRs show relatively low levels of fear in the novel extinction context. bLRs, however, display extremely high levels of freezing, indicating that they may be generalizing across the conditioning and extinction contexts despite the fact that they are different in odor, texture, lighting and location (Figure 5.2). Given this initial and limited evidence, we believe that bLRs might provide both a model of reduced extinction learning and retention, and possibly a model of fear generalization. Further studies will be needed to test fear generalization in bLRs and bHRs directly, to determine whether bLRs show fear generalization across both discreet stimuli and contexts, or just contexts, and how far this generalization extends.

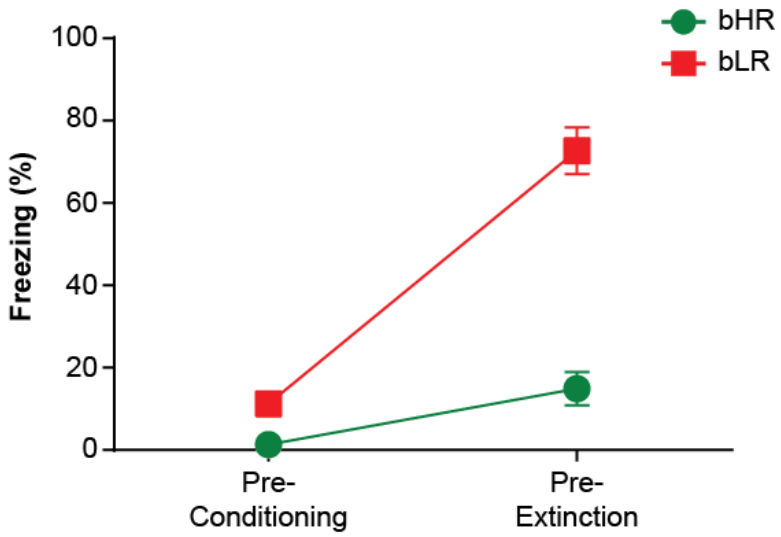


Figure 5.2 Freezing levels prior to conditioning and extinction indicate that bLRs may show some context generalization. *Although bHR and bLR freezing levels differ at the pre-conditioning timepoint, both groups demonstrate low levels of freezing (< 20%) in the novel context. In contrast, while bHR freezing increases mildly from pre-conditioning to pre-extinction, bLR freezing increases dramatically. This heightened fear response to a novel context after conditioning indicates that bLRs may show increased context generalization post-fear conditioning.*

Sleep Disturbances. Sleep disturbances are characteristic of two of the symptom categories of PTSD, with insomnia being a symptom of hyperarousal, and nightmares being a re-experiencing symptom (Friedman, Resick, Bryant, & Brewin, 2011; Friedman, Resick, Bryant, Strain, et al., 2011). Sleep disturbances have also been noted as one of the most prevalent symptoms of PTSD, and are therefore considered a hallmark of the disorder (Ross, Ball, Sullivan, & Caroff, 1989). PTSD patients appear to experience disrupted rapid eye movement (REM) sleep with increased periods of wakefulness, potentially due to nightmares that occur during REM sleep (Gilbert, Kark, Gehrman, & Bogdanova, 2015; Pigeon & Gallegos, 2015). Since sleep disorders are so prevalent in human PTSD patients, it is important that a good animal model of PTSD also display disrupted sleep, especially shorter or interrupted bouts of REM sleep or insomnia. Studies have demonstrated that even a single acute stress disrupts REM sleep, although in rats

REM was increased after stress (Hegde et al., 2008). This indicates that a stressor such as fear conditioning may also induce changes in the sleep architecture of rodents. In a rodent model of PTSD, rats exhibit increased REM and decreased theta waves during sleep (Vanderheyden et al., 2015). While these changes are the opposite of those that humans with PTSD experience, changes in sleep in a rodent model are an important step toward better modeling the development and symptoms of PTSD. We have not yet explored sleep in our selectively bred bHR/bLR rats, and therefore cannot say how individual differences might contribute to sleep disturbances after fear conditioning and whether those differences promote or inhibit resilience. Further studies investigating sleep after fear conditioning and extinction in these animals will provide additional insights into how individual differences might shape changes in sleep and how that leads to vulnerability or resilience.

Selective Autobiographical Amnesia. PTSD patients also display the symptom of selective amnesia for the traumatic event (Friedman, Resick, Bryant, & Brewin, 2011). Somewhat paradoxically, while PTSD patients appear to have enhanced memory for certain aspects of their traumatic memory (such as cues present during the trauma), often their conscious recall of the traumatic event itself is disordered and they are unable to recall the entire memory (Brewin, 2008; Desmedt, Marighetto, & Piazza, 2015). This has led to the hypothesis that sensory memory is enhanced and may be over-generalized in PTSD, while the conscious autobiographical memory of the trauma is impaired (Brewin, 2008; Desmedt et al., 2015). The latter part of this hypothesis was further refined by certain researchers to be a de-contextualization of the traumatic memory, so that the fear to cues is out of context and pervasive (Desmedt et al., 2015; Kaouane et al., 2012).

While, to our knowledge, the majority of animal models have not endeavored to observe enhanced cue-related memory coincident with reduced contextual memory, a recent study in mice has demonstrated a fear conditioning protocol that leads to these behaviors (Kaouane et al., 2012). As mentioned earlier, our bLR rats appear to generalize their fear across contexts, indicating that they may have difficulty contextualizing their fear; however, this will require further testing and more complicated paradigms to determine whether our animals show contextual amnesia for their fear conditioning experience. Additionally, it will be important for further studies to determine how well poor contextual memory for the traumatic event mimics the disorganized and poor autobiographical memory seen in PTSD patients.

Sex Differences. While many animal models are making progress in operationally defining PTSD symptoms and demonstrating similar behaviors across species, the evaluation of sex differences is one place where most animal models are lacking. In humans, men tend to experience more traumatic events during their lifetime; however, women are almost twice as likely to develop PTSD (Kessler, Sonnega, Bromet, Hughes, & Nelson, 1995). This difference may be because women are more likely to experience specific traumas such as rape that are more likely to cause PTSD (Kessler et al., 1995), but even so, these data indicate that there are strong sex differences that promote vulnerability to PTSD in females. It is therefore worth noting that while many human studies involving PTSD patients include females, the majority of rodent models described throughout this dissertation, including our own selectively bred rats, are studied exclusively in males. While the exclusive study of males is often done for convenience, as we begin to develop an understanding of the individual differences that might lead to

development of PTSD in males it should be a high priority to determine if these same individual differences also matter for the development of PTSD in females.

We believe that our selectively bred animal model provides important insights into the individual differences that may contribute to increased resilience or vulnerability to the reduced extinction learning seen in PTSD patients. Much research remains to be done, however, to determine if our model of individual differences also models other symptoms of PTSD such as generalization of fear, sleep disturbances, selective amnesia, and sex differences in vulnerability to PTSD. We believe that further characterization of our animal model may lead to discoveries about how individual differences influence the selective resilience or vulnerability to the development of PTSD. We look forward to future studies that will add to the discoveries discussed in this dissertation to enhance the field of individual differences research and better model how PTSD develops in humans.

Fibroblast Growth Factor 2 May Interact With Stress to Produce Task-Specific Anxiolytic Effects for Certain Individuals

As we begin to develop better animal models of specific vulnerability and resilience to developing PTSD, it is also important to test potential resilience-inducing factors such as fibroblast growth factor 2 (FGF2). FGF2 was originally found to be involved in mood disorders by the observation that it is strongly decreased across a variety of brain regions in post-mortem major depressive disorder (MDD) patients (Bernard et al., 2011; Evans et al., 2004; Gaughran, Payne, Sedgwick, Cotter, & Berry, 2006). Brain regions where FGF2 was found to be downregulated, such as the prefrontal cortex (PFC), and locus coeruleus are known to be dysfunctional in MDD, but at least

one (PFC) is also involved in fear and anxiety (Giustino & Maren, 2015; Malykhin & Coupland, 2015; Maren, 2001; Price & Drevets, 2012; Sotres-Bayon & Quirk, 2010).

FGF2 has anxiolytic properties on both spontaneous fear and learned fear in rodents. Tests of spontaneous anxiety-like behavior rely on a rodent's innate and evolutionarily conserved fears. These spontaneous fears are in contrast to the fear expressed during fear conditioning and extinction, which are learned responses to stimuli not innately feared by the animal. FGF2 has anxiolytic effects on tests of spontaneous anxiety-like behavior (Eren-Kocak, Turner, Watson, & Akil, 2011; Perez et al., 2009; Turner et al., 2011). For example, chronic administration of FGF2 decreases anxiety-like behavior, and knock-down of endogenous FGF2 increases anxiety-like behavior (Eren-Kocak et al., 2011; Perez et al., 2009). In the study of learned fear, acute administration of FGF2 facilitates within-session extinction, and enhances recall of that extinction 24 hours later (Graham & Richardson, 2009a, 2011), in addition to reducing the return of fear after extinction (Graham & Richardson, 2009a, 2010b, 2011). This enhanced extinction learning and better retention of the extinction memory could be interpreted as an anxiolytic effect, as animals display less learned fear subsequent to FGF2 administration. Our data also support this, with distinct anxiolytic effects for bLRs on the elevated plus maze (innate fear), and facilitated extinction learning for bHRs (learned fear) after early life FGF2 administration. While the effects were found in animals with different phenotypes, these data demonstrate that FGF2 can affect both innate and learned fear responses.

FGF2 also facilitates individual differences in behavior between animals in both spontaneous and learned forms of fear, and administration affects certain individuals

more. Animals selectively bred for differences in locomotor response to a novel environment show different levels of FGF2 in their dorsal hippocampus (Perez et al., 2009). Outbred animals also have different endogenous expression of FGF2 in dorsal hippocampus and, similar to our bHR/bLR model, lower levels of individual FGF2 expression correlates with both increased spontaneous (EPM) and learned fear (fear conditioning; Eren-Kocak et al., 2011; Graham & Richardson, 2016). Administration of FGF2 either early in life, or chronically in adulthood has anxiolytic effects solely for more anxious selectively bred animals on measures of spontaneous anxiety-like behavior, indicating that FGF2 administration may be more effective for certain individuals (Perez et al., 2009; Turner et al., 2011). The results of this dissertation also support this interpretation of individual differences in effectiveness. In contrast to the previous results, however, the findings described here demonstrate that FGF2 administration enhanced extinction learning in bHRs, the less anxious animals that have higher levels of endogenous FGF2 (Chapter 2). The results of previous studies along with those described in this dissertation demonstrate a strong interplay of individual differences of FGF2 endogenous expression and administration on both spontaneous and learned fear.

If there are such strong individual differences on the effects of administration of FGF2, the question then becomes why FGF2 might have effects on some individuals in one task and not others. For instance, administration of FGF2 either chronically or early in life decreases spontaneous anxiety-like behavior for the more anxious bred low responder (bLR) animals, while leaving the bred high responders (bHRs) unaffected (Perez et al., 2009; Turner et al., 2011). In contrast, during fear extinction, administration of FGF2 facilitated extinction and extinction retention in bHRs but not bLRs (Chapter 2).

There are several differences between these tasks that might influence the efficacy of FGF2 including the learning process and stress induced by the task.

While FGF2 influences learning, and in particular FGF2 administration enhances fear learning at an early age (Graham & Richardson, 2009b, 2010a), we believe it unlikely that learning is solely responsible for the individual differences in effects of FGF2 administration on fear extinction as seen here. First, individual differences in endogenous expression of FGF2 influence fear expression in ways consistent with our bHR and bLR rats, such that low levels of endogenous FGF2 increase fear expression during fear learning (Graham & Richardson, 2016). Secondly, previous studies demonstrated that administration of FGF2 facilitated learning across development in all individuals. FGF2 administration increased learning of conditioning at an age where rats normally would not retain contextual conditioning and enhanced learning and retention of fear extinction memories in young adult rats (Graham & Richardson, 2009a, 2009b, 2010a, 2010b). These studies indicate that administration of FGF2 should either enhance the learning of extinction across all individuals, or facilitate extinction in bLRs that have reduced levels of endogenous FGF2, neither of which match our results. Thus, the effects of FGF2 administration on learning are unlikely to explain the differential effects of FGF2 administration on spontaneous versus learned fear in certain individuals.

We believe it likely that the amount of stress an animal experiences as a result of a particular behavioral task impacts the ability of FGF2 to have anxiolytic effects. Tests of spontaneous anxiety-like behavior such as the elevated plus maze are considered to be a mild stressor to animals while fear conditioning is considered a relatively strong stressor (Coover, Sutton, Welle, & Hart, 1978; Pellow, Chopin, File, & Briley, 1985;

Stockhorst & Antov, 2015). The majority of studies where individual differences in the effectiveness of FGF2 administration were found have used mild stress tasks assessing spontaneous anxiety-like behavior (Perez et al., 2009; Turner et al., 2011). In all of those instances, FGF2 administration was anxiolytic for the bLR rats specifically, reducing anxiety behavior in high anxiety individuals. In contrast, during fear conditioning and extinction we observed that FGF2 administration had anxiolytic effects for bHRs but not bLRs (Chapter 2). In other unpublished data from our lab, administration of FGF2 was also anxiolytic for bHRs after social defeat (personal correspondence from C. A. Turner), a particularly stressful paradigm (Koolhaas, De Boer, De Rutter, Meerlo, & Sgoifo, 1997). This change in the ability of FGF2 to modulate anxiety in individuals between tasks indicates that the stress induced by the task may interact with FGF2 administration to predict which individuals will receive beneficial effects of administration.

We hypothesize that the ability of FGF2 to have anxiolytic effects is determined by the stress level of the individual on a given task (Figure 5.3). For example, the EPM may be a greater stressor for bLR rats that display high levels of spontaneous anxiety than for bHR rats. This increased stress level allows FGF2 to be effective at reducing anxiety for bLRs in tasks of spontaneous anxiety, while it is not effective for bHRs (Figure 5.3). During fear conditioning and extinction, however, the high levels of stress may send bLR animals into such a high stress state that FGF2 can no longer effectively modulate their anxiety, while the increased stress levels of bHRs allow FGF2 to moderate their anxiety responses (Figure 5.3). Therefore, we see anxiolytic effects of FGF2 administration on bHR but not bLR rats during fear conditioning and extinction learning. While further studies are needed to specifically assess the stress level of individual

animals using hormones and heart rate levels, we believe that the most likely candidate hypothesis is that stress interacts with FGF2 to mediate the efficacy of FGF2 administration on certain individuals.

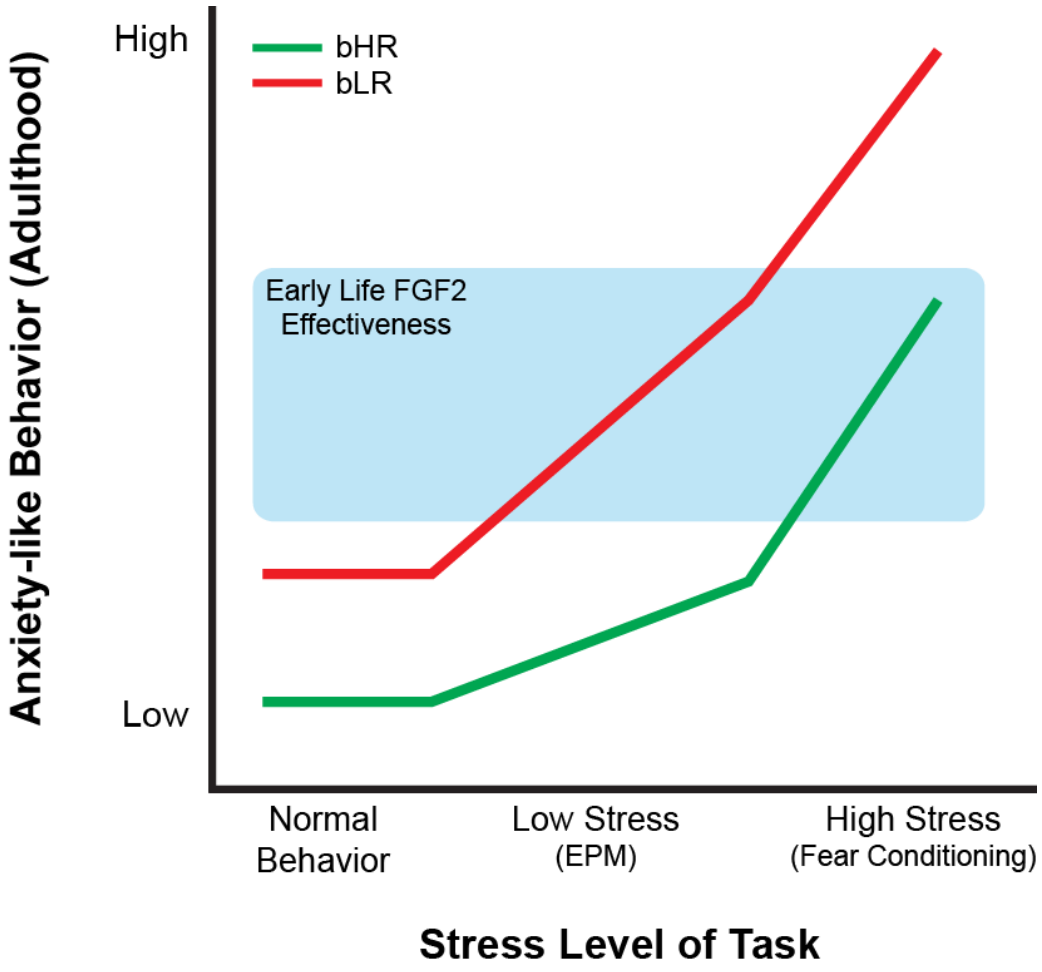


Figure 5.3 Hypothetical interaction of the effectiveness of early life FGF2 at eliciting an anxiolytic response with the stress induced by the task used. *We hypothesize that FGF2 interacts with stress to influence anxiety responses. Animals with stress levels above or below the blue rectangle where FGF2 is anxiolytic do not show the anxiolytic effects of FGF2, whereas animals whose stress levels place them inside the blue rectangle demonstrate anxiolytic effects of early life FGF2 administration. In the case of the bHR and bLR rats demonstrated here, FGF2 is anxiolytic for bLRs on tasks involving mild stress such as the elevated plus maze (EPM), while FGF2 is unable to provide anxiolytic effects for bHRs except during high stress tasks such as fear conditioning.*

Based on our data indicating that only bHRs are affected by early life administration during fear extinction learning, we hypothesize that the ability of FGF2 to

produce anxiolytic effects is likely mediated by stress induced by the task used during testing. Further studies will be needed to assess how much the stress induced by different tasks differs between bHR and bLR animals, and the mechanisms by which the glucocorticoid system and the FGF system interact. These studies will provide further insight into how the effects of FGF2 are modulated by individual differences, and may lead to more effective pharmacological interventions to promote resilience in vulnerable individuals such as bLR rats.

Social Support Provides a Novel Way to Facilitate Extinction Learning: Implications for Group Therapy in Humans

In this dissertation, we determined that social context has a significant influence on extinction learning both for outbred and for selectively bred animals. While previously discussed in terms of social buffering, where the presence of other individuals can reduce fear, these results demonstrating the impact of individual phenotype within groups also have implications for social support and group behavioral therapy in humans.

Social support decreases PTSD symptomatology through social interaction and perceived support from other individuals. Several studies have identified military unit cohesion, or perceived social support from within the military, as significant buffers against stress during military training and operations (Brailey, Vasterling, Proctor, Constans, & Friedman, 2007; Smith et al., 2013). This ability of unit cohesion to modulate stress may be similar to having in-group phenotype individuals together during fear conditioning, as like-individuals may be more cohesive, and therefore better able to buffer the stress. In post-deployment individuals, a recent study identified social support

from military peers as being more important for a reduction in PTSD symptoms than support from friends, indicating that different sources of social support may influence PTSD symptoms after deployment, and that military peers may be important for this effect (Wilcox, 2010). While it is clear that support from military peers can be influential, it is currently unknown how unit cohesion might alter the effects of social buffering that military peers can have on PTSD symptoms post-deployment. Thus, we do not have a direct comparison of how unit cohesion influences stress and PTSD symptoms to the timing of when in-group phenotype facilitates extinction learning in rodents. Further studies in humans that determine the influence of unit cohesion on PTSD and stress symptoms post-deployment will provide further insight into how social support can be used to buffer stress.

Social support can be provided by a number of sources including friends, family, significant others, and an extended network of individuals outside of those categories, possibly including group therapy partners (Wilcox, 2010). Group therapy was shown to be effective for PTSD patients, and to have a number of potential advantages, including reduced cost, accessibility of therapy with limited numbers of therapists, and the opportunity for social interaction with other individuals (Castillo, Lacefield, C'De Baca, Blankenship, & Qualls, 2014; Resick et al., 2015; Sloan, Bovin, & Schnurr, 2012). Interestingly, while demonstrating that group therapy for PTSD was highly effective, one study also showed that group cohesion, or the willingness of participants to engage with the group based on group dynamics, was a significant predictor of treatment outcome (Ellis, Peterson, Bufford, & Benson, 2014). This study demonstrates that the composition of the group might influence willingness to engage with treatment for some individuals

(Ellis et al., 2014). This same aspect of group cohesion has also been found to be predictive of treatment outcomes in group therapy for major depressive disorder, indicating that group cohesion may be crucial to the success of group therapy across disorders (Crowe & Grenyer, 2008). These studies indicate that group cohesion is important for the success of group therapy; however, they do not provide insights into what allows group cohesion to develop, or how group cohesion affects social support. We hypothesize that group cohesion could be influenced by individual differences in personality, perceived support, and ability to trust, indicating that individual differences may influence the efficacy of group therapy. Similar to our rodent studies, it may be that choosing groups that contain more similar individuals would allow enhanced group therapy participation and more effective reduction of PTSD and anxiety symptoms for individuals. Further studies are needed to determine whether this is indeed the case.

Amygdala-Medial Prefrontal Circuits May Provide a Biomarker for Individuals with High Anxiety Across Species

The Comparative Anatomy of Prefrontal Cortex, Amygdala, and Hippocampus

Certain emotions, such as fear, have been demonstrated in multiple species (Panksepp, 2011), and this cross-species study of emotions is particularly important to advancing the understanding of maladaptive fear in PTSD and anxiety disorders. However, before comparing brain activity across species it is first important to ascertain the anatomical similarities or dissimilarities of these brain regions. In Chapters 1 and 4, the literature on brain activity during fear conditioning and extinction was outlined for both humans and rodents. In both species, it appears that contextual information is

processed in the hippocampus and the amygdala is involved in fear memory storage (Delgado, Nearing, Ledoux, & Phelps, 2008; Kalisch et al., 2006; LaBar, Gatenby, Gore, LeDoux, & Phelps, 1998; LeDoux, 2000; Maren, 2001; Maren, Phan, & Liberzon, 2013; Orsini, Kim, Knapska, & Maren, 2011; Phelps, Delgado, Nearing, & LeDoux, 2004). In addition, distinct areas of the medial prefrontal cortex are involved in either expression of fear (ACC in humans, PL in rats) or the inhibition of fear during extinction (vmPFC in humans, IL in rats; Giustino & Maren, 2015; LeDoux, 2000; Maren, 2001; Milad, Quirk, et al., 2007; Milad, Wright, et al., 2007; Phelps et al., 2004; Quirk & Beer, 2006; Quirk et al., 2006; Sierra-Mercado et al., 2011; Sotres-Bayon & Quirk, 2010). While the consistent activity in these regions appears to indicate that they may have homologous functions across species, it is important to consider how similar these structures are anatomically.

The presence and homology of the prefrontal cortex in rodents is arguably one of the most controversial in the comparative anatomy to be discussed here (Brodmann, 1909; Preuss, 1995, 2000; Reep, 1984). We ascribe to the theory that the structures that make up the prefrontal cortex in the rat have some homology to the medial prefrontal cortex in primates, although there remains significant variation in these structures between species (Preuss, 1995). Specifically, the rat IL is thought to be relatively homologous to Brodmann's Area (BA) 25, or the subgenual cingulate area in primates (Preuss, 1995). The rat PL is thought to be relatively homologous to BA 32, or the rostral area of the ACC in primates, and the orbitofrontal cortex is homologous to orbitofrontal cortex in both species (Preuss, 1995). It should also be noted that the anatomical connections between these regions of PFC and structures such as the amygdala and

hippocampus are also relatively well conserved across species (Barbas & Blatt, 1995; Barbas & De Olmos, 1990; Ghashghaei, Hilgetag, & Barbas, 2007; McDonald, 1991a, 1998; McDonald, Mascagni, & Guo, 1996). While there are significant differences including the size, shape, and neuronal properties of the prefrontal cortex in different species (Preuss, 1995, 2000), the combination of cytoarchitecture, anatomical connectivity and preserved functions all lead to the conclusion that some aspects of medial prefrontal function are conserved in specific areas across both rats and humans (Seamans, Lapish, & Durstewitz, 2008).

The amygdala is relatively well conserved across species, and thus is observed to be similar in both rodents and humans. The amygdala is a heterogeneous collection of approximately 10 nuclei that perform a variety of functions and have incredibly diverse cellular composition and connectivity (Amaral, Price, Pitkanen, & Carmichael, 1992; Jolkkonen & Pitkanen, 1998; Pitkanen & Kemppainen, 2002; Pitkanen, Savander, Nurminen, & Ylinen, 2003; Pitkanen, Savander, & LeDoux, 1997; Schmitt et al., 2012). Although the nomenclature for the different nuclei varies across species, there are distinct similarities both in cellular composition and intrinsic connectivity that certain nuclei of the amygdala share (Pitkanen & Kemppainen, 2002). In particular, the lateral nucleus of the amygdala shares intra-amygdala connectivity as well as some specific neuronal markers in rats, non-human primates and humans (Pitkanen & Kemppainen, 2002). While the differing nomenclature between species makes direct comparison of different nuclei challenging, several studies have confirmed that connectivity between different relatively homologous nuclei of the amygdala and areas such as the prefrontal cortex are similar across species (McDonald, 1991a, 1991b). Based on the evidence from studies of

amygdala cellular properties and connectivity, the amygdala nuclei, particularly lateral and central nuclei, are relatively homologous between rodents and primates.

The hippocampus, like the amygdala, has several divisions based on connectivity and function (Bannerman et al., 2004; Chase et al., 2015; Fanselow & Dong, 2010). These divisions include the dorsal, intermediate and ventral hippocampus in rodents, and the anterior and posterior hippocampus in humans (Bannerman et al., 2004; Chase et al., 2015; Fanselow & Dong, 2010). A series of studies in rodents has given rise to the theory that dorsal hippocampus (posterior hippocampus in humans) is primarily involved in spatial navigation, context encoding and memory for these spatial and contextual items (Bannerman et al., 2003; Bannerman et al., 2004; Fanselow & Dong, 2010). In contrast, ventral hippocampus is implicated in emotion and anxiety functions (Bannerman et al., 2003; Bannerman et al., 2004; Fanselow & Dong, 2010). It was demonstrated using fMRI that the dorsal hippocampus in rodents bears many similarities in anatomical connection and laminar structure to the posterior hippocampus in humans (Sasaki et al., 2004). This, along with a recent study identifying functional differentiation between anterior and posterior hippocampus in humans (Chase et al., 2015), has further solidified the idea that different areas of the hippocampus in both rodents and humans underlie emotion versus cognitive functions. Together, these data have led to the conclusion that dorsal hippocampus is homologous to posterior hippocampus in humans, while ventral hippocampus in rodents is homologous to anterior hippocampus in humans (Bannerman et al., 2004; Chase et al., 2015; Fanselow & Dong, 2010; Sasaki et al., 2004).

Together, the data demonstrate that homology exists across species for the regions traditionally thought to be involved in fear conditioning and extinction (amygdala,

hippocampus, and medial prefrontal cortex). While it is understood that a rodent brain is not a human brain (Preuss, 2000), the cellular and connectivity homology across species gives us confidence in comparing the results of studies in humans and rodents to assess their similarities and differences.

Amygdala-Prefrontal Coactivation During Extinction Learning in Rats and Humans

We examined coactivation between brain regions in outbred animals, selectively bred bLR animals, and in humans with varying levels of trait anxiety during extinction. We found that outbred animals exhibited strong coactivation between IL and basolateral amygdala (BLA), as well as strong intra-hippocampal coactivation. Interestingly, this pattern was significantly altered in bLRs, with IL-BLA coactivation being absent and intra-hippocampal coactivation being reduced. These bLR rats display high spontaneous anxiety, and also reduced extinction learning (Chaudhury et al., 2014; Perez et al., 2009; Stead et al., 2006; Turner et al., 2011). The decrease in IL-BLA coactivation was not unexpected, given that bLRs do not learn extinction effectively, and that activation in IL and the connectivity of that structure to BLA are important for extinction learning (Dejean et al., 2015; Herry et al., 2008; Milad & Quirk, 2002; Senn et al., 2014; Sierra-Mercado et al., 2011; Vidal-Gonzalez et al., 2006). Interestingly, in bLR animals that did display some extinction learning, there were increased coactivations between orbitofrontal cortex (OFC) and piriform cortex with BLA and the hippocampus, as well as increased intra-hippocampal coactivation. Increased coactivations between OFC and hippocampus were particularly prevalent with ventral hippocampus, the portion of rodent hippocampus hypothesized to be involved in emotion (Bannerman et al., 2003;

Bannerman et al., 2004; Fanselow & Dong, 2010). This indicates that these coactivations between OFC and ventral hippocampus might be involved in the reduction of fear seen during extinction learning, despite the lack of IL-BLA coactivation that these animals display. We believe that the coactivations of OFC with BLA and ventral hippocampus might represent compensatory networks brought online to help the animals perform extinction since their IL-BLA coactivation is dysfunctional. Overall, we see decreased coactivation between expected regions of medial prefrontal cortex and amygdala (specifically IL-BLA) in rodents that have high spontaneous anxiety, and in situations where they do show some extinction, compensatory networks between OFC, amygdala, and ventral hippocampus may contribute to the reduction in fear.

Given that we demonstrated reduced medial prefrontal-amygdala coactivation between brain regions expected to be involved in extinction in rodents with high anxiety, we were curious to know whether humans would display a similar pattern of brain activation during extinction. Contrary to our expectations, we found increased anterior cingulate cortex (ACC) coactivation with BLA in humans with high trait anxiety when they learned extinction, perhaps similar to the compensatory networks we observed in highly anxious rodents. As discussed, rodent IL is thought to be homologous to subgenual cingulate in the human; we did not observe any reduction in coactivation between subgenual cingulate and BLA in humans that exactly matched that seen in our rodents. While we also did not observe increased coactivation between OFC and hippocampus or BLA in humans during extinction, we did observe increased coactivation between ACC and BLA. It would appear that our results in humans do not identically match our results in rodents; however, in both cases individuals with high anxiety

brought online networks that involved areas of the prefrontal cortex and amygdala not normally involved in extinction learning when they extinguished their fear, and in this way the results are similar across species. We therefore believe that these results are important because they demonstrate one commonality: a clear dysfunction between amygdala and prefrontal cortex across species in individuals with high anxiety.

Medial prefrontal cortex and amygdala dysfunction have long been implicated in a variety of anxiety and stress-related disorders, including PTSD and generalized anxiety disorder (Duval, Javanbakht, & Liberzon, 2015; Etkin & Wager, 2007; Shin & Liberzon, 2010). It is commonly found that the amygdala is hyperactive in patients with a variety of anxiety disorders (Duval et al., 2015; Etkin & Wager, 2007; Shin & Liberzon, 2010). In PTSD, this hyperactive amygdala is negatively correlated with activity in ventromedial prefrontal cortex (Shin et al., 2004; Shin et al., 2005), a technique similar to our analysis of coactivation. While the technique is similar, the results indicate a negative relationship between PFC and amygdala rather than the coactivation (positive relationship) seen in our studies. Interestingly, another study has demonstrated a positive correlation (coactivation) between amygdala and hippocampus in PTSD patients (Brohawn, Offringa, Pfaff, Hughes, & Shin, 2010). While we did not see a similar result in our analysis of human brain activity, this result parallels the increased coactivation between BLA and ventral hippocampus that we saw in our highly anxious bLR rats that also display PTSD-like behavior. These studies were all performed in PTSD patients, while our human cohort has a majority of generalized anxiety disorder patients, perhaps explaining the differing results seen in these studies. Studies using connectivity analyses in generalized anxiety disorder (GAD) patients have demonstrated increased connectivity

between the amygdala and ventrolateral prefrontal cortex (Heinz et al., 2005; McClure et al., 2007; Monk et al., 2008). While the ventrolateral prefrontal cortex is not one of the areas we chose to include in our analysis, these studies demonstrate that increased coactivation between amygdala and PFC regions in high anxiety participants, especially those with GAD, may be somewhat consistent. While diagnosis and techniques differ, the human studies, including ours, indicate that individuals with high anxiety demonstrate a general dysregulation of amygdala-PFC circuitry.

One major difference between these studies in humans is the use of a variety of different tasks, mostly emotional face viewing or script-driven imagery, while our work specifically looks at extinction learning. The difference between face perception and extinction learning could be dramatic, and lead to the differences in directionality of coactivation as well as regional differences in prefrontal subfield activation, as described above. We believe that studies examining threat detection may provide a unifying basis to examine how these different types of tasks intersect. Several studies have implicated ACC activity in threat detection and fear expression (Milad, Quirk, et al., 2007; Pissiota et al., 2003; Rougemont-Bucking et al., 2011). Additionally, studies that have examined electrophysiological signatures of the ACC and amygdala during extinction learning in monkeys have identified synchrony between these two regions as predictive of reduced extinction learning (Livneh & Paz, 2012). Reducing activity of ACC neurons during extinction learning reduced spontaneous recovery of fear after extinction (Klavir, Genud-Gabai, & Paz, 2012). Together, these studies suggest that activity in ACC, and synchrony between ACC and amygdala are critical for the formation of long-lasting extinction memories, perhaps by reducing the amount of threat detected in the environment by

reducing ACC activity. In our studies, both rats and humans with high anxiety demonstrated increased coactivation of amygdala with medial prefrontal cortex (in humans this was specific to ACC) when they learned extinction. These results indicate that high anxiety individuals may coactivate amygdala and medial prefrontal cortex as a threat detection mechanism. This coactivation of amygdala with ACC may be a compensatory measure to attempt to better assess the extinction contingencies and level of fear that should be expressed. Further studies are needed to assess how amygdala-prefrontal cortex coactivation changes based on levels of threat, and whether this additional threat detection is characteristic across patient groups and non-psychiatric individuals with high trait anxiety.

Interestingly, we have previously shown that in a group of high anxiety patients, amygdala – ACC connectivity was reduced across both a face perception task and at rest (Prater et al., 2013). This consistency of amygdala-PFC dysregulation across two mental states indicates that this circuit is important for anxious individuals. Our findings here in high anxiety participants during extinction identify dysregulation in the same regions as our previous study, suggesting that while the direction may change between tasks, dysfunction between amygdala and ACC in highly anxious humans may be a consistent and pervasive marker of anxiety across a variety of mental states.

It is important to note that there are a number of reasons why our rodent and human results are not identical. We identified ways in which the anatomy of these two species is similar; however, the prefrontal cortex of a human is significantly different and more complex than a rat (Preuss, 1995, 2000). Given the significant differences in anatomy of the medial prefrontal cortex across species, it is not surprising that our results

might differ. Additionally, spontaneous anxiety as measured in rodents and trait anxiety measured in humans are also not perfectly synonymous measures, and therefore coactivation may differ because we are not measuring high anxiety individuals on the same construct. Future studies will be needed to confirm our findings in high anxiety individuals and to further compare coactivation between species to determine whether amygdala-PFC circuitry can be effectively compared between them.

Despite the differences, both between our rodent and human coactivation results, and between our human results and the results of other studies in humans, all of these studies implicate dysregulated amygdala – prefrontal circuitry in high anxiety individuals. In humans, a network of regions including the dorsal ACC and anterior insula were identified as having both reduced grey matter and altered activity in a variety of mood and anxiety disorders (Goodkind et al., 2015). Studies like ours and Goodkind and colleagues' (2015) indicate that there may be similarities in dysfunctional brain networks across mental state or task, and potentially across different mental health disorders. Our results point toward dysfunction of amygdala-prefrontal circuitry in high anxiety individuals across species, and further work will confirm whether our identified network is robust across multiple anxiety disorders and tasks.

Remaining Questions and Future Directions

This dissertation has characterized a unique animal model of individual differences in extinction learning that may provide further insight into vulnerability to the development of PTSD, and identified that FGF2 affects solely more resilient individuals when given early in life. Additionally, we determined that social context significantly

impacts extinction learning in both outbred and selectively bred rats, and identified common patterns of dysfunctional activity of amygdala and prefrontal cortex in both rodents that do not extinguish well and in human patients with anxiety disorders. This dissertation displays a variety of strengths, including the use of human and animal models of anxiety to delineate brain circuitry dysfunction common across species, and the use of behavioral techniques to identify an animal model of individual differences in extinction learning that mimics certain aspects of PTSD. While we have provided a large volume of novel information, there remain a number of unanswered questions related to this work that are detailed below.

We demonstrated significant individual differences in the ability of FGF2 to facilitate extinction learning, and it may be that other pharmacological tools show similar effects on specific individuals. Pharmacological agents like d-cycloserine (DCS) and traditional selective serotonin reuptake inhibitors (SSRI) or benzodiazepines might be effective at facilitating extinction learning in more vulnerable animals (Difede et al., 2014; Ori et al., 2015). We propose that studies that administer chronic doses of DCS, benzodiazepines, and SSRIs to determine their respective effects on both bHR and bLR extinction learning should be completed. Further studies to determine which or whether these different pharmacological agents are effective at enhancing fear extinction specifically in bLRs will provide further information on how individual differences influence response to different therapeutics.

We demonstrated both in outbred and in selectively bred animals that social context influences how well an individual learns extinction. Since we chose to pursue an understanding of the brain mechanisms underlying how the social context might

influence extinction learning, we still do not know how the social context is impacting extinction. It could be that animals are communicating with each other via odor or ultrasonic vocalizations (Kim, Kim, Covey, & Kim, 2010; Klein et al., 2015; Takahashi et al., 2013); however, it is currently unclear which mechanism of communication is particularly important for extinction learning. Future studies can manipulate the use of odor, or record and playback USVs, to determine if and how they influence extinction learning. Studies that demonstrate the communication mechanism underlying facilitated extinction learning in social contexts will allow us to better manipulate and determine the neural correlates of how social context influences extinction learning.

We identified a set of brain regions that provide potentially compensatory coactivation networks to support extinction in highly anxious bLR rats. Further studies are needed to delineate whether these compensatory coactivations are specific to extinction learning and/or high anxiety individuals. Further experiments examining cFos coactivation in outbred rats can determine whether OFC and piriform cortex are normally coactivated with BLA and hippocampus during extinction learning. Our current study did not identify significant coactivation between these structures, but a greater sample size would significantly benefit this study. This comparison with bLR animals will allow further definition of whether these networks are compensatory for extinction learning in high anxiety individuals. Additionally, outbred animals could be stressed to increase their levels of spontaneous anxiety to assess whether OFC is involved in extinction for anxious animals or if it is specific to our selectively bred animals. In addition, the examination of cFos coactivation in bLR rats that do not undergo extinction but are placed in different social contexts (in-group phenotype, mixed phenotype, and isolated conditions) will

allow further interpretation of whether the OFC and piriform coactivation with BLA and hippocampus are specific to varying the social context rather than extinction learning in general. If OFC coactivation with BLA and hippocampus were found to be involved in either extinction learning or processing of social contexts, studies using optogenetics could further refine definition of its role. Studies could determine the direction of the connections by placing channel rhodopsins in either BLA, subregions of the hippocampus, or the OFC and then stimulate in the other areas during extinction learning to determine how increasing activity of the terminals of one region affects behavior during extinction. This would facilitate understanding what direction information flows between regions. The use of halorhodopsins that reduce the activity of cells when stimulated would provide further information about the types of activity that are occurring between regions, whether decreasing amygdala activity to OFC or increasing OFC activity to amygdala facilitates extinction learning or the identification of social contexts. Finally, optogenetic studies such as these could better define the subregions of OFC and differentiate their potential roles in extinction and social buffering. Together, these studies in both outbred and selectively bred rodents will build a better understanding of the role of OFC in fear extinction and social contexts and define how this network interacts to facilitate extinction learning, potentially specifically in highly anxious individuals.

Additionally, we would like to see future studies that examine the location and storage of fear memories in these selectively bred animals to determine if they differ in accordance with the individual differences seen in the lines. The basolateral amygdala has populations of “fear” and “extinction” neurons, and is believed to be the site of fear

memory storage (Dejean et al., 2015; Herry et al., 2008; Pape & Pare, 2010). We propose a series of studies using molecular markers and electrophysiology to identify which cells in the amygdala underlie fear and extinction memories in these lines of animals, whether the proportion of cells identified as "fear" or "extinction" or their activity differs between the lines (e.g. Herry et al., 2008). Differences in the number or firing of neurons in bHRs compared to bLRs and outbred animals will provide additional insights into the cellular mechanisms of fear and extinction memory encoding and retrieval in these animals. These studies will give us a better idea of the neural underpinnings of individual differences in fear and extinction learning displayed behaviorally by these animals. Further studies could then attempt to manipulate either "fear" or "extinction" neurons using optogenetics or DREADDs to inhibit or enhance extinction learning in these lines of rats. It is known that "fear" and "extinction" neurons have different long-range connections with prefrontal cortex (Herry et al., 2008), which could be used to specifically target extinction neurons, for example. We could manipulate extinction neurons during the extinction session, inhibiting their activity in bHRs and increasing their activity in bLRs to determine whether this would change behavior differently depending on temperament. Assessing how manipulation of these "fear" or "extinction" neurons changes behavior on other measures of spontaneous anxiety and depression such as the elevated plus maze, open field, and forced swim test would allow additional insight into how individual differences in anxiety and depression are influenced by the same neural mechanisms as fear and extinction learning.

Finally, determining how network connectivity differs during extinction learning in human PTSD patients as opposed to individuals diagnosed with other anxiety disorders

or high state anxiety non-psychiatric individuals would provide further insight into how these disorders impact neural circuitry. To our knowledge, no connectivity analyses have been performed during extinction learning in a population of PTSD patients. While previous fMRI studies often demonstrate no differences in activation during extinction learning for PTSD patients, it may be that their connectivity differs from other populations (Garfinkel et al., 2014; Milad et al., 2009). While our current participant pool does not provide enough participants with a primary diagnosis of PTSD to study this question, we encourage further research into the networks underlying extinction learning. Developing a better understanding of whether PTSD patients differ in their functional connectivity from other participant and patient groups during extinction learning will allow us to determine how similar or different networks are across species and across disorders.

Together, additional research to address the outstanding questions posed by the studies described here in this dissertation will build on the important insights found in this dissertation into how individual differences influence fear conditioning and extinction learning. These studies will further develop both our animal model of individual differences and knowledge of how circuits underlying fear and extinction learning are similar across species.

Conclusions

This dissertation has built upon the idea that identifying individual differences that lead to resilience or vulnerability to PTSD may provide additional avenues for prevention or therapeutic intervention. A recent review of prevention strategies for PTSD

called for the development of a prediction tool that would allow clinicians to better assess the individual vulnerability of a person to developing PTSD (Howlett & Stein, 2016). This dissertation provides a preclinical animal model of individual differences in temperament that predisposes certain individuals towards PTSD-like behavior, allowing us to predict with some certainty those individuals who are more vulnerable. While further studies are needed to fully assess the extent to which these selectively bred rodents model PTSD symptoms after fear conditioning, we have provided a well-characterized animal model that displays behavior similar to that of PTSD patients. We additionally describe how individual differences in temperament influence both pharmacological intervention and contextal manipulation in these animals, providing further information for clinical studies of how different interventions require appropriate tailoring for the individual. Finally, we have provided evidence that amygdala-prefrontal cortex dysfunction is present in highly anxious individuals across both humans and rodents during extinction of fear. These findings indicate that amygdala-prefrontal dysregulation may be a core deficit in individuals with high anxiety, and further studies are needed to confirm these results across anxiety disorders and tasks.

The studies contained in this dissertation provide evidence that individual differences in temperament are particularly useful tools to dissect variation in propensity for mental health disorders, pharmacological intervention efficacy, group cohesion and dynamics, and neural circuit coactivation. Further studies such as these that rely on differentiating the responses of individuals rather than averaging across groups may provide even greater progress toward identifying ways to prevent or better treat mental health disorders such as PTSD.

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