Chronic Stress Has Lasting Influences on Fear Extinction Cued Discrimination Early in Extinction That is Mediated by the Infralimbic Cortex

by

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ABSTRACT

Post-Traumatic Stress Disorder (PTSD) is characterized by intrusive memories from a traumatic event. Current therapies rarely lead to complete remission. PTSD can be modeled in rodents using chronic stress (creating vulnerable phenotype) combined with fear conditioning (modeling a traumatic experience), resulting in attenuated extinction learning and impaired recall of extinction. Studies typically investigate cognition soon after chronic stress ends; however, as days and weeks pass ("rest" period) some cognitive functions may improve compared to soon after stress. Whether a rest period between chronic stress and fear conditioning/extinction would lead to improvements is unclear. In Chapter 2, male rats were chronically stressed by restraint (6hr/d/21d), a reliable method to produce cognitive changes, or assigned to a non-stressed control group (CON). After chronic stress ended, fear conditioning occurred within a day (STR-IMM), or after three (STR-R3) or six weeks (STR-R6). During the first three extinction trials, differences emerged in fear to the non-shock context: STR-R3/R6 showed significantly less fear to the context than did STR-IMM or CON. Differences were unlikely attributable to generalization or to second-order conditioning. Therefore, a rest period following chronic stress may lead to improved fear extinction and discrimination between the conditioned stimulus and environment. In Chapter 3, the infralimbic cortex (IL) was investigated due to the IL's importance in fear extinction. Rats were infused with chemogenetics to target IL glutamatergic neurons and then assigned to CON, STR-IMM or STR-R3. During the rest period of STR-R3 and the restraint for STR-IMM, the IL was inhibited using CNO (1mg/kg BW, i.p., daily), which ended before behavioral testing. STR-R3 with IL inhibition failed to demonstrate a tone-shock association as spontaneous recovery was not

observed. CON with IL inhibition behaved somewhat like STR-IMM; freezing to the extinction context was enhanced. Consequently, inhibiting IL function during the rest period following chronic stress was particularly disruptive for learning in STR-R3, impaired freezing to a safe context for CON, and had no effect in STR-IMM. These studies show that time since the end of chronic stress (recently ended or with a delay) can interact with IL functioning to modify fear learning and response.

DEDICATION

This dissertation is dedicated to my parents, Michael and Rita, who have supported me throughout my educational journey, even when it meant I had to move away, across the country. They have been proud of each and every milestone. Without their love and support, this dissertation would not have been possible.

My dad passed away while I was conducting my dissertation experiment and he didn't get to see the end of this journey. Before his passing, he bragged to the nurses in the hospital about his "daughter getting her PhD in neuroscience."

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Lastly, as much as defending a dissertation is about looking back onto the last six years, it is also an event that requires looking into the future. While there is much about

the future that has yet to be determined, particularly with the uncertainty brought by pandemic, I am optimistic about the future and know it will be wonderful because it will include Manuel Padilla. Thank you, Amor, for your love and support, and for always being by my side. This day is more special because you are part of it.

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CHAPTER 1
GENERAL INTRODUCTION

Historical Background and Significance

The authors of the earliest recorded history recognized that traumatic experiences on the battlefield can change a person (Crocq & Crocq, 2000). One example is the Ancient Greek hero Odysseus who had difficulty adjusting to civilian life after first fighting in the Trojan War and then struggling ten years to get back home. When he finally returns home, he addressed his problems with the same violent responses he employed to survive in battle, killing hundreds of townspeople and servants (Homer, 1999; O'Donnell, 2015). In the U.S. Civil War and World Wars I and II, the ability of a trauma to alter a person's psyche was acknowledged with terms such as "war neurosis," "shell shock," and "battle fatigue." Unfortunately, these labels were accompanied with the judgement that the individual was to blame for their psychiatric issues (Wong & Cook, 1992). The post-World War II era saw the creation of the first edition of the American Psychiatric Association's Diagnostic and Statistical Manual (DSM-I), which included an entry for "Gross Stress Reaction," that was defined as an extreme stress response to an exceptional physical or mental stress. In 1968, the diagnosis of Gross Stress Reaction was removed from the DSM-II, during the Vietnam War, and was replaced with "Adjustment Reaction of Adult Life" in a "Transient Situational Disturbances" category, which defined the responses to intense trauma as "an acute reaction to overwhelming environmental stress." Like Gross Stress Reaction, the newer category was considered a "transient disorder" but again, specific diagnostic criteria were not provided. The DSM-III attempted to improve upon the limitations of the Gross Stress

Reaction and Adjustment Reaction of Adult Life diagnoses by including symptoms and trauma reactions from years of research since WWII on Vietnam Veterans and civilians alike. Consequently, the DSM-III introduced of the diagnosis of Post-Traumatic Stress Disorder, commonly known as PTSD, and this diagnosis has remined in subsequent versions of the DSM (Andreasen, 2010; North, Surís, Smith, & King, 2016; Ray, 2008; Wong & Cook, 1992).

In the DSM-III, PTSD is defined as a psychiatric condition that includes intrusive symptoms following either a personal experience with or witnessing of a traumatic event. Individuals with PTSD have intrusive recall of the event in flashbacks and nightmares. Often, they will avoid thinking or talking about the trauma. They may also avoid external reminders of the trauma. Patients with PTSD experience negative thoughts and feelings, such as hopelessness and anhedonia, or an inability to experience pleasure to events that were previously enjoyable. They may also have difficulty sleeping and concentrating. Symptoms have to be present for at least a month for diagnosis (American Psychiatric Association, 2013). This definition has remained practically the same to this day in the current DSM-5.

PTSD affects 6-9% of the population and up to 40% of those exposed to extreme trauma (American Psychiatric Association, 2013; Hoge & Warner, 2014; Sareen, 2014), but no universally targeted treatment for PTSD in particular exists. Current pharmacological treatments include antidepressants and anti-anxiety medications, but these treat the symptoms to improve daily functioning and do not cure the core of the condition (Steckler & Risbrough, 2012) (Difede, Olden, & Cukor, 2014). Some studies have even suggested that pharmacological treatments might be less effective in combat-

related trauma than they are for civilian-related traumas (Ravindran, et al 2009). Further, current prescribing guidelines make recommendations of pharmacotherapies by class of drug, even though individual drugs in a class might not be as effect as other drugs of that class for certain disorders (Friedman, Bernardo 2007). The other main type of treatment approach for PTSD is psychotherapies, but these also have issues. The main reason for some of these failures of psychotherapies is that they tend to be context-dependent, such as difficulties in generalizing the new responses to regions outside of the clinic (Bradley et al., 2005; VanElzakker, Kathryn Dahlgren, Caroline Davis, Dubois, & Shin, 2014). Further complicating the issue is that the intensity of symptoms can change over time, often fluctuating between more severe and less severe presentations. As a result, an individual might be misled into thinking that the symptoms are abating, when instead, the symptoms could return even stronger than the last episode (American Psychiatric Association, 2013). Consequently, understanding the mechanisms behind PTSD development and maintenance are vital to combat it.

Exposure to a traumatic event alone is typically insufficient to cause someone to develop PTSD and, as a result, not everyone who is exposed to a traumatic event will develop PTSD. There are some identified risk factors that make individuals more likely to develop PTSD, including genetic predisposition and environmental factors (Koenen et al., 2008; Sarapas et al., 2011; Skelton, Ressler, Norrholm, Jovanovic, & Bradley-Davino, 2012). Some of the risk factors for the development of PTSD include: a family or personal history of other mental health issues (American Psychiatric Association, 2013; Brewin, Andrews, & Valentine, 2000; Sareen, 2014), including anxiety and depression; lack of social support (American Psychiatric Association, 2013; Brewin et al.,

2000); previous trauma exposure (American Psychiatric Association, 2013; Breslau, Chilcoat, Kessler, & Davis, 1999; Sareen, 2014); and the type of trauma, with sexual abuse the most likely to result in PTSD (Kessler et al., 2017; Surís, Lind, Kashner, Borman, & Petty, 2004). Importantly for the current discussion, chronic stress is a risk factor for the development of PTSD as well (Breslau et al., 1999; Sareen, 2014). As such, understanding the risk factors that increase the susceptibility to PTSD development is a critical field to study to better comprehend the full milieu of PTSD.

Normal Stress Response is Important for Survival, but Chronic Stress Can Be Detrimental

The stress response is an important mechanism for survival when an organism encounters a real or perceived threat (De Kloet, Joëls, & Holsboer, 2005; Koolhaas et al., 2011; McEwen, 2000). During the stress response, glucocorticoids are released to mobilize extra energy and to increase attention to appropriately respond to and address the threat (McCarty, 2016; Sapolsky, Romero, & Munck, 2000). However, under chronic stress conditions, when the stress response is activated for an extended time, it can take a toll on an organism. Indeed, some of the earliest research into chronic stress found that chronic stress exposure leads to ulcers, metabolic disorders, cardiovascular issues, and psychiatric illness, including depression and anxiety (Levine, 2005; Selye, 1984). The current understanding of chronic stress suggests that chronic stress allows the organism to survive threats over extended durations by maintaining biological set points outside of homeostasis, a process known as allostasis (McEwen, 1998; McEwen & Wingfield, 2003). Being at these altered set points is not detrimental in the short term, but they can

take a toll on an organism over time, which is termed “allostatic load.” Consequently, research into risk factors for a variety of illnesses focus on the effects of chronic stress.

Chronic Stress Alters Brain and Behavior in Humans and Rodents

Chronic stress impacts the periphery, as well as the brain. In human patients with chronic stress-related psychiatric disorders, notable changes occur in key brain regions including the hippocampus, prefrontal cortex, and the amygdala. In the hippocampus, there is a reduction in volume (Bremner et al., 2000; S. Campbell, Marriott, Nahmias, & Macqueen, 2004; Gianaros et al., 2007; Mervaala et al., 2000; von Gunten, Fox, Cipolotti, & Ron, 2000), which corresponds to issues forming long term memories and navigating spatial locations (Bearden et al., 2006; Cornwell et al., 2010; Gould et al., 2007). Chronic stress also causes reductions in prefrontal cortex volume and activity (Arnsten, 2015; Drevets, 2000; Drevets, Price, & Furey, 2008), leading to poorer behavioral flexibility and emotional regulation (Hart, Wade, & Martelli, 2003; McKlveen, Myers, & Herman, 2015; Öhman, Nordin, Bergdahl, Birgander, & Neely, 2007; Sandström, Rhodin, Lundberg, Olsson, & Nyberg, 2005). Conversely, chronic stress causes an increase in amygdala volume and activity (Cacciaglia et al., 2017; Frodl et al., 2002; Tebartz Van Elst, Woermann, Lemieux, & Trimble, 2000), corresponding to heightened emotionality and strengthened aversive memory formation (Somerville, Kim, Johnstone, Alexander, & Whalen, 2004; Williams et al., 2009). These chronic stress-induced changes in the brain seem to enhance risk for the development and maintenance of psychiatric conditions, such as in the case of PTSD.

Interestingly, the brains of individuals who develop PTSD have notable differences compared to individuals who do not develop PTSD and these differences correspond to the brain changes caused by chronic stress. Individuals with PTSD commonly have a smaller and less active hippocampus (Astur et al., 2006; Bennett, Hatton, & Lagopoulos, 2016; Childress et al., 2013; Gilbertson et al., 2002; S. L. Rauch, Shin, & Phelps, 2006; Shin, Rauch, & Pitman, 2006), a larger and more active amygdala (Bennett et al., 2016; Bryant et al., 2008; Milad et al., 2009; Shin et al., 2006), and a smaller, less active medial prefrontal cortex (mPFC) (Bennett et al., 2016; Bryant et al., 2008; Milad et al., 2009; Shin et al., 2006). The majority of studies on the differences in the brains of individuals with PTSD have examined the brain after the occurrence of the traumatic triggering event or the diagnosis of PTSD. Consequently, such studies cannot tell us whether the observed brain differences are a result from the development of PTSD or a predisposition factor. At least one study on monozygotic twins, with identical genes and shared placenta, provided evidence that brain differences might exist prior to the development of PTSD. In this study, one twin was a trauma-exposed combat veteran, while the other was not. The combat veterans that developed PTSD had a small hippocampus. Interestingly, their non-combat twin also had a small hippocampus, which predicted the severity of PTSD to suggests that a smaller hippocampus predisposes one for PTSD, and is not an outcome from PTSD itself (Gilbertson et al., 2002). This research, along with findings on the impact of chronic stress in the brain, suggests that understanding the impact of chronic stress in the brain can increase our understanding of why some people develop PTSD, while others do not.

Helpfully for researchers of PTSD, the effects of chronic stress on the brain are conserved across species and can be modeled in rodents. In rodents, changes in dendritic complexity following chronic stress is often measured and the changes in dendritic complexity typically positively correlate with changes in volume in the brain region of interest (Kassem et al., 2013). In the brains of rodents that have undergone a chronic stress manipulation, neuronal dendritic retraction occurs in the hippocampus (C. D. Conrad, 2006; C. D. Conrad, Ledoux, Magariños, & Mcewen, 1999; C. D. Conrad, Ortiz, & Judd, 2017; Luine, Villegas, Martinez, & McEwen, 1994; McLaughlin, Gomez, Baran, & Conrad, 2007; Vyas, Pillai, & Chattarji, 2004). Following chronic stress, amygdala neuronal dendritic complexity increases (Govindarajan et al., 2006; Hoffman et al., 2015; Johnson et al., 2009; Leuner & Shors, 2013; Mitra, Vyas, Chatterjee, & Chattarji, 2005; Vyas, Jadhav, & Chattarji, 2006; Vyas, Mitra, Shankaranarayana Rao, & Chattarji, 2002; Vyas et al., 2004). Decreased neuronal dendritic complexity and functional activity is observed in the mPFC following chronic stress (Arnsten, 2015; Bloss, Janssen, McEwen, & Morrison, 2010; Cook & Wellman, 2004; Czéh, Perez-Cruz, Fuchs, & Flügge, 2008; Goldwater et al., 2009; Holmes & Wellman, 2009; McKlveen et al., 2016; Moench & Wellman, 2014; Radley et al., 2005; Wellman & Moench, 2019). These findings on neuronal complexity and functional activity in chronically stressed rodents parallel the changes in structure volume and activity observed in human patients with PTSD.

Rodents that have undergone chronic stress manipulations also show behavioral changes that correspond to the stress-induced changes in the dendritic arbors of the respective brain regions. Compared to non-stressed controls, chronically stressed rodents have difficulties in learning or recalling recent hippocampal-dependent spatial tasks

(Abidin et al., 2004; Bowman, Ferguson, & Luine, 2002; Ghiglieri et al., 1997; Kleen, Sitomer, Killeen, & Conrad, 2006; Luine et al., 1994; Ortiz et al., 2015; Song, Che, Min-wei, Murakami, & Matsumoto, 2006). Chronic stress leads to an increase in anxiety-like behaviors (Cordero, Venero, Kruyt, & Sandi, 2003; Vyas, Bernal, & Chattarji, 2003; Vyas et al., 2004) that reflects the heightened complexity and activity in the amygdala. Chronically stressed rodents also show impairments in flexible cognition and working memory tasks and a corresponding shift towards habit based behaviors (Arnsten, 2015; Baran, Armstrong, Niren, Hanna, & Conrad, 2009; Holmes & Wellman, 2009; Liston et al., 2006; Miracle, Brace, Huyck, Singler, & Wellman, 2006; Mizoguchi et al., 2000; T Arnsten, 2009) that is a reflection of less mPFC activation and more response-based behaviors dependent upon the striatum (Taylor et al., 2014). Consequently, chronic stress manipulations in rodents can create a model that parallels the structural abnormalities and cognitive profile of an individual that is at risk for development of PTSD.

Fear Conditioning Principles to Understand Fear Learning and Extinction in PTSD

Fear conditioning and extinction, which are based on Pavlovian conditioning principles (Maren, Phan, & Liberzon, 2013; Phillips & Ledoux, 1992), are particularly useful for studying fear memory formation in a "normal" system and for modeling the traumatic event requirement in PTSD development. In cued fear conditioning, a rat is placed in a small inescapable chamber and a tone (conditioned stimulus, CS) is temporally paired with a foot shock (unconditioned stimulus, US). After repeated presentations, the rat will begin to demonstrate freezing responses to the tone even before

the foot shock is presented (conditioned response, CR) because the rat expects that a foot shock will follow. Fear extinction training involves the presentation of the tone or CS without the foot shock or US. To dissociate the cued tone from the context where training occurred, extinction training is often provided in an environment where fear conditioning did not occur. With repeated presentations of the CS without the US, the robustness of the CR (i.e., freezing in this case) typically decreases and may even disappear. Extinction to the CS is due to new learning that the CS no longer signals danger (foot shock) and is not a form of forgetting the original CS-US association (Baron, 1951; Bouton & Bolles, 1979; Rescorla, 1967). Support for extinction being new learning and not forgetting comes from observing spontaneous recovery and other post-extinction effects, that show the original association is still present, as observed through renewed responding to the CS (Rescorla, 2004). Had the extinction process reflected forgetting, the original association would not have returned. Fear conditioning and extinction are useful methods to model PTSD, with the strong CS-US associations formed in fear conditioning that can provide a model for the strong memory of the trauma in PTSD (Daskalakis, Yehuda, & Diamond, 2013; Goswami, Rodríguez-Sierra, Cascardi, & Paré, 2013).

Fear conditioning is also an extremely useful tool to understand PTSD because many of the brain regions required for fear conditioning and extinction are also impacted by chronic stress and altered in patients with PTSD. The hippocampus is important for the consolidation of contextual information (Bennett et al., 2016; Ji & Maren, 2007; Lee & Kesner, 2004; Maren, 2001; Maren, Aharonov, & Fanselow, 1997; Maren et al., 2013; Phillips & Ledoux, 1992; Selden, Everitt, Jarrard, & Robbins, 1991). The amygdala is important for the acquisition of the CS-US fear memory formation and then the

subsequent CS-only fear extinction process (Amano, Unal, & Paré, 2010; Chaaya, Battle, & Johnson, 2018; Herman et al., 2003; Herry, Trifilieff, Micheau, Lüthi, & Mons, 2006; Laurent, Marchand, & Westbrook, 2008; Maren, 2001; Phelps & LeDoux, 2005; Rogan, Stäubli, & LeDoux, 1997). In addition, the mPFC is also vital for learning the CS-only fear extinction process (Fucich, Paredes, Saunders, & Morilak, 2018; Morgan, Romanski, & LeDoux, 1993; Peters, Kalivas, & Quirk, 2009; Quirk, Garcia, & González-Lima, 2006; Sotres-Bayon, Cain, & LeDoux, 2006; Sotres-Bayon & Quirk, 2010). Within the mPFC, the infralimbic cortex (IL) is particularly important for fear extinction learning and suppressing fear responses in the environment where extinction occurred (Bennett et al., 2016; Izquierdo, Wellman, & Holmes, 2006; Knapska & Maren, 2009; Mueller, Bravo-Rivera, & Quirk, 2010; Sierra-Mercado, Padilla-Coreano, & Quirk, 2010). Consequently, fear conditioning and extinction require a complex set of inputs from multiple brain regions that are all known to be impacted by chronic stress and altered in PTSD patients.

Fear conditioning and extinction can be used to study differences in fear response and retention in individuals with PTSD compared to individuals without PTSD. Patients with PTSD form particularly strong fear memories that are resistant to extinction (Blechert, Michael, Vriends, Margraf, & Wilhelm, 2007; Milad et al., 2009). Individuals with PTSD display impaired retention of fear extinction training to a trauma related cue, compared to their non-trauma exposed twin (Milad et al., 2008) and more generalization of their fear; expressing fearful responses to stimuli that even remotely remind them of the trauma (Grillon & Morgan, 1999; Morey et al., 2015). This impairment of fear inhibition in non-trauma related contexts is a distinct marker of PTSD over other

psychiatric issues (Jovanovic, Kazama, Bachevalier, & Davis, 2012) and seems to be due to impaired integration of contextual information into their responses, so that environments that are not associated with trauma, and should be considered "safe," are reacted to as though they are trauma associated (Garfinkel et al., 2014). Some research suggests that this is due to the strong, traumatic memories being repeatedly paired with safety signals, conditioning the safety signals to the trauma memory (Wessa & Flor, 2007). Additionally, individuals with PTSD also show alterations in brain activation patterns on a fMRI during fear extinction tasks, compared to non-PTSD trauma exposed controls. Those with PTSD show hypoactivation in the ventral medial prefrontal cortex, a region responsible for emotional inhibitory control over the amygdala (similar to the rodent IL), and hyperactivation of the dorsal anterior cingulate cortex, a region responsible for facilitating fear expression (Rougemont-Bücking et al., 2011). Consequently, PTSD patients are uniquely impaired in their abilities to differentiate their responding between safe environments and unsafe ones.

The failure to properly respond to non-trauma related environments and stimuli is a major issue for successful treatment of PTSD. The most prescribed treatment for PTSD is exposure-based therapy, which is similar to extinction training and involves reliving the traumatic event and the emotions associated with it in a safe context, such as the therapist's office. Repeated sessions (exposures) should decrease maladaptive responds to reminders of the trauma (Blechert et al., 2007; Bouton, Mineka, & Barlow, 2001; Helpman, Marin, et al., 2016; Helpman, Papini, et al., 2016; Rothbaum & Davis, 2003). Extinction therapy is considered the gold standard for treatment of PTSD (S. A. M. Rauch, Eftekhari, & Ruzek, 2012). However, exposure-based therapies suffer from a lack

of access to a qualified therapist, routine failure to complete all the required sessions, and, most important for the current discussion, failure to generalize responses learned in therapy to the outside world in many cases. Indeed, even with complete treatment, about half of patients will continue to experience persistent PTSD symptoms (Ponniah & Hollon, 2009). Finding techniques to make exposure-based treatments more successfully translated to outside of the clinic has the potential to improve PTSD patient outcomes.

Two-Hit Rodent Model of PTSD

In our lab we use a combination of chronic stress for a risk factor and fear conditioning to create a rat model of PTSD (Baran et al., 2009; Daskalakis et al., 2013; Hoffman, Lorson, Sanabria, Olive, & Conrad, 2014; Hoffman et al., 2015). Compared to rats that are not chronically stressed, chronically stressed rats show faster and more robust acquisition of fear conditioning (C. D. Conrad et al., 1999; Hoffman et al., 2015; Yehuda & LeDoux, 2007). During fear extinction training, they show impairments in the acquisition and recall of extinction (Baran et al., 2009; Hoffman et al., 2014; Izquierdo et al., 2006; Miracle et al., 2006; Rau, DeCola, & Fanselow, 2005). Chronically stressed rats also generalize their fear to non-shock contexts more readily (Blechert et al., 2007; Hoffman et al., 2014; Radulovic, Kammermeier, & Spiess, 1998). These results in rodents correspond to the overly robust fear memories that are observed in PTSD patients.

The combination of chronic stress and fear conditioning can be considered a "two-hit model" of PTSD because it is also translationally relevant to PTSD. Indeed, individuals with a history of chronic stress or previous trauma are widely recognized to

be at heightened risk for the development of PTSD (American Psychiatric Association, 2013; Breslau et al., 1999; Brewin et al., 2000; Kessler et al., 2017; Sareen, 2014; Surís et al., 2004). Previous stressful and traumatic experiences are additive in that each increases the likelihood that an individual will develop PTSD and that if PTSD manifests, then PTSD will likely be remission-resistant (D. Conrad et al., 2017; Kolassa et al., 2010; Wilker et al., 2015). Individuals with a history of chronic stress combined with a lack of social support seem to be particularly vulnerable to psychological distress when faced with stressful situations (Regehr, LeBlanc, Blake Jelley, Barath, & Daciuk, 2007). In addition to the vulnerability that chronic stress causes to prime the brain for developing PTSD (Bennett et al., 2016; Bryant et al., 2008; Childress et al., 2013; Gilbertson et al., 2002; Milad et al., 2009; S. L. Rauch et al., 2006; Shin et al., 2006), some theorize various biological cascades occur. Specifically, the first hit incident, such as chronic stress, may lead to a neuroimmune response (Deak et al., 2015; Georgopoulos, James, Christova, & Engdahl, 2018; Hodes, Kana, Menard, Merad, & Russo, 2015). With the second trauma, or the fear conditioning exposure in the rat model, the brain releases glutamate at intense levels (Averill et al., 2017; Georgopoulos et al., 2018; Reul & Nutt, 2008). The combination of the neuroimmune response with high levels of glutamate is proposed to create to a highly connected limbic network (Georgopoulos et al., 2018). Consequently, a "two-hit" model of PTSD in pre-clinical research leads to heightened and robust fear memory formation that would not occur for either situation alone, making it translational with high validity.

A Mild Stressful Experience Can Lead to Resilience Against Negative Impact of Future Stressful Experiences

Previous exposure to chronic stress doesn't always cause issues later in life and can in fact lead to resilience against future stressful events. Extensive, research has indicated that while severe stress and trauma, particularly in childhood, predisposes individuals to poor psychological health, exposure to mild or moderate stress can actually lead to resilience against later stressful events (Forest, Moen, & Dempster-McClain, 1996; Khoshaba & Maddi, 1999; Lyons, Parker, Katz, & Schatzberg, 2009; Lyons, Parker, & Schatzberg, 2010). Early exposure to stress may operate in a curvilinear function (Boyce & Ellis, 2005; Ellis & Boyce, 2008; Ellis, Essex, & Boyce, 2005), with low or high stress exposure leading to poorer outcomes, and mild to moderate stress exposure leading to better skills at navigating stress later in life (Ellis & Boyce, 2008). Individuals in the previous mild or moderate stress exposure category show better emotional regulation and lower stress response activation than do those with low or high stress exposure (Chorpita & Barlow, 1998; Koenig, Walker, Romeo, & Lupien, 2011; Rudolph & Flynn, 2007). These studies suggest that mild stress in childhood and adolescence may improve responses to stress challenges later in life as adults. Some research also suggests that mild stress in adulthood can also lead to stress resilience or improved cognitive outcomes in some cases (Bonanno, 2004; McEwen & Gianaros, 2011; Russo, Murrough, Han, Charney, & Nestler, 2012). However, the ability of mild stress to result in resilience against the development of PTSD following a traumatic event is less studied.

Recently, our lab and others have investigated whether the effects of chronic stress on brain and behavior in adult rats are long lasting, persisting even in the absence of the stressor. Specifically, rats are chronically stressed, usually for 21 days of restraint for 6 hours per day, and then given ten or more days without stress exposure before investigating potential changes in the brain and behavior. Interestingly, when chronic stress ends and rats are left unperturbed beyond normal handling requirements (a period we term "rest"), the brain and its function changes in some regions, but not others. In the hippocampus, there is a recovery of the dendritic branching and spatial memory ability is restored to, or even better than, pre-stress levels (Bian et al., 2012; C. D. Conrad et al., 2017; Hoffman et al., 2011; Luine et al., 1994; Ortiz et al., 2015; Sousa, Lukoyanov, Madeira, Almeida, & Paula-Barbosa, 2000). The amygdala maintains its chronic stress-induced dendritic hypertrophy and corresponding anxiety-like behaviors remain elevated (Adamec & Shallow, 1993; van Dijken, Mos, van der Heyden, & Tilders, 1992; Vyas et al., 2004). In the mPFC, the overall dendritic complexity return, but the organization of the complexity can be different than in the pre-stress state (Bloss et al., 2010; Goldwater et al., 2009; Radley et al., 2005). Taken together, following a post-chronic stress rest period, rat brain and behavior display a unique phenotype compared to non-stress controls or of rats tested immediately after enduring chronic stress. Since fear conditioning and extinction require all these brain regions to be learned and remembered, it is important to understand what effect a post-stress rest period has on fear conditioning and extinction and was studied in Chapters 2 and 3.

Rationale for Empirical Studies in Chapters 2 and 3

In Chapter 2, we combined our standard "two-hit" model of PTSD with a delay between the end of stress and the start of a fear conditioning and extinction paradigm to investigate the impact of a post-stress rest period on fear conditioning extinction. Since chronically stressed rats tend to generalize their fear between contexts, we used an acclimation procedure that is supposed to produce similar fear conditioning acquisition rates (Hoffman et al., 2014) to allow the study of the effects of chronic stress and the rest period on extinction processes. Rats were chronically stressed either with or without a rest period and then underwent fear conditioning and extinction. Rats that were allowed a rest period showed less fear responses early in extinction training compared to recently and even unstressed rats. Follow-up behavioral studies were performed to try to identify the behavioral mechanisms that were responsible for these observations. This series of studies showed that fear extinction mechanisms were enhanced in the post-stress rest period, leading to better recognition of safety signals in an environment not associated with a fearful memory.

The infralimbic cortex (IL) of the medial prefrontal cortex is a critical region for fear extinction acquisition and recall. Inactivation of the IL (Sierra-Mercado et al., 2010) or blockade of D2 dopamine receptors in the IL (Mueller et al., 2010) impairs fear extinction acquisition and memory. Less fear response (i.e. freezing behavior) during extinction is associated with more IL activity, better consolidation of extinction learning, and greater inhibition of fear on subsequent encounters with fear-related stimuli (Milad & Quirk, 2002). Signaling events that facilitate extinction in the IL involve a calcium-mediated cascade that triggers protein kinases and protein synthesis, processes that are

required for long-term extinction memory formation (Burgos-Robles, Vidal-Gonzalez, Santini, & Quirk, 2007; Hugues, Deschaux, & Garcia, 2004; Lin, Mao, Su, & Gean, 2009; Mueller, Porter, & Quirk, 2008; Santini, 2004; Sierra-Mercado, Corcoran, Lebrón-Milad, & Quirk, 2006). Further, the IL plays a critical role in the ability to discriminate between cues for danger and safety (Sangha, Robinson, Greba, Davies, & Howland, 2014), which is part of extinction learning. Thus, proper IL functioning is required for successful fear extinction consolidation and recall.

As it pertains for fear conditioning, the IL works primarily through its projections to the amygdala, a region of the brain critical for fear extinction memory consolidation and fear expression (Bennett et al., 2016). The IL sends projections to the intercalated cells of the amygdala (ITC), which have GABAergic projections onto the central amygdala (CeA), an important region for fear expression (Li, Amano, Pare, & Nair, 2011; Millhouse, 1986; Paré & Smith, 1993). IL inputs increase ITC activity (Berretta, 2005; Berretta, Pantazopoulos, Caldera, Pantazopoulos, & Paré, 2005). During fear extinction, the strength of the synapses from IL projecting neurons to the ITC GABAergic neurons increases, as IL burst firing increases predict the strength of extinction recall (Bennett et al., 2016; Burgos-Robles et al., 2007). Within the extinction context, the IL neurons excite the ITC interneurons, which then suppress CeA neuron activity and hence, fear expression (Knapska & Maren, 2009). Moreover, memory retrieval of fear extinction also relies on the IL projections to the ITC (Knapska et al., 2012). In parallel to the ITC pathway, the IL projects directly to the basolateral amygdala, producing downstream changes in BLA plasticity that further facilities extinction memory formation (Bloodgood, Sugam, Holmes, & Kash, 2018).

Consequently, the IL to amygdala circuits are clearly important in fear extinction circuitry.

Following chronic stress, the IL and amygdala circuit is perturbed. In the IL, chronic stress leads to a decrease in dendritic complexity and activity (Goldwater et al., 2009; Radley et al., 2005). Conversely for the basolateral amygdala, chronic stress leads to an increase in dendritic complexity and activity (Govindarajan et al., 2006; Johnson et al., 2009; Mitra et al., 2005; Vyas et al., 2006, 2002, 2004). The central amygdala appears to be unchanged in complexity or activity following chronic stress (Vyas et al., 2003), which suggests that upstream changes influence fear expression. The combination of a weaker inhibition circuit from the IL and a stronger fear consolidation circuit in the basolateral amygdala may lead to a system that favors overly robust fear memories, as observed with PTSD. However, when a delay occurs between the end of chronic stress and the start of the fear conditioning exposure, the IL regains its overall complexity and functionality (Goldwater et al., 2009; Radley et al., 2005). The improvements in the IL following a delay from chronic stress suggests that the inhibitory circuitry may have been strengthened and perhaps is responsible for the improvement in fear extinction that was observed in Chapter 2.

The goal of Chapter 3 was to investigate whether the IL was responsible for the improved fear extinction in chronically stressed rats that had a rest period before fear conditioning commenced. Glutamatergic cells in the IL underwent long-term inactivation using designer receptors exclusively activated by designer drugs (DREADDs). Long-term inactivation in the IL occurred during the weeks following the end of chronic stress in an attempt to prevent dendritic or functional recovery in the IL that may be responsible for

the improvements in context discrimination seen in the post-stress rest rats in Chapter 2. Following recovery from the stereotaxic infusion of DREADDS into the IL, rats underwent chronic stress with or without a post-stress rest period and were then exposed to fear conditioning and extinction as in Chapter 2. Though the goal was to target the post-stress rest modification in the IL, all stress groups had DREADDs infused into the IL and all rats received the activating ligand, clozapine-n-oxide (CNO). Following the chronic stress manipulation, all rats underwent fear conditioning and extinction. Importantly, the IL was not perturbed during behavioral testing, as CNO was not administered on behavioral testing days. Long-term inactivation of the IL leading up to but during fear conditioning altered behavior in chronically stressed rats with a rest period and in non-stressed controls, but not in chronically stressed rats tested soon after chronic stress ended. In the chronically stressed rats with rest period, long-term inhibition of the IL led to a failure to form a tone-foot shock association. In contrast, long-term inhibition of the IL in control rats led to high freezing to context in the early extinction trials, suggesting less discrimination between fear and safety cues. Ironically, the behavioral phenotype of the control rats showed extinction behavior that was similar to the behavior displayed by recently stressed rats, as reported in chapter 2. Consequently, the results of Chapter 3 provide some support for a role of the IL in the early stages of safety signal recognition and indicates that the IL might have a role in fear learning, not just in its traditional role in fear extinction.

CHAPTER 2

CHRONIC STRESS HAS LASTING EFFECTS ON IMPROVED CUED DISCRIMINATION EARLY IN EXTINCTION

Currently under revision for Learning and Memory

Post-traumatic stress disorder (PTSD) affects 6-9% of the U.S. population and nearly 40% of those exposed to extreme trauma, such as combat veterans (Hoge & Warner, 2014; Sareen, 2014). PTSD is characterized by persistent memories of the traumatic event, avoidance of potential triggers for memories of the traumatic event, and hyperarousal (American Psychiatric Association, 2013). Although the ability to form a fear memory is necessary for long-term survival and danger avoidance (Boissy, 2004), individuals with PTSD exhibit maladaptive and overly robust fear memories that can be debilitating (Milad et al., 2008).

A history of chronic stress is a risk factor for the development of PTSD in humans (Breslau et al., 1999; Sareen, 2014) and can create a PTSD-like phenotype in rodent models when chronic stress is paired with an aversive event (Daskalakis et al., 2013). The behavior of chronically stressed rats, as it pertains to fear conditioning, parallels observations from PTSD patients, including robust fear memories (American Psychiatric Association, 2013; C. D. Conrad et al., 1999; Cordero et al., 2003; Hoffman et al., 2015; Yehuda & LeDoux, 2007), generalization of fear responses to safe environments (Blechert et al., 2007; Hoffman et al., 2014; Radulovic et al., 1998), and resistance to extinction-based therapies (Blechert et al., 2007; Milad et al., 2009) or extinction training (Baran et al., 2009; Hoffman et al., 2014; Izquierdo et al., 2006; Miracle et al., 2006; Rau

et al., 2005). Consequently, chronically stressed rodents tested on fear conditioning paradigms may provide unique insights into the neurobiology of PTSD (Bryant et al., 2008).

A feature of cognitive outcomes following chronic stress is that the passage of time can modulate the influence of chronic stress on certain cognitive processes. For example, when behavioral testing occurs within days after chronic stress has ended, spatial memory is compromised (Abidin et al., 2004; Bowman et al., 2002; Ghiglieri et al., 1997; Kleen et al., 2006; Luine et al., 1994; Ortiz et al., 2015; Song et al., 2006). However, when chronic stress ends and a rest period ensues, spatial abilities rebound and may even improve above and beyond those of their non-stressed counterparts (Bian et al., 2012; Conrad, Ortiz, & Judd, 2016; Hoffman et al., 2011; Luine, Villegas, Martinez, & Mcewen, 1994; Ortiz et al., 2015). In contrast, chronic stress leads to elevated anxiety (Chiba et al., 2012; D'Aquila, Brain, & Willner, 1994; Eiland & McEwen, 2012; Huynh, Krigbaum, Hanna, & Conrad, 2011; Vyas et al., 2004), which is often maintained weeks after the termination of chronic or acute stress (Adamec & Shallow, 1993; van Dijken et al., 1992; Vyas et al., 2004). Consequently, time elapsed from the last stressor may differentially influence a range of cognitive and emotional processes.

Stressful events that might precipitate PTSD typically produce symptomology long after the stressful experience has ended (Schnurr, Lunney, & Sengupta, 2004; Xue et al., 2015). Consequently, investigating fear memories at time points further removed from the stressful event may be clinically relevant. Studies that have investigated a delay between the cessation of a stressor and the commencement of fear conditioning, using a single prolonged stressor or a chronic variable stressor a week before fear conditioning

and extinction testing commenced (Knox et al. 2012; McGuire et al. 2010a), show that a week after the end of the stressor manipulation, rodents resisted fear extinction. It is unknown whether this resistance to fear extinction persists weeks after the stressor has ended, but this is important to understand, considering the potential improvements that occur for spatial ability when three or four weeks elapses after chronic stress has ended (Bian et al., 2012; C. D. Conrad et al., 2017; Hoffman et al., 2011; Ortiz et al., 2015; Sousa et al., 2000). Consequently, the present study compares rats that had been recently chronically stressed with those that were remotely stressed three- or six- weeks prior to fear conditioning (termed "stress-rest"), to determine whether the longer gap between the end of chronic stress and the start of fear conditioning is an important factor in the maintenance of robust fear memories.

Materials and Methods

Subjects

Male Sprague-Dawley (Charles River Laboratories) rats weighing approximately 250 g upon arrival were pair housed in standard laboratory cages (21-22 °C, corncob bedding). Except where noted below, animals were allowed food and water *ad libitum*. Animals were housed on a reverse 12:12 light cycle, with lights off at 7AM. All procedures occurred during the dark phase of the light cycle and were performed in accordance with the Guide for the Care and Use of Laboratory Animals and the approval of the Arizona State University Institutional Animal Care and Use Committee.

Chronic Stress Procedure

Rats were chronically stressed by restraint for 6 hours/day for 21 days. Our previous work demonstrated that these restraint parameters were the minimum required duration for restraint stress to produce behavioral and structural changes (McLaughlin et al., 2007). Restraint took place between 9AM and 3PM and occurred in the animal's home cage. Sound-attenuating chambers were used to isolate animals undergoing restraint separated from animals not undergoing restraint. To keep food and water access similar between groups, the control group had their food and water removed during restraint hours. Additionally, control rats were handled at the start of each day to keep daily handling by the investigator consistent. Animals were initially restrained using a wire mesh tube (6.4 cm DIA × 26.7 cm L) that was made using grip guard sealer (Flynn and Enslow, San Francisco, CA) to keep the wire ends coated, but were upgraded to a larger restrainer (7.6 cm DIA × 29.2 cm L) as the rats grew. Body weights were recorded weekly to confirm stressor effectiveness.

Group Assignments and Timeline

In Experiment 1, rats were assigned to one of four groups (n=10/group, 40 rats total): control (CON), chronic stress with a six-week rest period (STR-R6), chronic stress with a three-week rest period (STR-R3), or chronic stress without a rest (i.e., tested within days or immediately, STR-IMM). Training on fear conditioning occurred six weeks (STR-R6), three weeks (STR-R3), or within days (STR-IMM) from the last day of restraint. The three- and six-week rest durations were selected because some behaviors, such as spatial ability, improve three weeks after chronic stress has ended (Bian et al.,

2012; Conrad, Ortiz, & Judd, 2016; Hoffman et al., 2011; Luine, Villegas, Martinez, & Mcewen, 1994; Ortiz et al., 2015), although anxiety may stay elevated (Mikics, Baranyi, & Haller, 2008). For Experiment 2, two stress groups were used, STR-IMM or STR-R6, and rats were further classified based on their conditioning environment for a total of four groups (n=8/group, 36 rats total). In Experiment 3, three stress groups were used, CON, STR-IMM and STR-R6, and rats were further classified based on whether they underwent second-order conditioning or not for a total of six groups (n=8/group, 48 rats total).

Fear Conditioning

Fear conditioning apparatus. Rat test cages were square and made of metal and plastic (30.5 cm W x 25.4 cm D x 30.5 cm H: Coulbourn Instruments, E10-18TC or H10-11R-TC) and were modified so that the top metal panel was replaced with clear Plexiglas for video recording. Both arenas were housed within a purchased sound-attenuating cabinet (Coulbourn, E10-23, white, 78.7 cm W x 53.3 cm D x 50.8 cm H) or a custom-made sound-attenuating cabinet (63.5 cm W x 61.0 cm D x 71.1 cm H: Melamine boards). Auditory tones (75 dB steady tone, 20 sec) were delivered through a speaker (Coulbourn, H12-01R) mounted on the inside of the sound-attenuating cabinet and were produced by a frequency generator (Coulbourn, E12-01 or H12-07). An animal shock generator (Coulbourn, H13-15) administered the shocks (0.8 mA, 1 sec) through a shock grid floor (Coulbourn, E10-18RF or H10-11RTC-NSF), with current equally distributed between parallel metal bars. Illumination was provided throughout testing by LED light

bulbs in porcelain lamp-holders (Pass & Seymour, Legrand) mounted to the ceiling of the isolation cubicles.

All stimuli were controlled using Graphic State software (v 4.0 GS4-UP). Graphic State was installed on a Dell computer (3.19GHz, Intel i5 CPU, 64 bit) running Windows 7 Enterprise (2009, Microsoft Corp.). The computer was connected to a linc system (Coulbourn, H02-08) that controlled the stimuli output via an USB interface (Coulbourn, U90-11H). Infrared lights (Coulbourn, H27-91R) were positioned to be observed by the video and were programed to denote the context and tone. The infrared lights could not be visually detected unless viewed on video.

Behavioral quantification. All behavior was digitally recorded on GoPro Hero 3 cameras (GoPro, Inc.) for offline analysis. Video from the GoPro cameras were monitored using a Quad Splitter Processor (Evertech), which allowed four videos to be viewed on one monitor (Samsung, 24”). The behavior from eight single chambers that were viewed on two monitors was also backed up on a VCR/DVD recorder (Funai). Behavior was manually scored by a trained observer. Freezing was defined as the lack of all movement, except those associated with respiration (Blanchard & Blanchard, 1969). Freezing to tone was defined as any freezing that took place during the 20 sec tone presentation and freezing to context was defined as any freezing that took place in the 20 sec immediately prior to the presentation of the tone. A fear conditioning difference score was calculated in order to assist in understanding how much of the freezing to the tone was due to associative processes over a more generalized, non-associative freezing response that may occur in the absence of the discrete cue. This was calculated as the amount of freezing to tone minus the amount of freezing to context 20 sec prior to the

tone (similar to Majchrzak et al. 2006). Inter-rater reliability was 97.3±6.4% and intra-rater reliability was 95.7 ± 2.0%.

Environments for fear conditioning procedures. Over the course of the three experiments, three different contexts were used. In one context, the testing cages were square metal and plastic and had a metal floor of parallel rods (Coulbourn, H10-11R-TC-SF), silver side panels (Coulbourn, H90-00R-M-KT01), and black and white striped panels on the clear plastic back wall. The sound-attenuating cabinet contained a 40-Watt equivalent LED bulb (450 Lumens; Osram Sylvania, Inc.) and a white-lit LED computer fan (Thermaltake, CL-F020-PL12WT-A or Coulbourn, ACT-130). The cleaning solution used after each rat was an all-purpose, grapefruit scented cleaner (Method, Inc.) and the room lighting of the overall holding room was white light. Experimenters wore a yellow wrap gown and black gloves. Rats were transported from the colony room to the testing room by hand-carrying the rats in their home cages. For a second context, the testing cages were round, plastic blue buckets (37 cm H x 30.5 cm DIA, Lowes). A 3-Watt, Red LED bulb (91 Lumens; Feit Electric) was used as illumination in the isolation cubical. A 35.6 cm, computer fan with red LED light (Thermaltake, TT-1425) provided white noise/ventilation in the cubicle. The cleaning solution used to clean the apparatus after each session was 70% isopropyl alcohol (Vi-Jon, Inc.). Experimenters wore a white lab coat and blue gloves. The rats were transported from the colony room to the testing room in their home cages on a cart and the room lighting of the overall holding room was red light. For the third context, the testing cages were modifications of the square testing cages (Coulbourn, H10-11R-TC-SF). A black semi-circular Plexiglas insert was placed in the testing cage to produce a curve in the back. The exposed side panels were covered in

black plastic. Room lighting, transportation method, isolation cubical door positioning, chamber lighting, and computer fan used were the same as in the second context. The cleaning solution used after each session was an all-purpose pine scented cleaner (Method, Inc.).

Experimental Procedures

Experiment 1: Influence of a rest period following the end of chronic stress on fear extinction. Six days before the chronic restraint procedure ended for the last cohort of rats (STR-IMM), acclimation to the contextual environments commenced. The goal of the acclimation sessions was to reduce conditioning to the environments and decrease possibilities for generalization between the contexts allowing extinction processes to be studied without *a priori* differences in baseline freezing (Hoffman et al., 2014, 2015; Jacobs, Cushman, & Fanselow, 2010). Acclimation occurred approximately one hour after restraint ended each day so they would have ample opportunity to access food and water prior to acclimation sessions. Rats were acclimated by being placed in a context for 10 minutes daily. Exposure to the two contexts alternated over the six days for a total of three exposures to each context. The day after the last acclimation session (Day 7), fear conditioning training occurred in Context "A". Training consisted of three tone-foot shock pairings (inter-trial interval (ITI) range between pairings = 80-170 sec), with the first tone was presented after 114 sec. The training session lasted 535 sec. One and two days after training, rats underwent extinction training sessions in Context "B". Extinction training consisted of 15 presentations of the tone (ITI range=85-120 sec). Seven days after the second extinction session, rats were exposed to three more

presentations of the tone in Context B to assess spontaneous recovery (ITI range=90-120 sec).

Experiment 2: Comparison of STR-IMM with STR-R6 on context generalization. In Experiment 1, there were indications that STR-IMM might be generalizing their fear responses to the non-shock environment, so a second experiment to test for generalization was performed. STR-R6 was used as a comparison group due to low freezing to context seen in this group, and because a goal of Experiment 2 was to better understand the differences between the chronic stress groups. A non-stressed group was not included here because the comparison in generalization between CON and STR-IMM has been previously reported (Hoffman et al., 2014). Acclimation and fear conditioning occurred as described in Experiment 1, whereby rats were acclimated to both environments over six days and then fear conditioned (three tone and foot shock pairings) the following day in either Context "A" or "B", which were counter-balanced across groups. A day after fear conditioning, all rats were given three tone-alone presentations in Context B (ITI=320 sec). One day later, the rats were placed in a novel Context "C", where they had no prior acclimation experience and then presented with three tone alone presentations.

Experiment 3: Investigation as to whether STR-IMM is more likely to form second-order conditioning than CON or STR-R6. This experiment was done to test whether STR-IMM were more likely to form a second-order association between the tone and the extinction context compared to STR-R6 or CON. Acclimation occurred over six days as described in Experiment 1. One day after acclimation ended, fear conditioning occurred in Context "A". Training consisted of three tone-foot shock pairings as in

Experiment 1. One day after training, all the rats were re-exposed to Context A in the morning, with half of them receiving three-tone presentations (A^+/B). In the afternoon, all rats were exposed to Context "B", with the half that did not get exposed to the tone in the morning receiving three-tone presentations (A/B^+) for the second-order conditioning manipulation. This led to a 3 x 2 design for stress group (CON, STR-IMM, STR-R6) and second-order conditioning or not (A/B+, A+/B). The following day, all groups were tested for contextual freezing behavior in Context B.

Statistical Analysis

Results were analyzed primarily using an one-way Analysis of Variance (ANOVA), with stress group as the factor. Experiment 3 utilized a two-way ANOVA, with stress group and training history as factors. Results that were significant at the $p<0.05$ level were additionally analyzed using the LSD (least significant difference) post-hoc test. Rats were excluded from further analysis if freezing to context exceeded 25% of the total freezing prior to the first presentation of tone during training (i.e., before tone or foot shock presentation). Three rats were excluded from both Experiment 1 (1 CON, 2 STR-IMM) and Experiment 2 (1 STR-R6 B+/B, 1 STR-IMM A+/B, 1 STR-IMM B+/B). Please note that due to equipment malfunction, some data were lost in Experiment 3 to produce n=7 or 8/group (n=7 for STR-R6-A+/B for Training Day 2 AM and PM sessions, for STR-R6-A/B+ for Training Day 2 AM and Test Day, for STR-IMM-A+/B for Training Day 2 AM and PM sessions and Test Day, for STR-IMM-A/B+ for Training Day 2 AM and PM sessions, for CON-A+/B for Training Day 2 AM and PM sessions, and for CON-A/B+ for Training Day 2 AM session). To correct for unequal variances,

data was transformed using $\sqrt{x+1}$ (Tabachnick & Fidell, 2007). Data analysis was done using SPSS Version 24 on an Apple iMac running macOS Sierra (v 10.12.6).

Results

Experiment 1: Do Rats Resist Fear Extinction Three- or Six-Weeks After Chronic Stress Ends?

Summary. All rats were fear conditioned to three tone-foot shock pairings in training Context A and then extinguished to the tone (30 trials, 15 trials/day) in the non-shock Context B over the next two days (Figure 1A). On the first three trials of Extinction 1, chronically stressed rats given a post-stress rest period (STR-R3, STR-R6) froze less to Context B than did CON and STR-IMM, but all groups froze similarly to tone, which suggested a generalization of context fear by CON and STR-IMM. Consequently, a difference score was calculated to determine how much freezing to tone occurred relative to the non-shock Context B; STR-R3 and STR-R6 discriminated between tone and context better than CON and STR-IMM.

Specific results. Over the course of the two days of extinction, freezing to tone decreased and was nearly extinguished by the last trial (Figure 1B, mixed factor ANOVA for group (CON, STR-IMM, STR-R3, STR-R6) by bins (three trials/bin with 5 bins/day) revealed a significant effect of bin on Extinction 1 ($F(4,132)=17.513$, $p<0.001$) and Extinction 2 ($F(4,132)=25.312$, $p<0.001$) with no other significant effects). A week after extinction ended, spontaneous recovery was performed with three tone presentations in the non-shock context to determine whether the freezing was due to associative properties, which was confirmed. A return of freezing to tone was statistically similar

across groups, although STR-R3 seemed to have a tendency towards less recovery than the other groups (Figure 1C, 1-way ANOVA for freezing to the first tone presentation). Freezing to tone ranged from 29.8±10.1% to 59.8±11.8%.

The first three trials in Extinction 1 were investigated to understand the tone-shock memory prior to extensive extinction presentations; they revealed that groups performed similarly (Figure 1D). A group (CON, STR-IMM, STR-R3, STR-R6) x trial (1, 2, 3) ANOVA showed a significant effect of trial, $F(2, 66)=24.996$, $p<0.001$, with no other significant effects. Freezing to tone increased from Trial 1 to Trial 2 ($p<0.001$), and became statistically similar between Trials 2 and 3. Importantly, all groups performed similarly. Freezing to Context B prior to the first tone (20 s prior to the first tone presentation, i.e. baseline freezing to Context B) was assessed separately from subsequent context measures to determine whether any *a priori* differences existed before tone presentation. Freezing to context was similar for all groups (Figure 1E, 1-way ANOVA for freezing to Context B, Baseline/Trial 1) and was relatively low, ranging from 12.7 ± 10.0 to 19.3 ± 12.9%. For subsequent Trials 2 and 3, rats given a rest after the end of chronic stress froze less to Context B than did STR-IMM or CON (mixed factor ANOVA for group (CON, STR-IMM, STR-R3, STR-R6) by trials (2, 3) showed significant effect of group $F(3, 33)=3.991$, $p<0.05$, with no other significant effects). Specifically, STR-R3 froze significantly less to Context B than did STR-IMM ($p<0.05$) or CON ($p<0.05$) and that STR-R6 froze significantly less to Context B than did STR-IMM ($p<0.05$) and froze less to Context B than did CON, but the difference was not statistically significant ($p=0.07$). This suggests that, compared to STR-IMM and CON, both STR-R3 and STR-

R6 froze less to a context that never involved foot shock and hence, could be considered a "safe" context.

To understand how much freezing to the tone was due to associative processes over the more generalized freezing responses occurring in the absence of a discrete cue, a fear conditioning difference score was computed and analyzed. The difference score in Trial 1 was much higher ($p<0.001$) than in Trials 2 and 3 (Figure 1F, mixed factor ANOVA for group (CON, STR-IMM, STR-R3, STR-R6) by trial (1, 2, 3) on the difference scores showed a significant effect of trial, $F(2, 66)=28.631$, $p<0.001$, which was followed by a 1-way ANOVA for the difference score in the first trial). The groups were statistically similar and showed high freezing to the tone over the context during Trial 1 (lowest difference score = 43.8±12.8%, highest difference score = 53.9±8.6%). This indicated low Baseline freezing to Context B in Trial 1. For Trials 2 and 3, however, a mixed factor ANOVA for group (CON, STR-IMM, STR-R3, STR-R6) by trial (2, 3) on the difference score, revealed a significant main effect of group, $F(3, 33)=5.557$, $p<0.05$, but no significant trial or interaction. Stress groups given a rest period after chronic stress, STR-R3 and STR-R6, froze selectively to tone over the non-shock Context B compared to STR-IMM ($p<0.05$) and CON ($p<0.05$), suggesting that they were better at learning that Context B was safe compared to STR-IMM or CON.

Experiment 2: Comparison of STR-IMM with STR-R6 on Context Generalization

In Experiment 1, an extended acclimation paradigm was implemented to reduce contextual conditioning and, consequently, reduce generalization between contexts. However, the STR-IMM group, but not the STR-R3 or STR-R6 groups, froze similarly to

the tone and the non-shock context, suggesting that STR-IMM had facilitated generalization of fear conditioning. Consequently, Experiment 2 was performed to test for differences in context generalization between STR-IMM and STR-R6.

As in Experiment 1, STR-IMM and STR-R6 were acclimated to Contexts A and B for six days and then underwent fear conditioning (3 tone/foot shock trials; Supplemental Figure 2). Half the rats were fear conditioned in Context A and the other in Context B (which were counterbalanced between groups). On the next day, rats were presented with three tones without foot shock in Context B, which gave rise to rats being trained and then tested in different contexts (A+/B) or in the same context (B+/B), Figure 2A.

Whether trained and tested in a different (A+/B) or in the same (B+/B) context, STR-IMM froze more to tone and context in the first trial than did STR-R6 (Figure 3B and 3B insert for tone, significant stress group by trial interaction, $F(2, 50)=5.304$, $p<0.05$, and significant trial effect, $F(2, 50)=5.304$, $p<0.05$; Figure 2C and 2C insert for context, stress group on Trial 1 before tone presentation (Baseline freezing to Context B), $F(1,25)=4.638$, $p<0.05$). Also, freezing to tone decreased as trials progressed for all groups (Figure 3B).

When the freezing to Context B was subtracted from the freezing to tone, no group differences were detected and average difference scores were above chance levels (Figure 2D). These data suggest that STR-IMM and STR-R6 discriminated between tone and context similarly, and that training context (A+/B or B+/B) did not influence tone-context discrimination. Consequently, the high freezing to tone and to Context B by STR-

IMM was indicative of a potentiated freezing response and not necessarily attributed to generalization.

On the next day, rats were placed into a novel context that was unfamiliar to them, in order to ascertain freezing in a non-acclimated, non-shock environment (see timeline in Figure 2A). Rats were presented with the tone cue (no shock) three times. Whereas the groups showed similar freezing to the tone across the trials (data not shown), differences were observed in Baseline freezing to Context C, prior to the first tone presentation (Figure 2E).Specifically, rats trained and tested in the same training condition (B+/B), froze significantly less to the novel Context C than did the rats who were trained and then tested in a different context (A+/B); stress history (STR-IMM, STR-R6) did not modify the outcome (Figure 2F, significant training context, $F(1,25)=4.762$, $p<0.05$). After the presentation of the tone, freezing to the novel Context C increased and was similar across all groups and across the remaining two trials ($F(2, 50)=11.989$, $p<0.001$), without any other significant main effects or interactions.

Experiment 3: Are STR-IMM Showing Second-Order Conditioning During Extinction?

Compared to STR-R6, STR-IMM showed heightened fear responses early in extinction in Experiment 1 and fear generalization was excluded as a possible interpretation in Experiment 2. Another possible explanation for the more robust fear responding in STR-IMM is that they more readily formed a strong second-order tone-context association, which is most likely to happen when the original tone-shock association is strong. Indeed, individuals with PTSD are more likely to form second-order

associations between trauma-related and neutral cues (Wessa & Flor, 2007). In Experiment 3, we tested for second-order conditioning with context as the second-order cue (Figure 3A).

On Day 1, rats (CON, STR-IMM, STR-R6) underwent fear conditioning (first-order) as described in Experiment 1 (Supplemental Figure 3A & B). On the next day, rats were returned back to Context A, the environment where they had been exposed to tone and foot shock. Half the rats were presented with three tones and the other half exposed to Context A without tones. Approximately 4 hours later, the rats were brought to Context B, which was used as the second order cue. The rats exposed to Context A earlier without tone presentations were now presented with three tones in Context B, while the remainder who received tones earlier in Context A were placed in Context B without the tone presentations. Therefore, all rats had equal exposure to both contexts and tones, but with different pairings of tone and environment: rats that received tone only presentations on Day 2 in the same context as training were designated as A+/B and those that received the second-order conditioning paradigm on Day 2 with tone in Context B were designated as A/B+. Rats in the A/B+ showed similar and high freezing to Context B by the end of the second-order conditioning session (1-Way ANOVA for freezing to Context Trial 3, $F(2,21)=0.668$, $p=0.524$; Supplemental Figure 3C). On the third day, rats were returned to Context B to assess their potential for second order conditioning.

When freezing to Context B was assessed for second-order conditioning on Day 3, groups trained in the A/B^{+} order showed more freezing to Context B than did rats trained in the A^{+}/B order, but stress history did not modify performance (Figure 3B, A 3 x 2 ANOVA for group (CON, STR-IMM, STR-R6) by training history (A/B+, A+/B) for

freezing to Context B showed a significant effect of training history, $F(1,44)=24.536$, $p<0.001$, with no other significant effects).

Discussion

The goal of this study was to investigate whether chronically stressed rats provided with a post-stress rest period would show facilitated fear extinction learning compared to chronically stressed rats without a rest period. We used an extended acclimation model (6 days) to the training and testing contexts because it leads to similar fear conditioning acquisition across groups (Hoffman et al., 2015). Consequently, differences in learning were minimized, allowing us to focus on extinction processes. During the early extinction trials in Experiment 1, the two chronically stressed groups given a post-stress rest period (STR-R3 and STR-R6) displayed lower freezing to the non-shock context than did non-stressed rats or recently chronically stressed rats. When the amount of freezing to the context was subtracted from the amount of freezing to the tone, both groups of chronically stressed rats with a post-stress rest period consistently demonstrated positive and high difference scores early in extinction during day 1 compared to non-stressed rats or recently chronically stressed rats. This reveals that early in extinction, both groups of chronically stressed rats with a post-stress rest period were better able to discriminate the tone from the context by freezing less to the context that never included a foot shock than did non-stressed rats or recently chronically stressed rats. As trials progressed, all groups showed similar extinction rates later on Day 1 and throughout Day 2.

Interestingly, the recently chronically stressed group appeared to freeze similarly and robustly to tone and context during the first few trials of extinction, suggesting a generalization of fear across contexts. Consequently, Experiment 2 was performed to assess potential context generalization by testing recently chronically stressed rats in an environment that differed (A+/B) or was the same as (B+/B) the conditioning environment, using chronically stressed rats with a six week rest period (STR-R6) as a comparison. The results revealed that recently chronically stressed rats discriminated between tone and context, but showed higher freezing to both tone and contexts (regardless of whether or not the context was associated with *a priori* exposure to foot shocks) than did chronically stressed rats with a rest period. Taken together, the recently chronically stressed group may be exhibiting higher freezing, reflecting hypervigilance, but not necessarily higher freezing due to generalization under this extended acclimation paradigm.

Another possible explanation for the heightened freezing to context in the non-shock context in Experiment 1 was that recently chronically stressed rats readily formed second-order associations with fear-related cues. Specifically, we hypothesized that the previously conditioned cue (tone), would be associated with the context in which it is presented, such that the context would then be indirectly associated with the aversive event (Gewirtz & Davis, 2000; Rizley & Rescorla, 1972). Indeed, results from human studies suggests that second-order conditioning in a safe context contributes to the maintenance of responding to trauma-related cues (Wessa & Flor 2007). In Experiment 3, we demonstrated that, when presented with the tone (the first-order conditioning stimulus) in a non-shock environment, the non-shock environment functions as a second-

order conditioning cue for recently chronically stressed rats. However, recently chronically stressed rats, chronically stressed rats with a six week rest period, and non-stressed rats all performed similarly. Our results did not support the idea that second-order conditioning contributed to the more robust freezing responses in recently chronically stressed rats. We suspect that instead of solely achieving second-order conditioning, we might have also encountered some other compound-stimuli phenomena, such as latent inhibition (discussed next, see also (Brembs & Heisenberg, 2001; Rauhut, Mcphee, & Ayres, 1999; Urcelay & Miller, 2009).

Latent inhibition is a phenomenon in which a neutral cue is paired with a meaningful stimulus, leading to two competing interpretations, neutral or meaningful. As it pertains to the current study, rats received extensive non-reinforced exposure to the non-shock context B during acclimation, and so context B likely had a "neutral" or "safe" meaning (see Fig. 1E, baseline, low freezing to Context B prior to the introduction of the first tone). Consequently, when the shock-paired tone was introduced to context B, the rat freezing response to context B may have reflected a combination of information: the previous information that context B was neutral and the new information coming from a predictive tone. In this view, freezing during Trials 2 and 3 of Extinction may have reflected the combination of an inhibitory response to Context B and an excitatory response to the tone, a test of which would be similar to a summation test for latent inhibition (Foilb, Bals, Sarlitto, & Christianson, 2018; Rescorla, 1969). While we did not explicitly test for latent inhibition, it is possible that the strength of latent inhibition was enhanced in chronically stressed rats provided with a rest period. Future studies should investigate the degree to which latent inhibition is able to control behavior in chronically

stressed rodent models, as this has important clinical implications for patients with PTSD (Grillon, Morgan, Davis, & Southwick, 1998; Jovanovic et al., 2012).

A defining feature of PTSD is a failure to recognize or appropriately respond to safety signals, cues that should indicate safety even in the presence of a trauma related cue (Grillon et al., 1998; Jovanovic et al., 2012). Extinction training, which here consisted of the shock-paired tone presented in a non-shock environment, should lead to a suppression of the fear response to the tone because of the new association that the tone in this environment does not predict shock. As such, the non-shock environment becomes a safety signal for when shock will not occur (Rothbaum & Davis, 2003). In the present study, we found that recently chronically stressed rats froze to the "safe" context in extinction more than chronically stressed rats with a rest period of three or six weeks, as seen in the first three trials of extinction in Experiment 1 and during the first context of Test 1 in Experiment 2. Perhaps the recently chronically stressed group had difficulty in identifying the safety signals as quickly as the chronically stressed rats with a rest period cohorts. A similar argument would suggest that chronically stressed rats with a rest period were better able to learn safety signals because of their lower freezing levels during extinction sessions. Future work should continue to investigate the behavioral mechanisms that lead to better, more flexible outcomes in the aftermath following chronic stress.

Taken together, the freezing responses to tone reflected associative learning in the present study. In Experiment 1, all groups showed spontaneous recovery seven days after the last extinction session, with freezing to tone ranging from 32.4 ± 9.8% to 52.7 ±10.7% in the first two trials. Moreover, groups showed statistically similar freezing to

the tone during spontaneous recovery. The return of freezing response in chronically stressed rats with a three week rest period showed a tendency to be less robust than the other groups, but this was unlikely to be attributed to *a priori* differences, as all groups showed similar and low levels of freezing by the end of Extinction 2. Consequently, the freezing to tone during the spontaneous recovery session likely reflected associative processes, as opposed to carry-over effects from extinction or non-associative effects (Ji & Maren, 2007). Additional supporting evidence was that, in Experiment 1, the freezing response was nearly absent in all groups prior to the first tone presentation in extinction using a non-shock context (i.e., baseline freezing), in which foot shock would be least expected. After the tone was presented in the non-shock context, freezing subsequently increased and this phenomenon was replicated in Experiment 2 with the two different contexts. Hence, this evidence suggests that freezing to tone reflected an association formed between tone and foot shock during conditioning.

This study is one of the first to investigate fear extinction processes based upon the timing from the end of chronic stress. In a prior study, rats given a single prolonged stressor and then tested for fear conditioning and extinction seven days later show poor retention of fear extinction (Knox et al., 2012). Another report used chronic variable stress and then tested rats on fear conditioning and extinction seven days later and found resistance to fear extinction (McGuire et al., 2010). The present study adds to this literature and revealed that chronically stressed rats given a three- or six-week rest period after chronic stress ended, show fear extinction that differs from either controls or stressed rats tested soon thereafter. Our results support the interpretation that exposure to

a traumatic event three- or six-weeks after a chronic stress history leads to a different fear extinction profile than had a traumatic event occurred soon after chronic stress ended.

A possible interpretation for the chronically stressed rats with a rest period improved discrimination of the conditioned tone from context is an inoculation effect. For example, stressor exposure early in life can lead to less anxiety and better cognitive flexibility when faced with stressors later in life (Katz et al., 2009; Lyons et al., 2009, 2010). In the present study, when young adult rats were chronically stressed, they demonstrated better discrimination between a conditioned cue and the safe context when they were permitted three- or six-weeks of rest following the end of chronic stress, compared to rats tested soon after stress ended. A similar finding has been observed for spatial memory in that a rest period following the end of chronic stress leads to better performance compared to a delay of a few days (Hoffman et al., 2011). This suggests that, under some circumstances, an earlier chronic stress experience can be helpful in navigating a later stressful experience.

A significant amount of PTSD patients do not seek therapy until much later after the traumatic event, giving the traumatic memory a chance to strengthen (Bryant, 2017b). Indeed, symptoms must be present for at least one month before PTSD can be diagnosed (American Psychiatric Association, 2013). In the present study, however, extinction training occurred in the days after fear conditioning (i.e., the traumatic experience). This was performed because it allowed for early assessment, as some patients exhibit PTSD-associated symptoms immediately after a traumatic event, called Acute Stress Disorder, or ASD (American Psychiatric Association, 2013). If left untreated, ASD could become PTSD (Bryant, 2017a). Our current study suggests that it is possible that early extinction

therapies in individuals showing ASD symptoms might lead to reduced PTSD rates. This is supported by the observation that the recently chronically stressed group was able to eventually extinguish their elevated freezing to the cue tone and context to a similar level as the other groups as extinction continued. Further supporting evidence is that recently chronically stressed performed similarly to the rest of the conditions during the measurement of spontaneous recovery. Future studies should investigate fear responding to extinction in weeks after fear conditioning to determine whether early extinction therapies could inoculate against PTSD.

The results of the present study suggest that when contexts are familiar, such as with our paradigm that included an extensive acclimation prior to training, chronically stressed individuals perform differently during the initial fear extinction acquisition process, depending upon whether extinction occurs soon after chronic stress ends or after a rest period. Chronically stressed individuals soon thereafter exposed to fear extinction discriminated between the conditioned stimulus and safe environment, but exhibited high freezing, perhaps due to being hypervigilant. Follow-up studies ruled out the possibility that chronically stressed subjects were generalizing or forming a stronger second-order context-tone association than the other groups. When chronically stressed individuals were exposed to fear extinction after a three- or six-week rest period, they also discriminated between the conditioned stimulus and safe environment, but without showing hypervigilance. Typically, clinical populations with PTSD show heightened responses to trauma related cues and impaired improvement with extinction-based therapies. In the present study, the stress group that best modeled these PTSD-like characteristics was the recently chronically stressed group, which displayed more

freezing to context during extinction training than either the chronically stressed with a rest period groups. This suggests that the time between chronic stress and the trauma exposure is a factor that may influence PTSD development.

CHAPTER 3

INHIBITING GLUTAMATERGIC NEURONS IN THE INFRALIMBIC CORTEX DISRUPTS ASSOCIATIVE LEARNING IN THE WEEKS FOLLOWING CHRONIC STRESS IN RATS, BUT ALTERS SAFETY LEARNING IN NON-STRESSED CONTROLS

The formation of fear memories is necessary for long-term survival (Boissy, 2004), but can become maladaptive. In a subset of individuals exposed to a trauma, memories may become overly robust and intrusive (American Psychiatric Association, 2013; Milad et al., 2008). The presence of overly robust fear memories following a traumatic event is known as Post-Traumatic Stress Disorder (PTSD) and occurs in about 6-9% of the general U.S. population and as many as 40% of those exposed to extreme trauma (Hoge & Warner, 2014; Sareen, 2014). Unfortunately, current therapies fail to be effective in the majority of cases (Bradley et al., 2005; Difede et al., 2014; Steckler & Risbrough, 2012; VanElzakker et al., 2014), necessitating additional research on mechanisms underlying PTSD to identify novel targets for intervention.

Chronic stress is a risk factor for the development of PTSD (Breslau et al., 1999; Sareen, 2014) and can be used to study PTSD in rodent models (Daskalakis et al., 2013). For both PTSD patients and chronically stressed rodents, the acquisition of fear memories is rapid and overtly robust (American Psychiatric Association, 2013; C. D. Conrad et al., 1999; Cordero et al., 2003; Hoffman et al., 2015; Yehuda & LeDoux, 2007). When fear memories are formed, PTSD patients often fail to show complete remission following treatment with extinction-based therapies, which involve the repeated presentation a

fearful stimulus in a safe environment (Blechert et al., 2007; Milad et al., 2009). Similarly, chronically stressed rodents commonly show impaired extinction learning, by resisting extinction to fearful stimuli (Hoffman et al., 2014; Izquierdo et al., 2006; Rau et al., 2005). When extinction does occur, chronically stressed rodents show impaired recall of extinction, in which the previously attenuated fearful responses return when presented with the aversive stimulus on another day (Hoffman, Armstrong, Hanna, & Conrad, 2010; Wilber et al., 2011). In addition, both PTSD patients and chronically stressed rodents demonstrate heightened anxiety (Grillon et al., 2009; Mikics et al., 2008; Vyas et al., 2004), which may manifest into anxiety generalization, which is when a safe environment or stimuli unassociated with the trauma now triggers the aversive response (Blechert et al., 2007; Hoffman et al., 2014; Radulovic et al., 1998). Consequently, chronic stress combined with fear conditioning parallel many of the characteristics described in PTSD patients (Bryant 2017) and can aid in parsing out the biological mechanisms underlying PTSD.

Recent research from our lab and others has investigated how the detrimental cognitive effects that occur following chronic stress can be time-dependent, in some cases. Numerous studies show that chronic stress leads to impairments in spatial memory (Abidin et al., 2004; Bowman et al., 2002; Ghiglieri et al., 1997; Kleen et al., 2006; Luine et al., 1994; Ortiz et al., 2015; Song et al., 2006), which requires the hippocampus (Moser, Moser, Forrest, Andersen, & Morris, 1995). However, when weeks pass between the end of the chronic stress manipulation and the start of spatial memory testing, spatial ability returns to "normal" or can be better than observed from unstressed control rats (Bian et al., 2012; Bloss et al., 2010; C. D. Conrad et al., 2017; Goldwater et al., 2009;

Hoffman et al., 2011; Luine et al., 1994; Ortiz et al., 2015; Radley et al., 2005; Sousa et al., 2000). We recently investigated whether a temporal component between the end of chronic stress and the start of behavioral testing also impacts fear extinction learning. Lower fear responses during extinction were observed in chronically stressed rats provided with a delay after stress ended and before the start of a fear conditioning paradigm compared to recently stressed or control rats (Chapter 2 of this Dissertation). This finding suggested that chronic stress' impact on fear extinction might be time-dependent; with fear extinction performance having the potential to improve in the weeks following the end of chronic stress. Consequently, chronically stressed rats that are cognitively tested after a delay from the end of chronic stress may represent a distinct phenotype from chronically stressed rats tested soon after stress exposure or even from non-stressed control rats.

An important structure in fear extinction is the infralimbic cortex (IL) of the medial prefrontal cortex (mPFC). In lesion studies, permanent removal of the IL impairs fear extinction memories, but the acquisition of the fear memory itself is unperturbed (Milad & Quirk, 2002). Temporarily inhibiting the IL using pharmacological methods during fear extinction leads to impairments in fear extinction learning (Sierra-Mercado et al., 2010) and retrieval (Mueller et al., 2010), which demonstrate that even transient inhibition of the IL prior to fear extinction can lead to impairments that last through to recall of fear extinction, despite the IL being fully functional at recall. The IL-mediated extinction process is a form of new learning, requires IL protein synthesis (Burgos-Robles et al., 2007; Hugues et al., 2004; Santini, 2004) and IL neuronal activity (Burgos-Robles et al., 2007; Milad & Quirk, 2002; Wilber et al., 2011). The IL essentially acts as

break on the emotional responses (Bennett et al., 2016; Bloodgood et al., 2018; Knapska & Maren, 2009), thereby allowing safe and un-safe cues to be differentiated.

Chronic stress negatively impacts the IL, both structurally and functionally with a temporal component. Soon after chronic stress, neurons in the IL show dendritic retraction (Cerqueira, Mailliet, Almeida, Jay, & Sousa, 2007; Goldwater et al., 2009; Izquierdo et al., 2006) and a reduction of dendritic spines (Moench & Wellman, 2014). The reduction in dendritic complexity in the IL following chronic stress is accompanied by decreased neuronal activity (Goldwater et al., 2009; Wilber et al., 2011). Concurrently, behavioral tasks that require the IL, such as fear extinction and the recall of fear extinction are impaired following chronic stress (Baran et al., 2009; Holmes & Wellman, 2009; Oualian & Gisquet-Verrier, 2010), Importantly, the IL also shows time sensitive changes following the end of chronic stress: when animals are given weeks of rest following the end of chronic stress before structural or functional assessments are made, the phenotype differs than compared to those assessed soon after chronic stress has ended. Chronically stressed rats given time to rest before assessment show enhanced complexity of IL dendritic arbors and increased IL neuronal activity (Bloss et al., 2010; Goldwater et al., 2009; Radley et al., 2005). Interestingly, although overall IL dendritic complexity recovers in the weeks after chronic stress has ended, the organization of IL dendritic complexity differs than compared to those assessed in close proximity to the chronic stress manipulation; dendritic complexity was more proximal to the soma than prior to stress (Goldwater et al., 2009). To our knowledge, this is the first investigation of IL functional ramifications following a delay period after the end of chronic stress. Taken together, the changes in the IL in the weeks after chronic stress has ended may represent

a unique phenotype that differs from the IL of an individual soon after chronic stress has ended, as well as from an individual who had not undergone chronic stress. Consequently, we hypothesized that the changes in the IL during this period of rest or "recovery" period after chronic stress has ended were responsible for optimal fear extinction learning.

In the present study, the goal was to interfere with modifications that take place in the IL following the end of chronic stress to understand the role that such modification(s) might play to improve fear and safety cue discrimination. The IL glutamatergic neurons were chronically inactivated for three weeks following the end of chronic stress and then allowed to function when behavioral testing started. The functional inactivation of the IL was achieved by using designer receptors exclusively activated by designer drugs (DREADDs). The goal of this timing was to block any alterations that may take place in the IL during the period between the end of chronic stress and the start of fear conditioning. Importantly, the IL was online during fear conditioning and extinction, which allowed us to focus on the role of changes in the IL during the rest period from when stress ended and when fear conditioning was to start. Finally, we used an extended acclimation paradigm prior to the start of fear conditioning so that the rats would show similar acquisition of fear conditioning (Hoffman et al., 2014, 2015; Jacobs et al., 2010) so that we could study the effects on fear extinction. We predicted that long-term inactivation of the IL following the end of chronic stress would lead to higher fear responses during fear extinction compared to chronically stressed rats that did not undergo long-term IL inactivation.

Materials and Methods

Subjects

Male Sprague-Dawley rats (Charles River Laboratories, Hollister, CA) weighing between 225 and 250 grams upon arrival were housed with 2 or 3 conspecifics in standard laboratory cages (21-22 °C). Except where noted below, animals were allowed food and water *ad libitum*. Animals were housed on a reverse 12:12 light cycle; lights off at 7AM. All procedures occurred during the dark phase of the light cycle and were performed in accordance with the Guide for the Care and Use of Laboratory Animals and the approval of the Arizona State University Institutional Animal Care and Use Committee.

DREADD Viral Infusion Surgery

A week after arrival, all rats underwent stereotaxic surgery to target the IL of the mPFC using biosafety level 2 surgical precautions. Surgeries were performed under aseptic sterile conditions by two surgeons. Rats were anesthetized with a ketamine/xylazine/acepromazine cocktail (ketamine=95mg/kg BW (Dechra, United Kingdom), xylazine=5mg/kg BW (Akorn, Illinois), acepromazine=1mg/kg BW, purchased from ASU animal care) in sterile 0.9% sodium chloride (0.2ml/100gm BW, i.p.). Once the rats no longer responded to tail pinch, they were given 1mg/kg BW of Meloxicam (Putney, United Kingdom; i.p.,) an anti-inflammatory drug, and 0.03mg/kg BW buprenorphine (Par Pharmaceutical, New Jersey; s.c.), for pain management. Ketamine boosters were given as needed during surgery (0.25ml, i.p.). A dab of Puralube ophthalmic ointment was applied over the eye to prevent eye dryness. Rats were secured

in a stereotaxic apparatus (David Knopf Instruments, Tujunga, CA). Rats received a local anesthetic s.c. injection of bupivacaine (2.5mg/mL, Pfizer) at the incision site to further manage pain. The rats' heads were next scrubbed 3x with alternating betadine (Perdue Products) and isopropyl alcohol (Vi-Jon, Inc.). A drape cloth was placed over the surgical area. An incision on the skin was made along the midline from around bregma to lambda. Connective tissue was cleared away and the head leveled to zero. Dedicated glass Hamilton syringes with a blunt tip (5μL; National Scientific Company, Rockwood, TN) were used for infusions. Two injections were given, one injection per hemisphere of 0.5μL per side. The targeted coordinates to the IL of mPFC from bregma and skull surface were A/P: +2.7; M/L: +0.5, -0.65; D/V: -5.2 (Paxinos and Watson 1997). The duration of the infusions was 2 to 3 minutes to allow the virus to spread and then syringes were left in place for at least another two mins after infusion was completed. When the second infusion was completed, the incisions were secured using coated vicryl sutures (Ethicon, Somerville, NJ). The area was cleaned with sterile water and then swabbed with triple antibiotic cream. Rats were placed in an empty cage over a heating pad until they awoke. Rats were individually housed for at least three days or until the scalp incisions healed. During post-operative care, rats received meloxicam and buprenorphine on post-surgery days 1 and 2. In some cases, rats had to be re-sutured during the post-operative period, prior to being re-pair housed. All rats had at least a week of recovery prior to the start of chronic stress. There was a 96% survival rate for surgery ($n_{\text{prior to surgery}}=72$, $n_{\text{survived surgery}}=69$).

Designer Receptors Exclusively Activated by Designer Drugs (DREADDs)

The following viral vectors were purchased from Addgene (Watertown, MA): active DREADD virus AAV8-CamKIIα-hM4Di-mCherry (Addgene #50477) and control virus AAV8-CamKIIα-EGFP (Addgene #50476). Vectors incorporating CaMKIIα as the promoter were chosen to allow for selective expression in cortical glutamatergic excitatory neurons (Liu, Jones, Mountcastle, & Hopkins, 1996). The volume injected was 0.5 µl per side. Virus titers were 3×10^{12} vg/mL. Clozapine-n-oxide (CNO) was obtained from the NIH Chemical Synthesis and Drug Supply Program (Batch 14073-1) and used to activate the DREADDs in those rats that received the active virus via daily injection at a dose of 1mg/kg BW, i.p.). Rats that received the control virus were also given CNO daily injection (1mg/kg BW, i.p.) to control for possible non-specific actions of CNO (Gomez et al., 2017; Mahler & Aston-Jones, 2018).

Chronic Stress Procedure

Rats were chronically stressed by restraint for 6 hours/day for 21 days. Our previous work demonstrated that these restraint parameters were the minimum required duration for restraint stress to produce behavioral and structural changes (McLaughlin et al., 2007) and were consistent with our past work on fear conditioning (C. D. Conrad et al., 1999; C. D. Conrad, Mauldin-Jourdain, & Hobbs, 2001). Restraint took place between 9AM and 3PM and occurred in the animal's home cage. Sound-attenuating chambers were used to isolate animals undergoing restraint from animals not undergoing restraint. To keep food and water access similar across treatment conditions, the control group had their food and water removed during restraint hours. Additionally, control rats

were handled at the start of each day to keep daily handling by the investigators consistent. Animals were initially restrained using a wire mesh tube (6.4 cm DIA × 26.7 cm L) that had the ends sealed using grip guard sealer (Flynn and Enslow, San Francisco, CA) to keep the wire ends coated. Rats were upgraded to a larger restrainer (7.6 cm DIA × 29.2 cm L) as they grew. Body weights (BW) were recorded weekly.

Groups and Timeline

Rats were assigned one of three stress groups. Rats in the stress immediate group were chronically stressed and underwent fear conditioning and extinction immediately (i.e., within days) after restraint ended (STR-IMM). Rats in the stress-rest group were chronically stressed and then had a three-week break or rest period without restraint prior to the start of fear conditioning and extinction (STR-R3). The control group did not undergo a stressor manipulation prior to fear conditioning and extinction (CON). During the three-week break for STR-R3, which overlapped with the stressor manipulation for STR-IMM, all rats (CON, STR-IMM, STR-R3) were given daily injections of CNO (1mg/kg BW, i.p.) for chronic activation of the DREADDs in those with the active virus ("active"). Those with the control virus (abbreviated GFP for its reporter) also received CNO to rule out any possible confounding effects of CNO actions (Gomez et al., 2017; Mahler & Aston-Jones, 2018). See timeline figure (Figure 4).

In summary, there were six total groups; three stress groups (CON, STR-IMM, STR-R3) and two virus types (GFP, active), creating a 3 x 2 design. Each stressor group had the control and active DREADD viral vector subgroups, leading to the final numbers of 8-14 rats per group at the start of the fear conditioning procedure.

Behavioral Testing

Fear conditioning.

Fear conditioning apparatus. Rat test cages were square and made of metal and plastic (30.5cm W x 25.4cm D x 30.5cm H: Coulbourn Instruments, E10-18TC or H10-11R-TC) and were modified so that the top metal panel was replaced with clear Plexiglas for video recording. Both arenas were housed within a sound-attenuating cabinet (Purchased: Coulbourn, E10-23, white, 78.7cm W x 53.3cm D x 50.8cm H, or custom-made: 63.5cm W x 61.0cm D x 71.1cm H: Melamine boards). Tones (75dB steady tone, 20 sec) were delivered through a speaker (Coulbourn, H12-01R) mounted on the inside of the sound-attenuating cabinet and were produced by a frequency generator (Coulbourn, E12-01 or H12-07). An animal shock generator (Coulbourn, H13-15) administered mild foot shocks (0.5mA, 1 sec) through a shock floor (Coulbourn, E10-18RF or H10-11RTC-NSF), with current equally distributed between parallel metal bars. Illumination was provided throughout testing by LED light bulbs in porcelain lamp-holders (Pass & Seymour, Legrand) mounted to the ceiling of the isolation cubicles.

All stimuli were controlled using Graphic State software (v 4.0 GS4-UP). Graphic State was installed on a Dell computer (3.19GHz, Intel i5 CPU, 64 bit) running Windows 7 Enterprise (2009, Microsoft Corp.). The computer was connected to a linc system (Coulbourn, H02-08) that controlled the stimuli output via an USB interface (Coulbourn, U90-11H). Infrared lights (Coulbourn, H27-91R) were positioned to be observed by the video and were programed to denote the context and tone. The infrared lights could not be visually detected unless viewed on video.

Environments for fear conditioning procedures. Two different contexts were used for training and testing. In one context, the testing cages were square metal and plastic and had a metal floor of parallel rods (Coulbourn, H10-11R-TC-SF), silver side panels (Coulbourn, H90-00R-M-KT01), and black and white striped panels on the clear plastic back wall. The sound-attenuating cabinet contained a 40-Watt equivalent LED bulb (450 Lumens; Osram Sylvania, Inc.) and a white LED computer fan (Thermaltake, CL-F020-PL12WT-A or Coulbourn, ACT-130). The cleaning solution used after each rat was an all-purpose, grapefruit scented cleaner (Method, Lowes) and the room lighting of the overall holding room was white light. Experimenters wore a yellow wrap gown and black gloves. Rats were transported from the colony room to the testing room by hand-carrying the rats in their home cages. For a second context, the testing cages were round, plastic blue buckets (37-cm H x 30.5-cm DIA, Lowes). A 3-Watt, Red LED bulb (91 Lumens; Feit Electric) was used as illumination in the isolation cubical. A 35.6-cm, computer fan with red LED light (Thermaltake, TT-1425) provided white noise/ventilation in the cubicle. The cleaning solution used after each rat was 70% isopropyl alcohol (Vi-Jon, Inc.). Experimenters wore a white lab coat and blue gloves. The rats were transported from the colony room to the testing room in their home cages on a cart and the room lighting of the overall holding room was red light.

Procedure. Rats were acclimated to Contexts A and B prior to the start of fear conditioning to prevent context generalization and to increase the likelihood that the groups would acquire fear conditioning similarly so that fear extinction could be studied (Hoffman et al., 2014). On the last 6 days of the stress manipulation, rats were exposed to the environments for ten minutes, alternating which environment they were exposed to

for three exposures per environment. On the day after the end of the stress manipulation for the STR-IMM cohort, all rats were fear conditioned in Context A with three tone and foot shock pairings. On the subsequent two days, rats were placed into Context B and were presented with 15-tone only presentations. The day after extinction training ended, anxiety-like behavior was measured on the elevated plus maze. All rats then underwent spontaneous recovery by being placed back into context B and getting three tone-only presentations. For cohort 1, this occurred a week after extinction training. For cohort 2, it took place 4-6 days after extinction training to allow spacing for sacrifice 90 minutes after behavioral assessment (Figure 4).

Behavioral quantification. All behavior was digitally recorded on GoPro Hero 3 cameras (GoPro, Inc.) for offline analysis. Video from the GoPro cameras were monitored using a Quad Splitter Processor (Evertech), which allowed four videos to be viewed on one monitor (Samsung, 24”). The behavior from eight single chambers that were viewed on two monitors was also backed up on a VCR/DVD recorder (Funai). Behavior was manually scored by trained observers. Freezing was defined as the lack of all movement, except those associated with respiration (Blanchard & Blanchard, 1969). Freezing to tone was defined as any freezing that took place during the 20-s tone presentation and freezing to context was defined as any freezing that took place in the 20-s immediately prior to the presentation of the tone. A fear conditioning difference score was calculated in order to assist in understanding how much of the freezing to the tone was due to associative processes over a more generalized, non-associative freezing response that may occur in the absence of the discrete cue. This was calculated as the amount of freezing to tone minus the amount of freezing to context 20 s prior to the tone

(similar to Majchrzak et al. 2006). Inter and intra-rater reliability was not explicitly measured for this study, but, rater-reliability for a study that was scored around the same time with the same scorers indicated that inter-rater reliability was 97.3±6.4% and intra-rater reliability was 95.7 ± 2.0%.

Elevated Plus Maze (EPM)

The elevated plus maze (EPM) was used to assess anxiety responses in rats by examining the conflict between exploration of novel environments and innate fear of heights and preference towards dark, enclosed areas (Walf & Frye, 2007). The EPM was a raised platform (50cm off ground) with two opposing open arms (50 cm long x 10cm wide) and two opposing closed arms (50 cm x 10 cm x 30 cm tall walls) that connected to a central area. A camera (GoPro Hero 3, GoPro, Inc.) mounted on the ceiling recorded behavior for offline quantification.

Rats were transported in their home cage with their littermate from the animal colony room to a novel testing room. Littermates were tested one at a time and could not see the apparatus while waiting in their home cage. At the start of a trial, a rat was placed in the center of the EPM and facing a closed arm. Rats were then given 5 minutes to explore. After the trial ended, rats were returned to their home cage. The EPM wiped of any debris and cleaned with all purpose, lavender scented cleaner (Method, Inc.).

EMP behavioral quantification. The video recordings of Elevated Plus Maze were scored for entries into the open arms, entries into the closed arms, total entries, and time spent in open arms. Time spent in closed arms was calculated as the total trial time

minus time in the open arms. An anxiety index was calculated by using the following formula:

$$1 - \frac{\frac{Time\ spent\ in\ open\ arms}{300} + \frac{Open\ Entries}{Total\ Entries}}{2}$$

Tissue Collection and Verification of Virus Placement

Due to the number of rats involved in these studies, behavioral testing occurred using two rat cohorts with all treatment groups represented in each cohort. The brain tissue was processed differently from each cohort. After behavioral testing concluded, rats were anesthetized with an overdose of sodium pentobarbital, i.p. (100mg/kg BW, Virbac, France) before cardiac perfusion. Rats were perfused with 0.1M phosphate buffered saline (PBS) followed by 4% paraformaldehyde (PFA) in 0.1M PBS. After perfusion, brains were removed and post-fixed in 4% PFA for 12-20 days (Cohort 1) and for 159-177 days (Cohort 2). Forty-eight hours prior to sectioning, brains were transferred to a 30% sucrose solution (in 0.1M PBS) for cryoprotection.

For the first cohort of rats, brains were prepared to visualize the virus placement. Brains were sectioned (60μm, -28°C) using a cryostat (Leica) and mounted onto 2% gelatin subbed glass slides. Fluorescence was protected with Vectashield (Vector Laboratories, California) and the slides cover slipped and sealed with clear nail polish. Fluorescence and bilateral placement in the IL was confirmed using a Leica MZ FLIII Stereozoom microscope equipped with a digital camera that was interfaced to a PC. The spread of the virus was visualized under green light excitation for the inhibitory DREADD (mCherry reporter) or blue light excitation (EGFP reporter) for control virus.

Placement was determined from corresponding coronal sections from a rat brain atlas (Paxinos and Watson 2014).

For the second cohort of rats, brains were examined for both DREADD placement and then saved for immunohistochemistry at a later date. Brains were sectioned (60μm, -28°C) using a cryostat (Leica) and wet mounted onto a glass slide. Slices were immediately examined for DREADD placement using a sectioned on a Leica MZ FLIII Stereozoom microscope equipped with a digital camera that was interfaced to a PC. Slices were then transferred to well plates filled with 0.1PBS with 0.01% sodium azide to prevent bacterial growth. Tissue was stored in a refrigerator (4°C) for later immunohistochemistry.

Using a Leica epifluorescence microscope, the brains from most animals showed that the IL was successfully targeted. Figure 5 shows a representative image of virus expression. Rats were included in the subsequent analyses when florescent placement was bilateral and within the IL (n=33) or a combination of being within the IL with some spread into the prelimbic cortex (PL; n=12). Rats were excluded from analysis when florescence was undetected, unilateral (one side only), or in the PL without being in the IL. Using these criteria, 46 out of 69 rats were included (for a 65% success rate), resulting in the final number of rats per group: (CON-GFP=5; CON-active=7; STR-IMM-GFP=9; STR-IMM-active=8; STR-R3-GFP=11; STR-R3-active=6).

Statistical Analysis

Results were analyzed using two-way ANOVA (Analysis of Variance) with stress group and viral type as independent factors. One-way ANOVAs were used where

described to probe interactions. Results that were significant at the $p \leq 0.05$ level or were in the predicted direction based upon past work ($p < 0.1$) were additionally analyzed using the LSD (least significant difference) post-hoc tests. After excluding rats based upon placement, sample sizes were unequal. In an experimental design with "subject dropout," correcting for unequal variances was necessary to allow statistical programs handle the inequality (Fidell & Tabachnick, 2007). To correct for unequal variances, data were transformed using $\log_{10} x + 1$ (Fidell & Tabachnick, 2007). Data analysis was performed using SPSS (Version 24, Apple iMac running macOS Sierra, v 10.12.6). Data are represented as means ± S.E.M.

Results

Fear Conditioning

Summary. All groups displayed low and minimal freezing behavior to tone and to context at the start of the fear conditioning session. As trials progressed, all groups increased freezing to tone and context. However, stress group differences emerged over the course of the session and, were apparent by the third and final trial, with STR-IMM freezing more to the tone than both CON and STR-R3. These differences at the end of fear conditioning acquisition suggest that the groups did not finish the fear conditioning session with similar associative strength of the tone-foot shock pairing (Figure 6).

Freezing to tone. The first tone trial of the fear conditioning session was analyzed for any initial differences in response to tone prior to the introduction of a foot shock. A 3x2 ANOVA for stress group (CON, STR-IMM, STR-R3) and virus type (GFP, active) for tone Trial 1 showed that all groups froze similar and low to the first

tone and that there were no differences based on stress group, virus type, and no stress and virus interaction. Therefore, the novel presentation of the tone did not create differences in freezing across groups.

Freezing across tone Trials 2 and 3 were assessed to determine behavior during fear conditioning acquisition, after the introduction of the foot shock. A repeated-measures ANOVA for stress group (CON, STR-IMM, STR-R3) and virus type (GFP, active) by conditioning trial (2, 3) showed there was a significant effect of trial ($F(1, 40)= 19.2$, $p<0.001$), with an increase in freezing from Trial 2 to Trial 3, as would be expected. No other significant effects or interactions were observed. To confirm that all groups had acquired similarly by the end of the session, Trial 3 was analyzed individually (Figure 6 C). A 3x2 ANOVA for stress group (CON, STR-IMM, STR-R3) and virus type (GFP, active) for 3rd tone revealed a significant main effect of stress group ($F(2, 40)= 3.264$, $p<0.05$) with no other significant outcomes. STR-IMM (16.61±0.85) froze more to the final training tone than did CON (13.27±1.95, $p=0.06$) and STR-R3 (11.94±1.46, $p<0.05$). Next, we analyzed the stress groups on Trial 3 individually to see whether virus type could account for stress group differences. One-way ANOVAs for virus type (GFP, active) for tone Trial 3 failed to reveal any significant differences based on viral type. However, the amount of freezing to Tone 3 by STR-R3-active (mean freezing time = 8.69±1.82 seconds) was noticeably lower than freezing by STR-R3-GFP (mean freezing time = 13.71±1.82 seconds) and seemed to be driving the low freezing in the STR-R3 stress group.

Freezing to Context A. Freezing to the context was measured prior to the introduction of either tone or foot shock, which provides an indication that acclimation to this context was similar across groups. A 3 x 2 ANOVA for stress group (CON, STR-IMM, STR-R3) and virus type (GFP, active) for context Trial 1 showed that all groups froze similar and low and that there were no differences based on stress group, virus type, and no stress by virus interaction.

Freezing across context Trials 2 and 3 were assessed to determine how freezing to Context A changed across acquisition trials, after the introduction of the tone and the foot shock. A repeated-measures ANOVA for stress group (CON, STR-IMM, STR-R3) and virus type (GFP, active) by conditioning trial (2, 3) showed that for all groups there was a significant effect of trial ($F(1, 40)= 58.24$, $p<0.001$), with an overall increase in freezing as trials progressed (more freezing to Trial 3 than Trial 2 ($p<0.001$). There was a marginal main effect of stress group ($F(2,40)=2.578$, $p=0.088$); STR-R3 froze less than STR-IMM ($p=0.029$). There were no other significant interactions or group differences.

By the end of the session, there were no significant differences among the groups in freezing to Context A. A 3 x 2 ANOVA for stress group (CON, STR-IMM, STR-R3) and virus type (GFP, active) for context Trial 3 showed no significant effects for stress group, virus type, nor interaction. However, while there were no significant main effects, post-hoc pairwise comparisons suggested that STR-R3 froze less than CON ($p=0.078$). One-way ANOVAs for virus type (GFP, active) for tone Trial 3 failed to reveal any significant differences based on viral type. However, the amount of freezing to Context A Trial 3 by STR-R3-active (mean freezing time = 8.54±3.49 seconds) was noticeably

lower than freezing by STR-R3-GFP (mean freezing time = 13.95±2.56 seconds) and seemed to be driving the low freezing in the STR-R3 stress group.

Extinction 1

Summary. Groups froze similarly to the first tone and Context B with differences emerging early in extinction on Trials 2 and 3 before extinction learning occurs. There were no significant differences in freezing to tone for stress group or virus type across trials in Extinction 1. For freezing to context in Extinction 1, STR-R3-active tended to freeze less during Trial 2 than did STR-R3-GFP, whereas CON-active froze more to Trial 2 than CON-GFP. These results suggest that IL inactivation may impact early extinction in opposite directions for STR-R3 and CON, by decreasing and increasing freezing respectively. Over the rest of the extinction trials, freezing to both tone and context B decreased and was similar across conditions (Figure 7).

Freezing to tone. Freezing to the first tone was assessed alone because it was the first time that the shock-associated tone was presented in this context (Context B) and the rats had not experienced the tone without a foot shock prior to the first extinction trial. No differences were found for stress group, virus type or interaction effects, as revealed by an ANOVA for stress group (CON, STR-IMM, STR-R3) and virus type (GFP, active) for tone Trial 1 (AVG 11.5 ± 0.7 s), suggesting that the differences during acquisition in the previous day did not carry over to the first extinction trial.

In our prior work on the effect of chronic stress on fear extinction, differences in freezing responses were observed on Trials 2 and 3 of extinction training (See Chapter 2 of this Dissertation). Consequently, freezing to tone during Trials 2 and 3 were assessed

and no significant effects of stress group, virus type or interactions were found (ANOVA for stress group (CON, STR-IMM, STR-R3) and virus type (GFP, active) by trial (2, 3)).

To determine whether extinction occurred over the 15 trials, the subsequent analysis used the average freezing over three trials for a total of 5 bins and supported that less freezing to tone occurred as bins progressed, with no significant effects from stress group, virus type or interactions (Fig. 7A, B, C). A repeated-measures ANOVA for stress group (CON, STR-IMM, STR-R3) and virus type (GFP, active) by extinction bin averaged freezing to three trials (1, 2, 3, 4, 5) showed a significant effect of bin ($F(4, 160)=18.02$, $p<0.001$), with no other significant effects. Freezing to tone decreased as bins progressed for all groups, starting with Bin 3 (vs Bin 2, $p=0.015$), and Bin 4 (vs Bin 3, $p=0.004$)).

Freezing to Context B. To understand whether the rats froze differently prior to the introduction of the shock associated tone, freezing to context B was measured prior to the presentation of the first tone and showed no differences among groups and that freezing to context B was low (3 x 2- ANOVA for stress group (CON, STR-IMM, STR-R3) and virus type (GFP, active) for context Trial 1).

Freezing to Context B during Trials 2 and 3 was analyzed as an assessment of how rats were responding to Context B after the introduction of the shock-associated tone, but before many extinction trials. Group differences were found for STR-R3 and CON, but not for STR-IMM (Fig. 7D, E, F). A repeated-measures ANOVA for stress group (CON, STR-IMM, STR-R3) and virus type (GFP, active) by trial (2, 3) for freezing to Context B showed a significant effect of trial ($F(2, 40)=9.831$, $p=0.003$), with increased freezing from Trial 2 to 3 ($p=0.003$), a significant stress group by trial

interaction ($F(1,40)=7.366$, $p=0.002$), and a significant three way interaction for stress group, virus type by trial ($F(2, 40)=15.54$, $p<0.001$), with the latter interaction probed further for each stress group condition. For the chronically stressed rats given a rest, STR-R3-active tended to freeze less to Context B during Trial 2 than did STR-R3-GFP (one-way ANOVA Trial 2, $F(1, 15) = 4.196$, $p=0.058$). For the controls, CON-active froze more to Context B during Trial 2 than did CON-GFP (one-way ANOVA Trial 2, $F(1, 10) = 5.985$, $p=0.034$). No significant virus type differences were observed for freezing to context B during Trial 3.

To assess whether freezing to Context B would show extinction as trials progressed, freezing to Context B was averaged over three trials/bin and analyzed across the five bins in the first Extinction. A repeated-measures ANOVA for stress group (CON, STR-IMM, STR-R3) and virus type (GFP, active) by extinction bin averaged for freezing Context B over three trials (1, 2, 3, 4, 5) showed a significant effect of bin ($F(4, 160)=6.36$, $p<0.001$) and a marginally significant interaction of stress group and virus type, $F(2,40)=2.811$, $p=0.072$. Freezing to Context B decreased across Extinction 1 for all groups; freezing to Context B during Bin 3 was less than freezing to Context B during Bin 2 ($p=0.036$), freezing to context B during Bin 4 was less than freezing to Context B during Bin 3 ($p=0.016$), and freezing to context B during Bin 5 was less than freezing to Context B during Bin 4 ($p=0.087$)). To probe the interaction, we analyzed each stress group separately. There were significant virus type differences in only the STR-R3 group. A repeated measures ANOVA for virus type (GFP, active) by extinction bin (1, 2, 3, 4, 5) for freezing to Context B revealed a significant main effect of virus type for STR-R3

($F(1,15)=4.466$, $p=0.052$). STR-R3-active froze less to Context B during Extinction 1 than did STR-R3-GFP (Figure 7G).

Extinction 2

Summary. Group differences emerged early in Extinction 2, during Trials 2 and 3, shortly after the reintroduction of the tone to context B. STR-R3-active froze less to tone than did STR-R3-GFP, while CON-active froze more to context than did CON-GFP. These results suggest that IL inactivation impacts early extinction in opposite directions for STR-R3 and CON (Figure 8).

Freezing to tone.

Overnight recall/forgetting. Freezing to the first tone presentation in Extinction 2 was assessed as a measure of extinction recall/overnight forgetting by comparing it to freezing from the previous Extinction's last tone presentation (trial 15). All groups performed similarly and showed higher freezing to the first tone in Extinction 2 than they did to the last extinction tone in Extinction 1 (repeated-measures ANOVA for stress group (CON, STR-IMM, STR-R3), virus type (GFP, active) by trial (Extinction 1 Trial 15, Extinction 2 Trial 1) revealed a significant effect of trial ($F(1,40)=17.262$, $p<0.001$), with no other significant effects). Freezing to the first tone on Extinction 2 averaged 11.5±0.8 s compared to freezing to the 15th tone on Extinction 1 with 6.4±.07 s. This reflected poor recall of the prior day's extinction session, but all groups performed similarly.

Tone trials. Trials 2 and 3 were examined to investigate the freezing to tone early in the Extinction 2 session to reveal effects with STR-R3, but not with CON or STR-

IMM. A repeated-measures ANOVA for stress group (CON, STR-IMM, STR-R3) and virus type (GFP, active) by trial (2, 3) revealed a marginally significant three way interaction for stress group, virus type by trial ($F(2,40)=3.02$, $p=0.06$), with no other significant main effects or interactions. The 3-way interaction was probed further by investigating each stress group separately. When analyzing STR-R3 only, the "active" group had reduced freezing to tone during Trials 2 and 3 (repeated-measures ANOVA for virus type (GFP, active) by trial (2, 3), with significant interaction, $F(1,15)=4.506$, $p=0.051$) compared to STR-R3-GFP ($p=0.02$). Looking at the trials individually revealed that this was primarily due to differences in tone Trial 2 (Fig. 8C, one-way ANOVA for virus type ($F(1,16)=8.804$, $p=0.01$). No other significant effects were found for CON or for STR-IMM (Fig. 8A, B).

Further analysis assessed whether freezing to tone would extinguish across trials, and showed that freezing to tone decreased as bins progressed and also revealed a significant interaction between stress group and virus type. A repeated-measures ANOVA for stress group (CON, STR-IMM, STR-R3) and virus type (GFP, active) by extinction bin averaged freezing to three trials (1, 2, 3, 4, 5) showed a significant effect of bin ($F(4, 160)=32.398$, $p<0.001$) and a marginally significant interaction of stress group and virus type by bin ($F(8,160)=1.906$, $p=0.063$). Freezing to tone decreased as bins progressed for all groups; there was less freezing to tone during Bin 5 than to tone during Bin 1 ($p<0.001$). Probing the stress group and virus type interaction showed that for the STR-R3, the "active" group showed decreased freezing to tone during Bin 1 compared to GFP (one-way ANOVA for virus type in STR-R3 for Bin 1 ($F(1,16)=4.893$, $p=0.043$).

No other bins were significant for STR-R3 and no significant effects were found for STR-IMM and CON.

Freezing to Context B.

Overnight recall/forgetting. Unlike freezing to tone, rats maintained the information that Context B was minimally aversive by showing nearly absent or low freezing in the first trial for context B in Extinction 2. A repeated-measures ANOVA for stress group (CON, STR-IMM, STR-R3) and virus type (GFP, active) by trial (Extinction 1 Trial 15, Extinction 2 Trial 1) showed no significant effects for trial, stress group, virus type or interactions. On average, freezing to trial 15 on Extinction 1 was (2.4±0.7 seconds) and comparable to freezing to trial 1 on Extinction 2 (1.0±0.3 seconds).

Context trials. In Extinction 2, stress group differences emerged after the first tone was presented for freezing to Context B during Trials 2 and 3. A repeated measure ANOVA for stress group (CON, STR-IMM, STR-R3) and virus type (GFP, active) by trial (2,3) was performed to assess freezing to context B after the reintroduction of the tone, but before many extinction trials were given on this day. It revealed a significant three way interaction for stress group, virus type by trial ($F(2,40)=5.229$, $p=0.01$) and a marginally significant stress group by trial interaction ($F(1,40)=2.868$, $p=0.069$). There were no other significant main effects or interactions. To probe the 3-way interaction further, each stress group was analyzed separately. CON-active froze more to Context B during trial 2 than did CON-GFP ($F(1,10)=5.466$, $p=0.041$). There were no other significant effects for freezing to context during Trial 3 nor for STR-IMM or STR-R3.

To assess whether freezing to Context B decreased across Extinction 2, freezing to context B during three trials was averaged for a bin and analyzed, showing decreased

freezing to context B during extinction 2. A repeated-measures ANOVA for stress group (CON, STR-IMM, STR-R3) and virus type (GFP, active) by extinction bin averaged freezing to three trials (1, 2, 3, 4, 5) showed that there was a significant effect of bin ($F(4, 160)=5.523$, $p<0.001$), with no other significant main effects or interactions. Freezing to Context B decreased as bins progressed for all groups, with less freezing to Context B during Bin 3 than during Bin 2 ($p=0.017$) and, importantly, less freezing to Context B on the last bin than compared to the first bin ($p=0.041$)).

Spontaneous Recovery

The sum of freezing to all three tones during spontaneous recovery was analyzed to determine whether freezing to tone reflected associated processes and whether differences across groups existed. A 3 x 2 ANOVA for stress group (CON, STR-IMM, STR-R3) and virus type (GFP, active) for the sum of freezing to all three tones revealed a significant stress group and virus type interaction ($F(2, 40) = 2.559$, $p=0.038$), with no significant main effects. Subsequently, each stress group was analyzed by an one-way ANOVA for virus type with STR-R3 showing a significant effect ($F(1,15) = 5.880$, $p=0.028$). STR-R3-active froze less (AVG = 23.3±5.6 s) than did STR-R3-GFP (AVG = 5.2±2.8 s). In contrast, CON and STR-IMM showed no significant effects, demonstrating that the active virus did not alter spontaneous recovery in all stress conditions.

It was notable that the CON-GFP group appeared to show low levels of spontaneous recovery and so a separate analysis was performed to determine whether freezing to tone during spontaneous recovery was greater than freezing to tone on the last (15^{th}) trial of Extinction 2. A repeated-measures ANOVA for stress group (CON, STR-

IMM, STR-R3) and virus type (GFP, active) by trial (Extinction 2 Trial 15, Spontaneous Recovery Trial 1) revealed a significant effect of trial ($F(1,40)=32.830$, $p<0.001$), a marginal stress group by virus type by trial interaction ($F(2,40)=2.689$, $p=0.080$) and a stress by virus type interaction ($F(2,40)=3.741$, $p=0.032$. Pairwise comparisons revealed that overall there was more freezing to Spontaneous Recovery Trial 1 than Extinction 2 Trial 15, with no other signification main. Probing the interaction by looking at each stress group individually revealed virus type differences. STR-R3 showed virus type differences (A repeated-measures ANOVA for virus type (GFP, active) by trial (Extinction 2 Trial 15, Spontaneous Recovery Trial 1) showed significant effect of trial ($F(1,15)=7.440$, $p=0.016$), with an overall increase in freezing to tone, and a main effect of virus type ($F(1,15)=4.981$, $p=0.041$)), with less freezing in STR-R3-active than in STR-R3-GFP. CON rats also show differences based on virus type. A repeated-measures ANOVA for virus type (GFP, active) by trial (Extinction 2 Trial 15, Spontaneous Recovery Trial 1) in only CON showed significant effect of trial ($F(1,10)=18.945$, $p=0.001$) with overall more freezing to at Spontaneous Recovery Trial 1. There was also an interaction with virus type and trial ($F(1,10)=7.020$, $p=0.024$), but no main effects. An one-way ANOVA for virus type on Spontaneous Recovery Trial 1 for CON only failed to reveal significant group differences ($F(1,11)=0.833$, $p=0.383$). STR-IMM did not show any differences based on virus type.

Elevated Plus Maze (EPM)

Differences in anxiety-like behavior on the EPM were not found. A two-way ANOVA for stress group (CON, STR-IMM, STR-R3) and virus type (GFP, active) was

performed for time in open arms, open arm entries, and the anxiety index and no significant main effects nor an interaction were observed. For closed arm entries (2 x 2 ANOVA, significant effect of stress group, (F(2, 40) = 3.649, p=0.035, no other significant effects), STR-IMM entered fewer closed arms than did CON (p=0.01) and nearly less than STR-R3 (p=0.08). As no other EPM measures were significant, then the significant differences in closed arm entries likely reflected STR-IMM moving around less, as opposed to showing differences in anxiety levels (Figure 10).

Body Weights

Body weight increased over the course of the experiment and weight gain was attenuated during chronic stress manipulation (Figure 11). A repeated-measures ANOVA for stress group (CON, STR-IMM, STR-R3) and virus type (GFP, active) by week (1, 4, 5, 6, 7, 8, 9, 10, 11) revealed a significant effect of week (F(8,312)=442.163, p<0.001) and a stress by week interaction ((F(16,312)=20.694, p<0.001). Note that weekly weights did not occur on weeks 2 and 3 because surgeries were occurring at that time. Across the weeks, all rats showed weight gain, weighing more on week 11 than week 1 (p<0.001). There was a significant main effect of stress group (F(2,32)=8.451, p=0.001), with STR-R3 (p<0.001) and STR-IMM (p=0.009) gaining less weight than CON. There were no other main effects or interactions. Next body weight was analyzed during the stress periods. A repeated-measures ANOVA for stress group (CON, STR-IMM, STR-R3) and virus type (GFP, active) by weeks that chronic stress occurred in STR-R3 (4, 5, 6) revealed a significant effect of week (F(2,80)=81.386, p<0.001) and a stress by week interaction ((F(4,80)=55.540, p<0.001). There was an overall increase in body weight in

all across these weeks, weighing more on week 6 than week 4 (p<0.001). There was a main effect of stress group (F(2,40)=5.873, p=0.006), with STR-R3 having lower body weight than STR-IMM (p=0.014) and CON (p=0.003). There were no other interaction and mean effects. A repeated-measures ANOVA for stress group (CON, STR-IMM, STR-R3) and virus type (GFP, active) by weeks that chronic stress occurred in STR-IMM (7, 8, 9) revealed a significant effect of week (F(2,78)=5.970, p=0.004) and a stress by week interaction ((F(4,78)=18.507, p<0.001). There was an overall increase in body weight in all across these weeks, weighing more on week 9 than week 7 (p<0.001). There was a main effect of stress group (F(2,39)=19.232, p<0.001), with both STR-R3 (p<0.001) and STR-IMM (p<0.001) having lower body weight than and CON. STR-R3 also had greater body weight than STR-IMM (p=0.011), showing that after the end chronic stress, weight gain increased in STR-R3. There were no other interaction and mean effects. Importantly there were no differences in weight gain based on viral type in any analysis.

Discussion

In the current study, glutamatergic neurons in the IL were inhibited with DREADDs in the 21 days prior to the start of fear conditioning, which corresponded to the post-stress rest period for chronically stressed rats given a rest. Fear conditioning and extinction was assessed when the IL should have been relatively unperturbed and functional because CNO administration was discontinued prior the start of fear conditioning. Unexpectedly, long-term inhibition of the IL during the post-stress rest period failed to enhance freezing to tone and context during extinction in chronically

stressed rats with a post-stress rest period. Instead, long-term inhibition of the IL in rats with a post-stress rest period led to less freezing tone and context compared to their counterparts without the long-term IL inhibition. Assessment of spontaneous recovery determined that inhibiting the IL of rats with a post-stress rest period likely led to a failure to form the association that tone predicted foot shock, so that the original fear memory acquisition was compromised. For the chronically stressed rats tested soon after stress ended, IL inhibition had no impact in fear conditioning or extinction. For the non-stressed rats, long-term IL inhibition disrupted fear extinction on both Days 1 and 2 by enhancing freezing to context in the early stages of extinction. These results indicated that the effect of long-term, inhibition of the IL on fear condition and extinction were dependent upon whether chronic stress immediately preceded fear conditioning (STR-IMM), had a delay before fear conditioning started (STR-R3) or stress never occurred (CON).

Long-term inhibition of the IL in chronically stressed rats with a rest period led to a failure to form a tone-foot shock association. Specifically, long-term IL inhibition in the rats with a post-stress rest period led to a failure to exhibit spontaneous recovery compared to rats with a post-stress rest period, but without IL inhibition. The lack of a cued-foot shock association in the chronically stressed rats with a rest period and IL inhibition likely contributed to the low levels of freezing at the end of the training trials and at various times in the extinction sessions. Consequently, *a priori* group differences in the strength of fear conditioning made it difficult to compare freezing behavior from the chronically stressed group given a rest and with IL inhibition to the other groups during extinction.

One potential area of concern is that some of the rats included in our data set had virus spread that extended into some of the PL region. In our study, twelve rats (26% of the total included) had virus expression in both the IL and in portions of the PL. The concern is that the PL has an opposing role than the IL in the expression of conditioned fear (Bennett et al., 2016; Perusini & Fanselow, 2015). For the PL, temporary inactivation suppresses the expression of fear (such as freezing), without affecting the acquisition of a fear memory (Corcoran & Quirk, 2007). As it pertains to our paradigm, any potential suppression of the PL should not have impacted fear memory formation or extinction. Thus, even if PL perturbation caused lower freezing to tone during fear conditioning and extinction, the expectation would be that the presence of a fear memory would still be revealed when tested during spontaneous recovery. For the other two conditions (chronically stressed rats tested soon after stress ends and controls), the mPFC inactivation process did not suppress the fear response during conditioning or, for the most part, extinction when compared to their respective non-mPFC suppressed controls (active versus GFP, respectively). For the non-stressed controls, the opposite was observed, with freezing to context being higher with mPFC inhibition than compared to those without mPFC non-inhibition. Moreover, the proportion of rats with virus spread in the PL was lower for the chronically stressed group given a rest (33%) than it was for the chronically stressed rats tested soon thereafter and the non-stressed controls (about 57%) to add more support that the potential influence of the PL on the freezing response was relatively minor, if at all. Consequently, the putative spread of the virus into the PL was unlikely to produce the results of the present study.

For chronically stressed rats with IL inhibition during the rest period, the association between a tone cue and foot shock did not form. Previous research identified the IL as being necessary in the formation of a new extinction memory, but not in the formation of the original cued-foot shock fear memory (Milad & Quirk, 2002; Mueller et al., 2010; Sierra-Mercado et al., 2010). One explanation may be that the IL is important any time that flexible learning is required, such as when a new strategy is needed in a familiar situation (Barker, Torregrossa, & Taylor, 2013; Mukherjee & Caroni, 2018; Oualian & Gisquet-Verrier, 2010). As to how flexible learning may pertain to the current study, all rats were extensively acclimated to both the fear conditioning and the extinction contexts (Contexts A and B, respectively) before fear conditioning started. As such, the rats would have learned that Context A was safe, which is supported by the lack of freezing to Context A by all groups prior to the tone-shock presentation. However, a new situation was created when the tone-foot shock pairing was introduced in Context A because the meaning of a familiar and safe environment had to change and became part of an aversive threat. The flexible response would require quick adaptation to the threat by higher freezing in the foot shock environment. However, chronically stressed rats with the IL inactivated during the rest period showed less freezing to the foot shock environment than did controls during fear conditioned training. Consequently, one interpretation is that rats with a post-stress rest period and the IL inactivated during the rest period suffered from impaired flexible learning that prevented them from expressing an adaptive response to a novel threat in a familiar environment. The fact that this impairment occurred with IL inhibition for the chronically stressed rats given a rest period and not the controls or the chronically stressed rats without a rest indicates that the

combination of the stress rest period combined with IL inhibition plays a vital role in fear memory formation.

The present study lent support to the growing evidence that chronically stressed rats provided with rest period are different from chronically stressed rats soon after stress ends. In chronically stressed rats with long-term IL inhibition during the rest period, the formation of the tone-foot shock association did not occur. One possible explanation is that the combination of chronic stress plus long-term inactivation in the IL led to more severe impairments on flexible learning than either manipulation alone. But this interpretation is unsatisfying because rats with long-term IL inactivation tested soon after chronic stress ended, formed the association and did not significantly differ from their counterparts without long-term IL inactivation and, differed from chronically stressed rats with long-term IL inhibition during the rest period. This suggests that the impairments in the formation of the tone-foot shock association in chronically stressed with long-term IL inhibition during the rest period require a history of chronic stress first, followed by the long term IL inactivation during the post-stress rest period. Perhaps, IL inactivation during the rest period of the chronically stressed rats prevented the acquisition of fear memory because the IL inactivation occurred in a compromised system. For the chronically stressed rats tested immediately, IL inactivation occurred at the same time as when the stressor started and hence, the IL was not compromised at the start of inactivation and may even have been protected against chronic stress effects, to some degree. Together, these findings suggest that a post-stress rest period is a critical window for when the IL functionality is uniquely vulnerable.

The literature increasingly shows that the effects of chronic stress on cognition depends upon the timing from when the stress exposure ends to when cognition is assessed. Studies on hippocampal-dependent spatial memory show that chronic stress impairs spatial memory (Abidin et al., 2004; Bowman et al., 2002; Ghiglieri et al., 1997; Kleen et al., 2006; Luine et al., 1994; Ortiz et al., 2015; Song et al., 2006). But, when a delay is inserted between the end of chronic stress and the start of behavioral training, chronic stress-induced spatial memory impairments do not persist. Indeed, rats with a sufficient delay after chronic stress has ended may even outperform non-stressed controls on spatial memory in some instances (Bian et al., 2012; C. D. Conrad et al., 2017; Hoffman et al., 2011; Luine et al., 1994; Ortiz et al., 2015; Peay et al., 2020). As in Chapter 2, there were behavioral differences based on the stress group. The present study also had differences based on when the IL was inhibited relative to when chronic stress occurred. Specifically, the IL was inhibited during the delay period in one set of chronically stressed rats (STR-R3), and during the stress manipulation for another (STR-IMM). We found that IL inhibition during the delay period prevented associative fear learning for chronically stressed rats, but did not alter fear conditioning acquisition or memory when chronically stressed rats had no delay. Together, this shows that long-term IL inhibition impacts fear processes differently, depending upon whether chronic stress concluded recently or weeks earlier.

In non-stressed control rats with long-term IL inhibition, parallels were seen with previous research on IL inactivation; IL inhibition altered fear extinction without disrupting fear memory acquisition (Rozeske, Valerio, Chaudun, & Herry, 2015; Sierra-Mercado et al., 2010). Specifically, IL inactivation during fear extinction resulted in

impaired fear extinction memory formation and the recall of fear extinction (Milad & Quirk, 2002; Mueller et al., 2010; Rozeske et al., 2015; Sierra-Mercado et al., 2010). Differences in our results from the published reports was that the non-stressed controls with IL inactivation showed enhanced freezing to context early in both extinction sessions, but an impairment in overall fear extinction was not observed. One important difference in the current study was that the IL was inhibited for three weeks leading up to the fear conditioning sessions, but was not manipulated, and presumably functional, during fear conditioning training and extinction. In prior published reports, the IL was inactivated during fear extinction. Our findings suggest that long term inactivation of the IL can lead to lasting changes that persist even when IL inactivation stopped. Indeed, control rats with the IL inactivation prior to the start of fear conditioning showed a behavioral phenotype somewhat resembling chronically stressed rats that were tested soon after stress ended, with heightened freezing to context during early extinction trials (Hoffman et al., 2014; See Chapter 2 of this Dissertation), which also resembled findings for when the IL was less active (Goldwater et al., 2009; Radley et al., 2005). Taken together, this suggests that inhibition of the IL for three weeks may have led to persistent changes in IL functioning that compromised early fear extinction, even if the IL was functional during behavioral testing.

On the elevated plus maze, no differences in anxiety-like behaviors among the groups was observed. Heightened anxiety-like behavior is often found following chronic stress and is typically measured through analysis of exploration of the open arms (Bondi, Rodriguez, Gould, Frazer, & Morilak, 2008; Mitra et al., 2005; Strekalova, Spanagel, Bartsch, Henn, & Gass, 2004; Vyas et al., 2004). No group differences were seen for

either open arm entries or time spent in open arms. However, elevated plus maze taps into both innate anxiety-like behaviors and exploratory behavior (Pellow, Chopin, File, & Briley, 1985). Some indication was revealed for recently chronically stressed rats (STR-IMM) showing less exploratory behavior by making fewer closed arm entries than did chronically stressed rats with a rest period or non-stressed rats (STR-R3 or CON). Typically, rats find exploration of novel areas rewarding and a lack of novelty exploration can be an indicator of a lack of motivation or anhedonia-like behavior (Rygula et al., 2005; Strekalova et al., 2004). As such, less exploration in the elevated plus maze by recently chronically stressed rats, could be indicative of a lack of motivation to explore or anhedonia-like behavior, which is a common outcome following chronic stress (C. D. Conrad, 2006; Huynh et al., 2011; Rygula et al., 2005). However, the possibility of less motivation to explore has some caveats. First, a change in locomotive ability can explain the reduced exploration. Second, an anxiety index that takes into account the entries into closed arms, open arms, and all arms, did not reveal any group differences, suggesting that any differences in motivation or locomotive ability was likely mild.

This experiment was also innovative in the way chemogenetic manipulation was used to study chronic stress effects on fear conditioning, but with some potential caveats. One concern was that the IL was inhibited for three weeks and then terminated a day before fear conditioning began. Consequently, the IL did not undergo chemogenetic manipulation during fear conditioning. Typically, chemogenetic manipulations are performed so that the brain region of interest is modified during or very close to when behavioral testing occurs (Mahler et al., 2014; Rapanelli, Frick, Bito, & Pittenger, 2017;

Roth, 2016; Smith, Bucci, Luikart, & Mahler, 2016; Whissell, Tohyama, & Martin, 2016). For the published reports that used chemogenetic activation over weeks, long-term changes were found in the manipulated cell populations even after chemogenetic activation ended, such as alterations in neuronal activity (E. J. Campbell & Marchant, 2018; Jain, Azua, Lu, White, & Guettier, 2013; Poyraz et al., 2016; Vetere et al., 2017). These results suggest that long-term changes could have continued in the IL region with our study, although additional explanation is needed. For example, the chronically stressed rats without a rest period (STR-IMM) failed to show differences in fear conditioning and extinction whether or not the IL was inhibited, although it is possible the IL inhibition may have protected against stress-induced changes too, as discussed earlier. Another concern is that published studies manipulating brain regions over an extended period with DREADDs typically administer the ligand (such as CNO) in the drinking water in order to produce a continuous source of the DREADD-activating ligand (Jain et al., 2013; Poyraz et al., 2016; Roth, 2016; Smith et al., 2016). In the present study, CNO was administered through daily injections, which may have allowed an opportunity for the IL to be unstimulated. However, CNO can continue to have behavioral effects up to 9 hours following injections (Alexander et al., 2009). Moreover, one study found that daily CNO injections produced similar outcomes on behavior as found with CNO given in the drinking water (Jain et al., 2013). Thus, the existing evidence suggests that the chronic activation of DREADDs via injection in our study likely were effective in creating long-term changes in the IL as desired.

Another potential concern is that CNO may not be as inert as initially thought. At issue is that CNO can reverse-metabolize into its parent drug, clozapine, an antipsychotic

medication with high affinity for dopaminergic receptors, but with actions on the serotoninergic system and other off-target sites (Baldessarini & Frankenburg, 1991). If CNO reverse metabolizes to clozapine and interacts with sites other than the intended DREADD, then it would be a major confound in our study. For example, dopamine plays an important role in fear extinction learning (Hikind & Maroun, 2008; Holtzman-Assif, Laurent, & Westbrook, 2010; Mueller et al., 2010; Pfeiffer & Fendt, 2006) and both dopamine and serotonin signaling are altered following chronic stress (Beck & Luine, 2002; Bekris, Antoniou, Daskas, & Papadopoulou-Daifoti, 2005; Haenisch & Bönisch, 2011; Thierry, Fekete, & Glowinski, 1968; Torres, Gamaro, Vasconcellos, Silveira, & Dalmaz, 2002). One way we addressed this issue was to use a low dose of CNO because CNO is less likely to reverse metabolize to clozapine at low doses (Gomez et al., 2017; Maclaren et al., 2016). In addition, all rats received CNO injections, whether they had an active DREADD or the inactive virus (Mahler & Aston-Jones, 2018; Roth, 2016). Consequently, had CNO/clozapine been responsible for the outcomes, then DREADD type infused would have been irrelevant, which was not observed in our study. Thus, CNO/clozapine was unlikely involved and suggests that CNO acted at the active DREADD to inhibit IL activity.

Another potential issue is that the present study implemented a different foot shock intensity than used previously, which was set at 0.8mA in Chapter 2 compared to 0.5mA for the current Chapter 3. In Chapter 2, non-stressed control and recently stressed rats froze similarly during extinction, which was surprising as prior studies showed chronically stress rats resist cued-fear extinction compared to non-stressed controls (Baran et al., 2009; Hoffman et al., 2014; Izquierdo et al., 2006; Miracle et al., 2006; Rau

et al., 2005). The lack of differences in cued fear extinction in Chapter 2 between non-stressed and recently stressed rats may have suggested that overtraining occurred because both non-stressed and recently chronically stressed rats showed similar and high freezing response after the shock associated tone was introduced to a context where shock had never occurred (95.3% and 87.0% for freezing to context in recently stressed rats non-stressed rats, respectively, versus 62.6% and 59.4% in chronically stressed rats with a 3 or 6 week rest period, respectively). In an attempt to avoid overtraining in the non-stressed rats, the foot shock intensity was lowered 0.5mA because it produced similar startle and jump response in non-stressed and recently stressed rats. Unexpectedly, differences in freezing to tone during the final training trial emerged in which chronically stressed rats froze more to tone during Trial 3 than did unstressed rats or chronically stressed rats given a rest period. The concern is differences in freezing to the final tone trial of training could have resulted from differences in tone-foot shock association; however, subsequent analysis of the first extinction trial demonstrated that all groups froze similarly to tone without showing a ceiling effect. Thus, the lower shock intensity used in the study for Chapter 3 allowed for the detection of differences to be observed in non-stressed control and recently chronically stressed rats.

The current research contributes to and expands on the existing literature on the role of the IL in fear conditioning and extinction following chronic stress. The failure to form a fear memory when the IL was inhibited during the post-stress rest period, in the weeks before the start of fear conditioning, suggests that the combination of chronic stress-induced changes to the IL followed by the inhibition of the IL during the post-stress rest period is particularly disruptive to fear learning. Unlike chronically stressed

rats with long-term IL inhibition during the rest period, chronically stressed rats that had concurrent long-term IL inhibition during stress were able to form a fear memory and, despite IL inhibition, were behaviorally identical to chronically stressed rats without long-term IL inhibition. Consequently, inhibiting the IL currently with chronic stress exposure does not produce alterations to IL-mediated fear extinction above and beyond the chronic stress effects alone. For unstressed, control rats, inhibiting the IL for the three weeks leading up to the start of fear conditioning led to enhanced freezing to context during the early trials of the two extinction sessions. The non-stressed controls with long-term IL inhibition showed some similarity to the PTSD-like, recently, chronically stressed group. In particular the non-stressed controls with long-term IL inhibition showed signs of hypervigilance to a safe environment, as the predominant effects occurred during freezing to context, as opposed to the tone. During chronic stress, a plethora of changes occur in the brain that may contribute to the way fear memories are formed and these could interact with the IL manipulation. However, for the controls, the IL was selectively inhibited for three weeks leading up to the start of behavioral training. Thus, we would not expect other brain regions to be impacted by our manipulation. This suggests that long-term IL inactivation can initiate a PTSD-like condition by causing elevated fear responding to a safe context early in the extinction sessions. It would be interesting to determine whether such generalization would become magnified had the animals not been extensively acclimated to the environments beforehand. This once again highlights the importance of understanding the impact of chronic stress or chronic stress followed by a delay on IL-mediated behaviors and underscores the importance of understanding the mechanisms by which chronic stress alters this critical structure.

CHAPTER 4

GENERAL DISCUSSION

Overarching Goals and Findings of the Current Studies

Post-traumatic stress disorder (PTSD) is a condition that develops in some people following a traumatic event. In individuals that acquire PTSD, the characteristic symptom is overly robust and distressing memories of the trauma. Memories of the trauma intrude onto daily life and flashbacks of the trauma are common (American Psychiatric Association, 2013). Robust fear memories from trauma in PTSD patients often resist therapeutic attempts to weaken their intrusive properties (Bradley et al., 2005; Difede et al., 2014; Steckler & Risbrough, 2012; VanElzakker et al., 2014). Animal models can be helpful to gain a greater understanding of the mechanisms of PTSD. In our lab we use chronic stress to create a phenotype that is vulnerable to developing PTSD-like symptoms followed by fear conditioning to simulate the traumatic event requirement. Previously we had shown that following chronic stress, conditioned fear memories are robust and resist attempts to weaken them (Hoffman et al., 2014, 2015).

The overarching goal of the present studies was to understand whether chronic stress has a lasting impact on fear memories through studying extinction processes. The first aim in Chapter 2 was to understand whether the effects of chronic stress on fear extinction learning would persist when weeks had passed between the end of chronic stress and the start of fear conditioning. After finding that fear responses during extinction depended on the passage of time from the end of chronic stress, the second aim in Chapter 3 was to understand the mechanism behind the differences in fear responses

during extinction. We hypothesized that the infralimbic cortex (IL) of the medial prefrontal cortex (mPFC) was responsible for the improvements in fear extinction of the chronically stressed rats given a rest period, as numerous other studies showed that the IL is required in fear extinction (Bennett et al., 2016; Milad & Quirk, 2002; Mueller et al., 2010; Sierra-Mercado et al., 2010). We found some evidence supporting the role of the IL in safety signal responding the non-stressed control rats during the early stages of extinction. Unexpectedly, IL inhibition during the rest period of chronically stressed rats disrupted the tone-foot shock association. Taken together, these studies indicate that time since the end of chronic stress is an important factor in an individual's fear response in a non-threatening environment and may involve the IL.

Findings from Chapter 2

In Chapter 2, we explored whether chronic stress would have a lasting impact on fear extinction. Previous research showed that when fear conditioning and extinction occur soon after chronic stress ends (STR-IMM), fear acquisition is more rapid, fear extinction and recall is impaired, and fear is more readily generalized than compared to non-stressed controls (Bryant et al., 2008; C. D. Conrad et al., 1999; Hoffman et al., 2014, 2015; Izquierdo et al., 2006; Miracle et al., 2006; Rau et al., 2005). However, whether chronic stress followed by a rest period would have lasting effects on fear extinction was unknown, although evidence suggested that chronic stress effects on cognition might change over time. In cognitive tasks that assess spatial memory, recently acquired memories are impaired in the days after chronic stress has ended (Abidin et al., 2004; Bowman et al., 2002; Ghiglieri et al., 1997; Kleen et al., 2006; Luine et al., 1994; Ortiz et

al., 2015; Song et al., 2006). However, spatial memory improves, and can be even better than in non-stressed control rats when assessed weeks after chronic stress has ended (Bian et al., 2012; Conrad, Ortiz, & Judd, 2016; Hoffman et al., 2011; Luine, Villegas, Martinez, & Mcewen, 1994; Ortiz et al., 2015). Conversely, anxiety-like behavior is elevated in the days following chronic stress (Chiba et al., 2012; D'Aquila et al., 1994; Eiland & McEwen, 2012; Huynh et al., 2011; Vyas et al., 2004) and appears to stay elevated in the weeks after chronic stress has ended (Adamec & Shallow, 1993; van Dijken et al., 1992; Vyas et al., 2004). In Chapter 2, we found that chronically stressed rats given a post-stress rest period prior to the start of fear conditioning expressed optimal fear discrimination, by showing low freezing to an environment that had never been associated with shock was lower than to a an environment where a foot shock had occurred. This optimal fear discrimination performance exceeded the performance of non-stressed controls and chronically stressed rats tested soon thereafter. Moreover, context generalization and second order conditioning were unlikely to explain these differences. We conclude that chronically stressed rats given a rest period before the start of fear conditioning leads to better discrimination of fear responding to the safety signal of the non-shock context versus the fear cue of the shock associated tone.

Findings from Chapter 3

In Chapter 3, the goal was to understand the mechanisms behind the improved discrimination between fear responses to the tone and the foot shock in the early extinction trials by chronically stressed rats given a rest period before fear conditioning began. We investigated the role that the IL might play in the differences in fear response

discrimination to tone and context in chronically stressed rats with or without a rest period and in non-stressed controls. Numerous previous studies have indicated that the IL is particularly important for fear extinction and is believed to act as a break on emotional (i.e., fear) responding (Bennett et al., 2016; Bloodgood et al., 2018; Knapska & Maren, 2009; Milad & Quirk, 2002; Mueller et al., 2010; Sierra-Mercado et al., 2010). The IL was additionally appealing as a target because it specifically plays an important role in discriminating between fear and safety cues (Sangha et al., 2014). Moreover, the IL is sensitive to chronic stress and shows improvements following a rest period, as measured in neuronal complexity and activity (Bloss et al., 2010; Cerqueira et al., 2007; Goldwater et al., 2009; Izquierdo et al., 2006; Moench & Wellman, 2014; Radley et al., 2005; Wilber et al., 2011). Consequently, the IL seemed like a promising region to investigate for the differences in fear extinction between rats given a rest period following the end of chronic stress than compared to rats without a rest following the end of chronic stress.

We targeted the IL during the post-stress rest period to hinder potential changes that may take place following the end of chronic stress that would lead to improved IL function, which is necessary for fear extinction. To investigate the role of the IL, we employed inhibitory chemogenetics that were activated long-term during the post-stress rest period in chronically stressed rats, but then stopped when fear conditioning procedures began. IL inhibition coincided with chronic stress period for the chronically stressed rats without a rest period. Since the IL was unperturbed during behavioral sessions, then it was presumed to be functional during the fear conditioning procedures. Each treatment group interacted differently to long-term IL inhibition. For the chronically stressed rats given a rest period, a surprising finding was that, combined with long-term

inhibition in the IL, the formation of the tone-foot shock associations was impaired. For the control rats, long-term IL inhibition led to less freezing to context in the early extinction trials, which lent some support for the IL's role in the discrimination of fear and safety cues. For the chronically stressed rats without a rest period, long-term IL inactivation had no effect on fear conditioning and extinction. In short, long term IL inhibition produced unique behavioral output in each stress group

Safety Signals and Post-Traumatic Stress Disorder

A hallmark of PTSD is a lack of discrimination between fearful and safety signals in that PTSD suffers will respond to cues that remind them of the traumatic event, even when they are in a safe environment, such as at home. Memories of the trauma are overly robust and intrude into daily life (American Psychiatric Association, 2013). When safety signals are properly learned, fear will be suppressed in the appropriate situation. However, PTSD patients often display a failure to respond to or recognize safety cues (Grillon et al., 1998; Jovanovic et al., 2012). The current studies successfully modeled this behavior with rats with recent chronic stress, showing high fear response to both the shock associated tone and the extinction context. Importantly, the extinction context had been extensively acclimated too and should have been able to act as a safety signal. However, in Chapter 2 once the tone was introduced to the extinction context (which had never included a foot shock), freezing to context by recently chronically stressed rats was high, suggesting that the extinction context was not successfully operating as a safety signal. Consequently, exposure to chronic stress in this case may skew fear learning so

that mechanisms that help recognize safe environments are over-ridden to favor mechanisms that signal danger is eminent.

In both studies, some groups showed better fear response discrimination to the shock associated tone and the context never associated with the tone. Chronically stressed rats with a post-stress rest period in Chapter 2 and the non-stressed controls without the chemogenetic IL inhibition in Chapter 3 showed low freezing to the non-shock extinction context during the early extinction trials, even after the introduction of the tone to this context. This suggests that these groups were successfully able to discriminate between fear and safety cues and respond appropriately during early extinction trials, prior to extinction effects occurring. However, in Chapter 3, we were able to mildly disrupt this appropriate discriminative responding. In the unstressed control rats with long-term IL inhibition, similar and high fear responses to tone and context was observed early in extinction, suggesting some lack of safety signal recognition. This is in contrast to the behavior of the unstressed control rats without IL inhibition, that successfully reacted to safety signals. This suggests a role of the IL in the ability to differentiate safety signals and respond to them. This finding is supported by prior work that found that disruption of the IL leads to disruption in cued safety discrimination (Sangha et al., 2014). Together, this supports continued investigation into the role of the IL in facilitating acquisition of safety signals and that this could be an important avenue for PTSD research.

The IL, a Complex Structure with Glutamatergic Projections to Both the Amygdala and the Periaqueductal Grey.

In Chapter 3, glutamatergic neurons in the IL were targeted using DREADDs for long-term chemogenetic inhibition during the post-stress rest period and then stopped when fear conditioning started. The idea being that the IL sends glutamatergic projections to the intercalated cells of the amygdala, and then this signaling cascade leads to inhibition of the amygdala and the associated emotionally-driven responses. In Chapter 3, we broadly targeted all the glutamatergic neurons in the IL as first pass to better understand the IL's role in the fear responding by different chronic stress conditions. However, projections from the IL to the amygdala are just part of the story. Glutamatergic neurons in the IL are a heterogenous population, projecting to different brain regions with different layers expressing differing chronic stress reactions. Research has indicated that layers II, III, and V are susceptible to chronic stress remodeling and recover in the post-stress rest period (Goldwater et al., 2009; Radley et al., 2005). One study investigated chronic stress with and without a rest period on dendritic complexity within the mPFC, although the IL and PL were combined (i.e., not differentiated). For layers II and III, chronic stress led to shrinkage of neuronal dendritic complexity, but neuronal dendritic complexity returned after a post-stress rest period and this complexity was similar to non-stressed controls (Radley et al., 2005). Another study that specifically quantified layer V of the IL, found that chronic stress also caused a decrease in dendritic complexity in this layer and that dendrites increased in complexity following a rest period after chronic stress ended. However, unlike in layers II/III, dendritic organization in layer V was different than before, with more complexity closer to the soma (Goldwater et al.,

2009). Differences in dendritic organization IL Layer V between chronically stressed rats with a post-stress rest period and non-stress controls could be important as to why rats with a post-stress rest period show better fear and safety discrimination than even non-stressed control rats (Chapter 2). Our current technique allowed us to target all glutamatergic neurons in the IL, but we were unable target specific layers. If different layers of the IL react differently to the post-stress rest period, then identifying ways to differentiate the IL layer roles in fear conditioning will be important.

Targeting specific neuronal populations of the IL is important both because of the different ways that neurons in the different layers of the IL react following a post-stress rest period, but also because the layers of the IL project to different regions. Neurons in the IL that project to the amygdala are located in layers II, III, and V, while neurons that project to the periaqueductal grey (PAG) are exclusively located in layer V (Cheriyan, Kaushik, Ferreira, & Sheets, 2016; Ferreira, Yousuf, Dalton, & Sheets, 2015). The studies that have investigated which neuronal populations project from the IL to the amygdala have largely focused on the basolateral amygdala and it has yet to be determined which layer of the IL contains the specific neurons that project to the intercalated cells of the amygdala (Cheriyan et al., 2016). The PAG is an important output structure for various fear responses (such as freezing and vocalizations) and fear response is impaired when the PAG is inactivated. (Helmstetter & Tershner, 1994; Johansen, Tarpley, Ledoux, & Blair, 2010; Ledoux, Iwata, Cicchetti, & Reis, 1988). Inactivation of the PAG also impairs fear conditioning acquisition (Johansen et al., 2010). In Chapter 3, the chronically stressed rats with a rest period and the IL inhibited failed to form a tone-foot shock association. Given the PAG's role in both the expression and

acquisition of conditioned fear (Johansen et al., 2010), the IL to PAG neurons might be the primary driving force behind the lack of fear conditioning association in rats with a post stress rest period and the active virus. Future studies should further delve into the role of the PAG in the post-stress rest period and how its role in fear circuitry changes over time after chronic stress occurs.

Closing Thoughts

The likelihood of developing PTSD is relatively low, with estimates that only 6-9% of the population will develop PTSD (American Psychiatric Association, 2013; Hoge & Warner, 2014; Sareen, 2014), despite approximately 50% of the population experiencing at least one traumatic event (Ozer, Best, Lipsey, & Weiss, 2003). Even in the face of situations that would seem to ensure the development of psychological issues, such as life threatening violence, war, and disasters, resilience is the most common outcome (Bonanno, 2004). Given that most people show resilience after encountering traumatic events, perhaps the findings in Chapter 2 are not surprising, whereby a delay after the end of chronic stress led to more appropriate behavioral responses to a context not associated with foot shock. Almost everyone will undergo a period of heightened stress (or a chronic stress period), whether caring for a sick family member (Bauer et al., 2000; Caswell, Vitaliano, Croyle, & Scanlan, 2003; Kiecolt-Glaser et al., 1987; Vedhara et al., 2002; Vitaliano et al., 2005), attending graduate school (Evans, Bira, Gastelum, Weiss, & Vanderford, 2018; Goplerud, 1980; Mousavi et al., 2018), or participating in another stressful situation. The prevalence of chronic stress and exposure to traumatic events appear to be a part of the human experience, making the development of a

disruptive psychiatric condition to be maladaptive. Thus, the relatively low occurrence of PTSD in the population seems evolutionarily reasonable because for chronic stress to be a risk factor for PTSD in perpetuity would be maladaptive.

Even in individuals who are at heightened risk for the development of PTSD due to recent chronic stress, the risk can be mitigated. As examples, exercise (Haglund, Cooper, Southwick, & Charney, 2007; Kochi et al., 2017; Silverman & Deuster, 2014), social support (Bonanno & Keltner, 1997; Haglund et al., 2007), and a focus on positive emotion (Keltner & Bonanno, 1997; Noll, Horowitz, Bonanno, Trickett, & Putnam, 2003; Ozbay et al., 2007) can increase resilience against PTSD or improve coping if PTSD does develop. These findings support the notion that PTSD is not inevitable. While chronic stress, a history of mental illness, or genetic predisposition will always lead to some individuals more prone to the development of PTSD (American Psychiatric Association, 2013; Breslau et al., 1999; Brewin et al., 2000; Koenen et al., 2008; Sarapas et al., 2011; Sareen, 2014; Skelton et al., 2012), perhaps interventions to promote resilience can lower the risk of PTSD in high risk individuals. In Chapter 2, just the passage of time since the end of chronic stress and the start of a traumatic experience or fear conditioning can reduce the risk of potentiated, PTSD-like fear memories. The old motto "an ounce of prevention is worth a pound of cure" seems appropriate here; the best way to reduce the impact of PTSD on an individual is to promote resilience.

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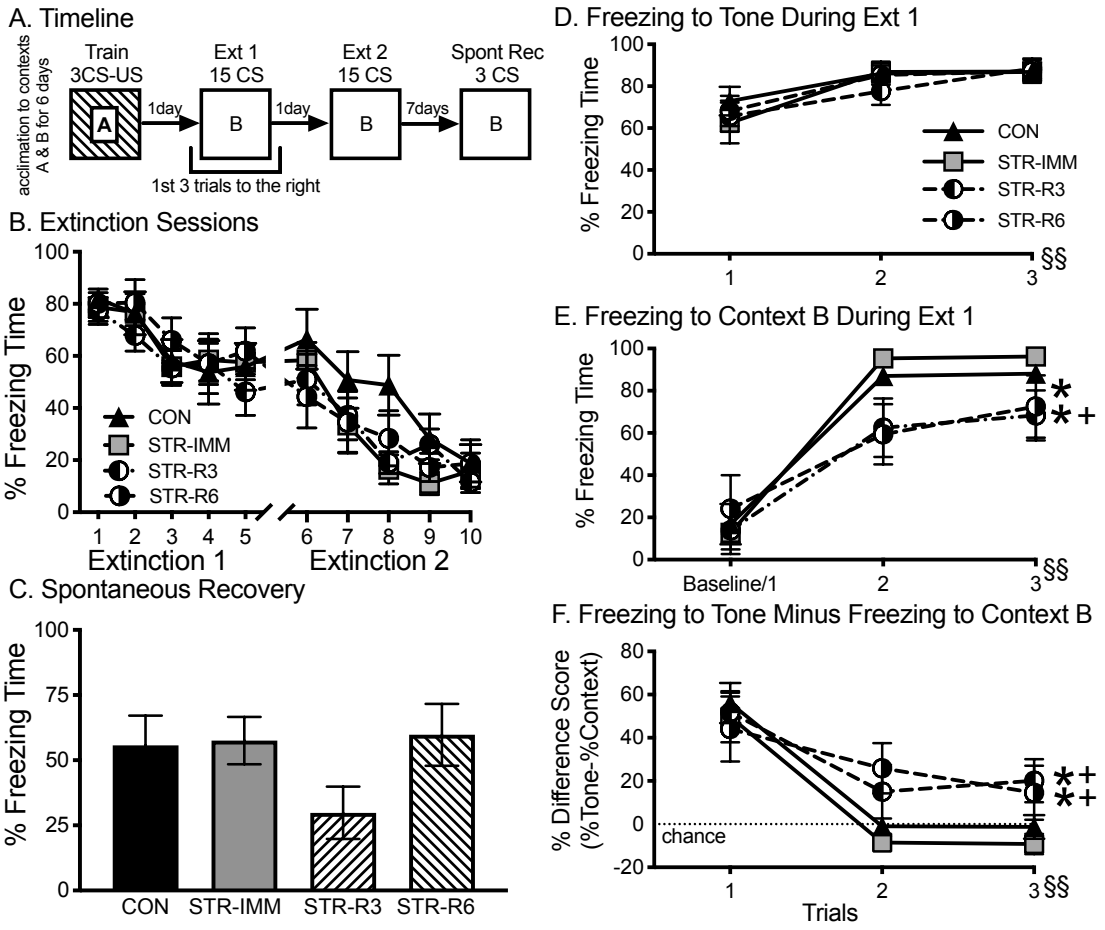

Figure 1, Chapter 2, Experiment 1. (A) Timeline of Experiment 1 (bracket denotes when Extinction 1 occurred). Rats were chronically stressed (6hr/day/21days), which terminated 1, 21, or 42 days prior to the start of fear conditioning. Six days prior to fear conditioning, rats were acclimated to both contexts (A and B) for 10 minutes daily, to reduce group differences in fear conditioning acquisition so that extinction processes could be studied (Hoffman et al., 2014). Fear conditioning occurred in Context A with 3 tone-foot shock pairings. Groups performed similarly by the end of fear conditioning acquisition (data not shown). Extinction to tone occurred in Context B on two subsequent days with spontaneous recovery occurring a week later. (B) Extinction to Tone Over Extinction 1 and 2. Freezing to tone decreased as trials progressed and groups performed similarly. Data are represented as bins of three trials. (C) Spontaneous Recovery. One week after the last extinction session, all groups were returned to Context B and presented with three tones. All groups showed a return of freezing to tone with no significant group differences to demonstrate they made the appropriate tone-foot shock associations. (D) Freezing to Tone During the First Three trials in Extinction 1. For the first tone presentation in Extinction 1 without foot shock, all groups froze robustly to tone, and freezing to tone increased over the first three trials, with no group effects. (E) Freezing to Context B During the First Three trials in Extinction 1. While all groups showed very little freezing in Context B prior to the first tone presentation in Trial 1 (Baseline freezing level), differences became apparent during Trials 2 and 3: STR-R3 and

STR-R6 froze less to context than did STR-IMM. Also, STR-R3 froze less to context than did CON, but did not reach significance for STR-R6. (F) Difference Score for the First Three Trials in Extinction 1. A difference score was calculated by subtracting freezing to Context B from freezing to tone to obtain a measure of selective fear memory to the cue. During Trials 2 and 3, STR-R3 and STR-R6 demonstrated higher difference scores than compared to both STR-IMM and CON. Moreover, STR-R3 and STR-R6 expressed difference scores that were above chance, showing selective freezing to tone than to Context B, whereas STR-IMM and CON froze at chance levels. Final number of subjects per group were n=8-10. $+p<0.05$ compared to CON, $*p<0.05$ compared to STR-IMM, $^{\S\S}p<0.01$ across trials.

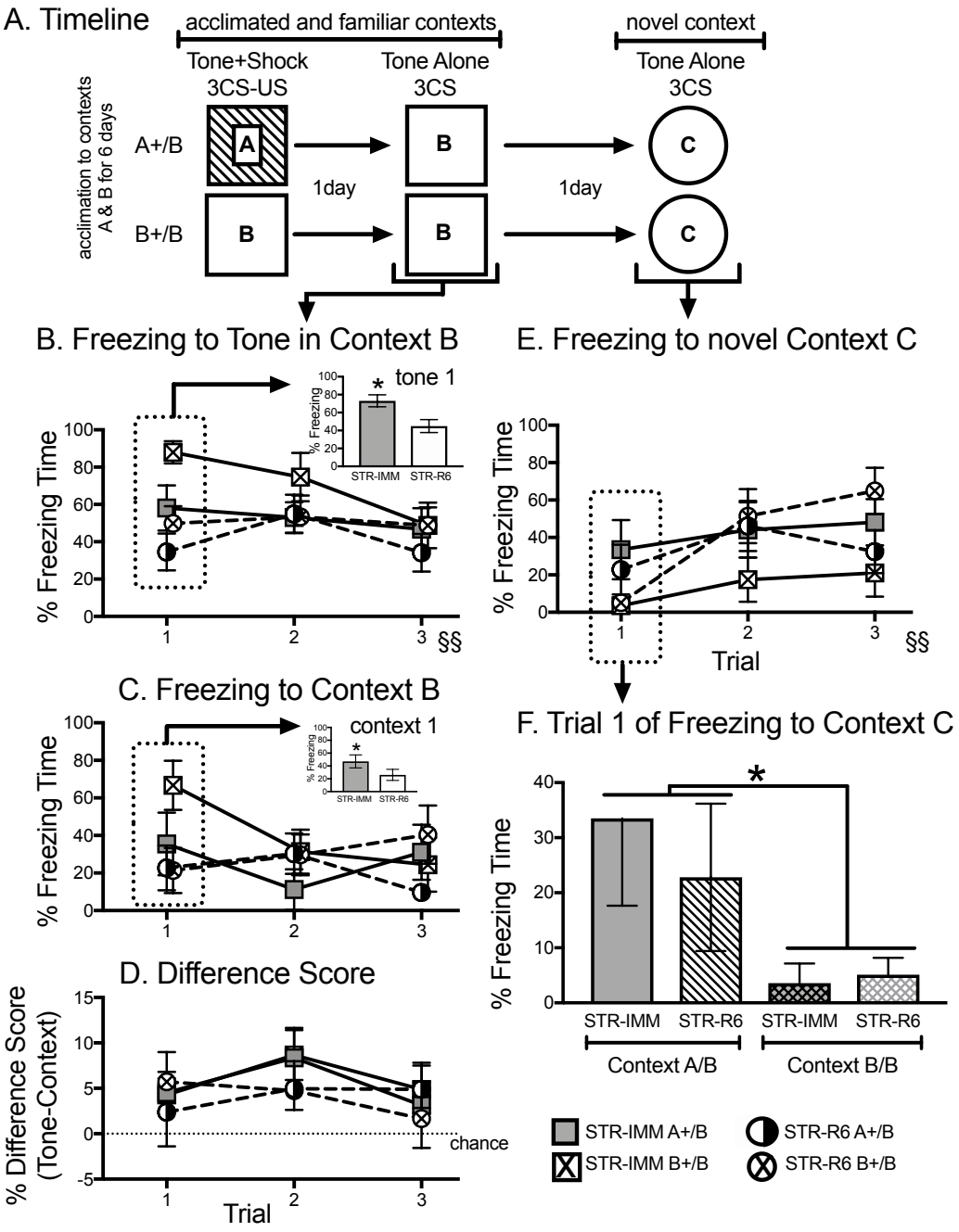

Figure 2, Chapter 2, Experiment 2 to Determine whether Chronic Stress Increases Generalization to Context. (A) Timeline of Experiment 2. Fear conditioning training consisted of 3 tone-foot shock pairings in either Context A or B (A+ and B+, respectively). The following day, rats were placed in Context B and presented with three tones, giving rise to being tested in a different context from training (A+/B) or the same context as training (B+/B). No differences in freezing levels were found during acquisition of fear conditioning for either the stress condition (STR-IMM, STR-R6) or the contexts (data not shown). (B) Tone-Alone Presentation in Context B: Freezing to Tone. Differences among groups were observed in the first trial, with STR-IMM freezing more to tone than did STR-R6 (Stress Effect,* $p<0.05$), and groups trained and tested in

the same Context B (B+/B) freezing more to tone than those trained and tested in a different context (A+/B) (Context Effect, $*p < 0.05$). (C) Tone-Alone Presentation in Context B: Freezing to Context. Differences among groups were observed in the first trial (Baseline Freezing to Context B), with STR-IMM freezing more to Context B than did STR-R6 (Stress Effect, $*p < 0.05$). (D) Tone-Alone Presentation in Context B: Difference Score for Freezing to Tone Minus Freezing to Context B. Groups performed similarly with no significant differences. (E) Tone-Alone Presentation in novel Context C: Freezing to Context. Freezing to novel Context "C" increased over trials for all groups, but rats trained and then tested in a different context (A+/B) froze more to the novel context prior to any tone presentation (Baseline Freezing to novel Context C) than did rats trained and tested in the same context (B+/B), which is illustrated in panel F. Freezing to tone in Context C was similar for all groups. Final number of subjects per group were n=7-8. For all graphs, §§ $p<0.01$ for significant effect of trial.

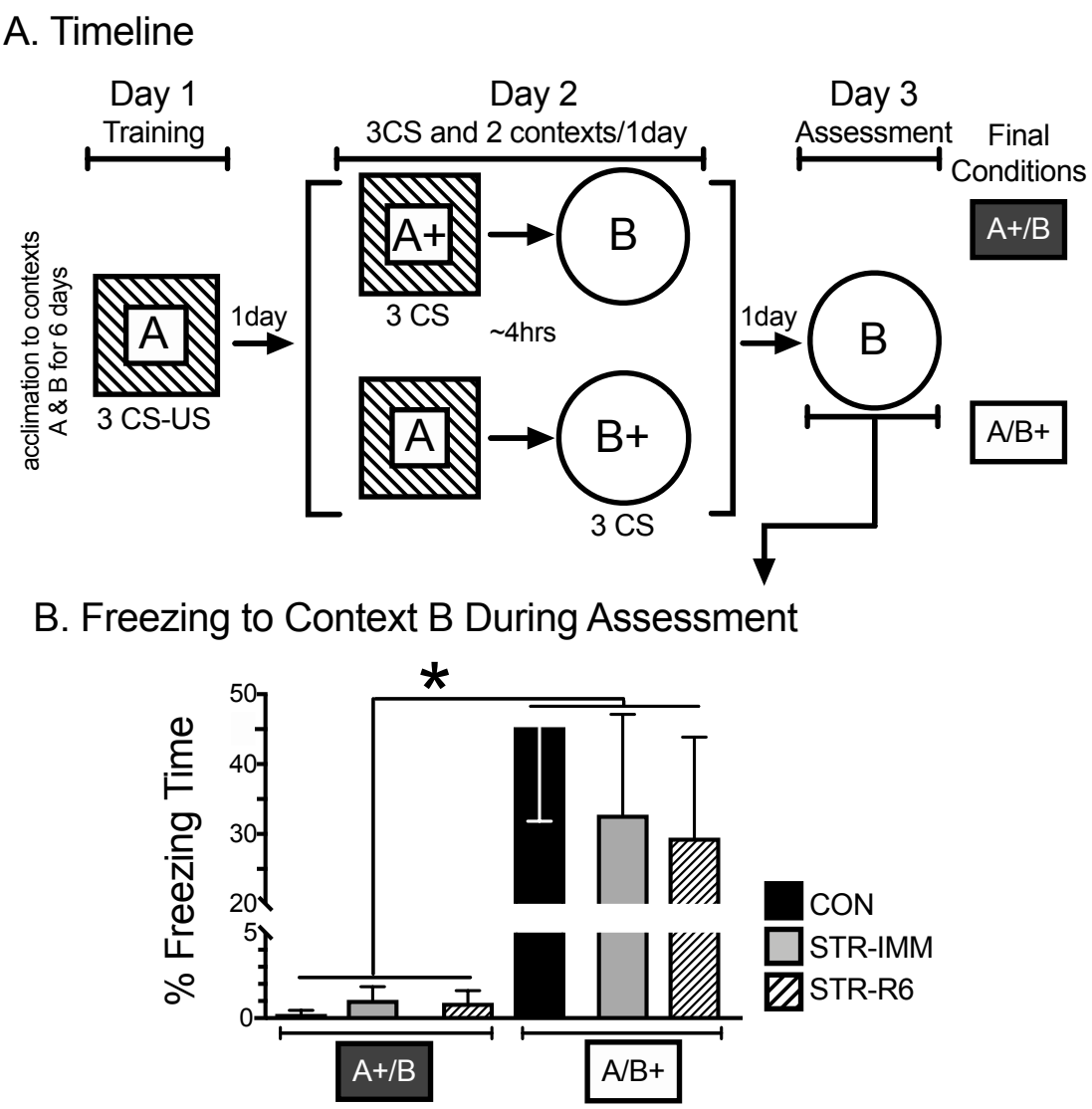

Figure 3, Chapter 2 Experiment 3 to Determine whether Chronic Stress Enhanced Second Order Conditioning. (A) Timeline of Experiment 3. Fear conditioned training consisted of 3 tone-foot shock pairings in Context A. One day later, rats were returned to Context A, with half of them exposed to the tone again in this environment and the other half not exposed. Later that same day, all the rats were placed in the non-shock Context B; rats that did not receive tone presentations in Context A were then exposed to three tones in Context B; those that received the tone presentations in Context A did not receive tones during this time. This led to the two conditions, A^{+}/B and A/B^{+}, respectively, based upon the context that had tone-alone presentations. Consequently, all groups received similar tone and context exposures leading into the assessment day. The following day, (day 3) the amount of freezing to Context B was assessed. (B) Freezing to Context B During Assessment. If the rats formed second-order conditioning, then rats that received tone presentations a day earlier in Context B (A/B+) would be expected to show higher freezing to Context B during the assessment. As expected, all groups in the A/B+ condition froze more to Context B than did the rats that never received a tone in Context B previously. There were, however, no other significant effects, indicating that

second-order conditioning was similar for CON, STR-IMM and STR-R6. Final number of subjects per group were n=7-8. *p<0.001 compared to A+/B.

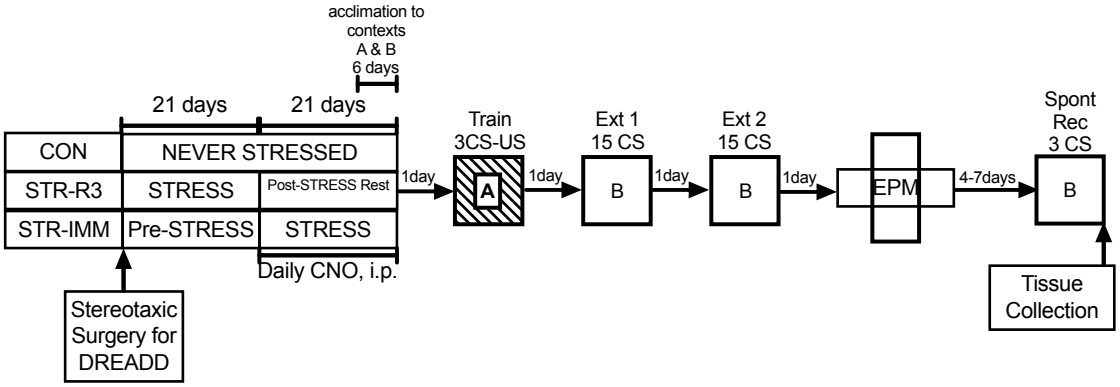

Figure 4, Chapter 3 Experimental Timeline to Determine Whether IL Inhibition Altered Fear Extinction in Chronically Stressed Rats given a Rest. Rats were assigned to one of three stress groups. Rats in the stress immediate group were chronically stressed and underwent fear conditioning within days after restraint ended (STR-IMM). Rats in the stress-rest group were chronically stressed and then had three weeks without restraint prior to the start of fear conditioning (STR-R3). The control group did not undergo a stressor manipulation (CON). During the three-week break for STR-R3, which overlapped with the stressor manipulation for STR-IMM, all rats (CON, STR-IMM, STR-R3) were given daily injections of CNO (1mg/kg BW, i.p.) for chronic activation of the DREADDs, which were stereotaxically placed in the IL prior to the start of any manipulation. Six days before the start of fear conditioning, rats were acclimated to Contexts A and B for 6 days for ten minutes per day, alternating which environment they were exposed. On the day after the end of the stress manipulation for the STR-IMM cohort and three weeks after the end of the stress manipulation for the STR-R3 cohort, CNO administration stopped and then all rats were fear conditioned in Context A with three tone and foot shock pairings. On the subsequent two days, rats were placed into Context B and were presented with 15-tone only presentations for extinction training. The day after the second extinction training ended, anxiety-like behavior was measured on the elevated plus maze (EPM). All rats underwent spontaneous recovery (Spont Rec) 4-7 days after the end of extinction training.

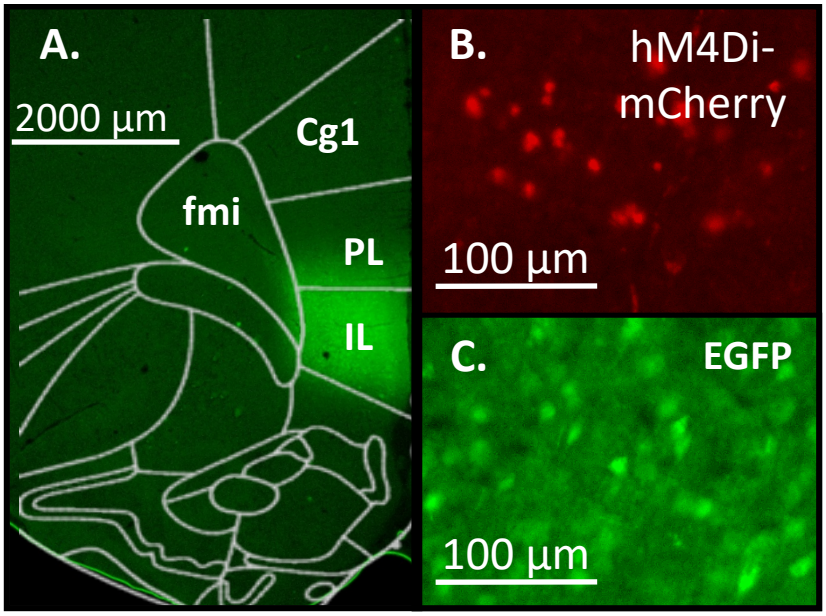

Figure 5, Chapter 3, Representative Viral Placement with Florescent Reporters in the IL Region. (A) Placement was primarily in the IL, but there were cases with some overlap of approximately 30% into PL. Rats were included in subsequent analyses when placement was bilateral and in the IL only, or when placement was bilateral and in the IL with some spread to the PL. (B) Close up image showing neurons with the active virus AAV8-CamKIIα-hM4Di-mCherry. (C) Close up image showing neurons with the control virus, AAV8-CamKIIα-EGFP.

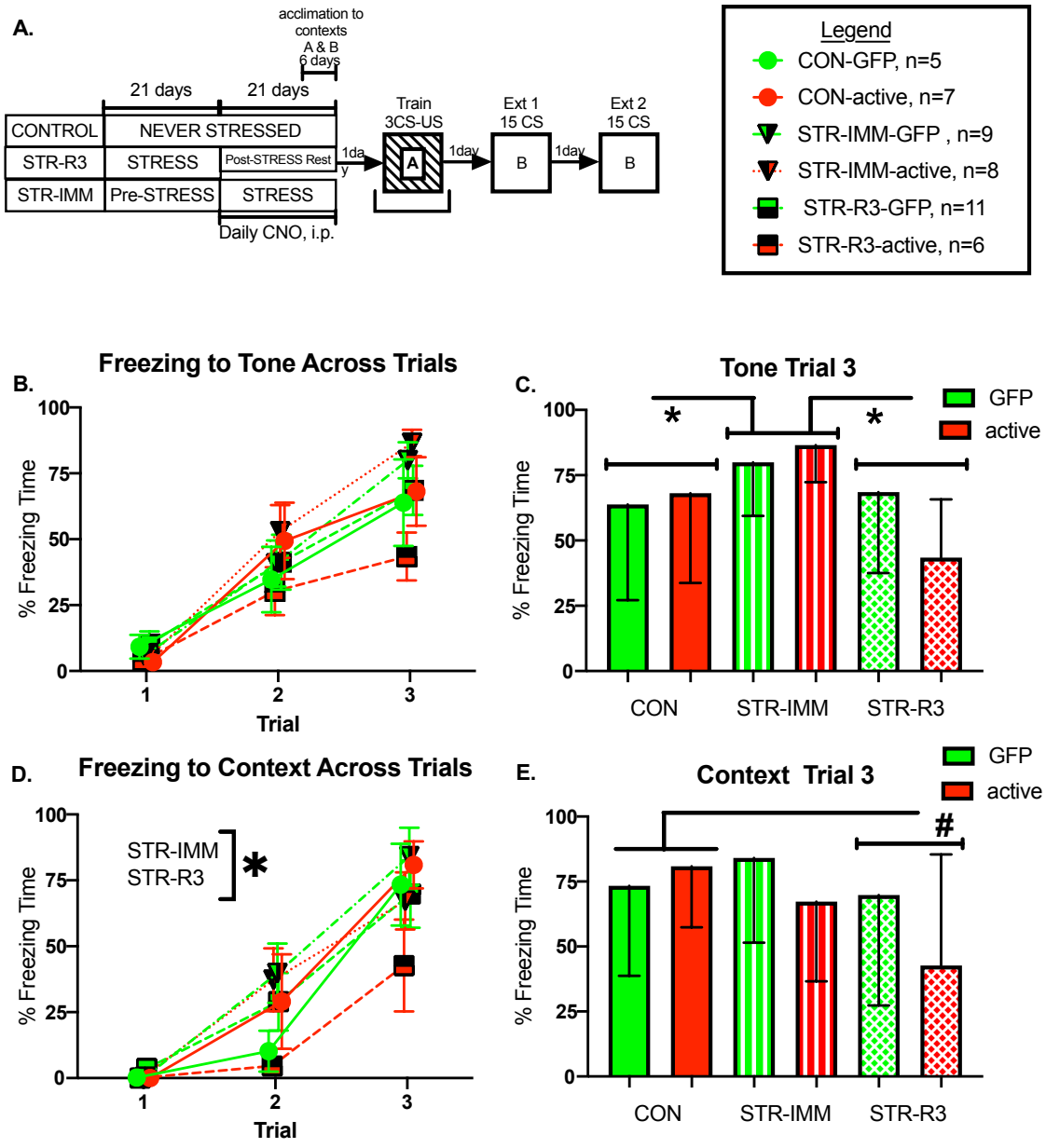

Figure 6, Chapter 3, Freezing Responses During Fear Conditioning. (A) Timeline of experiment with the freezing response quantified from the fear conditioning day (hatched box and bracket below it to denote the testing stage illustrated). (B) Groups performed similarly with minimal freezing to tone on the first trial, with increased freezing to tone as trials progressed. (C) Group differences emerged by Trial 3. STR-IMM froze more to the final training tone than CON (* $p<0.05$) and STR-R3 (* $p<0.05$). (D) Groups performed similarly with minimal freezing to context on the first trial, with increased freezing to context as trials progressed. STR-R3 froze less across trials than STR-IMM (* $p<0.05$) (E) By the final context STR-R3 froze less than CON ($p<0.10$).

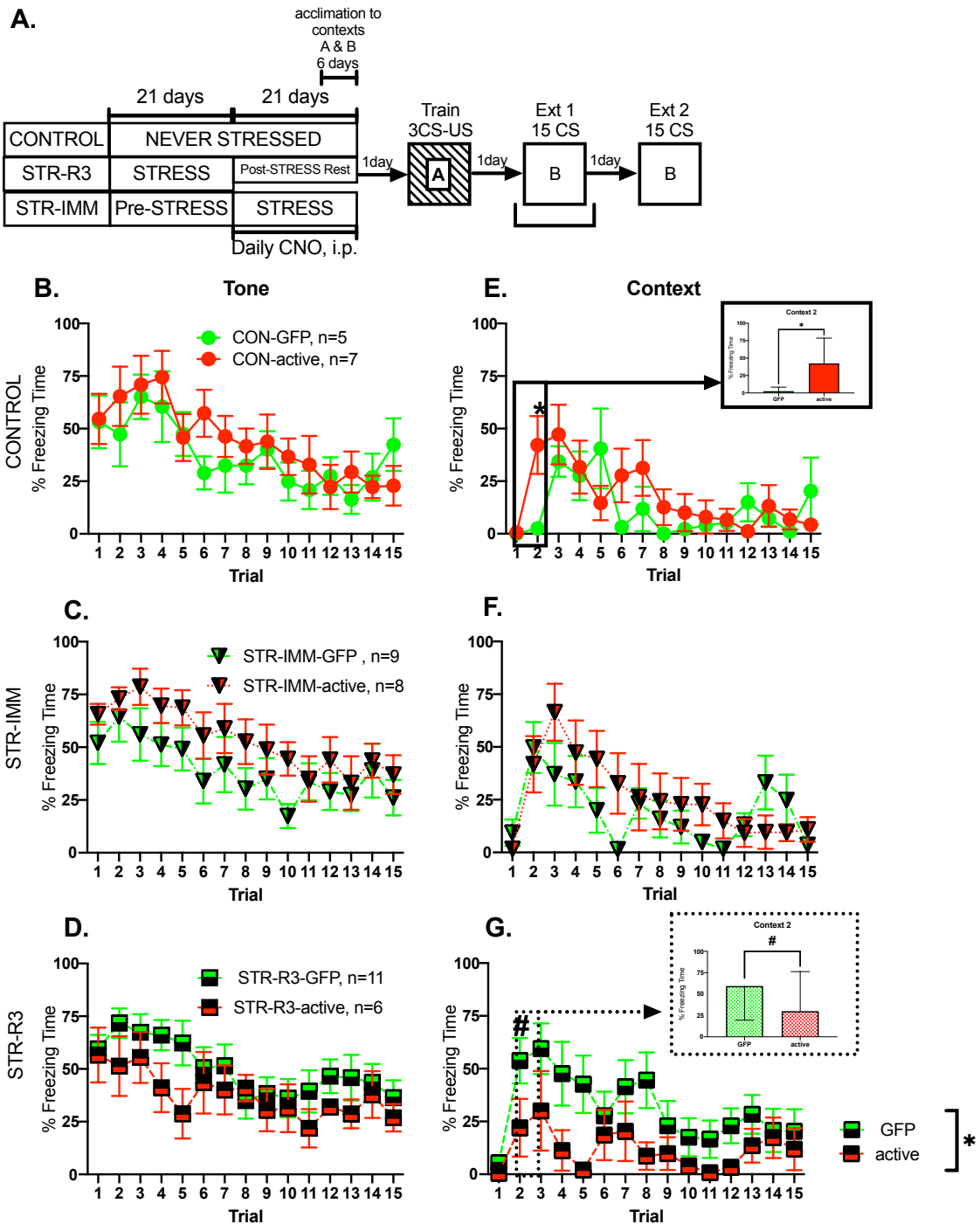
A.
acclimation to contexts A & B 6 days
21 days
21 days
CONTROL
NEVER STRESSED
STR-R3
STRESS
Post-STRESS Rest
STR-IMM
Pre-STRESS
STRESS
Daily CNO, i.p.
1day
Train 3CS-US
A
1day
Ext 1 15 CS
B
1day
Ext 2 15 CS
B
B.
Tone
CONTROL
% Freezing Time
CON-GFP, n=5
CON-active, n=7
Trial
E.
Context
Context 2
GFP
active
C.
STR-IMM
STR-IMM-GFP , n=9
STR-IMM-active, n=8
F.
D.
STR-R3
STR-R3-GFP, n=11
STR-R3-active, n=6
G.
GFP
active

Figure 7, Chapter 3, Freezing to Extinction 1. (A) Timeline of experiment with the freezing response quantified from Extinction 1 (bracket below it to denote the testing stage). (B,C,D) For freezing to tone, there were no initial differences in freezing to the tone and all groups showed a similar decrease in freezing response over the session. (E, F, G) For freezing to context, all groups had similar and low freezing prior to the introduction of the tone (context Trial 1). As trials progressed over Extinction 1, freezing to context decreased in all groups. (E) When examining the early extinction trials, before extinction effects occur, CON-active froze more to context than did CON-GFP on Trial 2. (G) STR-R3-active froze less to context across extinction 1 than did STR-R3-GFP (* $p<0.05$). Moreover, STR-R3-active froze less than did STR-R3-GFP to context on Trial 2 (# $p<0.10$).

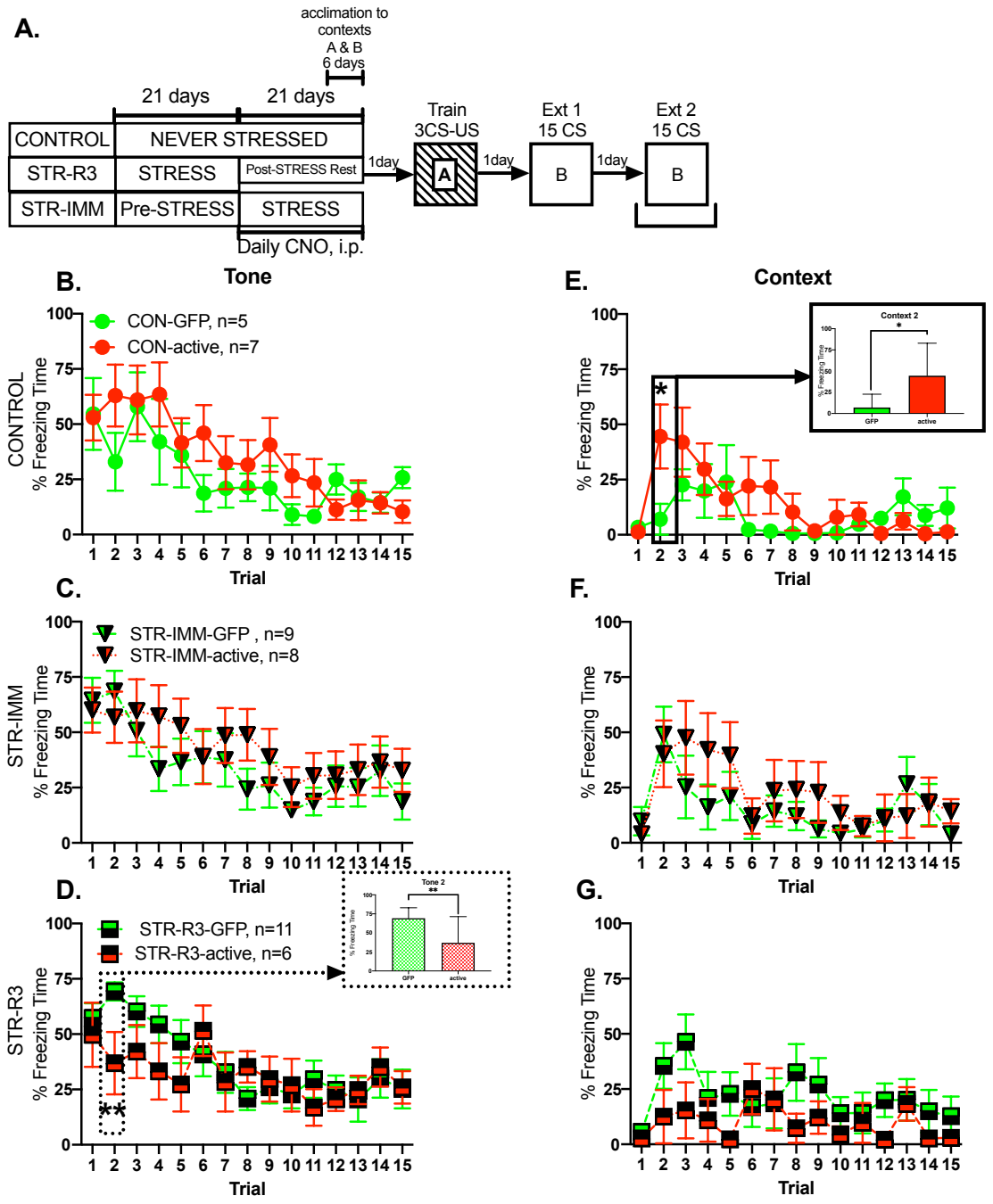

Figure 8. Chapter 3, Freezing to Extinction 2. (A) Timeline of experiment with the freezing response quantified from Extinction 2 (bracket below to denote the testing stage). (B,C,D) For freezing to tone, all groups showed a decrease in freezing response over the session. (D, insert) However, STR-R3-active froze less to tone on Trial 2 than did STR-R3-GFP (** $p<0.01$). (E, F, G) For freezing to context, all groups had similar and low freezing prior to the re-introduction of the tone (context Trial 1). (E, insert) When examining the early extinction trials, before extinction effects occur, CON-active froze more to context than did CON-GFP on Trial 2 (* $p<0.05$).

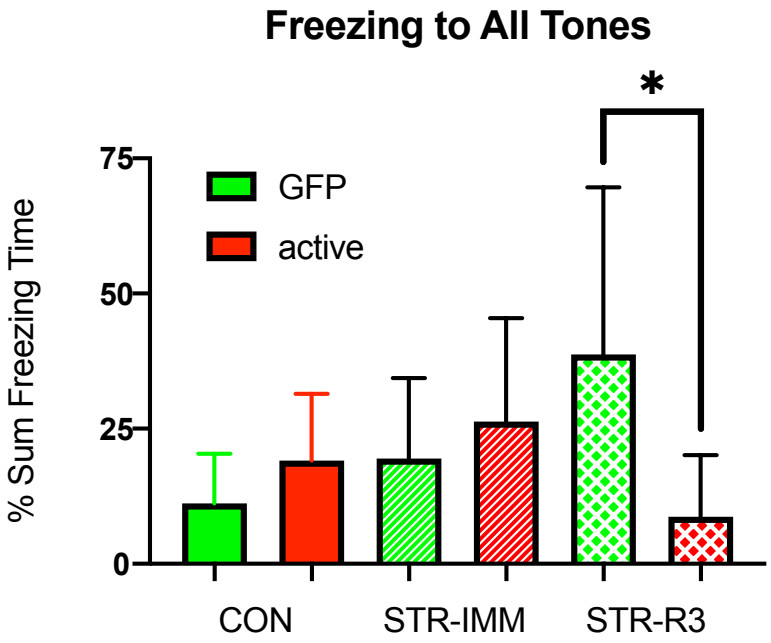

Figure 9, Chapter 3, Freezing During Spontaneous Recovery. Spontaneous recovery to tone was assessed 4-7 days after extinction. When analyzing the sum freezing time to all three tones presented, group differences emerged. STR-R3 interacted with virus type with STR-R3-active freezing significantly less and at chance levels compared to STR-R3-GFP. This low freezing to tone indicated that STR-R3-active failed to show spontaneous recovery and as such, did not form a tone-foot shock association. No other significant group differences were observed. * $p<0.05$ compared to same treatment condition with GFP.

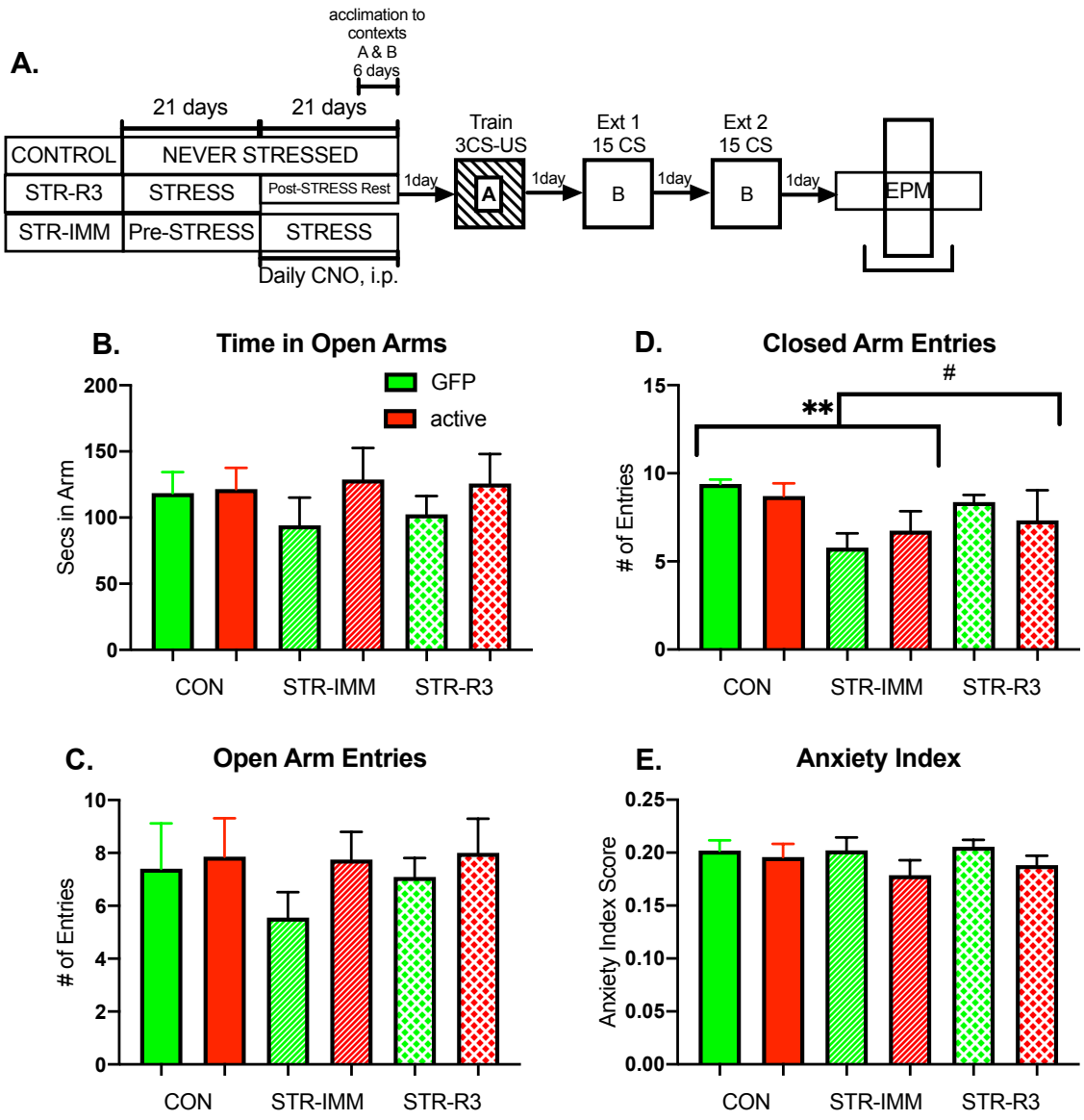

Figure 10, Chapter 3, Elevated Plus Maze. (A) Timeline of experiment with the elevated plus maze session denoted by the bracket below. There were no significant group differences on (B) time spent in open arms or (C) number of entries into open arms. (D) STR-IMM made fewer closed arm entries than CON (** $p<0.01$) or STR-R3 (# $p<0.10$). (E) An anxiety index that employs a calculation to account for both open and closed arm entries revealed no significant group differences.

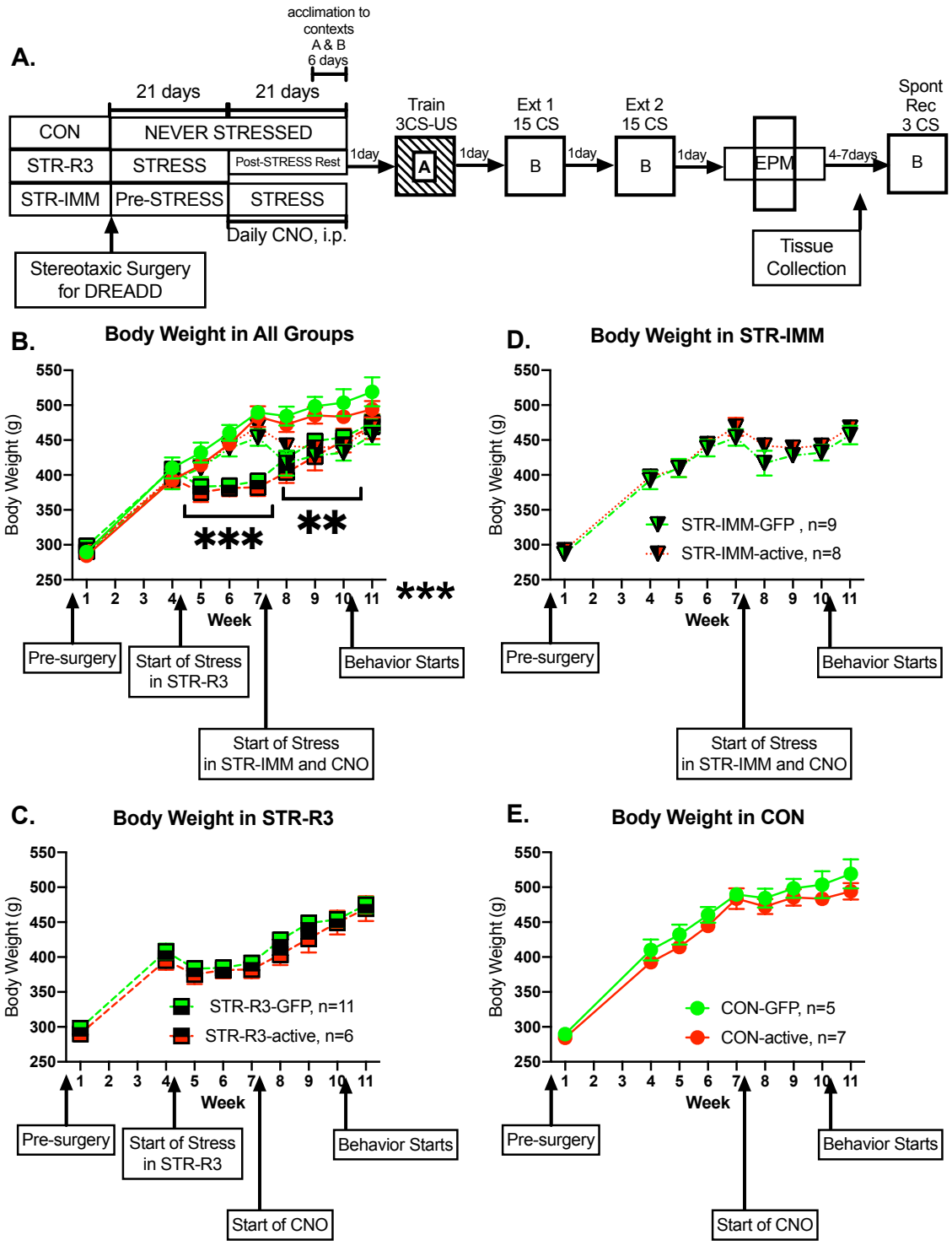
A.
acclimation to contexts A & B 6 days
21 days
21 days
CON
NEVER STRESSED
STR-R3
STRESS
Post-STRESS Rest
STR-IMM
Pre-STRESS
STRESS
Daily CNO, i.p.
Stereotaxic Surgery for DREADD
1day
Train 3CS-US
A
Ext 1 15 CS
B
Ext 2 15 CS
B
EPM
4-7days
Spont Rec 3 CS
B
Tissue Collection
B.
Body Weight in All Groups
Body Weight (g)
Week
***
**
***
Pre-surgery
Start of Stress in STR-R3
Start of Stress in STR-IMM and CNO
Behavior Starts
D.
Body Weight in STR-IMM
STR-IMM-GFP , n=9
STR-IMM-active, n=8
C.
Body Weight in STR-R3
STR-R3-GFP, n=11
STR-R3-active, n=6
Start of CNO
E.
Body Weight in CON
CON-GFP, n=5
CON-active, n=7

Figure 11, Chapter 3, Body Weight Measures. (A) Timeline of experiment for reference on how body weight changes over the course of the experiment. Body weight graphs highlight important timepoints that influence body weight gain, such as the start of chronic stress in a particular group. (B) Body weight increased over the course of the experiment in all treatment groups. (C) For STR-R3, body weight gain was attenuated during restraint compared to STR-IMM and CON (*** $p<0.001$), and was unaffected by virus type. (D) For STR-IMM, body weight gain was attenuated during restraint compared to STR-R3 and CON (** $p<0.01$), and was unaffected by virus type. (E) For CON, body weight gain occurred throughout the experiment and was unaffected by virus type.