Mechanisms of Recovery from Chronic Stress

by

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ABSTRACT

Chronic stress results in functional and structural changes to the hippocampus. Decades of research has led to insights into the mechanisms underlying the chronic stress-induced deficits in hippocampal-mediated cognition and reduction of dendritic complexity of hippocampal neurons. Recently, a considerable focus of chronic stress research has investigated the mechanisms behind the improvements in hippocampal mediated cognition when chronic stress ends and a post-stress rest period is given. Consequently, the goal of this dissertation is to uncover the mechanisms that allow for spatial ability to improve in the aftermath of chronic stress. In chapter 2, the protein brain derived neurotrophic factor (BDNF) was investigated as a mechanism that allows for spatial ability to show improvements following the end of chronic stress. It was found that decreasing the expression of BDNF in the hippocampus prevented spatial memory improvements following a post-stress rest period. Chapter 3 was performed to determine whether hippocampal CA3 apical dendritic complexity requires BDNF to show improvements following a post-stress rest period, and whether a receptor for BDNF, TrkB, mediates the improvements of spatial ability and dendritic complexity in a temporal manner, i.e. during the rest period only. These experiments showed that decreased hippocampal BDNF expression prevented improvements in dendritic complexity, and administration of a TrkB antagonist during the rest period also prevented the improvements in spatial ability and dendritic complexity. In chapter 4, the role of the GABAergic system on spatial ability following chronic stress and a post-stress rest period was investigated. Following chronic stress, it was found that male rats showed impairments on the acquisition phase of the RAWM and this correlated with limbic

glutamic acid decarboxylase, a marker for GABA. In chapter 5, a transgenic mouse that expresses a permanent marker on all GABAergic interneurons was used to assess the effects of chronic stress and a post-stress rest period on hippocampal GABAergic neurons. While no changes were found on the total number of GABAergic interneurons, specific subtypes of GABAergic interneurons were affected by stressor manipulations. Collectively, these studies reveal some mechanisms behind the plasticity seen in the hippocampus in response to a post-stress rest period.

DEDICATION

To my family for your love and emotional support throughout this process. To Anne N. Hoffman, Jennifer M. Colon, and Alex F. Bourne for keeping me sane in the last few months of this dissertation, I couldn't have done it without (grimacing face emoji).

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CHAPTER 1

GENERAL INTRODUCTION

Major depressive disorder (MDD) is a multifaceted mental disorder that affects a significant percentage of the population. Depression is one of the leading causes of disability worldwide (Organization, 2015) with approximately 20% of adults in the United States demonstrating symptomology for MDD (Kessler & Bromet, 2013). The symptoms of MDD include feelings of sadness, emptiness, or hopelessness, loss of interest or pleasure, significant changes in weight or diet, insomnia, psychomotor agitation, fatigue, and feelings of worthlessness; MDD involves drastic changes in affect and cognition (Association, 2013). Patients with MDD are at risk for other adverse health outcomes with higher than average co-morbidity for diabetes mellitus, heart disease, and stroke (Whooley & Wong, 2013). Furthermore, MDD commonly reoccurs with approximately 80% of patients experiencing at least one recurrent episode of MDD in their lifetime, even after successful remittance (Otte et al., 2016). More striking is that approximately 30% of patients do not respond to treatments and fail to recover from MDD (Rush et al., 2006; Thase et al., 2007; Vos et al., 2004). While treatments for MDD have existed for many decades, the resistance to remission and recurrence of MDD emphasize the need to identify novel therapies and treatments (Otte et al., 2016).

In preclinical work, MDD is difficult to mimic, due in part to the heterogeneous nature of the disorder. As such, animal models of MDD can be utilized to understand different neurobiological processes underlying specific symptoms. For example, administering chronic stress in rodents is a prevalent model currently utilized to study

MDD, due in part to the findings that chronic stress can lead to depressive-like behaviors that can be reversed by antidepressants (Willner, 2005). Depressive-like behaviors are tasks that are thought to be a measure of hopelessness and hedonic behaviors. For example, the forced swim test is a task that measures the time an animal spends actively swimming versus passively floating when they are placed in a tube filled with water. Chronic stress leads to rodents spending less time actively swimming and this is thought to reflect behavioral despair (Cryan, Markou, & Lucki, 2002; Nestler et al., 2002), although some may disagree with this interpretation as it may be an effective strategy to float rather than struggle (Nestler & Hyman, 2010). In the sucrose preference tasks, chronically stressed animals consume less sucrose compared to non-stress animals (Willner, Towell, Sampson, Sophokleous, & Muscat, 1987), but there are exceptions (Bai et al., 2014). In these tasks, anti-depressant medications can reverse the depressive-like phenotype, providing validity to use chronic stress in rodents as a model for some symptoms of MDD.

One brain area that is commonly found to be altered in patients with MDD is the hippocampus. The hippocampus is located in the limbic region of the brain and is involved in the formation of episodic and declarative memory (Scoville & Milner, 1957). Furthermore, the hippocampus is rich in receptors for the stress hormone, glucocorticoids (McEwen, Weiss, & Schwartz, 1968), and is involved in the regulation of the hypothalamic-pituitary-adrenal (HPA) axis (Sapolsky, Krey, & McEwen, 1984a). Consequently, the hippocampus is particularly positioned to be sensitive to stress. In depressed individuals, the volume of the hippocampus is reduced (Kaymak et al., 2010; McKinnon, Yucel, Nazarov, & MacQueen, 2009) and this accompanied by hippocampal-

mediated cognitive impairments (Gotlib & Joormann, 2010; Trivedi & Greer, 2014; Vythilingam et al., 2004) and a dysregulated HPA axis (Knorr, Vinberg, Kessing, & Wetterslev, 2010; Stetler & Miller, 2011). In rodents, a period of chronic stress can produce similar morphological and functional outcomes, as chronic stress reliably produces atrophy in the structure of the hippocampus and deficits in hippocampal-mediated cognition (Conrad, 2006; Kim & Diamond, 2002). Specifically, numerous studies found that chronic stress leads to dendritic atrophy in the apical branches of hippocampal CA3 principal neurons (Conrad, LeDoux, Magarinos, & McEwen, 1999; Hoffman et al., 2011; McLaughlin, Baran, Wright, & Conrad, 2005; Pavlides, Watanabe, Magarinos, & McEwen, 1995; Sousa, Lukoyanov, Madeira, Almeida, & Paula-Barbosa, 2000), and deficits on various spatial tasks, such as the Morris water maze (Wright, Lightner, Harman, Meijer, & Conrad, 2006), the radial arm land maze (V. Luine, Villegas, Martinez, & McEwen, 1994), and the radial arm water maze (Hoffman et al., 2011; Mika et al., 2012; Ortiz et al., 2014). Moreover, chronic stress also leads to a dysregulation of the HPA axis (Sapolsky, 1984; Joels, 2004; McEwen, 2007; Jankord, 2008). Collectively, these studies show that chronic stress in rodents can lead to many of the symptom profile observed in patients with MDD. While no one animal model can capture the full milieu of MDD symptomology, chronic stress rodent models are useful to understand the contribution of changes in the hippocampus to the pathology of MDD.

Historically, chronic stress work has focused on the functional, structural, and molecular changes in the hippocampus of chronically stressed rodents in order to understand the potential changes in cognitive processing from patients with MDD. The preclinical literature derived from chronic stress in rodents has led to many insights about

the mechanisms that produce poor hippocampal functioning. We know much about the factors by which chronic stress results in a pruning of dendritic arbors in the hippocampus (Watanabe, Gould, & McEwen, 1992), and this coincides with spatial learning and memory deficits (Conrad, 2006). Hippocampal dendritic atrophy is mediated in part by corticosterone, the main glucocorticoid in rodents. For example, chronic administration of corticosterone to rodents results in hippocampal CA3 apical dendritic retraction (Ortiz et al., 2013; Woolley, Gould, & McEwen, 1990) and inhibiting corticosterone in chronically stressed rats prevents this effect (Magarinos & McEwen, 1995). Moreover, chronic stress results in a downregulation of receptors for GCs in the hippocampus (Kitraki, Karandrea, & Kittas, 1999; Sapolsky, Krey, & McEwen, 1984b), leading to less negative feedback on the HPA axis from the hippocampus and resulting in prolonged glucocorticoid exposure following a stressor (Mizoguchi, Ishige, Aburada, & Tabira, 2003; Sapolsky et al., 1984a). The hippocampal excitatory/inhibitory tone becomes skewed towards hyperexcitability after chronic stress or prolonged corticosterone exposure (Gronli et al., 2007; Joels et al., 2004). Altering excitatory neurotransmission (Magarinos & McEwen, 1995) or lesioning the main excitatory input into the hippocampus (Sunanda, Meti, & Raju, 1997) can prevent stress-induced hippocampal dendritic atrophy. The GABAergic tone can also be manipulated, by enhancing hippocampal GABAergic activity via a benzodiazepine to block chronic stress-induced dendritic retraction (Magarinos et al., 1999). Reports support these outcomes as chronic stress decreases the concentration of GABA in the hippocampus (Gronli et al., 2007), increases the concentration of glutamate (Joels et al., 2004), and decreases the expression of markers for GABAergic neurons in the hippocampus (Czeh et

al., 2015). Brain derived neurotrophic factor (BDNF), a protein that is important for synaptic, morphological, and cognitive plasticity, can also impact hippocampal CA3 dendritic retraction. Chronic stress decreases BDNF in the hippocampus (Gourley, Kiraly, Howell, Olausson, & Taylor, 2008; Lakshminarasimhan & Chattarji, 2012; Murakami, Imbe, Morikawa, Kubo, & Senba, 2005; Smith, Makino, Kvetnansky, & Post, 1995) and administering BDNF directly into the hippocampus can prevent chronic stress-induced deficits in spatial ability (Radecki, Brown, Martinez, & Teyler, 2005). Furthermore, BDNF overexpression in the hippocampus can completely occlude stress-induced effects on hippocampal dendritic complexity (Govindarajan et al., 2006). In BDNF haploinsufficient mice, hippocampal dendritic complexity is reduced in the brains of unstressed mice, but these mice are unresponsive to a period of chronic stress as dendritic complexity is not further reduced following chronic stress (Magarinos et al., 2011). It would appear that BDNF is necessary for stress-induced changes to occur, and without BDNF, the brain becomes rigid or reduces its capacity to show plasticity. Clearly, much is known about the mechanisms producing hippocampal morphological changes and cognitive dysfunction in rodents soon after they are exposed to chronic stress. However, in the context of MDD, understanding how these stress-induced changes can be negated or improved following chronic stress is a critical step in helping find ways in which to ameliorate the disorder. As such, studying how rodents can show resilience to chronic stress, or how individuals recover from a period of sustained stress is a promising step forward.

The discussion up until this point referred to chronic stress effects on the hippocampus that have been reported during or soon after the chronic stress manipulation

has ended. But as days and weeks pass from the end of chronic stress, the hippocampus and cognitive outcomes show marked plasticity. Specifically, the chronic stress-induced decreases in hippocampal dendritic complexity and deficits in spatial ability can improve with the passage of time (usually several weeks in rodents) without interventions. This period of time is often referred to as a "recovery period", due to the improvement observed in the hippocampus that appear to recover to levels of non-stressed controls and as such, we have published using this terminology (Hoffman et al., 2011; Ortiz et al., 2014; Ortiz et al., 2015). But as we are learning more about this process, "recovery" implies that the system has returned to its previous state. While that may be the case in some situations, we will use the term "rest" because it avoids assumptions and accurately represents that the animals had an opportunity to "rest" following the end of chronic stress. The mechanisms underlying the changes that occur in the hippocampus during the post-stress rest period are gaining considerable interest. Understanding the processes that occur during the rest phase after chronic stress has ended will add critical new information for us to understand ways in which these adaptive plasticity mechanisms can be promoted. In the context of MDD, investigating these processes occurring during the post-stress rest period may help us to better understand how to increase the resilience of individuals and discover novel ways to treat the disorder.

The first study investigating the post-stress rest period found that the hippocampus showed structural changes with the passage of time after chronic stress ended. This report documented that 6 hours of daily restraint for three weeks led to a decrease in the number and length of apical dendritic arbors in the hippocampal CA3c region, and that when ten days elapsed from the end of chronic stress, the apical dendritic arbors increased in

complexity and were statistically similar to the non-stressed controls (Conrad et al., 1999). Rats that were given four days of rest following the end of chronic stress still showed CA3c apical dendritic retraction (Conrad et al., 1999). This report established a timeline for this phenomenon, by demonstrating that chronic stress-induced hippocampal CA3 apical dendritic retraction persists for at least four days, but from five to ten days, the CA3 dendritic arbors increased in complexity. This study was the first to show that chronic stress effects on dendritic complexity are not permanent, and that the hippocampus has the capacity to display plasticity even after chronic stress. In the following year, Sousa and colleagues (Sousa et al., 2000) observed that following 30 days of chronic unpredictable stress, the apical dendrites from the CA3b-c subregion of the hippocampus and the terminal segments of apical dendrites from the CA1 subregion showed significant atrophy. Moreover, these effects on the hippocampal dendritic arbors were accompanied by spatial learning and memory impairments in the Morris Water Maze (Sousa et al., 2000). The novelty of this work was that when the animals were provided with a post-stress rest period of 30 days, both structural and functional measures improved: CA3 dendritic arbors enhanced in complexity and spatial ability was improved. Consequently, this study was the first to demonstrate that post-stress rest period allowed for the cognitive deficits in response to chronic stress to improve and that the spatial memory improvements were concurrent with the dendritic structural improvements. Another novel feature was that these stress-induced outcomes could be reproduced with corticosterone treatment, the predominant stress steroid in rodents (Sousa et al., 2000). An important feature of the Sousa study showed that chronic stress-induced impairments in hippocampal-dependent spatial ability can improve in the weeks

during the post-stress rest phase. One of the first reports looking at spatial learning in the weeks after chronic stress has ended observed that spatial ability on a land version of the radial arm maze was no different than that of non-stressed controls (V. Luine et al., 1994). Since then, the finding that spatial memory deficits produced from chronic stress can be improved following a post-stress rest period has been reported in many laboratories using a variety of spatial tasks, such as the Morris Water Maze (Bian et al., 2012; McFadden et al., 2011; Sousa et al., 2000), radial arm water maze (Hoffman et al., 2011; Ortiz et al., 2014; Ortiz et al., 2015), and object placement (Luine, 2002). These studies led to an interest in the structural and cognitive plasticity that occurs in the brain following a period of prolonged stress, and whether stress-induced modifications persist beyond the end of stress.

These studies laid the groundwork for many others investigating the changes in the structural plasticity of neurons throughout the brain, and it is now known that the structural plasticity of neurons in the aftermath of chronic stress is not unique to the hippocampus. Many studies have replicated the findings that CA3 dendritic arbors can increase in complexity in the rest period after chronic stress has ended (Hoffman et al., 2011; B. S. S. Rao, Madhavi, Sunanda, & Raju, 2001). Moreover, several studies report structural changes in the CA1 region as well. Sousa and colleagues (Sousa et al., 2000) found that chronic unpredictable stress decreased the terminal length of hippocampal CA1 pyramidal neurons, an effect that returned to non-stress control levels following a post-stress rest period. Another study found that chronic administration of corticosterone in the drinking water led to a decrease in the number and the length of basal branches in CA1 pyramidal neurons and decreased the number of spines on basal branches in the

CA1 region, but these effects did not improve after a washout period (Gourley, Swanson, & Koleske, 2013; Shapiro, Omar, Koleske, & Gourley, 2017). Moreover, the improvement of stress-induced dendritic complexity occurs in other brain regions besides the hippocampus, such as the prefrontal cortex (Bloss, Janssen, McEwen, & Morrison, 2010; Goldwater et al., 2009; Radley et al., 2004). In contrast, the amygdala shows dendritic hypertrophy in response to chronic stress, and this affect is sustained following a post-stress rest period (Vyas, Pillai, & Chattarji, 2004). Taken together, these studies show that many regions of the brain, in addition to the hippocampus, display structural plasticity in the face of chronic stress and maintain this plasticity in the weeks following the end of chronic stress.

Since the first study on CA3 dendritic morphology in 1999 by (Conrad et al., 1999) and the spatial memory outcomes in 2000 by (Sousa et al., 2000), the outcomes following a post-stress rest period were thought to exhibit an organism's return to the non-stress condition. However, recent work is making it increasingly apparent that changes in the hippocampus following a post-stress rest period are not simply a reversal of the chronic stress effects. A study by Gray and colleagues (Gray, Rubin, Hunter, & McEwen, 2014) found that chronic stress leads to significant changes in gene expression in the hippocampus compared to a non-stressed group, with approximately 700 genes showing significant changes in expression. Furthermore, in chronically stressed animals given a post-stress rest period, hippocampal gene expression was also significantly different than controls with approximately 700 gene expression changes. Interestingly, an analysis of the 700 gene expression changes that occurred in both the chronic stress and post-stress rest groups showed that only 36 of the impacted genes overlapped. This indicated that the

majority of the changes in hippocampal gene expression occurring in response to chronic stress and a post-stress rest period were distinct (Gray et al., 2014; Kachemov et al., 2015). Another example of a post-stress rest period being different than a non-stressed state was demonstrated in the infralimbic cortex. At the structural level, infralimbic neurons show apical dendritic retraction mostly at the distal apical tree (Goldwater et al., 2009). Following a post-stress rest period, dendritic complexity becomes a statistically similar to that of non-stressed controls, but a closer look at dendritic branching shows that this increase in dendritic complexity occurs primarily in apical dendrites most proximal to the cell body (Goldwater et al., 2009). Consequently, the total measure of dendritic complexity was similar, but a more detailed level of analysis demonstrated that the post-stress rest condition differed than the non-stress control group. Together, these studies suggest that changes that occur during the post-stress rest period do not reflect a return to baseline conditions, but instead reflect novel processes. Understanding these processes may increase our understanding of how to increase brain plasticity following a period of prolonged stress, and in the context of MDD, may lead to novel targets and treatments for the disorder.

The goal of my dissertation was to investigate the mechanisms underlying the ability of spatial cognition to show improvements in the weeks after chronic stress ends, with a particular focus on the improvement in hippocampal CA3 dendritic morphology and its correspondence with spatial ability. The overarching hypothesis of the studies presented here is that BDNF and the TrkB receptor interact with the GABAergic system to promote the neuroplastic changes in the hippocampus using a post-stress rest paradigm. In chapter 2, hippocampal BDNF levels were downregulated prior to and

throughout the chronic stress and post-stress rest period and then spatial ability was assessed. The goal was to determine whether BDNF is necessary for the improvements of spatial ability following a post-stress rest period. The next step was to determine whether spatial ability following chronic stress and BDNF downregulation was mediated by hippocampal CA3 dendritic complexity. As such, the experiments in chapter 2 were followed up by a series of experiments outlined in chapter 3 where we utilize the same techniques and timelines as in chapter 2 and assessed hippocampal CA3 principal cell dendritic complexity. Furthermore, whether the decrease in hippocampal CA3 BDNF expression increased the vulnerability of CA1 neurons was investigated. As these studies addressed the importance of hippocampal BDNF for the post-stress rest period, we next wanted to determine whether the receptor for BDNF, TrkB, played a role and more specifically to determine its involvement during the rest period only. Therefore, chapter 3 also assessed the impact of administration of a TrkB antagonist during the post-stress rest period on spatial ability and hippocampal dendritic morphology. We then asked whether the GABAergic system was involved in the improvements of spatial ability following a post-stress rest period by investigating whether the synthesizing enzyme for GABA, glutamic acid decarboxylase (GAD65), is affected by chronic stress and a post-stress rest period, in chapter 4. While the prior chapters focus on the impact of chronic stress and a post-stress rest period in male rats, chapter 4 investigates whether there are sex differences in the response to a post-stress rest period. Finally, chapter 5 was conducted to address the role of GABAergic neurons in the post-stress rest period. We used a transgenic mouse, that expresses a permanent marker on all GABAergic neurons, to address a discrepancy in the literature of whether hippocampal GABAergic interneurons

are decreased following chronic stress. Additionally, while other studies show that various subtypes of GABAergic cells are impacted by chronic stress, we wanted to determine how a post-stress rest period affect these hippocampal GABAergic cell types. With these studies, we aim to improve our understanding of the behavioral, morphological, and molecular changes that occur following a post-stress rest period with the goal of identifying mediators that are critical to improve the resilience of individuals in the face of stressors.

CHAPTER 2

HIPPOCAMPAL BDNF MEDIATES RECOVERY FROM CHRONIC STRESS-INDUCED SPATIAL REFERENCE MEMORY DEFICITS

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Contributions: JBO and CDC designed the project. JBO led the project as follows: JBO, CMM, ANH performed surgeries, stressor manipulations, behavioral testing. JBO, CMM processed brain tissue and performed protein assays. PDH and JBO performed protein assays. EFT supplied viral vectors and interpretation. JBO and CDC analyzed the data and wrote the manuscript.

Introduction

Chronic stress impacts many structures in the brain with the hippocampus being particularly vulnerable. The hippocampus contributes to spatial learning and memory (Devan, Goad, & Petri, 1996; Eichenbaum, 1999), which is compromised following

chronic stress (Conrad; Conrad, Galea, Kuroda, & McEwen, 1996; E. Kitraki, O. Kremmyda, D. Youlatos, M. Alexis, & C. Kittas, 2004a; Song, Che, Min-Wei, Murakami, & Matsumoto, 2006; Wright et al., 2006). When chronic stress impairs spatial learning and memory, alterations in the architecture and physiology of the hippocampus often co-occur (reviewed in (Conrad)), as shown by hippocampal CA3 apical dendritic retraction (Hutchinson et al., 2012; McLaughlin, Gomez, Baran, & Conrad, 2007; Watanabe, Gould, & McEwen, 1992) and blunted long-term potentiation (Radecki et al., 2005), a form of neuronal plasticity that models learning and memory processes (Bliss & Collingridge, 1993). Together, these studies support the interpretation that chronic stress disrupts hippocampal function and the corresponding synaptic plasticity.

When chronic stress ends, the stress-induced spatial memory deficits recover in the following weeks, as shown in tasks, such as the Morris water maze (Sousa et al., 2000), radial arm maze (V. Luine et al., 1994), and radial arm water maze (Hoffman et al., 2011). Moreover, the chronic stress-induced alterations in the architecture of the apical CA3 dendrites follow a similar time course of restructuring over the recovery period (Hoffman et al., 2011; Sousa et al., 2000). These studies reveal that even in adulthood, organisms display remarkable behavioral and morphological plasticity in the hippocampus during the recovery from chronic stress.

Brain derived neurotrophic factor (BDNF) may contribute to the recovery from stress-induced spatial learning and memory deficits. BDNF is a neurotrophin that influences growth, differentiation, and survival of neurons in developing and adult brains (Karatsoreos & McEwen, 2013; Lu, Yang, Liu, Li, & Luo, 2005; Numakawa, Adachi, Richards, Chiba, & Kunugi, 2013). BDNF and its receptor, tropomyosin receptor kinase

B (TrkB), are especially concentrated in the hippocampus (Conner, Lauterborn, Yan, Gall, & Varon, 1997; Drake, Milner, & Patterson, 1999; Gray, Milner, & McEwen, 2013). Importantly, TrkB regulates neuronal morphology and synapse formation and plasticity (An et al., 2008; Levine, Dreyfus, Black, & Plummer, 1995; McAllister, Katz, & Lo, 1996, 1999; Verpelli et al., 2010), outcomes that are diminished with reduced BDNF expression (Bartoletti et al., 2002; Ninan et al., 2010). Within the hippocampus, chronic stress decreases BDNF expression (Lakshminarasimhan & Chattarji, 2012; Murakami et al., 2005; Smith et al., 1995), which contributes to poor hippocampal plasticity and function, such as impaired spatial learning and memory. These functional deficits can be prevented by BDNF infusions into the hippocampus before and throughout the stress paradigm (Radecki et al., 2005). Whether hippocampal BDNF plays a role in the recovery from stress-induced deficits in spatial ability remains to be determined.

The current study used RNA interference to temporally and spatially down-regulate hippocampal BDNF to investigate this consequence on spatial learning and memory following chronic stress and a post-stress recovery period. The dorsal hippocampal CA3 region was targeted because it is one of the first hippocampal areas to express chronic stress-induced dendritic retraction (Magarinos & McEwen, 1995) and these changes correspond to spatial memory deficits (Conrad, 2006). We hypothesized that hippocampal BDNF expression mediates spatial learning and memory following chronic stress and a post-stress recovery period.

Materials and Methods

This experiment was conducted in accordance with federal guidelines as described by the Guide for the Care and Use of Laboratory Animals (National Academy of Sciences, National Research Council, Institute of Laboratory Animal Resources on Life Science, Washington, D.C., 2011) and institutional guidelines set forth by the Arizona State University Institutional Animal Care and Use Committee. The Arizona State University Institutional Animal Care and Use Committee approved the procedures used in the study.

Subjects

Seventy-two young-adult male Sprague-Dawley rats approximately two months of age were purchased from Charles River Laboratories (Wilmington, MA, USA) and housed at Arizona State University housing facilities. All rats were paired housed, on a reverse light cycle (12:12, lights off at 0600), for the duration of the study in a colony room that contained animals in the same treatment condition (i.e. unstressed controls were housed with unstressed controls, stressed rats given a post-stress recovery period were housed with other rats given a post-stress recovery period, etc.). Water and food were available *ad libitum*, except during the restraint procedures. After arrival, rats were given one week to acclimate prior to surgery. Rats were weighed weekly throughout the duration of the experiment. All behavioral testing was conducted during the dark phase of the light cycle.

Surgery

One week after acclimation, all rats underwent stereotaxic surgery targeting the CA3 region of the dorsal hippocampus. The surgeries were performed under aseptic sterile conditions. In each case, rats were anesthetized with a ketamine cocktail (1ml/kg, i.p., 70 mg/kg ketamine, 6 mg/kg xylazine, 10 mg/kg acepromazine, in 0.9% sodium chloride), received a dose of the local anesthetic Marcaine (bupicvicaine, 0.25% Marcaine with sterile 0.9% sodium chloride) under the incision line 30 minutes prior to surgery (subcutaneous, dose of up to 0.4 mL), and a dose of the non-steroidal anti-inflammatory drug Meloxicam 30 minutes prior to surgery (subcutaneous, 1.0mg/kg of 5mg/mL of Meloxicam). Rats received boosters of the ketamine cocktail (0.5ml/kg) throughout the surgery. When the rats no longer responded to a foot pinch, they were placed on the stereotaxic apparatus (David Kopf Instruments, Tujunga, CA, USA) and the head was secured with a nosepiece and ear bars. The scalp was scrubbed with 2% chlorhexidine acetate surgical scrub (Fort Dodge Animal Health, Fort Dodge, IA, USA) and an incision was made along the midline of the scalp. The skin was held with tissue forceps while lambda and bregma were located and then leveled to zero. Glass Hamilton syringes (5 μL, National Scientific Company, Rockwood, TN, USA) were used to infuse viral vectors. Six injections (three in each hemisphere) each consisting of approximately 0.2 μL of the viral vector targeted to the CA3 region of the dorsal hippocampus, using coordinates from (Paxinos & Watson, 1997) as follows: In mm from Bregma: anterior/posterior (A/P): -3.3, Medial/Lateral (M/L): ±3.0, Dorsal/Ventral (D/V): -3.9; A/P: -3.8, M/L: ±3.4, D/V: -4.0; A/P: ±4.3, M/L: +4.0, D/V: -4.2. Viral vectors were infused over the course of three minutes, and syringes were left in place for five minutes

after each infusion. After the last injection, the incision was sutured using coated vicryl sutures (Ethicon, Inc., Somerville, NJ, USA) and swabbed with triple antibiotic ointment (E. Fougera & Co, Melville, NY, USA). Rats were placed under a heated lamp until they awoke. Rats were then individually housed for approximately three days until the scalp incision healed. During this healing period, rats received post-operative doses of Meloxicam for pain management as well as triple antibiotic ointment over the wound to reduce the risk of infection. Rats were then returned to their original cage mate for the remaining duration of the study.

Viral Vectors

An adeno-associated viral (AAV) vector packaged with an shRNA directed against BDNF, as well as a separate transcription cassette for enhanced green fluorescent protein (eGFP; together, abbreviated throughout the manuscript as 'shRNA') was used to knock down hippocampal BDNF levels. AAVs were specifically selected because of their documented history of successful transfection of neurons without producing neurotoxicity (Howard, Powers, Wang, & Harvey, 2008; Royo et al., 2008; Tenenbaum et al., 2004). Moreover, past work with this AAV showed no significant differences in protein levels of microtubule-associated protein levels or other growth factors in experimental treatments when manipulating BDNF (Sadri-Vakili et al., 2010). A murine U6 Pol III promoter controlled the shRNA. The eGFP sequence was transcribed by a CMV-IE element. The shRNA sequence used was 5'-ACCATAAGGACGCGGACTTGT-3'. This shRNA sequence targeted BDNF exon IX, which is common to all BDNF transcripts (from the National Center for Biotechnology

Information, reference sequence NM_012513.3). A viral vector delivering a "scrambled" shRNA coding sequence with no correspondence to any known rat mRNA and the coding information for eGFP (abbreviated throughout the manuscript as 'Scr') served as a control. The vectors were packaged in plasmids that provided AAV2 replicase and AAV-rh10 capsid functions, and a pHelper plasmid (Stratagene, La Jolla, CA, USA) provided adenovirus helper functions. The three plasmids were co-transfected into AAV-293 cells (Stratagene, La Jolla, CA, USA) at a molar ratio of 1:1:1. Two days post-transfection, the cells were harvested and suspended in Dulbecco's Modified Eagle Medium and intracellular virus particles were freeze-thawed three times in order to release the virus. Afterward, the cells were centrifuged (13,000 rpm for 10 min), and the supernatant was stored at 80 °C. Viruses were titered by real-time PCR (ABI Prism 7700 Sequence Detection System, PerkinElmer Applied Biosystems, Foster City, CA, USA). The average titer was approximately 10^{12} DNase Resistant Particles/ml. AAV-rh10-mediated expression of transgenes has previously been shown to achieve maximum expression in 1-2 weeks and can last at least 3 months post-transfection (Klein et al., 1998). Furthermore, pilot data from our lab has shown this viral vector decreased hippocampal CA3 BDNF levels in adult male rats by 62.7% ± 0.1% two weeks after transfection; and hippocampal BDNF remained downregulated by 67.2% ± 0.2% six weeks after transfection (Figure 2.1). Successful transfection and expression of eGFP from one of the rats used in the pilot study is shown in figure 2.1.

Figure 2.1. Pilot data on viral vector efficacy.

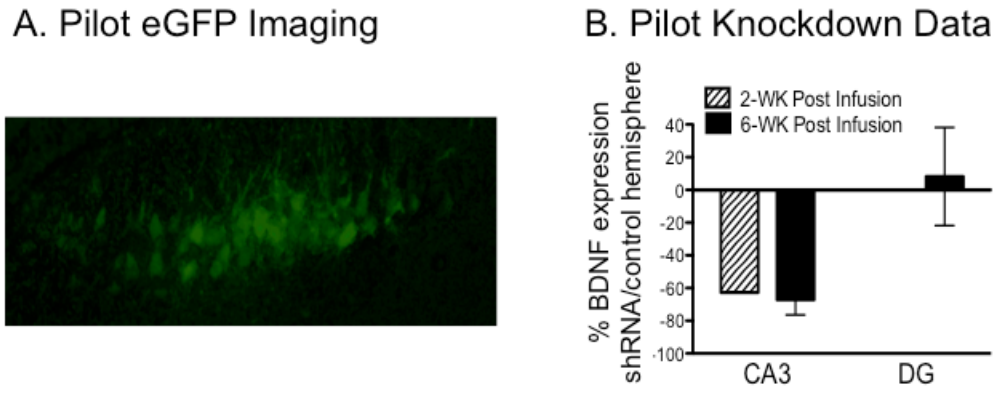

Figure 2.1. Verification of viral vector infusion accuracy and pilot assessment of BDNF knockdown. Neurons that were successfully infected with viral vectors expressed the coding information for green fluorescent protein (GFP). (A) Illustration of GFP-infected cells within the CA3 region of the hippocampus. (B) Preliminary BDNF knockdown data. Rats underwent stereotaxic surgery to target the CA3 region of the hippocampus. One hemisphere was infused with the shRNA against BDNF and compared with the non-infused hemisphere (control hemisphere). 2 weeks (n = 2) and 6 weeks (n = 2) later, a cohort of rats were killed, had their brains removed, and hippocampal CA3 BDNF expression was assessed by ELISA. It was found that the shRNA directed against BDNF knocked down hippocampal BDNF by 62.7% ± 0.1% at 2-week post-infusion and by 67.2% ± 0.2% at 6 weeks post-infusion compared with the control hemisphere.

Group Assignment

Rats were randomly divided into one of six groups, (n=12/group) based upon stress and viral vector condition: non-stressed controls infused with a scrambled virus (Con-Scr), non-stressed controls with the virus delivering the shRNA against BDNF (Con-shRNA), chronically stressed animals given a three-week pre-test recovery period from chronic stress and a scrambled virus (Str-Rec-Scr) or a shRNA against BDNF virus (Str-Rec-shRNA), and chronically stressed animals not given weeks to recover (i.e. tested 'immediately' after the restraint period) and infused with a scrambled virus (Str-Imm-Scr) or infused with shRNA against BDNF (Str-Imm-shRNA).

Chronic Stress Paradigm

Rats were placed in wire mesh restrainers, 18 cm in circumference and 24 cm in length, ends were sealed with grip guard, (Flynn and Enslow, San Francisco, CA, USA). The restrainers were secured at both ends with clips (Black Binder Clips, Staples Inc., Framingham, MA, USA). Rats that outgrew the smaller restrainers were then restrained with larger restrainers (23 cm in circumference and 28 cm in length). The restraint process occurred for 6 hours each day for 21 consecutive days between 09:00 h and 15:00 h. At the same time, the food and water were removed from the controls for 6 hours to keep the food and water access similar across groups.

Radial Arm Water Maze (RAWM)

Apparatus

The RAWM was implemented because of its well documented use in measuring spatial ability in rodents (Diamond, Park, Heman, & Rose, 1999; Hoffman et al., 2011). The RAWM was composed of black polypropylene, with eight symmetrical arms (27.9 cm long x 12.7 cm wide) emanating from a circular center (48 cm diameter). The maze was filled with water and allowed to come room temperature ranging from 20°C - 22°C. The water was rendered opaque with black powder tempera paint. Two testing rooms consisted of several salient extra-maze cues including the door to the room, shelves, heat lamps, and cues made of black and white construction paper located on the walls. Groups were counterbalanced between the testing rooms and were tested by two different experimenters.

Procedure

On the day of testing, a platform was placed at the end of one of the arms and positioned 2.5 cm below the surface of the water. Platform location was counterbalanced across and within groups, but was held constant across all trials for a given rat. Testing took place over three consecutive days between 0900 h and 1300 h. On day one and two of testing rats were given 8 trials (16 total), and one retention trial was administered on day 3. A trial started as soon as the rat was released into an arm that did not contain the platform (start arm), which varied across trials so that the start arm for a given trial differed from the start arm for the previous trial. Also, the start arm was never directly across from the arm that contained the platform to enhance the use of spatial search

strategies. Once the rat found the platform it was allowed to remain on the platform for 15 seconds for spatial localization and was then returned to its testing cage, which was located in the testing room under a heat lamp. If a rat failed to find the platform within three minutes, the rat was gently guided to the platform with a net. After each trial, a net was used to stir the water and collect bedding and feces to prevent rats from using non-spatial cues. In order to avoid exhaustion of the rats during testing, rats were tested in squads; rats from different groups were tested together in squads of six to eight (e.g. two rats from the Con-Scr group were tested with two rats from the Str-Rec-shRNA group and two rats from the Str-Imm-Scr group). Once a rat finished one trial, it was placed back in its testing cage while the other rats in the squad completed that trial. Therefore, the time between each trial for a given rat ranged from 5 minutes to 20 minutes. Reference memory errors were the number of first time entries into arms that did not contain the platform within a given trial. Working memory errors were the number of repeat entries into an arm that did not contain the platform within a given trial (i.e. repeat entries into an arm where a reference memory error was previously committed in the same trial).

Tissue and Brain Processing

Brain extraction

On the third day of RAWM testing, rats received one trial (retention trial) and after the platform was located, were placed back into their testing cage. After 30 minutes had elapsed from when the retention trial was given, rats were transported to a necropsy

room and were deeply anesthetized with isoflurane. When the rats no longer responded to a tail pinch (approximately 30 seconds to one minute), they were rapidly decapitated.

Brain sectioning and tissue collection

The unperfused brains were removed, flash frozen in 2-methylbutane kept chilled with dry ice, and stored in a -80°C freezer. The brains were sectioned and processed to determine BDNF levels in one hemisphere using a BDNF sandwich-style ELISA and to identify eGFP in the other hemisphere, with each side counter-balanced across subjects. Brains were blocked to target the dorsal hippocampus and cut in a series of two coronal sections (at 20 μm) using a cryostat (Microtome HM 500 OM cryostat, kept between -25 and -30°C). When the dorsal hippocampus was visible, a tissue punch (0.5mm diameter) was taken from one hemisphere (right or left counterbalanced across and within groups) and placed in a cold, pre-weighed and labeled Eppendorf tube and stored at -80°C to be later analyzed for levels of BDNF. Tissue punches were taken from the CA1, CA3 and dentate gyrus (DG) subregions of the hippocampus. One set of punches was taken from the anterior dorsal hippocampus and another from the posterior dorsal hippocampus (i.e. a total of two punches per subregion were obtained). Two tissue punches were also taken from the auditory cortex that served as a control for the BDNF assay. After being placed on subbed slides, one set of a series was stained using cresyl violet (Cresyl violet acetate, Gibbstown, NJ, USA) to ensure correct placement of the tissue punches. The sections were defatted, stained, and rehydrated through a series of solutions as follows: Hemo-De (Electron Microscopy Sciences, Hatfield, PA, USA), 100% ethanol to 95% ethanol to 70% ethanol to double distilled water, then stained in 0.5% cresyl violet for 15 minutes,

rehydrated in the reverse order of the solutions, and then coverslipped with permount (Fisher, Fair Lawn, NJ, USA). The second set was coverslipped with Vectashield Hard Set Mounting Medium (Vector Laboratories, Inc., Burlingame, CA, USA) and used to visually inspect eGFP fluorescence under a fluorescent microscope to confirm accuracy of viral injections as well as to confirm expression of viral vector.

BDNF protein levels

Hippocampal BDNF levels were assessed using a commercially available sandwich-ELISA kit (BDNF Emax ImmunoAssay System Kit, Promega Corporation, Madison, WI, USA) according to the manufacturer's instructions. Tissue punches from the CA1 region of the hippocampus were used to optimize the ELISA. It was determined that shearing of the tissue punches via an automatic tissue lyser was insufficient to extract enough protein for the assays. Thus, Dounce homogenizers (Kimble Chase Life Science and Research Products, Vineland, NJ, USA) were used to manually shear the tissue samples. This method of shearing the tissue led to higher levels of extracted protein and produced ELISA results that were within the detectable range. Due to optimizing the techniques with CA1 punches, there was insufficient CA1 sample data. For the remaining regions, 300 µl of hypotonic lysis buffer (0.05M Tris base in ultra-pure water, pH 8.0) containing a protease inhibitor cocktail (Protease inhibitor cocktail set 1, Calbiochem, EMD Millipore, Billerica, MA, USA) was pipetted into microcentrifuge tubes containing the tissue punches and incubated for at least one hour. After, the lysis buffer and tissue punch were transferred to a Dounce homogenizer and manually sheared. A bicinchoninic acid (BCA) protein assay kit (Micro-BCA assay, Pierce, Rockford, IL, USA) was used to

determine total protein concentration of the tissue punches. For the ELISA, samples from each region were assayed in duplicate and counterbalanced between and within groups across plates. The BDNF ELISAs had a sensitivity of 15.6 pg/mL and cross-reactivity with other neurotrophins that was less than 3%, as per kit instructions. Recombinant mature BDNF (supplied by the kit) was used to calculate the standard curve from which the concentration of BDNF in each tissue sample was interpolated. BDNF levels were normalized to total protein concentration for each sample. Data are represented as picograms of BDNF per micrograms of total protein.

Viral vector functional assessment

Transduction was confirmed using fluorescence microscopy to visually inspect for eGFP location. As each viral vector included an eGFP cassette, cells infected with viral vectors expressed eGFP protein and fluoresced at an excitation of 488nm. Vectashield Hard-Set mounting medium (Vector Laboratories, Burlingame, CA, USA) was dispensed on slides containing 20µm thick brain sections. Slides were then coverslipped and visually inspected for eGFP under fluorescence microscopy. To be included in the final analysis, we confirmed that eGFP expression was predominately in the CA3 region of hippocampus. Rats were excluded from the analysis when eGFP expression was outside the CA3 region. Figure 2.2 illustrates the most anterior and posterior eGFP expression within each treatment condition. These strict criteria resulted in a low subject number for the Con-Scr group. Since AAV packaged with the Scr sequence was not expected to alter behavior, a t-test was performed between the excluded and included subjects in the Con-Scr group. No statistical differences were observed in

errors committed in the domains for reference or working memory ($p > 0.2$ for both). Consequently, these originally excluded rats were added to the Con-Scr group to increase the statistical power.

Figure 2.2. Verification of viral vector spread.

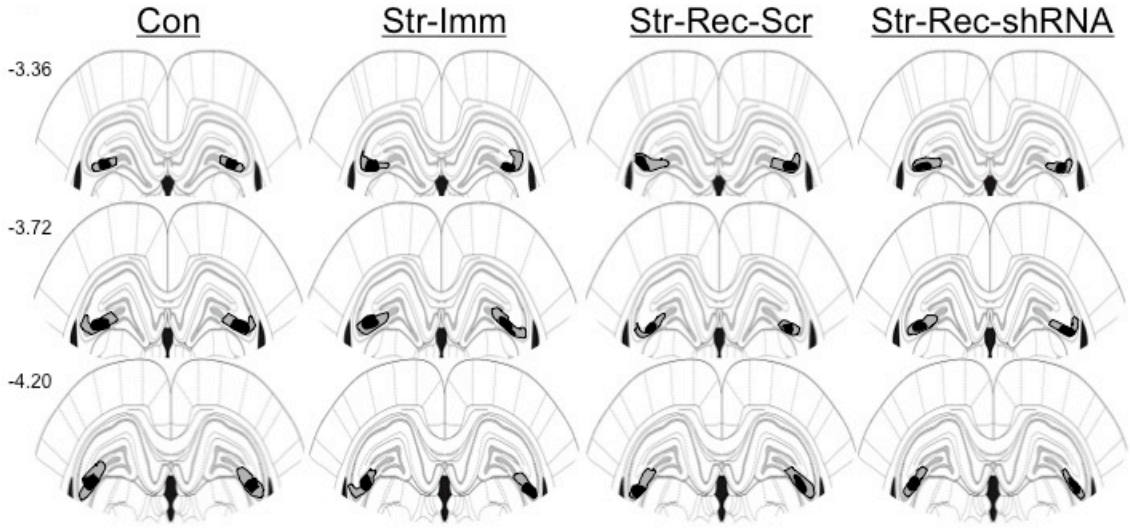

Figure 2.2. Illustration of eGFP spread. Verification of viral vector spread determined by eGFP expression. The illustration shows the most anterior and posterior eGFP expression within each treatment condition. Gray represents the largest spread and black represents the most localized spread.

Synopsis of Experimental Design

At least two weeks after stereotaxic surgery, rats in the Str-Rec group were placed in wire mesh restraints for 6h/d/21d. Matched control rats had their food and water

removed for the same duration as when the Str-Rec rats were in restraints. The matched controls were also picked up briefly each day in order to be consistent with the daily handling required for the stressed rats. The day after the 21st day of restraint for the Str-Rec rats, the restraint process began for the Str-Imm rats for 6h/d/21d. Again, another subset of control rats had their food and water removed for 6h/d to match the food/water restriction occurring with the Str-Imm rats. The day after the last day of restraint for the Str-Imm rats, RAWM testing began for all rats. Testing on the RAWM took place over three days, and on the last day animals were sacrificed thirty minutes after their first and only trial. Trunk blood was collected, as well as the adrenals and brains (Figure 2.3).

Figure 2.3. Timeline of the study.

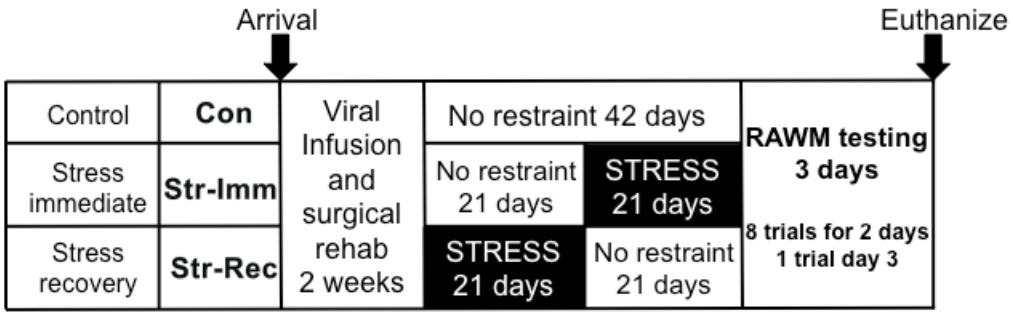

Figure 2.3. Timeline of the study. Rats were infused with viral vectors packed with either the coding information for an shRNA against BDNF or a scrambled sequence, with both viral vectors also delivering a transcription cassette for eGFP. The stress manipulations were started after rats had recuperated from surgery for 2 weeks. Rats in the stress recovery (Str-Rec) group were restrained in wire mesh restrainers for 6 h/day for 21 days, which was then followed by a 21-day recovery period without restraint. The opposite was provided for the rats in the stress immediate group (Str-Imm), in which they

were left in their home cage for the first half of the study and then restrained for 6 h/day for 21 days during the latter half of the paradigm. On the day after the last day of restraint for the Str-Imm group, all rats were tested on the RAWM, which took place over 3 days. After the single retention trial on day 3 of RAWM testing, rats were euthanized and adrenal glands and brain tissue were processed for analysis.

Statistical Analyses

The statistical software package, SPSS (version 19), the graphing and curve fitting software GraphPad Prism, and a Macintosh computer (OS X 10.7.5) were used for the data analyses. A four-parameter logistic non-linear regression model was used for standard curve fitting in the BCA assays and ELISAs. The unknown concentrations of the samples were interpolated based on the curve. The data were transformed to natural logarithms to validate the assumption of homoscedasticity**.** In order to assess the effects of hippocampal BDNF, chronic stress, and post-stress recovery period on spatial learning and memory, we decided, *a priori,* to run comparisons between groups on all factors measured in the study. Thus, omnibus analyses of variance (ANOVAs) were followed by one-way ANOVAs on group assignment and then followed by Fisher's LSD post hoc tests when $p \leq 0.05$. Data are represented by means ± S.E.M.

Results

Viral infection efficacy

The accuracy of tissue punch placement and spread of viral vector infusions was determined by cresyl violet stain and fluorescence microscopy, respectively. Since

viruses contained the coding sequence for eGFP, fluorescence microscopy allowed for visualization of infected cells in all treatment conditions. That is, cells that were infected with either the shRNA or Scr viral vectors fluoresced green. Rats whose brain slices had visible eGFP in the CA3 region of the hippocampus were included in the behavioral analyses. After excluding rats based on these criteria, the final group number per group were: Con-Scr=7, Con-shRNA=8, Str-Imm-Scr=10, Str-Imm-shRNA=10, Str-Rec-Scr=7, Str-Rec-shRNA=8.

Tissue punch assessment

After tissue punches were sampled from the brains of rats, one series of sectioned brain slices were mounted on slides and stained with a cresyl violet to evaluate the accuracy of the punches. Tissue punches were included in the BDNF ELISA if they were localized to stratum pyramidale in the CA3 region for CA3 tissue punches, and the stratum granulosum of the DG for DG tissue punches. Punches that were outside of these regions were excluded from the BDNF assay. Based on these criteria, we achieved an accuracy of 74% for the DG and 76% for the CA3 region. Final group numbers in the BDNF ELISA were: Con-Scr=4, Con-shRNA=4, Str-Imm-Scr=9, Str-Imm-shRNA=10, Str-Rec-Scr=3, Str-Rec-shRNA=7.

Effects of chronic stress and shRNA against BDNF on hippocampal BDNF protein levels

A viral vector delivering the coding information for an shRNA directed against BDNF significantly reduced hippocampal BDNF in the CA3 region, as revealed by a 2 X 3 ANOVA for viral vector contents and stress assignment (significant main effect of viral vector, $F_{(1,31)} = 15.32$, $p < 0.001$. Figure 2.4A). No other effects were found to be significant. CA3 BDNF expression was approximately 4.3 pg of BDNF per μg of total protein in the Str-Imm-Scr and approximately 5.6 pg of BDNF per μg of total protein in the Con-Scr, which reflected a nonsignificant decrease following chronic stress of approximately 23%. To determine whether our non-significant results were due to a lack of statistical power, we conducted a post-hoc power analysis using GPower (Faul, Erdfelder, Lang, & Buchner, 2007) with $\alpha = 0.05$ and power $(1 - \beta)$ set at 0.80, two-tailed. This power analysis indicated that a subject number of 15/group would be required to detect a significant difference between stress and controls in regard to hippocampal CA3 BDNF expression.

BDNF levels in the dentate gyrus and auditory cortex were unaffected by viral vector manipulations. A 2 X 3 ANOVA for viral vector contents and stress condition revealed no significant effects, as all groups had statistically similar BDNF levels in the DG ($p > 0.7$, Figure 2.4B), and auditory cortex ($p > 0.6$, Figure 2.4C).

Figure 2.4. BDNF protein expression.

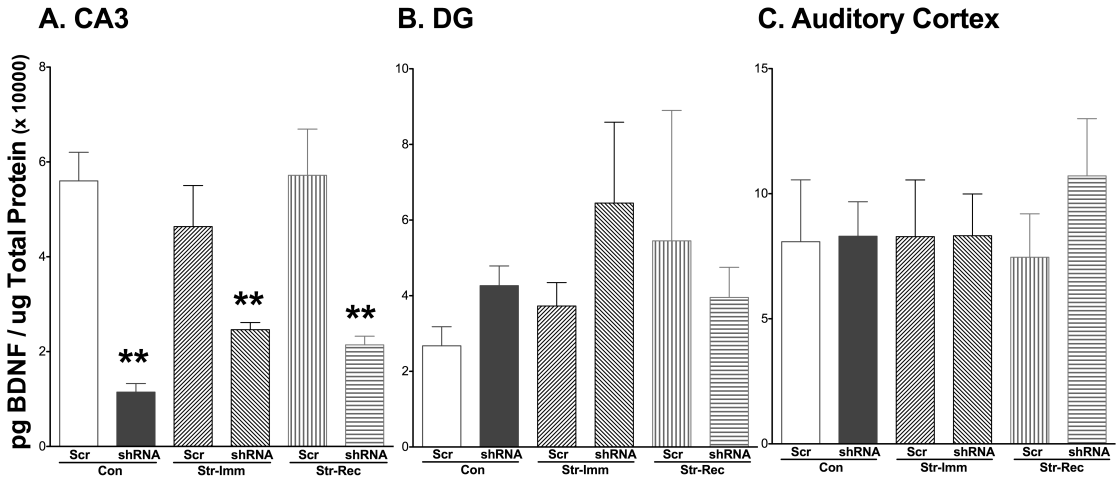

Figure 2.4. BDNF protein levels. Viral vectors packaged with either an shRNA against BDNF or a Scr sequence were injected bilaterally into the hippocampal CA3 region. An ELISA helped to assess BDNF protein levels in the hippocampal CA3 region and DG, as well as the auditory cortex. (A) In the CA3 region of the hippocampus, the viral vector packaged with the shRNA against BDNF decreased BDNF protein levels in all three stress conditions (Con-shRNA, Str-Imm-shRNA and Str-Rec-shRNA). In this paradigm, chronic stress did not significantly lower CA3 BDNF levels (compare Str-Imm-Scr and Con-Scr), although the Str-Imm-Scr mean levels were approximately 31% lower than those observed for the Con-Scr. Viral vectors or chronic stress did not significantly affect BDNF protein expression in the DG (B) or auditory cortex (C). Data are represented as pg of BDNF/μg of total protein X 10,000. ** $p < 0.001$, relative to Scr counterparts.

Effect of chronic stress and downregulated hippocampal BDNF on spatial reference learning and memory

During acquisition on days 1 and 2, all groups made fewer errors across trials in the reference memory domain to demonstrate task acquisition. A 2 × 3 × 8 omnibus ANOVA for viral vector contents (Scr, shRNA), and stress condition (Con, Str-Imm, Str-Rec) across trials (8 trials) revealed significant effect of trial on Day 1 ($F_{(7, 308)} = 9.65$, $p < 0.001$) and Day 2 ($F_{(7, 308)} = 4.86$, $p < 0.001$). At the start of training on day 1, the average errors in the reference memory domain ranged from 2.4 to 3.3 and decreased to around 0.4 to 1.8 by the end of the last training trial on the same day (Figure 2.5A, 2.5B). In addition, Day 2 revealed a significant effect of stress across trials ($F_{(14,308)} = 1.83$, $p < 0.05$) with no other significant effects. Posthoc tests on each trial of day 2 showed that Str-Imm made more errors than did Con on trial 11 ($p < 0.05$), but then Con made more errors than both Str-Imm and Str-Rec on trial 13 ($p < 0.01$). This latter outcome was unusual, given that Con made fewer errors than Str-Imm in a preceding trial. Consequently, trial 13 was probed further and revealed that none of subjects in the Str-Rec-Scr group committed an error, which likely contributed to this effect.

On the single retention trial on the third day, chronic stress significantly impaired reference memory, which was modulated by recovery from chronic stress and viral vector contents. A 2 × 3 ANOVA for viral vector contents and stress revealed a significant main effect of stress ($F_{(2,44)} = 5.05$, $p < 0.05$) without a significant main effect of virus ($p = 0.09$) or interaction on reference memory errors on the single retention trial on day 3. Rats that were chronically stressed and tested soon after restraint ended (Str-Imm) made more errors than did Con rats ($p < 0.005$). These data were probed further using a one-

way ANOVA to investigate our hypothesis that BDNF downregulation would differently affect the Str-Rec group. A significant effect of group was observed for reference memory errors ($F_{(5,44)} = 3.31$, $p = 0.01$). Post Hoc analyses revealed that Str-Imm-Scr and Str-Imm-shRNA made significantly more errors in the reference memory domain than did Con-Scr ($p < 0.05$), Con-shRNA ($p < 0.05$), or Str-Rec-Scr ($p \leq 0.01$). Importantly, downregulation of hippocampal BDNF impaired the recovery from chronic stress-induced spatial memory deficits; Str-Rec-shRNA rats committed statistically more errors in the reference memory domain than did the Con-Scr, Con-shRNA, and Str-Rec-Scr rats (all at $p < 0.05$) and performed similarly as the chronically stressed rats tested immediately (Str-Imm-Scr, Str-Imm-shRNA, Figure 2.5C). The two control groups, regardless of viral vector contents, were statistically similar on the retention trial ($p > 0.9$ for Con-Scr vs Con-shRNA).

Since the first trial on the second day is also a retention trial, an additional analysis was performed for Trial 9 (Figure 2.5B). A 2×3 ANOVA for the factors of viral vector contents and stress assignment revealed no statistically significant effects for stress, virus or the interaction ($p > 0.05$).

Figure 2.5. Errors in the reference memory domain.

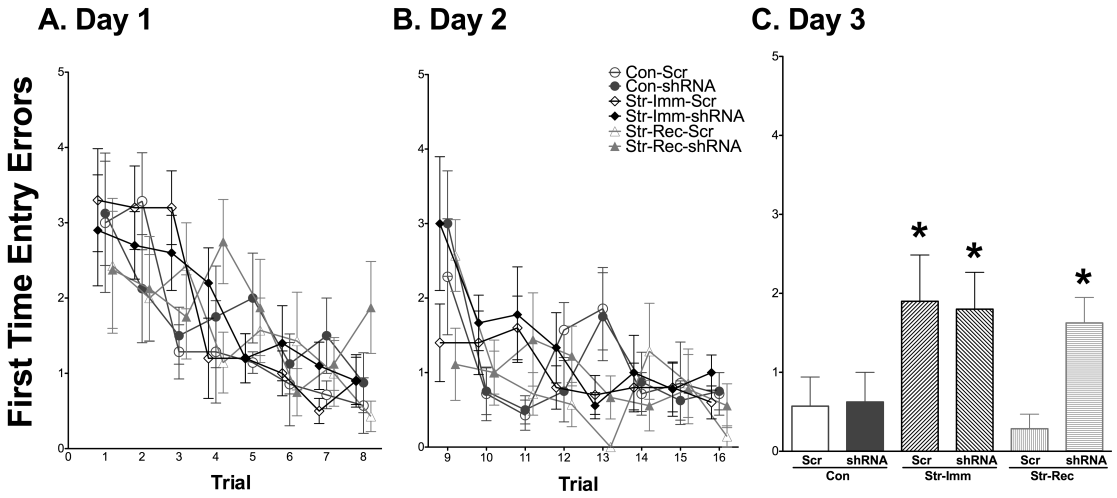

Figure 2.5. Errors within the reference memory domain on the RAWM. The number of errors committed within the reference memory domain was scored as first-time entries into arms that never contained a platform. (A and B) All groups showed that they learned the task and were statistically similar to each other, as demonstrated by decreased entry errors across days 1 and 2. Statistically significant differences were observed on trials 11 and 13, with Str-Imm committing more errors on trial 11 and Con committing more errors on trial 13. (C) During the single retention trial on day 3, statistically significant differences emerged, with rats that were chronically stressed and tested immediately after stressor cessation (Str-Imm) committing significantly more reference memory errors than did the Con-Scr, Con-shRNA, and Str-Rec-Scr groups, regardless of the content of the viral vector (Str-Imm-Scr and Str-Imm-shRNA). Importantly, rats given time to recover (Str-Rec) differed in their performance on the retention trial based upon viral vector contents. Rats infused with the shRNA against BDNF (Str-Rec-shRNA) made a high number of errors in the reference memory domain and were statistically similar to rats in

the Str-Imm group. In contrast, rats in the Str-Rec group infused with the scrambled virus (Str-Rec-Scr) made the fewest errors, on average, in the reference memory domain and were significantly better than Str-Rec-shRNA. #$p < 0.05$ Str-Imm compared to Controls, § $p < 0.05$ compared to Str-Imm and Str-Rec, * $p < 0.05$ compared with controls (Con-Scr and Con-shRNA) and Str-Rec-Scr. Note that data points were shifted along the x-axis to aid in viewing but the timeline is consistent across treatment conditions.

Effect of chronic stress and downregulated hippocampal BDNF on spatial working memory

All groups showed improvement in the working memory domain on the RAWM during acquisition on days 1 and 2. Two separate 3-way repeated measures ANOVA for viral vector contents and stress condition across trials for days 1 and 2 on repeat entry errors committed on the RAWM revealed a significant effect of trial (Day 1, $F_{(7, 308)}$= 9.39, $p<0.001$; Day 2, $F_{(7, 308)}$=7.90, $p<0.001$). The average errors in the working memory domain ranged from 1.7 to 3.6 on the first trial and dropped to 0 to 0.2 by the last trial of the first day, with a similar pattern on day 2 (Figure 2.6A, 2.6B). In addition, Day 2 revealed a significant effect of stress across trials ($F_{(14,308)} = 2.1$, $p < 0.05$) and a significant 3-way interaction for viral vector contents and stress across trials ($F_{(14,308)} = 2.3$, $p < 0.005$) with no other significant effects. The three-way significant interaction was investigated for effects on each trial. On trial 9, which is a retention trial for day 1, the critical comparison was that the Con-shRNA group made more repeat errors than did Con-Scr ($p = 0.01$). For trial 11, Str-Imm-shRNA made more repeat errors than did Con-Scr, Con-shRNA, Str-Rec-Scr, Str-Rec-shRNA ($p < 0.05$), but was statistically similar to

Str-Imm-Scr ($p = 0.1$). In summary, BDNF knockdown briefly disrupted working memory on the retention trial on day 2 for the non-stressed control condition.

On the retention trial for day 3, errors in the working memory domain were not affected by any of the treatments. A 2 × 3 ANOVA for viral vector contents and stress assignment on working memory errors committed in trial 17 revealed no significant effects ($p > 0.5$). Furthermore, a one-way ANOVA for group assignment on errors within the working memory domain during the retention trial also corroborated this outcome ($p > 0.9$; Figure 2.6C).

Figure 2.6. Errors in the working memory domain.

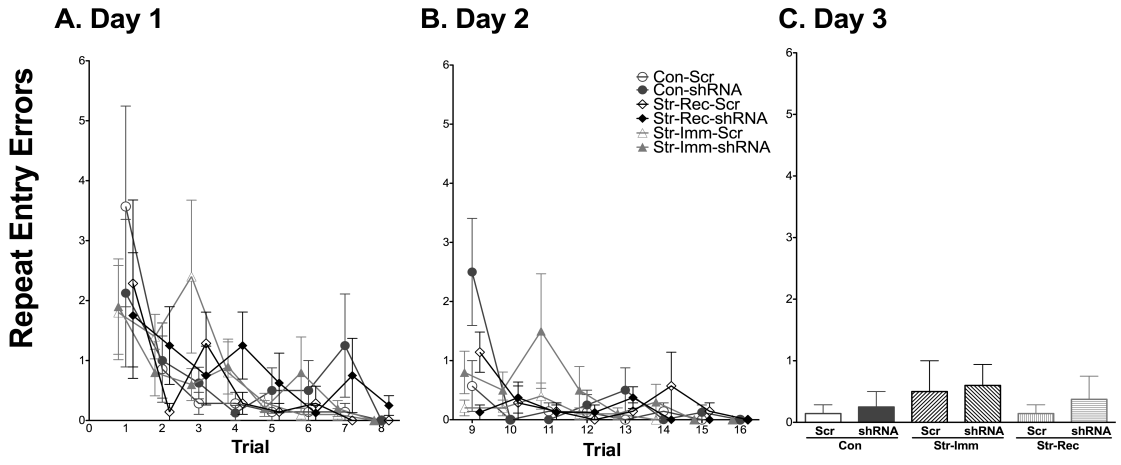

Figure 2.6. Working memory errors on the RAWM. The number of errors committed within the spatial working memory domain were scored as repeated entries into arms that never contained a platform. (A and B) All groups showed improved acquisition that was statistically similar to each other. Specifically, spatial working memory errors decreased across the first 2 days of testing on the RAWM. Furthermore, on day 2 of training, analyses revealed a significant effect of stress and a significant three-way interaction for

viral vector contents and stress across trials. On trial 9, Con-shRNA made significantly more errors than did Con-Scr. On trial 11, Str-Imm-shRNA made significantly more errors than did rats in the Con and Str-Rec groups regardless of viral vector contents, but were statistically similar to Str-Imm-Scr. (C) Similarly, on the single retention trial on day 3, all rats committed statistically similar amounts of errors within the working memory domain. † $p < 0.01$ Con-shRNA compared to Con-Scr, ‡ $p < 0.05$ Str-Imm-shRNA compared to Con-shRNA, Con-Scr, Str-Rec-shRNA, Str-Rec-Scr.

Effects of Chronic Stress and downregulated hippocampal BDNF on physiological measures

Body Weight

Chronic stress significantly altered body weight gain, which was unaltered by the content of the viral vector. A 2 × 3 ANOVA for viral vector contents and stress history on body weight gain from week 1 to 4, and on body weight gain from week 4 to 7 revealed a significant main effect of stress history on body weight gain ($F_{(2,44)}=63.2$, $p < 0.001$; and $F_{(2,44)}=151.6$, $p < 0.001$, respectively. Figure 2.7A). The effect was further probed to investigate differences in body weight gain between groups. Analysis on body weight gain from week 1 to 4, when the Str-Rec rats underwent restraint, showed that Str-Rec rats gained significantly less weight than did the rats in both the Con and Str-Imm groups ($F_{(5, 44)}=25.6$, $p < 0.001$). Likewise, an analysis on body weight gain from week 4 to 7 across groups demonstrated that the rats in the Str-Imm groups gained significantly less weight during their period of restraint than did both the Con and Str-Rec rats ($F_{(5,44)}=61.1$, $p < 0.001$). Rats in the CON group gained weight continuously throughout

the experiment. In contrast, rats in the Str-Rec group lost weight during the three weeks they were subjected to chronic restraint stress, but then gained weight rapidly after restraint ended. For rats in the Str-Imm group, they steadily gained weight during the three weeks they were not placed in restraints, but progressively lost weight during the three weeks that they were chronically restrained. Consequently, restraint slowed body weight gain when it was implemented and rats showed increases in body weight gain during the weeks that they were not restrained (Figure 2.7A).

Adrenal Weights

Restraint stress produced a significant increase in adrenal weights relative to body weights to demonstrate the increased demand on the adrenals. Adrenal weights were analyzed as total adrenal weights per 100 grams of body weight. A 2 × 3 ANOVA for contents of viral vector and stress assignment revealed a significant effect of stress on adrenal weights ($F_{(2, 43)} = 11.63$, $p < 0.001$, Figure 2.7B). Post Hoc analysis showed that rats in the Str-Imm group displayed significantly larger adrenal weights than both the Con rats ($p < 0.001$) and Str-Rec rats ($p < 0.05$). Furthermore, it appeared the rats in the Str-Imm-shRNA group had a very large adrenal to body weight ratio. In order to investigate this potential effect, a between subject's ANOVA on group assignment was performed and showed that there was a significant effect of group assignment on adrenal weights ($F_{(5, 43)} = 5.72$, $p < 0.001$, Figure 2.7B). Post Hoc analysis revealed that rats that were tested immediately after stress ended and infused with shRNA against BDNF (Str-Imm-shRNA) had significantly larger adrenal glands than did all other groups ($p < 0.05$).

Figure 2.7. Physiological data.

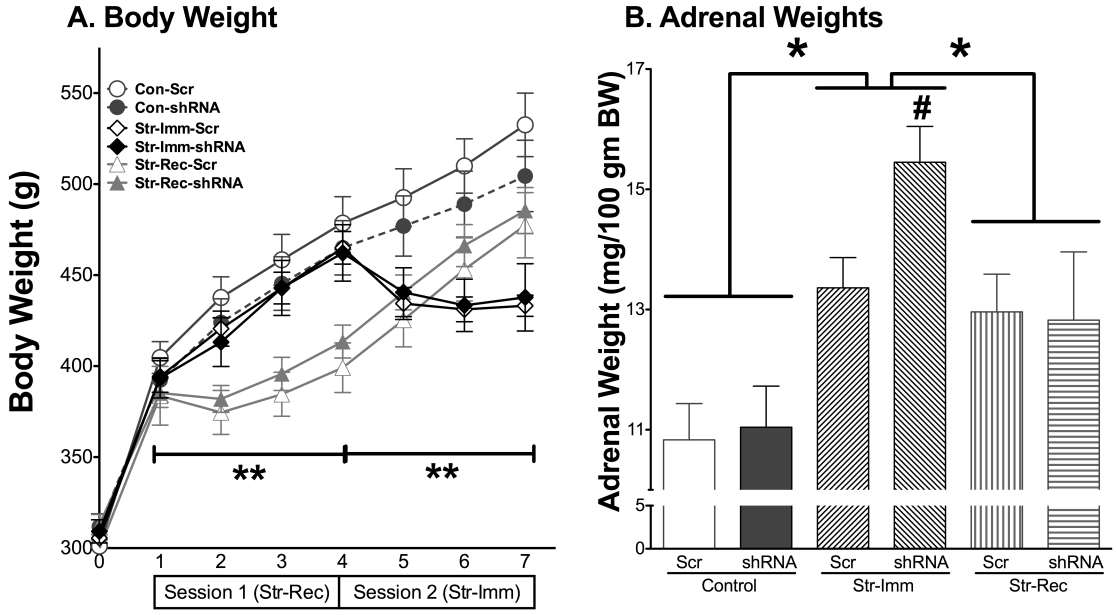

Figure 2.7. Physiological data. (A) Body weight gain. Chronic stress significantly altered body weight gain. All rats weighed the same at the time of surgery (Week 0). Rats in the Str-Rec group (Scr and shRNA) gained significantly less weight than did all other groups during the time that they were subjected to chronic restraint stress (weeks 1-4). Similarly, when restraint began for the rats in the Str-Imm group (Scr and shRNA), body weight gain was significantly attenuated (weeks 4-7). $^{**}p < 0.001$ for main effect of stress condition (regardless of viral vector contents) compared with all other groups. (B) Adrenal weight. Chronic stress significantly altered adrenal weight. Rats in the Str-Imm group had a larger adrenal : body weight ratio than did both the Con and Str-Rec rats. Furthermore, rats in the Str-Imm group (Scr and shRNA) had the largest adrenal : body weight ratio at the end of the study, with rats in the Str-Imm-shRNA group having larger adrenal glands than all other groups. Data are represented as adrenal weight/100 g of

body weight. * $p < 0.03$ compared with Con and Str-Rec, # $p < 0.03$ compared with all other groups.

Discussion

The current study investigated whether downregulating hippocampal BDNF in the dorsal CA3 region would exacerbate spatial memory deficits produced by chronic stress and hinder the recovery from these deficits after stress ended. With the latter point, we present novel findings that hippocampal BDNF is necessary for the recovery process from chronic stress-induced spatial reference memory deficits. Rats with downregulated hippocampal CA3 BDNF expression failed to show spatial reference memory improvements following a post-stress recovery period. With regard to the first prediction, we failed to find evidence that hippocampal BDNF downregulation exacerbated spatial learning and memory deficits of chronically stressed rats. Nonetheless, we corroborated past findings to demonstrate that chronic stress impairs spatial reference memory in rats tested soon after chronic stress ended and that these impairments were reversed following a post-stress recovery period (Hoffman et al., 2011). Finally, we found that hippocampal BDNF downregulation in nonstressed controls briefly disrupted spatial working memory. For the effectiveness of the restraint manipulation, altered body weight gain and adrenal size confirmed that chronically stressed rats perceived the restraint as a stressor. To our knowledge, this is the first study to show that hippocampal BDNF mediates the recovery from chronic stress-induced spatial reference memory deficits.

Effects of Hippocampal BDNF Downregulation on Spatial Memory

A novel finding was that hippocampal BDNF mediates the recovery of spatial reference memory following the end of chronic stress. This is supported by the observation that chronically stressed rats infused with shRNA against BDNF within the dorsal hippocampus failed to recover from chronic stress-induced deficits within the spatial reference memory domain. Specifically, rats in the Str-Rec-shRNA group displayed a high number of first time entry errors (i.e., entries into non-platformed arms) that were statistically similar to the rats that were tested immediately after stress cessation (Str-Imm-shRNA or Str-Imm-Scr). This outcome is consistent with spatial reference memory deficits that follow BDNF downregulation after intracerebroventricular infusions of antisense BDNF oligonucleotides in the hippocampus (Mizuno, Yamada, Olariu, Nawa, & Nabeshima, 2000). Furthermore, a specific area within the hippocampus was targeted (CA3) with shRNA against BDNF and this was the only area to display downregulation of BDNF following viral vector transduction. This implies that transfection of a relatively small number of neurons caused the marked reduction of BDNF to affect spatial memory. This finding is consistent with previous studies showing that specific deletion of BDNF in the hippocampus contributes to spatial memory impairments in the Morris water maze (Heldt, Stanek, Chhatwal, & Ressler, 2007; Taliaz et al., 2011). Moreover, in both of these studies, manipulations of BDNF resulted in a reduction in hippocampal BDNF expression of approximately 50%, consistent with the current study, suggesting that even relatively small changes in hippocampal BDNF can contribute to alterations spatial learning and memory. Together, these reports support the

interpretation that BDNF in the dorsal hippocampus is necessary for the recovery of spatial reference memory following chronic stress.

We also report that chronic stress impaired spatial memory in the reference memory domain without altering working memory. Specifically, chronic stress disrupted stable, long-term information that remained constant over trials (first time entry errors into non-platformed arms) and failed to alter flexible information that varied from trial to trial (repeated entries into non-platformed arms). We previously found chronic stress impaired both reference and working memory and that both recovered after chronic stress ended (Hoffman et al., 2011). However, some reports found that chronic stress disrupted reference memory without altering working memory (Srikumar, Raju, & Shankaranarayana Rao, 2006). Also, some studies suggest that stress-induced working memory deficits may be more fleeting than reference memory deficits (Krugers et al., 1997). Our research group also found that even when conditions are designed to study chronic stress effects on spatial working memory, the deficits could be transient (Mika et al., 2012). Thus, stress-induced effects in the working memory domain may be more difficult to detect than those in the spatial reference memory domain.

One purpose of the current study was to determine whether downregulated hippocampal BDNF would exacerbate the detrimental effects of chronic stress on spatial learning and memory. While, we found chronically stressed rats tested soon after stressor cessation (Str-Imm) displayed the most errors within the reference memory domain than did any other group, we failed to find that BDNF downregulation exacerbated spatial reference learning and memory deficits. Moreover, when hippocampal BDNF downregulation in the Str-Imm-shRNA rats impaired spatial working memory on trial 11,

significant effects were not detected for the Str-Imm-Scr, the critical comparison group. For both reference and working memory, Str-Imm-shRNA performed similarly as Str-Imm-Scr. Perhaps chronic stress lowered BDNF levels to produce a floor effect, such that any further decreases via the viral vector would have been ineffective.

It was unexpected that hippocampal BDNF downregulation failed to impair spatial reference memory in the unstressed controls, although, fleeting deficits in spatial working memory were detected. Some studies support the interpretation that BDNF is required for spatial memory (Cunha, Brambilla, & Thomas, 2010; Heldt et al., 2007; Mizuno et al., 2000), and one report observed deficits in both spatial reference and working memory in non-stressed controls (Mizuno et al., 2000). While our work did not support these outcomes, other studies indicate that working memory deficits are more transient than reference memory deficits (Krugers et al., 1997; Mika et al., 2012). Our finding that the Con-shRNA group expressed fleeting spatial working memory deficits on the first retention trial on day 2 is consistent with those latter reports. In addition, a failure of BDNF downregulation to impair spatial reference memory in nonstressed controls, despite successful hippocampal BDNF knockdown, raises the question as to whether BDNF is necessary for spatial reference memory? In reviewing the literature, two other studies found that manipulating hippocampal BDNF failed to alter novel environment exploration or depressive-like behaviors in non-stressed controls (Z. Y. Chen et al., 2006; Taliaz et al., 2011). One interpretation that is consistent with our current findings is that decreases in hippocampal BDNF play a more pronounced role in a compromised system, such as following exposure to chronic stress, but with less obvious effects in healthy controls.

Mechanisms of Hippocampal BDNF Downregulation

The mechanism by which hippocampal BDNF downregulation disrupted the recovery of spatial reference memory following chronic stress most likely involved the CA3 region. Viral vector infusions targeted the dorsal CA3 region of the hippocampus. If infusions missed the CA3 region, those animals were excluded from the analyses. Moreover, BDNF levels were statistically similar in the nearby DG region. Consequently, downregulated CA3 BDNF expression was the common denominator across animals in the Str-Rec-shRNA group. Given that CA3 apical dendritic arbors recover from stress-induced retraction within the timeframe that spatial memory deficits recover (Hoffman et al., 2011; Sousa et al., 2000), perhaps BDNF is involved in CA3 dendritic restructuring. Mechanisms involving BDNF regulating neuronal morphology seem plausible given BDNF's role in synaptic plasticity, especially in regulating dendritic and spine structure (Chao, 2003; Cowansage, LeDoux, & Monfils, 2010; Horch, Kruttgen, Portbury, & Katz, 1999; Koleske, 2013; McAllister, Lo, & Katz, 1995; Yacoubian & Lo, 2000). For example, transgenic mice expressing low BDNF levels show simplified dendritic arborization (Magarinos et al., 2011). BDNF's actions might involve its TrkB receptor, which is expressed in the CA3 region (Minichiello et al., 2002) and mediates axonal outgrowth (F. Jeanneteau, Deinhardt, Miyoshi, Bennett, & Chao, 2010), hippocampal long-term potentiation (Minichiello, 2009), and learning and memory (Cunha et al., 2010; Minichiello et al., 1999). Taken together, BDNF is a critical component in the detrimental effects of stress. Future studies are needed to investigate how a post-stress recovery period and BDNF interact to influence hippocampal CA3 dendritic complexity.

An unexpected observation is that chronic stress failed to significantly downregulate hippocampal CA3 BDNF levels (Str-Imm-Scr vs. Con-Scr). Past studies report that chronic stress or GCs decrease hippocampal BDNF mRNA and protein expression (Jacobsen & Mork, 2006; Lakshminarasimhan & Chattarji, 2012; Taliaz et al., 2011). However, there are exceptions as not all studies found chronic stress to decrease hippocampal BDNF (Gray et al., 2013; Gronli et al., 2007; Kuroda & McEwen, 1998). In the current study, behavioral training in the RAWM may have masked the full extent of stress-induced changes in BDNF expression, as others observed hippocampal BDNF mRNA levels to increase following maze training (Kesslak, So, Choi, Cotman, & Gomez-Pinilla, 1998; Mizuno et al., 2000). Alternatively, the lack of statistical significance might also be attributed to the data being underpowered. Or perhaps, the changes in hippocampal BDNF expression in response to chronic stress may be subtle and yet physiologically significant (Mizoguchi et al., 2003). Such relatively small changes have been documented in other systems, such as the relatively small 10% decrease in hippocampal GRs that lead to significant effects on spatial memory (Wright et al., 2006). Consequently, the chronic stress effects on CA3 BDNF levels may represent a testing effect, a subtle change or a null effect.

Unexpectedly, rats infused with shRNA, chronically stressed, and precluded from the recovery period (Str-Imm-shRNA) displayed significantly enlarged adrenals. The potential mechanism(s) behind this observation is unknown, but one possibility is that hippocampal BDNF downregulation combined with chronic stress hindered HPA axis function. It is well documented that chronic stress disrupts the HPA axis, perhaps through altered expression of glucocorticoid receptors (GRs) and/or mineralocorticoid receptors

(Dallman et al., 1992; Kitraki et al., 2004a; Mizoguchi et al., 2003; Ratka, Sutanto, Bloemers, & de Kloet, 1989; van Haarst, Oitzl, & de Kloet, 1997; Wright et al., 2006). Perhaps blunting BDNF expression via shRNA may exacerbate this HPA axis dysregulation further; the repeated activation of hippocampal GRs suppresses BDNF influence on glutamate release (Numakawa et al., 2013) and alters BDNF synthesis and activity (F. D. Jeanneteau et al., 2012). Future work will be needed to investigate the potential relationship between chronic stress and hippocampal BDNF in HPA axis regulation.

Summary

We demonstrated that hippocampal CA3 BDNF is necessary for the recovery of spatial memory following chronic stress. Specifically, low hippocampal CA3 BDNF levels impaired the ability to recover from stress-induced spatial reference memory deficits. Low hippocampal BDNF levels in non-stressed individuals produced a transient disruption of spatial working memory. Consequently, hippocampal BDNF expression appears to be important for spatial memory, and is especially important for the recovery of hippocampal function in individuals that have been compromised by chronic stress. Uncovering factors, such as BDNF, that are important for the process of recovery may help identify novel mechanisms underlying resilience in the face of adversity.

CHAPTER 3

BDNF AND TRKB MEDIATE THE IMPROVEMENT FROM CHRONIC STRESS-INDUCED SPATIAL MEMORY DEFICITS AND CA3 DENDRITIC RETRACTION

[Formatted for submission to: *Journal of Neuroscience*

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Contributions: JBO and CDC designed experiment 1 and CDC designed experiment 2. In experiment 1, JBO led the undergraduates as follows: JBO and EJD performed surgeries, JBO and the team implemented the stressors, JBO processed the brain tissue, JBO and EJD found the neurons, JBO and many undergraduates drew neurons, JBO and EJD checked the neuron tracings, quantified the data, and analyzed it. In experiment 2, JMA led the behavioral testing, JBO and JMA and many undergraduates implemented stressors, JBO trained JMA on tissue processing and both processed brain tissue, JMA found neurons, JMA and JBO traced neurons, PPO checked neurons, CDC and PPO quantified neurons, and CDC analyzed data. JBO, JMA, EJD and CDC wrote manuscript.

Introduction

Chronic stress leads to a multitude of changes in brain structure and function. In the hippocampus, chronic stress decreases apical dendritic complexity of hippocampal CA3 neurons and compromises hippocampal-dependent spatial ability. When chronic stress ends and a post-stress rest period ensues, in which animals are left experimentally

undisturbed, CA3 apical dendritic arbors increase in complexity and spatial memory improves (Hoffman et al., 2011; V. Luine et al., 1994; Ortiz et al., 2014; Sousa et al., 2000). Much research has investigated the mechanisms that alter hippocampal structure and function in response to chronic stress (Conrad, 2006; Gray et al., 2013; Magarinos & McEwen, 1995; Watanabe, Gould, Cameron, Daniels, & McEwen, 1992; Woolley et al., 1990), but the mechanisms that allow for the return of spatial ability and dendritic complexity following a post-stress rest period are becoming recent topics of investigation.

Findings garnered from studying the post-stress rest period imply that the factors contributing to hippocampal plasticity occurring during the rest period extend beyond merely reversing those mechanisms initiated by chronic stress. In particular, one study reported that chronic stress and the post-stress rest period had each changed approximately 700 genes in the hippocampus; but more importantly, only 5% of these genes overlapped (Gray et al., 2014). This finding is corroborated by a proteomics assay of hippocampal tissue from chronically stressed rats that were allowed a post-stress rest period or not (Kachemov et al., 2015). In another study investigating spatial memory following a post-stress rest period, rats exposed to chronic stress and then given a post-stress rest demonstrated better spatial memory compared to non-stressed controls (Hoffman et al., 2011). Taken together, these reports indicate that "recovery" from stress-induced hippocampal changes is not simply a return to baseline. Instead, studying mechanisms underlying the post-stress rest period may provide new insight into hippocampal plasticity and perhaps emphasizing those processes may help with resilience in the face of chronic stress.

We previously demonstrated that hippocampal BDNF was necessary for the improvement of spatial ability that occurs following a post-stress rest period (Ortiz et al., 2014). This is consistent with chronic stress altering hippocampal BDNF expression (Lakshminarasimhan & Chattarji, 2012; Smith et al., 1995) reviewed in (Gray et al., 2013; Suri & Vaidya, 2013). A caveat was that BDNF levels were downregulated during both the stress and the post-stress rest period. The goal of these experiments was to accomplish the following. 1) To determine whether downregulation of hippocampal BDNF that extends throughout the post-stress rest period would also lead to reduced CA3 apical dendritic complexity, as reduced CA3 dendritic complexity coincides with poor spatial memory (Conrad, 2006). 2) To investigate changes in the dendritic arbors of CA1 neurons following chronic stress and a post-stress rest period with and without BDNF downregulation. Chronic stress or chronic administration of glucocorticoids can lead to dendritic retraction of CA1 apical dendrites (Gourley et al., 2013; Sousa et al., 2000), but not always (D. N. Alfarez, Karst, Velzing, Joels, & Krugers, 2008; Woolley et al., 1990). Importantly, manipulation of BDNF expression in the CA3 region of the hippocampus can affect neurons in the CA1 region, as mice with a disruption of BDNF production in CA3 neurons show impaired plasticity in CA1 neurons (Zakharenko et al., 2003). 3) To determine whether administering a TrkB antagonist daily during the post-stress rest period would disrupt the improvements in the CA3 apical dendritic arbor complexity and spatial memory. Together, these experiments will test the hypothesis that hippocampal BDNF and its high affinity TrkB receptor are required for the improvement in hippocampal CA3 dendritic complexity and spatial memory during the post-stress rest period.

Materials and Methods

These experiments were conducted in accordance with federal guidelines as described in the Guide for the Care and Use of Laboratory Animals (National Academy of Sciences, National Research Council, Institute of Laboratory Animal Resources on Life Science, Washington, D.C., 2011), and institutional guidelines set forth by the Institutional Animal Care and Use Committee at Arizona State University who approved the procedures used in the study.

Subjects

Young-adult male Sprague-Dawley rats of approximately two months of age were purchased from Charles River Laboratories (Wilmington, MA, USA) and housed at Arizona State University animal housing facilities. All rats were paired housed, on a reverse light cycle (12:12, lights off at 0600), for the duration of the studies in a room that only contained rats of the same treatment condition (i.e. stressed rats were housed with stressed rats, stressed rats given a post-stress rest period were housed with other rats given a post-stress rest period, etc.). Food and water were available *ad libitum*, except during the restraint procedures. After arrival, rats were given one week to acclimate to their environment prior to surgery. Rats were weighed weekly throughout the duration of the experiment. All behavioral testing was conducted during the dark phase of the light cycle.

Chronic Stress Paradigm

Rats were placed in restrainers made of wire mesh, 18 cm in circumference and 24 cm in length, ends were sealed with Plasti Dip, (Plasti Dip International, Blaine, MN, USA). The restrainers were secured at both ends with black binder clips (Staples Inc., Framingham, MA, USA). Rats that outgrew the smaller restrainers were restrained with larger restrainers (23 cm in circumference and 28 cm in length). Chronically stressed rats were placed in restraint for 6 hours each day for 21 consecutive days between 09:00 h and 15:00 h. At the same time, the food and water were removed from the controls for 6 hours to maintain similar access to food and water across groups.

Golgi Stain

The brains were processed according to the manufacturer's instructions (FD Rapid Golgistain™ Kits FD NeuroTechnologies, Baltimore, MD, USA). After removal, fresh brains were rinsed in double distilled water and then immersed in the impregnation solution overnight at room temperature in a dark location. The impregnation solution was changed one day later and the brains remained in this solution undisturbed for 2 weeks at room temperature and in the dark. Then, brains were placed in the kit's fixing solution for 72 hours, which was replaced once after 24 hours. At this time, the brains were frozen rapidly using 2-methylbutane (that was chilled using dry ice surrounding the beaker) and stored in a -80°C freezer for sectioning at a later date. Frozen brains were blocked to target the hippocampus and cut (120µm, coronal sections) using a cryostat (Microtome HM 500 OM cryostat, kept between -25 and -30° C). Sections were mounted onto 2% gelatin-coated microscope slides with the kit's fixing solution. Excess solution was

removed from the slides, which were then stored in the dark at room temperature until the sections were dried (at least one day). Sections were then rinsed with double distilled water, placed in the kit's staining solution, rinsed with double distilled water, dehydrated in a series of increasing ethanol concentrations, and cleared with HemoDe (xylene substitute) before being cover slipped with Permount™ Mounting Medium (Thermo Fisher Scientific, Waltham, MA, USA).

Hippocampal pyramidal neurons were identified in the CA3b-c and CA1b-c region of the dorsal hippocampus (between Bregma -2.30 mm and -4.68 mm) (Conrad, Ortiz, & Judd, 2017). Neurons were randomly selected and used if they were fully impregnated by the stain, fully intact (i.e. no breaks in dendritic branches), and relatively isolated. Selected neurons were then traced using a light microscope and a camera Lucida drawing tube. The morphology of the apical and basal dendritic tree was quantified by counting the number of dendritic bifurcations and by using the Sholl analysis at every 20 μm from the soma (Sholl, 1953). A minimum of 3 long shaft (LS) and 3 short shaft (SS) neurons from the CA3b-c region and 3 neurons from CA1b-c region were required from each animal in order to be included in the analyses. For the CA3 neurons, the SS and LS numbers were averaged first before being combined to represent one final averaged number in order to avoid bias from differences in SS and LS dendritic complexity (Fitch et al., 1989).

Figure 3.1. Experimental Timeline and Body Weights

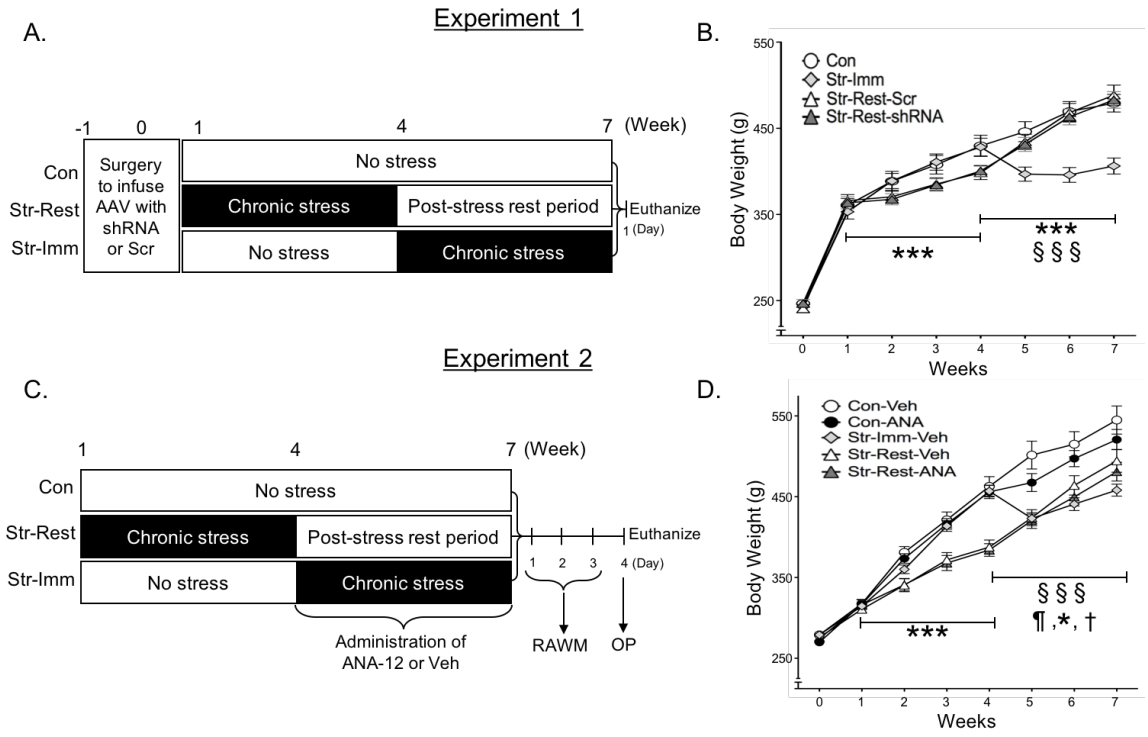

Figure 3.1. Experimental timeline and body weights. (A) Timeline for Experiment 1. Rats were chronically stressed with wire mesh restraint for 6h/d/21d and then given a post-stress rest period (Rest) of 21d or not (Str-Imm), a subset of rats were not exposed to the stressor manipulation (Con). For experiment 1, rats underwent stereological surgery to infuse the AAVs containing the coding sequence for an shRNA against BDNF (shRNA) or a scrambled sequence with no known mRNA complement (Scr). After the stressor manipulations, the brains were removed and processed for the Golgi stain. (B) Stress significantly attenuated body weight gain in Experiment 1. During weeks 1-3, Str-Rest rats, regardless of viral vector contents, gained significantly less weight than both Con and Str-Imm (*** $p < 0.001$). Likewise, during weeks 4-6 when stress was being implemented for the Str-Imm rats, Str-Imm rats gained significantly less weight

compared to Con and Str-Rest (§§§ $p < 0.001$), while Str-Rest rats gained significantly more body weight than unstressed controls to regain body weight (*** $p < 0.001$). (C) Timeline for experiment 2, rats received daily i.p. injections of the TrkB antagonist ANA-12 (abbreviated as 'A') or saline (Veh) during the 21-day post stress rest period. Afterwards, the rats were tested on the radial arm water maze (RAWM) for three days followed by testing on the object placement task (OP). Brains were then removed and processed for the Golgi stain. (D) Stress significantly attenuated body weight gain in Experiment 2. Rats that were being restrained showed significantly attenuated body weight gain compared to other groups, and ANA-12 administration also appeared to slow body weight gain. During weeks 1-3 of Str-Rest rats gained significantly less weight than both Con and Str-Imm rats (*** $p < 0.001$). When stress was implemented for Str-Imm rats during weeks 4-7, Str-Imm rats gained significantly less weight compared to Con and Str-Rest (§§§ $p < 0.001$), while Str-Rest-Veh rats gained significantly more weight than Con-Veh, Con-ANA, and Str-Imm-Veh (* $p < 0.05$). ANA-12 also affected body weight gain as Con-ANA gained less weight than Con-Veh († $p < 0.05$) and Str-Rest-ANA gained more weight than Con-ANA and Str-Imm-Veh (¶ $p < 0.05$).

Experiment 1: Effects of hippocampal BDNF downregulation on CA3 and CA1 dendritic complexity following a post-stress rest period

Treatment Conditions

The experimental design is illustrated (Figure 3.1A). Rats (n=48) were randomly divided into one of four groups, (*n*=12/group) based upon stress and viral vector

condition: non-stressed controls infused with a scrambled virus (Con), chronically stressed animals given a three-week post-stress rest period from chronic stress and a scrambled virus (Str-Rest-Scr) or a shRNA against BDNF virus (Str-Rest-shRNA), and chronically stressed animals infused with a scrambled virus and not given a rest period (i.e. brains removed 'soon' after the restraint period, Str-Imm). The viral vectors used were from the same stock as used in our previous study and confirmed to effectively downregulate BDNF levels for at least two months (Ortiz et al., 2014). Furthermore, our prior study demonstrated that downregulating hippocampal BDNF did not alter performance of the Str-Imm and Con groups compared to their respective scrambled cohort (Str-Imm-shRNA behaved similarly to Str-Imm-Scr, and Con-shRNA behaved similarly to Con-Scr). Consequently, the Str-Rest-shRNA group will be compared to the Str-Imm as a positive control for chronic stress-induced CA3 apical dendritic retraction and to the Str-Rest-Scr as a positive control for the improvement in CA3 dendritic restructuring following a post-stress rest period.

Surgery

Rats underwent stereotaxic surgery to have the CA3 region of their dorsal hippocampus infused with viral vectors. The surgeries were performed under aseptic sterile conditions as follows. Rats were anesthetized with a ketamine cocktail (1ml/kg, i.p., containing 70 mg/kg ketamine, 6 mg/kg xylazine, 10 mg/kg acepromazine, in 0.9% sodium chloride). The rats also received a concurrent dose of the non-steroidal anti-inflammatory drug, Meloxicam (1.0mg/kg of 5mg/mL, s.c.). When the rats no longer responded to a foot pinch, they were placed on the stereotaxic apparatus (David Kopf

Instruments, Tujunga, CA, USA) and the head was secured with a nosepiece and ear bars. Thirty minutes prior to the incision, the local anesthetic, Marcaine (bupicvicaine, 0.25% Marcaine with sterile 0.9% sodium chloride), was injected under the incision line 30 (up to 0.4 mL, s.c.). Rats received boosters of the ketamine cocktail (0.5ml/kg, i.p.) as needed throughout the surgery.

The scalp was scrubbed with 2% chlorhexidine acetate surgical scrub (Fort Dodge Animal Health, Fort Dodge, IA, USA) and an incision was made along the midline of the scalp. Lambda and bregma were located and leveled to zero. Glass Hamilton syringes (5 μL, National Scientific Company, Rockwood, TN, USA) were used to infuse viral vectors. A total of six injections (three in each hemisphere) were made, each consisting of approximately 0.2 μL of the viral vector targeted to the CA3 region of the dorsal hippocampus, using coordinates from Paxinos and Watson (1997) as follows: In mm from Bregma: anterior/posterior (A/P): -3.3, Medial/Lateral (M/L): ±3.0, Dorsal/Ventral (D/V): -3.9; A/P: -3.8, M/L: ±3.4, D/V: -4.0; A/P: ±4.3, M/L: +4.0, D/V: -4.2. Viral vectors were infused over the course of three minutes, and syringes were left in place for five minutes after each infusion. After the last injection, the incision was sutured using coated vicryl sutures (Ethicon, Inc., Somerville, NJ, USA) and swabbed with triple antibiotic ointment (E. Fougera & Co, Melville, NY, USA). Rats were placed under a heated lamp until they awoke. Rats were then individually housed for approximately three days until the scalp incision healed. During this healing period, rats received post-operative doses of Meloxicam for pain management as well as triple antibiotic ointment over the wound to reduce the risk of infection. Rats were then returned to their original cage mate and pair housed for the remaining duration of the study.

Viral Vectors

An adeno-associated viral (AAV) vector packaged with an shRNA directed against BDNF, as well as a separate transcription cassette for enhanced green fluorescent protein (eGFP; together, abbreviated throughout the manuscript as 'shRNA') was used to knock down hippocampal BDNF levels, as previously described (Ortiz et al., 2014). A murine U6 Pol III promoter controlled the shRNA. The eGFP sequence was transcribed by a CMV-IE element. The shRNA sequence used was 5'-ACCATAAGGACGCGGACTTGT-3'. This shRNA sequence targeted BDNF exon IX, which is common to all BDNF transcripts (from the National Center for Biotechnology Information, reference sequence NM_012513.3). A viral vector delivering a "scrambled" shRNA coding sequence with no correspondence to any known rat mRNA and the coding information for eGFP (abbreviated throughout the manuscript as 'Scr') served as a control. The vectors were packaged in plasmids that provided AAV2 replicase and AAV-rh10 capsid functions, and a pHelper plasmid (Stratagene, La Jolla, CA, USA) provided adenovirus helper functions. The three plasmids were co-transfected into AAV-293 cells (Stratagene, La Jolla, CA, USA) at a molar ratio of 1:1:1. Two days post-transfection, the cells were harvested and suspended in Dulbecco's Modified Eagle Medium and intracellular virus particles were freeze-thawed three times in order to release the virus. Afterward, the cells were centrifuged (13,000 rpm for 10 min), and the supernatant was stored at 80 °C. Viruses were titered by real-time PCR (ABI Prism 7700 Sequence Detection System, PerkinElmer Applied Biosystems, Foster City, CA, USA). The average titer was approximately 10^{12} DNase Resistant Particles/ml. AAV10-mediated

expression of transgenes has previously been shown to achieve maximum expression in 1-2 weeks and can last at least 3 months post-transfection (Klein et al., 1998). Furthermore, data from our lab has shown this viral vector decreased hippocampal CA3 BDNF levels in adult male rats by 62.7% ± 0.1% two weeks after transfection; and hippocampal BDNF remained downregulated by 67.2% ± 0.2% six weeks after transfection (Ortiz et al., 2014).

Tissue collection and Golgi staining

The unperfused brains were removed and hemisected. One hemisphere was flash frozen in 2-methylbutane and kept chilled with dry ice, and then stored in a -80C freezer. The other hemisphere was immediately processed for Golgi staining (described earlier). Each hemisphere was counter-balanced across subjects.

Experiment 2: Effects of the administration of a TrkB antagonist during the post-stress rest period on hippocampal CA3 dendritic complexity and spatial ability

Treatment Conditions

The experimental design is illustrated (Figure 3.1C). Male rats (n=50) were used to determine whether the administration of ANA-12 during the post-stress recovery phase could block the improvement in spatial ability and hippocampal CA3 dendritic complexity. Rats were assigned to one of five treatment conditions (n=10) as follows: non-stressed controls injected daily (i.p., 1ml/kg) with saline (Con-Veh), non-stressed controls injected daily (i.p., 0.5mg/kg) with ANA-12 (Con-ANA), chronically stressed

rats given a three-week post-stress rest period concurrent with daily saline injections (Str-Rest-Veh), chronically stressed rats given a three-week post-stress rest period concurrent with daily ANA-12 injections (Str-Rest-ANA), and chronically stressed animals not given time to recover (Str-Imm). For Str-Imm, they were further separated into two groups in which rats were either injected with daily saline (Veh, n=4) or not given any injections (n=6) and then averaged together (Str-Imm-Veh).

Drugs

ANA-12 (Tocris Bioscience, Bristol, UK), a TrkB inhibitor (Cazorla et al., 2011), was purchased from Tocris Bioscience (www.tocris.com) and dissolved in sterile saline to reach a concentration of 0.5mg/ml. The 0.5 mg/kg dose chosen was because active concentrations of ANA-12 could be detected in the brain within 30 min and up to 6 hr after i.p. injection (Cazorla et al., 2011).

Radial Arm Water Maze (RAWM)

Apparatus

The RAWM was implemented because of its well documented use in measuring spatial ability in rodents (Diamond et al., 1999; Hoffman et al., 2011; Ortiz et al., 2014). The RAWM was composed of black polypropylene, with eight symmetrical arms (27.9 cm long x 12.7 cm wide) emanating from a circular center (48 cm diameter). The maze was filled with water and allowed to reach room temperature ranging from 20°C - 22°C. The water was rendered opaque with black powder tempera paint. Two testing rooms

were used in order to test all of the rats and these consisted of several salient and similar extra-maze cues including the door to the room, shelves, heat lamps, and cues made of black and white construction paper located on the walls. Groups were counterbalanced between the testing rooms and were tested by two different experimenters.

Procedure

On the day of testing, a platform was placed at the end of one of the arms and positioned 2.5 cm below the surface of the water so that it could not be seen directly. Platform location was counterbalanced across and within groups, but was held constant across all trials for a given rat. Testing took place over three consecutive days between 0900 h and 1300 h. On day one and two of testing, rats were given 8 trials (16 total), and on day 3 a single retention trial was administered. A trial started as soon as the rat was released into an arm that did not contain the platform (start arm), which varied across trials so that the start arm for a given trial was different from the start arm in the trial that preceded it. Also, the start arm was never directly across from the arm that contained the platform to enhance the use of spatial search strategies. Once the rat found the platform, it was allowed to remain on the platform for 15 seconds for spatial localization and was then returned to its testing cage, which was located in the testing room under a heat lamp. If a rat failed to find the platform within three minutes, the rat was gently guided to the platform with a net. After each trial, a net was used to stir the water and collect floating debris to prevent rats from using non-spatial cues. In order to avoid exhaustion of the rats during testing, rats were tested in squads; rats from different groups were tested together in squads of six to eight (e.g. two rats from the Con-Veh group were tested with two rats

from the Str-Rest-ANA group and two rats from the Str-Imm-Veh group). Once a rat finished one trial, it was placed back in its testing cage while the other rats in the squad completed that trial. Therefore, the time between each trial for a given rat ranged from 20 minutes in the earlier trials to 5 minutes in the latter trials as the rats acquired the task. Reference memory errors were the number of first time entries into arms that did not contain the platform within a given trial. Working memory errors were the number of repeated entries into an arm that did not contain the platform within a given trial (i.e. repeated entries into an arm where a reference memory error was previously committed in the same trial).

Open Field and Object Placement

Apparatus

The open field was made from black Plexiglas to form a 61 × 61 × 38 cm box. Inside the open field for trial 1 of object placement, two identical objects (20.3 cm tall, weighing .32 kg) were located equidistant from the corners. These objects are tall enough that they could not be displaced nor climbed upon. In trial 2, these objects were replaced with two new, but identical objects, with one object located in the same position as in trial 1 and the second object located in a new location than used in trial 1.

Procedure

The protocol is similar as previously described (McLaughlin, Bimonte-Nelson, Neisewander, & Conrad, 2008). One day after RAWM testing ended, rats were transported in their home cage with their cage mate to the testing room and positioned so

that the rats could not see the maze or room cues from the home cage location. One rat was placed in a corner of the open field apparatus and allowed to explore for 10 min. After 10 minutes, the rat was returned to his home cage and the maze was cleaned with all-purpose cleaner (Method, Target) before its cage mate was tested. A day later, object placement testing was carried out in the same room and rats were placed in the same corner as they were previously, but now with two objects in the maze and given three minutes to explore. Trial 2 began 2-hours later with one of the objects moved to a novel location. The starting position of the rats and the placement of the objects in the trial were counterbalanced across rats. The testing arena and the objects were thoroughly cleaned after each trial. Object exploration was defined as a rat facing the object within 3 cm and actively sniffing or touching the object. Total exploration time of each object was recorded by an experimenter who was blind to the treatment conditions.

Tissue and Brain Processing

After the second trial on day 2 of object placement testing, rats were transported to a necropsy room and deeply anesthetized with carbon dioxide and, when they no longer responded to a foot pinch, decapitated. The unperfused brains were quickly removed and then processed for Golgi staining with FD Rapid Golgistain™ Kits, as described earlier.

Statistical Analyses

The statistical software package, SPSS (version 19), the graphing and curve fitting software GraphPad Prism (version 7), and a Macintosh computer (OS X 10.7.5) were used for the data analyses. Omnibus analyses of variance (ANOVAs) were followed by one-way ANOVAs on group assignment and then followed by Fisher's LSD post hoc tests when $p \leq 0.05$. Data are represented by means ± S.E.M.

Results

Experiment 1: Effects of hippocampal BDNF downregulation on hippocampal dendritic complexity following a post-stress rest period

CA3 Dendritic complexity (Figure 3.2)

Branch Points

First, we determined whether the viral vector mediated knockdown of hippocampal BDNF would impair the recovery of dendritic complexity following a post-stress rest period as measured by branch points. Representative neurons and tracings from the CA3 region of the hippocampus are shown in Figure 3.2A. The final analysis included a total of 355 neurons with an average from 4.1 short shaft neurons per rat for Con to 4.7 short shaft neurons per rat for Str-Imm; and 4.2 long shaft neurons per rat for Con to 4.8 long shaft neurons per rat for Str-Rest-shRNA. An ANOVA for CA3 apical dendritic complexity revealed a significant effect of group ($F_{(3,37)} = 3.65$, $p < 0.05$). Knockdown of hippocampal BDNF prevented the improvement of hippocampal CA3

apical dendritic complexity as Str-Rest-shRNA rats displayed significantly less apical dendritic material as Str-Rest-Scr rats ($p < 0.05$, Figure 3.2B) and were similar to Str-Imm rats. Str-Imm also displayed significantly less dendritic material than Str-Rest-Scr ($p < 0.01$). Given past work that Str-Imm and Con would differ in CA3 apical dendritic complexity (Hoffman et al., 2011; Watanabe, Gould, & McEwen, 1992), planned comparisons confirmed that chronic stress reduced apical dendritic complexity (Str-Imm vs Con, $F_{(1,19)} = 6.67$, $p < 0.05$).

Sholl Analysis

For CA3 neurons, a repeated measures ANOVA for group on apical branch intersections revealed a significant effect of distance from the soma ($F_{(19,684)} = 501.826$, $p < 0.001$) and a significant interaction for distance by group ($F_{(57,684)} = 1.598$, $p < 0.01$) with no statistically significant effect of group. Subsequent analyses investigated three separate regions, based upon previous work (Fitch, Juraska, & Washington, 1989), as dendritic complexity differs as a function of distance from the soma: 20 – 120 μm (proximal), 140 – 240 μm (middle), 260 – 400 μm (distal). Repeated measures ANOVAs for the proximal and distal apical regions failed to reveal significant effects or interactions with groups. However, the medial apical region (140um-240um) was significantly affected by the manipulations (significant main effect of group, $F_{(3,36)} = 2.935$, $p < 0.05$; no significant interactions). Post-hoc analyses revealed that the Str-Rest-Scr rats displayed significantly more apical dendritic intersections in this region than did both the Str-Imm ($p < 0.05$) and the Str-Rest-shRNA rats ($p < 0.01$, Figure 3.2C).

There were no statistically significant effects on CA3 basal branch points (Figure 3.2D) or basal dendritic intersections (Figure 3.2E) as determined by ANOVA. Moreover, repeated measures ANOVA on dendritic intersections most proximal to the cell body (20um-120um) or most distal to the cell body (140um-240um) failed to show statistically significant differences for group or the group by distance interaction.

Figure 3.2. Effects of chronic stress, a post-stress rest period, and knockdown of hippocampal BDNF on hippocampal CA3 dendritic complexity

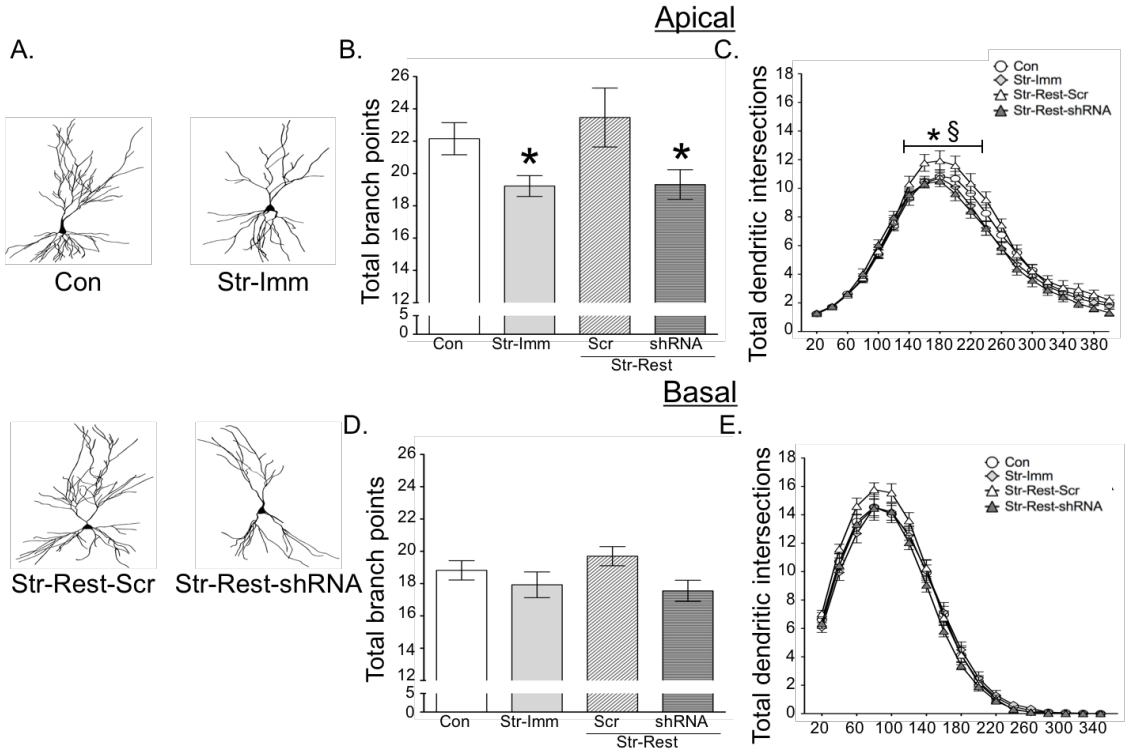

Figure 3.2: Experiment 1, effects of chronic stress, a post-stress rest period, and knockdown of hippocampal BDNF on hippocampal CA3 dendritic complexity. (A) Tracings of neurons from the CA3b-c region of the hippocampus. Short shaft and long

shaft neuronal types were combined to obtain a single value. (B) Chronic stress decreased CA3 apical dendritic complexity and rats given a post-stress rest period showed similar dendritic complexity as non-stressed control rats. Importantly, rats given the shRNA mediated knockdown of hippocampal BDNF displayed atrophy dendritic complexity (* $p < 0.05$ compared to Str-Rest-Scr and Con). (C) A repeated measures ANOVAs for apical dendritic intersections in the apical region most proximal (20-120 um) and distal (260-400) to the cell body failed to reveal significant effects or interactions with groups. However, the analysis showed that the medial region (140um-240um) was significantly affected by the manipulations, as animals in the Str-Rest-Scr group displayed significantly more apical dendritic intersections in this region than did both the Str-Imm group and the Str-Rest-shRNA group (* $p < 0.05$ for Str-Rest-Scr compared to Str-Imm, and § $p < 0.01$ for Str-Rest-Scr compared to Str-Rest-shRNA). (D-E) No statistically significant effects were found in the basal region.

CA1 Dendritic Complexity (Figure 3.3)

Branch Points

Tracings of CA1 neurons are represented in Figure 3.3A. A total of 120 neurons was included in the final analysis with a total of 3 neurons per animal per group. While no significant effects were observed for the CA1 apical dendritic branch points (Figure 3.3B), significant differences among groups were found for the CA1 basal branch points ($F_{(3,36)} = 5.19$, $p < 0.01$). Specifically, post-hoc analyses revealed that the number of dendritic bifurcations in the basal region of neurons obtained from rats in the Str-Rest-

shRNA group were statistically greater than those observed in the other rest group and control animals ($p < 0.001$ for Str-Rest-shRNA vs. Str-Rest-Scr; $p < 0.01$ for Str-Rest-shRNA vs. Con, Figure 3.3D). The analysis also showed that Str- Imm approached statistical significance by having less basal dendritic branch points than Str- Rec-shRNA ($p = 0.06$) and more basal branch points than Str-Rest-Scr ($p = 0.06$).

Sholl Analysis

For CA1 neurons, region-specific changes were found, with the distal most region of the apical dendrites neurons being affected. The apical data was further divided into three components based upon distance from the cell body: 20 – 100 μm (proximal), 120 – 200 μm (middle), 220 – 300 μm (distal). Repeated measures ANOVA for the apical distal region revealed a significant main effect of group ($F_{(3,41)} = 3.571$, $p < 0.05$) and a significant repeated effect for distance ($F_{(4,164)} = 105.5$, $p < 0.05$) with no significant interaction. Post-hoc analyses of the group effect showed neurons from Str-Imm rats had a statistically greater number of dendritic intersections in the distal 220-300μm region from the soma than the chronically stressed rats given a rest period ($p < 0.05$ for Str-Imm rats vs. Str- Rest-Scr or Str-Rest-shRNA rats, Figure 3.3C). For the proximal and medial apical regions, no significant main effect or interaction was found. Taken together, neurons from Str-Imm rats had statistically greater number of dendritic intersections in the CA1 distal apical regions compared to the two recovery groups (Str-Rest-Scr and Str-Rest-shRNA).

The analyses in the basal region were performed for the proximal (20 – 120 μm) and distal regions (140 – 240 μm, Figure 3.3E). A repeated measures ANOVA of the

proximal region, 20-120μm, identified a significant main effect of group ($F_{(3,41)} = 4.758$, $p < 0.005$), a repeated effect of distance ($F_{(5,205)} = 95.3$, $p < 0.05$), and a significant interaction ($F_{(15,205)} = 1.88$ $p < 0.05$). Post-hoc analyses in this region revealed that Str-Imm and Str-Rest-shRNA had more dendritic intersections than did Str-Rest-Scr ($p < 0.05$, Figure 3.3E). No significant effects were found in the distal basal region.

Figure 3.3. Chronic stress, a post-stress rest period with knockdown of hippocampal BDNF in the CA3 region affects dendritic complexity of CA1 neurons.

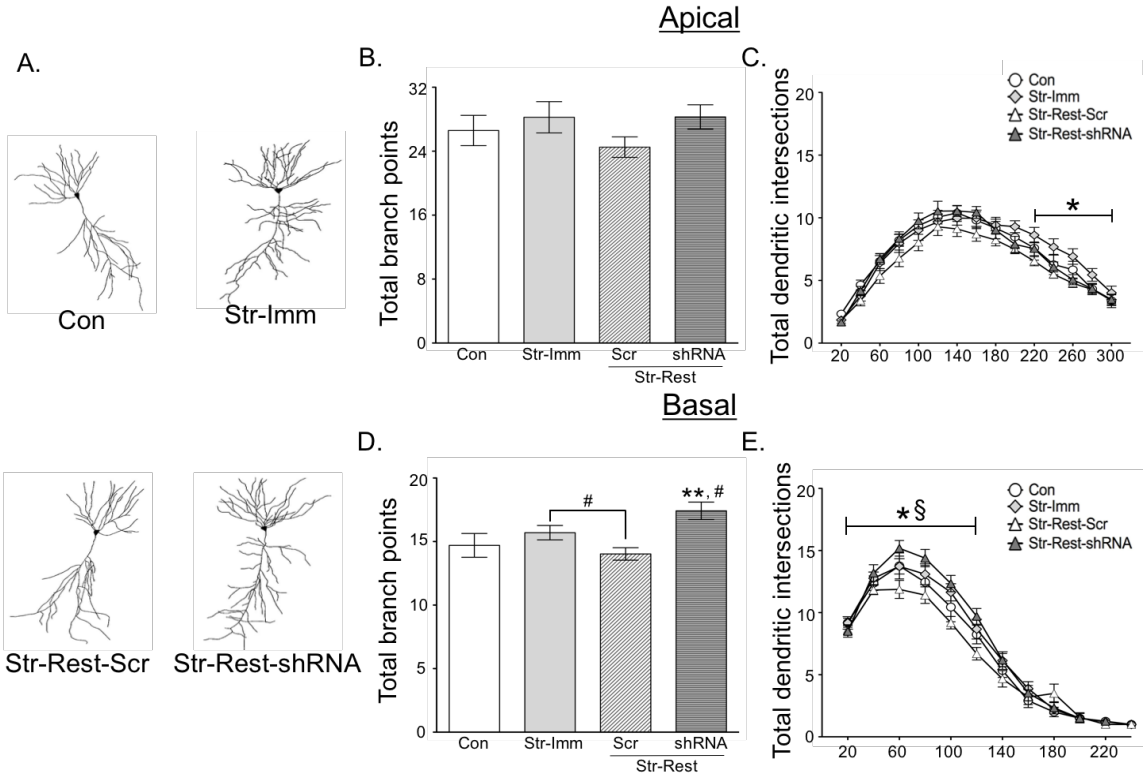

Figure 3.3: Experiment 1, chronic stress, a post-stress rest period with knockdown of hippocampal BDNF in the CA3 region affects dendritic complexity of CA1

neurons. (A) Tracings of neurons from the CA1b-c region of the hippocampus. (B) Chronic stress and knockdown of hippocampal BDNF had no statistically significant effect on the number of CA1 apical branch points. (C) Sholl analysis revealed that chronic stress increased the number of apical dendritic intersections at 220-300um from the soma compared to rats in the Str-Rest group (* $p < 0.05$ for Str-Imm compared to Str-Rest-Scr and Str-Rest-shRNA). (D) For basal branch points, rats given a knockdown of hippocampal BDNF and a post-stress rest period displayed significantly more dendritic branch points than control rats and the rats given the Scr virus and a post-stress rest period (** $p < 0.05$ for Str-Rest-shRNA compared to Con and for Str-Rest-shRNA compared to Str-Rest-Scr). Basal branch points of Str-Imm approached statistical significance by having less basal branch points than Str-Rec-shRNA and more than Str-Rest-Scr (# $p = 0.06$ for both). (E) Similarly, Str-Rest-shRNA rats, as well as Str-Imm rats displayed more dendritic intersections in basal branches more proximal to the cell body as measured by the Sholl analysis than did Str-Rest-Scr rats. (* $p < 0.05$ for Str-Rest-shRNA compared to Str-Rest-Scr and § $p < 0.05$ for Str-Imm compared to Str-Rest-Scr).

Correlation between CA3 apical dendritic complexity and CA1 basal dendritic complexity

A Pearson correlation was used to identify a potential relationship between CA3 and CA1 neuronal morphology. Comparison of the apical branch points of CA3 neurons and the basal branch points of CA1 neurons (including all treatment groups) resulted in a marginally significant inverse correlation (1-tail, $n = 45$, $r = -0.289$, $p = 0.05$).

Simplifications in CA3 apical dendritic complexity correlated to increases in CA1 basal dendritic complexity, as measured by branch points.

Physiological Measures

Chronic restraint, but not infusion of AAV into the hippocampus, significantly affected body weight gain (Figure 3.1B). A repeated measures ANOVA for treatment condition on body weight gain across weeks revealed a significant interaction for treatment across weeks ($F_{(18,246)} = 57.627.6$, $p < 0.001$) and a significant repeated effect for weeks ($F_{(6,246)} = 628.754$, $p < 0.001$) with no significant main effect for treatment. An ANOVA for body weight gain for weeks 1 through 4 during which restraint was administered to the Str-Rest cohort revealed a significant main effect of treatment ($F_{(3,41)} = 20.119$, $p < 0.001$). Post-hoc analyses showed that the Str-Rest-Scr and Str-Rest-shRNA groups gained less weight than did both Con and Str-Imm ($p < 0.001$). When restraint began for Str-Imm and ended for Str-Rest-Scr and Str-Rest-shRNA starting on week 4, the body weight gain changed accordingly. An ANOVA for body weight gain across weeks 4 through 7 indicated a significant effect for treatment condition ($F_{(3,41)} = 390.684$, $p < 0.001$). Post-hoc analyses revealed that Str-Imm- displayed significantly attenuated body weight gain compared to all other groups ($p < 0.001$) and that body weights from both Str-Rest groups (Str-Rest-Scr, Str-Rest-shRNA) returned to levels comparable to Con.

Experiment 2: TrkB antagonist administration on the recovery of spatial ability and hippocampal dendritic complexity

CA3 Dendritic Complexity (Figure 3.4)

Branch Points

Representative tracings of hippocampal CA3 neurons are displayed in Figure 3.4A. The final analysis included a total of 172 neurons drawn and averaged 4.7 (Con-ANA) to 4.9 neurons/rat (Str-Rest-Veh). The results demonstrated that daily administration of ANA-12 during the rest period in chronically stressed rats prevented the enhancement of dendritic complexity of the CA3 apical dendritic arbors without altering the dendritic arbors in non-stressed controls. For total apical branch points, a one-way ANOVA revealed a significant effect of treatment, $F_{(4,31)} = 8.627$, $p < 0.001$. Chronically stressed rats receiving daily ANA-12 injections for three weeks during the post-stress rest period displayed significantly reduced CA3 apical dendritic branch points (Str-Rest-ANA vs. Con-Veh, $p < 0.01$; Str-Rest-ANA vs. Str-Rest-Veh, $p < 0.001$). Moreover, Str-Rest-ANA expressed CA3 apical dendritic arbors similar to that of chronically stressed rats that were not provided with a post-stress rest period (Str-Imm-Veh, Figure 3.4B). As found in other reports, Str-Imm-Veh had fewer CA3 apical dendritic branch points compared to Con-Veh, Con-ANA, Str-Rest-Veh (all $p < 0.01$). ANA-12 had no significant effect on CA3 apical dendritic branch point counts in the non-stressed controls (Con-Veh = Con-ANA).

Sholl Analysis

A Sholl analysis of the CA3 apical branch intersections revealed that ANA-12 administered during the post-stress rest period blocked the CA3 apical dendritic arbors at the distal regions from increasing in complexity. A mixed factor ANOVA for treatment and intersections from the soma revealed a significant effect of treatment ($F_{(4,31)} = 5.518$, $p < 0.001$), distance from soma ($F_{(14,434)} = 353.7$, $p < 0.001$), and treatment by distance interaction ($F_{(56,434)} = 2.666$, $p < 0.001$). The significant interaction was probed further by performing one-way ANOVAs at each intersection. Statistically significant effects were observed at 160μm from the soma ($F_{(4,31)} = 7.35$, $p < 0.001$) and persisted to the end at 300 μm from the soma ($F_{(4,31)} = 3.19$, $p < 0.05$). The number of intersections were fewer for the Str-Rest-ANA and Str-Imm-Veh groups than compared with Con-Veh, Con-ANA, Str-Rest-Veh (Figure 3.4C). ANA-12 had no effect on the non-stressed controls, as the number of intersections were similar between Con-Veh and Con-ANA. No other significant effects were found at the proximal and mid-dendritic regions from the soma.

The effects of ANA-12 and chronic stress were specific for the CA3 apical dendritic arbors, as no statistical differences were observed in the CA3 basal dendritic arbors for branch points (Figure 3.4D) or for dendritic intersections from the soma (Figure 3.4E).

Figure 3.4. Effects of chronic stress, a post-stress rest period, and ANA-12 administration on hippocampal CA3 dendritic complexity.

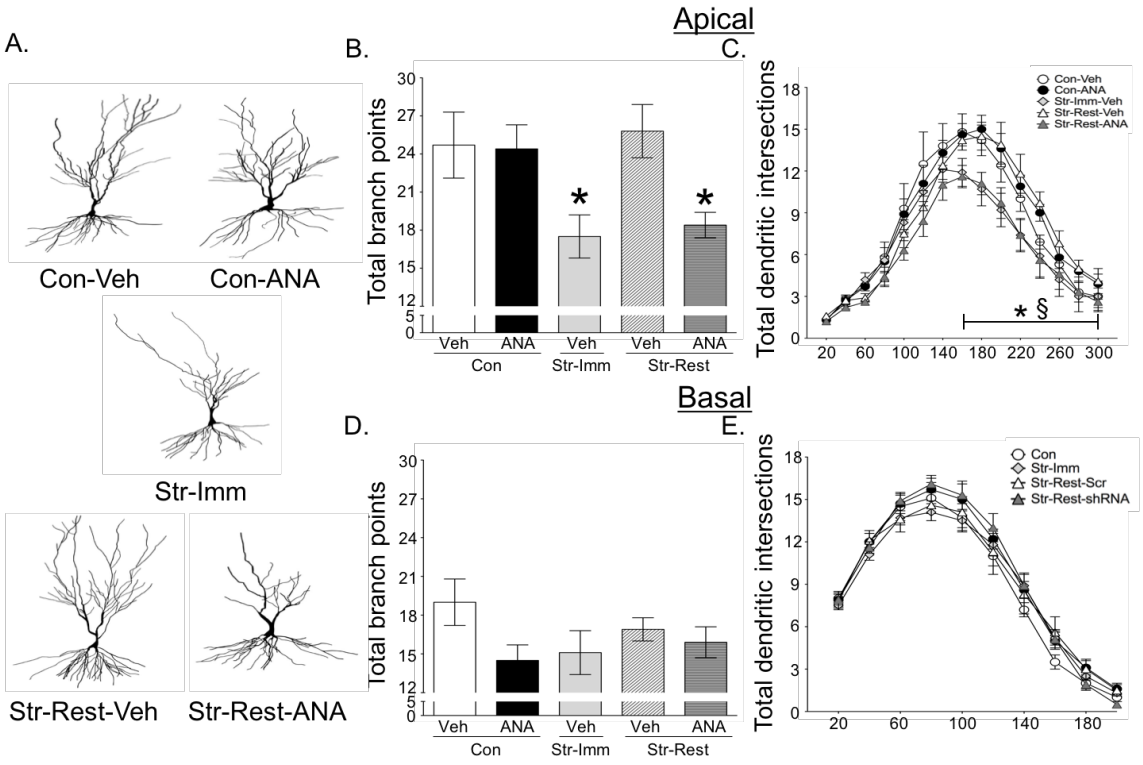

Figure 3.4: Experiment 2, effects of chronic stress, a post-stress rest period, and ANA-12 administration on hippocampal CA3 dendritic complexity. (A) Tracings of neurons from the CA3b-c region of the hippocampus. Short shaft and long shaft neuronal types were combined to obtain a single value. (B) ANA-12 administration during the post-stress rest period led to diminished hippocampal CA3 dendritic complexity in rats that were chronically stressed and given a post-stress rest period compared control rats and rats given a post-stress rest period but administered vehicle. Chronic stress also decreased dendritic complexity compared Con and Str-Rest-Veh rats (*$p < 0.05$ for Str-Rest-ANA and Str-Imm compared to Str-Rest-Veh, Con-Veh, and Con-ANA). ANA-12

administration did not significantly alter dendritic complexity in non-stressed control rats. (C) Sholl analysis on CA3 dendritic complexity revealed that ANA-12 administration led to diminished CA3 apical arbors despite a post-stress rest period, a similar pattern as was seen for the Str-Imm-Veh group. Probing further, it was found that the complexity of apical dendritic arbors of Str-Rest-ANA and Str-Imm-Veh was significantly decreased at 160μm from the soma to 300 μm compared to all other groups (*$p < 0.05$ for Str-Rest-ANA compared to Con-Veh, Con-ANA, Str-Rest-Veh and § $p < 0.05$ for Str-Imm compared to Con-Veh, Con-ANA, Str-Rest-Veh). (D-E) No statistical differences were observed in the CA3 basal dendritic arbors for dendritic intersections from the soma.

RAWM (Figure 3.5)

During the first two days of acquisition, all groups showed improvement in spatial ability as trials progressed. A 5 x 8 repeated measure ANOVA performed on total entry errors committed on Day 1 and Day 2 revealed a significant repeated effect of trials (Day 1, $F_{(7,315)} = 14.845$, $p < 0.001$; Day 2, $F_{(7,315)} = 12.513$, $p < 0.001$) with no significant treatment or interactions. Rats made fewer total entry errors as trials progressed on each day, demonstrating that the rats were acquiring the task during the course of the training days. On the first trial, the average number of entry errors committed averaged 4.2 to 8.0. By the last training trial on Day 2 (trial 16), all groups were making an average of less than one error.

The retention trial on Day 3 revealed that chronic stress (Str-Imm-Veh) and the ANA-12 treatment in the Str-Rest group resulted in an impairment of spatial memory (Figure 3.5A). A one-way ANOVA on total entry errors committed on the single retention trial on Day 3 revealed a significant main effect of treatment ($F_{(4,45)} = 2.753$, $p < 0.05$). Str-Rest-ANA performed significantly worse than the Str-Rest-Veh group ($p < 0.05$) and the Str-Imm-Veh performed significantly worse than Con-Veh, Con-ANA, and Str-Rest-Veh ($p < 0.05$ for all). Str-Rest-ANA and Str-Imm-Veh exhibited statistically similar performances on their Day 3 retention trial, committing approximately 2.4 and 3.1 errors compared to 1.0 or fewer errors from the Con-Veh, Con-ANA and Str-Rest-Veh groups.

Object Placement and Spatial Memory Index (Figure 3.5)

On trial 2 of the OP task, the Con-Veh and Str-Rest-Veh conditions tended to explore the object that was moved to a new location more than they did the object that remained in the familiar location, as revealed by an OP index with means and SEM above chance performance (Figure 3.5B). However, a one-way ANOVA on the OP discrimination score was not significant, due in part to the reduction in power from the exclusion of rats that failed to explore sufficiently. The time spent exploring objects in trial 1 was statistically similar across treatment conditions (Figure 3.5D), demonstrating that the rats were motivated to explore the objects. Consequently, the pattern detected on trial 2 most likely reflected mnemonic processes instead of differences in anxiety or motivation to explore.

A spatial memory index was computed by calculating a z-score from the total number of errors made on trial 17 of the RAWM and a z-score from the object placement index. The z-scores were summed together to form a spatial memory index with high numbers representing good spatial memory and were computed for rats in which OP performance could be measured (Figure 3.5C). A one-way ANOVA revealed a significant effect of treatment ($F_{(4,31)} = 3.920$, $p < 0.05$). The spatial memory index was significantly lower for Str-Rest-ANA compared to Con-Veh ($p < 0.01$) and Str-Rest-Veh ($p < 0.05$). The Str-Imm-Veh group also showed a spatial memory index that was significantly lower than Con-Veh ($p < 0.01$) and Str-Rest-Veh ($p < 0.05$). The spatial memory index for Con-ANA was also significantly lower than Con-Veh ($p < 0.05$) and appeared in between the levels observed for Con-Veh or Str-Rest-Veh and those of Str-Imm-Veh or Str-Rest-ANA. No other group differences were detected.

Figure 3.5. Spatial ability assessment on the radial arm water maze and object placement tasks.

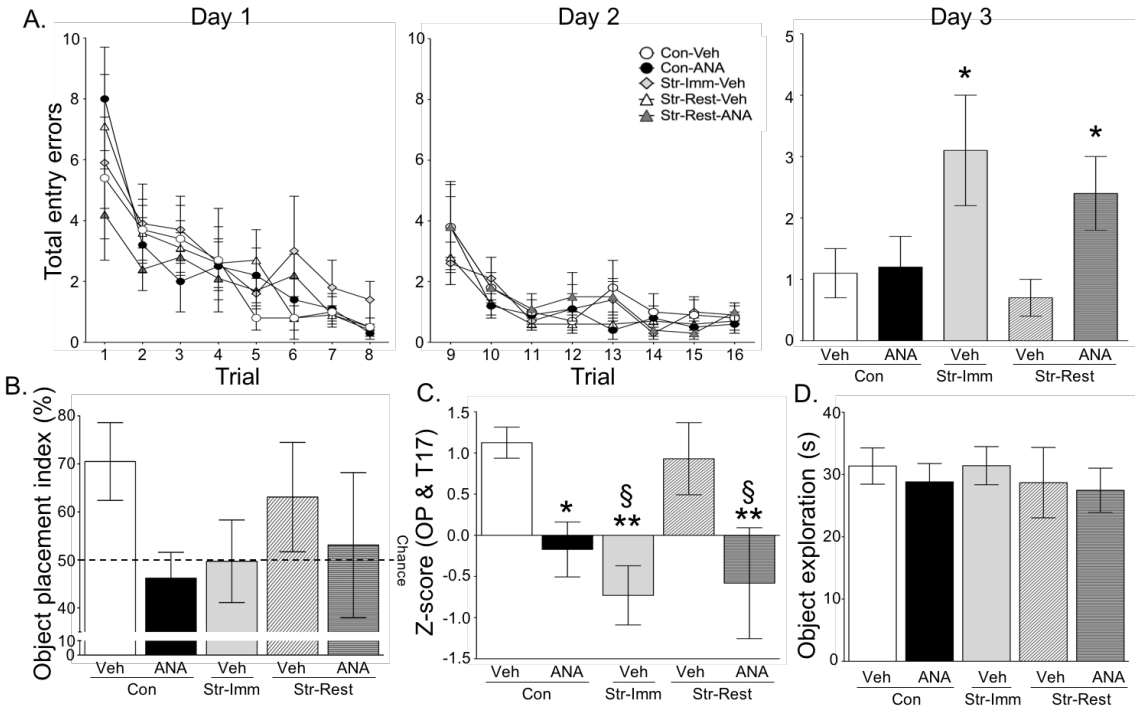

Figure 3.5: Spatial ability assessment on the radial arm water maze and object placement tasks. (A) Spatial ability was first assessed on the radial arm water maze (RAWM) and the total number of entry errors was assessed. Over days 1 and 2 all rats, regardless of stress history or drug administration, acquired the task as shown by decreased errors across trials 1-16. On the single retention trial (trial 17) on day 3, chronic stress led to significantly more errors compared to Str-Rest-Veh and Con rats. Rats that were given a post-stress rest period and administered ANA-12 displayed significantly more errors than chronically stress rats given a recovery period and administered saline (compare Str-Rest-ANA and Str-Rest-Veh). (B) Object exploration discrimination in the object placement task. On averages, Con-Veh and Str-Rest-Veh

explored the displaced object more than the object in the familiar location, but no statistically significant effects were found. (C) A Z-Score for spatial ability was calculated from the object placement discrimination index and the total number of errors made on trial 17 of the RAWM. The spatial ability index was significantly lower for Str-Rest-ANA and Str-Imm-Veh compared to both Con-Veh and Str-Rest-Veh. Last, the spatial memory index for Con-ANA was also significantly lower than Con-Veh.(D) The time spent exploring both objects in trial 1. All rats explored both objects for a similar amount of time demonstrating that the rats were motivated to explore the objects. * $p < 0.05$, ** $p < 0.001$ compared to Con-Veh, § $p < 0.05$ compared to Str-Rest-Veh.

Physiological measures

Changes in body weight was assessed to measure the effectiveness of restraint as a stressor. As expected, restraint significantly influenced body weight gain and unexpectedly, ANA-12 did too. For the first three-week restraint period, a repeated measure ANOVA for treatment over weeks for body weight gain revealed significant effects for treatment ($F_{(4,45)} = 7.355$, $p < 0.001$), significant repeated effect of weeks on body weight gain ($F_{(6,270)} = 1072.841$, $p < 0.001$), and significant interaction for treatment over weeks on body weight gain ($F_{(24,270)} = 17.98$, $p < 0.001$). A separate ANOVA for body weight gain from weeks 1 through 4, when Str-Rest rats were undergoing restraint, was performed and revealed a significant effect of treatment ($F_{(4,45)} = 39.056$, $p < 0.001$, Figure 3.1D). Post-hoc analyses showed that both Str-Rest groups (Str-Rest-Veh and Str-Rest-ANA) gained less weight during this time than did both Con groups (Con-Veh, Con-ANA) and the Str-Imm-Str group ($p < 0.001$).

For the second session of restraint during weeks 4 through 7, an ANOVA between treatment conditions was performed on body weight gain. This period included the procedures for injection and reflected when the Str-Imm-Veh group was being restrained, while the Str-Rest groups (Str-Rest-Veh, Str-Rest-ANA) were in their rest period. A significant main effect of treatment was detected ($F_{(4,45)} = 46.761$, $p < 0.001$). Post-hoc analyses showed that body weight gain of Str-Rest-Veh rats was significantly different than Con and Str-Imm rats with Str-Rest-Veh rats gaining more weight than these groups but with total body weight reaching a similar level of non-stressed rats ($p < 0.05$ for Str-Rest-Veh compared to Con-Veh, Con-ANA, Str-Imm-Veh). A similar effect was found in the Str-Rest-ANA group but with the drug having some effect on body weight gain ($p < 0.05$ compared to Con-ANA and Str-Imm-Veh). Furthermore, the Str-Imm-Veh group showed a significant attenuation of body weight gain ($p < 0.001$ for Str-Imm-Veh compared to all other groups), and the drug had some effect on body weight gain in the Con-ANA animals as they displayed a significant attenuation of body weight gain compared to Con-Veh ($p = 0.05$).

Discussion

These results indicate that hippocampal BDNF and the TrkB receptor are required for hippocampal CA3 apical dendritic retraction and spatial memory deficits to improve in the weeks after chronic stress has ended. Chronic stress led to an expected reduction in CA3 apical dendritic complexity and an impairment in spatial ability. Moreover, rats that were given a post-stress rest period without experimental manipulation of hippocampal

BDNF displayed a similar dendritic and functional profile as the control rats and an improvement of spatial ability compared the Str-Imm rats. The novel findings reveal that hippocampal BDNF knockdown or TrkB receptor antagonism hindered the improvement of CA3 dendritic architecture, as the stressed group provided with the post-stress rest period and infused with BDNF knockdown (Str-Rest-shRNA) in experiment 1 or ANA-12 (Str-Rest-ANA) in experiment 2 showed reduced dendritic complexity that was statistically similar to the Str-Imm rats. Moreover, novel findings in experiment 2 revealed that ANA-12 administration targeting the rest period was sufficient to block the enhancement of CA3 apical dendritic complexity that typically occurs during the post-stress rest period and that spatial memory deficits failed to improve in the same rats. Together, these data suggest that BDNF and its receptor TrkB mediate two forms of plasticity in the hippocampus following the termination from chronic stress: enhancement of CA3 dendritic arborization and improvement in hippocampal-dependent spatial cognition.

These findings provide new evidence that BDNF and its TrkB receptor are required for the changes necessary to increase CA3 apical dendritic complexity following a post-stress rest period and that this dendritic restructuring corresponds with improvements from spatial memory deficits following the end of chronic stress. Our chronic stress findings are consistent past work reporting that chronic stress leads to a retraction of hippocampal CA3 apical dendrites (Hoffman et al., 2011; Sousa et al., 2000; Watanabe, Gould, & McEwen, 1992) and deficits in spatial ability (Conrad, 2006; Hoffman et al., 2011; Sousa et al., 2000). Moreover, when chronic stress ends and a post-stress rest period is given, these findings corroborate other work showing that

hippocampal CA3 apical dendritic retraction reorganizes during the post-stress period and increases in complexity along with concurrent improvement in spatial ability (Hoffman et al., 2011; Ortiz et al., 2014; Sousa et al., 2000). In our past work, we first reported that hippocampal BDNF was necessary for the spatial memory improvements that occur in the weeks that follow the end of chronic stress (Ortiz et al., 2014). Specifically, a viral vector infused into the hippocampus to knockdown BDNF expression led to chronically stressed rats displaying a sustained impairment in spatial ability that persisted despite a three-week rest period following the end of chronic stress (Ortiz et al., 2014). This work demonstrated that BDNF within the hippocampus was necessary for the spatial memory improvements to occur after chronic stress has ended. While BDNF knockdown was localized to the hippocampus, the knockdown occurred throughout chronic stress and the post-stress rest period. Consequently, this clarified that BDNF acted in the hippocampus, but it was unclear as to whether BDNF actions were required during the chronic stress phase, the post-stress rest period, or both. In the current report, we provide new evidence that BDNF effects on hippocampal CA3 dendritic arbors and spatial ability occur in the post-stress rest period after chronic stress has ended. In experiment 2, ANA-12 was administered daily throughout the three-week rest period and that was sufficient to block the CA3 apical dendritic complexity enhancement and spatial memory improvement.

The daily administration of ANA-12 during the post-stress rest period prevented the return of spatial ability and hippocampal CA3 dendritic complexity in chronically stressed rats. ANA-12 was recently described as a TrkB antagonist (Cazorla et al., 2011) that has been used with success to study TrkB's involvement in various behaviors (Cazorla et al., 2011; Ren et al., 2015; Walsh et al., 2014). Advantages of using ANA-12

is that it was one of the first TrkB antagonists developed that can penetrate the blood brain barrier, allowing for systemic administration (Cazorla et al., 2011). Consequently, ANA-12 was a logical next step to test the relevance of the TrkB receptor during the post-stress rest period for effects on spatial ability and hippocampal dendritic complexity. This was especially relevant, given that BDNF and TrkB are essential for hippocampal-mediated learning (Minichiello, 2009; Minichiello et al., 1999; Yamada & Nabeshima, 2003), synapse maturation, plasticity and dendritic growth (Yacoubian & Lo, 2000; Yoshii & Constantine-Paton, 2010) and are expressed highly in the hippocampus (Conner et al., 1997; Drake et al., 1999). As such, we were able to use ANA-12 to target a specific time period, such as the post-stress rest period. Indeed, our initial work using shRNA began prior to the widespread availability of ANA-12. A caveat is that ANA-12 can impact any brain region with TrkB receptors, for which there are many, including the amygdala, cingulate and frontal cortex (Fryer et al., 1996). While, the administration of TrkB affecting other brain regions is one limitation, the strength of our report is the combined use of shRNA in the first experiment, which localized changes in BDNF expression to the hippocampus, and the use of ANA-12 in the second experiment, which targeted the post-stress rest period. These two experiments combined, along with our published work (Ortiz et al., 2014), provide very compelling support that BDNF within the hippocampus acting on its TrkB receptor in the period following the end of chronic stress is necessary to improve hippocampal dendritic complexity and spatial memory.

The results further extend findings on how chronic stress affects the dendritic complexity of hippocampal CA1 neurons. First, chronic stress had subtle effects on CA1 dendritic complexity that may not have been detected using traditional branch counts of

total bifurcations. As found in many reports (D. N. Alfarez et al., 2008; McLaughlin et al., 2005; Woolley et al., 1990), chronic stress did not lead to changes in total apical branch points within the apical or basal CA1 regions. However, regional changes were observed after performing Sholl analyses. In the apical CA1 region, chronic stress led to an increase in CA1 apical dendritic complexity at the most distal ends. Other reports have observed chronic corticosterone (CORT) or chronic stress administration to influence CA1 dendritic complexity, but how and to what extent differs (Lambert et al., 1998; Sousa et al., 2000). In studies investigating chronic CORT administration, one study found chronic CORT administration, that resulted in CORT levels similar to chronically stressed animals, to reduce the number and length of basal dendrites with the medial basal region being most affected (Gourley et al., 2013). Another study found chronic CORT administration, that resulted in higher levels of CORT than in rats exposed to chronic stress, reduced the length of CA1 dendrites, but did not account for differences in apical versus basal dendrites (Sousa et al., 2000). For chronic stress studies, the severity or duration of the stressor maybe relevant. Rodents housed with activity wheels and food restricted showed hippocampal CA3 and CA1 apical dendritic atrophy in about one week (Lambert et al., 1998), whereas a less robust stressor in the Sousa study (Sousa et al., 2000), resulted in a reduction in the total length of apical dendrites of CA1 neurons following chronic unpredictable stress for 30 days. It is important to note that the authors said the CA1 neurons were less vulnerable than the CA3 neurons (Sousa et al., 2000). Our finding is consistent with these reports that the CA1 neurons are less likely to show dendritic alterations than the CA3 neurons. We also acknowledge that the enhancement in CA1 basal complexity was detected using an extreme manipulation that knocked down

hippocampal BDNF, the correlation using all groups was significant and indicated that as CA3 apical dendritic complexity decreased, CA1 basal dendritic complexity increased. The mechanisms behind this phenomenon are unknown, but the CA3 and CA1 neurons are intimately connected, with CA3 neurons providing the primary inputs to the CA1 basal region (Kajiwara et al., 2008; Witter, 1993). Furthermore, BDNF release from CA3 neurons is required to induce presynaptic LTP in CA1 neurons (Zakharenko et al., 2003). Consequently, our findings support the interpretation that the diminished complexity of CA3 neurons may lead to alterations in the dendritic complexity of the CA1 region, perhaps as a compensatory mechanism. Further studies will be needed to test this hypothesis.

The mechanisms by which a post-stress rest period, BDNF and TrkB interact to promote the increase in CA3 dendritic complexity and improvement in spatial ability in the weeks after chronic stress ends may involve the hippocampal inhibitory system. There is a link between BDNF-TrkB and the GABAergic system. For example, BDNF application leading to reduced inhibitory transmission in the brain, with no effect on excitatory transmission (Frerking, Malenka, & Nicoll, 1998; Tanaka, Saito, & Matsuki, 1997). Furthermore, TrkB controls synapse formation of GABAergic neurons (A. I. Chen et al., 2011) and inactivating the TrkB receptor results in decreases of GAD65 expression, and decreases in GABAergic synaptic contacts in the hippocampus (Carmona et al., 2006). As such, it would appear chronic stress-induced decreases in BDNF – TrkB signaling in the hippocampus would also alter the hippocampal GABAergic system. This is relevant to the findings that chronic stress leads to alterations in GABAergic and glutamatergic activity in the hippocampus and alters the ratio of glutamate to GABA

favoring more glutamate (Gronli et al., 2007; Joels et al., 2004). Lesions of the main excitatory input into the hippocampus (Magarinos & McEwen, 1995), or preventing the synthesis of glutamate (Watanabe, Gould, Cameron, et al., 1992) can negate chronic stress induced dendritic retraction. For the GABAergic system, chronic stress or chronic corticosterone treatment decreased the expression of GABAergic interneurons in the hippocampus (Czeh et al., 2015) as well as specific subtypes of GABAergic interneurons (Czeh et al., 2005; Czeh et al., 2015). We have found that GAD65, one of the synthesizing enzymes for GABA, is correlated with spatial ability (Ortiz et al., 2015) and a single dose of a $GABA_A$ antagonist can rescue spatial ability following chronic stress (Nishimura, Ortiz, & Conrad, 2017). In the context of this study, a decrease in the expression of BDNF, or a disruption of TrkB signaling, during the post-stress rest period may lead to a sustained disruption in the balance of glutamate and GABA. Taken together, the influence of BDNF/TrkB on the GABAergic system may be especially important during chronic stress and the post-stress rest period in order for the brain to maintain its plasticity.

The current data and our past findings (Ortiz et al., 2014) demonstrate for the first time a potential mechanism that allows individuals to improve their spatial ability in the aftermath from chronic stress. Hippocampal BDNF is required during the post-stress rest period for hippocampal CA3 apical dendritic arbors to increase in complexity following the end of chronic stress. BDNF within the hippocampus is critical, as viral vector technology permitted spatial resolution to the CA3 region of the dorsal hippocampus in order to downregulate hippocampal BDNF levels. BDNF acting on its TrkB receptor must be present during the post-stress rest period, as the TrkB receptor antagonist, ANA-

12, injected daily during the post-stress rest period prevented the enhancement in CA3 apical dendritic complexity and spatial memory ability. By disrupting BDNF expression or TrkB receptor actions, the plasticity mechanisms that typically occur during a post-stress rest period are compromised and CA3 dendritic arbors fail to become complex and spatial memory remains poor. Given that many brain regions express BDNF and TrkB receptors, future studies will begin probing potential down-stream mediators.

CHAPTER 4

SEX-SPECIFIC IMPAIRMENT AND RECOVERY OF SPATIAL LEARNING FOLLOWING THE END OF CHRONIC UNPREDICTABLE RESTRAINT STRESS: POTENTIAL RELEVANCE OF LIMBIC GAD.

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Contributions: JBO, SBT, and CDC designed the experiment. ANC led the behavioral testing and then JBO took over the project to bring it to completion as follows: JBO, SBT, ANH, ANC implemented stressors and collected brain tissue. JBO, ANC did behavioral testing. LRL performed *in situ* hybridization. JBO, SBT and CDC analyzed data. JBO wrote the manuscript under the guidance of SBT and CDC.

Introduction

It is increasingly apparent that chronic stress impairs hippocampal-dependent spatial learning and memory in a sex-dependent manner. In males, chronic stress impairs performance on the radial arm maze (V. Luine et al., 1994; Sweis, Veverka, Dhillon, Urban, & Lucas, 2013), Y-Maze (McLaughlin et al., 2007), Morris water maze (Green & McCormick, 2013; Kitraki et al., 2004a; E. Kitraki, O. Kremmyda, D. Youlatos, M. N. Alexis, & C. Kittas, 2004b; McFadden et al., 2011; Radecki et al., 2005; Song et al., 2006; Sousa et al., 2000), and the radial arm water maze task (RAWM, (Hoffman et al., 2011; Hutchinson et al., 2012); For review see: (Conrad, 2010; Popoli, Yan, McEwen, & Sanacora, 2011). Conversely, several studies report that chronic stress enhances spatial memory ability in females on the radial arm maze (Bowman, Zrull, & Luine, 2001), Y-maze (McLaughlin et al., 2007), object placement task (Beck & Luine, 2002), and Morris water maze (Kitraki et al., 2004a; Kitraki et al., 2004b; V. N. Luine, Beck, Bowman, Frankfurt, & Maclusky, 2007; McFadden et al., 2011; Simpson & Kelly, 2012). These studies suggest that the sex of an individual influences the underlying neural mediators of spatial ability.

A putative target for chronic stress actions is the hippocampus, as it is essential for spatial memory (Conrad, 2010; Eichenbaum, 1999; Moser, Moser, & Andersen, 1993; O'Keefe & Dostrovsky, 1971) and contains an abundance of receptors for glucocorticoid stress hormones (McEwen et al., 1968). In male rats, chronic stress leads to a simplification of apical dendritic arbors within the CA3 region of the hippocampus (Conrad, 2006; Galea et al., 1997; Watanabe, Gould, Cameron, et al., 1992; Watanabe, Gould, & McEwen, 1992) and reduced glucocorticoid receptor number (Sapolsky et al.,

1984a, 1984b), both of which correspond to deficits in spatial tasks (Hoffman et al., 2011; Wright et al., 2006). When chronically stressed males are given time to recover following the end of chronic stress, spatial ability improves in parallel with the restoration of the CA3 dendritic architecture (Hoffman et al., 2011; Ortiz et al., 2014). In contrast, chronically stressed, gonadally-intact female rats show mild or negligible basal dendritic retraction in the CA3 region (Galea et al., 1997; McLaughlin et al., 2010), which does not necessarily correspond with spatial memory (McLaughlin et al., 2005). Mitigating variables that might contribute to chronic stress effects on the hippocampus and spatial memory in females include the type of stressor (McCormick, Smith, & Mathews, 2008; Park, Zoladz, Conrad, Fleshner, & Diamond, 2008), task (McCormick, Nixon, Thomas, Lowie, & Dyck, 2010; McLaughlin et al., 2008; Wood & Shors, 1998), estrogen status (Conrad, McLaughlin, Huynh, El-Ashmawy, & Sparks, 2012; Ortiz et al., 2013; Shansky, Rubinow, Brennan, & Arnsten, 2006) and even experimenter handling of the rats (Bohacek & Daniel, 2007; Dobrakovova, Kvetnansky, Oprsalova, & Jezova, 1993; Hoffman, Armstrong, Hanna, & Conrad, 2010). Whether females would return to their previous status in spatial ability following a post-stress recovery period has not been investigated. One goal of the present study is to investigate the potential sex differences arising from the effects of chronic stress and a post-stress recovery period on spatial learning and memory. Successful spatial memory recovery from chronic stress has been documented in male rats using the radial arm water maze (Hoffman et al., 2011; Ortiz et al., 2014) and so a similar task will be used in the current study.

An important variable that could impact sex differences in spatial memory performance is the type of stressor used. Restraint stress is commonly used in rodents due

to its relative ease of use, however chronic restraint elicits detriments in spatial learning and memory in male (Gomez, Lewis, & Luine, 2012; Hoffman et al., 2011; Ortiz et al., 2014; Sousa et al., 2000), but not female rats (Kitraki et al., 2004b; McLaughlin et al., 2010). Stressors can be categorized as being physical, psychological, or both and chronic restraint is thought to include components of both physical and psychological stressors (Buynitsky & Mostofsky, 2009). In humans, psychological stressors produce a stress response when the stressor consists of any one of three characteristics: loss of control, novelty, and unpredictability (Cohen, 1980; Mason, 1968; Miller, 1981). In rodents, administration of chronic restraint stress might consist of a loss of a sense of control, but lacks components in the other categories (i.e. novelty and unpredictability). Furthermore, repeatedly administering restraint leads to predictability, as male rats demonstrate habituation of the stress hormone response (Galea et al., 1997). Therefore, one purpose of the present study was to determine whether enhancing the unpredictable nature of chronic restraint would lead to impaired spatial ability in both male and female rats.

Another purpose of this study was to examine the role of inhibitory tone as it pertains to chronic stress-induced changes in spatial learning and memory. Glutamate and γ-aminobutyric acid (GABA) neurotransmitters are primarily responsible for regulating inhibitory tone. In male rats, chronic stress leads to significant increases in hippocampal extracellular glutamate (Joels et al., 2004) and decreases in hippocampal GABA (Gronli et al., 2007; O'Mahony, Clarke, Gibney, Dinan, & Cryan, 2011). Hippocampal GABA levels in males might be lowered following chronic stress, in part, due to decreases in glutamic acid decarboxylase (GAD) levels, the synthesizing enzyme for GABA (El-faramawy et al., 2009). Furthermore, other limbic areas may display a dysregulated

inhibitory tone following chronic stress, which may also impact spatial learning and memory. MeA input into the HPA axis is critical for HPA axis activation following restraint stress (Dayas, Buller, & Day, 1999). Cells in the MeA exhibit the greatest level of *c-fos* activation following a psychological stressor compared to other amygdala nuclei (e.g. central amygdala). These findings suggest that the MeA is implicated in the response to stressors. Taken together, these studies provide support for the notion that the spatial memory impairments induced by chronic stress are mediated by changes in inhibitory tone the limbic system. Whether these changes contribute to the sex differences observed in spatial learning and memory tasks following chronic stress are unknown and will be investigated.

The current study tested the hypothesis that the unpredictability of a chronic stress paradigm impacts the sensitivity of females to the immediate and long-term (i.e., recovery from) effects of chronic stress. The behavioral endpoint of spatial learning and memory was chosen due to the well-established immediate and long-term effects of chronic restraint on male rats. In addition, this study explored the effect of chronic unpredictable restraint stress on GAD expression in limbic regions, as a likely mediator of chronic stress effects and a potential mediator of sex differences.

Material and methods

Animals

Sixty young adult Sprague-Dawley male and female rats (males=30, females=30) approximately 2 months of age were purchased from Charles River Laboratories (Wilmington, MA) and were pair housed with a same-sex cage mate at Arizona State

University housing facilities. Male and female rats were housed in separate rooms and control and experimental groups were additionally separated into different chambers on a reverse light cycle (12:12; lights off at 06:00am). Rats were given one week to acclimate before any behavioral procedures were performed. Food and water were available *ad libitum* except during the restraint procedure when both control and stressed animals did not have access. Rats were weighed once a week throughout the experiment. Behavioral testing was conducted during the dark phase of the light cycle. All procedures were conducted according to federal guidelines outlined in the Guide for the Care and Use of Laboratory Rats (Institute of Laboratory Animal Resources on Life Science, National Research Council, 1996) and institutional guidelines set forth by the Institutional Animal Care and Use Committee at Arizona State University.

Group Assignments

Rats were randomly divided into six groups (n=10/group): male and female non-stressed control (Con), male and female stressed rats that were tested immediately (Str-Imm), and male and female stressed rats that were given a 21-day post-stress recovery period (Str-Rec). The experimental groups and timeline are depicted in (Figure 4.1A).

Figure 4.1. Timeline and Physiological data.

B.

	Body Weight Gained (g)		Adrenal Weight (mg/100g body weight)
	Weeks 1-3	Weeks 4-6	After RAWM Retention Test
Con male	85.2 ± 8.27*	58.6 ± 4.86***	9.81 ± 0.5
Str-Imm male	68.1 ± 14.53**	13.2 ± 10.46	12.32 ± 0.59**
Str-Rec male	32.8 ± 5.74	76.8 ± 6.62***	11.62 ± 0.58*
Con female	16.7 ± 2.75	15.7 ± 1.13#	26.22 ± 1.09
Str-Imm female	25.3 ± 3.38	9.6 ± 5.19##	27.21 ± 1.25
Str-Rec female	13.3 ± 3.97	33.7 ± 5.78	24.36 ± 0.82

Figure 4.1. Experimental Timeline and Physiological Data.

A. Experimental Timeline. Male and female rats were assigned to a chronic unpredictable restraint (CUR) stress for 21 days and then given a 21-day post-stress recovery period (Str-Rec) before radial arm water maze (RAWM) testing, or tested the day after restraint ended (Str-Imm), or left undisturbed (Con). All rats underwent 3 days of training and testing on the RAWM and were then sacrificed 30 minutes after the last testing session. Note that darkened blocks are shown to indicate when CUR stress occurred. **B. Stressor Effectiveness**. In males, CUR significantly attenuated body weight gain. Unstressed males gained significantly more weight than stressed males during CUR administration. During weeks 1-3, Str-Rec rats gained significantly less weight than both Con (* $p < 0.05$) and Str-Imm (** $p < 0.01$) males. During weeks 4-6, Str-Imm males gained significantly less weight than Con (*** $p < 0.001$) and Str-Rec (* $p < 0.001$)

males. In females, CUR did not significantly attenuate body weight gain. However, during recovery (weeks 4-6), Str-Rec females exhibited significantly increased body weight gain compared to both Con (# $p < 0.05$) and Str-Imm (## $p < 0.01$) females. In males, CUR significantly increased relative adrenal weight in both Str-Imm (** $p < 0.01$) and Str-Rec * $p < 0.05$) groups compared to Con males. In females, CUR had no effect on adrenal weight. Note that darkened blocks are shown to indicate when CUR stress occurred.

Chronic Unpredictable Restraint Stress Procedure

Rats were transported to different rooms each day and were restrained using restrainers made from wire mesh (purchased from Flynn and Enslow, San Francisco, CA, USA) with the ends sealed with grip guard sealer (ACE Hardware). The ends were secured with black binder clips. The chronic unpredictable restraint (CUR) schedule was comprised of different start time periods (ranges: 05:00-07:00, 08:00-10:00, 11:00-13:00, 14:00-16:00, 17:00-19:00), physical contexts (four different rooms), extract odors (almond, banana, orange), and restraint durations (no restraint, 30 minutes, 60 minutes, 90 minutes, and 120 minutes). After acclimation, the Str-Rec group was unpredictably restrained for 21 days then given a 21-day recovery period from restraint before behavioral testing. Str-Imm, however, were left undisturbed during the first 21 days, then unpredictably restrained during the subsequent 21 days and behaviorally tested the day after restraint ended. Con rats were left undisturbed until behavioral testing. Since the past literature indicates that even the subtle manipulation of handling to determine estrous status can impact female performance (Bohacek & Daniel, 2007; Dobrakovova et al.,

1993; Hoffman et al., 2010), the gonadally-intact male and female rats were not manipulated to determine female estrous stage status.

Radial Arm Water Maze (RAWM)

Apparatus

The RAWM was constructed of black polypropylene with eight symmetrical arms (27.9 cm long by 12.7 cm wide) protruding from a circular center (48 cm diameter). The water (20˚C-22˚C) was made opaque with non-toxic powdered black tempera paint in order to obscure the platform. Males and females were tested in separate rooms. Each room contained salient cues along the walls of the room. Groups were counterbalanced across experimenters.

Procedure

A testing platform was placed at the end of one arm, 2.5 cm below the surface of the opaque water. The platform location was kept constant throughout the trials for each rat, but was counterbalanced between rats. Testing occurred over three consecutive days between 9:00am and 3:00pm. The first two training days consisted of eight trials, with one retention trial given on the third day. A trial began as soon as the rat was released into a non-platformed arm (start arm), which varied across trials so that a rat did not start in the same start arm on consecutive trials. Additionally, a trial was never initiated in an arm directly across from the platformed arm. Rats were given a maximum of three minutes to find the hidden platform. If a rat failed to find the hidden platform, the experimenter guided the rat to it. Rats remained on the platform for 15 seconds before they were removed and returned to the testing cage underneath heat lamps. After each

trial, the maze was cleared of any debris, and the water was stirred to prevent subsequent rats from using odor cues. To avoid exhaustion, rats of different groups were tested in cohorts of six to eight at a time. After a trial was completed, rats were placed back into the testing cage to rest while the other rats in the cohort were tested. Time between each trial for an individual rat ranged from 5 minutes to 20 minutes. An entry into an arm was scored when the tip of the rat's nose passed 11 cm into an arm.

Brain Collection and Tissue Processing

Thirty minutes after the start time of the retention trial on the final day of testing, rats were transported to a necropsy room and deeply anesthetized with isoflurane and rapidly decapitated. Adrenal glands were dissected and weighed. Brains were removed, blocked for hippocampus and amygdala, flash frozen in 2-methylbutane chilled with dry ice, and stored in a -80^{o}C freezer until brains until processing for *in situ* hybridization.

***In Situ* Hybridization**

In situ hybridization was utilized to quantify GAD_{65} mRNA expression in the cornu ammonis 1 (CA1) and dentate gyrus (DG) regions of the hippocampus, as well as the ventromedial hypothalamus (VMH) and medial amygdala (MeA). Brains were maintained at -70°C until transfer to cryostat for sectioning (at -20°C) and subsequently kept at -20°C until used in the *in situ* protocol. Twenty micrometer coronal brain sections were thaw-mounted on to glass microscope slides (Superfrost Plus, VWR, West Chester PA) on a cryostat (Leica).

Antisense mRNA was generated from a cDNA plasmid (generously provided by Dr. A.J. Tobin, UCLA) linearized with HdIII and transcribed with RNA Polymerase T3 (Promega) with [35-S]-UTP (Perkin-Elmer) to a specific activity of 4.5×10^8 in a 550 nucleotide mRNA fragment. Unincorporated nucleotides were removed by Rneasy® MinElute™ Cleanup Kit (Qiagen). Prehybridization of tissue sections included a brief drying at room temperature after removal from -20°C storage, postfixation in 4% formaldehyde/PBS, rinsing in PBS, and acetylation in 0.25% acetic anhydride/0.1 M triethanolamine. Slides were passed through a dehydrating ethanol gradient and incubated with probe in hybridization solution at a saturated concentration (~ 28 Kcpm/µL) under glass coverslips at 45 °C for 18 h. Coverslips were removed in 4× standard sodium citrate and non-specifically bound probe was removed by treatment with RNase (Sigma-Alrich) for 30 min. Sections were run through stringency washes of 1× SSC and 0.5× SSC at 37 °C, and 0.1× SSC at 45 °C. Sections were then dehydrated, air-dried and exposed to Kodak BioMax X-ray film for 3 days along with microscale ^{14}C standards (Amersham Biosciences). Autoradiographic images were transferred to a desktop illuminator (Kaiser Fototechnik, Buchen, Germany) with a CCD video camera (Hamamatsu C8484) with a MacroNikkor lens (Nikon) attached. X-ray film brain sections were digitized using computer assisted densitometry (Compix Imaging Systems, Sewickly PA). Background illumination was recorded (and digitally subtracted from subsequent images) and optical density was plotted as a function of microscale calibration values. Regions of interest in the CA1, DG, VMH, and MeA were selected among 4 to 6 coronal sections bilaterally, digitized and a calibrated mean optical density

value was obtained for each location resulting in one averaged value of each region per animal.

Statistical Analyses

Statistical analyses were performed using SPSS (version 22). Physiological and behavioral data were analyzed with multifactorial or repeated measures analyses of variance (ANOVAs). Main effects and interactions were followed by Fisher's LSD post hoc tests when $p < 0.05$. Pearson's correlations were used to help determine the relationship between GAD_{65} expression and RAWM performance. Data are represented as means ± S.E.M. Results were considered statistically significant if $p < 0.05$.

Results

Physiological Measures

Body Weight

Attenuation of body weight gain throughout the study confirmed the effectiveness of CUR. Before experimental manipulation, the weight of male rats ranged from 282 grams to 332 grams, with an average weight of 317.5 grams. Female rats weight ranged from 194 grams to 230 grams, with an average weight of 210.3 grams. A multifactorial repeated measures ANOVA for stress history and sex over the two treatment periods (weeks 1-3: restraint on Str-Rec; weeks 4-6: restraint on Str-Imm) revealed significant main effects of stress ($F_{(2,54)} = 11.26$, $p < 0.001$) and sex ($F_{(1, 54)} = 194.69$, $p < 0.001$) and an interaction between treatment period and stress ($F_{(2, 54)} = 15.48$, $p < 0.001$). Unstressed males gained significantly more weight than stressed males during CUR administration

(weeks 1-3: Con > Str-Rec, $p < 0.01$, Str-Imm > Str-Rec, $p < 0.05$; weeks 4-6: Con/Str-Rec > Str-Imm, $p < 0.001$; Figure 4.1B). In addition, during the recovery period the Str-Rec males exhibited weight gain similar to Con males (Figure 4.1B). Interestingly, the same pattern was not evident in females. Unstressed females did not gain significantly more weight than stressed females during the restraint periods (Figure 4.1B). However, during the recovery period (weeks 4-6), the Str-Rec females exhibited a significant increase in weight gain compared to both Con ($p < 0.05$) and Str-Imm ($p < 0.01$) females.

Adrenal weight

Restraint stress produced a significant increase in adrenal weights relative to body weight in males, but not in females (Figure 4.1B). Adrenal weights (mg) were analyzed per 100 g body weight. A 2 × 3 ANOVA for sex and stress revealed a significant main effect of sex ($F_{(1, 50)} = 401.134$, $p < 0.001$) and a tendency for an effect of stress history ($F_{(2,50)} = 2.53$, $p = 0.09$) on adrenal weights, with no significant interaction of sex by stress ($F_{(2,50)} = 2.17$, $p = 0.125$). This analysis was followed up with separate one-way ANOVAs in males and females to examine stress history. In males, a significant effect of stress was found ($F_{(2, 26)} = 4.872$, $p < 0.05$; Figure 4.1B), while the females failed to show an effect of stress on adrenal weight (Figure 4.1B). Post hoc analyses in male rats revealed a significant increase in relative adrenal weight in both Str-Rec ($p < 0.05$) and Str-Imm ($p < 0.01$) groups compared to Con males (Figure 4.1B).

Radial Arm Water Maze

Acquisition

Significant acquisition effects of CUR were observed across training and this interacted with stress history. A repeated-measures ANOVA for stress history and sex across training blocks (two trials each, blocks 1-8) revealed a significant effect of block ($F_{(7,364)} = 32.41$, $p < 0.001$) on entry errors, indicating that all rats learned the task (Figure 4.2). In addition, an interaction between stress history and block was detected ($F_{(14,364)} = 1.84$, $p < 0.05$) indicating that not all groups performed similarly across time and this was probed further by day, as described next.

Training Day One: To reduce the impact of variability during the information gathering trials, the first block of day 1 was parsed out and analyzed separately. A two-way ANOVA for stress history and sex on the first block (2 trials) of training revealed no main effects of treatment or sex on entry errors (Figure 4.2A and 4.2B), confirming that all groups made a similar number of entry errors at the beginning of training. The subsequent blocks on day 1 (blocks 2-4) reflect behavior impacted by information gathered in the first and subsequent blocks. A two-way repeated measures ANOVA across blocks 2-4 on entry errors revealed a significant interaction between stress history and sex ($F_{(2,52)} = 4.72$, $p < 0.05$) with no significant main effects of stress or sex. To further probe the sex-specific effects of stress history on acquisition, subsequent analyses assessed day 1 performance in males and females separately. In males, a repeated measures ANOVA on blocks 2-4 revealed a significant main effect of stress history ($F_{(2,26)} = 7.58$, $p < 0.01$). Post hoc analyses revealed that Str-Imm males made more errors than did Con ($p < 0.05$; Figure 4.2A) and Str-Rec males ($p < 0.001$; Figure 4.2A). Con

and Str-Rec males performed similarly on blocks 2-4. In females, no main effect of stress history was detected on blocks 2-4 (Figure 4.2B).

Sex Differences on Day One: Additional ANOVAs were used to probe the stress by sex interaction on blocks 2-4. An effect of sex was probed for each stress cohort separately. A significant effect of sex was found between Str-Rec males and females on blocks 2-4 ($F_{(1,18)} = 8.57$, $p < 0.01$), such that Str-Rec females made more errors than did Str-Rec males (2.17 ± 0.34 vs. 1.1 ± 0.12; Figure 4.2C). For the Str-Imm comparison, a tendency for an effect of sex was also identified ($F_{(1,17)} = 3.00$, $p = 0.1$), such that Str-Imm females made fewer errors than did Str-Imm males (1.72 ± 0.33 vs. 2.67 ± 0.45; Figure 4.2C). No difference in performance between Con females and males was evident (2.09 ± 0.46 vs. 1.67 ± 0.22; Figure 4.2C).

Training Day Two: On the second day of training (8 trials; blocks 5-8), no significant main effects of stress or sex were identified with a repeated measures ANOVA on entry errors (Figure 4.2). However, an interaction between block and stress was observed ($F_{(6,156)} = 3.08$, $p < 0.01$). To further probe performance on day two, repeated measures ANOVAs were run separately for males and females. This analysis indicated that the stress effect was carried by the males (block by stress interaction; $F_{(6,78)} = 3.89$, $p < 0.01$) but not the females. Block by block analysis of male performance revealed a significant effect of stress on block 5 only. Post hoc analyses indicated that this effect was caused by the Str-Rec males, such that Str-Rec males made more errors on block 5 than both Con and Str-Imm males ($p < 0.05$; Figure 4.2A). No effect of stress history on day two was identified in females.

Retention

Twenty-four hours after the final day of training, all groups were given a single retention trial to investigate long-term memory. A two-way ANOVA for stress history and sex did not reveal a significant main effect of either stress history or sex on either memory domain (Figure 4.2). Further probing of the retention trial with separate ANOVAs for males and females did not reveal any sex-specific effects of stress history.

Sample Size: One female Con rat was a statistical outlier and excluded, and one male Str-Imm rat was excluded for health issues. The final sample sizes were as follows: Male Con: n=10, male Str-Rec: n=10, male Str-Imm: n=9, female Con: n=9, female Str-Rec: n=10, female Str-Imm: n=10.

Figure 4.2. RAWM Performance.

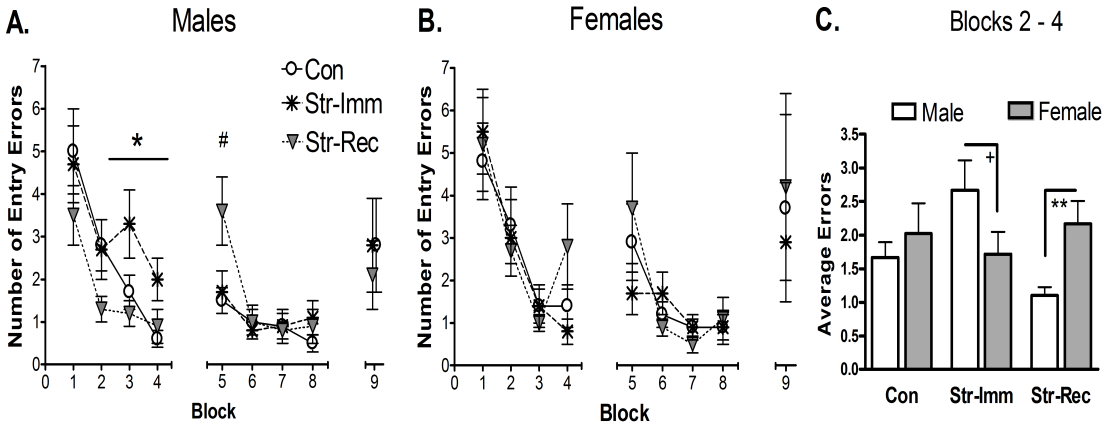

Figure 4.2. Radial Arm Water Maze.

A and B. All groups demonstrated improved performance during acquisition (Day 1: blocks 1-4, Day 2: blocks 5-8). Data points represent mean entry errors ± SEM for each block. **A.** In males, significant acquisition effects were found on day 1. After the first

block of information gathering (block 1), Str-Imm males made significantly more errors compared to both CON and Str-Rec males (* $p < 0.05$, blocks 2-4). Str-Rec and Con males showed statistically similar number of errors on day 1. However, at the beginning of the second day of training, Str-Rec rats made more errors than both Con and Str-Imm males ($^{\#} p < 0.05$, block 5), possibly indicating greater overnight forgetting. No other effects of stress history were found in males on day 2 of training (blocks 5-8) or on the retention trial (block 9). **B.** In females, no main effect of history was detected on any day or block. **C.** Sex differences were evident on day 1, blocks 2-4, where males demonstrated significant effects of stress history. Str-Rec females made significantly more errors than Str-Rec males (** $p < 0.01$) while Str-Imm females demonstrated a pattern of making fewer errors than Str-Imm males (+ $p = 0.1$). Con males and females did not differ. Bars represent mean ± SEM entry errors on blocks 2-4.

GAD_{65} expression

In a first pass analysis, an omnibus 2 × 3 ANOVA for the variables of sex and stress history on GAD_{65} expression was performed on each brain region analyzed. There were no significant main effects for sex, stress history, or interaction in any of the four areas examined (Table 1). Since the variable of stress was expected to alter GAD_{65} expression in limbic structures (El-faramawy et al., 2009), planned comparisons were performed. However, no significant effects of stress history on GAD_{65} expression were revealed in any of the four brain regions analyzed in either males or females (Table 1).

Table 1. Mean GAD_{65} mRNA Expression

	Mean GAD_{65} expression (nCi/g)			
	CA1	MeA	DG	VMH
Con male	437.7 ± 33.07	358.0 ± 26.16	533.6 ± 27.01	355.8 ± 44.34
Str-Imm male	459.5 ± 20.86	317.1 ± 31.00	538.4 ± 15.25	356.9 ± 57.69
Str-Rec male	438.7 ± 19.63	378.8 ± 31.78	553.0 ± 26.08	335.1 ± 37.90
Con female	444.1 ± 26.00	334.6 ± 31.45	542.1 ± 9.66	345.3 ± 56.82
Str-Imm female	423.8 ± 32.28	333.0 ± 24.28	543.1 ± 21.28	241.6 ± 38.16
Str-Rec female	415.8 ± 24.24	335.1 ± 28.12	555.6 ± 22.89	258.2 ± 30.39

Table 1. Mean GAD_{65} expression. Calibrated optical densities from *in situ* hybridization results of GAD_{65} mRNA (nCi/g) expression from all brain regions analyzed. No differences between sex or stress conditions were found to be significant in any of the brain regions analyzed. Data are represented as mean ± SEM.

We found a wide range of variability in GAD_{65} expression across animals. We took advantage of this variability and conducted correlation analyses to determine whether individual differences in GAD_{65} expression were associated with RAWM performance. Pearson's correlations were conducted to determine the extent to which GAD_{65} expression in the CA1, dentate gyrus (DG), ventromedial hypothalamus (VMH) and medial amygdala (MeA) correlated with performance on the RAWM (total errors on each day of training). GAD_{65} expression significantly correlated with total errors on Day 1 of training in a sex and region-specific manner. In males, a significant negative correlation was identified between GAD_{65} expression in the MeA and total errors on day 1 of training ($r = -0.41$, $p < 0.05$; Figure 4.3), such that increased errors were associated

with decreased GAD_{65} expression. In females, a significant positive correlation was identified between GAD_{65} expression in the CA1 and total errors on day 1 of training ($r = 0.59$, $p < 0.01$; Figure 4.3), such that increased errors were associated with increased GAD_{65} expression. In both males and females, no significant correlations were found between GAD_{65} expression and day 2 of training in any of the brain regions analyzed.

Figure 4.3. Correlation Between Limbic GAD_{65} Expression and RAWM Performance.

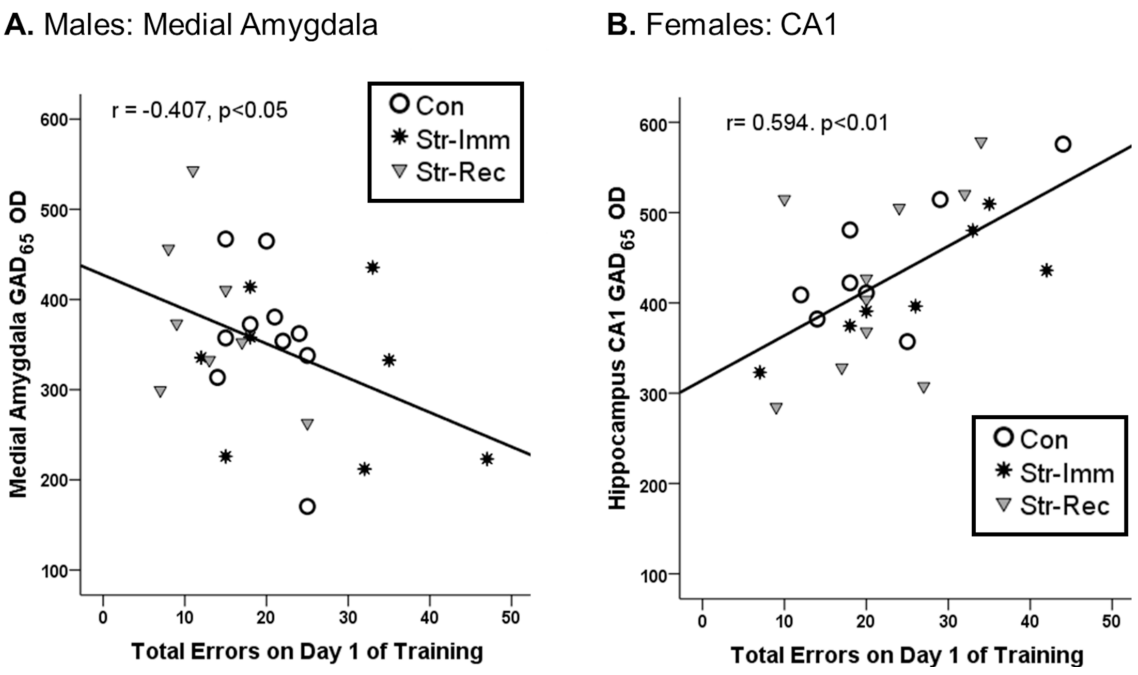

Figure 4.3. Correlation between GAD_{65} expression and Acquisition on the RAWM. **A.** In males, GAD_{65} expression in the medial amygdala was negatively correlated with total errors on day 1 of training ($r = -0.407$, $p < 0.05$), such that increased errors were associated with decreased GAD_{65} expression. **B.** In females, GAD_{65} expression in the CA1 was positively correlated with total errors on day 1 of training ($r = 0.594$, $p < 0.01$), such that increased errors were associated with increased GAD_{65} expression.

Discussion

The present study administered chronic unpredictable restraint (CUR) stress to investigate whether CUR would impact spatial learning and memory in male and female rats similarly with regard to the immediate and long-term effects of chronic stress. The effectiveness of CUR in male rats was confirmed by attenuated body weight gain and adrenal hypertrophy. In male rats that received CUR followed by a recovery period (Str-Rec), body weight gain returned to normal, while adrenal weights did not. Str-Imm female rats were resistant to the measured physiological effects of CUR administration, though Str-Rec females gained significantly more weight than did Con and Str-Imm females during the recovery period. Male rats that were tested on the RAWM immediately after CUR administration (Str-Imm) exhibited significant impairments during the acquisition phase of the task, as demonstrated by increased total errors on day one of testing. However, male rats that were given a 21-day recovery period (Str-Rec) performed better than Str-Imm males and similarly to non-stressed control males. CUR administration did not affect acquisition performance in females tested immediately (Str-Imm) or after a recovery period (Str-Rec). For the retention trial phase of the RAWM (day 3), the effects of CUR did not affect performance in either males or females. Interestingly, RAWM acquisition correlated with GAD_{65} mRNA expression in a sex- and region-specific manner. In male rats, GAD_{65} expression in the MeA was negatively correlated with total errors on day 1 of RAWM training. Conversely, in female rats GAD_{65} expression in the hippocampal CA1 region was positively correlated with total errors on day 1 of RAWM training.

Physiological effects, such as attenuated body weight gain and enlarged adrenal glands, are common metrics used to determine chronic stress effectiveness. In the current study, chronically stressed male rats displayed increased relative adrenal weights compared to non-stressed controls, an effect that persisted even after a post-stress recovery period. In female rats, the adrenal to body weight ratio and body weight gain were unaltered by CUR. (Figure 4.1B). This contrasts with other studies showing that chronic restraint stress (Conrad et al., 2012; McLaughlin et al., 2005) and chronic unpredictable stress (McFadden et al., 2011) attenuate body weight gain in female rodents. Thus, it appears that females are less sensitive to CUR when compared to other stress paradigms. The predictability of chronic restraint stress administration was decreased in order to increase the effectiveness of the stressor on altering spatial performance, because repeated exposure to the same stressor leads to habituation of the HPA axis (Viau & Sawchenko, 2002). Habituation to a stressor depends on an organism's ability to recognize the familiarity of stressor and if an animal encounters a novel stressor then the response of the HPA axis can be enhanced (Bhatnagar & Dallman, 1998). Indeed, some studies have shown that the physical context in which a repeated stressor is administered affects subsequent HPA responses to the stressor (Grissom & Bhatnagar, 2009). The present data suggest that manipulating the predictability in which a stressor is delivered, without changing the stressor itself per se, has little impact on altering body weight, adrenal size and spatial ability in female rats, but it does affect male rats in a manner consistent with other chronic stressors.

An extensive literature reveals that the effects of chronic stress on spatial learning and memory are determined by the sex of the animal (for reviews, see (Conrad &

Bimonte-Nelson, 2010; McLaughlin, Baran, & Conrad, 2009). In males, some reports show that chronic stress impairs the learning aspect of spatial tasks (V. Luine et al., 1994; Sousa et al., 2000; Wright & Conrad, 2008), while others show that chronic stress affects only the memory for the task (Hoffman et al., 2011; Ortiz et al., 2014) or both (Radecki et al., 2005; B. S. Rao & Raju, 2000). Despite these discrepancies, the data reported here corroborate the established pattern of chronic stress having detrimental effects on learning in males, as administration of CUR to male rats produced deficits during the acquisition of the RAWM. Consistent with the literature, the CUR stressor in this study did not lead to learning and memory deficits in female rats (Kitraki et al., 2004b; McLaughlin et al., 2010). Although no significant effects were found, if any pattern was to be generously discerned, CUR may have even potentiated spatial learning in female rats, as documented in other reports on learning and memory (see (Bowman et al., 2001; Conrad, Grote, Hobbs, & Ferayorni, 2003; Conrad et al., 2012; Kitraki et al., 2004b; McLaughlin et al., 2005). Thus, it appears that when restraint stress was made to be unpredictable, a non significant effect on spatial learning was observed in female rats. The common theme of these studies is that female rats appear to be resilient to the effects of chronic stress, especially when compared to male rats.

While CUR impaired spatial ability in the males, we found that a post-stress recovery period following chronic stress reversed the spatial learning deficits. The improvement of spatial ability in males following a post-stress recovery period is corroborated by other studies (Bian et al., 2012; Hoffman et al., 2011; V. Luine et al., 1994; Ortiz et al., 2014; Sousa et al., 2000), with outcomes that correspond to a restoration of hippocampal CA3 apical dendritic complexity following a post-stress

recovery period (Conrad et al., 1999; Hoffman et al., 2011; Sousa et al., 2000). Unexpectedly, Str-Rec males showed impaired performance on day 2 (trial 1), implying that they did not retain the previous day's information, an outcome that contrasts with our previous findings (Hoffman et al., 2011 2014) and others (Sousa et al., 2000). However, the Str-Rec male performance on day 2 is confounded by acquisition differences occurring on day one. Specifically, Con and Str-Imm males made more arm entries during acquisition on day one than did Str-Rec males, which means that all male groups had different arm entry exposures on day 1. Thus, these *a priori* behavioral differences on day 1 confound any interpretations for performance on day 2. Despite these discrepancies, we add to the growing body of literature to show that allowing a post-stress recovery period following chronic stress allows for the return of spatial learning and memory.

The mechanisms underlying chronic stress-induced spatial learning deficits, and subsequent recovery in males, are unclear, but may involve altered inhibitory tone in limbic brain regions. GABA, the main inhibitory neurotransmitter, works in concert with glutamate to maintain a balanced excitatory/inhibitory tone in many brain circuits (Herman, Mueller, & Figueiredo, 2004). Disruption of the delicate balance of these tones, as seen following chronic stress, is associated with impaired learning and memory and compromised neuronal integrity (Magarinos, Deslandes, & McEwen, 1999; Watanabe, Gould, Cameron, et al., 1992; Yoon, Okada, Jung, & Kim, 2008). In the current study, GAD_{65} expression was measured in the CA1 subfield of the hippocampus, the medial amygdala, the dentate gyrus, and the ventromedial hypothalamus; brain regions known to display sexual dimorphisms (Cooke, Tabibnia, & Breedlove, 1999; MacLusky, Hajszan,

& Leranth, 2004; Madeira, Ferreira-Silva, & Paula-Barbosa, 2001; Matsumoto & Arai, 1983), to be influenced by chronic stress (Deborah N. Alfarez, Joels, & Krugers, 2003; Heine, Maslam, Zareno, Joels, & Lucassen, 2004; Jankord & Herman, 2008; Radahmadi, Hosseini, & Nasimi, 2014) and/or to mediate spatial learning and memory (Goodrich-Hunsaker, Hunsaker, & Kesner, 2008; Hunsaker, Mooy, Swift, & Kesner, 2007; Moser et al., 1993; Whitlock, Heynen, Shuler, & Bear, 2006).We predicted that CUR would impact GAD_{65} expression in a region and sex dependent manner. However, we found no significant main effects of CUR or sex in GAD_{65} expression in any of the brain regions analyzed. We followed up with exploratory correlational analyses to determine the extent to which individual differences in GAD_{65} expression contributed to performance on the RAWM. It was observed that GAD_{65} expression in the medial amygdala (MeA) of males negatively correlated with spatial learning: increased errors were associated with less GAD_{65} expression in the MeA. This brain region is intriguing because it is responsible for emotional regulation (Phelps & LeDoux, 2005) and is impacted by stress (Dayas & Day, 2002; Prewitt & Herman, 1998; Rosenkranz, Venheim, & Padival, 2010; Vyas, Mitra, Shankaranarayana Rao, & Chattarji, 2002; Vyas, Pillai, & Chattarji, 2004). It is fascinating that the distribution of the data show the Str-Imm males clustered together and with low GAD_{65} expression. In contrast, spatial learning in females positively correlated with GAD_{65} expression in the CA1 region of the hippocampus: increased errors correlated with higher CA1 GAD_{65} expression and the groups (Con, Str-Imm, Str-Rec) are dispersed throughout the graph. The lack of a correlation between spatial learning and amygdalar GAD_{65} expression in females is consistent with CUR not impacting spatial ability in females. However, the CA1 region is involved with spatial

ability and shows plasticity with spatial learning (Moser et al., 1993; Whitlock et al., 2006). In sum, it appears that GAD_{65} expression in stress-sensitive limbic regions may be associated with sex differences on spatial ability tasks. A caveat in the current study is that animals underwent three days of RAWM testing, which can increase GAD expression in of itself (Bianchi et al., 2003; de Groote & Linthorst, 2007). Additionally, estradiol can regulate the inhibitory tone in the hippocampus of female rats through GAD_{65} and GAD_{67} mRNA expression (McCarthy, Kaufman, Brooks, Pfaff, & Schwartz-Giblin, 1995), without necessarily affecting on $GABA_A$ receptor subunit mRNA expression (Weiland & Orchinik, 1995). Thus, inhibitory tone in limbic regions is affected by a number of factors, with chronic stress being one, but with other mediators (e.g. RAWM testing and sex hormones) that were present in the current study also regulating GAD_{65} expression. Future studies are needed to elucidate the influence that inhibitory tone, chronic stress, and a post-stress recovery period have on spatial learning between the sexes.

Conclusions

In this study, administration of CUR induced spatial learning deficits in male rats and this negatively correlated with GAD_{65} mRNA expression in the MeA an area important for emotional regulation. Conversely, administration of CUR led to no effects on spatial learning in female rats. Consequently, increasing the unpredictability of chronic restraint stress did not impact the resilience of female rats to chronic stress on these outcomes. Nevertheless, female rat performance on RAWM correlated with GAD_{65} mRNA expression in the CA1 region, an area that is highly plastic and responsive to ovarian hormones (Conrad et al., 2012; McLaughlin et al., 2010). The present findings

represent an important first step in identifying the brain regions and neural mechanisms that contribute to sex differences in behavior and plasticity in response to chronic stress. Further investigations are necessary to determine how inhibitory tone in the amygdala and hippocampus contribute to the stress-induced behavioral differences observed between the sexes.

CHAPTER 5

INVOLVEMENT OF THE HIPPOCAMPAL GABAERGIC SYSTEM IN THE RECOVERY OF HIPPOCAMPAL PLASTICITY FOLLOWING CHRONIC STRESS

[To be submitted to: *Hippocampus*

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Contributions: JBO led the study in its entirety with consultation from Dr. Jason Newbern and Dr. Cheryl D. Conrad: JBO designed the experiment, JBO oversaw the mice breeding, JBO and many undergraduates implemented the stressors, JBO processed the tissue, JBO quantified the data, JBO analyzed the data under the guidance of Dr. Jason Newbern and Dr. Cheryl D. Conrad. JBO wrote the manuscript.

Introduction

Major depressive disorder (MDD) is a common and serious medical condition characterized by abnormalities in affect, mood, cognition, and psychomotor abilities. MDD is the leading cause of disability worldwide (Kessler & Bromet, 2013; Organization, 2015) and is a significant contributor to the global burden of disease. Individuals affected with MDD show a wide range of symptoms and despite an abundance of available therapies, many individuals suffering from MDD will relapse even after successful treatment. As such, a full understanding of the neurobiological

bases of the development and maintenance of MDD will elucidate possible novel approaches for diagnosing and treating the disorder.

The main inhibitory neurotransmitter gamma-aminobutyric acid (GABA; (Luscher, Shen, & Sahir, 2011) appears to be disrupted in patients with MDD. Postmortem assessment of GABAergic interneurons indicate that the size and density decrease in various brain regions of MDD patients (Maciag et al., 2010; Rajkowska, O'Dwyer, Teleki, Stockmeier, & Miguel-Hidalgo, 2007). Moreover, GABA levels are decreased in cerebrospinal fluid (Gerner & Hare, 1981), plasma (Petty & Schlesser, 1981; Petty & Sherman, 1984), and brain tissue (Honig, Bartlett, Bouras, & Bridges, 1988) in MDD patients. These studies suggest that the relationship between MDD and GABAergic changes in the brain may be an important mechanism to investigate.

One way the cellular and molecular underpinnings of MDD can be studied preclinically is through the use of rodent models of MDD. Chronic stress in rodents can mimic many features observed from MDD, such as increases in anhedonic and anxiety-like behaviors, cognitive dysfunction, changes in hippocampal morphology, and alterations in the hippocampal GABAergic system. The hippocampus is a commonly studied brain region in animal models of chronic stress. As such, much is known about the morphological, functional, and biochemical properties of the hippocampus immediately following the termination of chronic stress. For example, chronic stress results in a pruning of dendritic arbors in certain regions of the hippocampus (Watanabe, Gould, Cameron, et al., 1992) and this coincides with deficits in spatial learning and memory (Conrad, 2006). In addition, chronic stress skews the hippocampal excitatory/inhibitory tone towards hyperexcitability (Gronli et al., 2007; Joels et al.,

2004), which contributes to hippocampal dendritic retraction and spatial learning and memory deficits (Magarinos et al., 1999; Magarinos & McEwen, 1995). Moreover, chronic stress in rodents decreases the concentration of GABA in the hippocampus (Gronli et al., 2007) and the expression of markers for GABAergic neurons in the hippocampus (Czeh et al., 2015). Taken together, studying the hippocampal GABAergic system in a rodent model of chronic stress can mimic findings in individuals with MDD, and will increase our understanding of how the GABAergic system contributes to the disorder.

While much is known about the immediate effects of chronic stress on the hippocampus, less is known about the mechanisms that occur when chronic stress ends, and the stress-induced deficits in spatial ability and dendritic morphology improve. Indeed, chronic stress-induced dendritic retraction and cognitive deficits can show improvements in the weeks following the end of chronic stress (Hoffman et al., 2011; Ortiz et al., 2014; Sousa et al., 2000). Past work has shown that the GABAergic system may be involved in the improvements following a post-stress rest period (Ortiz et al., 2015). But a clearer understanding of how the GABAergic system is affected by chronic stress and a post-stress rest period is warranted. To study the impact of chronic stress and a post-stress rest period on the GABAergic system in the hippocampus, we determined whether these stress manipulations would result in a change in the total number of GABAergic neurons in the hippocampus and/or whether there were specific changes to certain sub-classes of GABAergic neurons. There are morphological, neurochemical, and functional differences in GABAergic neuron subtypes and identifying which subtypes, if any, are vulnerable to chronic stress and a post-stress rest period are a critical step to

understanding the involvement of the GABAergic system in the post-stress rest period. Past studies have found that chronic stress decreased several types of inhibitory neurons, such as the number of somatostatin (SOM) (Czeh et al., 2015), calretinin (CR) (Czeh et al., 2015), and parvalbumin (PV) (Csabai et al., 2017; Czeh et al., 2005; Czeh et al., 2015) neurons in the dorsal hippocampus. But, whether how these inhibitory neuron subtypes would be impacted by a post-stress rest period is unknown. Moreover, the literature has a discrepancy as to whether chronic stress decreases the total number of GABAergic neurons in the hippocampus, with some studies reporting chronic stress to decrease inhibitory neuron numbers (Lucassen, Fuchs, & Czeh, 2004; Lucassen et al., 2001; Milner et al., 2013), and others failing to find effects (Czeh et al., 2005; Czeh et al., 2015). This concern is that immunohistochemistry may not differentiate between whether GABAergic neurons die or merely reduce their expression of a particular protein. To address this discrepancy, we utilized a transgenic mouse model that contained an indelible marker for GABAergic neurons throughout the brain. This allowed us to determine whether chronic stress and a post-stress rest period alters the total number of GABAergic neurons and/or the expression of different neurochemical markers for various GABAergic cell types.

Materials and Methods

This experiment was conducted in accordance with federal guidelines as put forth by the National Academy of Sciences Guide for the Care and Use of Laboratory Animals (National Academy of Sciences, National Research Council, Institute of Laboratory Animal Resources on Life Science, Washington, D.C., 2011) and by the institutional

guidelines described by the Arizona State University Institutional Animal Care and Use Committee. The Arizona State University Institutional Animal Care and Use Committee approved of the procedures used in this study.

Animals

Male and Female homozygous *VGAT:Cre Ai9* mice were used in this study. These mice exhibited expression of red fluorescent protein (RFP) in all GABAergic neurons and provided an indelible label for assaying the entire hippocampal GABAergic population. These mice were bred at Arizona State University animal housing facilities. To achieve a VGAT:Cre Ai9 mouse line, mice carrying a vesicular GABA transporter (VGAT) promoter-driven Cre recombinase (VGAT:$Cre^{+/-}$) were crossed with a reporter mouse line (CAG-loxP-STOP-loxP-tdTomato; $Ai9^{+/+}$). Genotyping was done by Polymerase Chain Reaction analysis of DNA isolated from toe clips.

When mice reached approximately 3 months of age (ranged from post-natal day 90 to post-natal day 113), they were transferred to a room on a reverse light cycle (lights off at 0700) for the duration of the study. Mice were weighed weekly throughout the duration of the study.

Chronic stress paradigm

A chronic stress paradigm was chosen that reliably produces hippocampal CA3 dendritic retraction and impairments in spatial ability (Magarinos et al., 2011; Pawlak et al., 2005). Mice that were to be stressed were removed from their home cage, placed in a new cage, and transported to a separate room. There, the mice were placed in wire mesh restrainers (approximately 15mm in length and 4mm in diameter, with the ends sealed with Plasti Dip) and the ends secured with binder clips. Restraint sessions consisted of

restraint for six hours a day (10:00 a.m. to 4 p.m.) for 21 days. When six hours elapsed, the mice were released from the restrainers and transported back to the colony room and returned to their home cage. Mice in the stress recovery group were restrained for 6h/d/21d and then left undisturbed for an additional 21 days (Str-Rec), while mice in the stress "immediate" group were chronically stressed for 6h/d/21d and then were perfused in the immediate days after stress ended (Str-Imm). Unstressed controls were left in their home cages for the duration of the study (Con).

Tissue collection and preparation

One to three days after the last day of restraint for the Str-Imm groups, mice were anesthetized with avertin and, when animals no longer responded to a foot pinch, they were transcardially perfused with 1X PBS followed by 4% paraformaldehyde. Brains were removed and post-fixed overnight with 4% paraformaldehyde and then transferred to 1X PBS. The samples were then rinsed in 1X PBS and, for cryoprotection, incubated in a 15% sucrose followed by 30% sucrose at 4°C. The brains were then embedded in O.C.T. mounting medium, frozen, and placed in a -80°C freezer until cryostat sectioning. Cryostat sections of brain tissue were collected in a series at 50μm. The sections were placed in a 12-well plate containing 3mL of 1X PBS with 4-6 sections per well. After the sections were collected, they were rinsed three times with 1X PBS and then stored at 4°C overnight prior to immunolabeling.

Immunolabeling

For immunofluorescent labeling, 50 μm thick sections were rinsed three times in 1X PBS with 0.1% Triton X-100. The sections were then blocked in 1X PBS with 0.05%

Triton and 5% normal donkey serum for 1 hour. The primary antibodies were diluted in 1X PBS with 0.05% Triton and 5% normal donkey serum and then added to appropriate wells and incubated overnight at 4°C with gentle agitation. The primary antibodies utilized were goat anti-Parvalbumin (Swant, 1:1000 dilution) rabbit anti-Calretinin (Swant, 1:2000 dilution) rabbit anti-Somatostatin (Peninsula laboratories, 1:1000 dilution). The next day, the tissue was washed three times with 1X PBS with 0.05% Triton and then the secondary antibodies were diluted in 1X PBS with 0.05% Triton and 5% normal donkey serum and added to the appropriate wells. The secondary antibodies utilized were Alexa Fluor 488 donkey anti-rabbit IgG (Invitrogen, 1:1000 dilution) and Alexa Fluor 488 donkey anti-goat IgG (Invitrogen, 1:1000 dilution). The tissue was incubated overnight at 4°C with gentle agitation. The following day, the tissue was washed two times in with 1X PBS and 0.05% Triton and then the tissue was mounted on glass slides (Fisherbrand Superfrost Plus, Fisher Scientific) and coverslipped under Fluoro-Gel (Electron Microscopy Sciences) and allowed to dry before visualization.

Image analysis and quantification

Epifluorescent microscope images of the hippocampus were collected from brain sections for 3-5 animals per group. For GABAergic neuron cell counts, relative density of RFP labeled neurons was determined by averaging counts from six separate hippocampal sections per mouse. For specific subtypes of GABAergic neurons, two sections per antibody (PV+, SOM+, CR+) were counted per mouse. The number of RFP labeled cells was counted followed by the number cells that were co-labeled with the antibodies. Each section was separated by major hippocampal subregion using anatomical markers. The

major subregions that were assessed were the dentate gyrus (DG), the CA3 region, and the CA1 region.

Statistical analysis

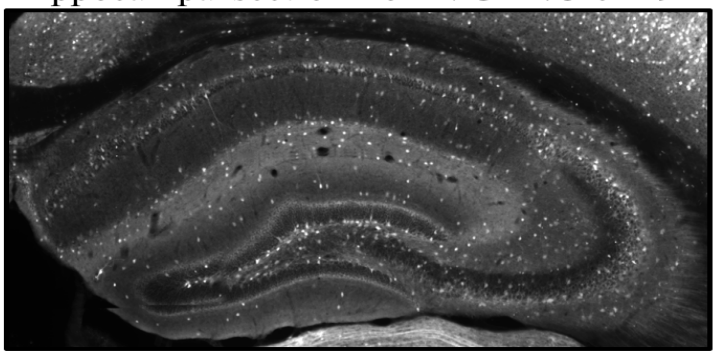
Data for the total number of labeled cells were averaged to obtain a single number per antibody per mouse, and the total number of RFP labeled neurons from all sections analyzed were averaged to obtain a single number per mouse. RFP labeled neurons are represented as the number of cells per mm^2 and SOM+, PV+, and CR+ neurons are represented as a proportion to RFP labeled cells. Results are presented as mean ± SEM. Data were analyzed by Analysis of Variance (ANOVA). When significant differences were detected at a p value of 0.05 or less, the analyses were followed by Tukey's HSD test as *post hoc* analysis to further test group differences.

Results

Figure 5.1 shows hippocampal sections from VGAT:Cre Ai9 mice that were immunolabeled with different antibodies. Cell counts were obtained by manually counting the number of RFP expressing neurons for total GABAergic cells and the number of fluorescently labeled cells for the three different antibodies.

Figure 5.1. Representative Hippocampal Sections from VGAT:Cre Ai9 Mouse and a Section Stained with an Antibody Against Somatostatin.

A. Hippocampal section from VGAT:Cre Ai9 Mouse



B. Hippocampal section showing SOM+ interneurons

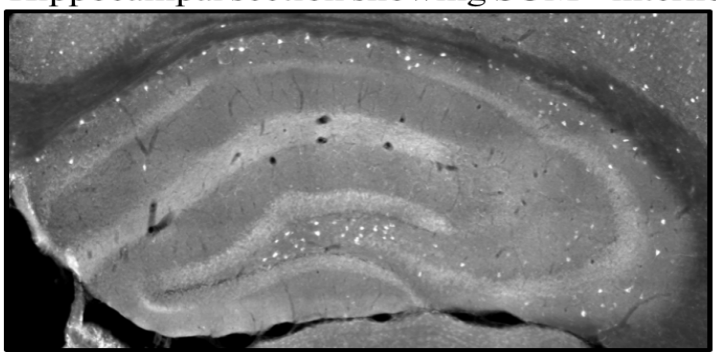


Figure 5.1. Representative hippocampal sections. (A) Representative hippocampal section from a VGAT:Cre Ai9 Mouse showing RFP labeled GABAergic interneurons. (B) Representative hippocampal section showing interneurons labeled with an antibody against somatostatin.

Total number of GABAergic cells

Chronic stress did not alter the total number of GABAergic cells in the hippocampus (Figure 5.2). A 2 X 3 ANOVA for sex and stress condition for each subregion of the hippocampus was conducted. The analysis failed to show significant

differences in the number of total GABAergic cell numbers among the sexes and treatment conditions in any of the subregions analyzed. Chronic stress or a post-stress recovery period had no statistically significant effect on the number of hippocampal GABAergic cells. The number of hippocampal sections from which the cell counts were obtained were as follows: for Males: Con – 21 counts from 4 mice, Str-Imm – 27 counts from 5 mice, Str-Rest – 18 counts from 3 mice. For Females: Con – 18 counts from 3 mice, Str-Imm – 18 counts from 3 mice, Str-Rest – 18 counts from 3 mice for all subregions.

Figure 5.2. Effects of chronic stress and a post-stress rest period on total number of hippocampal GABAergic interneurons.

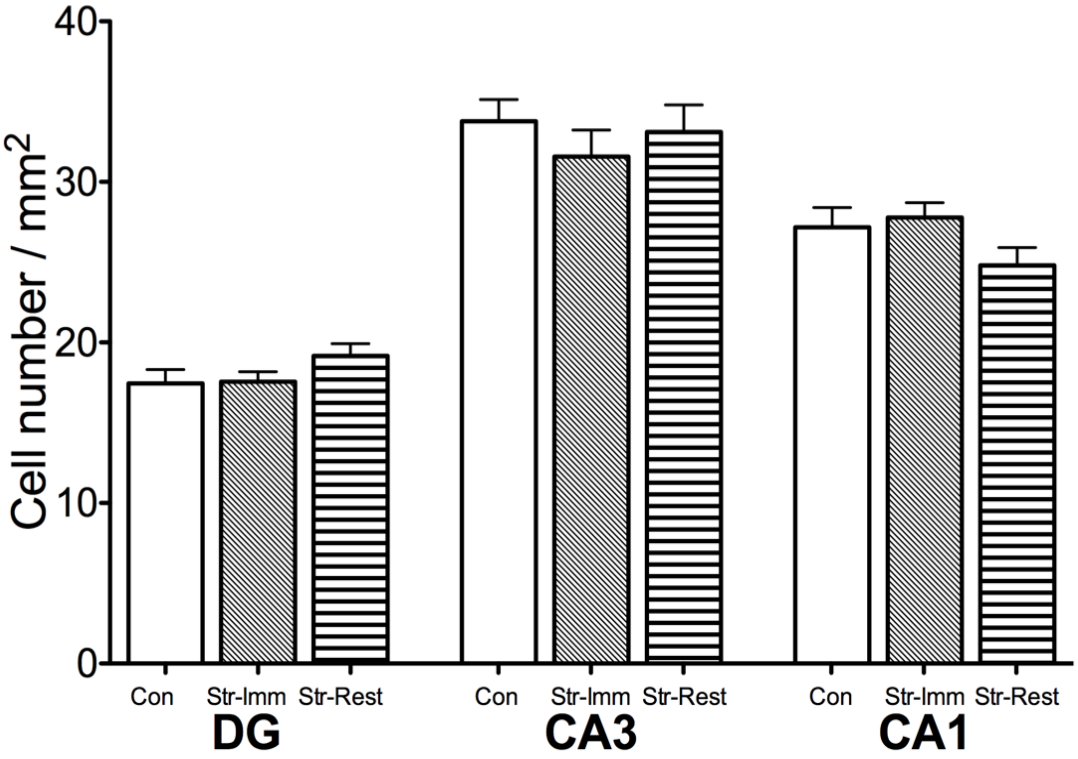


Figure 5.2. Effects of chronic stress and a post-stress rest period on total number of hippocampal GABAergic neurons. Chronic stress had no significant effects on the total number of hippocampal GABAergic neurons in any of the subregions investigated. Con n = 7, Str-Imm n = 8, Str-Rest n = 6.

Somatostatin+ interneurons

Chronic stress and a post-stress rest period did not have statistically significant effects on SOM+ interneurons, but a marginal effect was observed. A 2 X 3 ANOVA for sex and stress condition failed to reveal a significant main effect of sex or stress or a significant interaction on SOM+ interneurons in any of the brain regions analyzed (Figure 5.3). In the dentate gyrus, there was a tendency for stress (Str-Imm, Str-Rest) to decrease the proportion of SOM+ interneurons, but did not quite reach statistical significance ($p = 0.090$). The number of hippocampal sections from which the cell counts were obtained were as follows: for Males: Con – 7 counts from 4 mice, Str-Imm – 9 counts from 5 mice, Str-Rest – 6 counts from 3 mice. For Females: Con – 6 counts from 3 mice, Str-Imm – 6 counts from 3 mice, Str-Rest – 5 counts from 3 mice for all subregions.

Figure 5.3. Effects of Chronic Stress and a Post-Stress Rest Period on Hippocampal Somatostatin+ Interneurons.

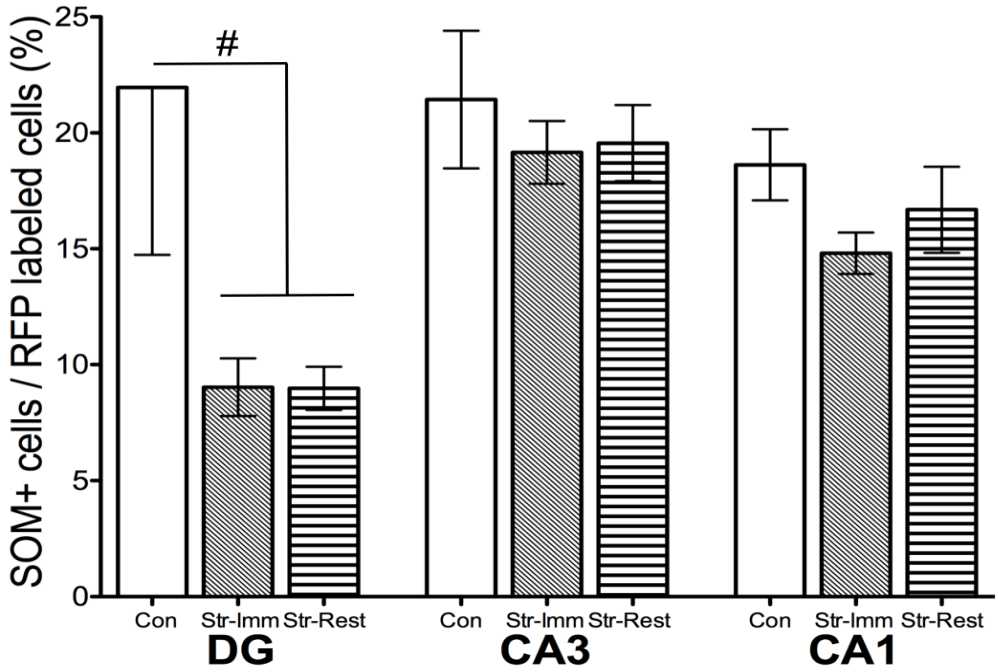


Figure 5.3. Somatostatin+ Interneurons in the hippocampus were unaffacted by chronic stress and a post-stress rest period. No significant differences in SOM+ interneurons were found in any of the hippocampal regions investigated in response to chronic stress or a post-stress rest period. Althougth there was a tendency for chronic stress to decreases SOM+ interneurons in the DG that did no reach statistical significance (# p = 0.09) and this decrease persisted despite the rest period. Con n = 6, Str-Imm n = 7, Str-Rest n = 5.

Parvalbumin+ interneurons

Chronic stress and a post-stress rest period had no significant effects on PV+ interneurons in any of the regions analyzed, but there was a tendency for stress to decrease PV+ interneurons in the DG that remained decrease despited a post-stress rest period. For PV+ interneurons, a 2 X 3 ANOVA for sex and stress condition failed to

reveal a significant main effect of stress or a significant interaction in any of the regions analyzed (Figure 5.4A), although a significant main effect of sex ($F_{(1, 19)} = 6.645$, $p < 0.05$) was observed in the CA1 region of the hippocampus, but not in the other regions. Although stress (Str-Imm, Str-Rest) appeared to decrease the proportion of PV+ interneurons to total GABAergic neurons in the DG, a main effect of stress did not reach statistical significance ($p = 0.075$). Because of the main effect of sex in the CA1 region, we analyzed the proportion of PV+ interneuron in the CA1 region of females and males separately (Figure 5.4B and 5.4C). However, no significant main effect of stress was found in either of these analyses, although stress (Str-Imm, Str-Rest) tended to decrease PV+ cells in the CA1 of male mice that did not reach statistical significance ($p = 0.094$) and this decrease seemed to be maintained depsite a post-stress rest period. The number of hippocampal sections from which the cell counts were obtained were as follows: for Males: Con – 6 counts from 3 mice, Str-Imm – 8 counts from 4 mice, Str-Rest – 6 counts from 3 mice. For Females: Con – 6 counts from 3 mice, Str-Imm – 6 counts from 3 mice, Str-Rest – 5 counts from 3 mice for all subregions.

Figure 5.4. Effects of Chronic Stress and a Post-Stress Rest Period on Hippocmapal Parvalbumin+ Interneurons.

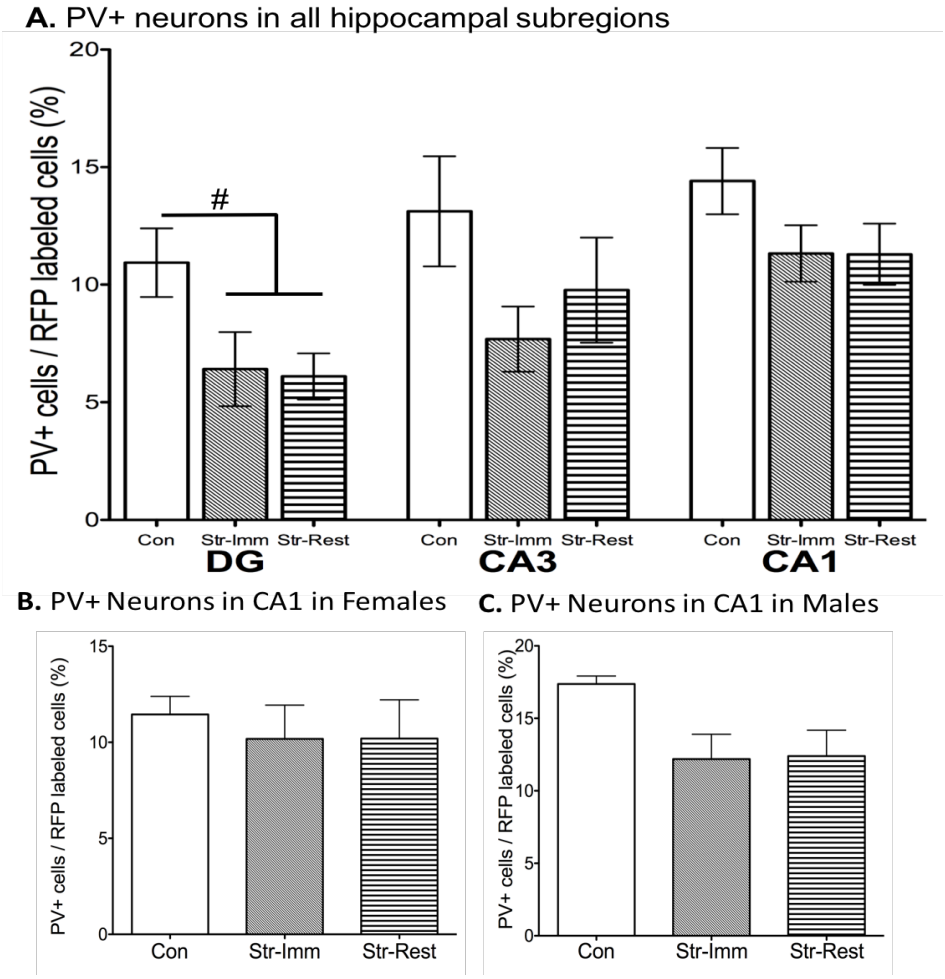


Figure 5.4. Chronic stress and a post-stress rest period had no statistically significant effect on PV+ interneurons. (A) Analysis of PV+ interneurons showed no siginificant main effect of stress in any hippocampal subregion. However, there was a marginal effect of stress ($p = 0.075$) showing decrease in the DG for the Str-Imm and Str-Rest mice compared to Con. Futhermore, a significant main effect of sex was found for CA1 interneurons for females (B) and males (C). As such, PV+ interneurons in the CA1

were analyzed separately for males and females. This analysis failed to reveal statistically significant effects. Con n = 6, Str-Imm n = 7, Str-Rest n = 6.

Calretinin+ interneurons

CR+ interneurons appeared to be the most responsive to a period of chronic stress and a post-stress rest period (Figure 5.5). A 2 X 3 ANOVA for sex and stress condition for the proportion of CR+ cells in the DG revealed a significant main effect of sex ($F_{(1,21)} = 14.65$, $p < 0.01$) and a marginal main effect of stress ($p = 0.078$; Figure 5.5A). This sex effect in the DG was probed further and males and females were analyzed separately. The ANOVA for females failed to reveal a significant main effect of stress (Figure 5.5B). On the contrary, in the DG of male mice, the proportion of CR+ interneurons was significantly decreased following chronic stress ($F_{(2, 12)} = 4.54$, $p < 0.05$ for a main effect of stress; Figure 5.5C) with post-hoc analyses showing that Str-Imm mice had significantly less CR+ interneurons than did Con ($p < 0.05$). A 2 X 3 ANOVA for sex and stress condition for the proportion of CR+ cells in the CA3 region of the hippocampus revealed a significant main effect of stress ($F_{(2,21)} = 4.67$, $p < 0.05$; Figure 5.5A), with post-hoc analyses showing that Str-Imm mice had significantly less CR+ interneurons than did both Str-Rec and Con mice ($p < 0.05$ for both). Finally, a 2 X 3 ANOVA for sex and stress condition on the CA1 region revealed a significant main effect of sex ($F_{(1,21)} = 11.04$, $p < 0.05$). However, when the proportion of CR+ interneurons in males and females were analyzed separately (Figure 5.5D and 5.5E), the analyses failed to reveal significant differences in stress conditions. No other significant

effects were found. The number of hippocampal sections from which the cell counts were obtained were as follows: for Males: Con – 8 counts from 4 mice, Str-Imm – 10 counts from 5 mice, Str-Rest – 6 counts from 3 mice. For Females: Con – 6 counts from 3 mice, Str-Imm – 6 counts from 3 mice, Str-Rest – 5 counts from 3 mice for all subregions.

Figure 5.5. Effects of Chronic Stress and a Post-Stress Rest Period on Calretinin+ neurons in the hippocampus.

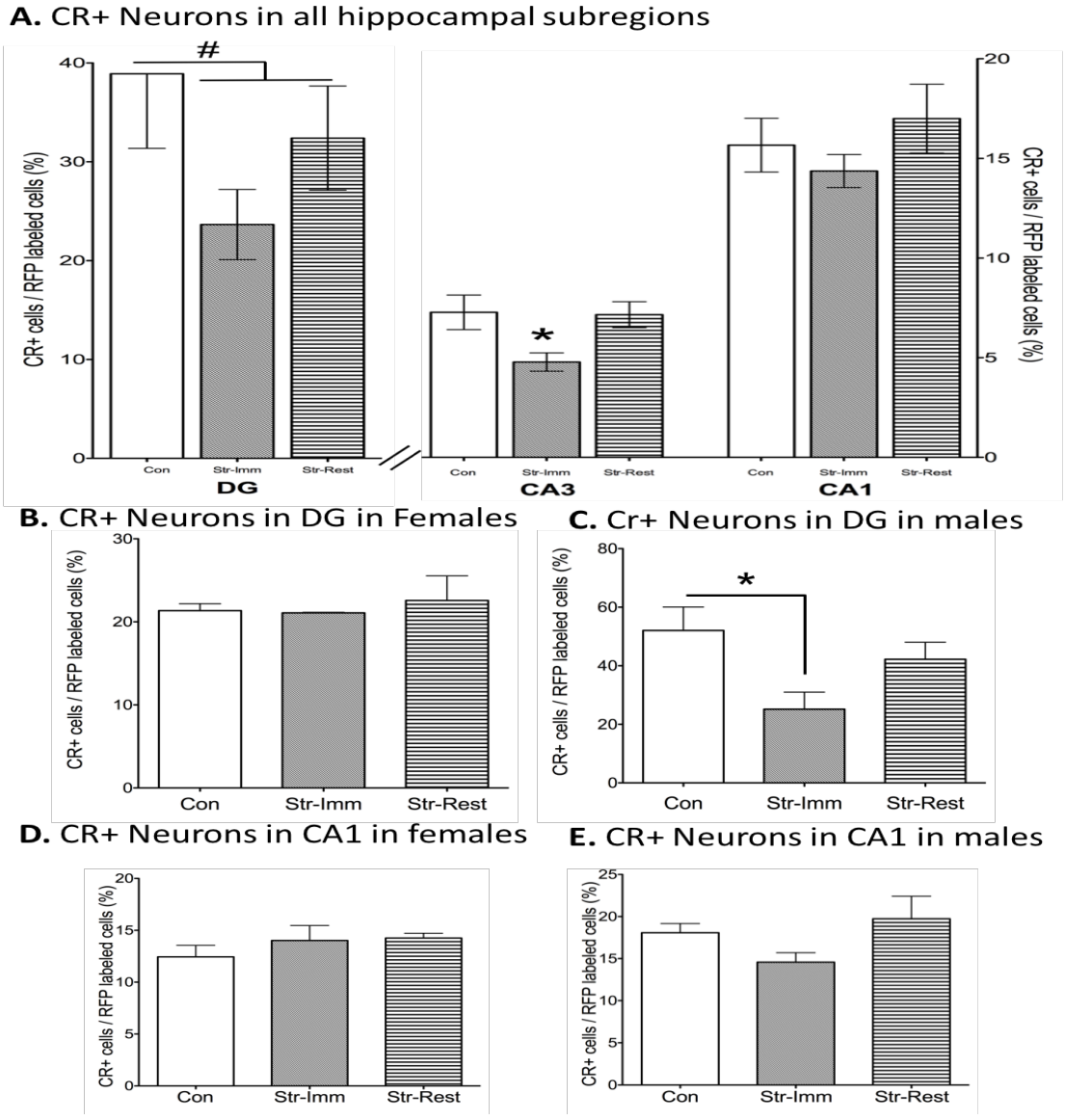


Figure 5.5. Effects of Chronic Stress and a Post-Stress Rest Period on Calretinin+ Interneurons in the hippocampus. (A) Chronic stress and a post-stress rest period affected the number of Calretinin+ interneurons in a region-specific manner. In the DG, there was a tendency for Str-Imm to reduce CR expression (#p = 0.078) and a significant sex difference with overall expression of CR being lower in the female DG than in the male DG. The sex effect was probed further by analyzing females and males separately (B and C). No significant effects were found in females, but in males a significant main effect of stress was found, with Str-Imm displaying significantly less CR+ interneurons in the DG than Con (* $p < 0.05$ for Str-Imm compared to Con). In the CA3 region, a significant main effect of stress revealed that Str-Imm had significantly less CR+ interneurons than did both Con and Str-Rest (* $p < 0.05$ for Str-Imm compared to Con and Str-Rest). A significant main effect of sex was found in the CA1 region, but separate analyses of females (D) and males (E) revealed no significant effects. Con n = 7, Str-Imm n = 8, Str-Rest n = 6.

Chronic restraint stress attenuates body weight gain

Body weight was recorded on the first day of restraint stress for each mouse and then weights were subsequently recorded once a week until animals were sacrificed. All mice that were utilized in the study were included in the analysis of body weight gain. As there were two restraint sessions (Str-Rec vs Str-Imm) body weight was analyzed as a measure of body weight gain during the two restraint session types. For body weight gain from weeks 1 to 4 when the Str-Rec mice were in restraint, a 2 X 3 ANOVA for sex and

stress condition revealed a significant main effect of stress on body weight gain ($F_{(2,56)}$ = 21.60, $p < 0.001$) without a significant main effect of sex or a significant interaction (Figure 5.6). The post-hoc analysis revealed that Str-Rec mice gained significantly less weight during this time than did both Con and Str-Imm ($p < 0.001$). Body weight gain during the next restraint session, week 4 to week 7, when the Str-Imm mice were subjected to restraint was analyzed. A 2 X 3 ANOVA for sex and stress revealed a significant main effect of stress ($F_{(2,56)} = 91.76$, $p < 0.001$) on body weight gain with no significant main effect of sex or a significant interaction. The post-hoc analysis revealed that the Str-Imm mice gained significantly less weight than did Con and Str-Rec mice ($p < 0.001$ for both), while the Str-Rec mice gained more weight than Con ($p < 0.001$) and reached a similar weight as Con by the end of week 7.

Figure 5.6. Effects of Chronic Stress on Body Weight Gain

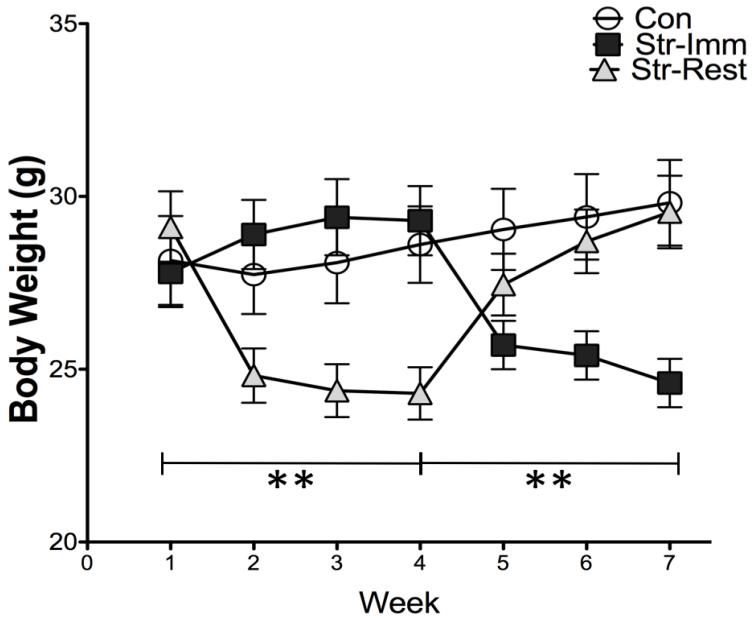


Figure 5.6. Effects of Chronic Stress on Body Weight Gain. Chronic restraint significantly attenuated body weight gain. In weeks 1 to 4, when Str-Rest mice were undergoing restraint, body weight gain was significantly reduced compared to Con and Str-Imm mice (** $p < 0.001$ for Str-Rest compared to Con and Str-Imm). Likewise, when restraint ended for Str-Rest mice and started for Str-Imm mice, body weight gain was altered accordingly. That is, from weeks 4 to 7, when Str-Imm mice were being restrained, Str-Imm mice showed a significant attenuation of body weight gain, while Str-Rest mice increased in body weight gain (**$p < 0.001$ for Str-Imm compared to Str-Rest and Con). All mice were included in this analysis. Con n = 19, Str-Imm n = 16, Str-Rest n = 19.

Discussion

This is the first study to investigate the impact of a post-stress rest period on the total number and selected subset of GABAergic interneurons in the hippocampus. Our findings demonstrated that the total number of GABAergic neurons in the hippocampus remained relatively unperturbed from chronic stress or a chronic stress rest period. In contrast, chronic stress resulted in a significant reduction in the amount of CR+ interneurons in the CA3 region of the hippocampus, which was observed to increase following a post-stress rest period. While no significant main effects of stress were found in other hippocampal regions or the PV+ or SOM+ neurons, a tendency was observed for chronic stress to decrease SOM+ and PV+ in the dentate gyrus. These results demonstrate that the total GABAergic number does not change in response to chronic stress or the weeks following the end of chronic stress and point to CR+ as being a potential new lead

to understand mechanisms facilitating the previous reported spatial memory improvements that occur in the weeks following the end of chronic stress.

Using a VGAT:Cre Ai9 mouse line, this is the first study to demonstrate that neither chronic stress nor a post-stress rest period alters the total population of GABAergic neurons in the hippocampus. Previously, the findings were mixed as to whether chronic stress led to neuronal death in the hippocampus. An early theory proposed that long-term exposure to glucocorticoids, such as that produced by chronic stress, can lead to irreversible neuronal death in the hippocampus (Sapolsky, Krey, & McEwen, 1986). Since then, several studies reported an increased incident of markers for apoptosis and degeneration in the hippocampus (Lucassen et al., 2001; Milner et al., 2013). However, the literature is mixed, as some studies show that chronic stress led to changes in hippocampal GABAergic subtypes, but the reports were unable to determine whether chronic stress also led to a reduction of total GABAergic neuronal numbers (Csabai et al., 2017; Czeh et al., 2005; Czeh et al., 2015). In other preclinical models of diseases, such as epilepsy and traumatic brain injury, several studies reported a loss of GABAergic neurons in the hippocampus (Almeida-Suhett et al., 2015; Buckmaster, Abrams, & Wen, 2017). However, these studies typically use glutamic acid decarboxylase (GAD), the synthesizing enzyme for GABA, as a marker for GABAergic interneurons when investigating neuron loss. While GAD67 can label GABAergic interneurons, GAD67 is subject to changes in expression following a variety of experimental manipulations, such as chronic stress (El-faramawy et al., 2009), behavioral training (Bianchi et al., 2003; de Groote & Linthorst, 2007), and traumatic brain injury (Reger et al., 2012). Consequently, whether the reduction in GAD reflects hippocampal

cell loss or not has been uncertain until now. The advantage of our current study is that we used a transgenic approach that provided a permanent label for GABAergic neurons and as such, GABAergic neurons could not be decreased by experimental manipulations unless it was caused by cell death. These findings also corroborate the hypothesis that chronic stress does not lead to neuron loss per se, but leads to a vulnerability (Conrad, 2008).

SOM+ interneurons may be a critical mediator of the effects seen following a post-stress rest period. While we failed to show that chronic stress or the post-stress rest period significantly altered the expression of SOM+ neurons, we did observe a non-significant tendency for chronic stress to attenuate SOM expression in the DG that did not show improvements following the post-stress rest period. Somatostatin is a neuropeptide expressed in GABAergic neurons that project to the distal part of apical dendritic tree in hippocampal CA1 and CA3 pyramidal cells (Freund & Buzsaki, 1996). This is in line with research showing that the immediate effects from chronic stress decreases apical dendritic complexity of hippocampal CA3 pyramidal cells in the distal apical tree (Conrad, Ortiz, & Judd, 2017). Interestingly, past work report reductions in SOM+ neurons from different regions the brain, including the hippocampus and prefrontal cortex, in chronically stressed rodents (Banasr et al., 2017; Czeh et al., 2015). Somatostatin is also implicated in MDD, as patients with MDD show reduced somatostatin in cerebrospinal fluid and in some regions of the brain, such as the anterior cingulate (Agren & Lundqvist, 1984; Tripp, Kota, Lewis, & Sibille, 2011). Moreover, intracerebroventricular administration of somatostatin can reduce anxiety and depressive-like profiles on various behavioral tasks in preclinical models (Engin, Stellbrink, Treit, &

Dickson, 2008). In contrast, chronic inhibition or ablation of SOM+ neurons in the frontal cortex can lead to a decrease in anxiety-like behavior and can prevent chronic stress-induced increases in anxiety-like behaviors (Soumier & Sibille, 2014). These studies suggest a complex relationship between SOM+ interneurons and depressive- and anxiety-like behaviors and chronic stress, such as too little SOM+ or too much SOM+ influence both being detrimental to brain function. While we did not find a statistically significant effect of chronic stress on SOM+ neuronal number, we did observe chronic stress (Str-Imm) to marginally decrease SOM expression in the DG, which did not appear to improve with a post-stress rest period. Due to this failure to show improvement following a post-stress rest period, an important next step would be to investigate how promoting SOM+ interneurons following chronic stress would impact hippocampal plasticity.

Chronic stress led to a small, but non-significant, decrease in PV+ hippocampal interneurons in the DG that did not improve following a post-stress rest period. The majority of studies investigating the impact of chronic stress on the hippocampus show a decrease in hippocampal PV+ interneurons (Csabai et al., 2017; Czeh et al., 2005; Czeh et al., 2015; Filipovic, Zlatkovic, Gass, & Inta, 2013; Harte, Powell, Swerdlow, Geyer, & Reynolds, 2007; Hu, Zhang, Czeh, Flugge, & Zhang, 2010; Milner et al., 2013), although there are some reports that report no effect like ours (Holm et al., 2011; Krugers, Koolhaas, Medema, & Korf, 1996; Nowak et al., 2010). PV+ neurons represent a large subset of GABAergic cells in the hippocampus (Freund & Buzsaki, 1996) and are classified as perisomatic inhibitory neurons, as a majority of their synapses form on the cell body and proximal dendrites of principal neurons (Freund & Buzsaki, 1996).

Following chronic stress, the complexity of proximal apical dendrites is largely unaffected (Conrad, Ortiz, & Judd, 2017), an effect that may involve PV+ interneurons. In one study, chronic stress decreased PV+ neurons in all subregions of the hippocampus, but perisomatic synapses were unaltered (Csabai et al., 2017). This showed that while chronic stress may reduce the expression of PV+ interneurons in the hippocampus, the axo-somatic synapses that PV+ interneurons form remain intact. These findings suggest that reducing parvalbumin content of interneurons does not necessarily compromise the synaptic count on primary neurons. While our current study did not show a significant effect from stress in PV+ interneuron expression in the DG, we did observe a tendency towards decreasing DG PV+ expression following chronic stress that remained low in the post-stress rest period. This potential reduction in DG PV+ neuronal number is intriguing because the TrkB receptor is abundantly expressed on PV+ hippocampal interneurons (Cellerino et al., 1996; Zheng et al., 2011) and is vital for PV+ neuron expression. Removing the TrkB receptor from hippocampal PV+ neurons disrupts hippocampal synaptic transmission and decreases the total number of hippocampal PV+ neurons (Zheng et al., 2011). Since chronic stress can reduce the expression of BDNF in the hippocampus (Lakshminarasimhan & Chattarji, 2012), and BDNF is necessary for spatial memory deficits to improve following a post-stress rest period (Ortiz et al., 2014), then a reduction of BDNF may compromise hippocampal PV+ neurons. Perhaps the decrease in hippocampal BDNF that has been reported in many studies (Smith et al., 1995; Lakshminarasimhan and Chattarji, 2012; Gray, Milner, & McEwen, 2013) plays a role in downregulating PV+ neuron number in the hippocampus. Future studies are necessary to address this prediction. However, based on the data here showing PV+ interneurons

remain decreased following a post-stress rest period, PV+ interneurons may not be necessary for the improvements in the hippocampus seen after the post-stress rest period.

In contrast to the effects on SOM+ and PV+ hippocampal interneurons, hippocampal CA3 CR+ interneurons displayed a significant and robust decrease following chronic stress, followed by an increase in the expression of CR+ interneurons with a post-stress rest period. This finding corroborates another study by Czeh and colleagues (2015) who found that chronic stress led to a significant decrease in CR+ neurons in the DG, CA3, and CA1 regions of the dorsal hippocampus (Czeh et al., 2015), although not all studies have replicated this finding (Banasr et al., 2017). Since we found no differences in the total number of GABAergic neurons, the decrease in CR+ is unlikely to occur from cell death. Consequently, the CR+ neurons reduced their cellular content of calretinin following chronic stress, which increased following a post-stress rest period. This decrease and then increase in CR+ expression may lead to an altered phenotype of CR+ neurons. For example, in mice with a global knockout of calretinin, there were no deficiencies in spatial ability or for LTP in the CA1 region of the hippocampus. However, deficits were region-specific and observed with LTP production in the DG. Moreover, the DG showed an increase in the amount of GABA release (Schurmans et al., 1997). Therefore, decreasing the amount of calretinin in neurons has functional implications. How decreases in calretinin may affect spatial ability and pyramidal cell neuronal morphology following chronic stress or a post-stress rest period needs to be explored further. As CR+ interneurons showed an increase following the post-stress rest period, they may be responsible for the improvements in hippocampal plasticity following the post-stress rest period. Therefore, investigating how promoting

these neurons, perhaps through pharmacogenetics manipulations, during the chronic stress period would be a critical step forward to understand their role in hippocampal plasticity.

GABAergic interneurons are highly heterogeneous in terms of their morphological, electrophysiological, and molecular properties. One-way to identify various subtypes of GABAergic neurons is through the expression of neurochemical markers. For example, the calcium-binding proteins parvalbumin and calretinin, and the neuropeptide somatostatin can be used to discriminate between three non-overlapping GABAergic interneuron populations. As such, we investigated these three GABAergic neuron populations in the hippocampus following chronic stress and a post-stress rest period. We found a tendency for PV+ and SOM+ expression to decrease in response to chronic stress and these levels stayed low with a post-stress rest period. In contrast, CR+ expression was significantly decreased following chronic stress and then subsequently increased following a post-stress rest period. Importantly we found that the total number of GABAergic interneurons in the hippocampus were unaltered following chronic stress, suggesting that the changes in the expression of GABAergic neuron subsets results from a decrease in the intracellular content of the immunohistochemical markers, rather than a reduction in the number of cells. Future studies are necessary to understand the role the reductions of these markers play in chronic stress-induced changes in the brain. Identifying the molecular and cellular vulnerabilities in the hippocampus that fail to show improvements following a post-stress rest period, will enhance our understanding of the mechanisms of plasticity that occur in the aftermath of chronic stress.

CHAPTER 6

GENERAL DISCUSSION

This dissertation summarizes a series of experiments designed to understand the underlying neurobiological mechanisms that allow individuals to improve functioning following the aftermath of chronic stress. Here we focused on the effects of chronic stress and post-stress rest period on the hippocampus by assessing hippocampal-mediated spatial ability, hippocampal dendritic complexity, and the hippocampal GABAergic system.

Chapter 2 demonstrated that chronic-stress induced deficits in spatial ability can improve when a post-stress rest period is given, and that BDNF within the hippocampus is vital for these improvements to occur. Specifically, rats with a knockdown of BDNF in the CA3 region specifically failed to show improvements in spatial ability despite a post-stress rest period. Chapter 3 followed up on this finding by asking whether these changes co-occur with changes in hippocampal dendritic morphology, whether they are mediated by BDNFs receptor TrkB, and whether targeting the rest period with TrkB manipulations leads to similar effects. Our findings on hippocampal BDNF as it pertains to CA3 dendritic complexity mapped on to the behavioral findings from chapter 2. Chronic stress-induced dendritic retraction remained atrophied despite a post-stress rest period in rats with downregulated hippocampal BDNF. Furthermore, these effects were reproduced by the administration of a TrkB antagonist throughout the post-stress rest period only, providing further support for the hypothesis that BDNF/TrkB signaling are necessary for chronic stress-induced effects to show improvements following a post-stress rest period. The administration of the TrkB receptor antagonist during the post-stress rest period was

important because the virally-mediated BDNF knockdown experiment was unable to discern whether the knockdown was crucial for the post-stress rest period or whether the effects were carry-over from the restraint period. Chapter 4 sought to determine whether there was a relationship between GAD65 and spatial ability of male and female rats following administration of a chronic unpredictable restraint stressor and a post-stress rest period. Correlational analyses found a significant positive relationship between errors on day 1 of training and GAD65 in the CA1 region of the hippocampus in female rats, and a significant negative correlation between errors on day 1 of training and GAD65 in the medial amygdala of male rats. This showed that the GABAergic system may be involved in spatial ability. This led us to ask whether the hippocampal GABAergic system is perturbed following chronic stress and a post-stress rest period. As such, we realized that there was no conclusive documentation in the literature as to whether chronic stress decreased total GABAergic neuron number and whether a post-stress rest period would change this. Consequently, in Chapter 5 and in collaboration with Dr. Newbern's team, we bred transgenic mice with a marker for all GABAergic neurons and determined that chronic stress has no effect on the total number of hippocampal GABAergic neurons. However, there were subtype specific effects with CR+ neurons decreasing in response to chronic stress and a post-stress rest period increasing the number of CR+ neurons. There were no statistically significant changes in SOM+ and PV+ neurons in response to chronic stress or a post-stress rest period, but we did find a pattern for chronic stress to decrease expression of SOM+ and PV+ neurons in the DG and that these levels appeared to stay low with a rest period after chronic stress. Collectively these studies demonstrate that chronic stress-induced changes in spatial

cognition and hippocampal dendritic morphology are plastic and can improve in the weeks after chronic stress ends. Importantly, we have uncovered some molecular mediators that are required in the aftermath of chronic stress in order for stress-induced changes to improve.

The data presented here show that the hippocampus is vulnerable to chronic stress with the structure of hippocampal dendritic arbors showing significant atrophy and hippocampal mediated behaviors showing deficits. These changes in the hippocampus are common features of the human hippocampus in individuals with MDD. Indeed, diminished hippocampal volume in the brains of MDD patients is a common finding in clinical research. Large scale meta-analyses of studies investigating the brains of individuals with MDD have consistently found the hippocampus to have a decreased volume compared to healthy controls without MDD (Schmaal et al., 2016). Similarly, meta-analyses have also revealed significant impairments of cognitive processes in patients with MDD (McDermott & Ebmeier, 2009; Rock, Roiser, Riedel, & Blackwell, 2014) including an impairment of cognitive processes thought to be mediated by the hippocampus. Relevant to these changes in the hippocampi of MDD patients, chronically stressed rodents in chapters 2, 3, and 4 displayed dendritic retraction of hippocampal CA3 apical dendrites and impairments of hippocampal-mediated spatial ability. Our lab and others have consistently found these effects following chronic stress in adult male rats (Hoffman et al., 2011; Hutchinson et al., 2012; Sousa et al., 2000). As such, chronic stress in rodents can be used to model two different aspects of MDD symptomology, i.e. the volume reduction in the hippocampus and hippocampal-mediated cognitive changes.

A novel way to understand MDD pathology proposed here is to investigate the post-stress rest period. As shown in chapters 2 and 3, chronic stress-induced deficits in spatial ability can show improvements when rats are allowed a post-stress rest period of 3 weeks. Furthermore, the data in chapter 3 also shows that the chronic stress-induced atrophy of hippocampal dendritic morphology can improve following a post-stress rest period of 3 weeks. Focusing on the mechanisms that allow for these stress-induced deficits to improve has been a recent topic of investigation in our lab and others, and findings from chronic stress research is beginning to shed light on the changes that occur during this period. In terms of human health, the phenomenon of structural and functional recovery following MDD or exposure to high amounts of glucocorticoids appear to be transient in the human brain as well. For example, in humans exposed to excess glucocorticoids (e.g. from Cushing's disease), ventricles are enlarged and there is a concomitant loss of total brain volume (Bourdeau et al., 2002; Heinz, Martinez, & Haenggeli, 1977). Furthermore, many of these patients display psychiatric illnesses, such as MDD disorder (Dorn et al., 1997). Upon correction of the hypercortisolism, incidences of MDD begin to subside and both brain atrophy and the enlargement of ventricles return to a normal-like state (Bourdeau et al., 2002; Dorn et al., 1997; Heinz et al., 1977). Similarly, psychological stress disrupts the functional connectivity of brain networks that regulate attention and decision making (Liston, McEwen, & Casey, 2009; Soares et al., 2013) and decreases the volume of some regions in the brain (Soares et al., 2012). In these reports, medical school students studying for critical exams and who self-reported high stress, exhibited reduced functional connectivity (Liston et al., 2009) and even atrophy in some subareas (Soares et al., 2012) in the prefrontal cortex. Weeks after the

medical school exams ended and the self-reported stress subsided, the connectivity and volumetric measures normalized and were similar to non-stress control levels. Taken together, these studies suggest that the brain houses the inherent plasticity to regain function from chronic stress.

Brain derived neurotrophic factor (BDNF) is an important protein for normal brain structure and function and is one of the mechanisms that are critical for hippocampal improvements in the weeks after chronic stress has ended. In our first study (chapter 2) investigating BDNF in the post-stress rest process, we found that downregulating the expression of hippocampal BDNF prior to and throughout the administration of chronic stress and the post-stress rest period prevented the improvements of spatial ability. This was soon followed up by another study (chapter 3) using the same manipulations to understand if hippocampal CA3 dendritic complexity is affected in the same manner. We found that downregulating hippocampal BDNF prevented stress-induced dendritic retraction from showing improvements after the post-stress rest period. One caveat of these two findings was that hippocampal BDNF was downregulated during both the rest period and the chronic stress period making the interpretations of whether BDNF is necessary solely during the rest period difficult to determine. Fortunately, a novel TrkB antagonist, called ANA-12, was synthesized and made available after the start of those experiments (Cazorla et al., 2011). This drug is able to be administered systemically and can cross the blood brain barrier to antagonize TrkB receptors in the hippocampus. As such, by administering the drug during the rest period only, we determined whether the TrkB receptor is necessary for the improvements from chronic stress-induced spatial memory deficits and chronic stress-induced dendritic

retraction. As shown in chapter 3, we found that systemic administration of a TrkB antagonist during the rest period prevented the improvements from both chronic stress-induced spatial memory deficits and hippocampal CA3 dendritic. Taken together, these studies clearly show that the improvements from chronic stress-induced spatial memory deficits and dendritic retraction are mediated by hippocampal BDNF and its TrkB receptor.

My work contributed to the knowledge that BDNF acting through its TrkB receptor is necessary for chronic stress-induced deficits in spatial ability, and chronic stress-induced dendritic retraction, to show improvements following a post-stress rest period. BDNF acts on its TrkB receptor to activate downstream signaling mechanisms such as the phosphatidylinositol 3-kinase (PI3K) pathway. This is one pathway that may be important in the dendritic restructuring seen after the post-stress recovery period as the PI3K pathway appears to control dendritic arborization. Several studies have demonstrated that BDNF via activation of the PI3K pathways can induce the formation of dendrites (Dijkhuizen & Ghosh, 2005) whereas inhibition of this pathway can lead to reductions in dendritic complexity (Jaworski, Spangler, Seeburg, Hoogenraad, & Sheng, 2005; Kumar, Zhang, Swank, Kunz, & Wu, 2005). Furthermore, studies have reported that chronic stress reduces components of the PI3K pathway (Fang et al., 2013). One simple explanation is that chronic stress reduces BDNF and when chronic stress ends hippocampal BDNF expression increases, allowing for BDNF-TrkB signaling to activate PI3K to eventually increase hippocampal dendritic architecture, and then spatial ability

Chronic stress has a significant impact on the balance of excitatory and inhibitory neurotransmission in the hippocampus, which contributes to the alterations seen in

cognition and dendritic morphology. For the excitatory system, chronic stress increases the concentration of glutamate (Joels et al., 2004). Lesions of the entorhinal cortex, the main glutamatergic input into the hippocampus, prevent chronic stress-induced atrophy of hippocampal CA3 neurons (Sunanda et al., 1997), and a similar effect is acquired when an NMDA antagonist is administered throughout the stress procedure and this prevents stress-induced atrophy of hippocampal CA3 neurons (Magarinos & McEwen, 1995). For the GABAergic system, exposure to chronic stress has been shown to increase glutamic acid decarboxylase (GAD) expression in certain subregions of the hippocampus (Bowers, Cullinan, & Herman, 1998) and alter the expression of subunits of GABA receptors (Cullinan & Wolfe, 2000). Moreover, administration of a GABA agonist throughout a chronic stress paradigm prevents chronic stress induced atrophy of hippocampal CA3 neurons (Magarinos et al., 1999). These findings show that hippocampal excitatory/inhibitory system is essential to proper maintenance of hippocampal neurons, and these systems appear to be disrupted following a period of chronic stress.

The change in hippocampal glutamatergic and GABAergic tone has some basis in the development of MDD (Nutt & Malizia, 2001). Patients with MDD have reduced concentrations of GABA (Sanacora et al., 2004; Sanacora et al., 1999) and a loss of GABAergic neurons in various brain regions (Maciag et al., 2010; Rajkowska et al., 2007). While, in postmortem tissue of individuals with MDD, there is a significant loss of GABAergic inhibitory neurons in certain areas of the brain (Rajkowska et al., 2007; Maciag et al., 2010). It appears that the changes observed in the GABAergic system of

rodents that are exposed to chronic stress align with those changes seen in the GABAergic system of individuals with MDD.

Few studies have investigated how a rest period following the end of chronic stress alters the hippocampal GABAergic system. In chapter 4, we found that, following chronic unpredictable restraint stress and a post-stress rest period, performance on the radial arm water maze correlated with GAD65 expression in limbic brain regions. In male rats, GAD65 expression in the medial amygdala negatively correlated with RAWM performance, while, in females, GAD65 expression in the CA1 region of the hippocampus positively correlated with RAWM performance. More studies are needed to understand the relationship between spatial ability and the expression of GAD65 in the hippocampus. However, this study piqued our interest in the role the GABAergic system plays in response to chronic stress and a post-stress rest period.

Many studies have noted the structural plasticity of hippocampal principal neurons following chronic stress, while the changes in the structure of hippocampal GABAergic interneurons following chronic stress have received sparse attention. Moreover, it is unknown how hippocampal GABAergic neurons are affected by a post-stress rest period. In the one study investigating the effects of chronic stress on the structure of hippocampal GABAergic neurons, the dendritic complexity of CA1 (but not CA3) interneurons decreased (Gilabert-Juan, Bueno-Fernandez, Castillo-Gomez, & Nacher, 2017). Moreover, chronic decreases the expression of markers for subsets of GABAergic neurons in the hippocampus (Csabai et al., 2017; Czeh et al., 2005; Czeh et al., 2015; Hu et al., 2010). In chapter 5, we showed that chronic stress decreased CR+ interneurons in the CA3 region of the hippocampus, and this expression increased

following a post-stress rest period. Although there were no statistically significant effects in parvalbumin and somatostatin positive interneurons, there was a small but non-significant decrease of these interneurons in the dentate gyrus and this decrease did not improve following the post-stress rest period. As such, it would appear that CR+ interneurons are more involved in the post-stress rest period, than are PV+ and SOM+ interneurons, to allow spatial ability and dendritic complexity to show improvements. How these alterations affect principal cells of the hippocampus and how these changes are important for the improvement of hippocampal function following a post-stress rest period will warrant future investigations. However, alterations in the inhibitory activity of various GABAergic interneurons can produce profound effects on the hippocampal network. For example, acute inhibition of somatostatin-positive inhibitory neurons in the frontal cortex can induce depressive- and anxiety-like behaviors, whereas chronic inhibition of these neurons leads to reduced depressive-like behaviors (Soumier & Sibille, 2014). Additionally, inhibiting PV+ neurons in the CA1 region of the hippocampus post-training can impair consolidation of fear learning (Xia et al., 2017). The small decreases we found in PV+ and SOM+ interneurons that fail to show improvements following the rest period, may contribute to chronic stress-induced changes in hippocampal plasticity, but do not explain the improvements in hippocampal plasticity following a post-stress rest period.

The decrease in expression of CR+ following chronic stress may be an adaptive response to protect against excitotoxicity. The decrease in calretinin expression is unlikely to be due from the death of neurons as we found no total differences in GABAergic neurons (chapter 5). The most probable explanation is a decrease in the

content of cellular calretinin. Decreasing cellular content of calretinin most likely changes the function of the cell, for example by altering its calcium buffering capacity, and this may be a form of neuroplasticity. It has been shown that the calretinin protein itself is protective from glutamate-induced, calcium-dependent excitotoxicity in cell culture (D'Orlando, Celio, & Schwaller, 2002). Moreover, in one study that utilized calretinin knockout mice, LTP in the CA1 region of the hippocampus was unaltered compared to wild type mice. Additionally, calretinin knockout had no effect on spatial ability when compared to wild type mice. However, deficits in LTP were observed in the DG, and, in this same region, there was an increase in the amount of GABA release (Schurmans et al., 1997). As such, there are regional differences in how changes CR+ neurons affect behaviors, and our findings on CR+ interneurons in the CA3 region of the hippocampus may be particularly relevant for spatial ability. A way to distinguish between CR+ interneurons in the CA3 region vs the CA1 region, for example, would be through chemogenetic manipulations. Stimulating CA3 CR+ interneurons throughout the chronic stress procedure, and then testing spatial ability would be one way to understand the role of CR+ interneuron hippocampal function. Regardless, given the wide variety of GABAergic neurons, distinguishing how the various subtypes play a role in the function of the hippocampus following chronic stress is an important step to understanding the contribution of these neurons to stress-related disorders such as MDD.

Investigating the post-stress rest period, when chronic stress ends and the stress-induced changes in hippocampal neuronal structure and function improve, is critical step forward in understanding MDD. Years of research have focused on the mechanisms that occur in the hippocampus in the immediate aftermath of chronic stress that lead to

deficits in spatial ability and decreases in dendritic complexity. The data presented in this provides insight into the mechanisms that allow the brain to improve from a chronically stressed state. BDNF, acting through its TrkB receptor, appears to be critically involved in the improvements from chronic stress induced hippocampal CA3 dendritic retraction and spatial ability (chapters 2 and 3). Moreover, the hippocampal GABAergic system may be involved (chapters 4 and 5). Future studies, looking at how promoting subtypes of GABAergic interneurons that are decreased in response to chronic stress are needed to determine the extent to which the GABAergic system plays a role in the improvements in the hippocampus that occur following the post-stress rest period. A promising future study would investigate changes in CR+ interneurons alongside manipulations of hippocampal BDNF to understand whether CR+ interneurons are affected by BDNF expression as well. Moreover, another study looking at how chemogenetically activating CR+ interneurons throughout a chronic stress paradigm would identify whether CR+ interneurons are sufficient to prevent stress-induced impairments in spatial ability and principal cell dendritic morphology. Identifying molecular substrates that aid in the recovery from chronic stress induced deficits in spatial ability and reduction in dendritic retraction is a promising way forward in chronic stress research and may illuminate new strategies to combat stress-related disorders such as MDD.

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