The Role of Adult Hippocampal Neurogenesis and Synaptic Changes in Mediating Extinction Retention Deficit in a Rodent Model of PTSD

By

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DEDICATION

This dissertation is dedicated to my daughter, Keany Rodriguez.

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ABSTRACT

Post-traumatic stress disorder (PTSD) is a severe and debilitating disorder that can emerge following exposure to a traumatic event. The development of effective rehabilitation strategies requires identification of specific and modifiable targets that can ease the burden of PTSD symptomatology. Functionally, prefrontal cortex (PFC)–hippocampus (Hpc) circuitry is critical for extinction retention (ER) and contextual processing, and PTSD patients have been shown to have impairments in these functions. PTSD patients have been shown to have smaller Hpc and PFC and show lower activation in these areas during ER compared to controls. We have developed and extensively studied an animal model of PTSD - Single Prolonged Stress (SPS) – characterized by context-dependent extinction retention and fear renewal deficits. My research focused on two possible cellular mechanisms that may underlie SPS-induced extinction retention deficit within contextual processing neuro-circuitry. Specifically, I investigated a possible role of adult hippocampal neurogenesis in contributing to SPS induced extinction retention deficits in chapter 2. In chapter 3, I investigated if SPS would change gene expression of a selected number of synaptic markers within key brain regions involved in contextual processing and fear responding: mPFC, Hpc and Amyg (amygdala). I found several lines of evidence supporting a role for adult hippocampal neurogenesis in extinction retention deficits. Specifically, elimination of neurogenesis through irradiation resulted in extinction retention deficit, SPS exposure decreased the numbers of proliferating dentate gyrus cells, immature neurons and surviving dentate gyrus

cells, and an intervention aimed at promoting neuronal cell survival rescued SPS-induced extinction retention deficit. I also found that expression of some synaptic markers was altered by SPS exposure. Specifically, several proteins involved in synaptic structure were decreased while several proteins associated with synaptic signaling and function were increased, suggesting possible changes in synaptic morphology accompanied by a functional compensation within these regions that could mediate extinction retention deficits.

CHAPTER 1

Introduction

Posttraumatic Stress Disorder

According to the American Psychiatric Association's Diagnostic and Statistical Manual of Mental Disorders (DSM-5), for a diagnosis of PTSD, a person must meet one or more requirements from 5 major criteria. Criterion A states that the person was exposed to death, threatened death, actual or threatened serious injury, or actual or threatened sexual violence by direct exposure, witnessing the trauma, learning that a relative or close friend was exposed to a trauma, or indirect exposure to aversive details of the trauma. For Criterion B, the traumatic event must be persistently re-experienced as intrusive thoughts, nightmares, flashbacks, emotional distress after exposure to traumatic reminders, or physical reactivity after exposure to traumatic reminders. For Criterion C, the person must avoid trauma-related stimuli, trauma-related thoughts or feelings and/or trauma-related reminders. The person must meet two requirements from Criterion D, which states that negative thoughts or feelings began or worsened after the trauma in the following ways: inability to recall key features of the trauma, overly negative thoughts and assumptions about oneself or the world, exaggerated blame of self or others for causing the trauma, negative affect, decreased interest in activities, feeling isolated,

or difficulty experiencing positive affect. Finally, the person must meet two requirements from Criterion E, which states that trauma-related arousal and reactivity began or worsened after the trauma in the following ways: irritability or aggression, risky or destructive behavior, hypervigilance, heightened startle reaction, difficulty concentrating, or difficulty sleeping. These symptoms must be present for more than a month, they must result in distress or functional impairment and the symptoms must not be due to medication, substance use or other illnesses [1]. Clinician-Administered PTSD Scale (CAPS) is the gold standard for diagnosing PTSD. The PTSD checklist (PCL) is a self-report and can be used as a provisional tool for PTSD diagnosis.

Nationally, PTSD is estimated to affect 3.5% of the U.S. adult population [2] and, of those, 36.6% (or 1.3% of U.S. adult population) are considered severe [3]. Between 12 and 20% of military personnel meets criteria for PTSD diagnosis at some point in their lives [4]; a more recent estimate puts the actual number closer to 23% [5]. For example, the prevalence of PTSD among Operation Iraqi Freedom (OIF) and Operation Enduring Freedom (OEF) U.S. military personnel is estimated to be 5-17% [6, 7]. With such high prevalence, it is no surprise that a significant portion of veteran's health-related costs goes to treating PTSD. The Congressional Budget Office (CBO) reports that as of September 2011, mental health diagnoses were the second largest diagnostic category among Overseas Contingency Operations (OCO) for those who seek medical treatment at Veteran Health Administration (VHA), affecting 52%. More specifically, a PTSD diagnosis represents four to six times the average cost for first year medical treatment compared to non-PTSD veterans. As reference, the overall cost of first year PTSD treatment for OCO is estimated to be about $2 billion per year [8]. The high prevalence and cost associated with PTSD necessitate

significant efforts towards improved understanding of the pathophysiology of PTSD and better strategies for preventing and treating this debilitating psychiatric disorder.

Even with our current costly treatments, PTSD patients have a high rate of symptoms relapse and others are treatment resistant. Of the various therapeutic strategies for treating PTSD, psychological interventions are considered first-line approaches by most mental health professionals [9]. Of these, cognitive behavioral therapy (CBT) has been shown to be the most effective type of psychotherapy for treating PTSD [10]. Within CBT, cognitive processing therapy (CPT) and prolonged exposure (PE) therapy are the most studied and preferred by therapists [9]. CPT focuses on the impact of trauma by helping the patient understand why trauma-related negative thoughts are stressful, then encourages patients to replace those thoughts and to cope with the negative feelings [11]. In contrast, PE therapy entails revisiting the trauma in a safe clinical setting while patients try to change their reaction to memories of the trauma. For reference, PE has a 60% efficacy treating combat-related PTSD [12]. Both of these CBT therapies aim to help the patient re-contextualize the trauma memory as less threatening by focusing on changing how the patient experiences trauma recollection. For PTSD patients who are non-responsive to CBT, medication is often their second option. From this second line of PTSD treatment, antidepressants are the most prescribed: with a large body of evidence indicating selective serotonin reuptake inhibitors (SSRIs) being most effective [13]. Interestingly, SSRIs match PE with 60% efficacy, however, fewer patients achieve full symptoms remission with the former [14]. Other drug treatments include non-classical (non-SSRIs) anti-depressants and mood stabilizers [11]. With increased prevalence and a high rate of treatment resistant PTSD patients and high probability of symptom relapse, there is a grave need for PTSD treatment

improvements. Consequently, this need has fueled research efforts into understanding the psychopathology of PTSD, with the specific aim of identifying better therapeutic brain targets in hopes of filling treatment gaps and minimizing or eliminating relapse.

Early studies into the pathophysiology of PTSD focused on indirectly assessing brain function-related biomarkers. Initially, the focus was primarily on assessing autonomic nervous system dysfunction by measuring stress hormones and catecholamine levels in the blood, saliva and urine. Of these early studies, two reliably replicated findings emerged and are still relevant to our understanding of PTSD today: hypothalamic-pituitary adrenal (HPA)- axis dysfunction [15] and abnormalities within the noradrenergic system [16]. Even with inconsistent findings, there is a high level of consensus that PTSD is most likely related to HPA-axis dysregulation and not hypercortisolemia [17]. Specifically, using dexamethasome suppression test, patients suffering from PTSD consistently show enhanced negative feedback sensitivity of the HPA-axis [18]. Dexamethasone suppression test involves measuring plasma cortisol concentration before and after injecting a low dose of this cortisol-derivative that acts as a glucocorticoid receptor (GR) agonist. Repeated studies using this test have consistently revealed that PTSD patients showed greater suppression of cortisol compared to controls [15, 18]. Although, mechanistically, this response is thought to be GR mediated [15], the specific mechanistic role of the dysregulated HPA-axis in the development of the disorder's specific symptom profile is yet to be understood. These and other, yet to be discovered, neural circuits underlie key PTSD dysfunctions and contribute to the development of better models.

Additional peripheral markers associated with PTSD include exaggerated catecholamine concentrations in response to trauma cues [18] and high norepinephrine concentration in urine [19]. However, categorizing catecholamine dysfunction as a relevant measure of PTSD pathology has been more complicated, highlighting the limitations of characterizing PTSD by measuring peripheral markers as a way of assessing brain dysfunction. Peripheral measures of brain function lack sufficient insight for understanding the mechanisms by which they emerge and their exact contribution to PTSD symptomology. Thanks to human neuro-imagining techniques, in conjunction with animal research, investigation into the pathophysiology of PTSD can begin to include direct assessments of brain structure/function abnormalities that may bear significance to understanding the disorder. For example, although the amygdala (Amyg) is a key region for fear processing, there is a growing body of evidence emerging from fMRI studies in humans supporting the hypothesis that fear learning and the Amyg might be unaffected in PTSD [20, 26-28], with some verification emerging from animal models of the disorder (e.g. single-prolonged stress; SPS) [29, 30]. From these studies emerges a new theory seeking to integrate what is known about PTSD-relevant brain functions during different functional tasks and PTSD symptomology.

Deficient contextual processing (CP) is an alternative model for PTSD that emerges from the overlapping neurocircuitry involved in context-dependent extinction retention/recall (ER) shown to be reduced or dysfunctional in PTSD [20]. Although some evidence suggests aberrant encoding of fear memories during conditioning [23] or abnormal safety learning during extinction [24, 25], the CP model suggests that PTSD fear processing deficits most likely emerge from faulty contextual processing [20], which normally assists in disambiguating neuronal patterns that share many overlapping features (i.e. pattern separation) [31]. Evidence for the CP model comes

primarily from studies of context-dependent processes like ER and fear renewal (FR) -- shown to be deficient in both PTSD patients [26, 27, 32] and in the Single Prolonged Stress (SPS) PTSD rodent model [29]. More specifically, in humans, appropriate ER requires uninterrupted communication between the ventromedial prefrontal cortex (vmPFC) and the Amyg [33], while intact FE (i.e. safety learning) requires intact connection between vmPFC and Hpc [34]. From animal research, these brain regions (mPFC and Hpc) have been identified as key players in CP [35, 36]. In rats, different parts of mPFC connect to the Amyg to modulate fear expression via efferent connections from the prelimbic (PL) to increase fear or the infralimbic (IL) to reduce it [37]. Brain circuits involved in contextual processing functions, shown to be abnormal in PTSD, include mPFC, Hpc, thalamus and locus coeruleus [19]. Further cellular and molecular inquiry into brain regions involved in CP (PFC-Hpc) related to modulation of fear response seems to be the best candidates for understanding key PTSD symptoms. Interestingly, PFC and Hpc have been shown to be dysfunctional among other psychiatric disorders (e.g. major depression disorder, bipolar disorder and schizophrenia) suggesting a possible common mechanistic role resulting in their shared symptomology with PTSD [39]. Additional brain regions shown to be abnormal in PTSD, specifically associated with exaggerated threat detection and part of the salience network, are the dorsal anterior cingulate cortex (dACC), insula and amygdala [20] as well as regions involved in executive function (mPFC and dorsal and ventral lateral PFC [21]).

Single Prolonged Stress

Single-prolonged stress (SPS) is a rodent model of PTSD originally designed with the primary objective of replicating PTSD-characteristic dysregulation of the HPA axis as evidenced by exaggerated dexamathosome suppression test, with the hopes of then studying resulting PTSD-relevant behavioral deficits and their underlying neuromechanisms [15, 18, 22]. SPS consists of serial exposure to three stressors: 2 hours of restraint, 20-minute forced-swim, and loss of consciousness with ether exposure -- during a single continuous session, with a 15-minute rest post swim. These stressors were originally chosen to cause a robust stress response through three different pathways – psychological (restraint), physiological (forced group swim), and pharmacological (ether). Interestingly, SPS exposure seems to require an incubation/sensitization period in order to observe HPA axis dysregulation [23] as well as the extinction retention deficit [24, 25]. In addition to the incubation time being important in order to observe SPS's effects, it has been shown that ommitting any one of the three stressor components will not result in PTSD-like phenotypes [25]. In the past two decades since its development, SPS has been widely used to study adaptations following a severe traumatic stressor, with great emphasis placed on characterizing behavioral changes following SPS and their relationship to underlying neurobiological processes.

The effects of SPS on PTSD-relevant fear and safety learning/processing can be studied during fear memory acquisition, retrieval of fear memory, acquisition of extinction memory, the retention/recall of the extinction memory, and the retrieval of the extinguished fear response through spontaneous recovery, reinstatement, or renewal [26]. Like with PTSD [27], SPS exposure leads to increased responses during testing for extinction recall and fear renewal [24] (Figure 1.1). The validity of SPS for modeling PTSD goes further than fear/safety learning and regulation.

Several studies have also shown that SPS exposure results in marked cognitive dysfunctions, such as memory impairment [28], deficits in mental processing speed [29], and inattention [30]. However, it is not yet known whether these cognitive impairments are predisposing factors for PTSD or part of the symptomology resulting from the disorder. In addition, sleep disturbances contribute to the development and maintenance of PTSD in humans [31], and is associated with diverse aspects of PTSD pathology, including sustained fear responses to trauma cues [32], hyperarousal [33], and suicidality [34]. Mirroring findings on disrupted sleep architecture in patients with PTSD [35], SPS causes increased wakefulness and REM sleep disturbances [36-38]. Importantly, the degree of sleep disruption predicted SPS-induced extinction retention deficits [37], which suggest that traumatic stress-induced sleep alterations likely contribute to development of other PTSD symptoms.

SPS has been a useful tool for investigating the mechanisms underlying PTSD-relevant deficits. Dysregulated HPA-axis seen in both PTSD patients and SPS exposed rats may correlate well with altered function and/or expression of glucocorticoid receptors like GR and mineralocorticoid receptor (MR). It is thought that a high GR/MR ratio leads to increased sensitivity to the stress-related effects of increases in circulating CORT, including negative feedback of the HPA axis [39]. Additionally, PTSD symptoms involve alterations of excitatory and inhibitory neurotransmission [40]. Interestingly, SPS has been shown to decrease glutamate (Glut) and its precursor in the mPFC, but not in the Amyg or Hpc [41, 42]. Less is known about SPS's effects on inhibitory neurotransmission. However, decreased GABA levels in the Hpc have been reported [43]. Alterations in serotonin have been linked to behavioral changes seen following SPS; for example, markers of serotonin turnover in the dorsal Hpc have been correlated

with increased fear generalization in mice exposed to SPS [44]. The behavioral effects of SPS can be ameliorated by a variety of SSRIs, including escitalopram [45], fluoxetine [46], and paroxetine [47-49]. SPS also regulates 5-HT receptor expression and signaling across diverse brain regions. For example, SPS increases expression of 5-HT2C receptors in the Amyg [50], where these receptors have been shown to regulate contextual fear and responses to anxiogenic stimuli [51]. In contrast, SPS decreases hippocampal expression of 5-HT3A receptors, which are thought to be involved in neurogenesis and antidepressant effects [52].

There is evidence suggesting that in SPS, neuropeptide-Y (NPY) interacts with brain monoamine systems and the HPA axis to orchestrate post-SPS behavioral changes. Neuropeptides like oxytocin and NPY have been implicated in stress responses and many are known to regulate HPA axis function. SPS exposure increases oxytocin receptor in the Amyg, hypothalamus, and Hpc [23]. SPS decreases NPY receptor subtype 2 within the central nucleus of the Amyg [53]. Intranasal NPY administration shortly after SPS can prevent these changes as well as rescue SPS-induced increases in hippocampal GR and SPS-induced behavioral dysfunctions [54-56]. Molecularly, MAPK signaling has been shown to play a role in coordinating transcriptional responses to SPS via activation of cFos [57], in regulating neuronal apoptosis [58], and in the behavioral effects of SPS including hyperalgesia [59] and increased anxiety-like behavior [60]. SPS increased levels of phosphorylation (activation) of Akt in the Hpc concurrently with increases in GR [61]. Both the MAPK and PI3K/Akt/mTOR pathways are involved in regulating synaptic plasticity and cellular morphology, changes underlying fear learning [62].

The SPS model of exposing animals to potentially traumatic stress has strong content validity as demonstrated by its ability to produce PTSD-relevant behavioral and neuro-hormonal syndrome, impairment of extinction recall and fear renewal, hyperarousal, changes in neurotransmitter and neuropeptide systems, and altered responsivity of the HPA axis. However, although SPS is a complex, multimodal stressor, it does not account for many of the complex features of trauma experienced by humans that may influence the development of PTSD. Nonetheless, in concert with other preclinical and clinical approaches to the study of traumatic stress and PTSD, SPS holds great potential to expand our basic knowledge of the relevant mechanisms, thereby potentially contributing to the discovery of better drug targets that will lead to treatment improvement and new treatment development.

SPS, as a rodent model of PTSD, makes for a power tool to study the underlying neuro-mechanisms for PTSD-relevant behavioral deficits. Adult hippocampal neurogenesis and synaptic changes are two such possible neuro-mechanisms that may underlie SPS-induced extinction retention deficit.

Adult Hippocampal Neurogenesis and Psychiatric Disorders

It is widely accepted that in mammals, including humans, adult neurogenesis occurs in the sub-granular zone (SGZ) of the dentate gyrus (DG) of the hippocampus (Hpc) [63], among other brain regions (Figure 1.3). These neural stem cells (NSCs) have astrocyte characteristics,

though in adulthood, only a fraction of specialized brain astrocytes function as stem cells and express glial fibrillary acidic protein (GFAP) [64, 65]. These GFAP expressing NSC give rise to new neurons by going through a multistep process involving proliferation, differentiation, maturation, survival and integration of new neurons from precursors (Figure 1.4). While these newly divided NSC remain immature (i.e. neuroblasts), they transiently express the microtubule binding protein – doublecortin (DCX) – making it a good marker for identifying young neurons [66]. As neurons mature, they stop expressing DCX and newly adult neurons can be identified by their expression of the neuronal marker NeuN [67]. These maturation steps are tightly regulated by both extrinsic and intrinsic factors, and by the existing neuronal network activity [65]. Interestingly, most newly divided NSCs die shortly after they are born while only a fraction become functionally integrated [63]. It is estimated that approximately seven hundred new neurons are incorporated into the human Hpc each day [68]. The exact roles these adult-born hippocampus neurons play in un/healthy brain function are not under consensus agreement. However, it is widely believed that these adult-born neurons play a role in learning and memory and that changes to normal rate of birth of these cells are associated with several psychiatric disorders [69]. Interestingly, a recent study raises questions of whether adult neurogenesis plays any role at all. They report that adult hippocampal neurogenesis drops to undetectable levels after early childhood [70]. More postmortem studies are necessary to validate these findings.

Because the hippocampus's central role in the process of pattern separation [70] -- required for proper contextual processing to facilitate information encoding and retrieval (i.e. learning and memory) [71] -- it is no wonder that Hpc has been identified as a potential key player in the pathophysiology of several psychiatric disorders [72], some of which share PTSD-relevant

symptoms (e.g. major depressive disorder: MDD) [73]. Furthermore, the fact that antidepressants are the most common pharmaceutical intervention for treating PTSD [9] and that their ameliorative effects are mediated, at least in part, by neurogenesis [74, 75] suggest that this may be a potential mechanism linking shared physiology and emergent symptomology between PTSD and MDD [73].

Of possible mechanistic relevance to PTSD, research in animals show that prolonged exposure to stress or stress hormones (e.g. cortisol/corticosterone), mediated by GR, reduces adult Hpc neurogenesis [76]. Interestingly, our animal model of PTSD – single-prolonged stress (SPS) – replicates PTSD-like context-dependent extinction retention deficit accompanied by increased GR/MR expression ratio in Hpc, including in the dentate gyrus [24, 25] – where Hpc neurogenesis takes place. Furthermore, SPS was designed with the aim of replicating PTSD characteristic HPA-axis dysregulation in order to study emergent PTSD-relevant fear processing deficits [77]. The confluence of evidence strongly suggests a possible role of adult Hpc neurogenesis as a contributing factor for PTSD-like SPS-induced ER deficit.

Synaptic Plasticity and Psychiatric Disorders

Synaptic remodeling/formation is another form of brain plasticity that could lead to susceptibility for development of psychiatric disorders [78], which therefore, has obvious relevance to PTSD [79]. At the cellular level, long-term potentiation (LTP) and long-term depression (LTD) are the substrates for learning [80]. LTP results from synaptic strengthening including long-lasting changes in the architecture of synapses while reduced/unsynchronized

synaptic activity and spine pruning characterize LTD [81]. These synaptic changes require coordination of gene expression related to all domains of synapse formation and function, many still unknown [82, 83]. Interestingly, synaptic remodeling can be induced with exposure to stress or stress hormones [84]. Acute cortisone treatment has been shown to result in dendritic atrophy in mPFC and hypertrophy within the amygdalae of rats. These neuronal changes were paralleled with heightened anxiety-like behavior in elevated plus maze [84]. Additionally, length of stress exposure was shown to modulate spatiotemporal patterns of spine formation within the amygdala [85]. Interestingly, MDD postmortem studies found fewer dendritic branching and reduced expression of synaptic markers within the PFC [86]. Additionally, fast-acting antidepressants' ameliorative action are thought to be mediated by increased synaptic remodeling [87]. Consequently, changes in synapses within mPFC-Hipp-Amyg circuitry (contextual processing to influence fear expression) could be a possible mechanism by which stress exposure, mediated by stress hormones, could alter the function of these brain regions and how they communicate with one another. In fact, the need to research synaptic plasticity as a possible contributor of PTSD has been proposed [88], yet not seriously addressed. The work contained in this thesis aims to investigate if two forms of brain plasticity – adult Hpc neurogenesis and synaptic changes – could play a mediating role in the PTSD-like extinction retention deficit in SPS.

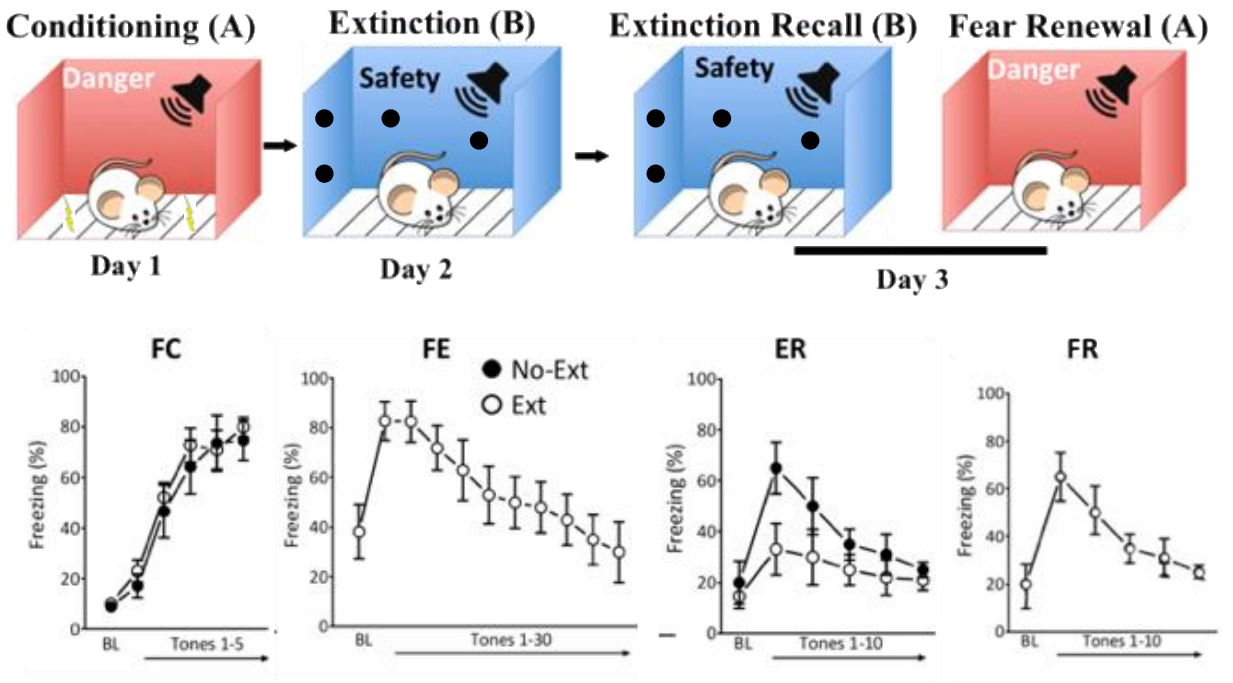


Figure 1.1 Fear/safety behavioral assessments: white/open circles represent animals that underwent an extinction session, while black/closed circles represent a group of animals that did not. All graphs depict mean (-/+ SEM) percentage freezing on the y- axis against tone trials on the x-axis. On day 1, fear conditioning (FC) consists of five trials presentations of 10-second tones co-terminating with a 1-second foot-shock every minute (1min10sec per trial) within context A. On day 2, fear extinction (FE) or safety learning, the same 10-second tone is presented for 30 trials with no shock reinforcement within context B. On day 3, rats are exposed to the same tone for 10 trials in either context A for fear renewal (FR) or Context B for extinction retention/recall (ER) test. Each behavioral paradigm has a three-minute baseline before the first trial starts.

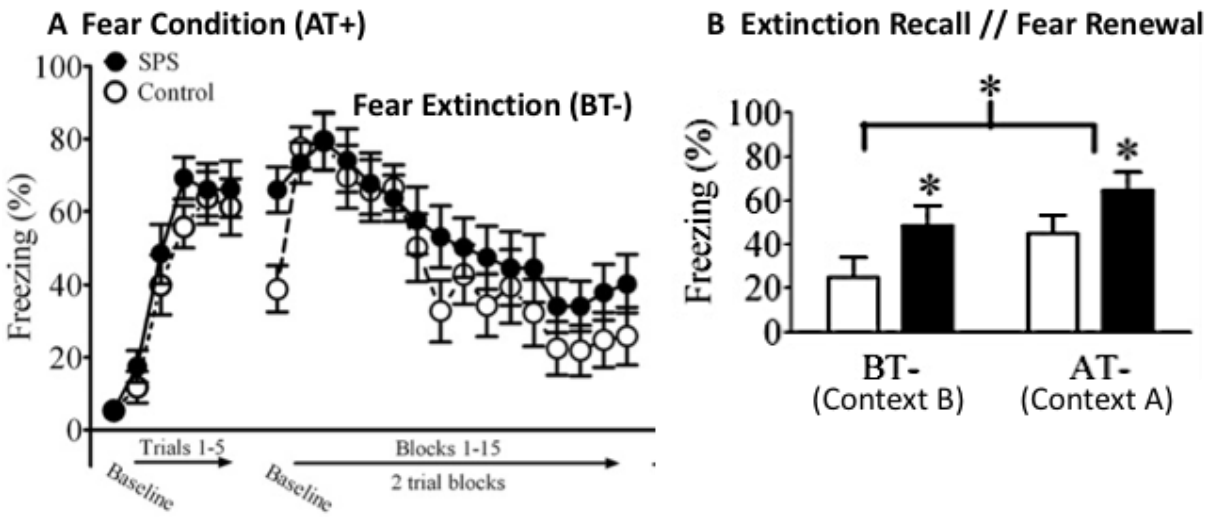
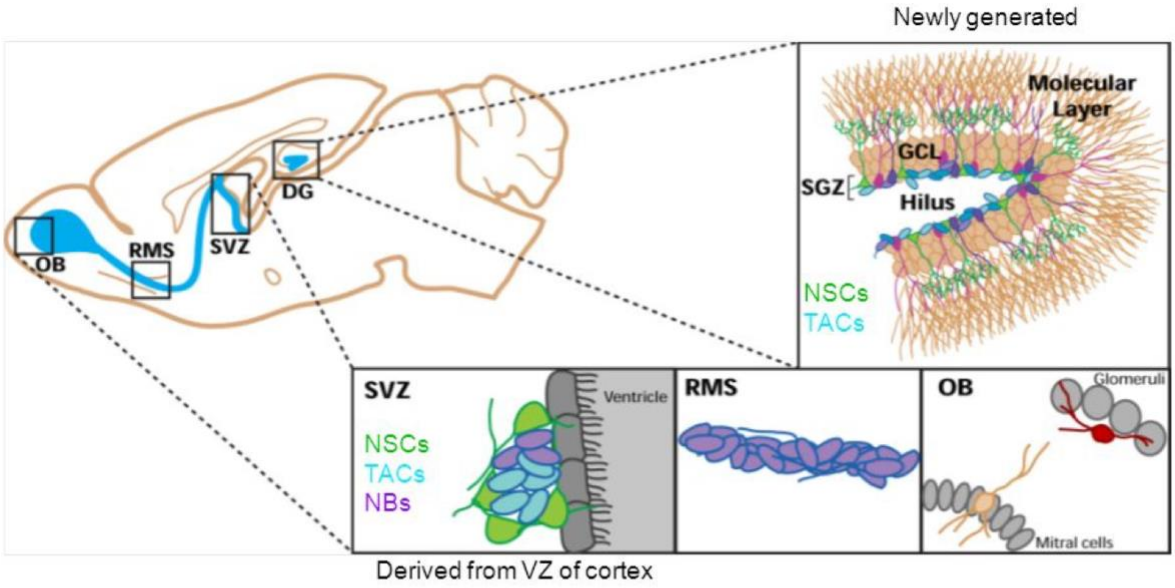


Figure 1.2 Effects of SPS on extinction retention and fear renewal. SPS (black) vs. Controls (white) average percent (+/- SEM) freezing is presented for each behavioral assessment. A) Fear conditioning and extinction occur on consecutive days are plotted together. There is no statistically significant difference between groups during these memory acquisitions days. B) Graphs depict bar graph of first five trials averaged for group means (+/- SEM). Extinction recall or fear renewal occurred on day three. SPS rats showed exaggerated fear (higher freezing) during both tests compared to controls. As expected controls showed higher freezing during fear renewal compared to extinction recall. Figure adapted from Knox et al., 2012 [24].



SVZ = Subventricular Zone, RMS = Rostral Migratory Stream, SGZ = Subgranular Zone of Dentate Gyrus
OB = Olfactory bulb, NSC = neural stem cell, TAC = transient amplifying cells (progenitors),
NB = neuroblast

Modified from: Madeleine A. Johnson, Jessica L. Ables & Amelia J. Eisch *Cell-intrinsic signals that regulate adult neurogenesis.* BMB Reports 2010

Figure 1.3 Niches of adult neurogenesis. Adult hippocampal neurogenesis takes place in the subgranular zone of the rat dentate gyrus. Newly divided neural stem cells in the sub-ventricular zone migrate via the rostral migratory stream to the olfactory bulb.

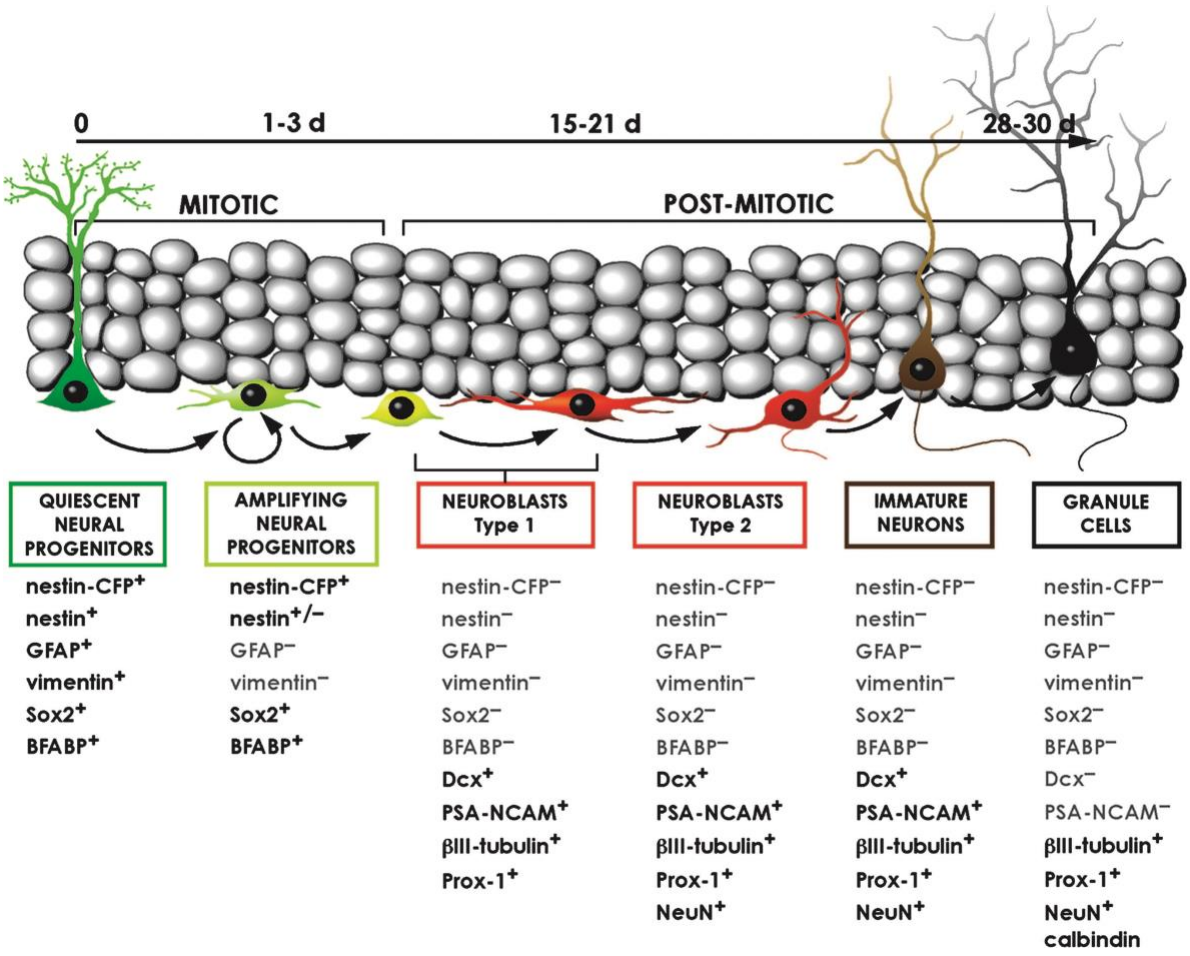


Figure 1.4 A schematic summary of the neuronal differentiation cascade in the dentate gyrus. "Quiescent neural progenitors generate, through asymmetric divisions, the amplifying neural progenitors that, after several rounds of symmetric divisions, exit the cell cycle within 1–3 days and become post-mitotic neuroblast-1 cells. Within next 15–21 days, neuroblast-1 cells mature into neuroblast-2 and then into immature neurons with apical processes and basal axons and the soma located in the granular cell layer. After an additional 10–15 days, immature neurons acquire the characteristics of mature granule neurons, develop extensive branching, and send long axonal processes, forming the mossy fiber." Figure taken from Encinas *et al.*, 2006 [89].

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Chapter 2

Role of Neurogenesis in SPS-Induced Extinction Retention Deficit

Abstract

Posttraumatic stress disorder (PTSD) has been functionally linked to impairments in context-dependent fear extinction retention (ER), a process that requires intact prefrontal cortex - hippocampal (Hpc) circuitry. PTSD patients show abnormalities in Hpc volume and function, and in contextual processing, generally. At the cellular level, adult Hpc neurogenesis plays a role in key Hpc functions, like pattern separation, and is sensitive to stress, but has not been examined in PTSD. Here we use an animal model of PTSD – single prolonged stress (SPS) – to examine adult Hpc neurogenesis and its involvement in context-dependent ER. We first demonstrated impaired ER in irradiated animals, associated with reduced Hpc neurogenesis (by 70%). Irradiated rats showed normal fear conditioning and fear extinction, but impaired ER (increased freezing) associated with the degree of immature cell loss within Hpc. Secondly, we examined the effects of SPS on neurogenesis, demonstrating decreased number of immature neurons in Hpc of SPS animals. To determine whether this effect was due to cell proliferation or survival, we injected BrdU (synthetic thymidine nucleoside, 5-bromo-2'-deoxyuridine) three

weeks before SPS (cell survival) or one day after SPS (cell proliferation). In both cases, SPS reduced the number of BrdU+ adult-born dentate gyrus granule cells. Finally, we rescued SPS ER effects by enhancing Hpc neuronal survival – via environmental enrichment. Together, these data strongly support a role for adult Hpc neurogenesis in mediating an SPS-induced extinction retention deficit. These results identify neurogenesis as a potential mechanism underlying PTSD contextual processing deficits.

Introduction

Post-traumatic stress disorder (PTSD) is a debilitating psychiatric condition characterized by increased fearfulness and heightened arousal [1] . Deficient contextual processing (CP) is a relatively novel hypothesis linking PTSD symptoms development to underlying neurobiology of hippocampal-prefrontal circuitry [2]. In PTSD, trauma-related memories incongruently intrude in "safe" contexts – a loud noise, for example, can elicit a trauma-related reaction even when heard in a "safe" home. This may result from an inability to use contextual information to regulate recollection of trauma memories [3]. In fear memory paradigms, extinction retention (ER) and fear renewal (FR) are context-dependent processes that require intact connections between medial pre-frontal cortex (mPFC) and hippocampus [4]. Consistent with CP hypothesis, PTSD patients demonstrate abnormalities in both ER and FR, and also display abnormalities in mPFC and Hpc [5, 6] suggesting that a CP neural circuit disruption could play a role in PTSD behavioral dysfunction.

Animal models of PTSD are required to elucidate specific neural circuit disruption on the cellular and molecular levels and identify possible therapeutic targets. Single prolonged stress (SPS) - a validated animal model of PTSD - produces alterations in physiology and behavior that mimic key features of PTSD. Specifically, it enhances fast negative feedback in the hypothalamic-pituitary-adrenal (HPA) axis [7] – a neuroendocrinological hallmark of PTSD [8] involving the Hpc [9]. Like PTSD patients, SPS-exposed rats also show inappropriate fear associated learning with abnormal ER and FR [10, 11]. They also have reduced mPFC glutamate and glutamate precursors [12], suggestive of altered mPFC activity. Dysfunctional PFC-Hpc neurocircuitry can disrupt regulation of amygdala activity, contributing to inappropriate fear responses in PTSD [13]. Both Hpc and mPFC are altered by SPS, adding to the model's utility for studying the neuronal basis of a PTSD-relevant CP deficit.

Human and animal studies demonstrate a critical role of the Hpc in CP [14, 15]. During memory acquisition, the Hpc helps develop memory representations that avoid overlap with old ones in a process known as pattern separation (PS). It also facilitates pattern completion (PC) – retrieval of old memories based on partial cues [16]. Cellularly, PS and PC require neurogenesis within the Hpc's dentate gyrus (DG). Eliminating adult Hpc neurogenesis produces PS impairments [17]. Conversely, mice with more functionally integrated adult-born dentate gyrus cells (DGCs) can better distinguish between two similar contexts [18, 19]. Appropriate behavioral responses to threat cues require an ability to differentiate a safe context from a dangerous one with overlapping features. This raises the possibility that diminished CP in PTSD is contributed at least partially by diminished Hpc neurogenesis.

Interestingly, Hpc, in general, and neurogenesis, in particular, are very sensitive to stress exposure. The Hpc is densely populated with glucocorticoid receptors [9], and stress and heightened circulating cortisol impact performance on Hpc-dependent tasks [20]. Stress and cortisol can alter synaptic plasticity and firing properties of Hpc neurons and can reduce adult Hpc neurogenesis [20]. Glucocorticoid (GC) blockade within Hpc can prevent reduction in adult neurogenesis from social defeat stress and rescue exaggerated anxiety-like responses [21]. Cortisol-induced neurogenesis reduction is likely mediated by low-affinity GC receptors (GR), while activation of high affinity mineralocorticoid receptors (MR) has opposing effects [22]. Interestingly, the SPS-induced ER deficit is uniquely associated with elevated Hpc GR/MR expression ratios [11], suggesting a potential mechanistic link between altered adult Hpc neurogenesis and ER deficits.

Together, the accumulating evidence suggests that impaired Hpc neurogenesis could play a mechanistic role in contributing to core CP deficits in PTSD. We herein report on a set of animal experiments designed to explore this possibility.

Methods

Overall design: In *Experiment I,* we first tested whether hippocampal neurogenesis is essential for the extinction retention (ER), and whether preventing neurogenesis by means of focal x-irradiation leads to an ER deficit, mimicking the SPS-induced ER deficit. In *Experiment II, we* sought to establish whether SPS exerts an effect on adult hippocampus neurogenesis. We first investigated if doublecortin (DCX) RNA expression was altered within the hippocampus following

SPS exposure. DCX is a neuronal migration microtubule-associated protein expressed almost exclusively by neuronal precursor and immature neurons [23]. Consequently, a change in the number of immature neurons would likely be reflected in the levels of DCX RNA expression. To this end, rats were randomly assigned to either SPS or control, their hippocampi dissected and processed for RT-qPCR. The experiment was repeated and DCX+ cells within the DG quantified. In *Experiment III,* we were particularly interested in investigating a possible effect of SPS on the survival of adult-born DG cells due to the timeline of the SPS-induced ER deficit. We have previously shown that SPS results in ER deficit ten days post SPS exposure (Knox et al., 2012). Newly born neurons do not show synaptic connectivity and functionality until approximately 21days after neurogenesis [24]. Therefore, SPS effects on neuronal proliferation could not explain the presence of behavioral deficits manifesting as early as 10 days following the SPS. To investigate whether SPS affects survival of adult-born DGCs (i.e. survival of neurons that were born prior to SPS exposure), rats were administered 5-bromo-2'-deoxyuridine (BrdU) - a synthetic nucleotide for tracking recently divided cells- for four days. Twenty-one days after the last injection, rats were exposed to either SPS or control. In *Experiment IV*, we investigated whether promoting proliferation of adult hippocampal neurogenesis through voluntary exercise would counter the effects of SPS on reduced proliferation, thereby rescuing SPS ER deficit. In *Experiment V*, we investigated whether adult Hpc neurogenesis plays a role in extinction retention (ER) by attempting to rescue ER in SPS-exposed animals by enhancing survival of adult Hpc neurogenesis. We exposed rats to environmental enrichment (EE) as a neurogenesis enhancing intervention following SPS-exposure, as EE has been previously shown to selectively promote neuronal survival [25].

Subjects: the subjects were 164 adult male Sprague Dawley rats (42 – 45days old; 150 g), obtained from Charles River (Wilmington, MA). Upon arrival, all rats were pair-housed for a minimum of 3 days and were then individually housed after exposure to stress or a control procedure. All rats had ad libitum access to water and standard rat chow. All experimental procedures were approved by the Veteran Affairs Institutional Animal Care Usage Committee.

Experiment I Does reducing adult hippocampal neurogenesis through irradiation lead to extinction retention deficits?

Irradiation: 20 animals (10 Sham and 10 Irradiated) were deeply anesthetized in a vented anesthesia chamber using 3-5% isoflurane. After 3-5minutes, the depth of anesthesia and analgesia was monitored by checking the following parameters: regular and relaxed respiratory rate, absent withdrawal reflex upon foot pinch, and no response to external stimuli. Rats were placed laterally on a treatment table, and the eyes, oropharynx and body shielded with lead. Rats received 9 Gy total irradiation split in 3 fractions of 3 Gy given over 3 days; each delivered at a rate of approximately 175 cGy per minute using a Phillips 250 kV orthovoltage unit. Half of each treatment was given, and then the animal was turned over and the remainder of the dose delivered to the other side of the head to reduce potential adverse effects. Sham-irradiated rats were handled and anesthetized, but not irradiated. All rats remained undisturbed for 28 days after the last exposure. After this quiescent period, all rats underwent fear learning (FL; Figure 2A).

Chambers: All FL sessions were conducted in identical rodent observation chambers constructed of aluminum and Plexiglas (30×24× 21 cm; MED Associates), situated in sound-attenuating chambers and located in an isolated room. The floor of each chamber consisted of 19 stainless steel rods (4 mm in diameter) spaced 1.5 cm apart (center to center). The grid floor was connected to a shock source and a solid-state grid scrambler (MED Associates) which delivered the foot-shock unconditioned stimulus (UCS). Mounted on one wall of the chamber was a speaker to provide a distinct auditory CS; on the opposite wall was a 15-W house light and a fan, which provided background noise (65 dB). Cameras mounted to the ceiling of the sound-attenuating chambers were used to record behavior, which was scored offline. Two unique contexts were created by manipulating auditory, visual, and olfactory cues: Context A comprised an odor- 1% acetic acid solution placed in trays at the bottom of the chambers, the house light on, chamber doors closed, and sound of fans on in the chambers; Context B comprised an odor- 1% ammonium hydroxide solution in chambers, red light on, chamber doors open, and fans off.

Fear learning paradigm: On the first day of FL, rats were placed in their individual chambers for fear conditioning in Context A and received five paired presentations of a tone (10 sec, 2 kHz, 80 dB) that co-terminated with a footshock (1.0 mA, 1 sec) beginning 180 sec after being placed in Context A. There was a 60-sec inter-trial-interval (ITI), and the rats remained in the chambers for 60 sec after the last foot-shock presentation. One day after conditioning, rats were placed into a novel context (Context-B) and were presented with 30 tone presentations (10 sec, 2 kHz, 80 dB, 60-sec ITI), in the absence of foot-shock, beginning 180 sec after being placed into the chambers in order to extinguish fear responding to the tone (i.e., extinction training). Two days after

conditioning, rats were placed back into Context-B and were presented with 10 tones beginning 180 sec after being placed into the chambers in order to assess extinction retention.

Brain collection and processing: The day following extinction retention testing, rats were deeply anesthetized with sodium pentobarbital and perfusion-fixed with saline followed by 4% paraformaldehyde (PFA). Brains were removed and post-fixed in 4% PFA overnight, then cryoprotected in 30% sucrose. Coronal serial sections were cut at 50 µm on a cryostat, every fourth section was collected into 24 well-plates containing tris-buffered saline (TBS, pH = 7.4), resulting in four sets of sectioned brain containing the Hpc (from bregma −2.30 to −5.20 mm). Ventral (v) and dorsal (d) hippocampus (Hpc) were defined here as the first and last 5 brain sections, respectively, within each set. The sets not immediately processed by immunohistochemistry (IHC) were transferred into cryoprotectant and stored at −20°C for later use.

Immunohistochemistry: To assess neurogenesis reduction, we quantified immature neurons expressing doublecortin (DCX). Diaminobenzidine IHC was performed with antibodies against DCX using previously published protocols [26]. Briefly, sections were rinsed in TBS, blocked with 10% normal horse serum (Invitrogen, Carlsbad, CA), and incubated overnight at 4°C with rabbit DCX antibody (1:1000, Roche Applied Science, Indianapolis, IN). The following day sections were rinsed, incubated in secondary antibody (1:200 biotinylated goat anti-rabbit IgG, Vector Labs, Burlingame, CA) for 1 hour, rinsed, signal amplified with Vector ABC kit, developed with stable diaminobenzidine (Invitrogen), mounted onto slides, dehydrated and cleared in ethanol and xylenes, then coverslipped using Permount (Sigma-Aldrich).

Imagining and analysis: Images were captured with an Olympus CX41 microscope equipped with INFINITY 1 camera (Lumenera; Ottowa, ON) using INFINITY ANALYZE software (Lumenera). Images were standardized by adjusting the minimum and maximum brightness to the ends of the optical density distribution (levels function, PHOTOSHOP; Adobe Systems, Mountain View, CA, USA). The hippocampus was identified by anatomical landmarks and both hemispheres were included in analysis. For DCX+ cell counts, three micrographs were taken within each dentate gyrus (DG) at a 100x magnification (Figure 1A). For BrdU-expressing cell counts, photomicrographs were captured at 10x and the granular cell layer was identified as a dark band of densely packed cells within the DG. Dark brown circular stainings were counted as BrdU-expressing cells only if directly underneath this cell layer in the SGZ (Figure 1B). All Photomicrographs were overlaid and counted using Adobe PHOTOSHOP by two observers blind to experimental group. Inter-rater reliability was assessed by Pearson correlation (R = 0.96).

Data analysis: Freezing behavior was manually scored by two observers blind to experimental conditions. All videos were first recorded and stored on a digital hard-drive, then copied to be viewed on the computer and scored using a stopwatch. Freezing was defined as the absence of movement, except that necessary for breathing, for >2 sec and quantified as a percentage of the total time recorded (i.e. Tone + ISI or 70sec). For fear extinction, freezing across three extinction trials were averaged into a block (baseline, blocks 1– 10 are graphed). For extinction retention/recall, freezing across five trials were averaged into a block (baseline, blocks 1 and 2 are graphed).

Statistical analysis: FC and ER were analyzed using repeated measures ANOVA for all experiments. Independent sample's t-Tests were used to compare effects of SPS where appropriate. The criterion for significance was set at $P<0.05$. Two rats that did not show a conditioned freezing response >30% at the start of a fear extinction session or exhibited freezing levels +/- 2 standard deviations from a group mean and were, therefore, excluded from final analyses. All data are represented as means +/- SEM.

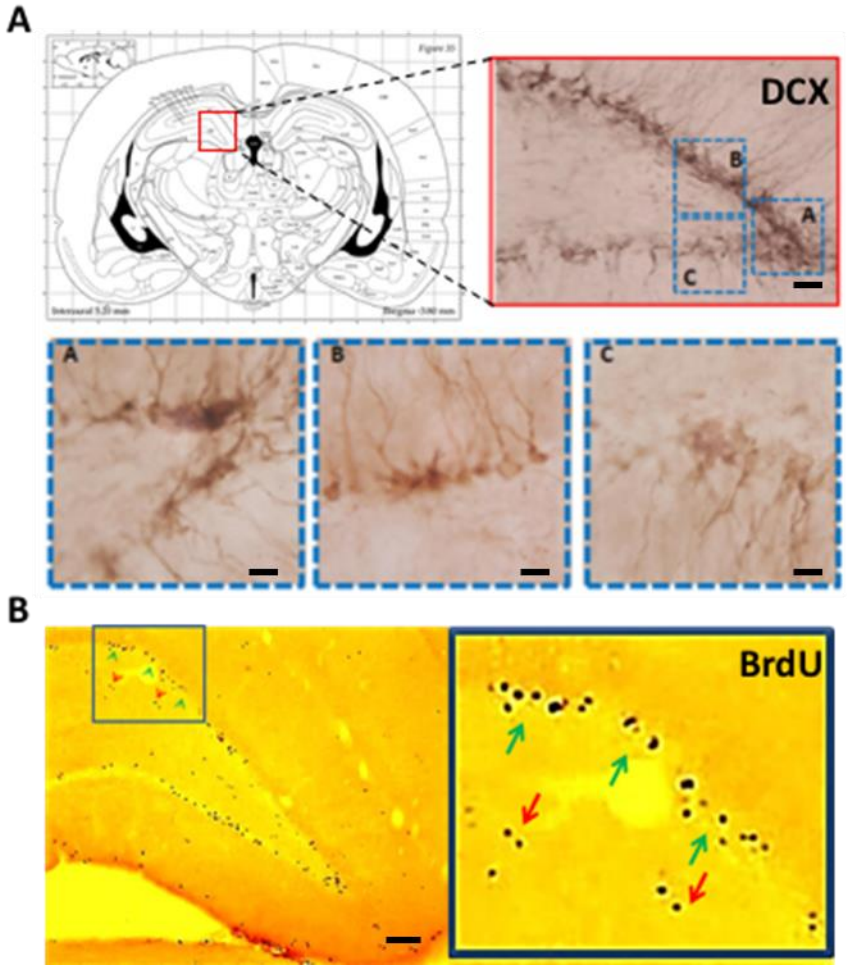

Figure 2.1 Methodological depiction of cell counts. All cell counts were done in the sub-granular zone (SGZ) of the dentate gyrus (DG) of the hippocampus (Hpc). (A) The blue boxes (top right micrograph) delineate the areas chosen for DCX counting (bottom row captured at 100x). Scale

bars = 15um and 150nm. (B) Micrographs were captured at 10x magnification (Left micrograph) and only BrdU+ cells immediately under the SGZ were counted (green arrows) while all staining outside of this area was excluded from analysis (red arrows). Scale bars = 15 um

Experiment II Does single-prolonged stress affect adult hippocampal neurogenesis?

SPS procedures were performed as previously described [27]. Briefly, animals in the SPS group were exposed to restraint for 2 h, followed immediately by 20 min of forced swim in a plastic tub (55.6-cm diameter, 45.4-cm height) filled two-thirds from the bottom with water (20–24°C). Fifteen minutes after the forced swim, rats were exposed to ether vapors in a glass desiccator until fully anesthetized displaying no toe or tail pinch response (5 min of ether exposure). Immediately after the induction of general anesthesia, rats were removed from the desiccator, housed singly, and left undisturbed for 7 days. Rats assigned to the control group were housed singly, left undisturbed and remained in the housing colony until euthanized along with SPS animals after 7 days undisturbed post SPS. their brains immediately flash frozen and later processed for RNA-isolation and real-time quantitative PCR (RT-qPCR). Briefly, brains were cut into 500um sections and ventral and dorsal hippocampi were identified using anatomical landmarks, dissected on both hemispheres and dorsal and ventral Hpc were processed separately (biological duplicates): homogenized, RNA was extracted and transcribed into cDNA using SuperScript III (Invitrogen) and random hexamer primers. cDNA was thereafter amplified on a Roche 96 light cycler using SYBR Green PCR Master Mix kits (Roche) and analyzed using the accompanying software (LightCycler 96 SW 1.1). Primer pairs were designed using Primer Blast. Doublecortin: Fwrd 5'- CAG TCA GCT CTC AAC ACC TAA G-3' REV 5'- CAT CTT TCA CAT GGA ATC

GCC-3'. Data were normalized by housekeeping transcript hypoxanthine-guanine phosphoribosyltransferase (Hprt): Fwrd 5'- GCG AAA GTG GAA AAG CCA AGT- 3' REV 5'- GCC ACA TCA ACA GGA CTC TTG TAG -3'

To verify that the decrease in DCX RNA expression was due to a decrease in the number of immature neurons and not due to an independent decrease in DCX RNA expression, this experiment was replicated changing how the animals' brains were processed; brains were fixed by cardiac perfusion of 4%paraformaldehyde, sectioned and processed for immunohistochemistry for DCX. The micrographs were analyzed and the number of immature neurons quantified as described in Experiment I.

Experiment III Does single-prolonged stress affect proliferation and/or survival of adult-born hippocampal neurons?

To test SPS's effect on proliferation, rats were randomly assigned to SPS or control (n = 12/group). After exposure to SPS or control condition as described in Experiment II, rats were all injected with 50mg/Kg of the synthetic nucleotide 5-bromo-2'-deoxyuridine (BrdU; Sigma Aldrich) intraperitoneally (IP) for seven days starting on day 9. All rats were euthanized on day 17 (Figure 2A). To assess SPS's effects on cell survival, all rats were injected with BrdU twice per day for four days then randomly assigned to SPS or control. 23 days after the first BrdU injection (Day 24) rats were exposed to SPS or the control condition and then euthanized on day 35 (Figure 3A). For both proliferation and survival, all brains were 4% PFA-fixed through cardiac perfusion and later processed for IHC, as described in Experiment 1, and probed for BrdU. For BrdU staining sections were rinsed in TBS, denatured in 2N HCl at 37°C for 30 min, neutralized in 0.1M boric acid for 10

min, rinsed, blocked with 10% normal goat serum (Invitrogen, Carlsbad, CA), and incubated overnight at 4°C with anti-BrdU antibody (1:1000 dilution; mouse monoclonal, Roche Applied Science, Indianapolis, IN). The next day, sections were rinsed in TBS then incubated for two hours in secondary antibody (1:200 biotinylated goat anti-mouse IgG, Vector Labs) and processed as with DCX IHC. The micrographs were captured at 10x magnification. The number of BrdU+ cells in the sub-granular layer (SGZ) of the DG was quantified as described above.

Experiment IV Does promoting proliferation of adult-born hippocampal cells through voluntary exercise rescue single-prolonged stress-induced extinction retention deficits?

After two days of acclimation to the locked wheel (14.0in or 35.6 cm diameter), half the wheels were unlocked and revolutions recorded using a bicycle speedometer (Wolfride Co., China) from day 3 to 42. Half of those (quarter overall) and another half of the group with the locked wheel were assigned to either SPS or Control on day 31 resulting in four experimental groups (2x2 design: Control, Control-VE, SPS and SPS-VE; n=8/group). SPS and control procedures were done as described in Experiment II. Fear learning took place day 43 through 45 as described in Experiment I. On day 46 all animals were euthanized, their brains harvested and prepared for BrdU IHC as described in Experiment III (see experimental design Figure 3.6A).

Experiment V Does promoting survival of adult-born hippocampal cells through environmental enrichment rescue single-prolonged stress-induced extinction retention deficits?

After acclimation, all rats were injected with BrdU twice per day for four days. 21 days after the last injection, the 32 rats were randomly assigned and underwent SPS or control procedure. On

day 35, half of each group was then randomly assigned to environmental enrichment (EE) or control, resulting in four experimental groups (two by two design with n = 8/group): Control, Control+EE, SPS, and SPS+EE. Rats in the EE conditions randomly received one of two versions of EE elements sequence (Table 1) in which EE elements were replaced every two days for a total of eight days. Control rats were left singly housed and undisturbed during this time. The day after the last EE exposure all rats went through fear learning as previously described (Figure 6A). After, all animals were euthanized, their brains fixed and processed for BrdU IHC as described above.

Statistical analysis: FC and ER were analyzed using repeated measures ANOVA for all experiments, and post hoc comparisons were done by creating four treatment groups by combining SPS and environmental enrichment (EE) exposure (e.g. Control-Control, Control-EE, SPS-Control, SPS-EE) for that experiment only. Additionally, a repeated measures ANCOVA with freezing scores for the last block from FE as a covariate was used to analyze SPS and EE effects on ER. Independent sample's t-Tests were used to compare effects of SPS where appropriate. The criterion for significance was set at $P<0.05$. Four rats that did not show a conditioned freezing response >30% at the start of a fear extinction session or exhibited freezing levels +/- 2 standard deviations from a group mean and were, therefore, excluded from final analyses. All data are represented as means +/- SEM.

Enrichment	Day 1	Day 3	Day 5	Day 7
Sequence A	Tunnel	PVC tube	Swing	PVC tube
	Jingle-weight	Jingle-ball	Hide-away	Rubber Bone
	Padding	jingle-ball	Chew-knot	Jingle-ball
Sequence B	Wooden cube	Swing	PVC tube	Tunnel
	Padding	Hide-away	hew-knot	Jingle-ball
	PVC tube	Rubber Bone	Chew-knot	jingle-ball

Table 2.1 Environmental enrichment element sequences. Details the order in which different enrichment component were introduced and replaced during the eight days.

Results

Hippocampal Irradiation mimics single prolonged stress-induced extinction retention deficit

Initially, to establish the functional link between effects of adult Hpc neurogenesis and the behavioral ER deficit independent of SPS exposure, we reduced Hpc cellular proliferation by x-ray irradiation and later tested for fear learning (FL) and processing deficits (Figure 2A). Irradiation resulted in a 70% average reduction of DG DCX+ immature neurons 28 days after the last irradiation treatment (independent sample's t-Test; $t_{(18)} = 15.17$, $p < 0.0001$; Figure 2C). This decrease in the number of neurons was accompanied by higher percentage of freezing during ER (Repeated measures ANOVA; $F_{(1, 18)} = 6.71$, $p = 0.019$), but not during FC or FE (Figure 2B), demonstrating significant reduction in extinction retention, with no effects on fear and extinction learning. These results mimic SPS's effects on FL, FE and ER reported by our group in earlier studies [10, 11]. Additionally, the number of Hpc immature neurons was negatively correlated to the percentage of time spent freezing during ER (Pearson's correlation; $r_{(19)} = -0.58$, $p = 0.009$; Figure 2D). In other words, greater decrease the adult Hpc neurogenesis was associated with greater deficit in the extinction retention - supporting the mediating role of decreased adult neurogenesis in the development of ER deficit.

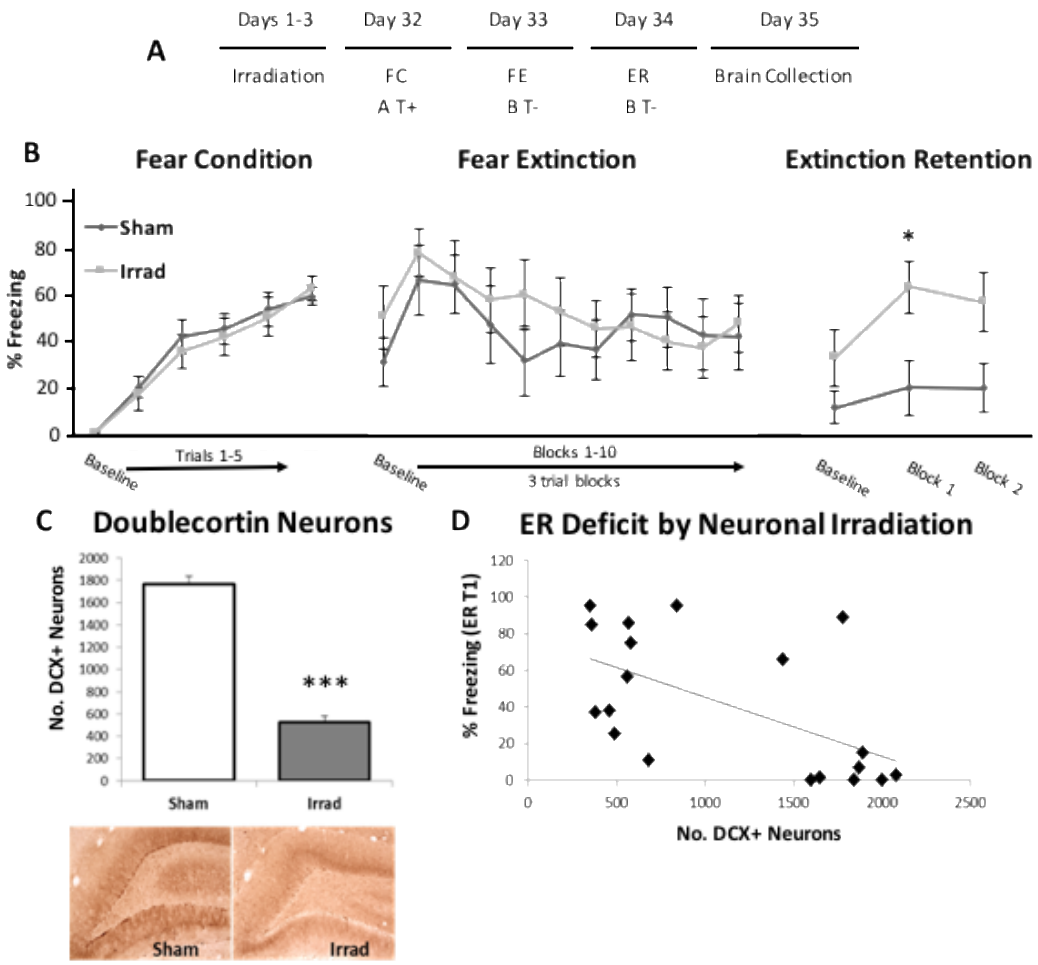

Figure 2.2. **Neuronal irradiation mimics SPS- induced extinction retention deficit.** (A) Illustration of the experimental design. (B) Averaged freezing percentages +/- SEM during FC, FE and ER are plotted in light (Irradiated) and dark (Sham) gray lines. Irradiated rats showed higher freezing percentage during ER ($T_{(17)}$= -2.73, p= 0.014), but not FC or FE. (C) Average plus SEM of total number of DCX+ DG neurons were graphed for Sham (white) and Irradiated (gray) rats with corresponding micrographs showing DCX expression in dark brown staining. Irradiation resulted in a significant reduction of DCX+ neurons ($t_{(18)}$ = 15.17, p< 0.0001). (D) Percent freezing during Block 1 of ER was negatively correlated to the number of immature neurons in the Hpc ($r_{(19)}$ = -0.58, p =0 .009).

Single prolonged stress exposure decreases the number of immature neurons in the dentate gyrus of adult rats

To explore whether SPS exposure influences adult Hpc neurogenesis, we first measured the relative DCX RNA expression within the Hpc of rats. We found that SPS resulted in an average 15% reduction in the relative (normalized to housekeeping gene HPRT) DCX RNA expression level as compared to Control group (independent sample's t-Test; $t_{(13)} = 2.91$, $p = 0.012$; Figure 3B). To verify that the decrease in DCX was due to a decrease in the number of immature neurons and not a decrease in RNA expression only, we replicated this experiment, substituting processing the brains for DCX immunohistochemistry instead of RT-qPCR. SPS exposure resulted in an average reduction in the number of DCX-expressing dentate gyrus cells (DGCs) of about 18% as compared to Control animals ($t_{(12)} = 2.7$, $p = 0.017$; Figure 3C). The decrease of immature neurons was found in both dorsal (d) and ventral (v) Hpc ($t_{(12)} = 2.26$, $p = 0.04$ and $t_{(12)} = 2.5$, $p = 0.025$, respectively; Figure 3C). This confirmed that the SPS decrease in DCX RNA was due to the decrease in the number of DCX-positive Hpc neurons.

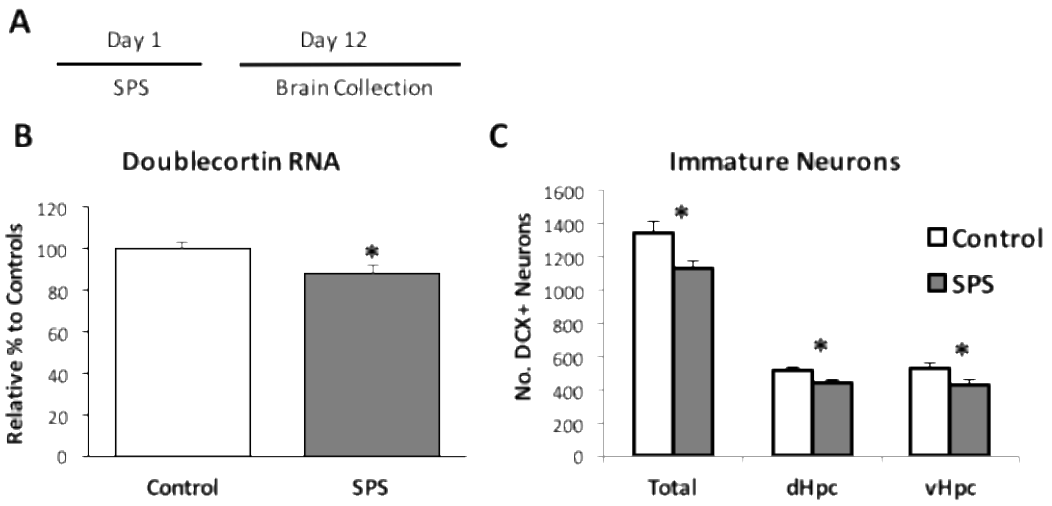

Figure 2.3 SPS decreases premature neurons in the dentate gyrus of adult rats. All bars represent the group average + SEM. (A) Illustration of the experimental design. In all panels: SPS (Grey) are compared to Controls (White). (B) Shows the relative percentage to Controls: where SPS had 85% of Control's DCX RNA expression ($t_{(13)} = 2.91$, $p = 0.012$). (C) In a different set of rats, the number of DCX expressing cells was counted. SPS significantly decrease the number of DG DCX+ neurons ($t_{(12)} = 2.7$, $p = 0.017$). The decrease was also seen in the dorsal and ventral Hpc ($t_{(12)} = 2.26$, $p = 0.04$ and $t_{(12)} = 2.5$, $p = 0.025$, respectively).

Single prolonged stress exposure decreases cellular proliferation in the sub-granular zone of the dentate gyrus of adult rats

To test if SPS affects adult hippocampal cellular proliferation, we exposed rats to SPS prior to BrdU injections (Figure 4A). We found that rats exposed to SPS had statistically significantly fewer total number of BrdU+ DGCs compared to Control animals (independent sample's t-Test; $t_{(21)}$ = 2.43, p = 0.024; Figure 4B). Furthermore, SPS resulted in fewer BrdU+ DGCs in the ventral (independent sample's t-Test; $t_{(21)}$ = 2.26, p= 0.035), but not dorsal Hpc compared to controls (Figure 4B).

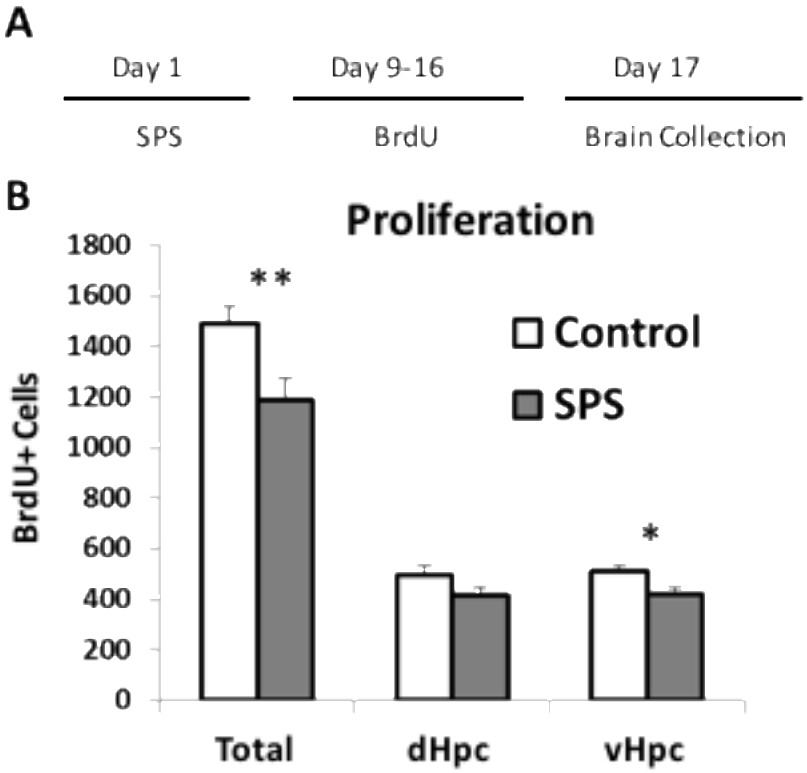

Figure 2.4. **SPS decreases proliferation of cells in the dentate gyrus of adult rats.** (A) Illustration of the experimental design. (B) Number of BrdU+ DGCs were counted and further divided into dorsal and ventral areas of the Hpc. The group's average + SEM are graphed here; SPS (Grey) are

compared to Controls (White). SPS reduced the number of BrdU+ cells in the Hpc and vHpc [($t_{(21)}$ = 2.43, p = 0.024) and ($t_{(21)}$ = 2.26, p= 0.035), respectively], but not the dHpc (P > 0.05).

Single prolonged stress exposure decreases survival of cells in the sub-granular zone of the dentate gyrus of adult rats

We found that rats that underwent SPS 21 days after the last BrdU injection had statistically significantly fewer total BrdU-stained DGCs as compared to non-exposed controls (independent sample's t-Test; $t_{(22)}$ = 2.72, p = 0.013). SPS rats also showed fewer BrdU positive cells in DG in vHpc, and a trending decrease in the dHpc compared to controls (independent sample's t-Test; $t_{(22)}$ = 2.53, p= 0.019 and $t_{(22)}$ = 1.76, p= 0.092, respectively; Figure 5B).

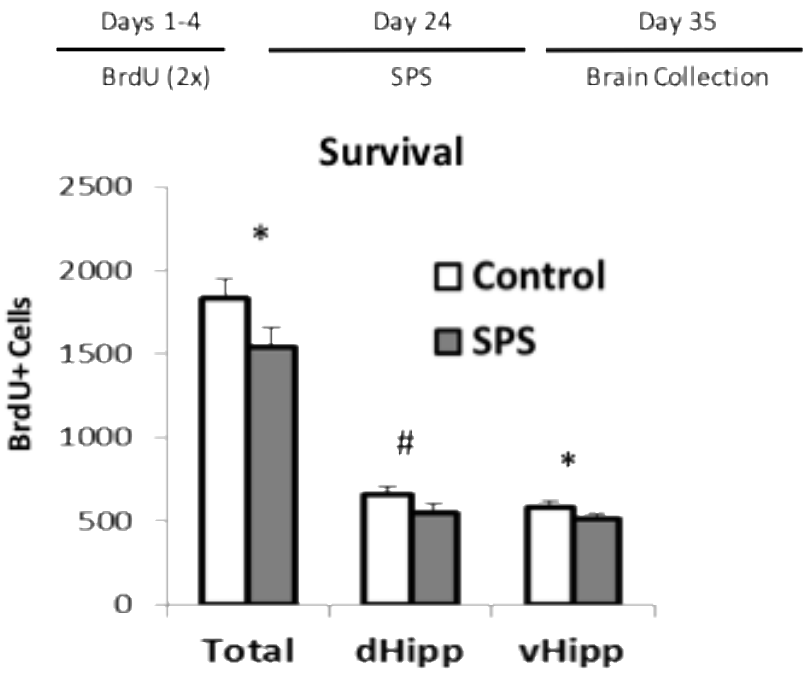

Figure 2.5. **SPS decreases survival of adult-born dentate gyrus cells of adult rats.** (A) Illustration of the experimental design. The group's average + SEM are graphed here; SPS (Grey) are compared to Controls (White). (B) Number of BrdU+ DGCs were counted and further divided into

dorsal and ventral areas of the Hpc. SPS reduced the number of BrdU+ cells in the Hpc and vHpc [($t_{(22)}$ = 2.72, p = 0.013) and ($t_{(22)}$ = 2.53, p= 0.019) respectively], and a trend level reduction in the dHpc (#0.1>P>0.05).

Voluntary exercise had no effects on neurogenesis or fear learning

In concert with previous findings, SPS had a significant effect on total and dHipp DGCs [ANOVA; F(1,29) = 11.41, P= 0.002; F(1,29) = 20.67, P= 0.00001]. Voluntary exercise had a trending effect on vHipp DGCs (ANOVA; F(1,28) = 3.26, P= 0.083). There was no interaction effect of SPS and VE (P> 0.05; Figure 3.6C). There were no effects of VE or SPS on fear learning (P> 0.05; Figure 3.6B). There was no difference in the amount of voluntary exercise between the two VE groups (P> 0.05; Figure 3.6D). Lastly, there was no difference in their body weights (P> 0.05; Figure 3.6E).

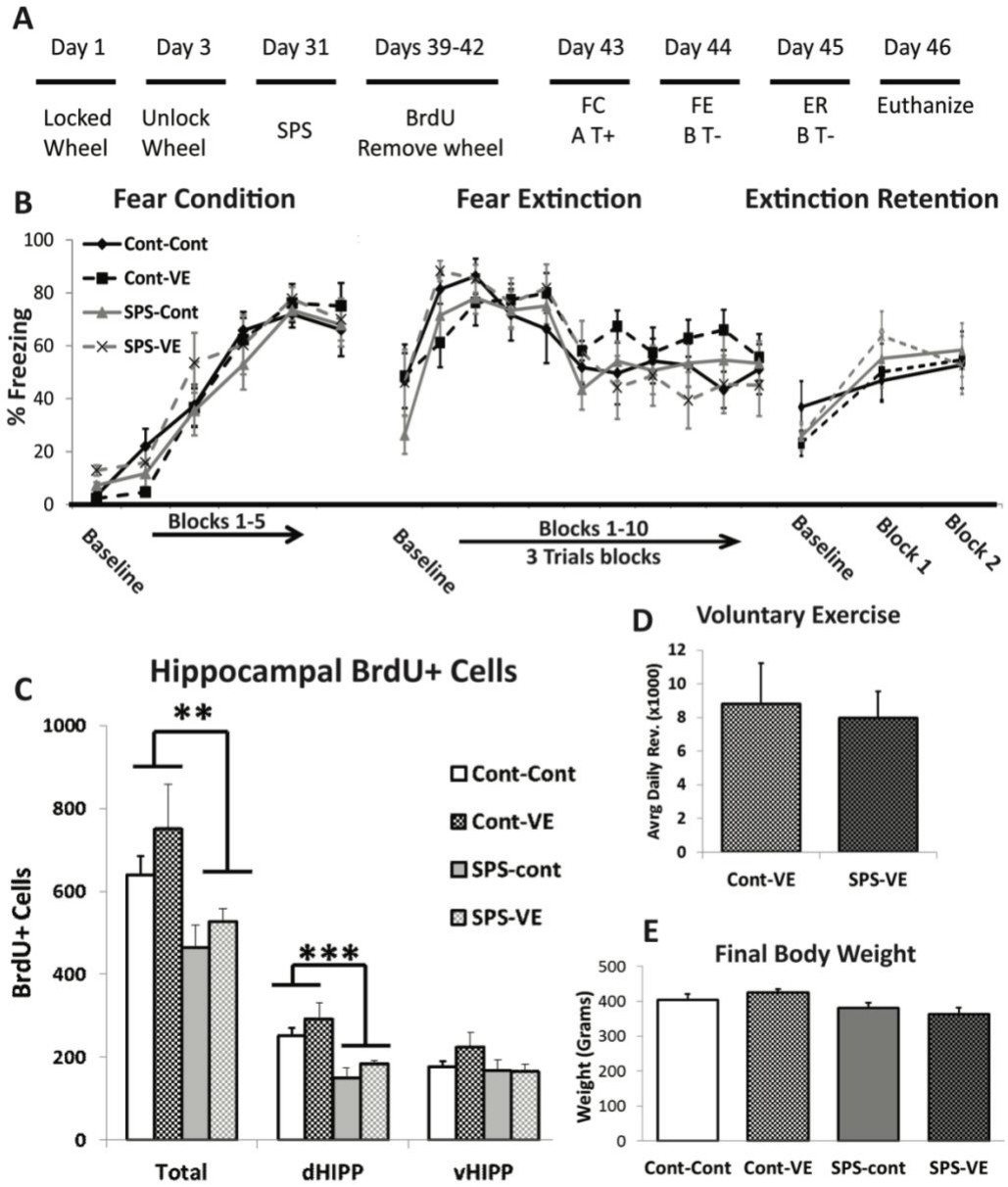

Figure 2.6 Voluntary exercise had no effects on any measures. (A) Illustration of the experimental design. (B) Averaged freezing percentages +/- SEM during FC, FE and ER are plotted for Controls (solid black), VE (dashed black), SPS (solid gray), and SPS plus VE (dashed gray). There were no statistically significant effects of SPS, VE or their combination. (C) Bar graph of average number of BrdU+ DGCs plus SEM are plotted for Controls (white), SPS (Gray), VE (checkered white), and SPS + VE (checkered gray). SPS had a significant effect on total and dHipp DGCs

[F(1,29) = 11.41, P= 0.002; F(1,29) = 20.67, P= 0.00001]. Voluntary exercise had a trending effect on vHipp DGCs (F(1,28) = 3.26, P= 0.083). There was no interaction effect of SPS and VE (P> 0.05). There were no statistically significant effects (P> 0.05). (D) Bar graph of average number of total daily revolutions. (E) Bar graph of average body weight per group. There were no statistically significant effects (P> 0.05).

Environmental enrichment promotes cell survival and reverses single prolonged stress-induced extinction retention deficits

In concert with previous findings, SPS exposure significantly decreased the total number of BrdU+ DGCs (ANOVA; $F_{(1, 23)} = 10.04$, $p = 0.004$). Furthermore, SPS reduced BrdU+ DGCs in vHpc and had a trend-level reduction in dHpc (ANOVA; $F_{(1, 23)} = 5.13$, $p = 0.033$ and $F_{(1, 23)} = 3.74$, $p = 0.066$, respectively). Environmental enrichment rescued these effects; there were statistically significant SPS by EE interaction effects on the number of BrdU+ DGCs in Hpc (ANOVA; $F_{(1, 27)} = 18.87$, $p < 0.0001$), dHpc ($F_{(1, 27)} = 19.80$, $p < 0.0001$), and vHpc (ANOVA; $F_{(1, 27)} = 6.47$, $p = 0.018$). There was no main effect of EE alone (Figure 6C). Behaviorally, there was no main effect of SPS or EE during fear conditioning (P>0.05, Figure 2.7B). Test of between-subjects effects revealed an interaction effect of SPS by EE during fear extinction (repeated measures ANOVA; $F_{(1, 27)} = 6.20$, $p = 0.022$), but no main effect of SPS or EE alone ($p > 0.05$). Post-hoc analysis among the four resulting groups revealed no significant differences between any of the groups compared (P>0.05). During ER, multivariate test in ANCOVA with the last FE block as a covariate revealed main effect of group (SPS) and main effect of EE ($F_{(1, 27)} = 4.58$, $p = 0.043$ and $F_{(1, 27)} = 7.19$, $p = 0.013$, respectively), while between-subjects effects analysis revealed a statistically significant

contribution from the covariate ($F_{(1, 27)}$ = 4.8, p = 0.038), main effect of EE and interaction effect of group by EE ($F_{(1, 27)}$ = 5.32, p = 0.03 and $F_{(1, 27)}$ = 5.65, p = 0.026, respectively), but no main effect of group alone (P>0.05). Repeated measures ANOVA analysis among the four resulting groups revealed statistically significant differences among the groups ($F_{(3, 27)}$ = 6.16, p = 0.003). Post-hoc Benferroni analysis revealed significant differences between Control-Control vs. SPS-Control (P = 0.008) and between SPS-Control vs. SPS-EE (P = 0.006). In this experiment, the number of BrdU positive cells in the DG was also negatively correlated to the percentage of time spent freezing during Block 1 of ER ($r_{(27)}$ = -0.634, p =0 .001; Figure 6D).

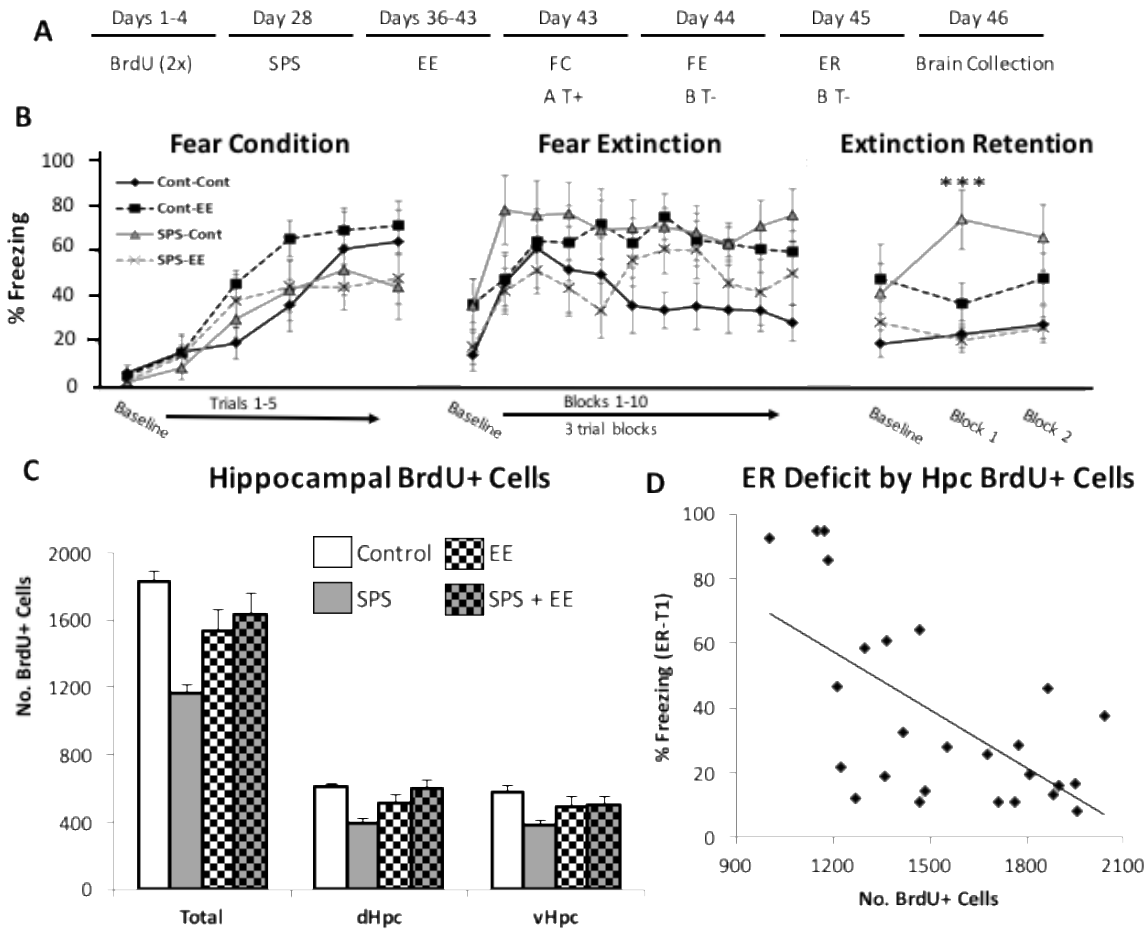

Figure 2.7 Environmental enrichment restored single prolonged stress reduction of adult-born dentate gyrus cells and rescued single prolonged stress -induced extinction retention deficit. (A) Illustration of the experimental design. (B) Averaged freezing percentages +/- SEM during FC, FE and ER are plotted for Controls (solid black), EE (dashed black), SPS (solid gray), and SPS plus EE (dashed gray). There was a statistically significant effect of SPS and an interaction effect of SPS by EE during FE [($F_{(1, 27)}$ = 7.22, p= 0.013) and ($F_{(1, 27)}$ = 10.0, p= 0.005), respectively], but not EE alone (P>0.05). There were statistically significant effects of SPS ($F_{(1, 27)}$ = 4.99, p= 0.037), EE ($F_{(1, 27)}$ = 5.00, p= 0.036) and interaction effect of the two ($F_{(1, 27)}$ = 19.59, p< 0.0001) during ER. These effects were no seen during FC (P>0.05). (C) Bar graph of average number of BrdU+ DGCs plus SEM are plotted for Controls (white), SPS (Gray), EE (checkered white), and SPS + EE (checkered gray). SPS had main effects on the number of BrdU+ DGCs ($F_{(1, 27)}$ = 10.04, p= 0.004) and vHpc ($F_{(1, 27)}$ = 5.13, p< 0.033), and a trending effect on dHpc ($F_{(1, 27)}$ = 3.74, p= 0.06). There were SPS by EE interaction effects on the number of BrdU+ DGCs ($F_{(1, 27)}$ = 18.87, p< 0.0001), dHpc ($F_{(1, 27)}$ = 19.801, p< 0.0001), and vHpc ($F_{(1, 27)}$ = 6.47, p= 0.018). (D) Percent freezing during Block 1 of ER was negatively correlated to the total number of BrdU+ DGCs ($r_{(27)}$ = -0.634, p =0 .001).

Discussion

Deficient contextual processing (CP) is a relatively new theoretical model seeking to link PTSD symptoms to underlying neurobiology [2]. Extinction recall (ER) is a context-dependent memory process that requires intact connections between medial pre-frontal cortex (mPFC) and hippocampus (Hpc) [4]. PTSD patients exhibit ER deficits accompanied by Hpc abnormalities in size and function [4-6]. Single prolonged stress (SPS) - a validated rodent model of PTSD mimics

key features of PTSD including extinction recall deficit [10, 11]. However, the exact molecular substrate of this deficit is not known. In a set of experiments utilizing adult Sprague Dawley rats, we have demonstrated that: 1) irradiation-induced decrease in neurogenesis correlates with ER deficits, while leaving intact fear learning and extinction learning capacity (similar to what we found in SPS model),(see Experiment I); 2) as hypothesized , animals exposed to SPS paradigm, (previously characterized to show deficient contextual processing and diminished ER [10]) demonstrate decreased hippocampal cellular proliferation, maturation and survival (cell loss shown in the Experiments II and III); 3) A behavioral intervention EE known to promote neuronal survival [25] rescued SPS-induced extinction retention deficits and the number of surviving young cells was inversely correlated with ER deficits. Taken altogether, our findings suggest that decreased adult hippocampal neurogenesis could mediate the effect of single prolonged stress exposure on extinction retention. These results thus implicate decreased neurogenesis as a potential mechanism underlying the extinction retention deficit seen in PTSD [6], and perhaps as a possible mechanism for the more general deficit in contextual processing that may play a critical role in the pathophysiology of the disorder [2]. Interventions aimed at enhancing survival of adult-born hippocampal neurons should be explored as a way to combat PTSD fear processing deficits.

Other psychiatric disorders, often comorbid with PTSD (e.g. depression), implicate decreased adult hippocampal neurogenesis [28], though the developmental stage of these targeted neuronal populations is not yet known. However, different psychiatric drugs and treatments have been shown to affect neurogenesis by promoting different stages of the cell cycle. For example, antidepressants promote increased neurogenesis by targeting all three

stages, while antipsychotics promote maturation and survival, but do not increase proliferation [28]. It is possible that neurogenesis is affected through unique mechanism depending on the disorder. In other words, depression may affect all stages while disorders involving psychosis may affect neurogenesis at the maturation and survival stages. In PTSD, sufferers often exhibit smaller Hpc volume [5]. If adult neurogenesis is part of the underlying etiology, it would be difficult to determine if decreased volume is attributed to decreased cell division (proliferation) or cell death (targeting maturation and cell survival). However, in this study, extinction retention deficit could only be explained by a decrease in neuronal survival.

Even though SPS exposure affected all stages of neurogenesis tested here - proliferation (Experiment III), maturation (Experiment II), and survival (Experiment III), SPS-induced behavioral deficits that manifest within 2 weeks of SPS exposure [10, 11]- could likely be explained by SPS's effects on the last stages of maturation and survival. Interestingly, this maturing neuronal population has unique properties compared to long matured DG neurons, especially during the last stages of maturation (4-6wks old). The effects of radiation-decreased Hpc neurogenesis in ER-deficits were observed around four weeks post treatment (Experiment I)- a time when these immature neurons would have been most active. In parallel, environmental enrichment (EE)'s rescuing effects on cell survival and ER deficits also occurred within this timeline (Experiment IV). During this developmental time, these young neurons are more prone to long-term potentiation (LTP), likely due to their higher probability to fire [29]. As they mature, they become more strongly inhibited resulting in their mature characteristic of low firing rate [30]. It is these unique properties that are theorized to give rise to two key Hpc cognitive functions: pattern completion (PC) and pattern separation (PS), which might be critical for contextual processing. Activation of

Hpc young neurons results in greater inhibition of mature granule neurons making it less probable for mature neurons to fire [31]. It is the mature granule neurons' very selective firing that upon partial activation of a neuronal ensemble, a memory can be quickly recalled (i.e. pattern completion). Therefore, a decrease in adult neurogenesis could result in lower inhibition and higher probably of firing in mature DG neurons leading to inappropriate pattern completion. On the other hand, higher firing probability of immature DG neurons leads to greater diversity of neuronal ensemble activation resulting in unambiguous encoding of similar yet novel cues presentations (i.e. pattern separation) [24]. A decrease in adult Hpc neurogenesis would also affect PS by limiting the diversity of neuronal encoding and possibly resulting in generalizations. Given the key role of adult Hpc neurogenesis in pattern separation and pattern completion and their key role in disambiguating similar contexts, the SPS-induced ER might result from decreased hippocampal neurogenesis.

Most of the reduction in the number of BrdU positive dentate gyrus cells (BrdU+ DGCs) was contributed by reduction in ventral and sometimes dorsal Hpc. In animals studies, the vHpc has been implicated in regulating anxiety-like behaviors; vHpc lesions decreased avoidance of open arms in elevated plus maze [32] and decreased freezing during exposure to predator odor [33]. Interestingly, neurons born within both location of the dorsoventral axis of the dentate gyrus have been shown to be functionally heterogenous. Specifically, adult-born cells within the dHpc have been shown to be important for discriminating contexts while vHpc were shown to be necessary for the ameliorative effects of fluoxetine [34]. However, appropriate responding, especially during challenging situations, requires both areas to act in concert.

Surprisingly, voluntary exercise failed to increase cellular proliferation or rescue SPS-induced reduction in the number of BrdU+ DGCs. Additionally, SPS did not result in fear learning deficits at any of the stages tested. Elsewhere, VE has been shown to increase proliferation of adult-born DGCs after as few as 12 days of access to the running wheel [35]. Additionally, the amount of daily exercise (average of 3.2 km/day) of our animals was comparable to other publications reporting high cap at 4 km/day [25]. Though rescuing of behavioral effects was not expected since these neurons would have been too young to contribute to Hpc function [29], we expected SPS to result in ER deficit as previously reported [10, 11]. However, it is possible that the presence of the locked running wheel in this experiments, which was supposed to serve as a control, may have actually served as EE and, therefore, prevented SPS effects on extinction retention [25].

In the EE experiment, during fear extinction, SPS-Control (i.e. SPS alone) animals appear to differ from other groups (Figure 2.7B) and that perhaps this could account for the elevated freezing during extinction retention test the following day. However, upon statistical scrutiny, repeated measures ANOVA during FE only revealed an interaction effect of SPS by EE, but no main effects of either intervention alone. Furthermore, the four resulting groups (e.g. Control-Control, Control-EE, SPS-Control and SPS-EE) were analyzed using repeated measures ANOVA, revealing no effect of group and no statistically significant difference between the pair-wise comparisons among the four groups in post-hoc tests. More importantly, when freezing percentage during the last FE block was used as a covariate to account for effects during ER, this measure could only partially account for some, but not all of the statistically significant differences. Furthermore, post-hoc Benferroni analysis of the four groups revealed statistically

significant differences when SPS alone was compared to controls and SPS that underwent EE (i.e. SPS-control vs. control-control and SPS-control vs. SPS-EE). These results support the conclusion that there was a SPS-induced ER deficit, which was rescued by EE exposure.

Taken all together, this body of evidence suggests a role for adult hippocampal neurogenesis in mediating extinction retention deficits in single-prolonged stress model of PTSD. Because of the key function of Hpc neurogenesis in pattern completion and pattern separation, it is likely that ER deficit is due to deficient contextual processing. Although this evidence contributes to understanding of the disorder and could eventually lead to better treatment, there is still much work to be done. For example, anti-depressants-- whose ameliorative actions depend on increased adult hippocampal neurogenesis [36], do not always result in symptoms improvement for PTSD patients [37]. It is likely that increased neurogenesis without being accompanied by other interventions, such as behavioral and cognitive therapies, limits the drugs' efficacy. Understanding the exact role adult neurogenesis plays in psychiatric disorders like PTSD still necessitates further scientific investigation. Nevertheless, studies like this one bring the field closer to being able to identify better and more precise therapeutic targets for treating and perhaps eventually curing PTSD.

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CHAPTER 3

Effects of SPS on Expressions of Synapse-Related Genes

Abstract

Post-traumatic stress disorder (PTSD) is characterized by overexpression of fear originally thought to result from exaggerated fear learning or the excessive encoding of fear memories (i.e. trauma memory) [1]. However, emerging work has begun to focus on core deficits that also include extinction retention (safety learning) and contextual processing (fear modulation) abnormalities [2, 3]. We have replicated some of these abnormalities in a rodent model of PTSD – Single Prolonged Stress (SPS) [4], however the specific cellular/molecular mechanisms leading to these abnormalities in SPS (and PTSD) remain unexplored. In the previous Chapter, I examined the potential contribution of adult hippocampal neurogenesis to these abnormalities, and in this chapter, I explored an alternative mechanism – synaptogenesis and synaptic remodeling. Synapses are the cellular substrates of learning and memory and are, therefore, highly plastic. Specifically, learning and memory require gene expression of many molecules involved in the formation of new synapses or the modification of existing ones [5, 6]. This level of brain plasticity creates opportunities for synaptic modifications/disruptions, which could potentially contribute to altered memory aspects of PTSD. Of relevance, prolonged exposure to stress or stress hormones leads to synaptic atrophy [7]. Interestingly, depression – a disorder highly comorbid with PTSD that shares some of its symptomology – has been associated with marked decreases in dendritic arborization, synapse numbers and synaptic genes expression, suggesting synaptic

changes could be a possible etiological factor the two disorders might share [8]. In this preliminary study, I investigated whether the expression of various synaptic markers within relevant fear processing/regulatory brain regions could be altered by our rodent model of PTSD (SPS). I found that SPS exposure altered mRNA levels of several important synaptic molecules within each fear processing region assessed. Specifically, SPS tended to decrease expression of markers related to synaptic morphology while increasing those related to function/signaling. These results suggest that synaptic remodeling within fear processing brain regions could play a role in the emergence of PTSD-like symptoms and might represent a modifiable therapeutic target for treating this disorder. However, more focused and comprehensive research into these possible synaptic changes is needed.

Introduction

The brain's sustained ability to adapt to extreme challenges requires the capacity for neurobiological changes (i.e. plasticity) that might also carry a potential cost in susceptibility to develop disruptions in cognition and affect that could contribute to psychopathology. A key feature of brain plasticity is the formation of new synapses and the modification of previously established ones. These synaptic changes are key molecular components in learning and memory [9-11], and they involve long-lasting changes in the efficacy of synaptic connections between two neurons: strengthening of connections via long-term potentiation (LTP) and weakening via long-term depression (LTD) [12]. For example, synaptic changes associated with early phases of LTP involve activation of effector molecules which phosphorylate several targets, including existing

receptors, thereby increasing their activity, as well as modulating insertion of new ready-made receptors into functional circuits [12]. Transitioning between the early- to late-phase LTP seems to be facilitated by transcription of brain-derived neurotrophic factor (BDNF), likely involved in the promotion of synapse-related genes expression [5]. Unlike early-LTP, late-LTP induction and maintenance require gene transcription and protein synthesis [13, 14]. Specifically, late LTP induction involves activation of effector molecules like MAPKs [15-18] leading to signaling cascades involving CaMKII and PKC which, in turn, activate the ERK pathway resulting in protein synthesis and morphological synaptic changes [15, 16]. Although the identities of all the proteins and molecules synthesized during LTP are not known, they contribute to the increase in dendritic spine numbers, cellular membrane size and altered morphology, as well as changes in neurotransmitter sensitivity post-synaptically [17]. All of these are of relevance to cognitive, affective and behavioral adaptations and of potential relevance to psychopathology due to the central role synapses play in learning and memory.

Synaptic changes within fear processing/regulatory neurocircuitry, including the medial prefrontal cortex (mPFC), hippocampus (Hpc) and amygdala (Amyg), provide an intriguing potential mechanism by which stress exposure, possibly mediated by stress hormones, could alter their internal circuitry and how they communicate with one another. Exposure to either stress or stress hormones has, in fact, been shown to result in reduction of dendritic branching and the number of synapses within these regions. Specifically, acute corticosterone treatment of rats can produce dendritic atrophy in the mPFC and hypertrophy within the Amyg, and these morphological changes were paralleled by heightened anxiety-like behavior in an elevated plus maze [19]. Additionally, length of stress exposure has been shown to modulate spatiotemporal

patterns of spine formation within the Amyg [20]. Accordingly, at the cellular level, exposure to various stressor types can impede ensuing induction of LTP in the Hpc [21].

Exposure to stress and altered activity in stress response systems (e.g. altered hypothalamic-pituitary-adrenal – HPA axis) thus appear to be able to change synaptic function and produce anxiety-like behavior. This has important potential relevance to psychopathology, and particularly to PTSD, a disorder that involves stress exposure and distinctive changes in HPA axis activity and in learning/memory processes. Specifically, although hypercortisolemia is not a feature of PTSD, the disorder is characterized by distinct dysregulation of the HPA axis, thought to be rooted in the disproportionally heightened expression of low-affinity glucocorticoid receptor (GR) compared to high affinity mineralocorticoid receptor (MR) in areas like the Hpc [22, 23]. Along with this HPA axis dysregulation [24], our animal model of PTSD has also replicated a distinguishing behavioral feature of PTSD: an extinction retention (ER) deficit [4], that is dependent on glucocorticoid receptor changes. We do not yet know how these are connected. However, partial SPS that does not produce increased Hpc GR expression also does not lead to extinction retention deficits that are normally seen in SPS exposure [25]. It is possible that SPS exposure and ER deficit are linked through increased GR thereby inducing synaptic changes, even in the absence of elevated circulating stress hormones [26].

Furthermore, disruption of mnemonic processes and synaptic indices are seen in a variety of stress-associated disorders that share some core symptomology, and perhaps some pathophysiology, with PTSD. Postmortem studies of major depressive disorder (MDD), for example, have revealed reduction in the size and cortical thickness of dorsolateral PFC (dlPFC) [7]

and the Hpc [27]. At the cellular level, dlPFC size reduction is accompanied by a decrease in the size and density of neurons [7] and post-mortem studies document decreased expression of synaptic function-related genes within this region, as well as increased expression of a transcriptional repressor of key synaptic genes [8]. The fact that antidepressants are the most common pharmacological treatment for PTSD [28] supports potential relevance of these depression findings to PTSD's pathophysiology. A recent randomized clinical trial with ketamine – a fast acting antidepressant whose effects are thought to be mediated via synaptic changes [29] – resulted in significant and rapid reduction of PTSD symptom severity [30]. Fast-acting antidepressants like ketamine and scopolamine increase expression of the growth factor, BDNF, accompanied by increased number and function of spine synapses in the mPFC of rats [29]. Interestingly, a recent study suggested that a polymorphism in the BDNF gene might be a risk factor for PTSD [31]. Altogether, these data suggest that synaptic changes could be a shared pathophysiological feature of depression and PTSD [32].

The examination of synaptic structure and function in PTSD is just beginning and further research is clearly needed. Early studies using animal models of the disorder have revealed some support for the hypothesis that synaptic changes leading to circuit disruption could be an important mechanistic pathway to PTSD symptoms in response to stress/trauma exposure. Our SPS model provides some support in that disruption in mPFC-Hpc connectivity may be critical to production of the FR (fear renewal) and ER deficits seen in this model [3, 4, 25]. Others have shown that SPS can also reduce BDNF mRNA levels in the Hpc [33] and increase activation of key molecules involved in MAPK and the mTOR cellular pathways – important for neuroplasticity – in the mPFC and Amyg [34-36]. SPS has also led to enhanced hippocampal LTD one day after

exposure and impaired LTP in the Hpc and Amyg seven days after [37]. Though these data are encouraging, more direct evidence is needed to clearly demonstrate that PTSD-relevant animal models produce synaptic changes. The resulting data, in turn, will lay the necessary foundation for a mechanistic link of stress/trauma exposure to specific synaptic function alterations and the associated PTSD-like behavioral abnormalities.

As a first step in our investigation of the possible role that synaptic changes within fear processing brain regions might potentially play in mediating SPS-induced fear processing deficits [4, 25], I tested whether SPS could alter the expression of synapse-related genes in the mPFC, Hpc and Amyg using real-time quantitative PCR (RT-qPCR). The synaptic functional categories assessed here included: 1) regulation of synaptic vesicles (CALM2, SYN1, SYN3, RAB3A, and SNAP25) [38-43]; 2) markers of dendritic formation (RAB4B, MAP2, and GAP43) [44-47]; 3) membrane-associated guanylate kinases (MAGUKs) involved in receptor scaffolding (PSD-95, DLG2, and DLG3) [48-50]; and 4) subunits of major excitatory and inhibitory neurotransmitter: GABAa receptor subunits (GABRA2, GABRB2), and glutamatergic AMPA (GluR1) and NMDA receptors (GluN1 and GluN2). I also assessed expression of a key promoter of synaptic plasticity (BDNF) [51, 52].

Methods

Overall design: We compared synaptic markers in various brain regions of SPS exposed and control animals using real time quantitative polymerase chain reaction (RT-qPCR) to test the effects of SPS on the expression of synapse-related genes. More specifically, we measured whether molecules involved in synaptic plasticity, synaptic maintenance, neuronal arborization,

as well as those associated with growth are altered in the medial prefrontal cortex (mPFC), hippocampus (Hpc) and amygdalae (Amyg) – brain regions essential for fear and extinction learning – as a result of SPS exposure.

Animals: After acclimatization, 48 male Sprague Dawley rats were randomly assigned to the single-prolonged stress (SPS) or control groups (n = 24/group) and underwent their respective procedure as described in Chapter 2. Half the animals of each group were randomly assigned to fear learning (FL), resulting in four experimental groups: Control-Control, Control-FL, SPS-Control, and SPS-FL. FL consisted of fear conditioning on day 8, fear extinction and extinction recall the next two consecutive days. All rats were then euthanized the following day, their brains flash frozen and later processed for real-time quantitative PCR (RT-qPCR). Due to time constraints, only animals that did not undergo FL were assessed for gene expression changes. However, effects of SPS on FL are reported here. Assessment of relative gene expressions were done against the housekeeping gene, Hprt. Additionally, SPS-induced differential expressions are reported relative to controls (i.e. a percentage of expression level of the Control group, where Control average is reported as 100%).

Real time quantitative PCR (RT-qPCR): Briefly, 24 brains were cut into 500um sections and medial prefrontal cortex (mPFC: infra-limbic and pre-limbic combined), whole amygdalae, ventral and dorsal hippocampi were identified using anatomical landmarks and dissected out on both hemispheres and combined; results were not different between dorsal and ventral Hpc subregions (i.e. biological duplicates), therefore their values were combined (averaged). Using PureLink RNA Mini Kit (ThermoFisher Scientific, New York), the samples were homogenized in

Lysis buffer and 2-mercaptoethanol (1% of final volume), equal volume of 70% ethanol was added and mixed, and then the sample was transferred into spin cartridge with a collection tube, spun at 12kg for 15 seconds and washed three times in Wash Buffer I.. RNA was quickly stored at -20c until processed to make cDNA using 2uL SuperScript III (Invitrogen), 4uL 5x Vilo mix, varying amounts of nuclease free water and RNA sample for resulting 20ng of RNA in 14uL (20uL total volume). For RT- (no superscript) controls, 20ng sample RNA diluted in 14uL of water or 20uL total volume with RNA concentration of 1ng/1uL. The plates were then placed in the PCR machine for cDNA synthesis with the following program: 15min at 25c, 45min at 42c and 5min at 85c. For RT-qPCR, cDNA was thereafter amplified on a Roche 96 light cycler using SYBR Green PCR Master Mix kits (Roche) consisting of 2uL of nuclease-free water, 1uL of 2uM primer(see Table 3.1 for list of primers used), 5uL of cyber-green and 2uL of 1:10 diluted cDNA stock solution (1ng/10ul). Melt-curve and relative concentrations were analyzed using the accompanying software (LightCycler 96 SW 1.1). Primer pairs were designed using Primer Blast choosing those whose sequence expanded more than one exon and no introns with a product length between 90 and 200 nucleotides. Refer to Table 4.1 for the list of primer sequences used.

Gene	Primer Sequence		Gene	Primer Sequence	
Hprt	Frwd	5'- GCG AAA GTG GAA AAG CCA AGT- 3'	DLG2	Frwd	5'- CCT TAC CTC GGC TAA CTC ATG -3'
	Rev	5'- GCC ACA TCA ACA GGA CTC TTG TAG -3'		Rev	5'- TGA CAA TTA TAG GAG CAG GGC -3'
Syn1	Frwd	5'- ATG TGC CAC CAC CCA TCA TT -3'	DLG3	Frwd	5'- CCC AGC CTA TGC GTG AAT GG -3'
	Rev	5'- CAG AGA CTG GGA TTT CTT GAG C -3'		Rev	5'- GAT GCC ACC TGC GAT ACT GA -3'
Calm2	Frwd	5'- GTG GTT GTC TGT TCT GGT CTC -3'	GluR1	Frwd	5'- GGA CAA CTC AAG CGT CCA GA -3'
	Rev	5'- TTG TTA TTG TCC CAT CCC CG -3'		Rev	5'- CAC AGT AGC CCT CAT AGC GG -3'
Rab3A	Frwd	5'- GCA GGA CAA GAG CGG TAC -3'	GluN1	Frwd	5'- ACA CAG GAG CGG GTA AAC AA -3'
	Rev	5'- CAT GAG TAA GTT TTG ATC TGA GTG G -3'		Rev	5'- CCA CAA TCA TGT CCG CTT GG -3'
Rab4B	Frwd	5'- TGG AGT TTG GAT CCA GGG TTG -3'	GluN2	Frwd	5'- GCT TTC CTC GAA CCC TTC AGT -3'
	Rev	5'- CCG TGT CAC CGA CCG AAA C -3'		Rev	5'- GCA ATG GCT GAG ACG ATG AG -3'
Syn3	Frwd	5'- GGT CCT GAG AAG TTT CCA CTA G -3'	GABRA2	Frwd	5'- CTT CTG GCT GTT CAG CTT C -3'
	Rev	5'- GTC GTG CTG GTT TT C TAC TTT G -3'		Rev	5'- AGC AGC GGA AAC CAT ACG TT -3'
SNAP25	Frwd	5'- GAC TTT GGT TAT GTT GGA TGA GC -3'	GABRB2	Frwd	5'- CTG GAT GAA CAA AAC TGC ACG -3'
	Rev	5'- AGC CCG CAG AAT TTT CCT AG -3'		Rev	5'- ACA ATG GAG AAC TGA GGA AGC -3'
GAP-43	Frwd	5'- GCA GAA AAG AGG TGG AGA GG -3'	MAP2	Frwd	5'- GAG AAG GAG GCC CAA CAC AA -3'
	Rev	5'- TTG TTC AAT CTT TTG GTC CTC ATC -3'		Rev	5'- TCT TCG AGG CTT CTT CCA GTG -3'
PSD95	Frwd	5'- CCC CCA ACA TGG ACT GTC TC -3'	BDNF	Frwd	5'- AGG CAC TGG AAC TCG CAA TG- 3'
	Rev	5'- TGC AAC TCA TAT CCT GG GC -3'		Rev	5'- AAG GGC CCG AAC ATA CGA TT -3'

Table 3.1 List of primer sequences

Data analysis and statistics: Freezing behavior was manually scored by two observers blind to experimental conditions. All videos were first recorded and stored on a digital hard-drive, then copied to be viewed on the computer and scored using a stopwatch. Freezing was defined as the absence of movement, except that necessary for breathing, for >2 sec and quantified as a percentage of the total time recorded (i.e. Tone + ISI or 70sec). For fear extinction, freezing across three extinction trials were averaged into a block (baseline, blocks 1– 10 are graphed). For extinction retention/recall, freezing across five trials were averaged into a block (baseline, blocks 1 and 2 are graphed). FC, FE and ER were analyzed using repeated measures ANOVA. Effects of group (SPS) on gene expression were analyzed in three different ways: independent samples' T-test with and without Bonferroni correction (P = 0.05 divided by the number of

comparisons within a gene category), and repeated measures three factors (i.e. group, genes within a category and region) ANOVA.

Results

As in previous experiments, exposure to SPS resulted in extinction retention deficit ($F_{(1, 23)} = 3.92$, $p = 0.036$, Figure 3.1) while sparing fear conditioning and fear extinction ($P > 0.05$).

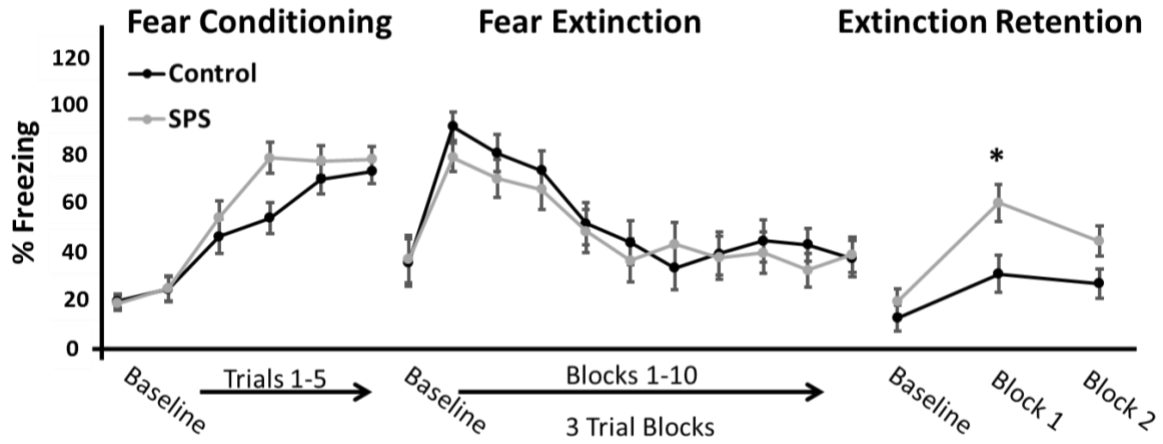

Figure 3.1 Fear Learning. Average +/- SEM percentage freezing for Controls (Black) and SPS (Gray) animals are plotted across three components of the behavioral paradigm. *P<0.05

Single-prolonged stress exposure leads to changes in synaptic markers mRNA levles in fear processing neural-network

In an effort to investigate a possible mechanistic link between SPS-induced extinction retention deficit and synaptic changes within contextual and fear processing brain regions, we first investigated changes in expression of genes with synaptic functions. In the medial prefrontal

cortex (mPFC), SPS exposure resulted in significantly decreased expression of the growth factor – BDNF ($T_{(20)}$ = 2.97, p = 0.008, Fig 3.1) and a trend-level decrease in arborization marker – MAP2 ($T_{(20)}$ = 1.88, p = 0.075, Fig 3.1), while increasing expression of vesicle regulator – Calm2 ($T_{(20)}$ = -2.25, p = 0.036, Fig 3.1). In the hippocampus (Hpc), SPS exposure resulted in statistically significantly decreased mRNA expression of two arborization markers – GAP43 and MAP2 ($T_{(22)}$ = 2.14, P = 0.044 and $T_{(21)}$ = 2.72, P = 0.013, respectively, Fig 3.1) while increasing expression of two vesicle regulators Syn1 ($T_{(19)}$ = -2.41, p = 0.027, Fig 3.1) and Calm2 ($T_{(21)}$ = -2.31, p = 0.031, Fig 3.1). In the amygdala, SPS resulted in opposing effects on expression of dendritic formation markers; SPS statistically significantly decreased expression of MAP2 ($T_{(18)}$ = 2.76, P = 0.013, Fig 3.1) while increasing Rab4B ($T_{(19)}$ = -2.70, P = 0.014, Fig 3.1). Additionally, SPS statistically significantly increased mRNA expression of receptor scaffolding protein (MAGUK) – DLG2 ($T_{(20)}$ = 3.70, P = 0.001, Fig 3.1). Similar to the two other regions assessed, in the Amyg, SPS significantly increased mRNA levels of vesicle regulator – Calm2 ($T_{(19)}$ = -2.68, P = 0.015, Fig 3.1). Interestingly, of the three regions assessed, SPS affected receptor expression only within the Amyg; SPS significantly increased expression of the GABAa receptor subunit B2 (GABRB2; $T_{(19)}$ = -3.83, P = 0.001, Fig 3.1) accompanied by a trend level decrease in AMPA receptor subunit A1 (GluR1; $T_{(18)}$ = 1.86, P = 0.079, Fig 3.1). After Bonferroni correction SPS effects on some of the gene expression remained statistically significant: 1) BDNF in the mPFC (P < 0.013); 2) MAP2 (P < 0.017) in the Hpc; 3) RAB4B, MAP2, DLG2 (P < 0.017), and GABRB2 (P < 0.01) expression within the Amyg. Repeated measures three factors ANOVA revealed statistically significant interaction effects of SPS by gene when looking at dendritic genes ($F_{(2,9)}$ = 9.16, P = 0.005), but no significant effects in any of the other gene categories (P>0.05).

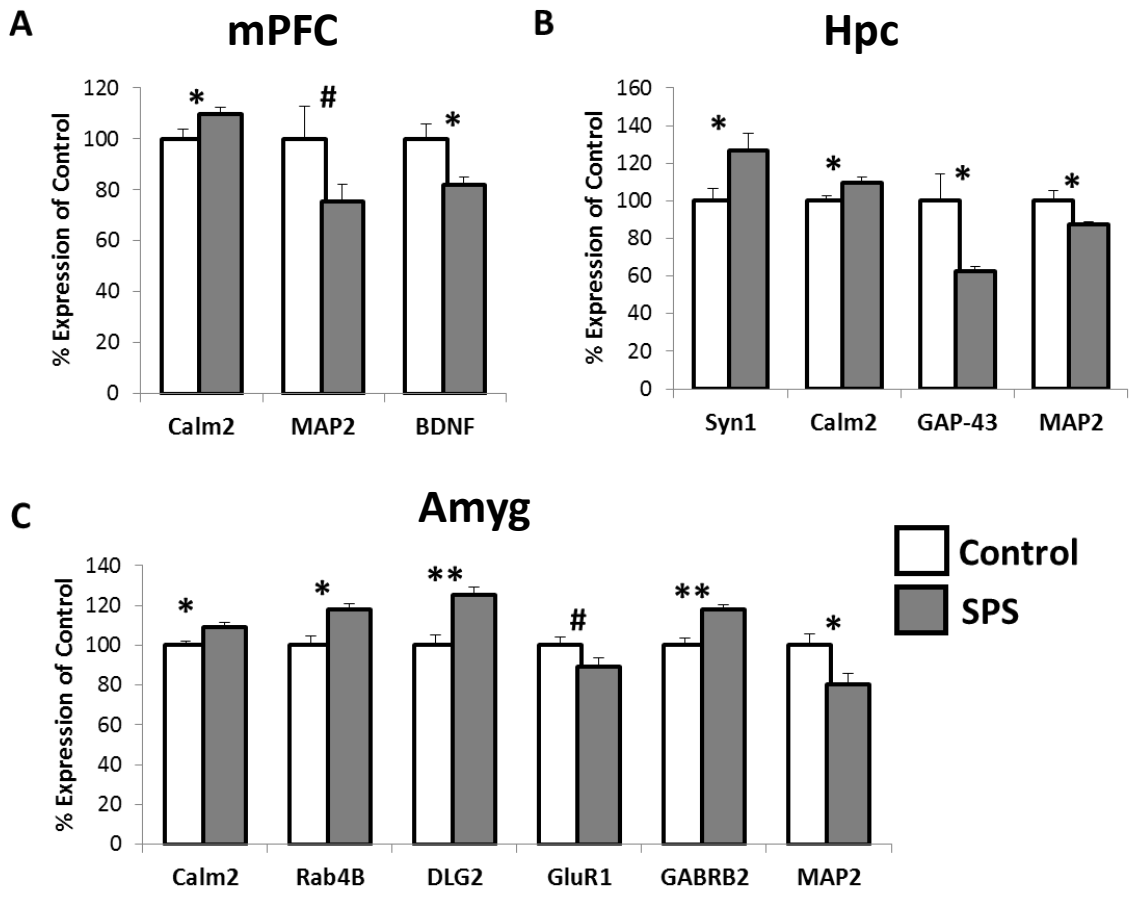

Figure 3.2 Bar graphs of average percentages relative to Controls plus standard error bars representing Controls (white) and SPS (gray). Here are only graphed the statistically significant differences of synaptic markers for medial-prefrontal context (A), hippocampus (B) and amygdalae (C). Independent samples t-Tests without Bonferroni correction, where *P<0.05 **P<0.01 and #0.1>P>0.05 (approaching significance).

		mPFC		Hpc		Amyg	
		% of Control	P- Value	% of Control	P- Value	% of Control	P- Value
Growth	BDNF	82 +/- 3	P = 0.008	107 +/- 3	P>0.05	93 +/- 6	P>0.05
Vesicle	Syn1	92 +/- 23	P>0.05	127 +/- 7	P = 0.027	94 +/- 19	P>0.05
	Calm2	109 +/- 2	P = 0.036	108 +/- 3	P = 0.031	109 +/- 2	P = 0.015
	Rab3A	111 +/- 5	P>0.05	95 +/- 4	P>0.05	99 +/- 3	P>0.05
	Syn3	108 +/- 19	P>0.05	100 +/- 7	P>0.05	108 +/- 6	P>0.05
	SNAP25	83 +/- 10	P>0.05	104 +/- 3	P>0.05	105 +/- 5	P>0.05
Dendritic Form.	Rab4B	110 +/- 4	P>0.05	104 +/- 3	P>0.05	118 +/- 3	P = 0.014
	GAP-43	102 +/- 4	P>0.05	96 +/- 3	P = 0.044	98 +/- 3	P>0.05
	MAP2	75 +/- 7	P = 0.075	60 +/- 5	P = 0.013	81 +/- 5	P = 0.013
MAGUK	PSD95	86 +/- 7	P>0.05	94 +/- 6	P>0.05	93 +/- 5	P>0.05
	DLG2	90 +/- 11	P>0.05	98 +/- 9	P>0.05	126 +/- 4	P = 0.001
	DLG3	102 +/- 7	P>0.05	109 +/- 8	P>0.05	112 +/- 8	P>0.05
Receptor S.U.	GluR1	103 +/- 3	P>0.05	96 +/- 4	P>0.05	89 +/- 4	P = 0.079
	GluN1	103 +/- 8	P>0.05	109 +/- 5	P>0.05	104 +/- 8	P>0.05
	GluN2	104 +/- 8	P>0.05	107 +/- 8	P>0.05	109 +/- 10	P>0.05
	GABRA2	94 +/-7	P>0.05	96 +/- 10	P>0.05	99 +/- 5	P>0.05
	GABRB2	100 +/- 7	P>0.05	103 +/- 4	P>0.05	118 +/- 2	P = 0.001

Table 3.2 Comprehensive list of P-values for all independent samples t-Tests comparing Controls and SPS for relative RNA expressions of the listed genes without Bonferroni correction. Statistically significant percentage expression increase listed in green and decrease in red.

Discussion

In this preliminary study, I investigated if SPS exposure produces changes in synaptic marker expression within fear processing brain regions thought to mediate SPS-induced ER

deficits [3, 4, 25]. Although SPS did not affect a large number of the synaptic markers assessed, there were some consistent changes across the brain regions examined; results suggests a tendency for SPS-exposure to reduce expression of markers related to synaptic growth/morphology while increasing synaptic markers related to function. For dendritic formation, SPS tended to decrease gene expression of MAP2 across all regions and GAP-43 in the Hpc and BDNF in the mPFC, with the exception of an increase of Rab4B within the Amyg. Of these, MAP2 is considered a key microtubule stabilizing protein [53]. Interestingly, SPS tended to increase expression of markers related to synaptic function and transmission: vesicle regulators, CALM2 (calmodulin 2) across all regions and Syn1 in the Hpc as well as a MAGUK (DLG2) within the Amyg. Pre-synaptically, calmodulin (e.g. CALM2) – a calcium-dependent effector protein [54]– has been shown to be an important component in synaptic neurotransmission and an effector in the establishment of LTP [49, 55]. Additionally, in the Amyg, SPS differentially changed expression of receptor subunits; SPS increased expression of GABAa receptor subunit GABRB2 and had a trend level decrease of the AMPA receptor subunit GluR1 suggesting a possible shift in input sensitivity from excitatory (glutamatergic) to inhibitory (GABAergic).

The amygdala is an essential fear processing brain structure with obvious significance to the study of PTSD [1, 56], and our experiment revealed the largest and most complex set of pre- and post- synaptic changes in this region, potentially reflecting its enhanced plasticity. Pre-synaptically, CALM2 (calmodulin 2) was the only molecule whose expression was increased by SPS exposure in the Amyg. Calmodulin dramatically accelerates replenishment of releasable vesicles pools [43]. Interestingly, at the same time MAP2 – a molecule that is so ubiquitous and essential to the structural integrity and formation of dendritic synapses that it is utilized as a way

to assess dendritic morphology [8, 45, 53] – was decreased within this region after SPS exposure. If MAP2 decrease, indeed, reflects decrease in synaptic integrity following SPS, the increase of CALM2 could represent compensatory increase in vesicle pools to maintain synaptic transmission. In the same region, expression of RAB4B, DLG2 and GABRB2 was increased by SPS exposure, while GluR1 expression was decreased. Post-synaptically, RAB4B regulates endosomal recycling required for spine maintenance and neurotransmitter receptor recycling [46], and in concert with increase in CALM2 pre-synaptically, increase in RAB4B might maintain synaptic transmission post-synaptically. It thus appears that SPS increased a number of synaptic markers associated with function while decreasing one related to structure. Related to excitatory/inhibitory neurotransmission, SPS exposure increased expression of inhibitory GABAa beta 2 sub-unit (GABRB2) -- involved in high affinity binding [57] -- and simultaneously decreased expression of excitatory GLuR1 (GluA1) in the amygdala. Interestingly, phosphorylation of this AMPA receptor subunit is important for LTP [38, 58, 59]. DLG2 (PSD-93) is an important MAGUK shown to mediate the insertion of a significant percentage of all synaptic AMPA receptors in the brain [48] and whose global deletion in mice results in learning impairments [60]. Altogether, these changes might suggest a potential shift toward enhanced inhibitory signaling after SPS exposure, which might also reflect a compensatory adaptation in SPS exposed animals.

mPFC and Hpc are key regions in the neurocircuitry that regulates fear expression [3] and are thought to be important for the psychopathology of PTSD and other stress related disorders [8, 29]. SPS exposure dampened mPFC BDNF expression. Interestingly, a BDNF polymorphism has

been linked to PTSD risk [31], and PTSD patients have lower levels of circulating peripheral plasma BDNF [61]. In rodents, infusing BDNF into the mPFC resulted in reduced fear expression after FC, even in the absence of extinction training. Additionally, animals with impaired extinction showed less mPFC BDNF expression, and infusing BDNF into mPFC prevented extinction failure [1]. The SPS-induced decrease in BDNF expression within the mPFC, thus has a potential relevance to the extinction retention deficit seen in PTSD, warranting further studies into BDNF-mediated synaptic plasticity. In the Hpc, SPS decreased two important components involved in transducing intra- and extracellular signals regulating cytoskeletal organization of axon terminals (GAP-43 and MAP2). Specifically, while GAP-43 overexpression leads to formation of new synapses and enhanced sprouting after injury, its null mutation disrupts axonal pathfinding and is generally lethal shortly after birth [45]. Thus, the SPS-related GAP-43 expression decrease observed in this study might be indicative of a reduction of Hpc synaptogenesis. By contrast, SPS-exposure increased expression of vesicle associated proteins (synapsin I and calmodulin 2). Taken all together, decreased expression of genes related to synapse morphology (GAP-43, MAP2 and BDNF) accompanied by increase expression of genes related to synaptic function/signaling (CALM2 and SYN1) within the mPFC and Hpc, similarly to amygdala, might suggest a possible functional compensation accompanied by a possible decrease in size or number of synapse. However, further research into synaptic morphological and functional changes is required to obtain more conclusive evidence to support this hypothesis.

This preliminary assessment of synaptic gene expression does suggest that there are possible synaptic changes in fear-relevant brain regions as consequence of exposure to SPS. Furthermore, decreased BDNF expression in the mPFC along with decreased expression of genes

shown to be essential to synaptic formation (GAP-43 and MAP2) within the Hpc are important indications that follow-up studies of SPS-exposed animals should include direct assessment of synapse numbers and synaptic morphology within these regions. Additional future studies will also need to include assessment of the influence SPS may have on the synaptic changes related to fear and extinction learning as well as extinction recall, separately. It is possible that SPS behavioral deficits may result from disruption of normal synaptic changes associated with each of these learning and behavioral processes. For instance, FC is associated with decreased inhibitory-excitatory (i.e. less inhibitory and more excitatory) connections between mPFC (PL) – Amyg (BLA) thereby promoting higher fear response when the conditioned stimulus is later encountered [62]. By contrast, FE is associated with increased disynaptic projections from mPFC (IL) to the inhibitory ITCs (intercalated cell masses in the Amyg) via the BLA [63]. However, these preliminary data hint at SPS possibly disrupting normal synaptic changes underlying each of these learning processes (FC and ER), leading to PTSD-like deficits. However, further work is worth pursuing to begin to establish a mechanistic link between SPS and PTSD-like deficits via disruption/alterations of synapses. Importantly, further research into these synaptic changes will contribute to possible development of interventions that promote synaptic formation and/or increased synaptic function. The prospect of which represents an exciting novel viable therapeutic target for treating PTSD [28, 30, 32, 64].

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CHAPTER 4

General Discussion and Future Directions

Result Summary and Discussion

An extensive body of work, including numerous studies emerging from the Liberzon laboratory at University of Michigan, has been reshaping research into the psychopathology of posttraumatic stress disorder (PTSD). Specifically, the focus has been shifting from fear learning and the amygdala (Amyg) to regulatory neural circuits that are critical in more complex processing of threat-related stimuli. The emerging circuits of central interest connect amygdala, prefrontal cortex, and hippocampus. This shifting neural focus has been shaped by growing awareness that replicable, fear-related processing deficits in PTSD primarily involve context dependent processes (e.g. extinction retention), leading to a context processing (CP) deficit model of PTSD [1]. This model highlights the central role of the Hpc and mPFC in this complex circuit as well as in PTSD, by focusing on their role in the contextualization processes. With the development of this model, and growing evidence of Hpc abnormalities in PTSD [1, 2], it becomes imperative to look at ways in which Hpc dysfunction could mechanistically contribute to PTSD biology and behavior. To this end, in Chapter II of this dissertation, I examined whether adult hippocampal neurogenesis might play a role in mediating the PTSD-like extinction retention deficit seen in the Liberzon lab's Single Prolonged Stress (SPS) animal model of PTSD. Additionally, the formation, strengthening and weakening of new synapses within brain circuits also play a role in learning and memory, and synaptic changes within the fear-related learning circuitry implicated in PTSD could provide an alternative or additional mechanism through which SPS produces PTSD-like biology or behavior. Therefore, in Chapter III, I preliminarily explored whether changes in the expression of genes involved in synaptic structure and function can be altered by

SPS exposure in order to determine whether they warranted further study as potential mechanisms in PTSD's pathophysiology.

In Chapter II, I demonstrated that: 1) irradiation-induced decreases in Hpc neurogenesis correlated with ER deficits, while leaving intact fear and extinction learning capacities; 2) animals exposed to SPS, which produces deficient contextual processing [1] and diminished ER [3, 4], show decreased hippocampal cellular proliferation, maturation and survival; 3) A behavioral intervention known to promote neuronal survival [5] rescued SPS-induced extinction retention deficits, and that higher numbers of surviving cells were associated with smaller ER deficits. Taken together, these findings support the hypothesis that reduced adult hippocampal neurogenesis could mediate the effect of single prolonged stress exposure on extinction retention. These results thus implicate neurogenesis as a potential mechanism underlying the extinction retention deficit seen in PTSD [6], and perhaps as a possible mechanism for the more general deficit in contextual processing that has been hypothesized to play a critical role in the pathophysiology of the disorder [1].

Though the collective evidence presented in Chapter II makes the fairly compelling case that neurogenesis may contribute to SPS-induced ER deficit, there are limitations to each experiment and follow-up work is, therefore, needed. To start, externally applied radiation to the head produces a global decrease in cellular proliferation, so reductions in cell populations other than neurons could mediate the ER-deficit observed. For one, glia cells have been shown to play different roles in brain functions related to learning and cognition [7]. Therefore, to circumvent the issues of specificity, repeating this experiment by directly targeting dentate gyrus' (DG) GFAP-

expressing progenitor neurons for elimination by targeting GFAP progenitor neurons using transgenic rats [8, 9] would provide more conclusive evidence into the role of adult hippocampal neurogenesis in possibly mediating ER deficits. Specifically, transgenic rats expressing the human herpes simplex virus thymidine kinase gene (HSV-TK, or TK rats) under the promotor for GFAP, thereby ensuring GFAP expressing cells are exclusively targeted for elimination with administration of antiviral drug valganciclovir – enzymatically converted to the active ganciclovir [9]. For this experiment, TK rats would be given valganciclovir for two weeks – this length of treatment has shown a 93% reduction in Hpc neuronal proliferation. After drug treatment to eliminate neurogenesis, they would be left undisturbed for four weeks and then assessed for extinction retention deficit.

Quantification of BrdU-containing cells within the sub-granular zone (SGZ) of the DG is not an exclusive measure of neurogenesis. Generally, GFAP-expressing progenitor cells of the SGZ can commit to either the neuronal or astrocytic cell-fate [10]. Because BrdU can incorporate into the DNA of any dividing cells, it is not possible to exclude other cell populations from being counted when BrdU is visualized alone [11]. For this reason, quantification of BrdU-containing cells does not provide the most accurate quantification of neuronal populations. However, in rats, most Hpc proliferating GFAP progenitor cells (over 70%) commit to the neuronal fate over glia [12, 13], making quantification of BrdU containing cells a reasonable estimation into the state of adult hippocampal neurogenesis. However, decreases in the number of astrocytes, their percentage of SGZ proliferating cells notwithstanding, could still mediate the assessed behavioral deficit. Therefore, as a future step, assessment into the role of adult hippocampal neurogenesis'

contribution to behavior should include BrdU co-localization with a mature neuronal marker like NeuN [14].

Behavioral interventions aimed at promoting neurogenesis, by themselves, do not provide conclusive evidence for a mediating role of neurogenesis. Here, I showed that environmental enrichment (EE) – shown to increase neurogenesis by promoting survival [15] – elevated the number of surviving SGZ BrdU+ cells and ameliorated the SPS-induced ER deficit. However, these results do not exclude other mechanisms by which EE could result in improved behavior. For example, EE could lead to ER deficit improvement through increases in synaptic plasticity and BDNF concentrations [16, 17]. Therefore, future studies should combine behavioral interventions with neurogenesis blockade (e.g. TK-GFAP rat). The resulting evidence would more conclusively support that it is neurogenesis and not another possible mechanism that mediates this behavioral improvement, and more strongly suggest a role of adult hippocampal neurogenesis in mediating SPS-induced ER deficits.

In Chapter III, I investigated the possibility that SPS could be linked to fewer synapses or decreased synaptic function within key brain regions in the CP neurocircuit: mPFC, Hpc and Amyg. In this exploratory experiment, I quantified expression of synaptic markers linked to synapse formation and function within these regions using real-time quantitative PCR. I found that SPS exposure altered mRNA levels of several important synaptic molecules within each fear processing region assessed. There were two markers that consistently changed across regions: the cytoskeleton stabilizing MAP2 [18] was decreased while CALM2 -- key effector molecule for neurotransmission [19, 20] -- was increased across regions. Of all the synaptic markers assessed

here, SPS exposure lead to the most marker expression changes in the Amyg. Interestingly, however, in all three regions, the expression of structural-related synaptic markers went down, while those involved in function and neurotransmission went up, hinting at a possible decrease in the number of synapses accompanied by a compensatory increase in function. However, the list of synaptic markers assessed here was qute small and only served to gauge whether we could find some evidence that further investigation into the role of synaptic changes resulting from SPS exposure is warranted. Though not extensive, the changes seen and the hint of a differential pattern regarding structure and function related markers, do support further investigation using synaptic markers and direct morphological and functional analysis of synapses within fear processing/regulatory brain regions.

Like most animal models used to study human disorders, SPS comes with limitations. PTSD can result from many different types of trauma, and no particular trauma always leads to the development of PTSD in those exposed to it. However, the primary focus in developing SPS was to maximally impact the HPA axis [21] in order to replicate PTSD's characteristic dysregulated/hyper-sensitive HPA axis function [22, 23], with the hope to then expand investigation into associated behavior and physiology. It has become a frequently used model because it does replicate the unique HPA biology of PTSD [21], and its utility has increased as it has been shown to also replicate context dependent learning abnormalities seen in PTSD (e.g., extinction retention and fear renewal abnormalities) [3, 4]. The confluence of neuroendocrine and behavioral abnormalities, and the fact that both involve mPFC-Hpc circuits, make it a particularly useful model for exploring Hpc-related mechanisms that might be relevant to the investigation of PTSD behavioral abnormalities. My data suggest that hippocampal neurogenesis

could, in fact, play a role in the PTSD-like behavioral abnormalities seen is PTSD. They also provide preliminary support for some ongoing examination of synaptic changes as a potential additional participating mechanism.

Future Directions

In light of the extensive body of evidence supporting the role of adult hippocampal neurogenesis in non-pathological key Hpc function (e.g. contextual processing) [10, 24-26], but especially because of neurogenesis' likely role in modulating stress response [27, 28] as well as possibly contributing to the psychopathology of various psychiatric disorders (e.g. depression and schizophrenia) [29-32] and their treatment with antidepressants [33], my results make a compelling case for a role of neurogenesis in mediating PTSD-like SPS-induced ER deficit. However, this conclusion needs to be balanced against new finding suggesting drastic decrease in hippocampal neurogenesis in humans after childhood [70]. This latest publication highlights the importance of human postmortem brain tissue assessment, especially those of former psychiatric patients. Nevertheless, neurogenesis will remain relevant until higher scientific consensus to the contrary is reached. Therefore, in addition to addressing the caveats of each of the experiments conducted, an important future step would be to investigate the mechanisms by which SPS-exposure dampens adult hippocampal neurogenesis. One possible mechanism worth exploring is the role stress hormones, possibly via glucocorticoid receptor (GR) action [34], might play in mediating SPS's effect on neurogenesis and behavior. Interestingly, SPS exposed rats show elevated expression of GR compared to MR (mineralocorticoid receptor; i.e. increased GR/MR ratio) in all areas of the Hpc, including DG [4]. It has been shown that cortisol leads to

opposing effects on neurogenesis depending on the receptor involved: GR decreases while MR increases neurogenesis [34]. Therefore, SPS' GR expression increase could mediate SPS's neurogenesis decrease I investigated here.

Further research is required to establish a mechanistic link by which stress hormones mediate decreased hippocampal neurogenesis from SPS exposure, thereby resulting in ER deficit. As a first step, one could selectively block GR during SPS exposure with intrahippocampal infusion of a GR antagonist (e.g. ru-43044), then assess if in the absence of GR action during SPS, exposure will still result in decreased neurogenesis and ER deficit. However, it is worth noting that GR-mediated dampening of neurogenesis is not the only mechanism by which SPS may result in decreased hippocampal neurogenesis. Other possible mechanisms include epigenetics influences on a variety of relevant genes [35], BDNF action [36], and neurotransmitters-regulation [37] of adult hippocampal neurogenesis.

Even in the face of overwhelming uniformity in mRNA expression changes of the synaptic markers assessed, it would remain necessary to investigate changes in dendritic morphology and synapse numbers by visualizing these structures and directly assessing them. As a future first step protein levels would need to be quantified to verified PCR results. More importantly, ER deficits likely result from changes in specific connections among brain regions within the neurocircuitry involving mPFC-Hpc-Amyg [1]. Therefore, assessment of changes in the expression of synaptic markers using whole-brain-structures is inadequate to address questions regarding possible changes in connectivity among these regions. Questions regarding synapses among neurons from one of these regions to another (e.g. mPFC-Hpc or Hpc-Amyg) would require the use of

anterograde tracers combined with visualization of tracer containing neurons. One such technique that has been garnering wider use in the study of morphology of connection between brain structures is the use of anterograde tracer Phaseolus vulgaris-leucoagglutinin (PHA-L) [38]. This type of techniques is a valuable tool for the study of anatomical mapping with specific advantages in the detection of circuit-based dendritic architecture, including spines [39]. As a future step in the study of synaptic changes resulting from SPS, these types of morphological and functional assessments are necessary to fully understand how they may underlie/mediate resulting behavioral deficits.

Very few studies investigate PTSD postmortem brain tissues. Even though this grave need was outlined over a decade ago [40], I could not find any studies directly assessing neurogenesis or dendritic morphology in these tissues. Like in depression, PTSD patients tend to have prefrontal cortex and hippocampal shrinkage [41]. Evidence from MDD postmortem studies reveals decreases in dendritic arborization and synaptic marker expression in the PFC [42, 43]. However, there is less consensus on the implications of decreased neurogenesis as a mechanism for why several psychiatric disorders are often accompanied by Hpc shrinkage (e.g. MDD, PTSD and schizophrenia). A study assessing Hpc Ki-67 expressing (proliferating) cells revealed no difference between MDD and controls [44], while another study showed that this cell population was decreased in schizophrenia [45]. Comparable direct assessment of hippocampal neurogenesis has not been conducted in PTSD, however. At present, the evidence for decreased neurogenesis as being a pathological feature of PTSD needs much more investigation. Although antidepressants are the most effective drug intervention for treating PTSD [46], the role of increased neurogenesis in mediating antidepressant effects [33] is not conclusive evidence for

decreased neurogenesis as part of PTSD pathology. Alternatively, it is possible for the promotion of neurogenesis by antidepressants to ameliorate symptoms as compensation for another, yet to be determined, mechanism [44]. Therefore, though PTSD shares features with other psychiatric disorders, direct assessment of PTSD postmortem brain tissues remains necessary. Interestingly, a new tool for measuring human hippocampal neurogenesis *in vivo* has been proposed [47]. However, this method remains in its early stages of implementation. Measures of neurogenesis with this tool will need to be verified by assessing neurogenesis of in postmortem brain tissue from a large percentage of the in-vivo neurogenesis participants -- further indication of the grave need to study PTSD in postmortem brain tissues, especially in light of the recent findings suggesting that human adult hippocampal neurogenesis may not occur [70].

In conclusion, understanding the pathophysiology of PTSD and the identification of modifiable therapeutic targets should include more research into adult hippocampal neurogenesis and synaptic changes within relevant brain regions. It is possible that limitations of existing pharmacological treatments known to target these processes do not lead to symptom improvement because it is not yet known how these drug-induced brain changes contribute to behavior and cognition in an individual whose brain has already been shaped by trauma. Therefore, further research can help better tailor treatment strategies to address gaps in treatment efficacy and to improve treatment outcomes, in general. The work contained in this thesis represents a very small contribution towards this end.

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