

# **Evaluation of the Role of Astrocyte Glutamate Transport and of Synaptic NMDA Receptor Subtype Representation in the Pathogenesis of PTSD**

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# Evaluation of the Role of Astrocyte Glutamate Transport and of Synaptic NMDA Receptor Subtype Representation in the Pathogenesis of PTSD

Thomas Rogers-Cotrone

## Abstract (Academic)

Post-traumatic stress disorder (PTSD) is a psychological disorder that can cause great social/economic hardship. Progress towards treating PTSD has been slow due to a lack of understanding of its pathogenesis. This dissertation aimed to address this issue by investigating the involvement of the astrocytic glutamate reuptake transporter, GLT-1, and regional differences in expression of NMDA receptor subtypes in the development of a rat model of PTSD. We hypothesized that impaired astrocytic glutamate reuptake inhibits long-term memory processes, and that concurrent presence of glucocorticoids (GCs) during situational trauma selectively inhibits fear extinction memory processes in the prefrontal cortex, but not of conditioned fear memory processes in the amygdala, due to differences between these brain regions in expression of NMDA receptor subtypes. The effect of GLT-1 manipulation was studied *in vivo*. Utilizing the Single Prolonged Stress (SPS) model of PTSD, rats were either exposed to SPS or not. Within these groups, rats were administered a saline sham, a GLT-1 facilitator (ceftriaxone (CEF)), or a GLT-1 inhibitor (dihydrokainic acid (DHK)). Using Classical Fear Conditioning (CFC) and Fear Extinction (EXT) paradigms, retention of fear extinction memories was measured to determine the effect of GLT-1 manipulation on SPS-induced behavior (i.e., impaired fear extinction retention). From the brain of each rat, the amygdala, hippocampus, and prefrontal cortex (PFC) were collected and expression of GLT-1, p-CREB (a molecular indicator of long-term memory), and glucocorticoid receptor (GR, a molecular indicator of a PTSD-like state) were quantified. Analysis of the behavioral data showed that SPS exposure alone reduced the retention of extinction memories, but CEF and DHK both eliminated this effect. Analysis of the brain tissues revealed that SPS induced an increase in GR expression in the hippocampus. SPS also increased GLT-1 expression, but not p-CREB, in the PFC and amygdala. To evaluate the involvement of regional differences in NMDA receptor subtype expression *ex vivo*, tissue sections of amygdala, hippocampus, and PFC were taken from SPS and non-SPS exposed rats. Synaptic transmission was stimulated in these tissues using bicuculline in the presence of glucocorticoids, NVP-AA077 (a NR2A NMDA receptor subtype inhibitor), or Ro-25 (a NR2B NMDA receptor subtype inhibitor). p-CREB was measured in the tissues treated with GCs to determine if GCs exert greater inhibition of long-term memory in the PFC (a region reported to express high NR2A) than in the amygdala (a region reported to express high NR2B). p-CREB was also measured in the tissues treated with NVP or Ro-25 to determine if these reported receptor profile differences could be demonstrated, and if they changed following SPS exposure. Contrary to the stated hypothesis, analysis of non-SPS exposed rats revealed that GCs, NVP, and Ro-25 decreased p-CREB in all three regions with no differences between regions. However, in the SPS exposed group, p-CREB was not decreased in PFC and hippocampal tissues treated with GCs, amygdalar and PFC tissues treated with NVP, and PFC tissue treated with Ro-25. Overall, the results of the *in vivo* experiment did not convincingly demonstrate a role of glutamate spill-over in the pathogenesis of PTSD, but did show that modulation of glutamate reuptake can mitigate some of the behavioral consequences of exposure to situational trauma. The results of the *ex vivo* experiment did not reveal evidence that regional differences in NMDA receptor profiles exist across the three regions analyzed, nor did they show that GCs exert a region specific inhibition of long-term memory formation. However, it was demonstrated that SPS may affect long-term memory by altering expression of synaptic NMDA receptors. This study provides evidence that glial cells may play a role in the pathogenesis of PTSD, and thus may serve as targets for future therapy.

# Evaluation of the Role of Astrocyte Glutamate Transport and of Synaptic NMDA Receptor Subtype Representation in the Pathogenesis of PTSD

Thomas Rogers-Cotrone

## Abstract (General Audience)

Post-traumatic stress disorder (PTSD) is a psychological disorder that can cause great social/economic hardship. Progress towards treating PTSD has been slow due to a lack of understanding of its pathogenesis. This dissertation aimed to address this issue by investigating the involvement of the glutamate reuptake transporter, GLT-1, and regional differences in expression of glutamate receptor subtypes in the development of a rat model of PTSD. We hypothesized that impaired glutamate reuptake inhibits long-term memory, and that concurrent presence of stress hormones (glucocorticoids (GCs)) during trauma selectively inhibits fear-suppressing memories but not fear-inducing memories, due to differences in expression of glutamate receptor subtypes between the two brain regions (prefrontal cortex and amygdala, respectively) that store these memories. Utilizing the Single Prolonged Stress (SPS) model of PTSD, rats were either exposed to SPS or not. Within these groups, rats were administered a saline sham, a GLT-1 facilitator (ceftriaxone (CEF)), or a GLT-1 inhibitor (dihydrokainic acid (DHK)). Retention of fear extinction memories was measured to determine the effect of GLT-1 manipulation on SPS-induced behavior (i.e., impaired fear extinction retention). From the brain of each rat, the amygdala, hippocampus, and prefrontal cortex (PFC) were collected and expression of GLT-1, p-CREB (a molecular indicator of long-term memory), and glucocorticoid receptor (GR, a molecular indicator of a PTSD-like state) were quantified. Analysis of the behavioral data showed that SPS exposure alone reduced the retention of extinction memories, but CEF and DHK both eliminated this effect. SPS also increased GLT-1 expression, but not p-CREB, in the PFC and amygdala. To evaluate the involvement of regional differences in glutamate receptor subtype expression *ex vivo*, tissue sections of amygdala, hippocampus, and PFC were taken from SPS and non-SPS exposed rats. Synaptic transmission was stimulated in these tissues using bicuculline in the presence of glucocorticoids, NVP-AA077 (a NR2A glutamate receptor subtype inhibitor), or Ro-25 (a NR2B glutamate receptor subtype inhibitor). P-CREB was then quantified following treatment. Contrary to the stated hypothesis, analysis of non-SPS exposed rats revealed that GCs, NVP, and Ro-25 decreased p-CREB in all three regions with no differences between regions. However, in the SPS exposed group, p-CREB was not decreased in PFC and hippocampal tissues treated with GCs, amygdalar and PFC tissues treated with NVP, and PFC tissue treated with Ro-25. Overall, our results did not convincingly demonstrate a role of glutamate spill-over in the pathogenesis of PTSD, but did show that modulation of glutamate reuptake can mitigate some of the behavioral consequences of exposure to trauma. Furthermore, it was demonstrated that SPS may affect long-term memory by altering expression of synaptic glutamate receptors. This study provides evidence that glial cells may play a role in the pathogenesis of PTSD, and thus may serve as targets for future therapy.

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## **Declaration of Work Performed**

I declare that I, Thomas Rogers-Cotrone, performed all the work reported in this dissertation except that which is reported below.

Dr. Stephen Werre wrote and performed all the codes and statistical analyses, as well as helped with the interpretation of the results.

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## LIST OF ABBREVIATIONS

ACH: Aceptlcholine

ACHE: Acetylcholinesterase

ACTH: Adrenocorticotropic Hormone

ANOVA: Analysis of Variance

ALS: Amyotrophic Lateral Sclerosis

BDNF: Brain-derived Neurotropic Factor

BLA: Basolateral Nucleus of the Amygdala

CamKII: Calcium/calmodulin Dependent Protein Kinase II

CBT: Cognitive Behavioral Therapy

CE: Central Nucleus of the Amygdala

CEF: Ceftriaxone

CFC: Classical Fear Conditioning

CNS: Central Nervous System

CREB: cAMP-Response Element Binding Protein

CRH: Corticotropin Releasing Hormone

CS: Conditioned Stimulus

DCS: D-cycloserine

DHK: Dihydrokainate

DSM: Diagnostics and Statistics Manual of Psychiatric Disorders

EAAT: Excitatory Amino Acid Transporter

E-LTP: Early Long-term Potentiation

EPM: Elevated Plus Maze

ERK: Extracellular Signal-Regulated Kinase

EXT: Extinction Training

ICM: Intercalated Cell Masses of the Amygdala

IL: Interleukin

GC: Glucocorticoid

GLAST: Glutamate Aspartate Transporter

GLT-1: Glutamate Transporter 1

GR: Glucocorticoid Receptor

LC: Locus Coeruleus

L-LTP: Late Long-term Potentiation

LTP: Long-term Depression

LTP: Long-term Potentiation

MAPK: Mitogen Activated Protein Kinase

mGLURs: Metabotropic Glutamate Receptors

MR: Mineralocorticoid Receptor

MRI: Magnetic Functional Imaging

NMDA: N-methyl-D-aspartate

NMDAR: NMDA Receptor

NMJ: Neuromuscular Junction

NVP: NVP-AAM077

NR2A: NMDA Receptor Subunit 2 (Subtype A)

NR2B: NMDA Receptor Subunit 2 (Subtype B)

PAG: Periaqueductal Grey Matter

p-CREB: Phosphorylated CREB

PE: Prolonged Exposure

PFC: Prefrontal Cortex

PKC: Protein Kinase C

PTSD: Post-traumatic Stress Disorder

PVN: Paraventricular Nucleus of the Hypothalamus

REC: Extinction Training Recall

RIPA: Radioimmunoprecipitation Assay Buffer

Ro-25: Ro-25-6981

ROS: Reactive Oxygen Species

SAM: Situationally Accessible Memory

SPS: Single Prolonged Stress

TBST: tris-buffered saline and tween-20

TROCs: Transducers of Regulated CREB Activity

US: Unconditioned Stimulus

VAM: Verbally Accessible Memory

# CHAPTER I

*JUSTIFICATION FOR SPECIFIC AIMS*

*AND*

*STATEMENT OF HYPOTHESIS*

## ***I. JUSTIFICATION FOR SPECIFIC AIMS AND STATEMENT OF HYPOTHESIS***

### **SECTION A: BACKGROUND INFORMATION AND JUSTIFICATION FOR SPECIFIC AIMS**

Post-traumatic stress disorder (PTSD) is a psychological illness that affects ~7% of the average U.S. population, and ~30% of combat veterans and sexual assault victims. For people with the disorder, the consequences of PTSD include increased suicide and divorce rates, lower productivity at work, increased aggression towards others, and difficulty raising children (Keane et al., 2009). Beyond these, it is estimated that about \$3 billion are lost each year merely as a result of PTSD-related loss of productivity in the work force (Keane et al., 2009; Kessler, 2000). As such, PTSD is a disorder that is not only debilitating for those that are directly afflicted, but one that also has a significant impact on society as a whole.

Although it has been over thirty years since it was first identified as a legitimate psychological disorder by the Diagnostic and Statistical Manual III of the American Psychiatric Association, progress towards treating PTSD has been slow and incomplete. Attempts have been made to develop effective pharmaceutical treatments for PTSD. However, a lack of understanding of the exact pathogenesis of the disease has made it difficult to achieve this goal, necessitating more research in this area to allow for development of better pharmaceutical interventions.

This research aimed to improve our understanding of PTSD pathogenesis through the evaluation of the underlying mechanism behind PTSD-associated persistent fear memories. In this regard, it has been established that this component of PTSD symptomology is attributed to alterations in the processing and maintenance of memories associated with fear conditioning (i.e., classical conditioning) (Keane et al., 2009; Milad et al., 2007; Milad et al., 2009). PTSD subjects exhibit an impaired ability to overcome the conditioned memories of previous trauma, and demonstrate this symptomatically

through chronic, persistent states of heightened arousal and anxiety (Keane et al., 2009). Interestingly, this is not so much a consequence of enhanced fear conditioning, but rather the result of an inability to retain memories of fear extinction (which normally suppress the expression of fear memories) when the threatening situation is no longer present (Milad et al., 2007; Milad et al., 2009).

Current studies have identified the amygdala and prefrontal cortex (PFC) as two regions of the brain that are implicated in the disruption of fear extinction memories characteristic of PTSD (Milad et al., 2007; Milad et al., 2009). These two regions share interconnecting circuits through which they can regulate the functional output of the other (Eisenhardt and Menzel, 2007; Rao et al., 2009). Diagrams 1, 2.1-2.4, 3.1-3.4, 4.1-4.4, and 5.1-5.7 (see pages 182, 183-184, 185-186, 187-188, and 189-192) depict the relationships between these regions and the formation, and subsequent suppression through extinction, of fear memories. In general, both neutral and noxious stimuli stimulate similar neural circuits in the amygdala, but only noxious stimuli activate circuits that can elicit a fear response (Diagrams 2.1-2.4 and 3.1-3.4; see pages 183-184 and 185-186). Conditioned fear memories form in the amygdala when a neutral stimulus (the conditioned stimulus, or CS) is temporally paired with a noxious stimulus (the unconditioned stimulus, or US) (Diagram 4.1; see page 187). Following pairing, the noxious circuit will potentiate (i.e., strengthen) the neutral circuit (Diagrams 4.2 and 4.3; see pages 187 and 188). As a consequence, the neutral circuit will be strong enough to elicit a fear reaction on its own, resulting in fear expression following future re-exposure to the neutral stimulus; even without concurrent re-exposure to the noxious stimulus (Diagram 4.4; see page 188). However, if the neutral stimulus is presented enough times without the noxious stimulus, the substrates for an opposing memory form in the PFC. Efferent projections from the PFC to the amygdala then indirectly inhibit amygdala output and subsequently suppress fear expression (Diagrams 5.1-5.7; see page 189-192). This process by which fear expression is suppressed by repetitive CS-US disassociation is termed “fear extinction”.

Regarding PTSD, it is the above mentioned inhibition by PFC of amygdala output, constituting fear extinction memory, that appears to be deficient in this disorder. Functional MRI has shown that PTSD is associated with an increase in amygdala activity and a concurrent decrease in PFC activity (Milad et al., 2007; Milad et al., 2009); a finding that is concordant with the fact that the amygdala is the region heavily involved in the formation and expression of fear memories, and the PFC is the analogous region for development of fear extinction memories. Diagrams 5.1 through 5.6 (See page 189-191) depict the process of extinction memory formation and Diagram 5.7 (see page 192) depicts the proposed change that occurs in the relationship between the PFC and amygdala in PTSD. The goals of this research were aimed at investigating pharmacological and molecular mechanisms of glutamatergic function in an animal model of PTSD that might help to clarify why memories encoding fear extinction are deficient in PTSD subjects while those encoding conditioned fear remain intact.

## SECTION B: PURPOSE AND STATEMENT OF HYPOTHESES

As mentioned earlier, the following research aimed to better understand the cellular and molecular mechanisms underlying the pathogenesis of post-traumatic stress disorder. This work focused on investigating the mechanism of one of the core PTSD symptoms: the persistence of intrusive fear memories of a psychologically traumatic experience. Because of its integral role in memory formation and maintenance, glutamate has been the target of many studies that have tried to elucidate the mechanisms underpinning this particular symptom of PTSD. We therefore concentrated on synaptic glutamatergic substrates in PTSD that could account for a deficiency of long-term fear extinction memory processes in the PFC without a concomitant deficit in long-term fear memory formation in the amygdala. Given their involvement in the body's stress reactions, the regulation of brain glucocorticoids has also garnered a great deal of attention regarding its role in producing characteristic PTSD symptoms. We theorized that disruption of synaptic glutamate and glucocorticoid regulation following situational trauma, combined with normal differences in synaptic glutamate receptor profiles between the amygdala and PFC, plays a role in the development of persistent fear memories. This research tested two principal hypotheses: **(a) Following situational trauma, impaired astrocytic glutamate reuptake leads to inhibition of long-term extinction memory processes within the pre-frontal cortex. (b) The concurrent presence of glucocorticoids selectively exacerbates the inhibition of fear extinction memory processes in the prefrontal cortex, but not of conditioned fear memory processes in the amygdala, due to differences between these brain regions in expression of NMDA receptor subunits.** To test these hypotheses, we relied on a rat model of PTSD: the Single Prolonged Stress model (SPS). Having been used in numerous animal studies aimed toward investigating the pathogenesis of PTSD, SPS has been shown to replicate many of the behavioral (e.g., persistent fear memory) and biochemical changes (e.g., increased hippocampal glucocorticoid receptor expression) associated with this disorder observed in humans (Yamamoto et al., 2009).

## SECTION C: SPECIFIC AIMS

### **c.i. Specific Aim 1: Determine how SPS-induced persistent fear is altered by manipulation of astrocytic glutamate transport**

The alterations in fear regulation associated with PTSD can be considered as a deficit of long-term fear extinction memory retention with consequent inability to suppress conditioned fear memories. In other words, this aspect of PTSD can be summarized as a disruption of long-term memory formation that selectively affects mechanisms of extinction memory retention of the PFC but not mechanisms of conditioned fear memory retention of the amygdala. Though an exact mechanism has yet to be defined, glutamate has been proposed to be an important component of PTSD pathogenesis as it is critically involved in the molecular basis of memory formation and retention (Izquierdo and Medina, 1997). As such, astrocytes may play a role in the mechanism that links glutamate to the development of PTSD due to the fact that extracellular concentration of glutamate following neuronal release is tightly regulated via reuptake by astrocytes. Evidence of astrocyte involvement can be found in studies that have demonstrated that interleukin 1 $\beta$  (IL-1 $\beta$ ) can decrease expression of astrocytic glutamate transporter proteins (Prow and Irani, 2008). This is interesting considering that PTSD is associated with several aberrations of the immune system including elevation of circulating pro-inflammatory cytokines, like IL-1 $\beta$  (Lindqvist et al., 2014). Therefore, our first specific aim focused on determining how manipulating astrocytic glutamate transport affects the behavioral components of persistent fear: fear memory and fear extinction memory in an animal model of PTSD.

Briefly, glutamate strengthens neuronal synapses by initiating gene transcription, ultimately leading to cellular changes that increase sensitivity of the postsynaptic neuron to presynaptic neurotransmission (Izquierdo and Medina, 1997). This process is termed “long-term potentiation” (LTP). A central transcription factor involved in LTP is cyclic AMP Response Element Binding Protein (CREB),

which must be phosphorylated in order to stimulate gene transcription (Diagram 6.1; see page 193) (Izquierdo and Medina, 1997). Upon binding to postsynaptic NMDA receptors and while the postsynaptic neuron is depolarized, glutamate induces an influx of calcium that results in a protein synthesis-independent early phase (associated with short-term memory) and a protein synthesis-dependent late phase (associated with long-term memory) of LTP (Malenka and Bear, 2004). During late-phase LTP, activation of Ca/Calmodulin dependent protein kinase II (CamKII) by increased  $Ca^{++}$  influx initiates an extracellular signal-regulated kinase (ERK) 1/2-dependent signaling cascade that ultimately phosphorylates CREB (Bito et al., 1996; Cortes-Mendoza et al., 2014; Hasegawa et al., 2014) (Diagrams 6.2, 7.1, and 7.2; see pages 193 and 194). Downstream, this late-phase CREB phosphorylation can lead to increased synthesis of synaptic elements, including new glutamate receptors.

While glutamate promotes LTP when acting at synaptic NMDA receptors, it has an inverse effect when acting at extrasynaptic NMDA receptors (those adjacent to the synaptic cleft). Extrasynaptic NMDA receptors are functionally linked to different signaling molecules, and have been shown to impair late-phase LTP via a competing p-38 mitogen activated protein kinase- (MAPK) dependent pathway that ultimately leads to CREB de-phosphorylation (Jiao and Li, 2011; Li et al., 2010; Mulkey et al., 1994; Parvez et al., 2010) (Diagram 6.2, see page 193). Thus, glutamate promotes CREB phosphorylation and LTP through stimulation of synaptic NMDA receptors, while conversely promoting de-phosphorylation of CREB and inhibiting late-phase LTP through stimulation of extrasynaptic NMDA receptors (Diagrams 7.3 and 7.4, see page 195).

Due to the resulting impairment of LTP following extrasynaptic NMDA receptor stimulation, we theorized that the pathogenesis of PTSD may involve this process in the PFC consequently leading to impaired long-term extinction memory formation. As major regulators of synaptic glutamate concentrations, astrocytes prevent glutamate from spilling over into extrasynaptic spaces through glutamate-specific reuptake transporters (i.e., GLT-1 and GLAST) (De Pitta et al., 2011) (Diagrams 7.3 and

7.4, see page 195). Thus, if astrocyte regulation of glutamate in the PFC is impaired by inflammatory cytokines following situational trauma, then this would lead to activation of extrasynaptic NMDA receptors, increased de-phosphorylation of CREB, and impaired long-term extinction memory formation. This would then lead to the persistent fear memories characteristic of PTSD.

If the scenario just described is correct, we hypothesized that facilitating astrocyte glutamate transport would mitigate the situational trauma-induced impairment of long-term fear extinction memory formation. Conversely, inhibition of astrocyte glutamate transport would exacerbate the trauma-induced memory impairment. To test this, we proposed to compare the retention of fear extinction memory of control rats that have undergone Classical Fear Conditioning (CFC) to that of rats exposed to CFC seven days after being exposed to the Single Prolonged Stress (SPS) protocol. The SPS protocol is commonly used to produce an animal model of PTSD. These two main groups (i.e., SPS and CFC) were each subdivided into three subgroups that were administered a glutamate transporter promoter (ceftriaxone), a transporter inhibitor (dihydrokainate, or DHK), or a sham treatment, as a means of evaluating the role of astrocyte glutamate reuptake in the formation of fear memories and retention of fear extinction memory following SPS exposure. Classical fear conditioning and extinction procedures were employed in all groups in order to assess fear memory and fear extinction memory.

**c.ii. Specific Aim 2: Evaluate the effect of SPS on glutamate transporter expression and on downstream CREB phosphorylation in the amygdala and prefrontal cortex.**

Although the neural substrates of conditioned fear memories and fear extinction memories are formed and stored in different regions of the brain, they both rely on very similar neurochemical and molecular events (refer to discussion above). More specifically, both depend upon the strengthening of synapses via a glutamate-mediated molecular signaling cascade that ultimately results in the phosphorylation of CREB (Mamiya et al., 2009; Tronson et al., 2012). Similarly, the mechanisms

responsible for the possible inhibition of these substrates, such as activation of extrasynaptic NMDA receptors (mentioned previously) are also conserved between these regions. However, it was noted earlier that PTSD can be thought of as a condition where fear extinction memory retention in the PFC is impaired, while fear memory retention in the amygdala remains intact (Milad et al., 2007; Milad et al., 2009). This raises the important question of how two regions that share these similar basic mechanisms are differentially affected by PTSD.

As Specific Aim 1 proposed to determine, astrocytic glutamate transport may play a crucial role in the retention or loss of fear extinction memories associated with PTSD. Although that work would show whether or not a connection exists between altered astrocyte glutamate transport and the impairment of extinction memory retention in PTSD, it would not directly address the PTSD-induced biochemical or molecular changes that we believe underlie the hypothesized outcomes of Specific Aim 1. Moreover, it would not explain what different changes are occurring between the PFC and amygdala that account for concomitant fear memory retention and fear extinction memory loss.

As such, part of Specific Aim 2 aimed to determine molecular changes induced by SPS that could lead to altered astrocytic glutamate transport and whether this change is differentially expressed between the amygdala and PFC. One possibility could be changes in expression levels of the astrocyte glutamate transporter proteins. Imbe et al. (2012) demonstrated that chronic restraint stress can lead to a decrease in GLT-1 expression in the periaqueductal gray matter. Interestingly, Reagan et al. (2004) reported an opposite effect of chronic restraint stress in the hippocampus; demonstrating an up regulation of GLT-1 expression. These two studies were conducted under very similar conditions using the same strain of rats, raising the possibility that glutamate transport expression may not be effected equivalently across all brain regions following psychological stress. A comparison of glutamate transporter expression has yet to be made between the amygdala and PFC under conditions of SPS.

In light of these reports, and the aforementioned relationship between PTSD, pro-inflammatory cytokines and glutamate transporter expression, we hypothesized that SPS will cause a decrease in glutamate transporter expression in the PFC without a concomitant decrease (or possibly an increase) in expression within the amygdala.

Under Specific Aim 1 we presented a theoretical scenario whereby impaired glutamate re-uptake could lead to enhanced activation of extra-synaptic glutamate receptors which would, in turn, activate a molecular signaling pathway that facilitates LTP-suppressing CREB de-phosphorylation. Therefore, we hypothesized that the SPS-induced decrease in glutamate re-uptake transporter expression in the PFC will coincide with a decrease in phosphorylated CREB (indicating impaired LTP), and that the concordant lack of change or increase in transporter expression within the amygdala will coincide with no change or elevation of CREB phosphorylation (indicating normal or improved LTP, respectively). These molecular changes would be concordant with the fear extinction memory deficit and fear memory maintenance observed in PTSD.

Finally, it has been reported that ceftriaxone can facilitate the glutamate re-uptake transport process. We therefore hypothesized that improved levels of phosphorylated CREB in the PFC should underly the recovery of extinction memory retention in SPS-exposed rats treated with ceftriaxone.

This analysis was based on micro-punch samples collected from the brains of the rats used in the behavioral study outlined for Specific Aim 1. Western blot analysis or ELISA was performed to quantify the expression of proteins in the amygdala and PFC. Two primary astrocyte glutamate transporters (GLT-1 and GLAST) and phosphorylated CREB (p-CREB) was evaluated as our primary test proteins of interest.

**c.iii. Specific Aim 3: Determine if differences in synaptic NMDA receptor subtype expression among the amygdala and PFC results in differential resistance to the detrimental effects that glucocorticoid has on NMDA-activated CREB phosphorylation**

While differences in expression of glutamate transporters between the amygdala and PFC could theoretically explain why fear memories and fear extinction memories have different retention levels in PTSD, there is another dimension to the interplay between synaptic and extrasynaptic glutamate transmission that could further explain the occurrence of this characteristic phenomenon of PTSD. Along these lines, it is important to note first that there are multiple NMDA receptor subtypes (Diagram 8, see page 196), and the molecular events that occur within a neuron after glutamate binds to them have been associated with the relative composition of the receptor subtype population. Several studies have linked NMDA receptor subtypes to different intracellular molecular pathways that influence cell survival and function (Cull-Candy and Leszkiewicz, 2004; Ewald et al., 2008; Sepulveda et al., 2010). For example, in the cortex and hippocampus, it has been reported that most synaptic NMDA receptors contain NR2A subunits, and activation is linked to molecular pathways associated with promotion of LTP (i.e., CamKII/ERK signaling cascade and CREB phosphorylation) (Cull-Candy and Leszkiewicz, 2004) (Diagrams 9.1 and 9.2, see page 197). Conversely, it has also been reported that most extrasynaptic NMDA receptors in these brain regions contain NR2B subunits, and activation is linked to long-term depression- (LTD) promoting pathways (i.e., p-38 MAPK pathway and CREB de-phosphorylation) (Cull-Candy and Leszkiewicz, 2004) (Diagram 9.3, see page 198). However, determining a clear cut relationship between NMDA receptor subtype and post-synaptic intracellular molecular pathways can be complicated by factors such as developmental stage, activity pattern of post-synaptic receptor activation, and synaptic/extrasynaptic segregation pattern of receptor subtypes.

There is also evidence that not all brain regions share similar NMDA receptor subtype profiles. For example, in the amygdala, the NR2B subunit has been shown to be expressed within many synaptic NMDA receptors and these receptors share similar intracellular molecular functions with synaptic NR2A-containing NMDA receptors of the hippocampus and PFC (Diagrams 10.1 and 10.2, see page 199). In fact, there is evidence that in the amygdala NR2B receptor stimulation is required for fear conditioning to occur (Walker et al., 2008), and the mechanism of this action is linked to the same molecular pathway (i.e., the CamKII/ERK pathway) (Zhang et al., 2008) that the NR2A subunit activates to induce LTP in the hippocampus and cortex. Thus, it has been suggested that NR2A is the dominant synaptic NMDA receptor subtype in the cortex and hippocampus, but that the NR2B subtype contributes significantly to amygdala synaptic transmission (Szinyei et al., 2003). It should be kept in mind, however, that there is a significant amount of variability in the literature regarding the synaptic and extrasynaptic NMDA receptor subtype composition even within a given brain region. Nevertheless, this potential difference in NMDA receptor subtype composition between the amygdala and PFC could prove to be a relevant contributing substrate for the selective deficit in fear extinction memory in PTSD.

This distinction could prove particularly important when considering, 1) a report by Xiao et al. (2010) that showed a difference between NR2A and NR2B subtype sensitivity to glucocorticoid exposure in hippocampal neurons and, 2) that PTSD patients have been shown to be in a persistent state of glucocorticoid sensitivity (with GC receptors being up-regulated in many regions of the brain) and dysregulation. In hippocampal culture, Xiao et al. (2010) found that glucocorticoids selectively blocked the intracellular molecular functions associated with receptors containing NR2A, but not those of receptors containing NR2B. More specifically, the ERK mediated signaling cascade associated with NR2A activation in this study was impaired by glucocorticoids, while the signaling pathway associated with NR2B in this study was left intact (Diagram 11, see page 200). This study did not investigate this phenomenon in neurons of the amygdala or the PFC. However if the above mentioned suggestion that

there is greater synaptic representation of NR2B subunits in the amygdala compared with the cortex or hippocampus is true, then LTP-promoting synaptic transmission in the amygdala might be expected to be less susceptible to the detrimental effects of glucocorticoids compared with the PFC. This could represent a significant molecular substrate for the differential retention of amygdala-associated fear memories and PFC-associated fear extinction memories in PTSD (Diagrams 12 and 13, see pages 201 and 202).

We therefore hypothesized that glucocorticoids will produce greater impairment of NMDA-activated CREB phosphorylation in a synaptic tissue preparation from the PFC compared with a similar preparation from the amygdala. Given that this hypothesis is based upon still equivocal evidence for a greater proportion of NR2B subunits at amygdala synapses compared with those of the PFC or hippocampus, we also hypothesized that NMDA-induced CREB phosphorylation in synaptic tissue samples from the amygdala will respond differently to NR2A or NR2B antagonism compared with similar tissue samples from the PFC or hippocampus. The assumption that the relative representation of synaptic NR2A and NR2B NMDA receptor subtypes does not change in PTSD underlies the clinical significance of our hypothesis regarding the interaction of glucocorticoids with these subtypes. Therefore, we also hypothesized that the effects of NR2A and NR2B antagonists on NMDA-activated CREB phosphorylation in synaptic tissue samples from the amygdala, PFC and hippocampus will be the same in SPS exposed rats as in controls. These hypotheses were addressed by making three different preparations of adult rat brain tissue sections that contain either amygdala, PFC or hippocampus from control or SPS treated rats. Tissues sections were incubated in appropriate combinations of NMDA, NR2A antagonist, NR2B antagonist or glucocorticoid following synaptic activation via administration of bicuculline. ELISA was used to measure levels of phosphorylated CREB.

# CHAPTER II

## *LITERATURE REVIEW*

## **II. LITERATURE REVIEW**

### **SECTION A: A REVIEW OF POSTTRAUMATIC STRESS DISORDER**

#### **a.i. History of PTSD**

Historically, people have often observed behavioral changes in individuals that experienced traumatic events. Most commonly noticed in those with combat exposure, these behavioral changes had been seen in survivors of events including the American Civil War, World Wars I and II, and the Vietnam War. Prior to the 1980's, these conditions had been given a variety of names including "stress syndrome", "shell shock", and "battle fatigue" (Shiromani et al., 2009), but had yet to be recognized as legitimate medical/psychiatric conditions. However, in 1980, the American Psychiatric Association officially recognized these trauma-induced behavioral alterations as a single anxiety disorder termed "posttraumatic stress disorder" (PTSD). Since then, PTSD has been diagnosed in 6% to 8% of the general U.S. population and in 30% to 50% of individuals within high risk groups like combat veterans and assault victims (Kessler et al., 2000). PTSD has been shown to have significant social and economic consequences. For example, an estimated \$3 billion are lost each year merely as a result of PTSD-related loss of productivity in the work force (Keane et al., 2009; Kessler, 2000), while higher divorce rates, increased aggression towards others, and difficulty raising children are linked to PTSD patients as well (Keane et al., 2009).

#### **a.ii. Symptoms and Risk Factors**

According to the 5<sup>th</sup> edition of the Diagnostic and Statistical Manual of Psychiatric Disorders (DSM), PTSD is defined as an anxiety disorder induced by exposure to a traumatic event, one that involves serious threat of death or injury to oneself or to another individual (American Psychiatric

Association, 2013) (APA, 2013). It is characterized by recurrent, intrusive memories of the trauma with concurrent hyper-arousal, cognitive deficits, negative emotional thoughts, and avoidance symptoms (such as emotional numbing and inability to remember details of the traumatic event) that have persisted for over one month (APA, 2013; Keane et al., 2009). These symptoms must have had a disruptive effect upon the patient's personal or professional life, and could not be otherwise explained by other factors (e.g., drug use, head injury, etc.) (APA, 2013). It is notable that, for a given individual, not every traumatic event will lead to the development of PTSD (Friedman, 2002). Moreover, when multiple individuals experience the same traumatic event, only a subset of these may go on to develop PTSD. Together, these two facts suggest that two categories of risk factors are associated with PTSD: 1) factors that are intrinsic to the trauma victims and 2) factors that are intrinsic to the traumatic experience.

Regarding risk factors that are intrinsic to an individual, there have been large discrepancies among different reports regarding which factors have significant influences on the development of PTSD. For example, some studies have found correlations between the onset of PTSD with factors that include female gender (Ryb et al., 2009), very young or old age (O'Connor, 2010), family history of psychological illness (Inslicht et al., 2010), and depression prior to the trauma (Ryb et al., 2009). On the other hand, for many of these (particularly gender and age) an equally strong body of data reports that there are no significant correlations with onset of PTSD (Christiansen and Elklit, 2008; Brewin et al., 2000). More recently, however, studies have identified PTSD risk at the molecular level, and have found that particular single nucleotide polymorphisms in the *fkbp5* gene (a modulator of glucocorticoid receptor sensitivity), polymorphisms in the promoter of the serotonin transporter 5-HTT gene, and variants in the gene encoding protein kinase C can serve as predictors of the onset of PTSD (Kenna et al., 2012; Liu et al., 2013; Mehta et al., 2012).

Unlike the factors associated with trauma victims, factors that characterize the traumatic event have been shown to have a much more consistent effect on the incidence of PTSD. Studies have consistently reported that the intensity of the event (Ryb et al., 2009), its proximity to the victim (Riviere et al., 2008), and lack of availability of psychological/emotional support immediately following the trauma (Brewin et al., 2000) are linked to the likelihood that a trauma victim will develop PTSD. In fact, the above mentioned risk factors have been used to develop models for the prediction of this disorder. One such model was developed in a series of studies using structural equation modeling; the results of which suggested that the strongest predictors of PTSD include previously experienced trauma, post-trauma support, and intensity of the trauma (in the form of serious threat of loss of life) (King et al., 1996; King et al., 1999).

### **a.iii. Treatments and therapies for PTSD**

#### **a.iii.i Psychological Therapy**

Currently, the most efficacious treatment for PTSD victims is cognitive behavioral therapy (CBT) (Klein et al., 2010; Powers et al., 2010), a process that has been effective in reducing PTSD symptoms associated with exposure to a variety of traumatic experiences (e.g., combat, rape, car accidents, and child abuse) (Harvey et al., 2003). Prolonged exposure (PE) therapy (a specific type of CBT) is considered the “gold standard” for PTSD treatment, and is used to gauge the efficacy of new methods of treating the disorder (Morkved et al., 2014; Powers et al., 2010). PE involves educating the victim regarding the symptoms of the disorder and the treatment methods that will be employed to overcome it. These methods involve exposing subjects to relevant trauma-associated cues (e.g., narration of the trauma or writing about the trauma) for ~50 minutes, aiding them in recognizing the adverse impacts of their negative thoughts/feelings regarding the trauma (i.e., cognitive restructuring), and developing their

ability to cope with fear and anxiety (Harvey et al., 2003; Powers et al., 2010). The overall goal of CBT is to legitimize the disorder in the mind of the individual, and subsequently resolve the symptoms by extinguishing the associated conditioned fear/anxiety response. This can take up to as many as twelve 60-90 minute sessions to achieve (Harvey et al., 2003; Powers et al., 2010).

The downside of CBT, however, is its low availability to many individuals either due to high financial cost or a lack of qualified, trained professionals to conduct the therapy (Klein et al., 2010). For this reason, alternative behavior therapies are being developed. For example, studies are being performed to evaluate the usefulness of self-help and therapist-assisted internet-based CBTs. Reports have shown that, by replacing traditional, face-to-face sessions with interactive on-line programs, the financial cost and the fear of social stigmatism associated with psychiatric treatment can be reduced without decreasing clinical efficacy (Klein et al., 2010). Thus, more PTSD sufferers can have access to treatment, and with more privacy, compared to traditional therapy programs.

#### **a.iii.ii. Pharmacological Therapy**

Due to the limitations of psychological therapy, there is an interest in developing pharmacological treatments for PTSD that can manage the disorder with similar efficacy as CBT. For example, treatment of the brain's over-active noradrenergic system, which contributes to the PTSD-associated hyper-arousal and anxiety, is a major target for pharmacotherapy (Friedman, 2002). Administration of anti-adrenergics, like  $\alpha_1$  and  $\beta$  receptor antagonists (prazosin and propranolol, respectively),  $\alpha_2$  receptor agonists (clonidine), neuropeptide Y agonists, and substance P antagonists have been shown in some studies to alleviate symptoms of PTSD (Raskind, 2009). Unfortunately, studies have shown that these compounds will not consistently have an effect on PTSD symptoms, or will only treat a narrow range of symptoms (Raskind, 2009).

Because of their effectiveness in treating similar symptoms in other psychiatric disorders, selective serotonin reuptake inhibitors, opioids, benzodiazepines, anticonvulsants, and atypical anti-psychotics have also begun to be considered as potential therapeutics for PTSD. However, of all these drug classes, only two selective serotonin reuptake inhibitors have received FDA approval for use as treatment of PTSD (Friedman, 2002; Han et al., 2014; Raskind, 2009; Rothbaum et al., 2014). The true efficacy of the other drug classes is questionable considering most data were collected from small sample groups, and there are varying reports regarding which are most effective (Friedman, 2002; Raskind, 2009). In addition, all of the above mentioned pharmacological treatments only address a subset of the symptoms of PTSD; thus failing to resolve the syndrome entirely.

Due to these short-comings of pharmacological options for PTSD treatment, CBT remains superior to other methods of treating PTSD (Morkved et al., 2014; Powers et al., 2010). The major factor behind the short-comings of the current pharmacotherapies is believed to be a result of a lack of knowledge regarding the neurochemical basis of this disorder (Friedman, 2002). As mentioned above, most of the current drugs being tested are selected on the basis of their efficacy in other psychiatric illnesses that have similar symptoms (e.g., schizophrenia, panic disorder, depression). Consequently, these treatments are used to ameliorate select symptoms rather than target the specific pathological mechanism causing the disorder. This fact underscores the necessity for conducting more research that is geared toward determining the neurochemical basis of PTSD and, subsequently, finding novel pharmacological therapeutic approaches.

## **SECTION B: LEARNING AND MEMORY BASICS**

As mentioned above, a diagnosis of PTSD is contingent upon an individual's presentation of multiple symptoms, of which recurring recollections of the traumatic event is one. This research aims to address this specific symptom, which can be generally considered as an aberration in normal memory formation and/or maintenance. As such, this section will review the theories of learning and memory formation, as well as discuss how these concepts are implicated in PTSD.

### **b.i. Different types of memory**

Memories can be defined as internal representations of previous experiences, and are the products of learning (a process by which new information and/or knowledge is acquired and retained). When describing memories we often categorize them as one of two basic types: declarative and non-declarative (also known as explicit and implicit, respectively) (Milner et al., 1998). Declarative memories are those that store information regarding facts and events and are recalled via conscious recollection (Milner et al., 1998). They can form from direct experiences (e.g., those that are personally experienced) as well as from indirect experiences (e.g., those that not experience personally, but learned about through other media, like oral or written communications) (Milner et al., 1998).

Conversely, non-declarative memories are those that are not available upon conscious recall. They are the type of memories that retain skills, habits, and behaviors (Milner et al., 1998), and are subdivided into procedural, conditioned, and non-associative memories. Procedural memories form when an action or activity is performed repeatedly (Milner et al., 1998). Dependent upon the striatum and cerebellum for acquisition and storage, this is the type of memory that results from learning to play sports or musical instruments (Squire and Zola, 2002). Conditioned memories are those that make an associative link between actions/behaviors with their outcomes/consequences (Milner et al., 1998).

There can be emotional conditioned memories (such as fear responses; governed mostly by the amygdala), and musculoskeletal conditioned memories (governed by the cerebellum) (Squire and Zola, 2002). Finally, non-associative memories are those related to habituation or sensitization to a particular stimulus after repeated presentation, and are stored in reflex pathways (Milner et al., 1998; Squire and Zola, 2002). Procedural and non-associative memories are exclusively the result of direct experiences. Conditioned memories are also usually the result of direct experiences as well (Milner et al., 1998; Squire and Zola, 2002).

### **b.ii. Neural substrates of PTSD-associated memory alterations**

As discussed in *Section A*, PTSD is characterized, in part, by abnormal fear memories of a past trauma. These fear memories can be considered abnormal in two general ways. Firstly, they are not suppressed by processes that normally extinguish the expression of fear when the individual is no longer being threatened. A consequence of this is that any reminder of a previous trauma can continuously act as a cue to reinstate a fear even if the subject is in no danger.

It should be noted that cued fear memory itself is not indicative of dysfunction. It was mentioned above that fear memories are a type of associative memory; those that specifically connect certain events or cues of an experience with an aversive outcome. If, for example, a normal subject is presented with a visual cue (such as a light being turned on) followed shortly by an electric shock, the subject will begin to express fear any time the light is turned on. This is due to the fact that the subject has made an associative memory between the light (the conditioned stimulus or CS) and the shock (the unconditioned stimulus or US) that predicts an aversive outcome (i.e., pain caused by the shock) when the light is turned on. Thus, even in normal individuals, a non-threatening stimulus (i.e., CS) can invoke fear when paired to a threatening stimulus (i.e., US) (Milad et al., 2002; Milad et al., 2007).

Under normal circumstances, this associative fear memory can be reversed by repeatedly exposing the subject to the CS in the absence of the US, through a process known as fear extinction (Milad et al., 2007). However, the fear memories of PTSD patients that are associated with traumatic experience have been shown to be resistant to the reversing effects of extinction, and it is in this aspect that PTSD subjects present dysfunction of fear regulation. It has been shown that normal extinction of a conditioned fear memory is not the result of memory erasure. Rather, extinction occurs when a new, non-threatening memory exerts a suppressive effect over the conditioned fear memory (Eisenhardt and Menzel, 2007). In other words, after extinction therapy, the individual has two opposite memories: one that associates the CS with the US, and another that contradicts and subsequently suppresses the memory of the CS-US association. Whether or not an individual has a fear reaction to the CS is thus dependent on how effectively the extinction memory suppresses the fear memory (Eisenhardt and Menzel, 2007).

Functional MRI has shown that there are no detectable differences in neuronal activity between healthy individuals and PTSD individuals during fear acquisition and fear extinction (Milad et al., 2009). However, it has been shown that healthy subjects retain extinction memories indefinitely while PTSD subjects do not retain extinction memories (Milad et al., 2009). In other words, healthy subjects recall fear extinction memories while PTSD subjects show impaired recall of extinction memories. Functional MRI showed that this deficit was correlated with a decrease in activity (indirectly detected via the observation of decreased blood flow) within the hippocampus and prefrontal cortex (Milad et al., 2009; Shvil et al., 2014). It has been suggested that this impaired extinction recall prevents the suppression of the conditioned fear memory by the extinction memory, and thus could serve as the basis for the recurrence of traumatic memories typically seen as a symptom of PTSD (Eisenhardt and Menzel, 2007; Milad et al., 2009; Shvil et al., 2014).

The second abnormal feature of PTSD-associated memories is that they are not normal recollections of past events. Rather, they are intrusive experiences that seem to be occurring in the present (APA, 2013). This suggests that PTSD is not only due to dysregulation of fear memory extinction, but is also the result of errors in processing spatiotemporal context (Brewin and Holmes, 2003). A characteristic feature of memories, in general, is that we can identify them as representations of something that has occurred in the past. Though we may be conscious of them in the present, we are aware that they are distinct from events that are occurring in the present. PTSD subjects seem to have lost this ability to place their traumatic memories in appropriate spatial and temporal contexts, allowing the reinstatement of a memory to feel like an event that is taking place in the present (APA, 2013).

Studies have shown that the cerebral cortex and the hippocampus may play a role in this spatiotemporal aberration of memory processing as well. It has been proposed that traumatic memories are stored in two functionally and structurally distinct systems. Briefly, it is theorized that reinstatement of traumatic memories can occur through situationally accessible memory (SAM) or verbally accessible memory (VAM) (Brewin and Dalgleish, 1996; Brewin and Holmes, 2003). SAM networks are thought to be found in subcortical structures (e.g., the amygdala) and primary sensory cortices that send projections to the cortical areas that originally encoded the traumatic event (Brewin and Dalgleish, 1996; Brewin and Holmes, 2003). Due to the direct involvement of primary sensory cortices and the relative simplicity of their networks, SAMs can be involuntarily reinstated by discrete sensory cues without perception of appropriate context, resulting in what is considered sensory-based representation (Brewin and Dalgleish, 1996; Brewin and Holmes, 2003). VAM networks, on the other hand, involve higher order cortical regions (e.g., the PFC and association cortices) and the hippocampus, and can be voluntarily reinstated with appropriate spatiotemporal contexts (Brewin and Dalgleish, 1996; Brewin and Holmes, 2003). As such, these memories are thought of as contextually-based memories. It is theorized that the basis of the intrusive memories of PTSD is the product of expression of these sensory-

based memories without the expression of the contextually-based memories (Brewin and Dalgleish, 1996; Brewin and Holmes, 2003). This might explain why discrete sensory reminders of a traumatic event can elicit experiences without proper perception of context (Brewin and Dalgleish, 1996; Brewin and Holmes, 2003).

Currently, the mechanisms behind these two general abnormalities of PTSD, extinction memory recall deficits and intrusive reinstatement, are still unknown. But, as the above discussion shows, three regions of the brain have been identified as having a critical role in the formation, regulation, and expression of PTSD-associated memory alterations: the amygdala, the hippocampus, and the prefrontal cortex (Rao et al., 2009). Each region has projections to the other two, and thus can modulate (and be modulated by) the other two regions (Rao et al., 2009). Since reciprocal regulation occurs between these regions, it has been suggested that the relative activity among them determines which memory is expressed: dominance of amygdala activity promotes a fear response, while dominance of hippocampal and PFC activity inhibits this promotion (Hugues and Garcia, 2007; Mamiya et al., 2009). Interestingly, PTSD-related deficits in extinction recall are associated with decreased activity of the hippocampus and the PFC relative to that of the amygdala, and may account for the dominance of the fear memory (Milad et al., 2009). Several stress and anxiety related alterations in these regions have been identified that might explain these PTSD-related changes in activity: decreased volume of the hippocampus and PFC, atrophy of hippocampal and PFC dendrites, increased arborization of amygdala neurons, increased spine density of amygdala neuron dendrites, and decreased spine density of PFC neuron dendrites (Rao et al., 2009). The following sections will describe how each of these regions encode and store memory, and provide details on how these functions might be adulterated by situational trauma in ways that could result in the development of PTSD.

### **b.iii. The Neural Substrates of the Fear Response**

In *Section a.ii.*, it was stated that PTSD is the result of experiencing a traumatic situation normally associated with intense fear, and that one of the primary symptoms of the disorder is persistent recollection of this feared event. It was also mentioned previously that fearful memories can be classified as a product of associative learning. As such, PTSD can be considered, in part, to be an aberration of the associative learning process as it pertains to fear. Considerable research has been done to determine the neural substrates of this aberration, but before this can be discussed, the general principles of the processing and storage of experiences that result in the state of fear must first be understood.

The state of fear is characterized by the expression of a variety of responses after exposure to an aversive stimulus (Steimer, 2002). Many of these responses are autonomic changes in physiological states such as elevation of heart and respiratory rates, increases in perspiration, and elevation of circulating glucocorticoids (Steimer, 2002). However, the nature of an organism's fear response can also be behavioral (such as the freezing response seen in rodents) and cognitive (i.e., feeling a sense of dread). The physiological, behavioral, and cognitive fear responses can be generated/regulated by the CNS, with the hypothalamus, the periaqueductal grey matter, and the locus coeruleus being particularly important regions (Steimer, 2002).

The hypothalamus is a structure of the ventral diencephalon that regulates the functions of the autonomic, somatic motor, and endocrine systems (Milner et al., 1998). Its main purpose is to maintain homeostasis by linking the behavioral/physiological status of an animal with appropriate autonomic, motor, and endocrine responses (Milner et al., 1998). It plays a significant role in the expression of certain regulatory behaviors (e.g., reproductive) and emotions (e.g., fear) (Milner et al., 1998). It is divided into distinct nuclei which have reciprocal connections with thalamic and brainstem nuclei (Sternson, 2013). It is through these connections that the hypothalamus can receive sensory

information, integrate it, and subsequently influence autonomic, motor, and endocrine functions in response to environmental stimuli.

Though there are multiple sources of input to the hypothalamus, the amygdala is one of the more prominent regions involved in affecting hypothalamic-mediated responses associated with fear. Projections from the amygdala directly innervate the paraventricular nuclei of the hypothalamus and induce a release of corticotropin releasing hormone (CRH) from the hypothalamus (Denver, 2009). CRH then circulates to the adenohypophysis of the pituitary gland and stimulates the release of adrenocorticotrophic hormone (ACTH) (Denver, 2009). Upon release, ACTH enters the general circulation and initiates a series of hormonal releases into circulation that are directly responsible for the physiological responses seen during fear (Steimer, 2002). For example, ACTH acts on the cortex of the adrenal glands to produce and secrete glucocorticoids (GCs) which subsequently induce the release of epinephrine and norepinephrine from the adrenal medulla (Steimer, 2002). Together, these three hormones act directly on various tissues throughout the body and can alter cellular metabolic states (e.g., increase glucose metabolism and fat break-down) and over-all organ function (e.g., increase contractility and contraction rate of the heart) in ways that are characteristic of the fear response (Mezzasalma et al., 2004; Steimer, 2002).

Regarding the behavioral aspects of the fear response, the most common behavior used in rodent models to determine the presence of fear is “freezing”. This is a state in which a rodent remains completely motionless (except for movements associated with respiration) for a period of time (usually longer than one to two seconds). “Freezing” is an intermediate step in a sequence of fear reactions that starts with 1) heightened alertness, 2) followed by cessation of movement (i.e., the “freezing response”), and then 3) flight (Steimer, 2002). Studies have shown that the freezing response is not dependent on the hypothalamus, but rather is mediated by the periaqueductal grey matter (PAG) (Koutsikou et al., 2014; Mezzasalma et al., 2004; Steimer, 2002). The PAG, which surrounds the cerebral

aqueduct of the mesencephalon, plays a significant role in descending CNS modulation of nociceptive stimulus processing (Steimer, 2002). It receives inputs from spinal tracts carrying thermal and nociceptive information, and in turn sends inhibitory projections to spinal motor neurons (either directly or indirectly via brainstem nuclei (e.g., the raphe nuclei) and the cerebellum (Koutsikou et al., 2014). These descending inhibitory efferents from the PAG to the spinal motor neurons cause a temporary state of motor immobility. Thus, following PAG stimulation, motor function is impaired and the freezing response manifests (Koutsikou et al., 2014).

Finally, studies have demonstrated that the cognitive changes that occur during the fear reaction are mediated primarily by the locus coeruleus (LC) (Mezzasalma et al., 2004; Samuels and Szabadi, 2008). Located in the dorsal aspect of the pons, on the lateral aspect of the floor of the fourth ventricle, the LC is the brain's major source of noradrenergic neurons (Samuels and Szabadi, 2008). Its projections extend into a variety of brain regions including other brain stem nuclei, various regions of the cortex, the cerebellum, and hypothalamus (Samuels and Szabadi, 2008). The release of norepinephrine from the LC has been shown to have an excitatory effect upon the regions that receive LC innervation, and this release has been documented to increase arousal (Samuels and Szabadi, 2008; Sanford et al., 2015). More specifically, the LC has been shown to be activated under stress conditions associated with fear (Mezzasalma et al., 2004; Samuels and Szabadi, 2008). When this occurs, its projections to the prefrontal cortex and the nucleus accumbens induce the cognitive and motivational reactions, respectively, that are characteristic of the fear response (Mezzasalma et al., 2004; Samuels and Szabadi, 2008).

#### **b.iii.i. Amygdala**

In the discussion above, it was mentioned how afferent signals entering the hypothalamus from the amygdala are critical for the physiological response of fear. Consequently, the amygdala plays a

prominent role in the formation and expression of fear, making it an important region of the brain to consider when discussing the possible neural substrates of PTSD. Located bilaterally in the ventral portion of the temporal lobes, the amygdala is a sub-cortical structure that is composed of several distinct nuclei. The networks of connections associated with these nuclei are extensive and complex. Studies have shown that the amygdala receives inputs (either directly or indirectly) from a myriad of structures throughout the CNS, including multiple brainstem nuclei, various regions of the cortex, the thalamus, the hippocampus, and the spinal cord, to name a few (Pitkanen, 2000). Through these inputs, the amygdala is provided with information from every sensory modality, and relays that input to specific regions of the brain like the cortex, hypothalamus, hippocampus, and brainstem nuclei (Pitkanen, 2000). It is these efferent signals from the amygdala that serve as the neural substrates through which sensory stimuli elicit a fear response.

Relevant to PTSD, the amygdala has been shown to be one of the most critical regions of the brain involved in the formation, storage, and expression of fear memories. Though there are several nuclei within the amygdala, studies have demonstrated that there is a group of amygdalar nuclei that is primarily involved in the memory-related functions of this region of the brain. These nuclei include the basolateral complex (BLA) (which is comprised of the lateral, basal, and accessory nuclei), the central nucleus (CE), and the lateral and medial intercalated cells masses (ICMs) (Pitkanen, 2000). The neurons of these nuclei are positioned within neuronal networks such that they bridge the gap between brain regions associated with sensory processing (e.g., cerebral cortex, spinal cord, thalamus, etc.) and those regions that govern the body's physiological and behavioral fear responses (e.g., cerebral cortex, periaqueductal gray matter, hypothalamus, etc.) (Pitkanen, 2000).

More specifically, information from all sensory modalities enters the amygdala primarily via afferents that project into the BLA (particularly the lateral nucleus) (Pitkanen, 2000) (Diagrams 2.1 and 2.2, see page 183). Glutamatergic efferents then project to the central nucleus (which is considered to

be the primary source of output from the amygdala) (LeDoux, 2000) (Diagram 2.3, see page 184). Projections from the central nucleus subsequently relay the information to aforementioned regions of the brain that govern the fear response, particularly the paraventricular nuclei of the hypothalamus, the LC and the PAG, which results in fear expression (LeDoux, 2000; Pitkanen, 2000) (Figure 2.4). In addition to this direct route of excitation of the CE, the BLA can indirectly stimulate the CE via the ICMs (Pitkanen, 2000). For example, the central nucleus receives inhibitory input from the medial ICM, which in turn, receives inhibitory input from the lateral ICM (Busti et al., 2011). In addition to the direct excitatory projections to the central nucleus, the BLA also sends excitatory glutamatergic projections to the lateral ICM that induce an inhibition of the medial ICM resulting in a dis-inhibition of the CE (Busti et al., 2011; LeDoux, 2000; Pitkanen, 2000). As such, sensory input received by the lateral nucleus can simultaneously activate and dis-inhibit the CE, and consequently induce fear expression.

As mentioned above, the BLA receives all forms of sensory information; both noxious and innocuous. Although both types of stimuli are received, it is important to note that innocuous stimuli induce a significantly lower excitation of CE neurons than noxious stimuli (Bear, 2001; LeDoux, 2000), and thus do not illicit a fear reaction (Diagram 3.1 – 3.4, see pages 185-186). However, since the BLA receives this wide array of incoming information, a convergence of sensory input occurs within this nucleus (LeDoux, 2000). It has been demonstrated that axon collaterals from neurons encoding noxious stimuli form synapses with axon terminals of neurons encoding innocuous stimuli (Bear, 2001; LeDoux, 2000) (Diagrams 4.1 and 4.2, see page 200). As a consequence, when noxious and innocuous stimuli are received by the BLA simultaneously, the noxious input causes a potentiation of the synapses encoding the innocuous stimuli (Bear, 2001; LeDoux, 2000) (Diagram 4.3, see page 188). With this increase in synaptic strength, innocuous stimuli can excite CE neurons to a high enough degree that a fear reaction is induced without the presence of the initial noxious stimulus (Bear, 2001; LeDoux, 2000) (Diagram 4.4,

see page 188). The end result is the formation of associative memories that link previously neutral stimuli to the adverse outcomes (e.g., pain) of noxious stimuli.

In Section b.ii., it was discussed how the persistent fear memories exhibited by PTSD subjects is believed to be the result of a failure of extinction memories to suppress fear memories. Considering its involvement in the formation of fear memories, the amygdala has become a likely candidate for having a role in PTSD development. In fact, data from animal studies using PTSD models have supported the notion that the amygdala plays a critical role in the development of this disorder. For example, it has been demonstrated that injection of cannabinoids directly into the BLA of rats can reverse the PTSD-like symptoms (e.g., extinction recall inhibition, enhanced fear conditioning, etc.) that are induced by exposure to single prolonged stress (a situational trauma protocol that induces a rodent model of PTSD) (Ganon-Elazar and Akirav, 2011). In addition, it has been shown that single prolonged stress causes a significant elevation of pro-apoptotic proteins relative to anti-apoptotic proteins in the rat BLA; suggesting that apoptosis in the amygdala is correlated with the disorder (Ding et al., 2010). Though the exact mechanisms by which these changes occur (and subsequently induce the behavioral and physiological changes associated with PTSD) have yet to be determined, these results indicate that alterations occur within the amygdala in this disorder, and that there is a connection between amygdala function and PTSD symptoms.

### **b.iii.ii. Prefrontal Cortex**

The discussion from *Section b.ii.* implicated abnormalities in the PFC as playing a significant role in the pathogenesis of PTSD. Though it should not be assumed that the PFC is the only cortical region involved in the disorder, most studies of PTSD focus on the PFC due evidence of its involvement in the formation and storage of extinction memories (i.e., the form of memory that is suggested to be abnormal in PTSD subjects (Milad et al., 2007; Milad et al., 2009). As such, this research (and,

subsequently, this review) will only go as far as to address the involvement of the PFC with regard to the role of the cerebral cortex in the development of PTSD.

It was previously mentioned that extinction memories have an inhibitory effect on the expression of fear memories. Though newly emerging data suggests that multiple regions of the brain are involved in the formation and storage of both of these types of memories (e.g., the amygdala), a much larger body of literature exists that describes how extinction memories are formed and stored in neuronal circuits of the PFC. For example, Milad and Quirk (2002) reported that rat PFC neurons (particularly in the infralimbic region) demonstrate a significant increase in firing rates after fear conditioned animals undergo extinction training. It was also shown that the degree of freezing behavior (i.e., fear expression) was found to be negatively correlated with the degree of PFC neuronal firing rates (Milad and Quirk, 2002). Interestingly, this decrease in fear expression could even be artificially simulated in fear conditioned rats that had not undergone extinction training, simply by stimulating the infralimbic PFC with electrodes (Milad and Quirk, 2002). This role of the infralimbic PFC has been further supported by more recent studies that have also demonstrated how fear expression is diminished by activity within this region of the cortex (Vidal-Gonzalez et al., 2006; Morawska and Fendt, 2011; Guhn et al., 2012).

While these studies provided strong evidence that the infralimbic PFC has a prominent role in extinction memory, they did not detail the specific circuits nor the exact mechanisms involved. Later studies, however, have addressed this issue and provide some more in-depth insight into the details of fear extinction memory. Neuronal circuits have been identified that connect the infralimbic region of the PFC with the medial ICM of the amygdala (Cho et al., 2013; Pinard et al., 2012) (Diagrams 5.1 through 5.6, see pages 189-191). In this network, glutamatergic projections from the infralimbic PFC run through the medial forebrain bundle to the amygdala (Cho et al., 2013; Dobi et al., 2012). Among the nuclei that are innervated by these fibers is the medial ICM which, as described above, contains GABAergic neurons

that project to and subsequently inhibit the CE of the amygdala (Cho et al., 2013; Li et al., 2011; Pinard et al., 2012) (Diagram 5.6, see page 191). Thus, infralimbic PFC activity has the potential to reduce fear expression by direct excitation of the amygdala's medial ICM which, in turn, inhibits the amygdala's CE output. Therefore, it is thought that the substrate of extinction memories (at least, in part) is an increase in neuronal firing in the excitatory networks that extend from the infralimbic cortex to the medial ICM of the amygdala.

In addition to defining the networks involved, studies have also aimed to determine the molecular mechanisms behind extinction memory, and have attempted to apply their findings to the pathogenesis of PTSD. Though current studies do not provide in depth detail regarding the exact mechanisms, they have identified molecular changes within the PFC in rat models of PTSD that could reasonably be associated with the pathogenesis of the disorder. For example, it has been demonstrated that after undergoing SPS conditioning, rats exhibit a significant increase in intracellular mineralocorticoid receptor (MR) expression in the PFC (Zhang et al., 2012), as well as a significant decrease in the levels of glutamate, glutamine, and creatinine (Knox et al., 2010). These studies did not address whether these changes were essential for the development of PTSD symptoms, however, the alteration in MR expression and the decrease in glutamate, glutamine, and creatinine suggest that the PFC can be functionally altered by PTSD-associated trauma (Zhang et al., 2012) and that this alteration is likely to be one of decreased neuronal activity (Knox et al., 2010). These findings are significant in the sense that they are consistent with findings from human PTSD subjects that show PFC activity is diminished in individuals with this disorder (Milad et al., 2007; Milad et al., 2009), however a causative link between these events and the development of PTSD has yet to be shown. Consequently, a need remains for further investigation into the molecular events that occur within the PFC under PTSD conditions.

### **b.iii.iii. Hippocampus**

The hippocampus has been widely studied in the field of learning and memory. Copious amounts of data show that the hippocampus is involved in both declarative and associative forms of memories, and a complete discussion of hippocampal function as it pertains to memory would be quite extensive and beyond the scope of this review. As such, hippocampal function will only be covered as it pertains to PTSD, for example, the contextual aspect of fear memory formation/regulation.

One of the most significant components of hippocampal function is detecting and storing information of the temporal characteristics and spatial orientation of an experience (Burgess, 2007; Morris, 2007). As such, it is not surprising that studies have demonstrated hippocampal involvement in learning and memory formation. Particularly of relevance for PTSD, data suggests that the hippocampus is involved in both fear conditioning (Kenney et al., 2012; Nalloor et al., 2012; Wang et al., 2012) and extinction (Lattal et al., 2007; Fiorenza et al., 2012; Garin-Aquilar et al., 2012).

Regarding fear conditioning, studies have established the necessity of the hippocampus in fear memory retention by demonstrating deficits in fear conditioning as a result of disruption of hippocampal function, such as through administration of NMDA receptor antagonist (Schemberg and Oliveira, 2008) or through ablation (Arruda-Carvalho et al., 2011). It has been proposed that theta rhythms generated by the hippocampus during context exposure serve as the substrate for hippocampal involvement in fear memory formation. Theta rhythm is a phenomenon characterized by synchronous firing of neurons (at a frequency between 4 and 7 Hz in humans) that can be detected in the hippocampus during certain activities including movement through space and exposure to arousing stimuli (Hasselmo and Stern, 2014). It has been shown that theta rhythm generated by the CA1 and subicular region of the hippocampus during exposure to a noxious stimulus causes a subsequent synchronous firing of principle neurons within the BLA of the amygdala (Bienvenu et al, 2012). This is relevant to memory formation, since neuronal synchrony at the theta frequency has been shown to be positively correlated with the

induction of long-term potentiation and subsequent memory formation (see *Section c.i.* below) (Hasselmo and Stern, 2014). Thus, it is proposed that when exposed to noxious stimulation, theta rhythm generated by the hippocampus creates synchronized neuronal firing in the BLA of the amygdala; promoting memory formation.

However, recent studies have shown that the role of the hippocampus in fear conditioning may not be as simple as previously thought. For example, there is evidence that suggests differing roles of the ventral and dorsal hippocampus in fear memory formation. It has been shown that infusion of nicotine into the ventral hippocampus before, during, or after fear conditioning causes a deficit in fear expression in mice when tested 24 hours later (Kenney et al., 2012). Interestingly, the same study went on to demonstrate that infusion of nicotine into the dorsal hippocampus induced a significant increase in expressed fear 24 hours after conditioning (though this was only noted if administration occurred before fear conditioning). These findings suggest that there are regional differences in the functionality of the hippocampus, and that there are more neurotransmitters involved than glutamate alone, which is the neurotransmitter classically considered to be most directly involved in long-term potentiation and formation of memory. See *Section c.i.* below).

To complicate matters further, it has also been reported that different mechanisms within the hippocampus govern fear memory formation depending upon whether the subject has been previously fear conditioned or not. Studies have shown that NMDA receptors and voltage-gated calcium channels in the dorsal hippocampus are required to achieve conditioned fear of a particular stimulus in naïve rodents (Bannerman et al., 1995; Saucier and Cain, 1995, Wang et al., 2012). However, in rodents that have been conditioned to fear similar stimuli in the past, NMDA and voltage-gated calcium channel blockade within the dorsal hippocampus is not sufficient to prevent subsequent fear conditioning (Wiltgen et al., 2010; Wang et al., 2012). For these previously trained subjects, it was demonstrated that

fear conditioning could be blocked only if both the dorsal and ventral hippocampus were functionally compromised via GABA agonists or NMDA antagonists (Wang et al., 2012). These findings again emphasize the functional diversity of the dorsal and ventral hippocampus, and additionally indicate that the role of the hippocampus in fear conditioning changes depending on the experiences of the subject.

Regarding extinction, less is known about the exact involvement of the hippocampus. It has been demonstrated that infusion of the GABA agonist muscimol into the dorsal hippocampus impairs context specific fear extinction (Corcoran and Maren, 2001), and that extinction of contextual fear memory is associated with AMPA receptor endocytosis and subsequent LTD (Bai et al., 2014). This latter finding suggests that contextual fear extinction requires plastic changes within the hippocampus and that decreased glutamate-dependent transmission is at least partly involved. In addition, there is evidence that the hippocampus is primarily involved in extinction of memories that have a strong association with context; having little involvement in extinction of memories that are independent of context (Morris, 2007).

Regarding hippocampal involvement in extinction and the pathogenesis of PTSD, Yamamoto et al. (2008) demonstrated that rats exposed to single prolonged stress exhibit up regulation of NR2 glutamate receptor subunit mRNA that was associated with impaired retention of contextual fear extinction memories. It was proposed that this was a compensatory response to decreased glutamate transmission (a theory that is consistent with the findings of Bai et al. (2014)) (Yamamoto et al., 2008). It was also shown that administration of D-cycloserine (DCS), a compound that binds to the glycine binding site of NMDA receptors and increases the receptors efficiency, reversed both the perturbations in NR2 mRNA levels and the alterations of extinction memory retention (Yamamoto et al., 2008). Interestingly, this was also consistent with the findings of Bai et al. (2014) who showed that DCS suppressed the endocytosis of AMPA receptors. Together, these findings support the concept that the hippocampus is

required for contextual fear extinction memory retention and that the mechanism is at least in part dependent upon modulation of NR2 glutamate receptor subunit function.

Data from current studies noted changes in hippocampal anatomy and function in PTSD subjects; lending credibility to the theory that hippocampal dysfunction is implicated in the development of the memory alterations associated with PTSD. It was previously mentioned that fMRI has revealed a negative correlation between hippocampal activity and the presence of PTSD (Milad et al., 2009). However, in addition to these findings, studies using human subjects have noted that individuals with PTSD exhibit decreased levels of N-acetyl aspartate (i.e., an indicator of decreased neuronal viability and metabolic function) within the hippocampus (Ham et al., 2007; Guo et al., 2012) and decreased hippocampal volume (Wignall et al., 2004; Zhang et al., 2011). Similarly, studies done with rodents have demonstrated hippocampal alterations after exposure to SPS, including increased neuronal apoptosis (Liu et al., 2012), increased BDNF/TrkB signaling (Takei et al., 2011), increased levels of mitochondrial-dependent neuronal apoptosis (Li et al., 2010), and decreased extracellular glycine (Iwamoto et al., 2007; Yamamoto et al., 2010).

Unfortunately, though a considerable number of studies (such as those cited above) have identified associative links between PTSD symptoms and various hippocampal alterations, few data have been presented that have demonstrated causal links between these associations and the development of PTSD. As such, it is still unclear as to whether or not these hippocampal changes are in fact components of PTSD pathogenesis, or merely coinciding phenomena that have little relevance to the pathogenesis of the disorder. Consequently, further research is needed to effectively describe the exact role of hippocampal dysfunction in the pathogenesis of PTSD.

## **SECTION C: THE MOLECULAR MECHANISMS OF MEMORY**

Up to this point, this review has addressed the cellular basis of learning/memory and how they are implicated in PTSD. However, if appropriate pharmacological treatments are to be determined for this disorder, the molecular mechanisms of PTSD must be elucidated. As such, this section describes the molecular bases of learning and memory formation, focusing on those that are most relevant to PTSD.

It is important to note that proper memory and learning cannot simply be considered as incorporation of new information into neuronal networks. An individual is bombarded with sensory information during any given experience, but only relevant information can be incorporated into a memory if it is to function correctly. Since the relevance of information is an issue, there must be molecular mechanisms in place between neurons during memory formation that increase synaptic transmission for relevant information as well as decrease synaptic transmission for irrelevant information.

### ***c.i. Increasing the strength of existing synapses through Long-term Potentiation***

Long-term potentiation (LTP) is the leading theory that details how memories are stored in the brain at the molecular level via synaptic strengthening. It is a cascade of molecular events that links neuronal action potential firing to an increase in synaptic sensitivity. LTP has classically been described as a process that begins when a presynaptic neuron releases neurotransmitter (typically glutamate) across a synapse at a high frequency (Malenka and Nicoll, 1999). The required frequency of stimulation received by the post-synaptic neuron for LTP to occur is somewhat variable, and has been demonstrated to be influenced by dopamine and brain derived neurotrophic factor (BDNF) (Li et al., 2011). Once a sufficient threshold of stimulation of the post-synaptic neuron has been exceeded, depolarization of the post-synaptic neuron occurs with enough magnitude to allow for an influx of calcium through NMDA

channels (see below for more details). This calcium influx, in turn, activates a multitude of kinases (Malenka and Nicoll, 1999). The various phosphorylating actions of these kinases can modulate protein function, ultimately resulting in alterations of the profile of receptors present at the synapse; thus increasing its sensitivity (Malenka and Nicoll, 1999). LTP is a broad term that actually incorporates two related, yet distinct, phases of molecular events: early-phase LTP and late-phase LTP. When considering the molecular basis of memory formation, it is more appropriate to delineate between these phases since each has a unique role in memory formation.

### **c.i.i. Early-phase LTP**

Early-phase LTP (E-LTP) is characterized as being both rapidly induced and transiently expressed. It is initiated when high intensity synaptic transmission (typically in the form of high frequency release of neurotransmitter from a pre-synaptic neuron) stimulates a post-synaptic neuron. The importance of the intensity of synaptic transmission is related to differing functional activities of the two glutamate receptors found on the post-synaptic neuron: AMPA receptors and NMDA receptors (Malenka and Bear, 2004).

AMPA receptors are ionotropic channels. They can be activated by glutamate alone, and they predominantly allow for an influx of sodium ions (Malenka and Nicoll, 1999). In regular synaptic transmission, the depolarization of the post-synaptic neuron (and its subsequent generation of an action potential) is largely due to this  $\text{Na}^+$  influx through AMPA receptor channels (Malenka and Nicoll, 1999).

Regarding their function in LTP, NMDA receptors differ from AMPA receptors in some critical ways. Firstly, though they are activated by glutamate, NMDA receptors will not allow ion influx upon binding of glutamate alone. This is due to the presence of  $\text{Mg}^{++}$  within the pore of the channel that blocks ion flow (Malenka and Nicoll, 1999; Malenka and Bear, 2004). This block can be removed, however, if the post-synaptic neuron is depolarized to a certain degree (Malenka and Nicoll, 1999;

Malenka and Bear, 2004). Thus, ion influx through NMDA receptors is dependent on concurrent depolarization of the post-synaptic neuron and glutamate release from the presynaptic neuron. The second important difference between AMPA and NMDA glutamate receptors is that NMDA channels preferentially allow for the influx of  $\text{Ca}^{++}$  (instead of  $\text{Na}^+$ ) into the post-synaptic neuron; an event that has a variety of functional consequences other than simply contributing to EPSPs (Malenka and Nicoll, 1999; Milner et al., 1998). Thus, E-LTP initiation is due to  $\text{Ca}^{++}$  influx through NMDA receptors, which can only occur after high frequency stimulation of AMPA receptors has allowed the post-synaptic membrane to depolarize, removing the  $\text{Mg}^{++}$  block of the NMDA channel.

Once  $\text{Ca}^{++}$  has entered the post-synaptic neuron, it induces molecular changes within the neuron that both E-LTP and late-phase LTP (L-LTP) depend upon. Regarding E-LTP, the  $\text{Ca}^{++}$  influx leads to a rapid increase in kinase phosphorylation. Two important examples are  $\text{Ca}^{++}$ /calmodulin-dependent protein kinase II (CamKII) and protein kinase C (PKC) (Malenka and Nicoll, 1999; Malenka and Bear, 2004). Once activated in the post-synaptic neuron, these enzymes phosphorylate AMPA receptors currently positioned on the neuron's post synaptic membrane. This induces a conformational change in each receptor that increases its conductance of  $\text{Na}^+$  (Malenka and Nicoll, 1999; Malenka and Bear, 2004). In addition, CamKII induces the fusion of AMPA-containing vesicles (AMPA receptors that have been synthesized and reside in their Golgi apparatus-produced vesicular packaging) with the neuron's post-synaptic membrane, leading to the incorporation of new AMPA receptors at the synapse (Malenka and Nicoll, 1999; Malenka and Bear, 2004). Both of these events, increased numbers and functional capability of post-synaptic AMPA receptors, result in a relative increase in the efficiency by which the synapse can transduce information and the strength (magnitude) of the pre-synaptic neuron's effect upon the post-synaptic neuron. Because E-LTP only involves phosphorylation events and trafficking of already-produced proteins, these effects can be seen very rapidly after  $\text{Ca}^{++}$  influx. However,

phosphatases and natural receptor turn-over can start significantly reversing the effects of E-LTP in a matter of minutes (Malenka and Bear, 2004).

### **c.i.ii. Late-phase LTP**

The strengthening of synapses via E-LTP is relatively brief (in the order of minutes or hours) (Malenka and Bear, 2004). This component of memory storage is sufficient to allow for short-term memory, but additional events have to take place if a memory is to be maintained long-term. Formation of long-term memories, those that last for hours to days, is dependent upon stabilization of the changes that occur during the late-phase of LTP (L-LTP). This can be achieved through persistent activation of CamKII and synaptic (or cellular) consolidation.

CamKII is composed of 12 subunits, each of which has a catalytic and regulatory domain (Malenka and Bear, 2004). The catalytic domains of the subunits are responsible for the kinase activity of the enzyme while the regulatory domain inhibits the actions of the catalytic domain (Malenka and Bear, 2004). However, this inhibitory action of the regulatory domain over the catalytic domain can be blocked following calcium influx. When calcium enters the neuron, such as when NMDA receptors are opened, the ion binds with calmodulin. This  $\text{Ca}^{++}$ /Calmodulin ( $\text{Ca}^{++}$ /Cam) complex, in turn, can bind to a regulatory domain of a CamKII subunit (Coultrap and Bayer, 2012; Lucchesi et al., 2010). When this occurs, the catalytic domain of the bound subunit is liberated from the inhibition of its associated regulatory domain, thus allowing that subunit to exhibit its kinase activity (Coultrap and Bayer, 2012; Lucchesi et al., 2010).

While this kinase activity allows for the phosphorylation of other proteins, active CamKII subunits can also phosphorylate sites on neighboring subunits within the same enzyme (Coultrap and Bayer, 2012; Lucchesi et al., 2010). This autonomous kinase activity, however, can only occur when two neighboring subunits are bound by  $\text{Ca}^{++}$ /Cam (Coultrap and Bayer, 2012; Lucchesi et al., 2010). In

addition to the liberation of the catalytic domain, binding of  $\text{Ca}^{++}/\text{Cam}$  to a CamKII subunit results in a conformational change that exposes the subunit's T286 phosphorylation site. Thus, if two neighboring subunits are bound by  $\text{Ca}^{++}/\text{Cam}$ , the catalytic domain of one subunit will be able to phosphorylate the exposed T286 site of its neighbor (Coultrap and Bayer, 2012; Lucchesi et al., 2010). The consequence of T286 phosphorylation includes strengthening the subunit's affinity for bound  $\text{Ca}^{++}/\text{Cam}$ , and prevention of the regulatory domain from inhibiting the catalytic domain even after  $\text{Ca}^{++}/\text{Cam}$  dissociates from the subunit (Coultrap and Bayer, 2012; Lucchesi et al., 2010). The result of this event is prolonged kinase activity that continues after  $\text{Ca}^{++}$  concentrations have returned to baseline levels (Coultrap and Bayer, 2012; Lucchesi et al., 2010).

Regarding LTP, the prolonged kinase activity of CamKII following autonomous phosphorylation has been shown to strengthen glutamate transmission of the affected synapse (Coultrap et al., 2014). For example, Coultrap et al. (2014) demonstrated that  $\text{Ca}^{++}/\text{Cam}$ -bound, autonomously phosphorylated CamKII selectively phosphorylated the S831 site of the GluA1 AMPA receptor subunit. As a result, the conductance of AMPA receptors at the synapse was increased, promoting early-phase LTP (Coultrap et al., 2014). Additionally, CamkII under these conditions has also been shown to promote late-phase LTP through translocation of new AMPA receptors to the postsynaptic membrane, as well as through activation of molecular cascades that ultimately lead to memory consolidation (discussed below) (Chen et al., 2014; Lucchesi et al., 2010). In contrast to the phosphorylation of existing AMPA receptors, which can reverse within an hour, these CamKII-associated late-phase LTP modifications to the synapse can last for hours to days (Malenka and Bear, 2004).

Synaptic consolidation is a protein synthesis-dependent process that results in long lasting structural modifications in both the pre-synaptic and post-synaptic neurons. For example, studies have shown that L-LTP leads to increased concentrations of synaptic vesicles in pre-synaptic boutons (Bell et al., 2014), as well as increased spine density in post-synaptic dendrites (Hasegawa et al., 2014). The basis

of these structural modifications is a change in neuronal gene expression mediated primarily by the activation of a gene transcription factor called c-AMP response element binding protein (CREB) (Bito et al., 1996). Once activated by phosphorylation, CREB binds to genomic c-AMP response elements (CREs) and subsequently promotes or inhibits the transcription of a multitude of genes, like Arc, c-fos, and Elk-1 (Bito et al., 1996; Cortes-Mendoza et al., 2014). Studies have shown that the effects of these CREB-dependent transcripts include, but are not limited to, increased dendritic arborization, increased spine density, and increased expression post-synaptic AMPA receptors (Flavell and Greenberg, 2008; Saito et al., 2013). The phosphorylation of CREB occurs following similar molecular events seen during E-LTP: namely high frequency stimulation of NMDA receptors and the subsequent influx of calcium and binding to calmodulin. Bound calmodulin activates CamKII and CamKIV, which then translocate to the nucleus and directly phosphorylate CREB (Cortes-Mendoza et al., 2014; Sun et al., 1994).

Once it occurs, CREB activation results in the expression of a variety of immediate early genes such as c-FOS, c-JUN, EGR1, and JUNB (Flavell and Greenberg, 2008; Saito et al., 2013). These genes encode for a variety of transcription factors that alter the protein expression of neurons such that AMPA receptors are produced and inserted into the post-synaptic membrane, production of dendritic spines is enhanced, and dendritic elongation is promoted (Flavell and Greenberg, 2008). Thus, through CREB phosphorylation a long term change occurs in neurons undergoing LTP that leads to a prolonged enhancement of their synaptic strength.

### **c.ii. Decreasing the strength of existing synapses through Long-term Depression**

Proper memory formation is not just dependent on the strengthening of relevant synapses, but also on the removal of irrelevant synapses. Consequently, long-term depression (LTD) must also be considered when attempting to understand learning and memory. Interestingly, the mechanism of LTD has a key similarity to LTP, even though they have opposite outcomes. It has been shown that an influx

of  $\text{Ca}^{++}$  is the basis for LTD just as it is for LTP (Mulkey et al., 1994; Parvez et al., 2010). However, unlike LTP, LTD is initiated under conditions of low frequency depolarization of the post-synaptic neuron (Malenka and Bear, 2004). Consequently, rather than large amounts of  $\text{Ca}^{++}$  entering the neuron as in the case of LTP, only a small increase in intracellular  $\text{Ca}^{++}$  concentration occurs (Mulkey et al., 1994; Parvez et al., 2010). This small increase leads to the selective activation of enzymes that function as phosphatases, as opposed to the kinases that are activated during LTP (Mulkey et al., 1994). As a result of phosphatase activation, the above described molecular events of LTP are reversed. This reversal is manifested by a decrease in sensitivity of AMPA receptors to glutamate, an increase of their internalization from the post-synaptic membrane, and the inhibition of CREB-dependent protein synthesis (Mulkey et al., 1994; Parvez et al., 2010).

Finally, recent studies have revealed that the activation of these phosphatases produce LTD through a BAD/BAX/caspase-3 cascade (Jiao and Li, 2011; Li et al., 2010). Li et al. (2010) showed that caspase-3 activation was required for endocytosis of AMPA receptors during LTD. Jiao and Li et al. (2011) went on further to demonstrate that this caspase-3 activation was in turn dependent upon the activation of BAD and BAX, two pro-apoptotic proteins, and that the phosphatases activated by low  $\text{Ca}^{++}$  influx were responsible for activation of the cascade. Interestingly, the data from these studies revealed that during NMDA receptor-dependent LTD induction, BAD levels were only elevated to a small degree and BAX did not translocate to the mitochondria; consequently leading to only a slight, momentary rise in caspase-3 (Jiao and Li, 2011). This was sufficient to lead to AMPA receptor endocytosis, but not sufficient to induce apoptosis (which required a high, more sustained rise in caspase-3) (Jiao and Li, 2011).

It should be noted, that LTD is not only activated through NMDA receptor associated pathways. Within the hippocampus, it has been shown that activation of metabotropic glutamate receptors (mGluRs) leads to LTD as well (though less is known about this particular pathway) (Huber et al., 2001).

Though the exact mechanism is not completely elucidated, there is evidence that suggests that hippocampal mGluRs induce AMPA receptor endocytosis and local protein synthesis within the post-synaptic terminal that ultimately lead to synaptic potentiation (Bernard et al., 2014; Huber et al., 2000; Luscher and Huber, 2010).

### **c.iii. Reconsolidation of memories**

It was mentioned above that formation of memories requires a protein-synthesis dependent process called consolidation, and that this process results in memory retention over a long-term period of time. However, it should be noted that consolidated long-term memories are not permanent, and that there are mechanisms by which they can become labile and susceptible to change. For example, Nader et al. (2000) demonstrated that administration of a protein synthesis inhibitor into the basolateral amygdala results in loss of an intact conditioned fear memory if the drug is given immediately after memory retrieval. It was also shown that retrieval of the memory was required for the amnesic effects to occur (Nader et al., 2000). Additionally, a 6-hour delay in administration of the inhibitor after memory retrieval failed to elicit the amnesic effects as well (Nader et al., 2000). Together, these findings suggest that retrieval of a consolidated memory initiates a cellular process that requires protein synthesis in order for the memory to remain intact. Thus, disruption of this process leads to loss of the memory entirely; indicating that retrieval of consolidated memories renders them labile and susceptible to alteration.

It has been proposed that this process exists so that old memories can be updated with new information (Besnard et al., 2012). The destabilization of a consolidated memory during retrieval allows for a window of plasticity during which a memory trace can incorporate new relevant information and/or eliminate information that is no longer relevant (Besnard et al., 2012). Under physiological conditions, this is followed by a restabilization process, after which new information is included as part

of the original memory trace (Besnard et al., 2012). This momentary destabilization and then re-stabilization of memories has been called reconsolidation. Contrary to what the name suggests, however, the process is not simply another consolidation process. Barnes et al. (2010) demonstrated that of the 163 genes involved in consolidation and the 248 genes involved in reconsolidation, only 12 were involved in both processes. Moreover, among these 12 genes, those that were upregulated in consolidation were down-regulated in reconsolidation, and vice versa, further substantiating the distinction between these two processes. (Barnes et al., 2010).

The concept of memory disruption via manipulation of reconsolidation has recently been proposed as a method of treating PTSD (Jones et al., 2013; Auber et al., 2013). In these studies, it was shown that a significant decrease in spontaneous fear recovery, renewal, and reinstatement was present in rats if they were given extinction training during the reconsolidation window of the conditioned stimulus. These findings suggest that the knowledge of how consolidated memories are processed (particularly during reconsolidation) could be useful when designing and implementing therapies for the persistent fears associated with PTSD.

#### **c.iv. Directions towards treating PTSD**

Throughout this section, some general mechanisms of memory formation and regulation have been discussed, and it is clear that there are a number of factors and events that play a role. These have been, for the most part, physiologically normal events that occur within healthy individuals. Thus, each can be attributed to the process of normal memory formation and regulation. PTSD, on the other hand, has been shown to be associated with a disruption of this process. As such, it is possible that some of the mechanisms discussed in this section are altered by the disorder, thereby leading to the characteristic memory dysfunction.

It was mentioned above how effective pharmaceutical therapies have been lacking due to our insufficient knowledge of these altered mechanisms of PTSD pathogenesis. Thus progression towards effective pharmaceutical therapy depends on not only understanding the molecular mechanisms of memory formation and retention, but also identifying which of these mechanisms change in PTSD and how these changes contribute to the disorder.

## **SECTION D: NMDA RECEPTOR SUBTYPES AND SYNAPTIC GLUTAMATE SPILL-OVER**

Considering the above discussion of neuronal plasticity, it is clear that the activation of NMDA receptors is a critical component of the processes associated with learning and memory formation. As mentioned above, PTSD is largely the result of altered memory function. In the case of PTSD, studies have shown that blocking glutamate transmission via glutamate receptor antagonists within the amygdala, or systemically, prior to either fear conditioning or extinction training, can effectively prevent both the acquisition of conditioned fear memories (Blair et al., 2001) and the retention of fear extinction memories (Santini et al., 2001; Suzuki et al., 2004), respectively. Furthermore, studies with D-cycloserine (DCS) (a NMDA partial agonist that increases  $Ca^{++}$  influx through NMDA channels) have demonstrated that enhancement of fear extinction memory formation or retention can be achieved when administration occurs before or shortly after extinction training, respectively (Davis et al., 2006). Consequently, attention has been directed towards discovering what roles NMDA receptors may play in the pathogenesis of diseases/conditions associated with altered memory states, such as PTSD.

### **d.i. Differential roles of NMDA receptor subtypes in memory formation**

Along these lines, it is important to note that there are multiple NMDAR subtypes, each of which is structurally and functionally unique. Any given NMDAR is composed of four protein subunits (Cull-Candy, 2004). The most common configuration consists of two glycine-binding NMDAR1 (NR1) subunits and two glutamate-binding NMDAR2 (NR2) subunits (a NMDAR3 subunit exists, but is considerably less common than the other two in the adult CNS) (Cull-Candy, 2004). In addition, the NMDAR2 subunits have 4 different subtypes: NR2A, NR2B, NR2C, and NR2D, each of which varies in channel kinetics and affinity for glutamate (Cull-Candy, 2004). These subtypes alter the function of their respective receptors by changing kinetic qualities of the receptor (Cull-Candy, 2004). For example, when NR2A-containing

receptors are activated they open rapidly, but for a short period of time, whereas NR2B-containing receptors open more slowly but remain open for longer (Cull-Candy, 2004). In addition, different receptor subtypes are linked to different intracellular molecular pathways resulting in varying cellular response depending upon which NR2 subunits make up a stimulated NMDA channel (Cull-Candy, 2004). For example, in the cortex, activation of NR2A-containing receptors is linked to molecular pathways associated with promotion of LTP, while activation of NR2B-containing receptors is linked to LTD-promoting pathways (Choo et al., 2012; Cull-Candy and Leszkiewicz, 2004). This opposing function of NR2A and NR2B-containing receptors has also been shown in molecular pathways that influence cell survival and apoptosis, where activation of NR2A-containing receptors improved cell survival and decreased apoptosis, while the inverse was true of NR2B-containing receptors (Cull-Candy and Leszkiewicz, 2004; Ewald et al., 2008; Sepulveda et al.).

Interestingly, studies have demonstrated that different regions of the brain express different profiles of NMDAR subtypes (Cull-Candy and Leszkiewicz, 2004; Wenzel et al., 1996), and that this expression can be influenced by environmental factors, such as substance abuse and caloric intake (Fontan-Lozano et al., 2007; Loftis and Janowsky, 2000). In fact, alterations in NMDAR subtype expression have been linked to psychiatric disorders (Bi and Sze, 2002; Loftis and Janowsky, 2003; Karolewicz et al., 2005) and impaired memory formation (Bi and Sze, 2002).

Another dimension of NMDAR receptor subtype variability that determines its ultimate effect on neural function is cellular localization of the NMDA receptors. For example, activation of NR2B within a synapse has been shown to promote NR2A-like events such as LTP (Gardoni et al., 2009), but activation of NR2B outside of the synapse, i.e., in extra-synaptic locations, induces neuronal apoptosis (Sinor et al., 2000). In general terms, however, studies have reported that NR2A receptors are the primary synaptic NMDAR subtype and promote neuronal survival, LTP, and stability of dendrites, while NR2B receptors

are the primary dendritic extra-synaptic subtype and have the opposite effects of NR2A (Cull-Candy and Leszkiewicz, 2004; Ewald et al., 2008; Sepulveda et al., 2010; Sinor et al., 2000).

Interestingly, while studying the effects of ischemic injury of the brain, Choo et al. (2012) showed that when synaptic NR2A and extrasynaptic NR2B are activated simultaneously in the same neuron, the ultimate response to glutamate is dependent on the balance of NR2A activation relative to NR2B activation. In other words, if there is a relatively high activation of NR2A-associated molecular pathways compared to that of NR2B-associated pathways, JNK and ERK MAPKs will be activated: promoting cell survival (Choo et al., 2012). However, predominant activation of NR2B leads to inhibition of the JNK/ERK pathway, and alters mitochondrial physiology such that apoptosis is promoted (Choo et al., 2012). Thus, due to the fact that the two pathways counteract each other's outcomes, the dominance of one pathway over the other proved to be the ultimate determinant of how the neuron responded to the glutamate administration (Choo et al., 2012). The importance of this balance between activation of synaptic NR2A and extrasynaptic NR2B pathways was further substantiated by Duan et al. (2013 and 2014) in studies that showed that exposure to low-frequency electromagnetic fields causes learning and memory deficits in mice undertaking the Morris Water Maze task. Upon deeper investigation, this type of exposure was shown to cause elevation of NR2B expression; leading to excessive intraneuronal calcium concentrations followed by a decrease in JNK, ERK, and CREB phosphorylation (Duan et al., 2014). Together, these studies suggest that learning/memory function is impaired when there is dominance of extrasynaptic NR2B activity.

#### **d.ii. Role of Glutamate Spill-over in NR2B stimulation**

It was previously noted that excessive  $\text{Ca}^{++}$  influx through NR2B receptors has a detrimental effect on memory acquisition and retention. It was also described how NMDA receptors with NR2B subunits are prevalent at extrasynaptic locations. Thus, it is reasonable to conclude that in an in vivo

system, there could be a negative correlation between extrasynaptic glutamate concentration and memory acquisition/retention. This conclusion has been supported by studies that have linked impaired learning and memory to extrasynaptic NMDA receptor stimulation (Kervern et al., 2012; Sanz-Clemente et al., 2013). Subsequently, these reports make it apparent that there can be considerably different neuronal responses to glutamate depending upon the degree to which this neurotransmitter is prevented from diffusing to extrasynaptic locations following its release into a synapse.

Extracellular glutamate concentration at the synapse is regulated primarily by astrocytes which form close associations around near-by synapses (De Pitta et al., 2011). At these synaptic locations, astrocytes express two major glutamate transporters named EAAT1 and EAAT2 (also known as GLAST and GLT-1, respectively, in the rat) that are responsible for moving glutamate out of the synaptic cleft and into the cytoplasm of the astrocyte (Shigeri et al., 2004). Once inside the astrocyte, glutamine synthetase converts the glutamate to glutamine, which in turn is released back into the extracellular environment for neuronal reuptake (Shigeri et al., 2004). Numerous studies have shown that this astrocyte-mediated glutamate transport is paramount for maintaining sub-toxic levels of extracellular glutamate, and that compromise of this regulatory mechanism is associated with a variety of pathologies (Napier et al., 2012; Shen et al., 2014; Weng et al., 2014).

For example, studies have shown that when astrocytic glutamate reuptake is sufficiently impaired, glutamate is allowed to diffuse out of the synapse and into extrasynaptic locations (Camacho et al., 2007; Sinor et al., 2000). Once this happens, glutamate stimulates NR2B receptors located on the extrasynaptic membrane of neurons, and subsequently leads to the previously mentioned neuronal apoptosis (Camacho et al., 2007; Sinor et al., 2000). In addition to neuronal apoptosis, GLT-1 expression has been shown to influence LTP (Pita-Almenar et al., 2012). It has been shown that inhibition of GLT-1 with dihydrokainate results in a decrease in hippocampal LTP (Pita-Almenar et al., 2012). Furthermore, decreased GLT-1 expression (and subsequent elevation of extrasynaptic glutamate) was shown to cause

memory deficits in rats performing the Morris Water maze (Zhu et al., 2014), which is consistent with the effects of extrasynaptic NR2B stimulation on memory described earlier. Altogether, these studies demonstrate a link between dysfunction of astrocyte-mediated glutamate reuptake, extrasynaptic glutamate spillover, NR2B stimulation, and impaired learning/memory and neuronal survival.

#### **d.iii. Inflammatory cytokines**

Though impaired glutamate reuptake can be associated with decreased learning and memory, implicating such a mechanism in the pathophysiology of PTSD would require the identification of a biochemical link between experience of a traumatic event and impaired glutamate reuptake. Though there are no current data that have demonstrated such a link, there are studies that provide evidence that support this concept. For example, while studying glial response during spinal cord injury, Yan et al. (2014) demonstrated that the release of interleukin 1-beta (IL-1 $\beta$ ) reduced glutamate reuptake by stimulating endocytosis of GLAST and GLT-1. Similarly, Prow and Irani (2008) showed that blockade of IL-1 $\beta$  prevented GLT-1 suppression and excitotoxicity in the mouse spinal cord following the inflammatory reaction produced during viral infection. Additionally, Fang et al. (2012) and Sitcheran et al. (2005) reported that other pro-inflammatory cytokines (macrophage inflammatory protein-1 gamma (MIP1 $\gamma$ ) and tumor necrosis factor alpha (TNF $\alpha$ ), respectively) decreased the expression of GLT-1 mRNA, and subsequently increased glutamate-induced neurotoxicity (presumably by activating extrasynaptic NR2B).

These findings are relevant to PTSD due to findings of altered immune function in patients with the disorder. Studies have reported increased risk of autoimmune disease (O'Donovan et al., 2014), elevated levels of circulating pro-inflammatory cytokines (Lindqvist et al., 2014), and increased production of pro-inflammatory cytokines from peripheral blood mononuclear cells *ex vivo* (Gola et al., 2013). These findings have been further supported by animal experiments that have demonstrated elevated pro-inflammatory cytokine (including IL-1 $\beta$ ) production in the prefrontal cortex and

hippocampus of mice exposed to predator scent (a rodent model of PTSD) (Wilson et al., 2013 and 2014). This elevation was correlated with an increase in anxiety behavior as demonstrated by performance in an elevated plus maze (Wilson et al., 2013 and 2014). Interestingly, the increase in pro-inflammatory cytokines and anxiety were reversed when subjects were given anti-inflammatory agents (Wilson et al., 2014). The combination of data from human and animal studies provides an intriguing (albeit unproven) connection between PTSD-associated pro-inflammatory states, glutamate reuptake inhibition, stimulation of extrasynaptic NMDA receptors, and subsequent impairment of learning/memory function. Further study into this potential link may yield insight into the underlying mechanism of PTSD, and uncover novel approaches for treating this disorder.

## **SECTION E: HYPOTHETICAL CONNECTIONS BETWEEN GLUTAMATE SPILL-OVER, GLUCOCORTICOID DYSREGULATION, AND PTSD**

This review has discussed a multitude of topics pertaining to PTSD. Starting with a general overview of the disorder in humans, this review detailed the fundamentals of memory formation and retention at the cellular and molecular level, and eventually discussed how normal memory function can be disrupted via perturbations in astrocyte glutamate reuptake. This final section will tie many of these concepts together into a framework that will describe a hypothetical mechanism through which PTSD develops.

### ***e.i. Stress and disruption of astrocyte glutamate reuptake***

The previous section discussed how disruptions in astrocytic glutamate transport can lead to glutamate spill-over and impairment of memory formation and retention, thus serving as a possible molecular mechanism through which PTSD develops. However, to support the hypothesis that astrocytic glutamate reuptake plays a role in PTSD pathogenesis, a link between experiencing psychological trauma and impaired glutamate transport should be made. In this regard, studies have reported evidence that suggests such a link via microglial activation. Though the exact mechanism has yet to be determined, stress has been documented to down-regulate anti-inflammatory neuronal proteins, such as CD200 (Blandino et al., 2009; Frank et al., 2006). CD200 is expressed on neuronal membranes and has been shown to be a component in the process of keeping microglia in a quiescent state (Blandino et al., 2009; Frank et al., 2006). Upon its down-regulation under conditions of high stress, neuronal CD200 inhibition diminishes and microglia begin to exhibit a pro-inflammatory state, secreting numerous cytokines including interleukin-1 beta and calcineurin (Blandino et al., 2009; Frank et al., 2006; Sama et al., 2008). Both of these cytokines have been shown to suppress astrocytic GLT-1 and GLAST glutamate transport (Blandino et al., 2009; Frank et al., 2006; Sama et al., 2008), which could lead to glutamate spill-over into extrasynaptic sites.

Considering this together with the previous discussion of the effects of glutamate spill-over on memory, it is possible that the stress experienced during psychological trauma initiates a sequence of events leading to impaired memory retention. The first event would be a diminished capacity of the brain to suppress the pro-inflammatory state of microglia via down-regulation of CD200. Microglia would then begin secreting cytokines like interleukin-1 beta and calcineurin that impair astrocytic glutamate transport. As a consequence of this impairment, glutamate would begin to spill-over into extrasynaptic locations, where stimulation of NMDA receptors activates the molecular pathways associated with LTD and suppression of long-term memory formation.

As mentioned above, this is strictly a hypothetical mechanism that is based on findings of several separate studies, and has consequently not been proven directly. Demonstrating the validity of this hypothetical link would require numerous experiments, and is beyond the scope of this proposed research project. However, it does help to justify further research into the involvement of impaired glutamate reuptake in the pathogenesis of PTSD, which is a key aim for this project.

### **e.ii. Glucocorticoids in PTSD**

According to the above theory, the memory deficits of PTSD are attributed at least in part to glutamate spill-over into extrasynaptic regions. However, the molecular mechanisms that lead to LTD and impaired memory formation/retention are conserved in both the amygdala and the PFC, suggesting that fear memory and extinction memory, respectively, would be disrupted by such an event. This, however, is not consistent with PTSD, where fear memories are retained and extinction memories are lost. If glutamate spill-over functions as a mechanism of PTSD development, there must be an explanation for why the PFC is affected to a greater degree than the amygdala. It has been mentioned in Section D that insults that disrupt glutamate transport do not always equivalently affect different regions of the CNS. This could prove true in the case of psychological trauma as well. However,

glucocorticoids may serve as a second explanation for how psychological trauma differentially affects the PFC and amygdala.

Aberrations of glucocorticoid regulation have been one of the most widely studied features of PTSD. Subjects with PTSD have been shown to express a heightened sensitivity to glucocorticoids, which results in altered levels of circulating GCs. This could prove important when considering the pathogenesis of PTSD since GCs have a very diverse effect on memory formation and retention. In this regard, a multitude of factors determine if GCs promote or impair memory formation and regulation of an individual, including the individual's age, the duration and/or magnitude of GC exposure, and the sensitivity of the individual's glucocorticoid receptors (GRs) to GCs (Lapiz-Bluhm 2014; Popoli et al., 2011). For example, it has been demonstrated that acute stress induces a GC-dependent increase in glutamate release at PFC and amygdalar synapses (Lapiz-Bluhm 2014; Popoli et al., 2011; Zschocke et al., 2005). This finding is consistent with studies reporting that acute exposures to GCs improve declarative and fear memory formation and retention (Lapiz-Bluhm 2014; Popoli et al., 2011). Conversely, glucocorticoids can have opposite effects under conditions of chronic stress compared to acute stress (Popoli et al., 2011). More specifically, chronic stress has been demonstrated to decrease working memory in adult rats, and is coincident with decreased volume, decreased neuronal number, and impaired induction of LTP in the PFC (Cerqueira et al., 2007). Another example of GC diversity can be found when considering the magnitude of the stress or GC exposure. Ninomiya et al. (2010) showed that low doses of GCs improve the retention of fear extinction memories in rats. However high doses of GCs showed no such effect (Ninomiya et al., 2010), and in humans, moderate stress prior to fear extinction has been shown to significantly decrease fear extinction memory recall (Raio et al., 2014).

Due to the complexity of their effects on memory, it is difficult to determine what role GCs may have in PTSD. However, this research aims to investigate a potential mechanism through which GCs act

as a component of the pathogenesis of PTSD that not only ties in with the hypothesized alterations in glutamate reuptake described above, but also provides an explanation for the selective deficits in extinction memory. In Section D, it was discussed how synaptic NMDA receptor subunit profiles are not identical among brain regions. This could prove important when considering the effects of GCs in PTSD since Xiao et al. (2010) demonstrated that glucocorticoids can selectively disrupt the synaptic transmission of NR2A receptors with no effect that of NR2B receptors. With these factors in mind, it stands to reason that synaptic transmission in regions of the brain that have high concentrations of synaptic NR2A will be disrupted by GCs to a greater extent than in regions that have relatively higher concentrations of synaptic NR2B. Though reports are conflicting, there is some evidence to suggest that the PFC has greater dependence on synaptic NR2A than the amygdala. Adding the fact that PTSD subjects have elevated sensitivities to GCs, the regional differences in synaptic NMDA subtype expression together with the subtype specific disruption by GCs could explain why extinction memory and fear memory are not affected to equivalent degrees in PTSD.

As such, even if glutamate spill-over affected the PFC and amygdala equivalently, a PTSD subject's increased sensitivity to GCs could hypothetically lead to selective disruption of fear extinction memory. According to this theory, in a PTSD subject under conditions of glutamate spill-over in the PFC, extrasynaptic pathways linked to LTD would be activated while GCs simultaneously disrupt the LTP-promoting pathways associated with synaptic transmission. The consequence of this is an impairment of fear extinction memory. However, in the amygdala under the same conditions, the synaptic LTP-promoting pathways remain intact, and could potentially counter balance the extrasynaptic LTD-promoting pathways, resulting in preserved fear memory. It is this interplay between impaired glutamate reuptake, elevated GC sensitivity, and regional differences in NMDA receptor subtype expression that this research proposed as a pathological mechanism for the development of PTSD and subsequently attempted to demonstrate experimentally.

## **SECTION F: SINGLE PROLONGED STRESS AND EX VIVO USE OF BICUCULLINE**

In the next chapter (Chapter V: Experimental Methods), it will be described how the three specific aims of this dissertation will be addressed in two different experiments. The first experiment will be an *in vivo* experiment that addresses Specific Aims 1 and 2, while the second experiment will be an *ex vivo* experiment addressing Specific Aim 3. While discussion of the methodological details will be saved for the next chapter, it is necessary to provide some background information on the Single Prolonged Stress model, as well as the use of bicuculline to stimulate synaptic transmission in brain tissue slices, since these two methodologies serve fundamental roles in the experiments.

### **f.i The Single Prolonged Stress Model (SPS)**

The SPS model was first described by Liberzon et al. (1997), and has since been recognized as a valid model of PTSD in rats (Yamamoto et al., 2009). It involves exposing a rat to three successive stressors: 2 hours of restraint, 20 minutes of forced swim, and ether anesthetization (Liberzon et al., 1997). Following this series of stressors, the rat is given 7 days before it undergoes classical fear conditioning and at least two subsequent fear extinction sessions (Liberzon et al., 1997). This model was used in both experiments of this dissertation with the intent of inducing a PTSD-like state in rats, and thus serves as a foundation for the project. Due to this fundamental role, it is important to discuss the validity of SPS as a model for human PTSD.

In their review of animal models of PTSD, Goswami et al. (2013) describe how any model should be assessed based on face validity, construct validity, and predictive validity. Face validity is the ability of a model to reproduce symptoms associated with the human disease/syndrome/disorder (Goswami et al., 2013). In this regard, rats exposed to SPS do in fact share many behavioral changes that are associated with PTSD in humans. For example, SPS-exposed rats have been shown to exhibit impaired retention of fear extinction memories (Knox et al., 2012), hyperarousal/hypervigilance (Khan and

Liberzon, 2004), and impaired learning of spatial tasks (Harvey et al., 2006). These could translate to the PTSD symptoms of recurring intrusive memories, hyperarousal/hypervigilance, and cognitive deficits, which are three of the four symptomatic criteria for its diagnosis in humans (APA, 2013). The fact that SPS induces in the rat behavioral changes that might be considered analogous to diagnostic criteria of PTSD in people provides strong evidence that SPS has face validity as an animal model of PTSD.

The next assessment of an animal model is construct validity. This reflects the homology between the substrates that underlie the symptoms induced by the model and those that cause similar symptoms in the human disease/disorder (Wilner, 1986). In the case of PTSD, there is still much that is unknown in regard to the exact pathogenesis of the disorder. However, it was discussed in Section b.ii of this literature review that deficits in retention of fear extinction memories have been shown to be the underlying cause of the intrusive fear memory symptoms associated with PTSD (Milad et al., 2007; Milad et al., 2009). Due to the roles of the amygdala, hippocampus, and PFC in fear memory and extinction memory formation and modulation, PTSD-associated impaired retention of fear extinction memories implicate abnormal function of these regions in the pathogenesis of PTSD (Milad et al., 2007; Milad et al., 2009). Consistent with this, studies have shown that PTSD subjects have decreased hippocampal and PFC volumes (Milad et al., 2009; Shvil et al., 2014). A decrease in PFC activity during fear extinction training has also been documented (Milad et al., 2007; Milad et al., 2009). Similarly, studies have demonstrated that SPS induces an increase in apoptosis within the hippocampus (Han et al., 2013) and the PFC (Yu et al., 2014) of rats, as well as a decrease in neuronal activity in the PFC (Knox et al., 2010). In addition to these substrates of recurring intrusive memories, similarities between PTSD subjects and SPS-exposed rats can also be seen in the substrates that underpin the hyperarousal/hypervigilance symptoms. For example, increased activity of CNS sympathetic pathways, particularly the locus coeruleus, is involved in the hyperarousal/hypervigilance exhibited by PTSD subjects (Geraciotti et al., 2001; O'Donnell et al., 2004). Consistent with this, SPS has been shown to induce a similar increase in

neuronal activity within the locus coeruleus of rats (George et al., 2013). As such, the SPS model shares at least two different neuronal substrates in common with those that are suspected to underpin the pathology of PTSD. This provides some construct validity for the model.

The final assessment of an animal model is predictive validity. This is the ability of a model to accurately predict treatment outcomes seen in the human syndrome (Goswami et al., 2013). As mentioned in Section A of this literature review, there are few accepted treatments for PTSD. Consequently, finding extensive evidence that the SPS model meets this criterion is difficult. However, it was mentioned in Section A that some SSRIs have been approved by the FDA to alleviate the symptoms of PTSD in humans. As such, the effect of these drugs on SPS-exposed rats is likely to be the best assessment of the model's predictive validity. In this regard, studies have shown that chronic administration of the SSRI, paroxetine, can prevent the enhanced fear and anxiety behaviors induced by SPS exposure (Takahashi et al., 2006; Wang et al., 2008). Although more research still has yet to be done for a thorough assessment, the fact that SSRIs provide symptomatic relief to both humans with PTSD and rats that are exposed to SPS suggests that this model does have some degree of predictive validity.

From this analysis, it can be argued that the SPS model satisfies to some degree the three validation standards for assessing an animal model. Though there is still much that is unknown about PTSD pathology, and thus a limitation to our ability to make thorough assessments, the above mentioned studies have provided a strong basis for the assertion that SPS serves as an acceptable model of PTSD.

#### **f.ii The ex vivo Use of Bicuculline**

The next methodology that warrants discussion is that of the use of bicuculline in the *ex vivo* experiment that addressed Specific Aim 3. A key goal of Specific Aim 3 was to characterize the profiles of NMDA receptor subtypes at the synapses within the amygdala, PFC, and hippocampus, and to

determine how these profiles influence changes in p-CREB following synaptic transmission in the presence of GCs. As described earlier, CREB is phosphorylated following activation of synaptic NMDA receptors, but dephosphorylated following activation of extrasynaptic NMDA receptors (Choo et al., 2012; Duan et al., 2014). Consequently, if we were to investigate the changes in p-CREB following synaptic transmission specifically, we needed to utilize a method that limited extrasynaptic spill-over of glutamate and the subsequent confounding effects on p-CREB expression that would occur due to activation of NMDA receptors outside of the synapse.

The method selected for this purpose was administration of bicuculline to brain tissue slices. After they have been prepared, brain tissue slices maintain some intact neuronal networks that contain glutamate releasing neurons (Bruckner et al., 2000). Activation of these neurons is locally suppressed, however, by inhibitory potentials generated by GABAergic neurons within the networks (Bruckner et al., 2000). Bicuculline is a GABA A receptor antagonist, and can indirectly induce synaptic glutamate release by impairing the inhibitory control that these GABAergic neurons exert upon the glutamatergic neurons (Johnston, 2013). Numerous studies have used this method of removing local inhibitory influence of local GABAergic neurons to examine effects of glutamate release that is mainly confined to the synapse (Zhang et al., 2011; Steigerwald et al., 2000; Johnson and Seutin, 1997; Lovinger et al., 1990; Ge et al., 2006; Rossi et al., 2000). Studies have even demonstrated that incubating brain tissue slices with bicuculline is capable of increasing intracellular signals associated with long-term LTP induction, including p-CREB (Zhang et al., 2011) and TORC1 (Zhou et al., 2006). Since it has been shown to both selectively induce synaptic glutamate transmission as well as increase p-CREB expression, we were confident that bicuculline administration could stimulate synaptic NMDA receptors in our brain slices with limited confounding activation of extrasynaptic NMDA receptors.

As the above discussion shows, the use of bicuculline is an indirect method of inducing synaptic transmission. It should be noted that alternative approaches for inducing neuronal activation exist. For example, incubation of brain slices with depolarizing agents like potassium chloride will directly induce neuronal firing. While the mechanism of action of potassium chloride (i.e., prevention of neuronal hyperpolarization) is different from that of bicuculline (i.e., blockade of GABA receptors), studies have shown that they also can induce LTP in neuronal tissue through activation of NMDA receptors the associated ERK/CREB pathway (Ch'ng et al., 2012; Macias et al., 2001; Zhou et al., 2009).

# CHAPTER III

## *EXPERIMENTAL METHODS*

### III. Experimental Methods

#### EXPERIMENT 1: EVALUATING HOW SPS-INDUCED PERSISTENT FEAR IS ALTERED BY MANIPULATION OF ASTROCYTIC GLUTAMATE TRANSPORT

Sixty adult male Sprague Dawley rats (150 – 200 grams) were used in this experiment. Rats were singly housed under a 12-hour light/dark cycle (lights are on from 7 AM to 7 PM) with *ad libitum* feed and water, and no environmental enrichment. Rats were assigned to one of two behavioral treatment groups: 1) the “SPS” group which was exposed to the single prolonged stress and the classical fear conditioning procedures (see below), and 2) the “CFC” group which was only exposed to the classical fear conditioning procedure. Within each of the two behavioral treatment groups, rats were then assigned to one of three drug treatment groups: 1) the “Sham” group which was dosed with isotonic saline, 2) the “CEF” group which was dosed with ceftriaxone (see below), and 3) the “DHK” group which was dosed with dihydrokainic acid (see below). Power analysis was performed to calculate sample size using JMP software with a value of 0.8 as the cutoff for the lowest acceptable power. This analysis resulted in a requirement of 10 rats per group. This yielded 6 total distinct groups as shown in Table 1 below with each containing 10 rats:

Table 1. Treatment Design

	SPS	CFC
Sham (saline)	Grp 1	Grp 2
Ceftriaxone (200 mg/kg) (Glu re-uptake facilitator)	Grp 3	Grp 4
DHK (10 mg/kg) (Glu re-uptake inhibitor)	Grp 5	Grp 6

DHK = dihydrokainate; SPS = Single Prolonged Stress; CFC = Classical Fear Conditioning

The 10 rats in each group were then divided into one of two blocks (5 rats/group/block). For practical reasons, all procedures could not be performed on all ten rats within a single day, and so the groups were blocked in this manner to account for different starting dates among rats (see below). Immediately following a 7-day quarantine period the treatments were administered to, and behavioral measures assessed for, the specified groups of rats in Block 1 over a 10-day period according to the timeline in Table 2 below:

**Table 2: Treatment Timeline**

	<b>Day 1</b>	<b>Day 2</b>	<b>Day 3</b>	<b>Day 4</b>	<b>Day 5</b>	<b>Day 6</b>	<b>Day 7</b>	<b>Day 8</b>	<b>Day 9</b>	<b>Day 10</b>
SPS	Grps 1, 3, 5									
Ceftiaxone		Grps 3, 4	Grps 3, 4	Grps 3, 4	Grps 3, 4	Grps 3, 4	Grps 3, 4	Grps 3, 4	Grps 3, 4	
DHK									Grps 5, 6	
Saline Sham		Grps 1, 2, 5, 6	Grps 1, 2, 5, 6	Grps 1, 2, 5, 6	Grps 1, 2, 5, 6	Grps 1, 2, 5, 6	Grps 1, 2, 5, 6	Grps 1, 2, 5, 6	Grps 1, 2	

Starting on Day 8, behavioral assessments were conducted to assess the effects of the above treatments on fear expression/memory and fear extinction memory. In Table 3 below, the timeline and descriptions of these assessments are provided.

**Table 3: Behavioral Assessment Timeline**

	<b>Day 1</b>	<b>Day 2</b>	<b>Day 3</b>	<b>Day 4</b>	<b>Day 5</b>	<b>Day 6</b>	<b>Day 7</b>	<b>Day 8</b>	<b>Day 9</b>	<b>Day 10</b>
EPM								All Grps		
Classical Conditioning										
i. CFC								All Grps		
ii. EXT1									All Grps	
iii. EXT2										All Grps

EPM = Elevated Plus Maze; CFC = Classical Fear Conditioning; EXT1 = First Extinction; EXT2 = Second Extinction (See below for details)

The same timeline was used for the groups of rats in Block 2. Day 1 of treatments for Block 2 was started on Day 4 of treatment for Block 1.

Single Prolonged Stress (SPS) Treatment:

SPS, a regimen that is often used to produce an animal model of PTSD (see Section f.i. of the Literature Review), was performed using the rats from Groups 1, 3, and 5 starting at 7 AM. For two hours, these rats were physically restrained in a tail-vein restraining device (diameter = 2.75 inches) from Braintree Scientific Inc (Braintree, MA). that had one open end and one closed end. The tube was slotted down its length to allow a rat to be pulled in by the tail, and the closed end had a hole in the center to accommodate the tail during restraint. The device utilized a plunger inserted into the open end to decrease the amount of space for the rat to move within the tube. Rats were pulled into the restraint devices by the tail, and the plunger was inserted into the open end. The plunger was pushed down the length of the tube until the rat was completely immobilized.

Immediately after this two hour period of restraint, rats were placed together (in groups of three with one rat each from Group 1, 3, and 5) into a cylindrical plastic container (30 cm in diameter and 60 cm in height) filled with water (~25 degrees Celsius). The water level was high enough to ensure that the rats' tails could not reach the bottom of the container while swimming at the surface. Rats were allowed to swim for 20 minutes or until any sign of imminent drowning were noted. A sign of "imminent drowning" included 1) the subject making continuous physical efforts to reach the surface but failing to do so within 5 seconds while unimpeded by other subjects, 2) the subject failing to attempt to reach the water surface for a duration of more than 5 seconds while unimpeded by other subjects, or 3) the subject beginning to choke while underneath the water surface.

Following this, rats were towel dried and given a 10 minute rest period. Together they were then placed into a bell jar that had a removable false bottom (bell jar dimensions: 20 cm diameter X 25

cm height from false bottom to top of jar). Prior to placing the rats into the jar, five gauze pads were soaked with diethyl ether and placed under the false floor. Rats remained in the jar until they lost the righting reflex and did not respond to tail pinch. Once these signs were noted, rats were returned to their home cages. Rats from Groups 2, 4, and 6 were transported to the treatment area, but remained in their cages and received none of the above treatments.

#### Drug Administrations (Ceftriaxone and DHK):

All injections were given via the subcutaneous route between the shoulder blades, and doses were calculated following daily body weight measurement. Ceftriaxone (ceftriaxone disodium salt hemi(heptahydrate), Sigma-Aldrich, St. Louis MO, #1098184), a glutamate re-uptake facilitator, was reconstituted in enough saline to yield a 80 mg/mL ceftriaxone solution, which was administered to Groups 3 and 4 once daily at a dosage of 200 mg/kg on Days 2 – 9 at 7 AM. The volumes of CEF doses ranged from 0.25 mL to 0.5 mL.

DHK (dihydrokainic acid >98% HPLC powder, Sigma-Aldrich, St. Louis MO, #D1064), a glutamate re-uptake inhibitor, was reconstituted in enough saline to yield a 4 mg/mL DHK solution, which was administered to Groups 5 and 6 at a dosage of 10 mg/kg on Day 9 (thirty minutes prior to the Extinction Trial of that day). The volumes of DHK doses ranged from 0.25 mL to 0.5 mL depending on rat weight. Rats in any group not receiving either ceftriaxone or DHK on a given day between Days 2 – 9 received a subcutaneous sham injection with isotonic saline. The volumes of isotonic saline were calculated by determining the volume of CEF that a given rat of the same weight assigned to the CEF-treated groups would have received, and thus ranged from 0.25 mL to 0.5 mL. It should be noted that the calculated volume of saline would have been the same if DHK had been used for this purpose instead of CEF.

#### Elevated Plus Maze (EPM) Assessment:

All rats were tested for general anxiety behavior at 8 AM using an Elevated Plus Maze (EPM) to determine if SPS effectively induced a state of increased general anxiety in exposed rats. The maze consisted of an elevated platform (60 cm off the floor) that had four arms (50 cm long and 10 cm wide) in the shape of a “+” sign. Two of the opposing arms were enclosed by 40 cm high, opaque walls and dimly lit while the other two opposing arms remained open without any walls and well lit. This variation in lighting was achieved by turning off the overhead fluorescent lights in the room and turning on a 75-watt incandescent light at each of the open ends. These lights were positioned 30 cm away from the open arms at a level that would illuminate the open arms directly, but only provide dim indirect light to the closed arms. Individually, rats were placed in the center square (10 cm X 10 cm) of the “plus” facing an open arm, and allowed to move freely about the maze for 5 minutes. A vertically mounted digital camera was used to record activity over the entire 5 minute period using Any-Maze software. For each rat, these videos were used to calculate the time spent within open arms, time spent within enclosed arms, and time spent in the central square. An index of general anxiety behavior was then calculated by dividing “time spent in open arms” by the “time spent in open arms + time spent in closed arms”.

## Classical Conditioning Assessment

### i. Classical Fear Conditioning (CFC):

On Day 8, at 10 AM (after dosing), all rats were exposed to Classical Fear Conditioning training. Four rats were trained concurrently in individual training chambers that were each enclosed within a sound attenuating box (Habitest Modular System, Coulbourn Instruments, Holliston MA). The training chambers had clear polycarbonate walls and a stainless steel grid floor that was attached to a current source (the overall dimensions of a single chamber are 25(H) X 25 (W) X 30 (L) cm). Each was illuminated with a red incandescent house light.

Rats were placed into the conditioning chamber and allowed to explore/acclimate for 180 seconds. After this period, the rats were presented with a white LED light (Coulbourn Instruments, Holliston MA, H11-01R-LED), mounted to the side of the test chamber for 10 seconds. Nine seconds after the beginning of this light presentation, a 1-second shock (1 mA) was delivered through the floor such that the last full second of the light presentation coincided with a shock. This 10 second light/shock presentation was followed immediately by a 50 second rest interval. This was repeated four additional times to yield five total light-shock pairings (each separated by a 50 second rest interval).

#### ii. First Extinction (EXT1):

Twenty-four hours after the start of CFC (Day 9), at 10 AM (after dosing), all rats were exposed to a nine-minute Fear Extinction session. They were re-introduced to the training chambers without presentation of shocks. Animals were placed in training chambers and allowed to acclimate for 180 seconds. Following this period, animals were exposed to the same visual cue (LED light) used during fear conditioning (10 second duration), but without the presentation of any shock. The visual cue was presented 6 times with 50 seconds separating each presentation.

#### iii. Second Extinction (EXT2):

Twenty-four hours after the start of EXT1, at 10 AM (Day 10), all rats were exposed to a second nine-minute Fear Extinction session (EXT2). This was performed in an identical fashion to that of the previous day's Fear Extinction session.

#### Analysis of Fear Behavior:

During CFC (8 min), EXT1 (9 min), and EXT2 (9 min) sessions, the behavior of each rat in the training chambers was recorded by a vertically-mounted digital camera. Digital files were analyzed using

FreezeFrame software (ActiMetrics, Wilmette, IL) to quantify duration of freezing behavior of rats. Changes in freezing behavior were used to infer a change in the state of fear in this project. Freezing behavior was defined as a complete lack of movement (aside from that associated with respiration) that persisted for longer than 1 second. In the CFC, EXT1, and EXT2 sessions, duration of expressed freezing behavior was measured during the 180 second Acclimation period and during each of the 10-second light-on intervals.

For Acclimation, freezing behavior duration was measured during the first, second, and third minute of this period. The freezing behavior duration was then divided by 60 seconds, yielding fear expression data in the form of “percentage of time exhibiting freezing behavior” (i.e., % freezing) for each of the three minutes of Acclimation of the respective CFC, EXT1, and EXT2 sessions.

For each light-on interval, freezing behavior duration was measured during the 10 seconds of light presentation. The freezing behavior duration was then divided by 10 seconds (i.e., the total duration of a light-on interval), yielding fear expression data in the form of “percentage of time exhibiting freezing behavior” (i.e., % freezing) for each of the light-on presentations of the respective CFC, EXT1, and EXT2 sessions. The 10-second intervals were grouped so as to divide each respective CFC, EXT and EXT2 session into subdivisions (see chart below). For CFC, only the % freezing time during the first light presentation was used in the analysis (see below for justification). For each rat in EXT1 and EXT2, % freezing across the three 10-second time intervals within each subdivision was averaged to yield a mean % freezing for each subdivision.

For simplicity, the subdivisions were eventually used for statistical analysis were given the following letter designations in Table 4 below:

Table 4. Letter Designations for Behavioral Session Sub-divisions

Experimental Day	Sub-divisions of Session	Designation
Day 8: CFC	Acclimation Period (three 60-second intervals)	
	“Light-shock” Pairing Period (only the first light-shock pairing was used for analysis)	A
Day 9: EXT1	Acclimation Period (three 60-second intervals)	
	Early “Light-no shock” Period (30 seconds; first three exposures to “light-no shock”)	B
	Late “Light-no shock” Period (30 seconds; last three exposures to “light-no shock” pairing)	C
Day 10: EXT2	Acclimation Period (three 60-second intervals)	
	Early “Light-no shock” Period (30 seconds; first three exposures to “light-no shock”)	D
	Late “Light-no shock” Period (30 seconds; last three exposures to “light-no shock” pairing)	

The primary aim of this analysis was to assess the effects of treatment on fear extinction memory retention. A fear extinction memory index for each rat was calculated by subtracting the % freezing of “C” from “D”, representing the change in fear expression between the late part of the first extinction session on Day 9 (EXT1 (late)) and the early part of the second extinction session Day 10 (EXT2 (early)). The smaller the value of this fear extinction memory index the greater the retention of fear extinction memory. The mean of this calculated dependent variable was found across rats, within each treatment group, and subsequently used in the statistical analysis described below.

It should be noted that an alternative approach could have been used to calculate this index. More specifically, the index could have been calculated by subtracting EXT1 (early) from EXT2 (early) (i.e., subtracting “B” from “D”). In this case, a larger value index would represent a greater retention of fear extinction memory. However, this approach does not take into account the possibility of variations in extinction learning. If there was a difference in magnitude of extinction learning during EXT1 between

the SPS groups and the CFC groups, this could potentially contribute to a significant difference in % freezing time between the SPS groups and the CFC groups at the beginning of EXT2. For example, if fear expression decreased significantly less during EXT1 for SPS rats compared to CFC rats, then it might be expected that the SPS rats would exhibit significantly more fear at the beginning of EXT2 compared to CFC rats. This could potentially result in a significant difference between “D-B” indices regardless of how well those memories were being retained after 24 hours. Since a “D-C” index is dependent on how much fear is expressed at the end of EXT1 and at the beginning of EXT2, it is less susceptible to the potentially confounding effects of differences in extinction learning magnitude. Furthermore, by employing late EXT1 in our index, extinction learning has greater chance to develop before its retention is evaluated. As such, the “D-C” calculation was selected as the preferred method.

To validate our model, additional comparisons were also made. Firstly, to determine if CFC induced a condition fear of the light presentation, “A” was compared to “B”. The rationale for this comparison was based on the fact that the first “Light-shock” pairing of CFC was the only time rats were exposed to the light without ever being previously exposed to a shock, thus representing their baseline reaction to this stimulus. It was expected that if the CFC procedure successfully induced a conditioned fear memory, then expression of freezing behavior in “B” would be significantly greater than that expressed in “A”.

Secondly, to determine if the parameters of our extinction sessions reduced fear expression over time, “B” was compared to “C”. It was expected that the repeated presentation of the light without a shock would extinguish the conditioned fear of the light, and thus expression of freezing behavior during “C” would be significantly less than that expressed in “B”.

Finally, to determine if the parameters of the extinction sessions reduced expression of freezing behavior equivalently between groups, “C” was subtracted from “B” for each treatment group. This

yielded indices of “extinction learning” that represented the magnitude of change in expression of freezing behavior over time during an extinction session. These indices were then compared between groups with the expectation that no significant difference would be found, indicating that there was no effect of treatment on a rat’s response to extinction training.

Statistical Analysis:

To determine the effect of treatments on fear extinction memory and conditioned fear memory, comparisons were made between treatment groups using the corresponding mean calculated values (described above) as dependent variables. According to our hypothesis, 1) SPS treated rats should exhibit impaired fear extinction memory compared to the CFC group, 2) ceftriaxone should have an attenuating effect on this SPS-induced memory impairment, and 3) DHK should exacerbate the SPS-induced impairment of fear extinction memory recall. As such, the following key comparisons were made to test this hypothesis (note that Table 1 has been provided again for reference):

Table 1. Treatment Design

	SPS	CFC
Sham (saline)	Grp 1	Grp 2
Ceftriaxone (200 mg/kg) (Glu re-uptake facilitator)	Grp 3	Grp 4
DHK (10 mg/kg) (Glu re-uptake inhibitor)	Grp 5	Grp 6

1. Group 1 was compared to Group 2 to determine the effect of SPS, respectively, on retention of fear extinction memory. Between these two groups, it was expected that the fear extinction memory index values for Group 1 would be significantly more positive than Group 2, which would represent an SPS-induced impairment of fear extinction memory retention

(recall that a smaller fear extinction memory index value reflects greater fear extinction memory).

2. Group 1 was compared to Group 3 to evaluate the potential attenuating effect of ceftriaxone, a glutamate re-uptake facilitator, on SPS-induced extinction memory deficits. Between these two groups, it was expected that the fear extinction memory index values for Group 1 would be significantly more positive than Group 3, which would represent improved fear extinction memory retention in SPS rats treated with ceftriaxone.
3. Group 1 was compared to Group 5 to evaluate the potentially exacerbating effect of DHK on the SPS-induced extinction memory deficit. Between these two groups, it was expected that the fear extinction memory index values for Group 5 would be significantly more positive than Group 1, which would represent poorer fear extinction memory in DHK-treated rats compared to SPS-treatment alone.

A statistician was consulted regarding the most appropriate tests for statistical significance to analyze the data generated from this study. A Two-Way, mixed model ANOVA was performed to evaluate the differences between the “fear extinction memory” indices. Post-hoc Holm-Tukey pair-wise comparisons were then performed to determine the effect of SPS on expression of freezing behavior within each drug treatment group. The threshold of statistical significance was set at  $p = 0.05$ , where  $p$ -values of less than 0.05 were considered an indication of statistical significance.

A Two-Way, mixed model ANOVA was performed to evaluate the differences between the expression of freezing behavior between “B” and “C”. Post-hoc Holm-Tukey pair-wise comparisons were then performed to determine if significant differences existed between “B” and “C” within each of the six treatment groups. The threshold of statistical significance was set at  $p = 0.05$ , where  $p$ -values of less than 0.05 were considered an indication of statistical significance.

A one-way non-parametric test (Kruskal-Wallis test) was performed to compare the “extinction learning” indices. Post-hoc Dunn’s Procedure of Multiple Comparisons was then performed as a pairwise comparison between groups. The threshold of statistical significance was set at  $p = 0.05$ , where  $p$ -values of less than 0.05 were considered an indication of statistical significance.

## **EXPERIMENT 2: EVALUATION OF THE EFFECT OF SPS ON GLUTAMATE TRANSPORTER EXPRESSION AND ON DOWNSTREAM CREB PHOSPHORYLATION IN THE AMYGDALA AND PREFRONTAL CORTEX**

### Tissue Collection:

Immediately following EXT2 (on Day 10), the rats used for Specific Aim 1 were anesthetized with isoflurane gas and decapitated. Immediately after, whole brains were rapidly removed (<5 minutes). Using a coronal rat brain matrix (a brain blocking mold; ASI Instruments, Warren MI, RBM4000C), each brain was cut with a razor blade into coronal sections (2-mm in thickness). A rat brain atlas of coronal brain sections and stereotaxic coordinates was used to confirm locations of the regions of interest, using the rostral border of the frontal lobe as a landmark. The infralimbic region of the PFC was collected from a 2-mm thick coronal section which had a rostral border that started 2-mm from the rostral border of the frontal lobe. The basolateral complex of the amygdala and the dorsal hippocampus were collected from a 2-mm coronal section which had a rostral border that started 9-mm from the rostral border of the frontal lobe. Sections were mounted on lysine coated slides using Tissue-Tek O.C.T. compound (VWR, Radnor PA, #25608-930), and flash frozen by submerging the slides into liquid isopentane containing dry ice.

Within the coronal sections, the dorsoventral and mediolateral positions of the structures of interest were also determined using the brain atlas. The position of the structures was noted relative to anatomical landmarks that would be easily identified in the frozen unstained tissue. All instruments, buffers, and slides were kept on ice to decrease tissue degradation. For the infralimbic region of the PFC, a 1-mm diameter microbiopsy punch (Ted Pella Inc., Redding CA, Harris Uni-Core, #15074) was used to take a single sample from both cerebral hemispheres. The dorsal border of the olfactory bulbs served as the landmark for the ventral border of the ventromedial PFC and the medial longitudinal fissure served as the landmark for the medial border (Diagram 14A, see page 203). The two individual tissue samples were then pooled together into a single sample.

For the BLA of the amygdala, a 1-mm micro biopsy punch was used to take a single sample from both cerebral hemispheres. The external capsule served as the landmark for the dorsal border, and the cerebral cortex served as the landmark for the lateral and ventral borders (Diagram 14B, see page 203). The two individual tissue samples were then pooled together into a single sample.

For the hippocampus, a 1-mm micro biopsy punch was used to collect multiple samples containing the CA1 region. For each hemisphere, collection began at the midline immediately ventral to the dorsal hippocampal commissure. Collection of tissue continued laterally along the ventral border of the dorsal hippocampal commissure until the lateral ventricle was reached (Diagram 14C, see page 203). All individual tissue samples were then pooled together into a single sample.

Immediately following collection, all samples were placed in 300 microliters of a premade lysis buffer (RIPA buffer, Sigma, St. Louis MO, R0278), and homogenized yielding an approximate 1:150 v/v homogenate. These samples were stored in 1.5 mL Eppendorf tubes at -80 degrees C for future biochemical analysis.

#### Biochemical Analysis:

The frozen homogenized samples were placed on ice until they were completely thawed. Samples were then centrifuged at 15000 x *g* for 20 minutes. All of the supernatant was collected, and the pellet was discarded. Total protein concentration of each test sample was determined using a BCA protein assay kit (ThermoFisher Scientific, Grand Island NY, #23225). Ten microliters of each sample were prepared following the manufacturers' instructions. Samples were loaded into a 96-well plate along with the nine BSA standards of different known concentrations provided in the kit. Using spectrophotometry, absorbance at 562 nm was measured for each sample and standard. A standard curve was plotted using the data points of the nine standards with "protein concentration" in micrograms/mL on the x-axis and "Net Absorbance" at 562nm on the y-axis. This standard curve was

then used to extrapolate the total protein of a given test sample using the absorbance data of that sample. Once total protein was determined, the samples were divided evenly into two Eppendorf tubes, one to be used for western blot analysis and the other for ELISA analysis (see below).

Western blot analysis was performed to quantify the expression of the astrocyte glutamate transporters GLT-1 and GLAST in the amygdala and PFC, as well as glucocorticoid receptor (GR) in the hippocampus. Volumes of a KRH sample buffer (116 mM NaCl, 4 mM KCl, 1 mM MgCl<sub>2</sub>, 1.8 mM CaCl<sub>2</sub>, 25 mM Glucose, 10 mM HEPES, pH = 7.4) were calculated and added to each in order to yield 3 micrograms of protein per microliter of sample. Samples were then divided into 50 microliter aliquots and 50 microliters of premade 2X Laemmli buffer (Bio-Rad, Hercules CA, #1610737) was added to each aliquot, yielding a final protein concentration of 1.5 micrograms of protein per microliter of sample. Samples were placed in a water bath at 100 degrees C for 5 minutes to denature proteins. At this point, samples were placed on ice and used immediately for protein quantification using western blot or ELISA (see below) or stored for later use at -80 degrees C.

Western blot was performed to quantify the two major astrocyte glutamate transporters in the brain, GLT-1 and GLAST. Rabbit anti-rat primary antibodies (Millipore, Billerica MA) against GLT-1 and GLAST were used with amygdala and PFC samples. Additionally, rabbit anti-rat primary antibodies against glucocorticoid receptor (Abcam, Cambridge MA) were also used with hippocampal samples to quantify changes in GR expression as a biochemical marker of successful induction of a PTSD-like state by SPS that has been previously observed (Knox et al., 2012). While efforts were made to ensure that equivalent amounts of total protein were tested per sample, beta-tubulin was also quantified to control for any variations in GLT-1 or GLAST that might be due to technical errors. Beta-tubulin was selected as a control since it is a ubiquitous cellular protein that should be expressed equivalently across brain regions. As such, variations in beta-tubulin measurements would be indicative of variations in the amount of total protein that was loaded between samples. Rabbit anti-rat primary antibodies against

beta-tubulin (Abcam, Cambridge MA) were used as a control during the quantification of the test proteins to determine relative changes in protein expression. All primary antibodies were prepared by dilution with tris-buffered saline (TBS) buffer (19 mM Tris-Cl, 137 mM NaCl, 2.7 KCl, pH = 7.4) to a 1:500 concentration. IRDye 800CW goat anti-rabbit IgG secondary antibodies (Li-Cor, Lincoln NE, 925-32211) with fluorescent dye tags with excitation wavelengths of 780 nm and emission wavelengths of 820 nm were used. All secondary antibodies were prepared by dilution with TBS buffer to a 1:10,000 concentration.

Fifteen microliters of sample containing 15 micrograms of total protein, as previously determined by the BSA assay kit, was loaded into a precast SDS-PAGE gel (Bio-Rad, Hercules CA). Gels were loaded with samples from one rat from each of the six groups. Gels had 15 lanes, and samples were run in duplicate, so only samples from one brain region could be run per gel. Gels ran at 100 V in a mini-PROTEAN Tetra Vertical Electrophoresis Cell (Bio-Rad, Hercules CA) until enough separation was achieved between bands so that each could be individually identified (about 1 to 2 hours). Proteins were then transferred to a nitrocellulose membrane (Bio-Rad). The nitrocellulose membrane was placed in 10 mL of a pre-made IRDye Blocking Buffer (Tris-based solution, pH = 7.6, Rockland, Pittsburg PA) for 1 hour at room temperature, followed by three 5-minute washes with TBS buffer plus tween 20 (TBST) (19 mM Tris-Cl, 137 mM NaCl, 2.7 KCl, 1 mL/L of tween 20, pH = 7.4). The membrane was then incubated in 10 mL of the diluted primary antibodies (see description above) at 4 degrees C overnight. The membranes were washed three times with TBST for 5 minutes each, and then incubated in 10 mL of the diluted secondary antibodies (see description above) for one hour at room temperature. Membranes were washed 3 times again with TBST for 5 minutes each.

An Odyssey Infrared Imaging system (LiCor, Lincoln NE) was used to detect and quantify the relative protein concentration of each test protein. This was accomplished by measuring the fluorescence intensity (in pixels) of the test sample band and the associated beta tubulin control band.

The fluorescence of the test band was divided by the fluorescence of the beta tubulin band, resulting in a data point that represented the test protein expression relative to beta tubulin expression. These data points were then used in the statistical analysis described below.

For quantification of p-CREB expression in the amygdala and PFC, a commercial ELISA kit (Abcam, Cambridge MA) was used. Sample supernatants were individually mixed with enough extraction buffer (provided by the ELISA kit) to make a final total protein concentration of 1  $\mu\text{g}/\mu\text{L}$  per sample. The ELISA plate and samples were prepared following the manufacturer's instructions along with eight positive control samples (provided in the kit), each with different pCREB concentrations. Test samples and control samples were loaded onto the plate in 50 microliter duplicates. All samples were incubated with the provided antibody cocktail for 1 hour at room temperature, after which the entire plate was washed three times with a provided wash buffer. Samples were then incubated with a tetramethylbenzidine substrate at room temperature for 15 minutes in the dark. A Stop solution was then added to each well and allowed to incubate for 1 minute. At that time, a spectrophotometer was used to measure absorbance at the 450 nm range and the resulting read outs of duplicate samples were averaged. A standard curve was plotted using the data points of the eight control samples with "protein concentration" in micrograms/mL on the x-axis and "Net Absorbance" at 450 nm on the y-axis. This standard curve was then used to extrapolate the p-CREB expression of a given test sample using the absorbance data of that sample.

For both the Western Blot and ELISA analyses, a Two-Way, mixed model ANOVA was performed to assess the effects of behavioral treatment (i.e., SPS exposure or no SPS exposure) and drug treatment (i.e., CEF, DHK, or Sham). Post-hoc Stepdown Dunnett pair-wise comparisons were then performed to determine the effect of SPS on expression of freezing behavior within each drug treatment group for each brain region. The threshold of statistical significance was set at  $p = 0.05$ , where p-values of less than 0.05 were considered an indication of statistical significance.

**EXPERIMENT 3: DETERMINING IF REGIONAL DIFFERENCES IN NMDA RECEPTOR SUBTYPE EXPRESSION CONFER DIFFERENTIAL PROTECTION OF P-CREB FROM EFFECTS OF GLUCOCORTICOIDS**

Sixteen male Sprague Dawley rats (150 – 200 grams) were used in this experiment. Rats were singly housed under a 12-hour light/dark cycle with ad libitum feed and water, and no environmental enrichment. Rats were assigned to either a “SPS” group (Group 1, N=8) which was exposed to both SPS and CFC paradigms, or a CFC control group (Group 2, N=8) which was exposed to CFC only.

After 7 days of quarantine, the treatments and behavioral assessments were administered to the specified groups over a ten day period according to the timeline below (see descriptions given above in “Experiment 1” for more detail):

Table 5. Treatment Design for Specific Aim 3 (*in vivo*)

	<b>Day 1</b>	<b>Days 2 - 7</b>	<b>Day 8</b>	<b>Day 9</b>	<b>Day 10</b>
SPS	Group 1	No treatments for either group			
EPM			Both Groups		
CFC			Both Groups		
EXT1				Both Groups	
EXT2					Both Groups

SPS = Single Prolonged Stress; EPM = Elevated Plus Maze; CFC = Classical Fear Conditioning; EXT1 = First Extinction Session; EXT2 = Second Extinction Session (See Specific Aims 1 above for details)

Tissue Collection:

Immediately following EXT2 (on Day 10), the rats used for Specific Aim 1 were anesthetized with isoflurane gas and decapitated. Immediately after, whole brains were rapidly removed (<5 minutes). Using a coronal rat brain matrix (a brain blocking mold; ASI Instruments, Warren MI, RBM4000C), each brain was cut with a razor blade into coronal sections (2-mm in thickness). A rat brain atlas of coronal

brain sections and stereotaxic coordinates was used to confirm locations of the regions of interest, using the rostral border of the frontal lobe as a landmark. The infralimbic region of the PFC was collected from a 2-mm thick coronal section which had a rostral border that started 2-mm from the rostral border of the frontal lobe. The basolateral complex of the amygdala and the dorsal hippocampus were collected from a 2-mm coronal section which had a rostral border that started 7-mm from the rostral border of the frontal lobe.

Samples of interest were then dissected from both hemispheres using a #15 scalpel blade. The PFC sample was taken from cerebral cortex that runs along the medial longitudinal fissure from the olfactory bulbs ventrally to the dorsal aspect of the cerebrum (Diagram 15A, see page 204). Amygdala samples were collected by cutting a wedge from the ventrolateral aspect of the section. This wedge had a dorsal border that extended from the rhinal fissure to the ventral aspect of the caudate putamen and a medial border that extended from the ventral commissure of the brainstem and cerebrum to the caudal aspect of the caudate putamen (Diagram 15B, see page 204). The hippocampus was identified by visual inspection using the dentate gyrus, CA1, and CA3 regions as characteristic morphological features, and was dissected out entirely using the cerebral cortex as the dorsal boundary and the thalamus as the ventral boundary (Diagram 15C, see page 204).

#### Thin Section Preparation and Biochemical/Pharmacological Analysis:

Immediately following dissection from the 2-mm coronal sections, tissues were mounted to a vibratome cutting block with the tissue oriented such that the blade of the vibratome cut the tissue in a dorsal-to-ventral direction. Once mounted, 300- $\mu$ m thick coronal sections were cut from each sample while in ice-cold artificial cerebrospinal fluid (119 mM NaCl, 26.2 mM NaHCO<sub>3</sub>, 2.5 mM KCl, 1 mM NaH<sub>2</sub>PO<sub>4</sub>, 1.3 mM MgCl<sub>2</sub>, 10 mM glucose, pH = 7.4). This yielded twelve 300- $\mu$ m sections (six sections per hemisphere) for each of the PFC, amygdala, and hippocampus for each rat. Using a small paint

brush, three sections of a given region were placed into a 15mm polyester mesh membrane insert (Capitol Scientific, Austin TX, COR-3477) that was then inserted into a 12-well plate. Enough calcium-deficient MEM media (Sigma, St. Louis MO, M8028) was then added to each well to just cover the tissues on top of the inserts. Plates were incubated for 30 minutes at 4 degrees C before biochemical analysis. Oxygenation of the media during incubation was not performed due to a concern that it would decrease the physical integrity of the slices prior to exposure to GCs, NVP, or Ro-25. However, it should be noted that the lack of oxygenation could have increased physiological injury to the tissue due to hypoxia. In an attempt to decrease the overall duration of the ex vivo experiment and thus limit the extent of hypoxic injury, tissues were exposed to GCs, NVP, or Ro-25 immediately following the 30 minute incubation at 4 degrees C. However, a downside to this counter measure was that sufficient time was likely not allowed for the damaged cells at the tissue surfaces to completely die. As such, the following analysis included the response to treatment of undamaged tissue at the core of the slices in addition to that of damaged tissue at the slice surfaces.

Next, while remaining in the inserts, the thin sections were transferred to a new 12-well plate. For each region (i.e., amygdala, hippocampus, and PFC), the thin sections were exposed to one of the following pharmacological treatments:

Table 6. Treatment Group Designations for Specific Aim 3 (*ex vivo*)

Drug TX	Behavioral Group	Group #
Sham (bicuculline only)	CFC	1
	SPS	2
Bicuculline + NR2A Inhibitor	CFC	3
	SPS	4
Bicuculline + NR2B Inhibitor	CFC	5
	SPS	6
Bicuculline + Corticosterone	CFC	7
	SPS	8

The temporal sequence for drug application for each group was as presented below in Table 7:

Table 7. Drug Exposure Design for Specific Aim 3 (*ex vivo*)

Group #	20 minute Pre-treatment	15 minute Bicuculline Treatment
1	Vehicle	50 $\mu$ M Bicuculline + 10 $\mu$ M Glycine (Groups 1-8)
2	Vehicle	
3	0.5 $\mu$ M NVP	
4	0.5 $\mu$ M NVP	
5	1.0 $\mu$ M Ro-25	
6	1.0 $\mu$ M Ro-25	
7	100 nM GC	
8	100 nM GC	

GC = Corticosterone; NVP = NVP-AAM077 (NR2A-specific inhibitor); Ro-25 = Ro-25-6981 (NR2B-specific inhibitor); Vehicle = 0.1% ethanol (v/v) in MEM media

The vehicle for all pre-treatments was 0.1% ethanol (v/v) in MEM. Pre-treatment solutions of corticosterone (Sigma, St. Louis MO, #27840), NVP-AAM077 tetrasodium hydrate (NVP) (Sigma, St. Louis MO, #459836-30-7), and Ro-25-6981 hydrochloride hydrate (Sigma, St. Louis MO, R7150) were prepared fresh using this vehicle to final concentrations of 100nM, 0.5  $\mu$ M, and 1.0  $\mu$ M, respectively. Enough pre-treatment solution was added to each well to just cover the tissues on the well inserts, and tissues were allowed to incubate for 20 minutes at 37 degrees C in a hot water bath. Immediately following this 20-minute period, tissues were washed three times in room temperature calcium-containing MEM, and then transferred to another 12-well plate sitting at room temperature. A treatment solution was prepared by adding bicuculline and glycine to calcium-containing MEM to a final concentration of 50  $\mu$ M bicuculline (Sigma, St. Louis MO, #14343) and 10  $\mu$ M glycine. This solution was kept at room temperature once prepared. Once pretreatment was completed, as described above, the treatment solution was added to each well such that the tissues in the inserts were just covered. The plate was allowed to incubate at room temperature for 15 minutes. All samples were then washed three times in PBS at room temperature.

Immediately after this treatment, each set of three thin slices, which had been treated together in a given insert, were pooled together. One mL of a Cell Extraction Buffer from a commercial ELISA kit (Abcam, Cambridge MA) was added to each pooled sample. Samples were homogenized, transferred to

Eppendorf tubes, and incubated on ice for 20 minutes. Samples were then centrifuged at  $18000 \times g$  for 20 minutes at 4 degrees C. All of the supernatant was collected, and the pellet was discarded. Total protein concentration of each test sample was determined using a BCA protein assay kit (ThermoFisher Scientific, Grand Island NY, #23225). Ten microliters of each sample were prepared following the manufacturers' instructions. Samples were loaded into a 96-well plate along with the nine BSA standards of different known concentrations provided in the kit. Using spectrophotometry, absorbance at 562 nm was measured for each sample and standard. A standard curve was plotted using the data points of the nine standards with "protein concentration" in micrograms/mL on the x-axis and "Net Absorbance" at 562nm on the y-axis. This standard curve was then used to extrapolate the total protein of a given test sample using the absorbance data of that sample.

A commercial ELISA kit (Abcam, Cambridge MA) was then used to measure p-CREB expression in each sample. Sample supernatants were individually mixed with enough extraction buffer (provided by the ELISA kit) to make a final total protein concentration of  $1 \mu\text{g}/\mu\text{L}$  per sample. The ELISA plate and samples were prepared following the manufacturer's instructions along with eight positive control samples (provided in the kit), each with different p-CREB concentrations. Test samples and control samples were loaded onto the plate in 50 microliter duplicates. All samples were incubated with the provided antibody cocktail for 1 hour at room temperature, after which the entire plate was washed three times with a provided wash buffer. Samples were then incubated with a tetramethylbenzidine substrate at room temperature for 15 minutes in the dark. A Stop solution was then added to each well and allowed to incubate for 1 minute. At that time, a spectrophotometer was used to measure absorbance at the 450 nm range and the resulting read outs of duplicate samples were averaged. A standard curve was plotted using the data points of the eight control samples with "protein concentration" in micrograms/mL on the x-axis and "Net Absorbance" at 450 nm on the y-axis. This

standard curve was then used to extrapolate the p-CREB expression of a given test sample using the absorbance data of that sample.

#### Statistical Analysis:

To determine the effect of these treatments on the molecular mechanisms of memory formation in which we are interested, comparisons of the p-CREB protein expression dependent variable was made between treatment groups. According to our hypothesis, 1) glucocorticoids should decrease p-CREB in the hippocampus and PFC significantly more than in the amygdala in CFC rats, 2) NR2A inhibitors should decrease p-CREB expression in the hippocampus and PFC significantly more than NR2B inhibitors in CFC rats, 3) NR2B inhibitors should decrease p-CREB expression in the amygdala significantly more than they do in the hippocampus and PFC in CFC rats, and 4) the effect of NR2A and NR2B inhibitors should not change between CFC and SPS-exposed rats.

As such, the following key comparisons were made to test this hypothesis:

1. Within the two behavioral groups (SPS and CFC), p-CREB expression of the Sham-treated group was compared to that of the NVP, Ro-25, and GC drug treatment groups in each of the three regions. This comparison aimed to determine the effect of drug treatment upon p-CREB expression within the two behavioral treatment groups.
2. To determine regional differences in the magnitude of change in p-CREB as a result of drug treatment, comparisons were made between brain regions within the CFC tissues. For example, to determine regional differences in the magnitude of change in p-CREB expression as a result of NVP administration in CFC tissues, values representing the change in p-CREB expression were calculated in the following manner:

$$\underline{(NVP_{\text{Hippo}} - \text{Sham}_{\text{Hippo}}) \text{ and } (NVP_{\text{Amyg}} - \text{Sham}_{\text{Amyg}}) \text{ and } (NVP_{\text{PFC}} - \text{Sham}_{\text{PFC}})}$$

These three values were then compared to determine if there were regional differences in the effects of NVP upon p-CREB expression. This process was repeated for GC and Ro-25.

Table 8. Magnitude of Change Index Designations

Comparison	Represents:	Index Designation	Designation with Region
Group 1 - Group 3	The magnitude of change resulting from NVP administration within CFC	Index <sub>NVP/CFC</sub>	Index <sub>NVP/CFC/Hippo</sub>
			Index <sub>NVP/CFC/Amyg</sub>
			Index <sub>NVP/CFC/PFC</sub>
Group 1 - Group 5	The magnitude of change resulting from Ro-25 administration within CFC	Index <sub>Ro-25/CFC</sub>	Index <sub>Ro-25/CFC/Hippo</sub>
			Index <sub>Ro-25/CFC/Amyg</sub>
			Index <sub>Ro-25/CFC/PFC</sub>
Group 1 - Group 7	The magnitude of change resulting from GC administration within CFC	Index <sub>GC/CFC</sub>	Index <sub>GC/CFC/Hippo</sub>
			Index <sub>GC/CFC/Amyg</sub>
			Index <sub>GC/CFC/PFC</sub>

GC = Corticosterone; NVP = NVP-AAM077 (NR2A-specific inhibitor); Ro-25 = Ro-25-6981 (NR2B-specific inhibitor); CFC = Classical Fear Conditioning; SPS = Single Prolonged Stress; Hippo = hippocampus; Amyg = amygdala; PFC = prefrontal cortex

For the analysis of the effect of drug treatment on p-CREB expression within the two behavioral treatment groups, a Two-Way, mixed model ANOVA was performed. Post-hoc Step-down Dunnett pairwise comparisons were then performed to determine the effect of drug treatment on expression of p-CREB within each behavioral treatment group for each brain region. The threshold of statistical significance was set at  $p = 0.05$ , where p-values of less than 0.05 were considered an indication of statistical significance.

To determine if regional differences in the magnitude of change in p-CREB expression exist following drug treatment, a Two-Way, mixed model ANOVA was also performed. Post-hoc Holm-Tukey

pair-wise comparisons were then performed to determine the effect of drug treatment on the magnitude of change in expression of p-CREB for each brain region of CFC rats. The threshold of statistical significance was set at  $p = 0.05$ , where p-values of less than 0.05 were considered an indication of statistical significance.

# CHAPTER IV

## *RESULTS*

## **IV. Results**

### **SECTION A: SPECIFIC AIM 1: EVALUATING HOW SPS-INDUCED PERSISTENT FEAR IS ALTERED BY MANIPULATION OF ASTROCYTIC GLUTAMATE TRANSPORT**

#### **a.i. Validation of SPS model: Demonstration of induced fear extinction memory recall deficit**

The primary goal of this research was to use the single prolonged stress paradigm as an animal model of PTSD with the purpose of better understanding the pathophysiology of memory alterations associated with this disorder. As mentioned earlier, one characteristic memory alteration seen in PTSD subjects is the inability to maintain long-term extinction memories, a phenomenon termed “impaired fear extinction memory recall” (Milad et al., 2009). This study was designed to use impaired fear extinction memory recall as a behavioral indicator of the presence and/or severity of a PTSD-like symptom in our rats in order to study its neural substrates.

To illustrate the concept of impaired fear extinction memory recall, Diagrams 1 and 2 depict the fear expression of a single control rat from the “CFC-Sham” group (i.e., no SPS exposure, saline-treated) and a single rat from the “SPS-Sham” group (i.e., SPS exposure, saline-treated), respectively. These individual examples conform most closely to our hypothesized similarities/differences between groups when the SPS procedure is used. Fear expression, as indicated by exhibition of freezing behavior, of these two rats was measured during each 10-second “Light-on” time interval of the first and second Extinction sessions and the Fear Conditioning session. As described in the Methods section, for each 10-second interval, the time (in seconds) a rat spent exhibiting freezing behavior was then divided by ten seconds to calculate “% Freezing Time”. In Figure 1, fear expression over the course of the experiment from one rat of the “CFC-Sham” group is shown. In this example, fear expression was absent during the acclimation period but sharply increased during the light-on periods following the paired presentation of the conditioned (i.e., the light) and unconditioned (i.e., the shock) stimuli (Figure 1.a).

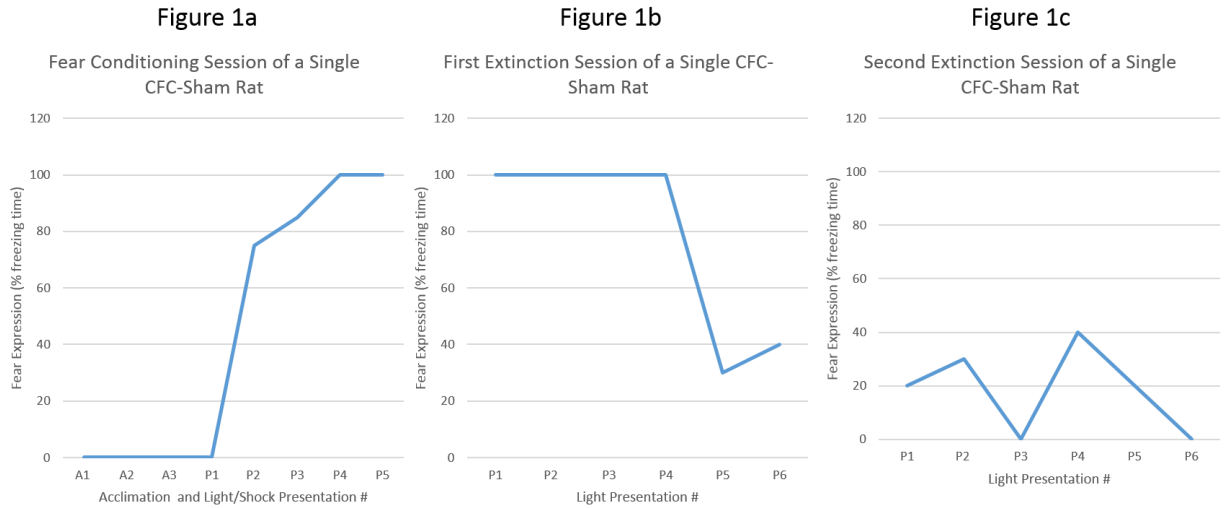


Figure 1. Depiction of fear expression in a single CFC-Sham treated rat during Fear Conditioning (1a), an initial Fear Extinction session 24 hours after fear conditioning (1b), and a second Extinction session 24 hours after the first Extinction session (1c). During Fear Conditioning, the rat was allowed 180 seconds to acclimate to the conditioning chamber (represented by the three 60s intervals designated A1, A2, A3) where no conditioned or unconditioned stimuli were presented (1a). As expected, no fear was exhibited at these acclimation points. However, delivery of a light paired with a shock (represented by the five 10s intervals designated P1, P2, P3, P4, and P5) induced strong fear expression, as seen in the last 5 time points of conditioning (1a). Twenty four hours later, the rat was placed in the chamber again and was exposed to 6 light presentations without concurrent shock (represented by the six 10s intervals designated P1, P2, P3, P4, P5, and P6) (1b). The rat showed high levels of fear expression early in the session, consistent with maintaining a fear memory induced by the conditioning process. Later in the session, however, fear expression diminishes which is consistent with fear extinction learning (1b). Finally, when the Extinction session was repeated 24 hours later, the rat exhibited low levels of fear early in the session that were comparable to the levels exhibited towards the end of the first Extinction session (1c). This is consistent with maintaining the memory of extinction learning from 24 hours earlier. SPS = single prolonged stress; CFC = classical fear conditioning.

After 24 hours, this rat demonstrated high levels of fear expression during the early presentations of the light without the shock, indicating that this rat formed an associative fear memory between the light and the shock (Figure 1.b). However, as more light presentations were made without shock, freezing behavior decreased, indicating that the rat began to learn that the light no longer predicted a shock delivery. In other words, this rat began suppressing the fear of the light, indicating the process of fear extinction learning (Figure 1.b). Twenty four hours after this first Extinction session, a second Extinction session was performed to see how well this learned fear extinction was remembered (i.e., fear extinction memory recall) (Figure 1.c). In the case of this rat, the fear expression at the

beginning of the second Extinction session was at comparable levels as that of the end of the first Extinction trial. This suggested that this rat had remembered that the light no longer predicted a shock delivery even after 24 hours had passed (i.e., fear extinction recall) (Figure 1.c).

Conversely, impaired fear extinction recall, relative to the CFC-Sham rat, is depicted in Figure 2. In this figure, behavior over the course of the experiment from one rat of the “SPS-Sham” group is shown. In this example of an SPS-exposed rat, fear expression increased following the paired presentation of the light and shock (Figure 2.a), similar in manner as the “CFC-Sham” rat.

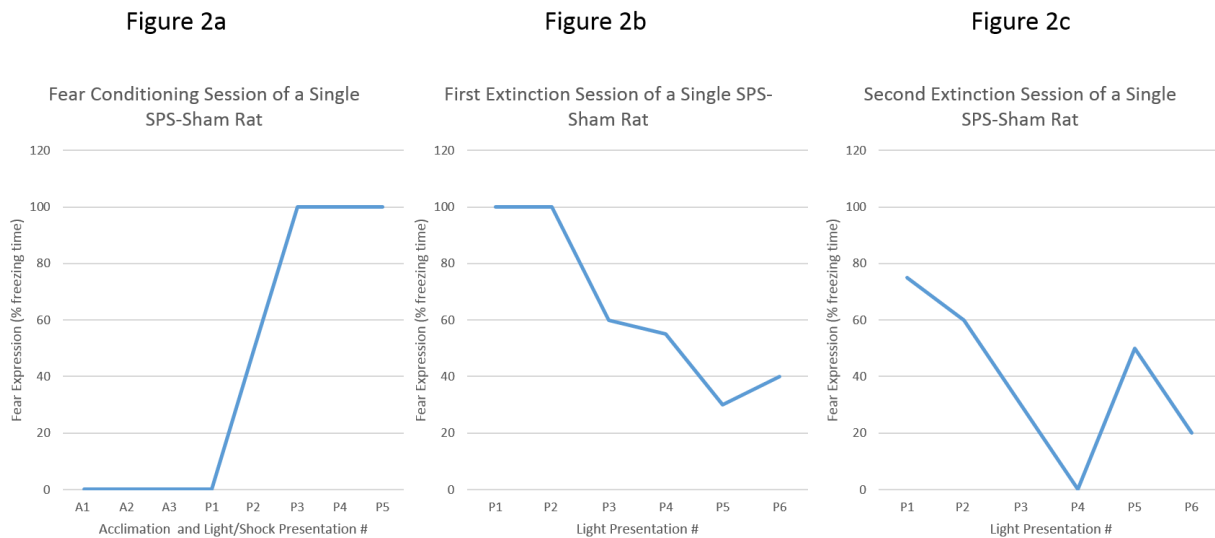


Figure 2. Depiction of fear expression in a single SPS-Sham treated rat during Fear Conditioning (2a), an initial Fear Extinction session 24 hours after fear conditioning (2b), and a second Extinction session 24 hours after the first Extinction session (2c). During Fear Conditioning, the rat was allowed 180 seconds to acclimate to the conditioning chamber (represented by the three 60s intervals designated A1, A2, A3) where no conditioned or unconditioned stimuli were presented (2a). As expected, no fear was exhibited at these points. Delivery of a light paired with a shock (represented by the five 10s intervals designated P1, P2, P3, P4, and P5) induced strong fear expression, as seen in the last 5 time points of conditioning (2a). Twenty four hours later, the rat was placed in the chamber again and was exposed to 6 light presentations without concurrent shock (represented by the six 10s intervals designated P1, P2, P3, P4, P5, and P6) (2b). The rat showed high levels of fear expression early in the trial, consistent with maintaining a fear memory induced by the conditioning process. Later in the trial, however, fear expression diminishes which is consistent with fear extinction learning (2b). Finally, when the Extinction trial was repeated 24 hours later, the rat exhibited higher levels of fear expression in the early part of the session compared to the levels exhibited towards the end of the first Extinction trial (2c). This is consistent with poorer maintenance of the memory of extinction learning from 24 hours earlier, compared with the CFC-Sham rat from

Figure 1, and is evidence of impaired fear extinction memory recall relative to the CFC-Sham rat. SPS = single prolonged stress; CFC = classical fear conditioning.

After 24 hours, this SPS-Sham rat also demonstrated high levels of fear behavior during the early presentations of the light without the shock, indicating that this rat also formed an associative fear memory between the light and the shock (Figure 2.b). Also similar to the “CFC-Sham” rat, repeated light presentations without the presence of a shock resulted in a progressive decrease in fear expression, indicating that this rat was also capable of fear extinction learning (Figure 2.b). However, a critical difference between these two rats is demonstrated in the second Extinction session. Compared to the “CFC-Sham” rat, the SPS-treated rat exhibited a notable increase in fear expression at the beginning of the second Extinction session relative to the end of the first Extinction session. This suggests that this rat’s ability to recall the fear extinction learning from the previous session was impaired (Figure 2.c) compared to the rat that was not subjected to SPS.

In Figure 3, mean fear expression for each light-on trial was calculated across rats, respectively, for the “SPS-Sham” and “CFC-Sham” groups. Plotted together, it can be seen that each group respectively exhibited the same fear extinction memory recall trend (Figure 3) as their single rat counterparts depicted in Figures 1 and 2. Additionally, the “SPS-Sham” group exhibited the same trend of impaired fear extinction memory recall (Figure 3), compared to the CFC-Sham group, as for the single rats depicted in Figures 1 and 2.

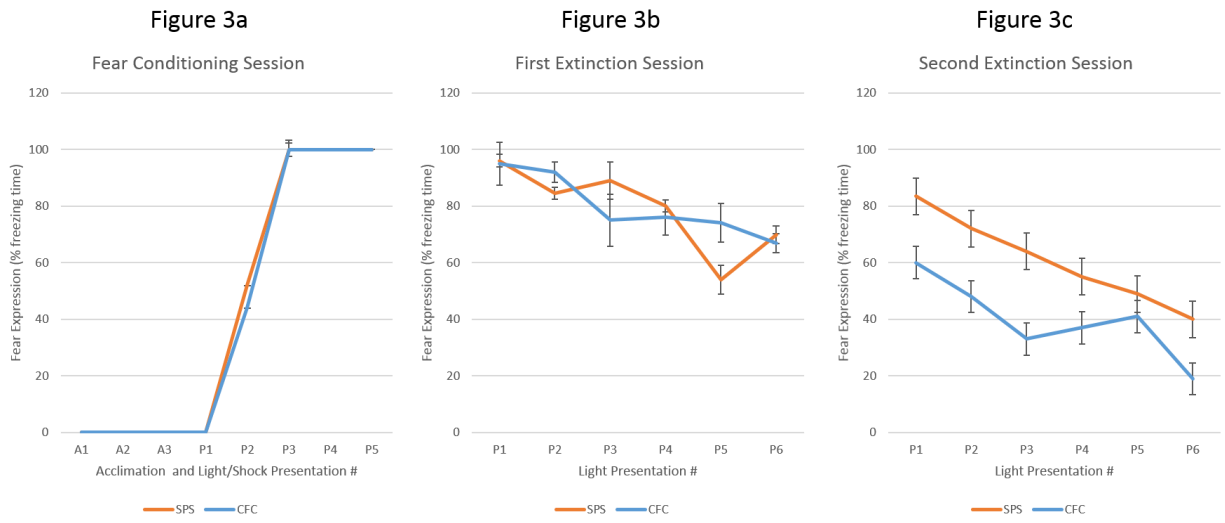


Figure 3. Depiction of mean fear expression across rats, for each time point, for the CFC-Sham and SPS-Sham groups during Fear Conditioning (3a), an initial Fear Extinction session 24 hours after fear conditioning (3b), and a second Extinction session 24 hours after the first Extinction trial (3c). During Fear Conditioning, both groups were allowed 180 seconds to acclimate to the conditioning chambers (represented by the three 60s intervals designated A1, A2, A3) where no conditioned or unconditioned stimuli were presented (3a). As expected, no fear was exhibited at these points in either groups. Delivery of a light paired with a shock (represented by the five 10s intervals designated P1, P2, P3, P4, and P5) induced strong fear expression, as seen in the last 5 time points of conditioning (3a). Twenty four hours later, the rats of both groups were placed in chambers again and exposed to 6 light presentations (represented by the six 10s intervals designated P1, P2, P3, P4, P5, and P6) without concurrent shock (3b). Both groups showed high levels of fear early in the session, consistent with maintaining a fear memory induced by the conditioning process. Later in the session, fear expression diminishes in both groups which is consistent with fear extinction learning (3b). Finally, when the Extinction trial was repeated 24 hours later, the CFC group exhibited levels of fear expression that were comparable to the levels exhibited towards the end of the first Extinction session, while the SPS group exhibited levels of fear expression that were higher compared to the levels exhibited towards the end of the first Extinction session (3c). This suggests that the CFC-Sham rats were more capable of maintaining fear extinction memories than the SPS-Sham rats, indicating that SPS induces an impairment of fear extinction memory recall. SPS = single prolonged stress; CFC = classical fear conditioning. Error bars represent standard deviations.

Though SPS is a well-established model of PTSD that has been reported to reliably induce impairment of fear extinction memory recall, this behavioral change was such an essential element to our study that it was necessary for us to demonstrate that we could reproduce this effect in a statistically significant fashion. Consequently, our first statistical analysis included a comparison of fear

extinction memory between the CFC-Sham group and the SPS-Sham group to determine if SPS treatment alone could induce an impairment of fear extinction memory recall as expected.

As mentioned above, determination of a fear extinction memory recall deficit is dependent upon conducting two Extinction sessions 24 hours apart from each other, comparing the fear expression near the end of the first session to the fear expression near the beginning of the second session, for each rat, to look at fear extinction recall, and then comparing this fear extinction recall measure between groups. A basic way of characterizing fear extinction recall, within a rat, would be to simply compare the fear expression of the last light presentation during the first Extinction trial to the first light presentation of the second Extinction trial. However, given the small number of light-on presentation trials within each session, and the inherently variable nature of behavioral dependent variables, this approach could yield a misleading representation of an individual rat's results. Figures 4 and 5 depict additional examples of single rats from each of the CFC-Sham and SPS-Sham groups, respectively. In Figure 4, this CFC-Sham rat exhibited the decrease in fear expression that was typical for a sham subject during the first Extinction session (Figure 4b).

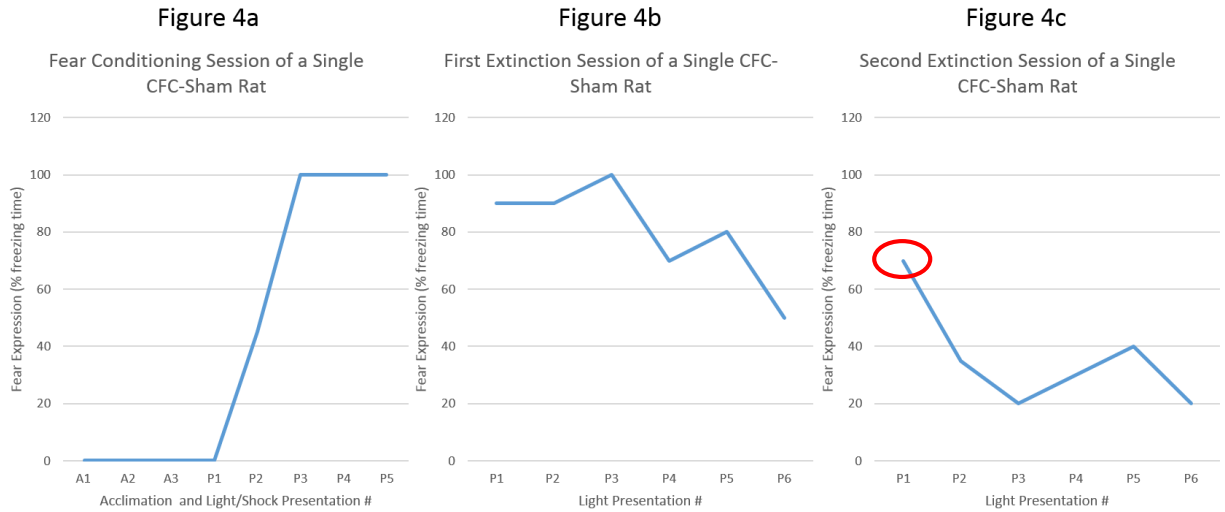


Figure 4. Depiction of fear behavior in a another CFC-Sham rat during Fear Conditioning (4a), an initial Fear Extinction session 24 hours after fear conditioning (4b), and a second Extinction session 24 hours after the first Extinction session (4c). Fear expression exhibited a similar pattern to that seen in the case depicted in Figure 1 during Fear Conditioning and the first Extinction session (4a and 4b, respectively). However, the fear expressed during the first presentation in the second Extinction session (circled in red) is considerably higher than that during the remaining presentations (4c). If we were to use the simple approach of comparing behavior of the last presentation of the first Extinction session to that of the first presentation of the second session, it would appear as if this CFC-Sham rat exhibited poorer fear extinction recall than might be the case. SPS = single prolonged stress; CFC = classical fear conditioning; A1, A2, A3 = Acclimation periods 1, 2, 3, respectively.

In the second Extinction session (Figure 4c) the fear expression, for the most part, was lower compared to that of the first Extinction session. However, the first light presentation of the second Extinction session was much higher than the subsequent 5 presentations (Figure 4c). If we were to use the simple approach of comparing behavior of the last presentation of the first Extinction session to that of the first presentation of the second Extinction session, it would appear as if this CFC-Sham rat exhibited considerably poorer fear extinction recall than might be the case.

Similarly, Figure 5 demonstrates how using just one presentation from each session can lead to an uncharacteristic representation of an individual rat’s fear extinction recall for an SPS-Sham treated rat.

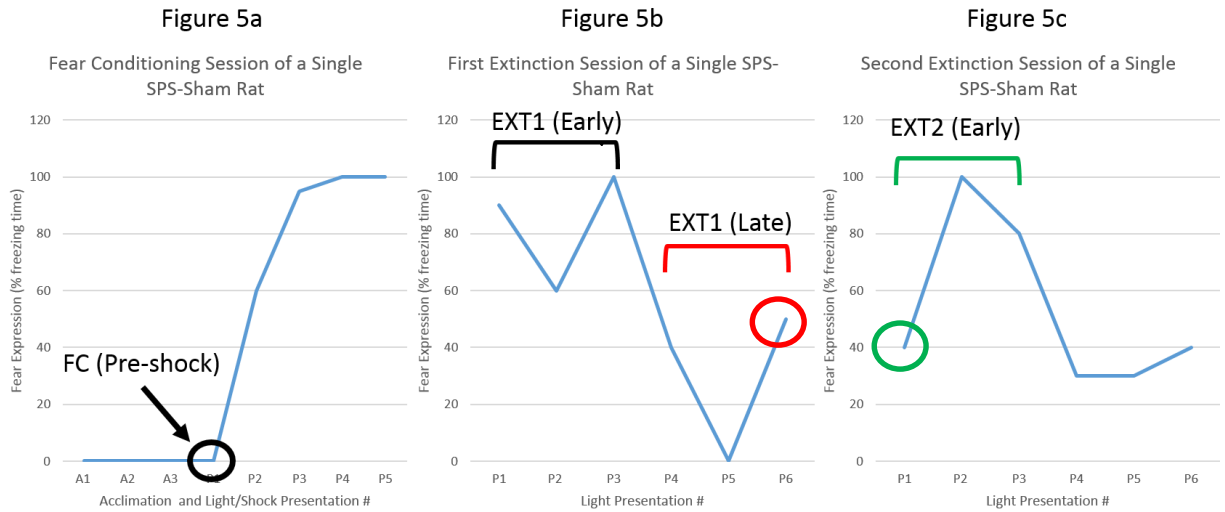


Figure 5. Depiction of fear behavior in a another SPS-Sham rat during Fear Conditioning (5a), an initial Fear Extinction session 24 hours after fear conditioning (5b), and a second Extinction session 24 hours after the first Extinction session (5c). Fear expression exhibited a similar pattern to that seen in the case depicted in Figure 2 during Fear Conditioning. However, there was a large spike in fear expression during the last light presentation of the first Extinction session (Figure 5b, highlighted by the red circle) and a much lower level of fear expression at the beginning of the second Extinction session compared to the subsequent two light-on trials (Figure 5c, highlighted by the green circle). If these data points alone were used to characterize fear extinction recall, it would appear as if fear extinction recall were better than might be the case. Therefore, to better represent fear expression near the end of the first Extinction session and near the beginning of the second Extinction session, we respectively averaged fear expression for the last three light-on trials of the first Extinction session (indicated by the red bracket labeled EXT1(late) in Fig. 5b) and for the first three light-on trials for the second Extinction session (indicated by the green bracket labeled EXT2(early) in Fig. 5c) for each rat. Using a similar rationale, we averaged fear expression for the first three light-on trials of the first Extinction session to characterize the beginning of extinction (indicated by the black bracket labeled EXT1(early) in Fig. 5b). There was only a single trial for each rat that could be used to represent fear expression to the light prior to association with shock (indicated by the black circle labeled FC (pre-shock) in Fig. 5a). SPS = single prolonged stress; CFC = classical fear conditioning; A1, A2, A3 = Acclimation periods 1, 2, 3, respectively.

In this case, a SPS-Sham rat exhibited a large spike in fear expression during the last light presentation of the first Extinction session (Figure 5b) and a much lower level of fear expression at the beginning of the second Extinction session compared to the subsequent two light-on trials (Figure 5c). If these data points alone were used to characterize fear extinction recall, it would appear as if the recall was uncharacteristically better than might be the case. To avoid having a limited sample of data points obscure the overall trend in behavior, we decided not to characterize fear extinction recall by comparing

two single points. Instead, we averaged the fear expression across the last three light presentations of the first Extinction session (EXT1(late); Fig. 5b) to more accurately represent the trend in behavior during the latter half of this session. Similarly, we averaged the fear expression across the first three light presentations of the second Extinction session (EXT2(early); Fig. 5C) to more accurately represent the trend in behavior during the early half of this session.

As mentioned in the Materials and Methods section, for each individual rat, the percentage of time spent freezing during the last three Light-on exposures of the first Extinction session were averaged (EXT1(late)) and subtracted from the average percentage of time spent freezing during the first three Light-on presentations of the second Extinction session (EXT2(early)) resulting in a Fear Extinction Recall Index ( $= \text{EXT2(early)} - \text{EXT1(late)}$ ) (see Figure 3). These indices for each rat were then used for the statistical analysis. As an interpretive note, indices with positive values represent rats that showed a fear expression increase from the last half of the first Extinction session to the first half of the second Extinction session, while indices with negative values represent a decrease in fear expression from the late part of the first Extinction session to the early part of the second Extinction session. However, when comparing fear extinction recall between rats or groups, those exhibiting poorer fear extinction memory recall will have indices significantly higher than the indices of those with better fear extinction memory recall (see example in left panel of Figure 6).

To determine the effects of SPS (as well as the effects of pharmacological modulation of astrocytic glutamate reuptake, which will be discussed below) upon fear extinction recall, a two-way ANOVA was performed that examined the potential interaction of SPS exposure with drug administration. Again, as we were first concerned with verifying that our SPS model effectively produced a behavioral alteration typical of PTSD, we initially used this analysis to compare the Fear Extinction Recall Index of the “CFC-Sham” group to that of the “SPS-Sham” group (Figure 6). ANOVA

revealed a significant interaction ( $F(2,53)=4.15, p=0.021$ ) of trauma exposure with drug treatment. Post-hoc analysis revealed that for the sham (saline) treated groups, rats exposed to SPS had significantly worse fear extinction recall compared to the CFC-only rats ( $t(53)= -2.73, p=0.0085$ ). As such, we concluded that our use of SPS was an effective method of inducing a PTSD-like state in rats.

#### **a.ii. Effect of Biochemical Modulation of Astrocytic Glutamate Reuptake upon SPS-Induced Fear**

##### **Extinction Recall Deficits**

The next step was to address the Specific Aim 1 of this research, which was to determine how SPS-induced fear extinction recall impairment is affected by pharmacological manipulation of astrocytic glutamate reuptake. We hypothesized that SPS exposure decreases astrocyte reuptake of glutamate such that synaptic glutamate, released during neuronal transmission, spills over into extrasynaptic locations. This would impair long-term potentiation in the PFC, a region which plays a role in fear extinction memory. To test this, we administered two drugs reported to modulate astrocyte glutamate reuptake: ceftriaxone (Cef), which increases expression of the primary astrocyte glutamate reuptake transporter (GLT-1), and dihydrokainate (DHK), which selectively blocks the activity of GLT-1 (Bechtholt-Gompf et al., 2010; John et al., 2015). If our hypothesis is correct, we would expect to see ceftriaxone administration exhibit a protective effect against SPS-induced impaired extinction recall, while DHK administration should exacerbate the symptom. These drugs were each administered to SPS and CFC rats, in addition to the SPS and CFC saline treated (Sham) rats, resulting in four more groups within this study: a “SPS-Cef” group, a “CFC-Cef” group, a “SPS-DHK” group, and a “CFC-DHK” group. Fear Extinction Recall Indices, for each rat, were calculated in the same manner as described above for the “SPS-Sham” and “CFC-Sham” groups (Figure 6). As mentioned above, a two-way ANOVA was performed that examined the potential interaction of SPS exposure with drug administration. Again, ANOVA revealed a significant interaction ( $F(2,53)=4.15, p=0.021$ ) of trauma exposure with drug treatment, but post-hoc

analysis revealed no significant differences in pair-wise comparisons other than the previously mentioned difference between the “SPS-Sham” group and the “CFC-Sham” group.

The results of the ceftriaxone and DHK treated groups are contrary to our expectations that CEF, as a GLT-1 facilitator, would mitigate SPS-induced effects on fear extinction recall while DHK, as an inhibitor of GLT-1, would exacerbate them. The data showed that ceftriaxone and DHK administration both eliminated the SPS-induced effect on fear extinction recall. However, among groups without SPS exposure, both ceftriaxone and DHK treated groups exhibited a numerical impairment in fear extinction recall compared to the Sham-treated group.

Furthermore, among the SPS-exposed groups, both ceftriaxone and DHK treated groups exhibited a numerical improvement in fear extinction recall compared to the Sham-treated group. Together, these numerical trends may have contributed to the elimination of the effect of the significant effect of SPS exposure seen in the saline treated groups.

These findings suggest that our methods of inducing a PTSD-like state using SPS are sufficient, but contrary to our expectations, both ceftriaxone and DHK can reverse this SPS-induced effect. The latter is potentially attributable to the concurrent numerical trends noted in the prior paragraph.

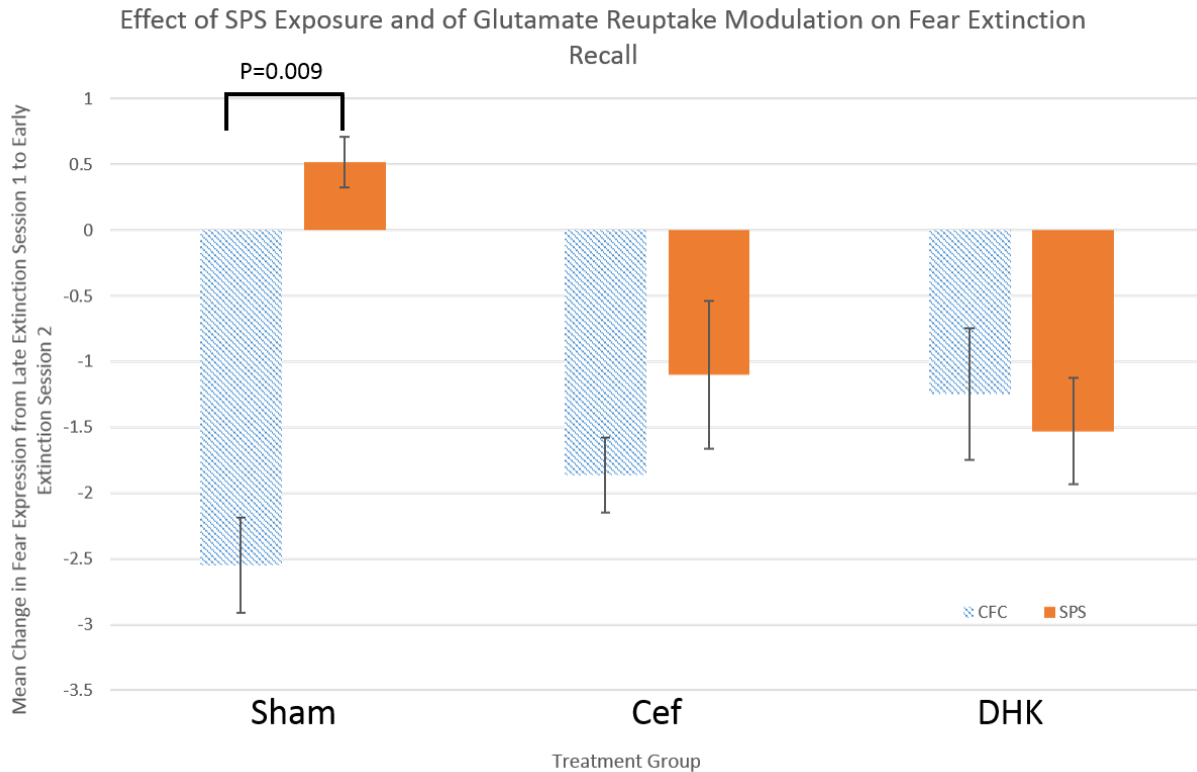


Figure 6. Depiction of the change in fear expression between the end of the first Extinction session and the beginning of the second Extinction session as a result of SPS exposure and drug treatment. For each individual rat within each treatment group, the percentage of time spent freezing during the last three Light-on exposures of the first Extinction session were averaged (EXT1(late)) and subtracted from the average percentage of time spent freezing during the first three Light-on presentations of the second Extinction session (EXT2(early)), resulting in an index representing the change in fear expression between the two (the Fear Extinction Recall Index). A two-way ANOVA was performed that examined the potential interaction of SPS exposure with drug administration, and revealed a significant interaction ( $F(2,53)=4.15$ ,  $p=0.021$ ) of trauma exposure with drug treatment. Post-hoc pairwise analysis revealed that for the sham treated groups, rats exposed to SPS had significantly worse fear extinction recall compared to the CFC-only rats ( $t(53)=-2.73$ ,  $p=0.0085$ ). No other pair wise comparisons between groups revealed significant differences. The results of the SPS-Sham comparison to CFC-Sham show that our SPS model effectively induces impaired fear extinction recall. Additionally, these results show that both Cef and DHK eliminated the statistical significance of SPS-induced impairment of fear extinction recall demonstrated in the sham (saline) treated rats.. SPS = single prolonged stress; CFC = classical fear conditioning; Cef = ceftriaxone; DHK = dihydrokainate. Error bars represent standard errors.

### **a.iii. Further validation of the SPS model**

Though not a direct part of Specific Aim 1, more analyses of the data were performed in order to confidently conclude that our SPS model was working appropriately. It was mentioned earlier that PTSD induces an impairment of maintaining long-term fear extinction memories, and that this can be replicated by the SPS paradigm in rats. The SPS procedure used in this study is not commonly associated with a significant effect on the formation of conditioned fear memories, nor should it have an effect on fear extinction learning. In other words, a SPS-exposed rat should be able to form conditioned fear to an innocuous stimulus (e.g., a light) when it is paired with a noxious stimulus (e.g., a shock) in a manner comparable to that of a rat that was not exposed to SPS. Furthermore, 24 hours after fear conditioning, if exposed to an Extinction session where the light is presented without concurrent shock, a SPS rat should demonstrate a decrease in fear expression over the course of the trial in a comparable manner to that of a rat not exposed to SPS. To reiterate, the critical difference in behavior between a SPS-exposed rat and a rat without SPS exposure is seen when a second Extinction session is performed, where the SPS rat should show a significantly higher return of fear expression compared to the rat without SPS exposure. Consequently, in order to conclude that the results we reported above were principally attributable to a disruption of the fear extinction recall process, we expected that the groups would exhibit comparable levels of fear conditioning during the Fear Conditioning session, as well as exhibit comparable fear extinction learning during the first Extinction session.

To first examine whether our method of fear conditioning induced a significant associative fear memory in the rats of all 6 treatment groups, a comparison was made between the fear expression of rats before fear conditioning and 24 hrs after fear conditioning. To represent fear expression before fear conditioning, % freezing time was measured during the first light presentation of the Conditioning session (FC(pre-shock); see Fig. 5a). Justification for this was that it was the only point where the rats

were exposed to the light stimulus without having any prior exposure to the shock. To represent fear expression in the presence of the light after fear conditioning, average % freezing time for each rat was calculated during the first three light presentations of the first Extinction session (EXT1(early); see Fig 5b). Pairwise post-hoc analysis within a two way ANOVA of light presentation phase by treatment group revealed that, for each group, there was a significant increase in fear expression during the first three light presentations of the first Extinction session relative to that during the first light presentation (prior to any shocks) of the Conditioning session (all p-values < 0.0001). This result suggested that for each group, the parameters of the Conditioning trial induced an associative fear memory between the light and the shock.

Next, we were concerned with demonstrating that the parameters of our Extinction session were adequate in inducing fear extinction learning. To do this, we made a comparison of fear expression early in the first Extinction session with that of fear expression later in the same session. To represent fear expression in early Extinction, the average % freezing time was calculated during the first three light presentations of the first Extinction session (EXT1(early)). To represent fear expression during late extinction, the average % freezing time was calculated during the last three presentations of the same session (EXT1(late)). Results of a two way ANOVA of light presentation phase by treatment group revealed a significant main effect of presentation phase ( $F(1,54) = 45.7, p < .0001$ ). Post-hoc pairwise comparisons showed that for each treatment group, the fear behavior of EXT1(early) was significantly higher than the fear behavior of EXT1(late) (Figure 7). This drop in fear expression over the course of the first Extinction session is consistent with the formation of a learned disassociation between the conditioned and unconditioned stimuli that competes against the association learned during fear conditioning. As such, we concluded from these results that for each group, the parameters of the Extinction session induced fear extinction learning.

### Change in Fear Expression During Extinction Session 1

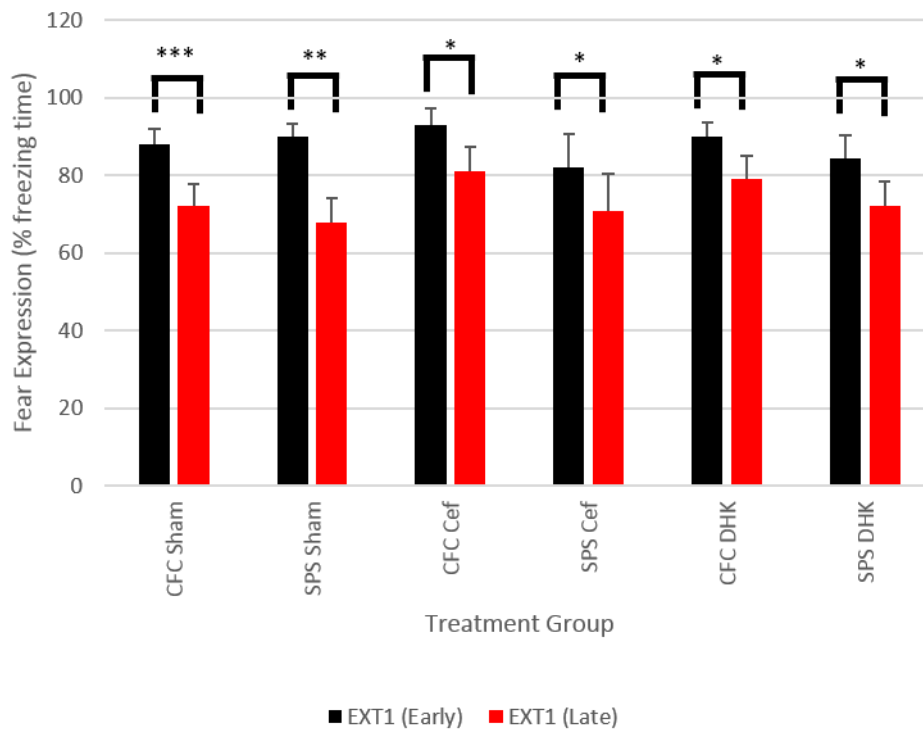


Figure 7. Depiction of fear expression during the first Extinction session, emphasizing the relationship between expression of fear during early and late extinction within each treatment group. To represent fear expression in early Extinction (EXT1(early)), the average % freezing time was calculated during the first three light presentations of the first Extinction session. To represent fear expression during late extinction (EXT1(late)), the average % freezing time was calculated during the last three presentations of the same session. Results of a two way ANOVA revealed that for each treatment group, the fear behavior of EXT1(early) was significantly higher than the fear behavior of EXT1(late) (\* $p < 0.05$ ; \*\* $p < 0.005$ ; \*\*\* $p < 0.0001$ ). This drop in fear expression over the course of the first Extinction session is consistent with the learned disassociation between the conditioned and unconditioned stimuli that is characteristic of fear extinction learning. As such, we concluded from these results that for each group, the parameters of the Extinction session adequately induced fear extinction learning. SPS = single prolonged stress; CFC = classical fear conditioning; Cef = ceftriaxone; DHK = dihydrokainate. Error bars represent standard errors.

The previous two analyses demonstrated that the parameters of our study produced conditioned fear memory as well as a process of fear extinction learning. However, as mentioned earlier, we not only had to examine whether our parameters were inducing these processes, but we also had to

examine whether these processes were comparable among the treatment groups. In our aforementioned analysis of conditioned fear memory, where we compared pre-shock light presentation to light presentations during early Extinction session 1, mean fear expression during the pre-shock light presentation for each treatment group was zero. Furthermore, post-hoc pairwise comparisons of fear expression during early Extinction session 1 (EXT1(early)) failed to reveal a significant difference between treatment groups. This suggests that exposure to SPS (and to drugs known to affect glutamate reuptake) does not affect formation of conditioned fear memory. To examine whether the process of fear extinction learning was affected by these treatments, a Fear Extinction Learning Index was calculated for each rat. This was done in a similar manner as described above for the Fear Extinction Recall Index, except in this case the average % freezing behavior during the last three light presentations of the first Extinction session (EXT1(late)) was subtracted from that of the first three light presentations of the first Extinction session (EXT1(early)) (as opposed to being subtracted from the first three presentations of the second Extinction session in the case of determining the Fear Extinction Recall Index) (Figure 8). Therefore, the positive values represent the magnitude of fear expression decreases from the first half of the session to the last half of the session. A nonparametric one-way procedure (Kruskal-Wallis test) revealed no significant differences between the Fear Extinction Learning Indices of any of the groups ( $p$ -value > 0.05) (Figure 8). The fact that the indices were not significantly different suggests that the decrease in fear expression for each group was comparable over the course of the first Extinction session. This is consistent with equivalent fear extinction learning, and so we can conclude that the rats in our study were not exhibiting alterations in this respect as a consequence of exposure to SPS or any of the drug treatments.

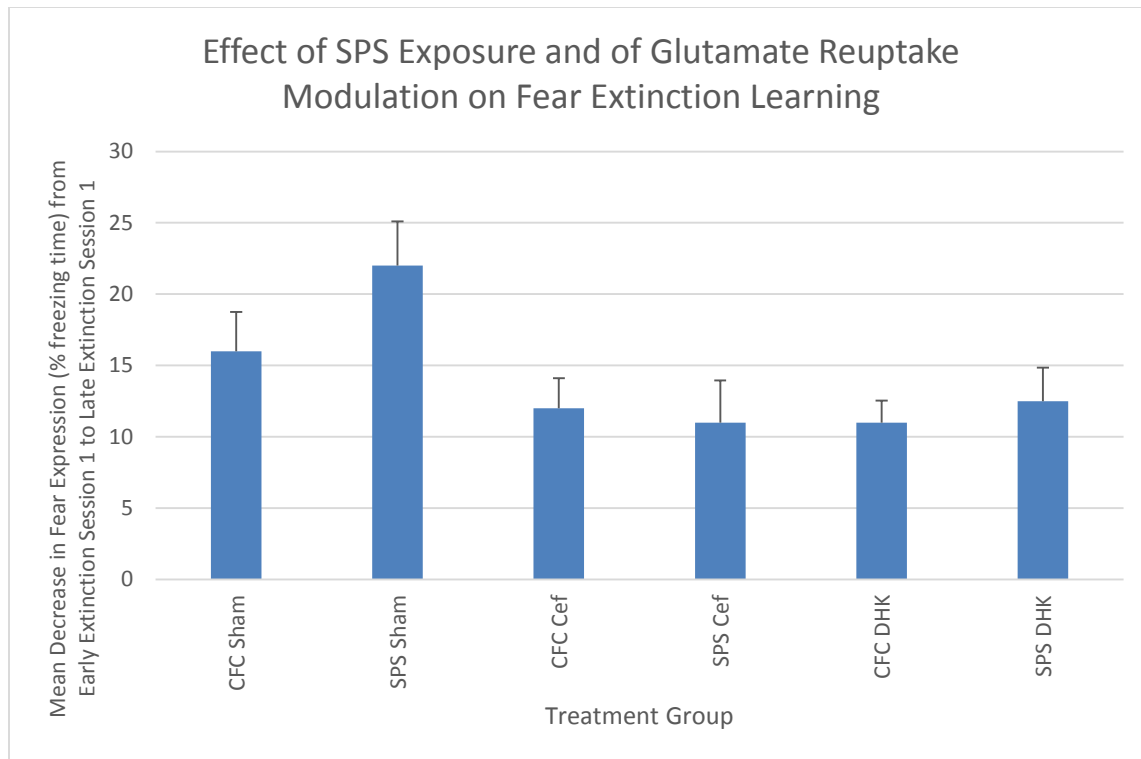


Figure 8. Depiction of the decrease in fear expression from the early part of the first Extinction session to the late part of the same session as a result of SPS exposure and drug treatment. For each individual rat within each treatment group, the percentage of time spent freezing during the last three Light-on exposures of the first Extinction session were averaged (EXT1(late)) and subtracted from the average percentage of time spent freezing during the first three Light-on presentations of the same session (EXT1(early)), resulting in an index representing the decrease in fear expression between the two (the Fear Extinction Learning Index). A nonparametric one-way procedure (Kruskal-Wallis test) revealed no significant differences between the Fear Extinction Learning Indices of any of the groups ( $p$ -value > 0.05). The fact that the indices were not significantly different suggests that the decrease in fear expression for each group was comparable over the course of the first Extinction session. This is consistent with relatively equivalent fear extinction learning, and so we can conclude that the rats in our study were not exhibiting alterations in this respect as a consequence of exposure to SPS or any of the drug treatments. SPS = single prolonged stress; CFC = classical fear conditioning; Cef = ceftriaxone; DHK = dihydrokainate. Error bars represent standard errors.

Finally, an analysis of the effects of SPS exposure on general anxiety behavior was performed using the data from the elevated plus maze (Figure 9). A two-way ANOVA (behavior x drug) was performed and failed to reveal a significant interaction ( $F(2,54) = .994, p = .377$ ) or main effect of either behavioral treatment ( $F(1,54) = .341, p = .562$ ) or drug treatment ( $F(2,54) = .599, p = .553$ ). Post-hoc pairwise comparisons showed that within each drug treatment group, SPS exposure did not significantly

change the anxiety index. This indicates that the increase in freezing behavior following CFC, as well as the SPS-induced alteration in fear extinction recall (Figure 6), is not likely to be due to an increase in general anxiety behavior. Rather, the results of this analysis support the conclusion that the increase in freezing behavior demonstrated at the beginning of EXT1 was likely due to formation of a conditioned fear to the light stimulus as a consequence of it being paired with a shock during CFC. Likewise, the higher levels of freezing seen in the SPS-Sham group at the beginning of EXT2 compared to the CFC-Sham group was likely due to differences in fear extinction memory recall.

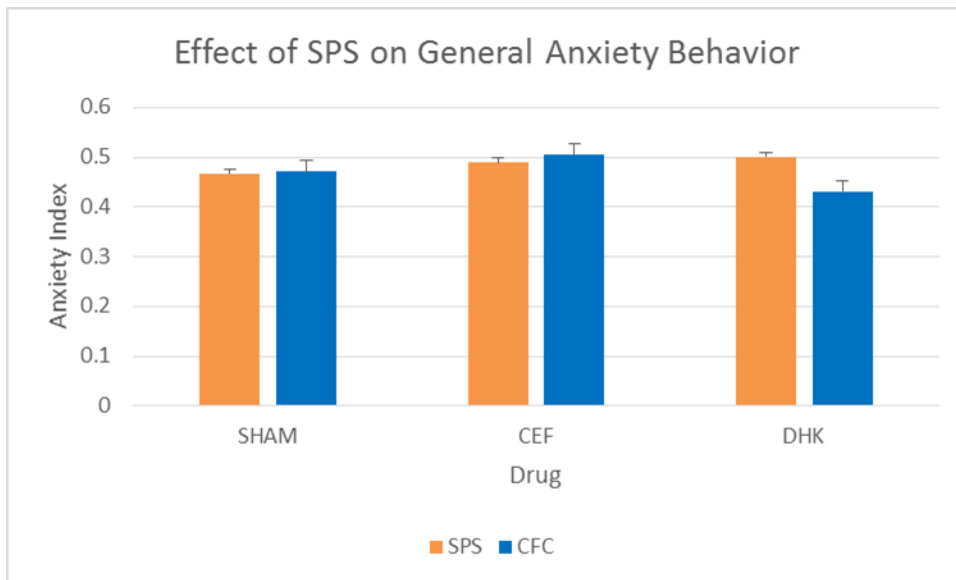


Figure 9. Depiction of the results from the elevated plus maze showing the effect of SPS on general anxiety behavior. A two-way ANOVA (behavior x drug) was performed and failed to reveal a significant interaction ( $F(2,54) = .994, p = .377$ ) or main effect of either behavioral treatment ( $F(1,54) = .341, p = .562$ ) or drug treatment ( $F(2,54) = .599, p = .553$ ). Post-hoc pairwise comparisons showed that within each drug treatment group, SPS exposure did not significantly change the anxiety index. This indicates that the increase in freezing behavior following CFC, as well as the SPS-induced alteration in fear extinction recall (Figure 6), is not likely to be due to an increase in general anxiety behavior. SPS = single prolonged stress; CFC = classical fear conditioning; Cef = ceftriaxone; DHK = dihydrokainate. Error bars represent standard errors.

## **SECTION B: SPECIFIC AIM 2: EVALUATION OF THE EFFECT OF SPS ON GLUTAMATE TRANSPORTER EXPRESSION AND ON DOWNSTREAM CREB PHOSPHORYLATION IN THE AMYGDALA AND PREFRONTAL CORTEX**

The goals of Specific Aim 1 were to validate our ability to reproduce the behavioral effects of the SPS model of PTSD and to assess whether, and how, modulation of astrocytic glutamate transport affected the expression of these behavioral effects. The goal of Specific Aim 2 was to determine the molecular changes induced by SPS that underpin our hypothesized alterations in glutamate reuptake, and in one of its downstream targets, induced in PTSD. This aim also sought to examine differences in these molecular changes that might be responsible for differential effects of SPS upon fear memory and fear extinction memory. As mentioned in Section III (Specific Aims), this involved 1) comparing changes in GLT-1 expression in the amygdala and PFC between CFC and SPS rats, 2) comparing changes in p-CREB expression in the amygdala and PFC between CFC and SPS rats, and 3) comparing p-CREB expression in the amygdala and PFC between CFC and SPS rats following ceftriaxone or DHK administration to determine if these drugs can mitigate or exacerbate, respectively, a hypothesized SPS-induced reduction of p-CREB. However, before addressing these goals, we first analyzed results from Specific Aim 2 to further confirm the validity of our behavioral and pharmacological treatments.

### ***b.i. Molecular Confirmation of the SPS Model***

Firstly, as a final step in confirming the efficacy of using SPS to induce a PTSD-like state in rats, a biochemical analysis was performed to demonstrate that our model produced previously reported PTSD-associated biochemical changes in addition to the behavioral changes discussed earlier. Studies have shown that elevated glucocorticoid receptor (GR) expression in the hippocampus is a typical finding in SPS-exposed rats (Knox et al., 2012), and is consistent with the elevated glucocorticoid sensitivity exhibited in PTSD individuals. Immediately following the second Extinction trial described

above, all rats were sacrificed and brains were collected. The dorsal hippocampus was dissected out and used for measurement of GR expression using ELISA. A mixed model ANOVA was performed and revealed a significant main effect of trauma exposure ( $F(1,54)=p,0.0001$ ). Post-hoc analysis showed SPS-Sham ( $t(54)= 6.82, p<0.0001$ ), SPS-CEF ( $t(54)= 4.65, p<0.0001$ ), and SPS-DHK ( $t(54)= 5.71, p<0.0001$ ) treated rats had significantly higher GR expression compared to their corresponding drug treatment groups not exposed to SPS. However, no significant interaction ( $F(2,54)=1.18, p=0.3161$ ) of trauma exposure with drug treatment was found. Figure 10 below shows the results from that analysis with the purpose of showing the effects of SPS-exposure on GR expression across drug treatment groups. The results demonstrate that hippocampal GR expression was significantly increased in SPS-exposed rats compared to rats not exposed to SPS, regardless of drug treatment. As such, we can conclude that our use of SPS successfully induced biochemical changes previously reported as characteristic of PTSD. Pairing this with the behavioral findings reported in the validation sections for Specific Aim 1, we conclude that our SPS procedure successfully produced an animal model of PTSD in our lab. Our data also suggest that treatments intended to modulate astrocytic glutamate reuptake did not affect the SPS-induced increase in hippocampal GR expression.

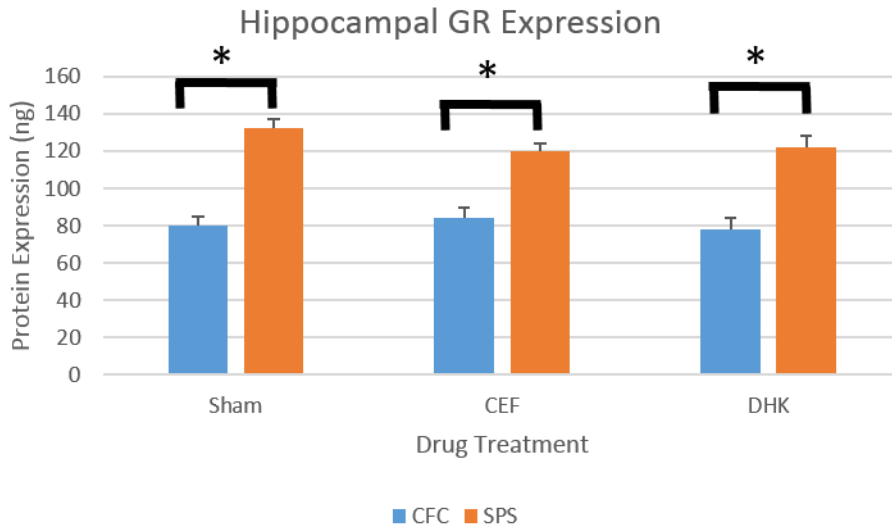


Figure 10. This figure shows GR expression in the hippocampus of each treatment group. Groups connected by brackets were significantly different ( $p < 0.0001$ ). These results demonstrate that hippocampal GR expression was significantly increased in SPS-exposed rats compared to rats not exposed to SPS, regardless of drug treatment. As such, we can conclude that our use of SPS successfully induced biochemical changes characteristic of PTSD, and that our model sufficiently models the disorder. SPS = single prolonged stress; CFC = classical fear conditioning; Cef = ceftriaxone; DHK = dihydrokainate. Error bars represent standard errors. “\*” represent  $p$ -values  $< 0.0001$ .

### **b.ii. Validating the Effects of Ceftriaxone and DHK on Glutamate Reuptake Transporters**

Our second validation step was to show that ceftriaxone and DHK induced the expected changes in GLT-1 glutamate transporter expression in animals not exposed to SPS. To help understand the role of astrocytic glutamate reuptake in the pathophysiology of PTSD, these drugs were selected because they modulate the function of the GLT-1 in opposing manners. Ceftriaxone has been shown to selectively up-regulate the expression of GLT-1 (Sari et al., 2009; Sari et al., 2013), thus enhancing uptake of extracellular glutamate by astrocytes, while DHK is a GLT-1 selective antagonist that decreases astrocyte glutamate reuptake but does not change GLT-1 expression (Bechtholt-Gompf et al., 2010; John et al., 2015).

For this analysis, the infralimbic prefrontal cortex (PFC) and the basolateral complex of the amygdala of rats were removed following collection of the brain immediately after the second Extinction

trial. As mentioned earlier, the infralimbic PFC is the region of the prefrontal cortex shown to be associated with extinction memory retention, while the basolateral complex is known to be critical for the retention of fear memories. Western blot analysis for GLT-1 was then performed on the above mentioned sub-regions of the PFC and amygdala. A mixed model 2(trauma) x 2(region) x 3(drug) ANOVA was performed and a significant interaction of trauma exposure with drug treatment was found ( $F(2,54)=11.15$ ,  $p<0001$ ). Consistent with our expectations post-hoc pair-wise analysis showed that, within rats not exposed to SPS, CEF administration resulted in a significant elevation in GLT-1 expression compared to Sham-treated rats in both the PFC ( $t(91.34)= 4.06$ ,  $p=0.0003$ ) and the amygdala ( $t(91.34)= 3.84$ ,  $p=0.0006$ ). Also as expected, there was no significant difference found in either region between the DHK-treated group and the sham-treated group. Figure 11 below shows the results from that analysis. A supplemental mixed model ANOVA was performed to examine the magnitude of the CEF-induced increase in GLT-1 protein across the two brain regions that were sampled (amygdala vs. PFC). No significant difference in this magnitude was observed between the two regions.

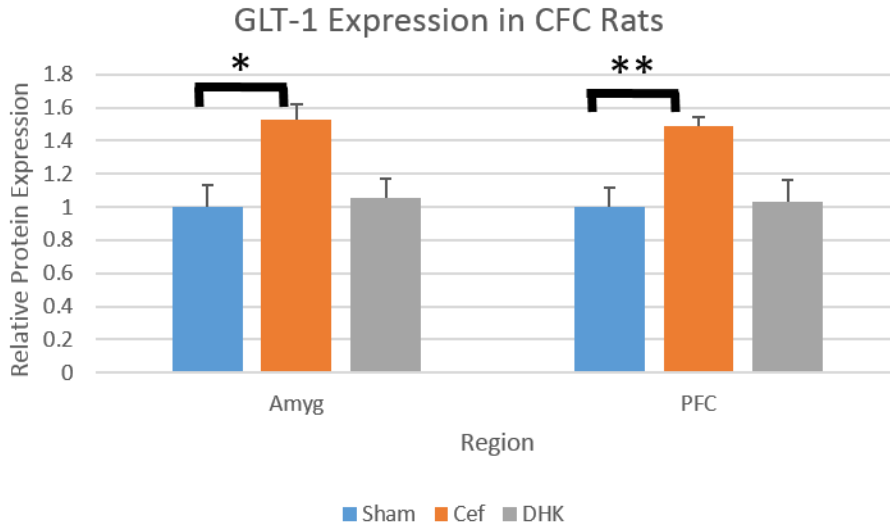


Figure 11. This figure shows GLT-1 expression in the PFC and amygdala of CFC rats only (i.e., rats that were not exposed to SPS) with the purpose of showing the effects of drug treatment on GLT-1 expression. Consistent with our expectations, a significant elevation in GLT-1 expression was found in both the PFC and amygdala of ceftriaxone-treated rats compared to sham-treated controls. Conversely, there was no significant difference found in either region between the DHK-treated group and the sham-treated group. . SPS = single prolonged stress; CFC = classical fear conditioning; Cef = ceftriaxone; DHK = dihydrokainate. Error bars represent standard errors. “\*” represent p-values=0.0006. “\*\*” represent p-values=0.0003.

Furthermore, the specificity of this drug for the up-regulation of GLT-1 expression was confirmed by analyzing the expression of the GLAST glutamate transporter. Western blot was performed to determine GLAST expression, and a mixed model 2(trauma) x 2(region) x 3(drug) ANOVA revealed no significant main effects of, or interactions between, the independent variables. Post-hoc pair-wise comparison confirmed that drug treatment did not change GLAST expression within CFC animals in either brain region.

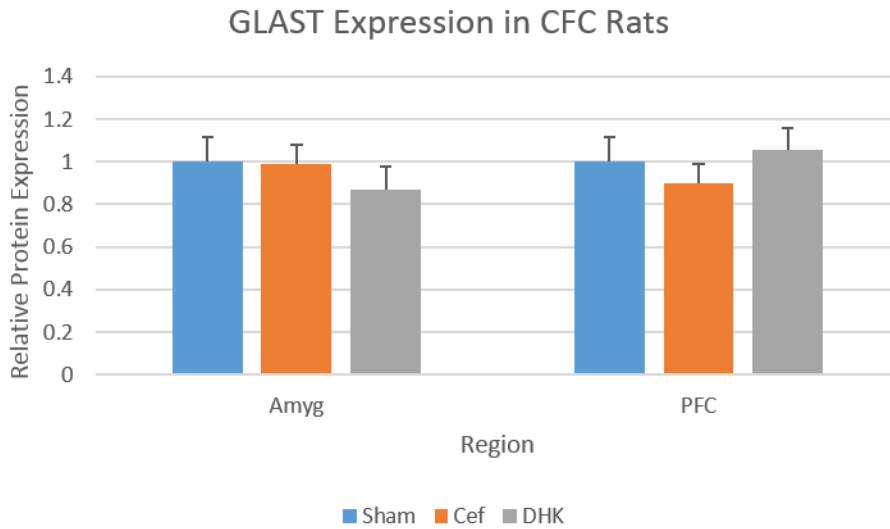


Figure 12. This figure shows GLAST expression in the PFC and amygdala of CFC rats only (i.e., rats that were not exposed to SPS) with the purpose of showing the effects of drug treatment on GLAST expression. Consistent with our expectations, no significant elevation in GLAST expression was found in either the PFC or amygdala of groups treated with ceftriaxone or DHK compared to sham-treated controls. SPS = single prolonged stress; CFC = classical fear conditioning; Cef = ceftriaxone; DHK = dihydrokainate. Error bars represent standard errors.

Taken together, these data support the assertion that ceftriaxone functioned in this experiment as a selective promoter of GLT-1 glutamate reuptake transporter expression, without concurrent up-regulation of the GLAST reuptake transporter. Additionally, DHK administration demonstrated no change in GLT-1 or GLAST expression, which is consistent with the activity of a transporter functional inhibitor. As such, in regard to glutamate reuptake transporter expression, we can conclude that these drugs are acting in a manner that is consistent with our expectations.

**b.iii. Determining the Effect of SPS on GLT-1 Expression**

A core aim of this study was to determine if impairment of astrocytic glutamate transport plays a part in the pathophysiology of PTSD. In Specific Aim 2, we first hypothesized that psychological trauma would reduce expression of GLT-1, and consequently impair the capability of astrocytes to sequester neuronal glutamate released during synaptic transmission. This would theoretically result in spillover of glutamate into extrasynaptic spaces, and could then activate molecular pathways, via extrasynaptic

NMDA receptors, that would impair long-term memory formation. Furthermore, we suggested that such a process could occur differentially in the amygdala and PFC resulting in differential effects of trauma upon fear memory and fear extinction memory. During Specific Aim 1, we had pharmacologically manipulated astrocytic glutamate reuptake to determine the effects upon fear memory and fear extinction memory. However, that work did not address the role of situational trauma in potential modulation of glutamate transport in the amygdala versus PFC and the resulting downstream effects upon a molecular substrate of LTP. As such, to achieve this second aim, we first needed to determine if SPS decreased GLT-1 expression in either the amygdala or the PFC compared to rats not exposed to SPS. To do this, a comparison of GLT-1 expression was made between the SPS-Sham and CFC-Sham rats using western blot to quantify the protein expression in the tissues described above. Using the mixed model ANOVA mentioned in the above section post-hoc pairwise analysis showed that, within Sham-administered rats, exposure to SPS resulted in a significant elevation in GLT-1 expression compared to rats not exposed to SPS in both the PFC ( $t(91.34) = 5.18, p < 0.0001$ ) and the amygdala ( $t(91.34) = 3.54, p = 0.0008$ ). Figure 13 below shows the results from that analysis. A supplemental mixed model ANOVA was performed to examine the magnitude of the SPS-induced increase in GLT-1 protein across the two brain regions that were sampled (amygdala vs. PFC). No significant difference in this magnitude was observed between the amygdala and PFC. The results show that SPS induces a significant change in GLT-1 expression, of a similar magnitude in the PFC and the amygdala, compared to rats not exposed to SPS. However, contrary to our hypothesis, this SPS-induced change was an increase in expression rather than a decrease.

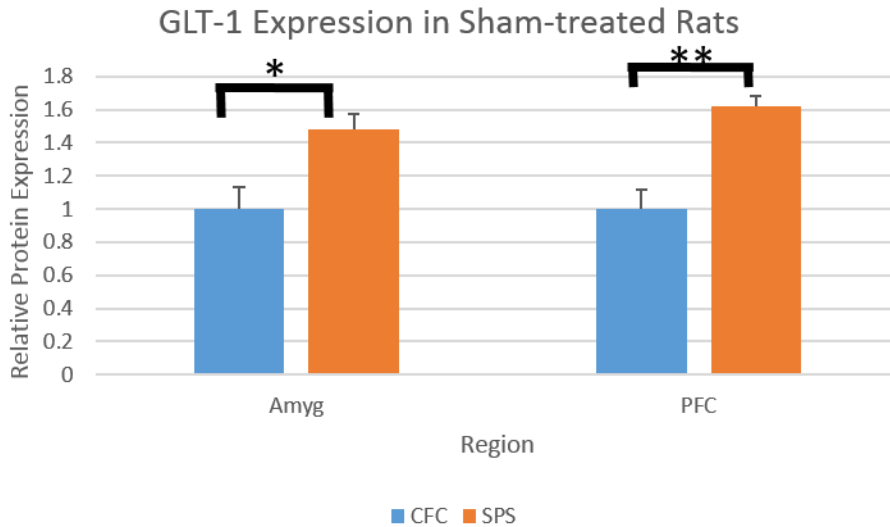


Figure 13. A subset of the results containing only the SPS-Sham and CFC-Sham groups with the purpose of showing how SPS effects GLT-1 expression. The results show that SPS does induce a significant change in GLT-1 expression in both the PFC and the amygdala compared to rats not exposed to SPS. However, contrary to our hypothesis, this SPS-induced change was an increase in expression rather than a decrease. SPS = single prolonged stress; CFC = classical fear conditioning alone; Cef = ceftriaxone; DHK = dihydrokainate. Error bars represent standard errors. “\*” represent p-values=0.0008. “\*\*\*” represent p-values<0.0001.

#### **b.iv. Determining the Effect of SPS on p-CREB expression in the Amygdala and PFC**

The next goal of Specific Aim 2 was to evaluate the effect of SPS on molecular indicators of long-term memory formation. It was discussed earlier that phosphorylation of CREB (p-CREB) is an important step in the formation of long-term memories, and that stimulation of extrasynaptic NMDA receptors can impair this process. Consequently, we selected p-CREB as the molecular indicator of long-term memory formation in this experiment. We hypothesized that SPS would induce spillover of glutamate into extrasynaptic spaces, and that this would subsequently cause a reduction in p-CREB expression. Furthermore, since SPS is associated with impaired long-term fear extinction memory, we further hypothesized that this effect may be more pronounced in the PFC compared to the amygdala.

To test this, a mixed model 2(trauma) x 2(region) x 3(drug) ANOVA was performed, and post-hoc pair-wise comparisons were made. For simplicity, Figure 14 below depicts only the comparisons of p-

CREB expression between CFC- and SPS-Sham treated rats in the amygdala and the PFC with the purpose of showing the effects of SPS on p-CREB without concurrent uptake-modulating drug treatment. While SPS induced a larger numerical decrease in p-CREB in the PFC compared to the amygdala, in neither region was there a significant difference in p-CREB expression between the CFC and SPS groups ( $t(107.6) = -1.89$ ,  $p = 0.0635$  for the PFC, and  $t(107.6) = -0.07$ ,  $p = 0.9428$  for the amygdala).

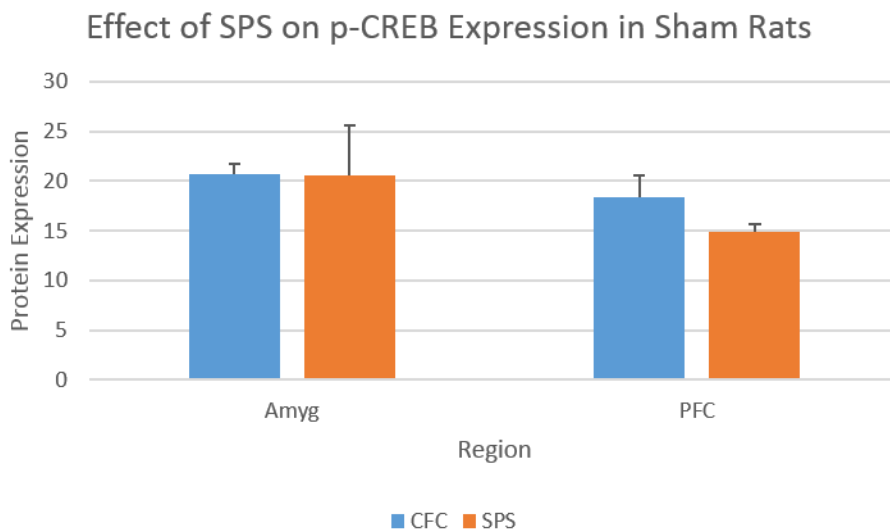


Figure 14. A subset of the results containing only the SPS-Sham and CFC-Sham groups with the purpose of showing how SPS affects p-CREB expression. The results show that SPS does not induce a significant change in p-CREB expression in either the PFC ( $p = 0.0635$ ) or the amygdala ( $p = 0.9428$ ) compared to rats not exposed to SPS. However, a numerically greater decrease in p-CREB expression was observed in the PFC compared to the amygdala. SPS = single prolonged stress; CFC = classical fear conditioning; Cef = ceftriaxone; DHK = dihydrokainate. Error bars represent standard errors.

**b.v. Determining the Effect of GLT-1-Modulating Intervention on p-CREB Expression in the PFC and Amygdala Following SPS Exposure**

As mentioned before, we did not only aim to determine the effect of psychological trauma on GLT-1 and p-CREB expression in the amygdala versus PFC, but also to determine if pharmacological modulation of GLT-1 could alter the effects of SPS on p-CREB expression. Consequently, the final component of Specific Aim 2 was to determine if treatment with ceftriaxone and DHK could mitigate or

exacerbate, respectively, the effects of SPS on p-CREB expression. This also addresses the potential mechanistic substrate for the mitigating effects of these drugs upon the SPS-induced deficit in fear extinction recall observed in Specific Aim 1.

To do this analysis, ELISA was used to determine p-CREB expression in the amygdala and PFC of each group (similarly in fashion as described above for the GR analysis). A mixed model ANOVA was performed, and a significant 3-way interaction of trauma exposure x drug treatment x brain region was found ( $F(2,54)=3.22$ ,  $p=0.0476$ ). To determine the potential mitigating or exacerbating effects of Cef and DHK, respectively, on changes in p-CREB expression following SPS exposure, post-hoc pairwise comparisons were made between CFC and SPS groups within each drug treatment group. Figures 15 and 16 below show the results of these comparisons within the PFC and amygdala, respectively. Similar to the results depicted in Figure 14 for Sham-treated rats, no significant differences were found between the CFC and SPS groups of Cef- or DHK-treated rats (CFC-Sham vs SPS-Sham in Amyg,  $t(107.6)= -0.07$ ,  $p=0.9428$ ; CFC-Sham vs SPS-Sham in PFC,  $t(107.6)= -1.89$ ,  $p=0.0635$ ; CFC-Cef vs SPS-Cef in Amyg,  $t(107.6)= -1.74$ ,  $p=0.0882$ ; CFC-Cef vs SPS-Cef in PFC,  $t(107.6)= 0.97$ ,  $p=0.3387$ ; CFC-DHK vs SPS-DHK in Amyg,  $t(107.6)= 1.08$ ,  $p=0.3092$ ; CFC-DHK vs SPS-DHK in PFC,  $t(107.6)= -0.20$ ,  $p=0.8436$ ). Therefore, it did not appear that intervention with the astrocytic glutamate reuptake modulators Cef or DHK altered the consequences of SPS exposure for p-CREB.

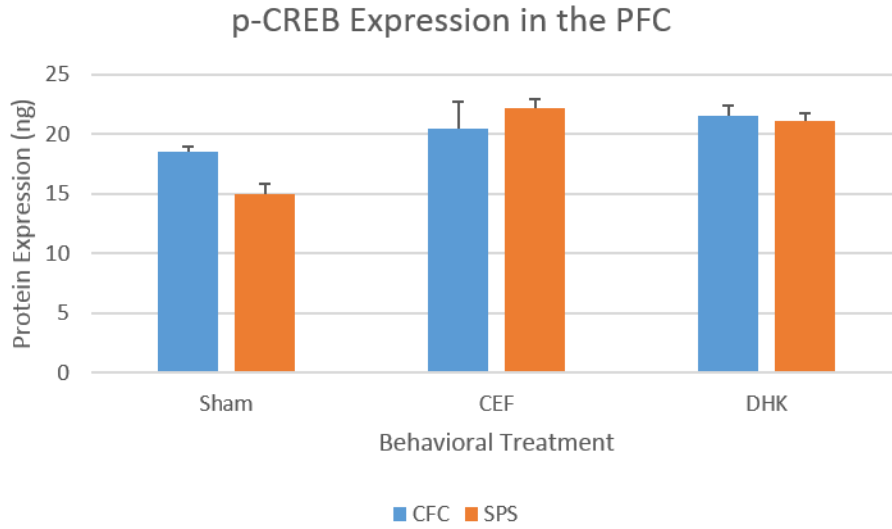


Figure 15. These data represent the results from the comparison of p-CREB expression in the PFC between CFC and SPS rats within each drug treatment group. The results show that no significant difference was found between the CFC and SPS groups treated with the Sham ( $p=0.0635$ ), Cef ( $p=0.3387$ ), or DHK ( $p=0.8436$ ). SPS = single prolonged stress; CFC = classical fear conditioning; Cef = ceftriaxone; DHK = dihydrokainate. Error bars represent standard errors.

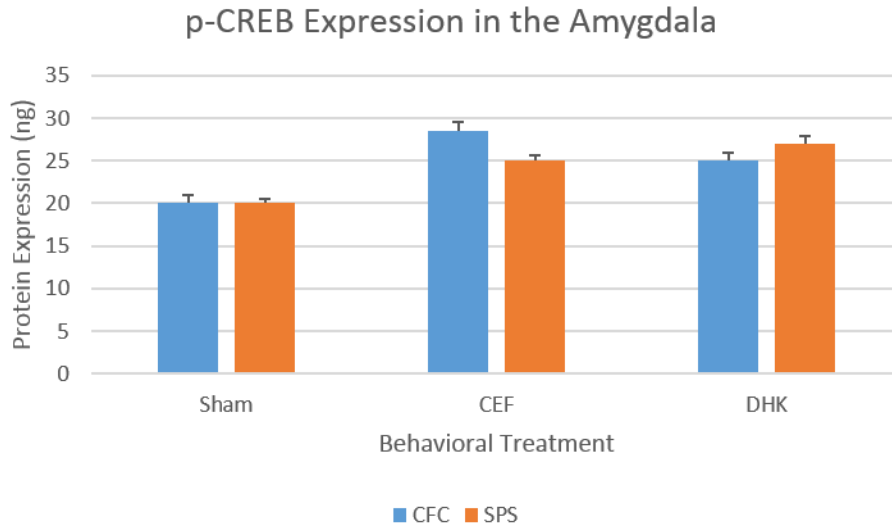


Figure 16. These data represent the results from the comparison of p-CREB expression in the amygdala between CFC and SPS rats within each drug treatment group. The results show that no significant difference was found between the CFC and SPS groups treated with the Sham ( $p=0.9428$ ), Cef ( $p=0.0854$ ), or DHK ( $p=0.3092$ ). SPS = single prolonged stress; CFC = classical fear conditioning; Cef = ceftriaxone; DHK = dihydrokainate. Error bars represent standard errors.

The post-hoc analysis also included comparisons between drug treatments within the CFC and SPS groups for both regions. Although it might be expected that the glutamate reuptake-modulating drugs would each operate in the same fashion, regardless of trauma exposure, we explored the possibility that some consequence of SPS exposure might interact with these drugs to change their expected synaptic actions and downstream consequences. Figures 17 and 18 show the results of these comparisons for the PFC and amygdala, respectively. These results showed that within the PFC of rats not exposed to SPS, neither CEF ( $t(107.6) = 1.07$ ,  $p = 0.4625$ ) nor DHK ( $t(107.6) = 1.61$ ,  $p = 0.1952$ ) administration resulted in a significant change in p-CREB expression compared to rats given the Sham (Figure 17, left half) suggesting that neither drug alters p-CREB expression in rats not exposed to SPS. However, the data show a different response of p-CREB expression in the PFC of SPS-exposed rats (Figure 17, right half). Using the above mentioned mixed model ANOVA, post-hoc analysis showed, within the PFC of SPS-exposed rats, both CEF ( $t(91.34) = 3.93$ ,  $p = 0.0005$ ) and DHK ( $t(91.34) = 3.31$ ,  $p = 0.0032$ ) administration resulted in a significant elevation in p-CREB expression compared to rats given the Sham. Together, these data suggest that both DHK and ceftriaxone increase p-CREB expression in the PFC, but that this effect is dependent upon trauma exposure.

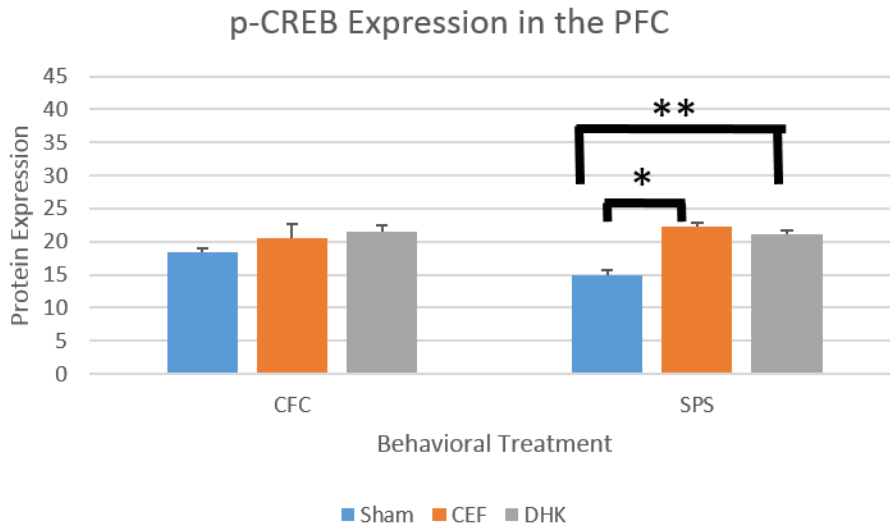


Figure 17. In the PFC of animals not exposed to SPS, there is no significant difference in p-CREB expression between the Sham-treated group and either the ceftriaxone- or DHK-treated groups. Conversely, in the PFC of SPS-exposed rats, both ceftriaxone and DHK administration resulted in a significant elevation of p-CREB expression relative to the Sham-treated group. These data show that the effects of the glutamate reuptake-modulating drugs upon the PFC appear to be dependent upon exposure to trauma. SPS = single prolonged stress; CFC = classical fear conditioning; Cef = ceftriaxone; DHK = dihydrokainate. Error bars represent standard errors. "\*" represent p-values=0.0005. "\*\*\*" represent p-values=0.0032.

The results generated from the amygdala tissue were somewhat different from those of the PFC described above. Using the above mentioned mixed model ANOVA, post-hoc analysis showed that, within the amygdala of rats not exposed to SPS, both CEF ( $t(91.34)= 4.23, p=0.0002$ ) and DHK ( $t(91.34)= 2.40, p=0.0366$ ) administration resulted in a significant elevation in p-CREB expression compared to rats given the Sham. Figure 18 (left half) shows these results which differ from those seen in the PFC for rats not exposed to SPS.

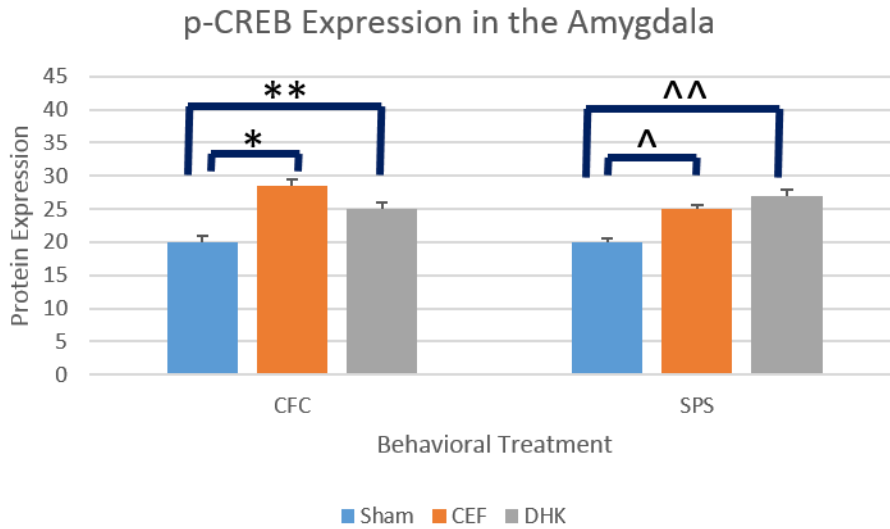


Figure 18. In the amygdala of animals, both exposed and not exposed to SPS, there is a significant difference between the Sham-treated group and both the ceftriaxone- and DHK-treated groups. These findings show that, regardless of trauma exposure, CEF and DHK both increase the level of p-CREB expression in the amygdala. SPS = single prolonged stress; CFC = classical fear conditioning; Cef = ceftriaxone; DHK = dihydrokainate. Error bars represent standard errors. “\*” represent p-values=0.0002. “\*\*” represent p-values=0.0366. “^” represent p-values=0.0118. “^^” represent p-values=0.0007.

Regarding SPS-exposed rats, the data also show an increase in p-CREB expression in the amygdala (Figure 18, right half). Using the above mentioned mixed model ANOVA, post-hoc analysis showed that, within the amygdala of SPS-exposed rats, as in the PFC, both CEF ( $t(91.34)= 2.56$ ,  $p=0.0118$ ) and DHK ( $t(91.34)= 3.50$ ,  $p=0.0007$ ) administration resulted in a significant elevation in p-CREB expression compared to rats given the Sham. Again, this shows that both DHK and ceftriaxone increase p-CREB expression in SPS-exposed rats.

Overall, these data show that following SPS exposure, both DHK and Cef administrations result in an increase in p-CREB expression in the PFC that is not seen in CFC rats. This indicates that the effects of these drugs on p-CREB expression in the PFC are dependent upon trauma exposure. This is contrary to what was demonstrated in the amygdala, where both drugs elevated p-CREB regardless of trauma exposure. This increase in p-CREB for both drugs, following trauma-exposure, which was observed in the

PFC, a region associated with the formation of fear extinction memories, seems concordant with the mitigation of the SPS-induced deficit in fear extinction recall observed for both drugs in Specific Aim 1. Thus, these results show that Cef and DHK action on p-CREB can vary not only upon exposure to trauma, but also among brain regions. Furthermore, these results show that in regard to p-CREB expression, when significant effects of both drugs occur, they occur in the same direction. This is contrary to our expectations that these drugs would have opposing effects on p-CREB considering previous reports of their opposite actions upon GLT-1 function.

### **SECTION C: SPECIFIC AIM 3: DETERMINING IF REGIONAL DIFFERENCES IN NMDA RECEPTOR SUBTYPE EXPRESSION CONFER DIFFERENTIAL PROTECTION OF P-CREB FROM EFFECTS OF GLUCOCORTICOIDS**

While the first two experiments of this dissertation revolved around describing the involvement of astrocytic glutamate reuptake and extrasynaptic glutamate spill-over in PTSD pathogenesis, this final stage aimed to identify additional brain region-specific factors that might explain why exposure to psychological trauma may preferentially affect fear extinction memory as compared with fear memory. As mentioned earlier, we theorized that one such factor might be regional differences in expression profiles of synaptic NMDA receptor subtypes. Though there is still a lack of consensus regarding the true nature of synaptic NMDA receptor subtype profiles, some studies have found that different regions of the brain express different levels of the NR2A subtype relative to the NR2B subtype. For example, it has been reported that the hippocampus and the PFC express relatively higher levels of NR2A in the synapse, while the amygdala expresses relatively higher levels of NR2B. This could prove significant considering it has been shown that GCs act as inhibitors of molecular mechanisms downstream of NR2A, and could thus differentially affect the respective functions of various regions of the brain of an animal under psychological stress depending upon the relative level of NR2A expression in a given region. As such, we hypothesized that elevation of GCs during the extreme stress of SPS would preferentially impair synaptic transmission, and thus memory formation, in regions of the brain with greater relative expression of NR2A (i.e., the hippocampus and the PFC) compared to those that have relatively higher expression of synaptic NR2B (i.e., the amygdala). This would subsequently translate into a greater inhibition of fear extinction memory compared to fear memory, and the subsequent impairment of fear extinction memory recall exhibited by SPS-exposed rats. Furthermore, we predicted that the regional differences in NMDAR profile expression that underpin this hypothesized role of GCs in PTSD pathology remain the same regardless of whether or not a subject was exposed to situational trauma. As such, we

also hypothesized that the effects of NR2A and NR2B antagonists on NMDA-activated CREB phosphorylation would not be changed following exposure to SPS.

To do this, we performed an *ex vivo* study using portions of coronal brain slices containing the amygdala, hippocampus, or the PFC. The basic design involved administering bicuculline to specifically stimulate synaptic transmission without spill-over of glutamate into the extrasynaptic space. Bicuculline is an antagonist of GABA receptors which, upon administration to tissue slices, decreases inhibitory control of presynaptic neurons and subsequently causes increased synaptic release of neurotransmitter that remains isolated to the synapse. Consequently, bicuculline administration induces phosphorylation of CREB in post-synaptic neurons. The relative expression profiles of synaptic NMDA NR2A to NR2B receptors of the aforementioned brain regions were evaluated indirectly by administering bicuculline with either a Sham (MEM media), NVP (a relatively specific inhibitor of NR2A), or Ro25 (a relatively specific inhibitor of NR2B) and then measuring the subsequent levels of p-CREB expression. We made the prediction that tissues administered these NMDA receptor inhibitors would show decreased p-CREB expression compared to those treated with the Sham due to inhibited synaptic NMDA receptor-dependent transmission. Furthermore, we made the assumption that a significant difference between Sham-treated tissue and an inhibitor-treated tissue would only be present if the synapses within that tissue depended upon the subtype that was specific to that inhibitor. For example, we assumed that if administration of NVP significantly decreased p-CREB expression compared to the Sham-treated tissue, then that would mean the synapses of that tissue significantly utilize NR2A subtypes for synaptic transmission. Conversely, we assumed that if administration of NVP did not significantly decrease p-CREB expression compared to the Sham-treated tissue, then that would mean the synapses of that tissue do not significantly utilize NR2A subtypes for synaptic transmission. Moreover, if this decrease in p-CREB expression following NVP treatment is shown to be greater in one region compared to another, then we are also making the assumption that synaptic transmission in the region with the greater

decrease depends on NR2A more than it does in the region with the smaller decrease. We predicted that (i) the magnitude of p-CREB decrease produced by NR2A inhibition (with NVP) would be smaller in the amygdala compared with the PFC or hippocampus, and (ii) that the magnitude of p-CREB decrease produced by NR2B inhibition (with Ro25) would be larger in the amygdala compared with the PFC or hippocampus. Such findings would reflect the prediction that the amygdala has a higher NR2B to NR2A ratio in the synapse compared with the PFC or hippocampus. A GC treatment was included in the above factorial design to determine whether this differential synaptic NMDA sub-type composition would result in a smaller GC-induced decrease in p-CREB in the amygdala compared with the PFC or hippocampus. This finding would address the putative ability of GCs to preferentially inhibit NR2A associated p-CREB induction. Finally, SPS treatment was also incorporated into the factorial design to assess whether the NR2B to NR2A ratio is affected by situational trauma.

### **c.i. Comparison of GC-induced inhibition of p-CREB between Brain Regions**

As outlined in the Specific Aims Section, our first goal towards addressing Specific Aim 3 was to compare the effect of GC administration on p-CREB expression among the hippocampus, PFC, and amygdala. As mentioned above, GCs have been shown to be inhibitors of cellular pathways specifically associated with NR2A receptor activation (Xiao et al., 2010) This is a central concept that connects this stage of work to the pathogenesis of PTSD, since we are predicting that the release of GCs during stress will selectively impair memories formed in regions of the brain that have higher expression of synaptic NR2A (like the PFC). However, this activity of GCs was demonstrated only in cultured hippocampal neurons. Since our model utilized tissue sections rather than cell cultures, we first wanted to demonstrate that GC administration in tissue sections reduces p-CREB as reported in cell culture. Following the complete treatment described above, tissues were processed, and ELISA was performed

to quantify the levels of p-CREB expression in each group. A mixed model ANOVA (trauma x region x drug) was performed to analyze the results, and a significant interaction ( $F(3,154)=10.62$ ,  $p<0.0001$ ) of trauma exposure with drug treatment was found. Figure 19 below shows a subset of the results of this analysis of p-CREB that focuses on the comparison of the effects of GC administration to Sham administration in the three regions of interest.

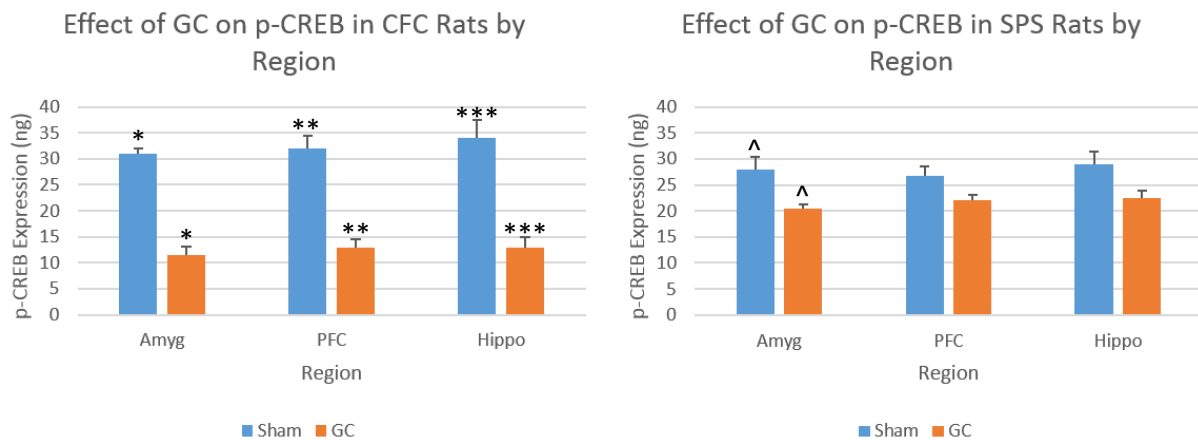


Figure 19. Subset of the results that focus on the effects of GC administration, compared to Sham administration, in the three regions of interest. On the left, GC administration results in significantly lower levels of p-CREB expression in all three regions of CFC rats. On the right, only the amygdala demonstrated a significant decrease in p-CREB expression following GC treatment in SPS rats, suggesting that SPS causes a change in susceptibility to GC-induced p-CREB inhibition in the PFC and hippocampus. SPS = single prolonged stress; CFC = classical fear conditioning only; GC = glucocorticoid. Error bars represent standard errors. Groups sharing the same symbols are significantly different from each other. “\*”, “\*\*”, and “\*\*\*” represent p-values less than 0.0001, and “^” represent a p-value of 0.028.

Post-hoc pair-wise comparisons show that in tissues from CFC rats, GC administration results in significantly lower levels of p-CREB expression in all three regions (amygdala ( $t(154) = -6.65$ ,  $p<0.0001$ ); hippocampus ( $t(154) = -7.17$ ,  $p<0.0001$ ); PFC ( $t(154) = -6.43$ ,  $p<0.0001$ )). Though this cannot be conclusively attributed to interactions with the molecular pathways associated with synaptic NMDA receptors, the immediate effect seen here is consistent with a GC action that is independent from its classical mechanism of action via GR binding and subsequent alteration of cellular gene transcription that can take hours to become evident (Makara and Haller, 2001).

Since it was unexpected that GCs decreased p-CREB expression in all three regions, further analysis was performed to see if the magnitudes of change induced by GCs were the same for each region. The rationale behind this approach was that if GCs exerted NR2A-specific inhibition of p-CREB, then the regions with greater dependence on NR2A transmission (i.e., hippocampus and PFC) would demonstrate a more pronounced inhibition relative to regions with greater NR2B dependence (i.e., the amygdala). This could translate into greater negative impact upon PFC associated fear extinction memory than upon amygdala associated fear memory. As such, for each region, the change in p-CREB expression was calculated by subtracting the value of the GC treated tissues from that of the Sham treated tissue. A mixed model ANOVA (region x drug) ( $F(2,14)=0.14$ ) was then performed to compare the magnitudes of these calculated differences. No significant difference was found between the three regions, suggesting that GCs exerted similar degrees of p-CREB inhibition across all three regions of interest. While this result could indicate that GCs do not exert a NR2A-specific action as seen in the Xiao et al. (2010) cell culture experiment, it does not rule out the possibility that our assumption is wrong about these regions expressing differing profiles of synaptic NMDA receptor subtypes.

Though the above analyses did not show a difference in the magnitude of GC inhibition of p-CREB across brain region in CFC animals, we did find that exposure to SPS changes how GCs affect p-CREB expression. This finding was unexpected as we had predicted that SPS would not change the synaptic NMDAR subunit profiles in these regions, and thus the role of GCs in the pathology of PTSD would remain the same regardless of whether or not a subject was exposed to situational trauma. Figure 19 shows that among the three regions of interest, pairwise post-hoc comparisons revealed that only the amygdala demonstrated a significant decrease in p-CREB expression following GC treatment ( $t(154)= -2.6, p=0.024$ ). This indicates that following exposure to trauma, only the amygdala remains significantly susceptible to GC inhibition of p-CREB expression. This could be an indication that SPS

causes a change in the proportional representation of NR2A/NR2B across regions or the downstream cellular mechanisms with which they are associated.

**c.ii. Relative Representation of Synaptic NR2A and NR2B Receptor Representation Across Amygdala, PFC and Hippocampus**

The next goal of Specific Aim 3 was to determine if synaptic transmission among the three regions of interest have differing dependence on one NMDA receptor subtype over another by demonstrating the effects of NR2A and NR2B inhibitors (NVP and Ro-25, respectively) on p-CREB expression across these regions. As the above analysis gave unexpected results in light of previous work regarding the subtype specificity of GC effects or the regional representation of NR2 receptor subtypes, this analysis provides further insight into the nature of the profiles of synaptic NMDA receptor subtypes within these regions. In so doing, we might shed more light onto whether the lack of regional differences in GC inhibition of p-CREB was due to a lack of subtype specificity of GC activity or a lack of a difference in subtype profiles across the regions.

Figures 20 and 21 below show the remaining results from the mixed model ANOVA (mentioned above that analyzed the effects of trauma x region x drug in this experiment), depicting the post-hoc pair-wise analysis of the effects on p-CREB of NVP and Ro-25 administration, respectively, compared to Sham administration in the three regions of interest.

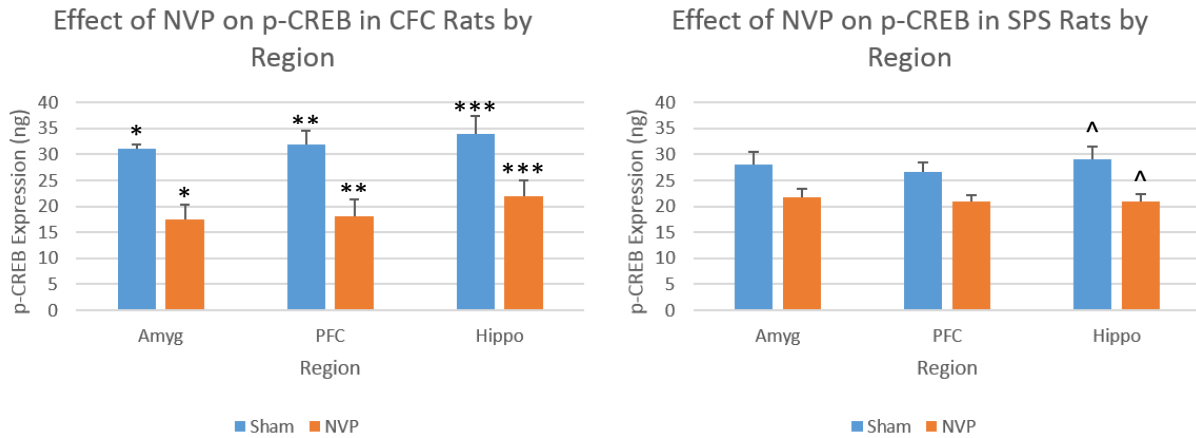


Figure 20. Subset of the results that focuses on the effects of NVP administration (NR2A inhibitor), compared to Sham administration, in the three regions of interest. On the left, NVP administration results in significantly lower levels of p-CREB expression in all three regions of CFC rats. On the right, only the hippocampus demonstrated a significant decrease in p-CREB expression following NVP treatment in SPS rats, suggesting that SPS causes a change in susceptibility to NVP-induced p-CREB inhibition in the PFC and amygdala. This is also suggestive of an SPS-induced change in the synaptic NR2A/NR2B subtype profiles among the three regions. SPS = single prolonged stress; CFC = classical fear conditioning only. Error bars represent standard errors. Groups sharing the same symbols are significantly different from each other. “\*”, “\*\*\*”, and “\*\*\*\*” represent p-values of <0.0001, 0.0003, and 0.0002, respectively, and “^” represent a p-value of 0.017.

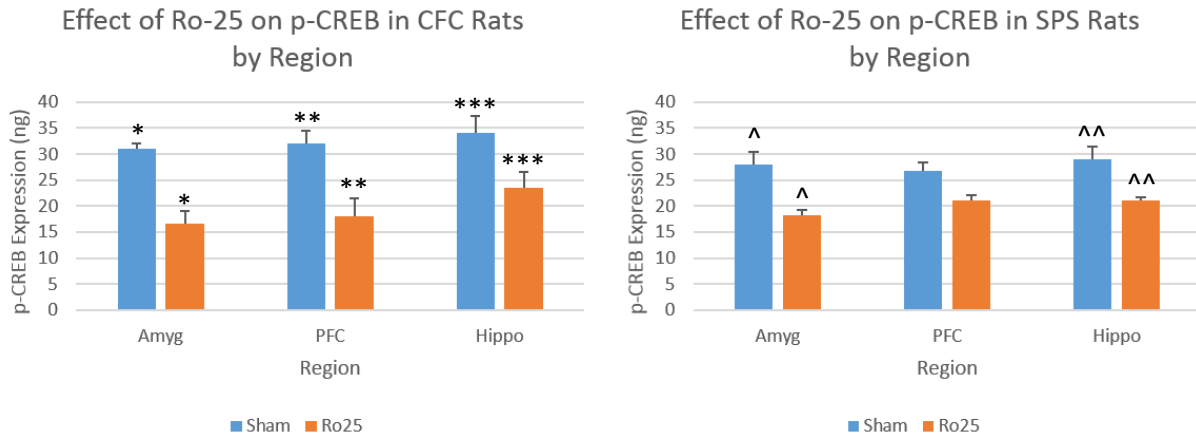


Figure 21. Subset of the results that focuses on the effects of Ro-25 administration (NR2B inhibitor), compared to Sham administration, in the three regions of interest. On the left, Ro-25 administration results in significantly lower levels of p-CREB expression in all three regions of CFC rats. On the right, the amygdala and hippocampus demonstrated a significant decrease in p-CREB expression following Ro-25 treatment in SPS rats, suggesting that SPS causes a change in susceptibility to Ro-25-induced p-CREB inhibition in the PFC. This is also suggestive of an SPS-induced change in the synaptic NR2A/NR2B subtype profiles among the three regions. SPS = single prolonged stress; CFC = classical fear conditioning only. Error bars represent standard errors. Groups sharing the same symbols are significantly different from each other. “\*”, “\*\*\*”, and “\*\*\*\*” represent p-values of <0.0001, <0.0001, and 0.0015, respectively, and “^” and “^^” represent p-values of 0.0034 and 0.0188, respectively.

Post-hoc pair-wise comparisons showed that within the CFC groups both NVP (amygdala ( $t(154) = -4.61$ ,  $p < 0.0001$ ); PFC ( $t(154) = -4.02$ ,  $p = 0.0003$ ); hippocampus ( $t(154) = -4.16$ ,  $p = 0.0002$ )) and Ro-25 (amygdala ( $t(154) = -4.85$ ,  $p < 0.0001$ ); PFC ( $t(154) = -4.58$ ,  $p < 0.0001$ ); hippocampus ( $t(154) = -3.55$ ,  $p = 0.0015$ )) significantly decreased p-CREB expression in all three regions. This suggests that both NR2A and NR2B subtypes have significant representation within the synapse, and provide significant contributions to CREB phosphorylation following synaptic transmission, in all three regions of CFC rats.

Exposure to SPS changed the effects of NVP and Ro-25 administration in some of the brain regions of interest. For example, Figure 20 shows that when rats are exposed to SPS, only the hippocampal tissues continued to demonstrate a significant decrease in p-CREB expression following NVP administration ( $t(154) = -2.78$ ,  $p = 0.017$ ), in contrast with the CFC condition. This suggests that synaptic NR2A significantly contributes to p-CREB expression in the hippocampus of SPS rats, but not in

the amygdala or PFC. Additionally, Figure 21 shows that when rats are exposed to SPS, only the hippocampal ( $t(154) = -2.74, p = 0.0188$ ) and amygdala ( $t(154) = -3.31, p = 0.0034$ ) tissues continued to demonstrate a significant decrease in p-CREB expression following Ro-25 administration, in contrast with the CFC condition. This suggests that synaptic NR2B significantly contributes to p-CREB expression in the hippocampus and amygdala of SPS rats, but not in the PFC. These findings are in contrast to those for the CFC condition, where both NR2A and NR2B appeared to be making a significant contribution to p-CREB expression in all three regions.

Similar to the analysis described earlier for GCs, a short-coming of the above analyses is that they are limited to only determining whether or not a change in p-CREB occurred as a consequence of treatment. While we can use this information to determine which NMDAR subtypes provide significant contributions to p-CREB expression following synaptic transmission, we cannot determine if one subtype contributes to p-CREB expression more than the other. It was rationalized that if a given inhibitor induced a greater decrease in p-CREB expression in one region compared to another, then this would indicate that synaptic transmission in that region depends more heavily on the corresponding NMDAR that the drug inhibits. For example, if NVP induced a greater decrease in p-CREB in the PFC than in the amygdala, we would infer from this that synaptic transmission in the PFC exhibits greater dependence on NR2A receptors than that in the amygdala. As depicted in Diagrams 9 and 10, we predicted that the synapses of the PFC and hippocampus would contain greater numbers of NR2A compared to NR2B, with the reverse relationship being true for the amygdala. Subsequently, following the above described rationale, we predicted that NVP (a NR2A inhibitor) would induce a greater decrease in p-CREB in the hippocampus and the PFC compared to the amygdala, while Ro-25 (a NR2B inhibitor) would induce a greater decrease in p-CREB expression in the amygdala compared to the PFC and hippocampus.

As such, further analysis was performed by calculating an index representing the magnitude of change in p-CREB expression induced by a given drug treatment group. As described above for GCs, these indices were calculated by individually subtracting the p-CREB expression of the NVP and Ro-25 groups within a given region of CFC rats from that of the sham-treatment rats for that region. A mixed model ANOVA (region x drug) (for NVP,  $F(2,14)=0.06$ ; for Ro-25,  $F(2,14)=0.23$ ) was then performed with no significant main effect of treatment for either group (NVP,  $p=0.9388$ ; Ro-25  $p=0.7939$ ). Post-hoc pair-wise comparisons were then performed and are depicted in Figure 22 below. Contrary to our expectation, no significant differences were found between any of the regions of the NVP treated groups (amygdala vs hippocampus ( $t(14)= -0.26$ ,  $p=0.8004$ ); amygdala vs PFC ( $t(14)= -0.34$ ,  $p=0.7376$ ); hippocampus vs PFC ( $t(14)= -0.08$ ,  $p=0.9342$ )) or Ro-25 (amygdala vs hippocampus ( $t(14)= -0.65$ ,  $p=0.5271$ ); amygdala vs PFC ( $t(14)= -0.13$ ,  $p=0.8956$ ); hippocampus vs PFC ( $t(14)= 0.52$ ,  $p=0.6146$ )). Thus, if our rationale is accurate, these findings suggest that synaptic transmission in these three regions have equivalent dependencies on NR2A and NR2B receptors.

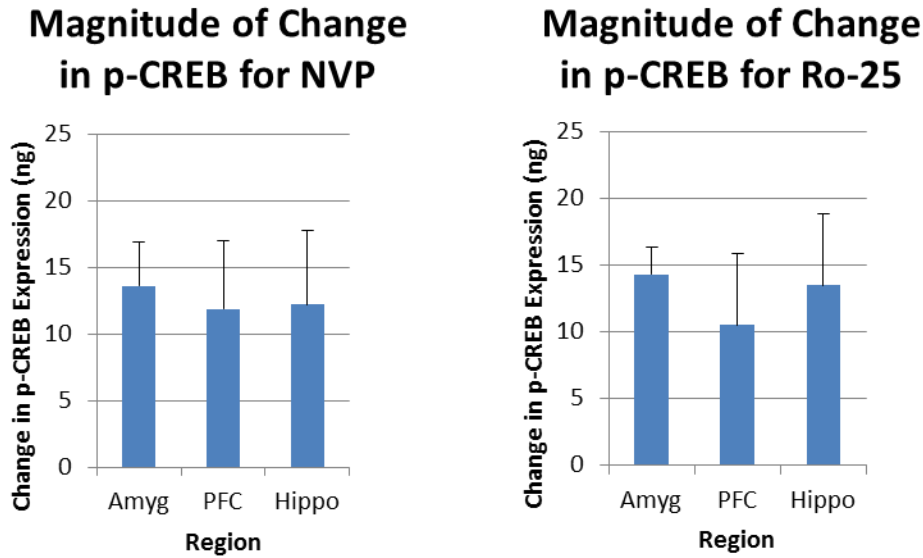


Figure 22. Depiction of the comparison between regions of CFC rats in the magnitudes of change in p-CREB expression induced by NVP (NR2A inhibitor) and Ro-25 (NR2B inhibitor). For each inhibitor, no significant differences were found between any of the regions. These findings suggest that synaptic transmission in these three regions have equivalent dependencies on NR2A and NR2B receptors, which is contrary to our expectation that there would be regional differences in these changes in magnitude. Amyg = amygdala; PFC = prefrontal cortex; Hippo = hippocampus. Error bars represent standard errors.

Overall, the findings of this stage of the experiment support the idea that both NR2A and NR2B subtypes have a significant representation within the synapse, and make a significant contribution to p-CREB expression, in all 3 regions of interest. We also demonstrated that exposure to SPS results in changes in regional susceptibility to either NR2A or NR2B inhibition. This suggests that exposure to SPS results in alterations in synaptic profiles of these NMDA receptor subtypes, and that these alterations are different among the three regions analyzed in this experiment. The latter finding is concordant with the observation that the effect of GCs upon p-CREB expression changed in SPS exposed rats.

# CHAPTER V

*DISCUSSION  
AND  
CONCLUSIONS*

## V. DISCUSSION AND CONCLUSIONS

### **SECTION A: DISCUSSION OF SPECIFIC AIMS 1 AND 2**

This research aimed to test two principal hypotheses. The first was that following situational trauma, impaired astrocytic glutamate reuptake leads to inhibition of long-term extinction memory processes within the pre-frontal cortex. The second was that the concurrent presence of glucocorticoids during situational trauma selectively exacerbates the inhibition of fear extinction memory processes in the prefrontal cortex, but not of conditioned fear memory processes in the amygdala, due to differences between these brain regions in expression of NMDA receptor subtypes.

Regarding the first principal hypothesis, Figure 23 below provides a visual representation of the sequence of proposed interactions (indicated by the straight dashed arrows) that potentially link situational trauma, glutamate transporter expression, p-CREB expression, and fear extinction memory recall (denoted as “Behavior”). Briefly, we hypothesized that SPS-induced situational trauma would decrease astrocytic glutamate reuptake transporter expression, specifically GLT-1. This diminished expression would lead to a spill-over of synaptic glutamate to extrasynaptic regions and a subsequent stimulation of extrasynaptic NMDA glutamate receptors that causes an inhibition of p-CREB and long-term memory formation.

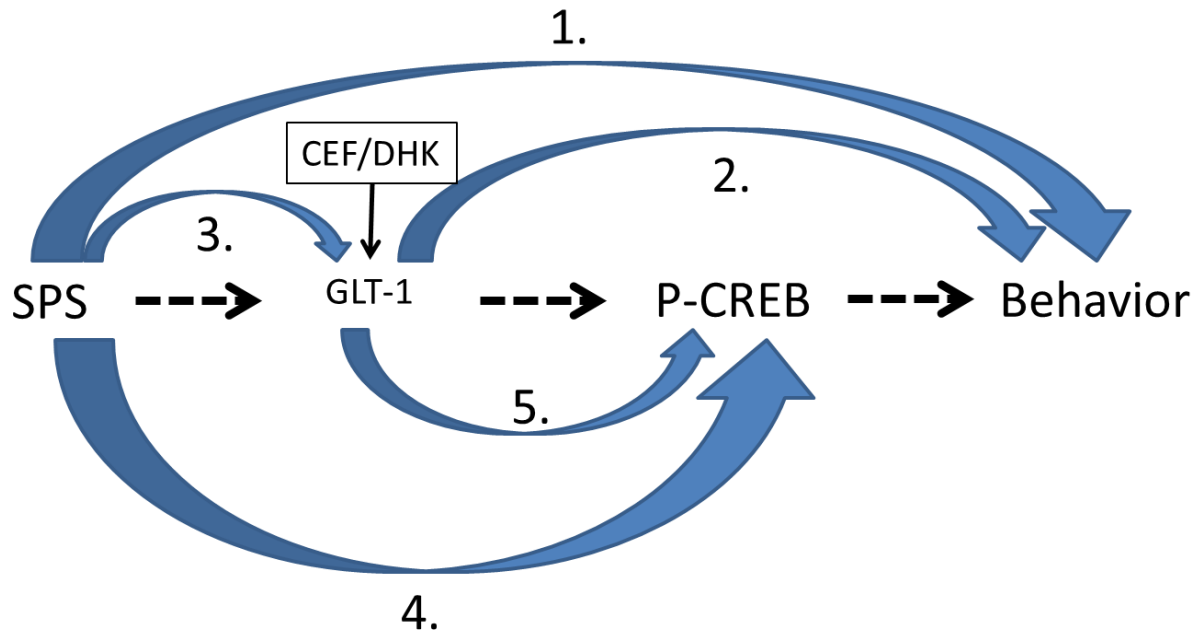


Figure 23. A visual representation of the proposed model representing the first of our two principle hypotheses. The dashed arrows represent the sequence of proposed interactions that potentially links situational trauma, glutamate transporter expression, p-CREB expression, and fear extinction memory recall (denoted as “Behavior”). The curved solid arrows represent assessments that were made to test the validity of the proposed sequence of interactions. These assessments are defined as: 1. Effects of SPS on fear extinction memory recall and GR expression, 2. Effect of GLT-1 modulation upon fear extinction memory recall, 3. Effects of SPS exposure upon GLT-1 expression, 4. Effect of SPS on p-CREB expression, and 5. Effects of GLT-1 modulation on p-CREB expression. Abbreviations: CEF = ceftriaxone, DHK = dihydrokainate, SPS = single prolonged stress; GLT-1 = glutamate transporter 1; p-CREB = phosphorylated cAMP Response Element Binding protein.

The approach used in this research to test the validity of this proposed chain of events was to perform several assessments, depicted in Figure 23 by curved solid arrows, targeting specific points in the hypothetical sequence. The first of these (Arrow 1) was to assess the effects of SPS on fear extinction memory recall and GR expression with the purpose of validating that our SPS model, in our hands, affected fear extinction memory recall in a manner consistent with that reported in the literature. Next, we aimed to assess the effect of GLT-1 modulation with ceftriaxone (CEF) or dihydrokainate (DHK) (indicated by the solid black arrow) upon fear extinction memory recall (Arrow 2)

to determine if GLT-1 function can in fact influence behaviors associated with situational trauma exposure. More specifically, we administered a facilitator (i.e., CEF) or an inhibitor (i.e., DHK) of GLT-1 activity, and measured the effect these manipulations had on the fear extinction memory recall endpoint. We then assessed the effect of SPS exposure upon GLT-1 expression (Arrow 3) to determine if situational trauma can change GLT-1 expression in a manner that might lead to modulation of glutamate spillover onto the extrasynaptic region. We also assessed the effect of SPS on p-CREB expression (Arrow 4) to establish how situational trauma is linked with a biochemical substrate of long-term potentiation of fear extinction memory and how the trauma-induced change in GLT-1 (see Arrow 3) affects this LTP substrate. Finally, we examined the effect of manipulating GLT-1 function with CEF or DHK upon p-CREB expression (Arrow 5) to address how indirectly altering the potential for synaptic glutamate spillover might affect this substrate of LTP, and how such an intervention might modulate the effects of SPS on p-CREB expression.

The data generated from these assessments yielded a combination of expected and unexpected results, some of which contradicted the hypothetical model described above, suggesting a possible revision of the model may be needed.

***a.i. Effects of SPS on Fear Extinction Memory Recall and GR Expression: Validation of the SPS Model (Arrow 1 from Figure 23)***

As stated above, the first step of our approach toward addressing the first principal hypothesis was to assess the effect of SPS upon fear extinction memory recall (see Fig. 23, Arrow 1). Extensive efforts were made to ensure that the rats exposed to SPS in our study exhibited similar behavioral and biochemical features compared to those reported in other studies using rodents to model PTSD (Yamamoto et al., 2009). From a behavioral standpoint, we used an index of fear extinction recall as the behavioral indicator of the presence of PTSD-like symptoms in our rats. In human studies, individuals

with PTSD exhibit a decreased ability to retain fear extinction memories for more than 24 hours (Milad et al., 2007; Milad et al., 2009). It has been suggested that this impaired retention of extinction memories prevents the long term suppression of fear memories, thus leading to recurrent intrusive recollection of the traumatic event (Eisenhardt and Menzel, 2007; Milad et al., 2009; Shvil et al., 2014). Similarly, following exposure to SPS, it has been consistently shown that rats will exhibit a decrease in fear extinction memory recall (Yamamoto et al., 2008; Yamamoto et al., 2009; Knox et al., 2010). As such, because it is relevant to PTSD symptomology, seemingly conserved between rats and humans, and has been consistently reported to be induced by SPS, we selected fear extinction memory recall deficit as our behavioral endpoint for validation of our behavioral model.

As expected, our results from Specific Aim 1 showed that rats exposed to SPS had significantly worsened fear extinction memory recall compared to rats that were not exposed to SPS, and that this change in behavior could not be attributed to other factors such as failure to develop fear memory during the conditioning process, failure of fear extinction learning, or differences between groups in level of extinction learning. We concluded from this finding that we were successful in using SPS to induce behavioral changes in our rats that are consistent with those seen in other studies that have used SPS to model PTSD (Yamamoto et al., 2009).

From a biochemical perspective, we selected GR expression in the hippocampus as an endpoint for determining the efficacy of our SPS model to induce a PTSD-like state in the rats of this study. Although this parameter has not been directly measured in human brain samples, people with PTSD can exhibit signs of increased glucocorticoid sensitivity, such as low basal circulating cortisol levels, increased GR expression in circulating leukocytes, and exaggerated cortisol response during and in anticipation of stressful situations (Meewisse et al., 2007; van Zuiden et al., 2013; Bremner et al., 2003). In a similar fashion, alterations to rat GC sensitivity can be seen following exposure to SPS such as an elevation in

hippocampal GR expression (Zhe et al., 2008; Knox et al., 2012). Subsequently, hippocampal GR expression was selected as an endpoint to validate our model due to its relevance to the symptomology of PTSD, its comparability with symptoms observed in human studies, and its consistency as a consequence of SPS exposure. As we reported in the Results for Specific Aim 2, our study found that, as expected, all groups exposed to SPS demonstrated this elevation in hippocampal GR expression (see Fig. 10).

Taken together, the behavioral and biochemical results support the conclusion that the methods used in this experiment sufficiently replicated critical features of the rodent SPS model of human PTSD. This is an important point, as some findings in this study were contrary to our hypotheses and expectations. One could argue that these unexpected outcomes may be the result of a faulty model. However, the fact that previously reported features of this model were diligently validated within this experiment suggests that this is not the case. As such, we are confident that any results that were inconsistent with our hypotheses and expectations were legitimate consequences of our treatments, and not the consequence of a failure to model the disorder.

**a.ii. Effect of GLT-1 modulation upon fear extinction memory recall: Effects of CEF (GLT-1 facilitator) and DHK (GLT-1 inhibitor) Administration (Arrow 2 from Figure 23)**

The second step of our approach towards addressing the first principal hypothesis was evaluating the effect of GLT-1 manipulation upon the behavioral consequences of SPS exposure (see Fig. 23, Arrow 2). As mentioned above, GLT-1 was manipulated via administration of ceftriaxone, which is purported to increase GLT-1 expression (Rawls et al., 2010; Althobaiti et al., 2016), and DHK, purported to be a GLT-1 functional antagonist (Rawls et al., 2010). Following these manipulations, effects on fear extinction memory recall were evaluated. Our original hypothetical model proposed that exposure to trauma would diminish the expression of GLT-1 and cause a spill-over of glutamate into extrasynaptic

spaces. This spillover would, in turn, activate extrasynaptic NMDA receptors, decrease downstream p-CREB expression, and subsequently impair long-term memory retention. As such, we predicted that facilitation of GLT-1 function through CEF administration would reduce SPS-induced deficits in fear extinction memory recall by reducing glutamate synaptic spillover, while DHK would exacerbate the deficits by increasing spillover.

However, contrary to our expectations, our findings showed that CEF and DHK had similar effects upon SPS-induced changes instead of opposing ones. More specifically, from the results of Specific Aim 1 (see Figure 6) we showed that when administered a saline sham, rats exposed to SPS exhibited worse fear extinction memory recall compared to rats that were not exposed to SPS. However, when administered either CEF or DHK, rats exposed to SPS exhibited no significant difference in fear extinction memory recall compared to rats not exposed to SPS. These results are consistent with other studies that have evaluated the effect of CEF administration upon memory retention. For example, Yang et al. (2013) and Li et al. (2012) demonstrated that chronic CEF administration in mice can reverse inhibition of spatial memory and associative fear memory, respectively, induced by GLT-1 down-regulation. Similarly, Hota et al. (2008) found that chronic CEF administration lessened the spatial memory deficits of rats induced by glutamatergic excitotoxicity associated with exposure to hypoxic conditions. Though these studies were not evaluating fear extinction memory recall, in each of these cases, GLT-1 dysfunction or excessive synaptic glutamate release caused an impairment of long-term memory formation that could be reversed with chronic CEF administration. As such, if SPS were inducing a similar depression of GLT-1 function and consequent synaptic spillover in our study as we hypothesized, we would expect to see, as we did, the elimination of SPS-induced impairment of fear extinction memory recall exhibited by our CEF-treated rats.

However, the results of DHK administration were contrary to our expectations. As already noted, DHK is a selective GLT-1 blocker (Kawahara et al., 2002) that should increase the likelihood of glutamate spillover to extrasynaptic regions and should exacerbate the hypothesized effect of any SPS-induced decrease in GLT-1 expression. DHK has been shown to impair spatial memory retention in rats (Bechtholt et al., 2010), and it was expected that it would similarly enhance our observed SPS-induced impairment in fear extinction memory recall. As Figure 6 shows, however, DHK administration seemed to eliminate SPS-induced impairment of fear extinction memory recall in manner comparable to that of CEF. While this discrepancy between our findings and that of Bechtholt et al. (2010) could be explained as a consequence of the differences in study design and type of memory being evaluated, the effect of DHK was still opposite of what we would expect to see in a condition where GLT-1 function is already being impaired. As such, the elimination of SPS-induced fear extinction memory deficits following DHK administration conflicts with some of the details we proposed for the model depicted in Figure 23, and raises the possibility that GLT-1 involvement is not what we predicted in our hypothesis. However, our results with CEF do show that manipulating the astrocytic glutamate reuptake transporter, GLT-1, can affect SPS-induced changes in fear extinction recall (see Fig. 23, Arrow 2). Potential explanations for the unexpected effects of DHK upon the fear extinction recall deficit, and the similarity between the effects of CEF and DHK in this regard, are further discussed below.

**a.iii. The Effects of SPS on GLT-1 Expression (Arrow 3 from Figure 23)**

The third assessment used to evaluate the hypothetical model depicted in Figure 23 aimed to determine how SPS exposure affected GLT-1 expression. It was described above how the effects of DHK administration upon fear extinction memory recall could be indicating that GLT-1 does not function in our hypothetical model as we predicted. As such, an assessment of how SPS changes GLT-1 expression becomes particularly important when trying to interpret this unexpected finding. Furthermore, this

assessment needed to compare the GLT-1 expression in the PFC with that in the amygdala. As discussed in the Literature Review, extinction of a fear memory occurs when fear memory formation strongly associated with the amygdala is suppressed by extinction memory formation strongly associated with the PFC (Hughes and Garcia, 2007; Mamiya et al., 2009). Since reciprocal regulation occurs between these regions, it has been suggested that the relative activity among them determines which memory is expressed: dominance of amygdala activity promotes a fear response, while dominance of PFC activity inhibits this promotion (Hughes and Garcia, 2007; Mamiya et al., 2009). In the context of our hypothetical model where we are hypothesizing that decreased GLT-1 expression leads to impaired long-term memory formation, we predicted that fear extinction memory recall may be impaired because GLT-1 expression is decreased in the PFC, but not in the amygdala. An alternative explanation could also be that GLT-1 expression is decreased in both regions, but more so in the PFC compared to the amygdala. Regardless, the need for evaluation of GLT-1 expression in both the PFC and amygdala is further highlighted by the unexpected effects we observed for DHK on fear extinction memory recall.

Our prediction that SPS would decrease GLT-1 expression in the PFC (and possibly the amygdala) was supported by the fact that stress has been shown to promote a pro-inflammatory state in the brain (Wilson et al., 2013 and 2014), and that many pro-inflammatory cytokines are associated with impairment of glutamate reuptake (Yan et al., 2014; Fang et al., 2012; Sitcheran et al., 2015). Furthermore, Imbe et al. (2012) demonstrated that chronic restraint stress can lead to a decrease in GLT-1 expression in the periaqueductal gray matter. However, contrary to these studies and our expectations, we found that SPS induced a significant elevation of GLT-1 expression in both regions. This finding is consistent with a study performed by Reagan et al. (2004) that reported an increase in GLT-1 mRNA and protein expression following chronic restraint. The experimental design of the Imbe et al. (2012) and Reagan et al. (2004) studies were very similar, as they consisted of the same type and duration of restraint as well as the same strain and size of rat. The only significant difference between

the two studies was that Imbe et al. (2012) measured GLT-1 in the periaqueductal grey matter while Reagan et al. (2004) measured GLT-1 in the hippocampus. This would indicate that stress-induced changes in GLT-1 expression may vary across different regions of the brain. As such, our study may have just included regions of the brain that respond to stress by increasing GLT-1 expression.

With this said, our findings do not necessarily contradict our hypothesis that SPS impairs GLT-1 function. For example, it has been shown that pharmacological antagonism of GLT-1 can lead to a compensatory up-regulation in GLT-1 expression (Lau et al., 2010). It is possible that the SPS-exposed rats did undergo a pro-inflammatory event as a result of their trauma, and that this event blocked the function of GLT-1 as we theorized. Since our tissues were not collected and analyzed until 10 days after SPS exposure, the increase in GLT-1 expression could be the result of the compensatory response to the SPS-induced acute inhibition, which may have been identified if tissues had been collected and analyzed at an earlier time point.

Although we suggest a possible explanation above for the increased GLT-1 expression following SPS exposure, this increase does not fit with our hypothesis that fear extinction memory recall is impaired by diminished GLT-1 expression and subsequent glutamate spill-over. Regardless of the explanation for the elevated GLT-1 expression, the fact remains that towards the end of the experiment when fear extinction memory recall was being tested, GLT-1 expression was significantly higher in SPS-exposed rats compared to the CFC rats. This increased expression of GLT-1 in SPS-exposed rats, if anything, might indicate an equivalent or greater capacity in these animals for glutamate uptake and for prevention of glutamate spill-over. Furthermore, since there was no significant difference in GLT-1 expression between the PFC and amygdala, region specific changes in GLT-1 expression induced by SPS could not serve as an explanation for the deficits in fear extinction memory compared to fear memory. Taking this together with the unexpected results following DHK administration, we concluded that

changes in GLT-1 expression do not play the role we hypothesized in the impairment of fear extinction memory recall exhibited after SPS-exposure. Consequently, the fact that our prediction of how SPS would affect GLT-1 expression did not match our results calls into question the validity of our predictions regarding the effect of SPS upon p-CREB expression.

**a.iv. The Effects of SPS on p-CREB Expression (Arrow 4 from Figure 23)**

The next step in evaluating our hypothetical model was to assess the effect of SPS upon p-CREB expression. Because it serves as a gene transcription factor integral to inducing the changes in protein expression responsible for LTP induction (Bitto et al., 1996), p-CREB was selected in this study as a biochemical indicator of long-term memory formation.

Since PTSD has been associated with impaired fear extinction recall memory (i.e., a form of long-term memory of the PFC) (Milad et al., 2007; Milad et al., 2009), we hypothesized that p-CREB expression in the PFC of our rats would be decreased following SPS exposure, relative to the p-CREB expressed in the amygdala, although our results already discussed suggest a mechanism different from our original hypothetical model. As shown in Figure 14, p-CREB expression in neither the PFC nor the amygdala of SPS rats revealed a statistically significant difference from that of the CFC rats. However, a numerical decrease was noted in the PFC of SPS rats compared to that of CFC rats which was not noted in the amygdala. Likewise, Figures 15 and 16 showed that even for the groups treated with either CEF or DHK, no significant differences were found in either region between SPS and CFC rats.

Evaluation of p-CREB following SPS exposure has not been reported before, however animal studies using various other stress paradigms have shown that the response of p-CREB to stress exposure can vary greatly. For example, it has been shown that mild chronic stress reduces p-CREB expression in the dentate gyrus of the hippocampus (Gronli et al., 2006), acute ether stress increases p-CREB in the hypothalamus (Kovacs and Sawchenko, 1996), and 5 minutes of acute forced swim stress has no effect

on hippocampal p-CREB (Molteni et al., 2009). Bilang-Bleuel et al. (2002) further demonstrated a biphasic time course of cortical p-CREB expression following forced swim stress exposure where p-CREB increased, decreased, and then increased again 15 min, 60 min, and 120 min following exposure, respectively.

Though the lack of a significant decrease in p-CREB expression within the PFC relative to the amygdala was not what we expected to find, it does not necessarily contradict our overall hypothesis that long-term memory formation is being impaired in the PFC following SPS exposure. Alberini (2009) has demonstrated that gene transcription essential for long-term potentiation, such as *C/EBP* and *zif268*, is increased when CREB is phosphorylated at Ser-133, but decreased when CREB is phosphorylated at Ser-142. Our evaluation of p-CREB only measured the Ser-133 form, raising the possibility that SPS is not impairing LTP through a decrease in Ser-133 phosphorylation, but via an increase in Ser-142 phosphorylation. It is also important to note that p-CREB is a transcription factor used in numerous intracellular pathways, and that different external stimuli can use p-CREB to induce different outcomes in cellular response (Mayr and Montminy, 2001). How this versatility of p-CREB occurs is still largely unknown (Mayr and Montminy, 2001), however, it is possible that SPS might be decreasing Ser-133 p-CREB expression in the pathways that promote long-term memory formation, while simultaneously increasing Ser-133 p-CREB expression in non-memory associated pathways. Such a phenomenon could result in a masking of the SPS-induced decrease in p-CREB expression modulating long-term memory in the PFC.

#### ***a.v. The Effect of GLT-1 Manipulation on p-CREB Expression (Arrow 5 from Figure 23)***

The final step in evaluating the hypothetical model depicted in Figure 23 was to assess the effect of GLT-1 manipulation by CEF and DHK upon p-CREB expression in the PFC and the amygdala. As discussed above, a key component of this hypothetical model was that SPS would induce a decrease in

PFC p-CREB expression relative to that of the amygdala, and that this phenomenon would in part serve as the molecular basis for why fear memories, but not fear extinction memories, are maintained following situational trauma. It was hypothesized that an SPS-induced alteration in GLT-1 expression serves as the mechanism by which this phenomenon occurs, thus making GLT-1 expression the molecular link between exposure to SPS and its ultimate effect upon p-CREB expression. As such, it was the goal of this step of the analysis to explore this link. We predicted that facilitation of GLT-1 expression with CEF would increase p-CREB expression by reducing any glutamate spillover to extrasynaptic regions, reducing the potential for CREB dephosphorylation (See Diagram 7.3). Conversely, we expected that antagonizing GLT-1 function with DHK would decrease p-CREB expression by increasing the aforementioned spillover, increasing the potential for CREB dephosphorylation. This step in the analysis also explored the potential for these GLT-1 manipulating drugs to mitigate our hypothesized SPS-induced effects upon the p-CREB molecular substrate of the fear extinction recall deficit. However, contrary to our expectations, Figures 17 and 18 show that CEF and DHK administration failed to exhibit opposite effects upon p-CREB expression in both CFC and SPS rats relative to sham (saline) treated control rats. For example, in the PFC, there was no significant difference in p-CREB expression for CEF- and DHK-treated CFC rats compared to the sham-treated CFC rats (Figure 17). Furthermore, within the SPS group, both CEF and DHK administration resulted in a significant increase in p-CREB expression compared to the sham-treated rats (Figure 17). For the amygdala, Figure 18 shows that for CFC and SPS rats, CEF and DHK administration both resulted in a significant increase in p-CREB expression compared to their corresponding sham-treated controls.

Within each of these situations, CEF and DHK had the same qualitative effect upon p-CREB expression instead of the opposite one we predicted. If CEF and DHK are manipulating GLT-1 as we intended, this finding suggests that our original hypothesis regarding the control of p-CREB expression by changes in astrocytic GLT-1 expression may require revision. The failure of SPS to change p-CREB

expression in light of the SPS-induced increases in GLT-1 expression also points to the need for this revision. Though these findings of similar action of CEF and DHK upon p-CREB expression were unexpected, they were ultimately consistent with some of the findings from our earlier analyses. More specifically, it was discussed above that CEF and DHK also had qualitatively similar effects on the SPS-induced fear extinction memory recall (Figure 6) even when it was predicted that they would have opposite ones. Furthermore, Figure 6 showed that this effect was one that apparently mitigated the SPS-induced impairment of long-term retention of fear extinction memories. This last finding could prove to be important since an improvement in long-term fear extinction memory following SPS would be expected to be induced by an increase in p-CREB expression in the PFC. Consistent with this, Figure 17 shows that both DHK and CEF administration did in fact induce a significant increase in PFC p-CREB expression compared to sham administration in SPS-exposed rats. As such, though administration of CEF and DHK did not have the effect upon p-CREB expression that we predicted, this effect upon the p-CREB substrate of fear extinction recall was at least consistent with the behavioral outcomes that we demonstrated in our earlier analysis. However, as alluded to above, this facilitation of p-CREB expression by CEF and DHK in the PFC may not have been mediated by changes in GLT-1 in the manner we originally conceived.

Another point to consider from this analysis is that the effects upon p-CREB of the GLT-1 modulating drugs CEF and DHK appear to be dependent upon both exposure to trauma and upon brain region. As mentioned above, within CFC rats, CEF and DHK administration both increased p-CREB expression relative to sham (saline) treated rats in the amygdala but neither drug increased p-CREB relative to sham rats in the PFC (Figures 17 and 18). However, within SPS rats, CEF and DHK administration increased p-CREB expression relative to controls in both the amygdala and the PFC (Figures 17 and 18). This would suggest that the mechanisms through which these drugs exert their effects upon p-CREB in the PFC are influenced by whether or not the rats are exposed to situational

trauma. As such, when evaluating a hypothetical model of the molecular mechanisms that underpin PTSD pathogenesis, our findings suggest that the model should offer explanations for why CEF and DHK exert similar effects on SPS-induced fear extinction memory recall behavior and p-CREB expression, as well as an explanation for why the effects on p-CREB may vary depending upon trauma exposure. The model we proposed in Figure 23 fails to provide both of these. As such, we offer alternative explanations below that might explain our results from Specific Aims 1 and 2 that may better reflect the pathogenesis of PTSD.

**a.vi. Possible Explanation for the Effects of DHK**

The results mentioned above were unexpected considering CEF and DHK were selected based on their previously reported, opposite effects on GLT-1 function (i.e., CEF facilitates GLT-1 function by inducing its expression, while DHK impairs its function by direct antagonism). Given what is known about the mechanisms of action of these drugs, the fact that both CEF and DHK seem to eliminate the SPS-induced fear extinction memory recall deficit is consequently difficult to explain if our hypothesis regarding GLT-1 involvement in PTSD pathogenesis is correct. As such, these results raise the possibility that GLT-1 may not be contributing to the behavioral and molecular changes associated with exposure to situational trauma as we predicted. As mentioned earlier, we predicted that DHK would exacerbate SPS-induced fear extinction memory recall deficits by promoting glutamate spill-over and activation of extrasynaptic NMDA receptors that are associated with intracellular pathways that inhibit long-term memory formation. However, the results of this study showed the opposite consequence where DHK administration eliminated the effects of SPS upon fear extinction memory recall.

One possible explanation for this unexpected result could be that PTSD pathogenesis is more involved with diminished synaptic NMDA transmission than glutamate spill-over and extrasynaptic NMDA receptors activation (evidence for this could be seen in the results of Specific Aim 3 that are

discussed in the next section below). For example, if exposure to situational trauma reduced expression of synaptic NMDA receptors in the PFC but not the amygdala, administration of DHK might actually help the formation of memory by increasing the amount of time that glutamate remains in the synapse. This would be analogous to administration of acetylcholinesterase (ACHE) inhibitors in the treatment of myasthenia gravis. Myasthenia gravis is a disease characterized by symptoms of skeletal muscle weakness due to loss of nicotinic receptors in the neuromuscular junction (NMJ) and subsequent diminished NMJ transmission (Grob, 1981). ACHE inhibitors slow the breakdown of acetylcholine (ACH), resulting in prolonged ACH within the NMJ (Grob, 1981). This increased persistence of ACH compensates for the loss of nicotinic receptors, increases transmission at the NMJ, and restores muscle strength (Grob, 1981). In a similar manner, DHK may be performing an analogous function in the synapses of the brain as the ACHE inhibitors do in the NMJ as described in the example given above. By slowing the elimination of glutamate from the synapse and allowing it to persist there for longer, DHK could have a compensatory effect on memory deficits associated with decreased expression of synaptic NMDA receptors. Admittedly, in this hypothetical scenario, there may be some spillover of glutamate following DHK administration that would stimulate extrasynaptic NMDA receptors and downstream intracellular pathways that inhibit long-term memory formation. However, it is possible that the improvement in synaptic transmission is enough to improve p-CREB expression even in the face of some glutamate spillover, and ultimately result in a net improvement in long-term memory formation.

**a.vii. Possible Explanation for the Comparable Effects of DHK and CEF Administration**

While this interpretation provides a possible explanation for why DHK administration unexpectedly eliminated SPS-induced fear extinction memory recall deficits, it still does not explain why CEF administration resulted in a similar outcome. We proposed a hypothetical scenario above where diminished expression of synaptic NMDA receptors could explain why DHK administration had effects

opposite to those we predicted. If this scenario is accurate, it would then stand to reason that increasing GLT-1 expression might actually worsen the effects of SPS if inhibiting its function leads to an improvement. However, our results showed that CEF, which should increase astrocytic glutamate re-uptake and synaptic glutamate removal, eliminated the effects of SPS in a manner comparable to that of DHK. The explanation for this phenomenon might lie in the fact that CEF is not as specific in its actions compared to DHK, which has a very narrow range of action. For example, in addition to its ability to increase GLT-1 expression, CEF has also been shown to reduce microglial activation, decrease IL-1 $\beta$  production, and inhibit bacterial cell wall formation function (Lujia et al., 2014; Yin et al., 2015). Conversely, DHK has only been reported to act as a GLT-1 inhibitor. As such, the effects on fear extinction memory recall that we observed following CEF administration in this study might be the consequence of its actions outside of inducing GLT-1 expression. One example of CEF effects that is relevant to our study is the inhibitory effect it has been demonstrated to exert on microglial activity (Lujia et al., 2014). In Section E of the Literature Review, it was discussed how microglia could be involved in the hypothetical effect of SPS on GLT-1 expression, which involved deactivation of mechanisms of microglial inhibition (Blandino et al., 2009; Frank et al., 2006). Once released from inhibition, we posited that microglia would then induce diminished expression of GLT-1 through the production of pro-inflammatory cytokines (Blandino et al., 2009; Frank et al., 2006; Sama et al., 2008). During our preliminary studies, we investigated the possibility that SPS altered microglia by measuring the expression of a generic marker of microglial activity, Iba 1/2, but did not pursue analysis further in this study when our preliminary results failed to show a significant SPS-induced change in Iba 1/2 expression. With that said, however, it is important to note that Iba 1/2 is a generic marker of microglial activity (Ito et al., 1998). Studies have shown that microglial activity can be characterized as a heterogeneous spectrum that ranges from a pro-inflammatory phenotype to an anti-inflammatory phenotype (Selenica et al., 2013; Italiani and Boraschi, 2015). In fact, it has been demonstrated in some

pathologies (like traumatic brain or spinal injury) that microglia initially exhibit a pro-inflammatory phenotype in the early stages of injury that causes further damage to surrounding tissues and promote the continuation of the pathology (Harvey et al., 2015; Kigerl et al., 2009). Conversely, resolution of traumatic injuries is promoted if a shift occurs in microglial phenotype towards one that is more anti-inflammatory in character (David and Kroner, 2011). These studies show that microglia play a diverse role in resolution of neural injury, and that the specific phenotypes these cells express upon activation are an important consideration when determining their overall physiological impact. Specifically for this study, it is important to consider that both the CFC groups and the SPS groups were exposed to CFC, EXT1, and EXT2. It is possible that this exposure caused an increase in microglial activity in both groups that was not significantly different from each other in IBA 1/2 expression. In other words, microglial activity may have “generically” increased in both groups, but this increase may have not been significantly different between the CFC and SPS groups. However, as discussed above, while there may not have been a significant difference in the increase in general microglial activity between these groups, it is possible that the character of the activity in the SPS group may have been more pro-inflammatory in nature compared to that of the CFC group.

Considering this heterogeneous nature of microglial activity, it is possible that SPS promotes a shift towards a more pro-inflammatory microglial phenotype from an anti-inflammatory one. This shift in SPS-exposed rats could occur without inducing a significantly different increase in total Iba 1/2 expression in the microglial population compared to non-exposed rats. This pro-inflammatory phenotype has been characterized by the production of many compounds that can adversely affect memory, such as IL-1 $\beta$  and reactive oxygen species (ROS), which have been shown to decrease expression of intracellular kinases, like ERK, that are associated with LTP induction (Donzis et al., 2014; Massaad and Klann, 2011). As described in Section E of the Literature Review, it is possible that the stress from SPS stimulates a microglial pro-inflammatory phenotype via down regulation of regulatory

proteins, like CD200 (Blandino et al., 2009; Frank et al., 2006). In this pro-inflammatory state, microglia could be producing the cytokines and ROSs mentioned above that can subsequently impair long-term memory (Donzis et al., 2014; Massaad and Klann, 2011). Hypothetically, this could mean that CEF administration would mitigate SPS-induced impairment of long-term memory by suppressing the pro-inflammatory activity of microglia and not through its up-regulation of GLT-1 as we hypothesized.

In summary, the results of Specific Aims 1 and 2 revealed data that did not completely fit into the hypothetical model we proposed. Consistent with our predicted model, SPS exposure decreased fear extinction recall and increased hippocampal GR expression. However, contrary to our model, SPS exposure increased GLT-1 expression and did not have an effect on p-CREB expression. Furthermore, manipulating GLT-1 expression in opposite directions with CEF and DHK did not result in opposing effects upon fear extinction recall and p-CREB expression.

These findings are not consistent with a pathogenesis that depends upon diminished GLT-1 function and subsequent glutamate spillover. We have described above a possible alternative model where SPS may be inducing a decrease in GLT-1 expression immediately after exposure to SPS, and that the GLT-1 increase that we measured 10 days after exposure was the compensatory response to that initial decrease. Furthermore, the memory deficits induced by SPS exposure may be a consequence of CREB modification other than Ser-133 phosphorylation, like phosphorylation at Ser-142. Such a situation would explain why we were not able to detect an SPS-induced change in p-CREB phosphorylation, since Ser-133 phosphorylation was the only CREB modification that we measured.

Finally, we also raised the possibility that synaptic NMDA receptor expression within the PFC but not in the amygdala is altered by SPS exposure. In such a situation, decreased synaptic NMDA receptors in the PFC would decrease LTP following Extinction training, and thus decrease long-term extinction memory formation. Behaviorally, this would manifest as the impaired fear extinction memory recall that

is characteristic of the SPS paradigm. This might explain why CEF and DHK had similar effects, as DHK may have been able to reverse the effects of SPS upon fear extinction memory recall by increasing synaptic glutamate and thus compensating for the decrease in synaptic NMDA receptors. Conversely, CEF administration may have alleviated the SPS-induced symptoms by suppressing a microglial pro-inflammatory response over the course of the study, thus decreasing the presence of factors like IL-1 $\beta$  and ROS that could impair memory formation.

Overall, a more accurate model might be one where SPS exposure induces an immediate, but temporary, decrease in GLT-1 function that the brain attempts to compensate for by increasing its levels of expression. SPS may instead be inducing a prolonged decrease in synaptic NMDA receptors, which may be more prominent in the PFC. As a result, LTP in the PFC may be selectively impaired, resulting in a subsequent impairment of long-term memory formation in that region.

### **SECTION B: DISCUSSION OF SPECIFIC AIM 3**

Regarding the pathophysiology of PTSD, it remains to be determined why a traumatic event selectively impairs long-term fear extinction memory without simultaneously impairing long-term fear memory. The second principal hypothesis of this study addressed a possible explanation for this phenomenon. More specifically, it was hypothesized that the concurrent presence of glucocorticoids, that has been associated with situational trauma, selectively exacerbates the inhibition of fear extinction memory processes in the prefrontal cortex, but not of conditioned fear memory processes in the amygdala, due to 1) differences between these brain regions in expression of NMDA receptor subtypes and 2) differences in the downstream effect of glucocorticoids on these different subtypes.

It is important to note that a critical component of this hypothesis was the assumption that NMDA receptor subtype profiles are not the same in synapses of the PFC/hippocampus compared to the amygdala. The validity of this assumption is supported by some studies (Walker et al., 2008; Liu et al., 2004; Zhang et al., 2008) but not others (Luo et al., 1997; Rauner and Kohr, 2011). Another significant facet of our hypothesis is evidence that GCs do not directly inhibit NMDA receptors (Xiao et al., 2010), but they can inhibit the downstream molecular pathway that is associated with the NR2A NMDA receptor subtype, and can selectively block some of the effects of NR2A stimulation by glutamate (Xiao et al., 2010). This could be relevant to the pathogenesis of PTSD as glucocorticoid regulation has been shown to be disrupted in individuals with PTSD. For example, studies have shown that PTSD subjects exhibit a hypersensitive hypothalamus-pituitary-adrenal axis characterized by enhanced negative feedback regulation of cortisol (Yehuda et al., 1995), which can manifest as low basal circulating cortisol levels under certain conditions (Meewisse et al., 2007), as well as an exaggerated cortisol response during and in anticipation of stressful situations (Bremner et al., 2003).

To further clarify the implications of these findings Figure 24 depicts a model of how preferential disruption of the synaptic NR2A NMDA receptor pathway, by the increased GC response following trauma, could preferentially reduce p-CREB expression in the PFC/hippocampus relative to the amygdala. This could potentially lead to a greater disruption of PFC-associated long-term fear extinction memory compared to amygdala-associated long-term fear memory. The figure depicts region specific NMDA receptor subtype profiles where it is assumed that the PFC and hippocampus express more synaptic NR2A compared to NR2B, and the amygdala expresses more synaptic NR2B compared to NR2A (Cull-Candy and Leszkiewicz, 2004; Walker et al., 2008; Zhang et al., 2008). Bicuculline-stimulated synaptic glutamate release is used to activate all NMDA receptors, but this is not pictured (Terasaki et al., 2010; Steigerwald et al., 2000; Flint et al., 1997). The model depicted in Figure 24 is the foundation for the second general hypothesis of this study and assessments performed under Specific Aim 3 were designed to test the model's validity. This figure shows how p-CREB expression in the presence of GCs can differ between regions due to the presence of different profiles of synaptic NMDA receptor subtypes in the PFC/hippocampus and the amygdala. The first of these assessments of Specific Aim 3, represented in the figure by the blue arrows labelled with a "1", was to determine whether glucocorticoids produce greater impairment of glutamate-activated CREB phosphorylation in a synaptic tissue preparation from the PFC, where stimulation leads to lower increases in p-CREB compared with a similar preparation from the amygdala, where a greater presence of NR2B at the synapse would be expected to allow for a higher increase in p-CREB.

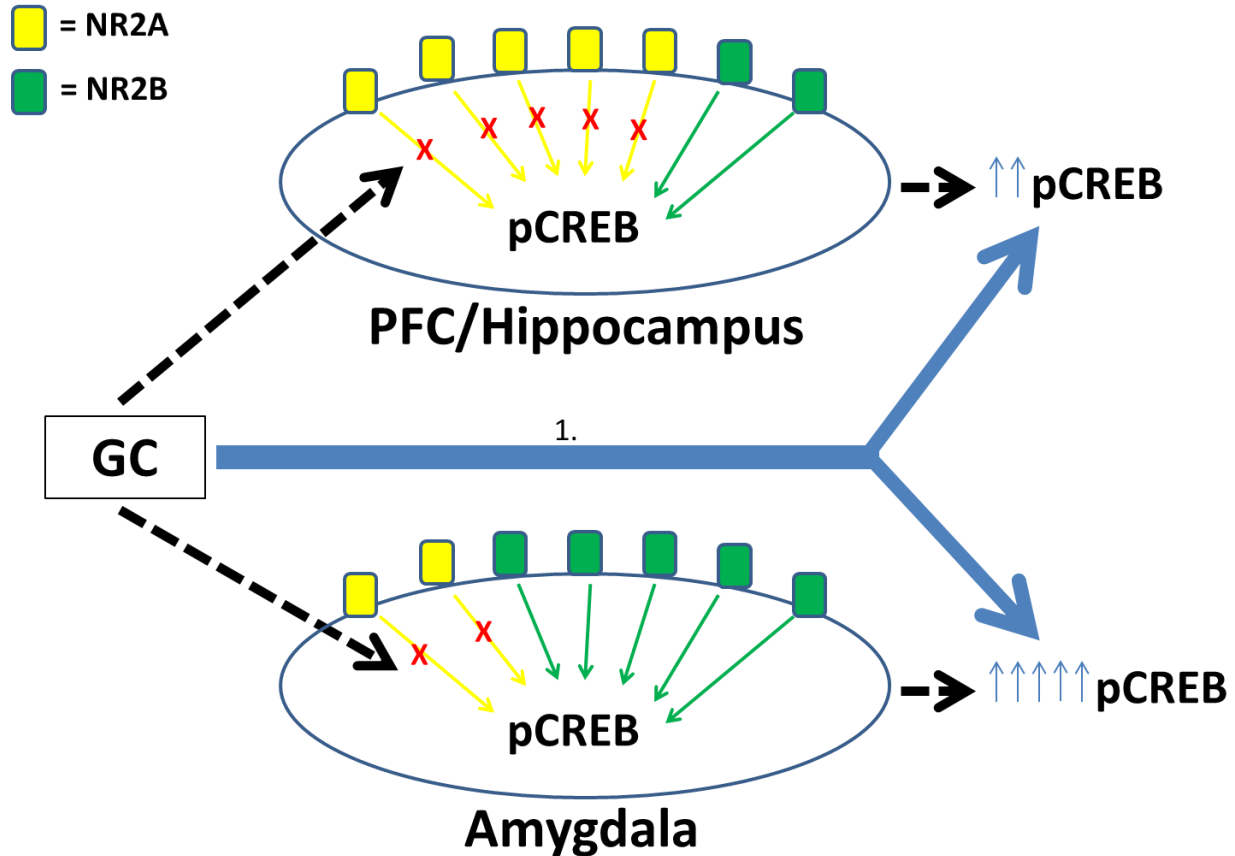


Figure 24. A visual representation of the hypothesized effect of glucocorticoid (GC) administration on p-CREB expression, assuming previously reported regional differences in NMDA receptor subtype expression. This figure depicts the hippocampus and PFC expressing more synaptic NR2A receptors (yellow blocks) compared to NR2B receptors (green blocks). Under these configurations, administration of GCs would cause greater interference with synaptic stimulation by glutamate (represented by the red "X"s covering the arrows connecting the receptors to p-CREB activation) in the hippocampus and PFC compared to the amygdala due to its ability to selectively impair downstream molecular pathway that is associated with NR2A receptors. This would translate to less CREB phosphorylation following synaptic glutamate release in the hippocampus and PFC compared to the amygdala, and would consequently lead to a greater disruption of long-term fear extinction memory compared to long-term fear memory. The first goal of Specific Aim 3, represented in the figure by the blue arrows labelled with a "1", was to assess whether glucocorticoids produce greater impairment of glutamate-activated CREB phosphorylation in a synaptic tissue preparation from the PFC compared with a similar preparation from the amygdala. GC = glucocorticoids, p-CREB = phosphorylated cAMP Response Element Binding protein; dashed arrows represent hypothesized mechanistic pathway, blue arrows represent experimental assessment.

The second assessment of Specific Aim 3 was designed to address the underlying assumption of our model regarding the differential representation of NMDA receptor subtypes across the brain regions of interest. This assessment determined whether 2) glutamate-induced CREB phosphorylation in synaptic tissue samples from the amygdala responds differently to known NR2A (Fig. 25A) or NR2B (Fig. 25B) antagonists compared with similar tissue samples from the PFC or hippocampus. This would be expected if the amygdala has a greater representation of the NR2B synaptic NMDA receptor subtype compared with the PFC/hippocampus, as suggested by some studies (Walker et al., 2008; Liu et al., 2004; Zhang et al., 2008) and refuted by others (Luo et al., 1997; Rauner and Kohr, 2011). Figure 25A shows how p-CREB expression would be affected in the hippocampus/PFC and amygdala by selective NR2A inhibition if our assumption regarding NR2A versus NR2B NMDA receptor subtype expression is correct. Application of NVP-AAM077 (NVP), that is known to preferentially block NR2A receptors, would result in fewer synaptic NMDA receptors being activated by glutamate in the PFC and hippocampus compared to the amygdala. This would translate to less post-synaptic CREB phosphorylation following synaptic glutamate release in the PFC and hippocampus compared to the amygdala.

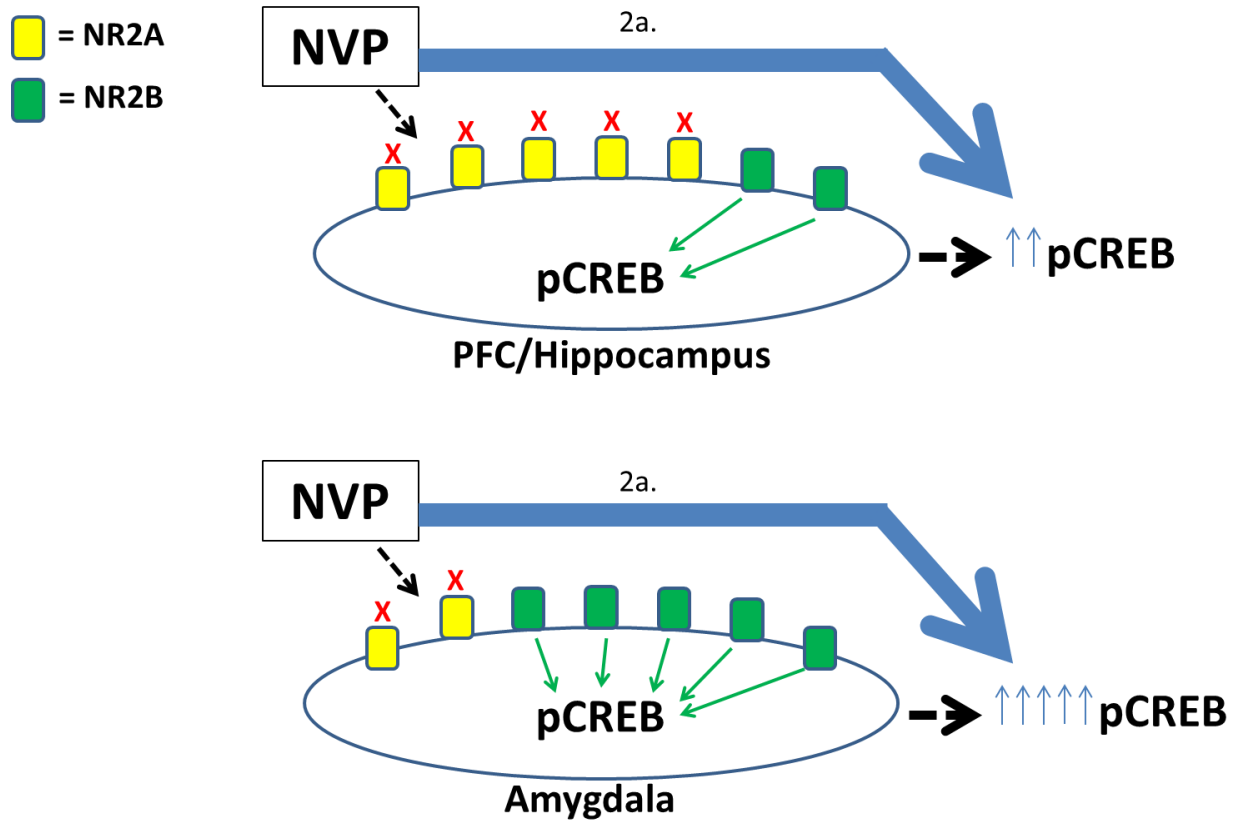


Figure 25A. A hypothetical representation of the effect of NR2A selective inhibition by NVP-AAM077 (NVP) on p-CREB expression, assuming previously regional differences in NMDA receptor subtype expression. This figure assumes the hippocampus and PFC express more synaptic NR2A receptors (yellow blocks) compared to NR2B receptors (green blocks) with the reverse relationship existing in the amygdala. Under these configurations, administration of NVP would block more synaptic receptors (represented by the red X's) in the hippocampus and PFC compared to the amygdala. This would translate to less CREB phosphorylation following synaptic glutamate release in the PFC and hippocampus compared to the amygdala. Therefore, this assessment represented by the blue arrow labelled "2a" determined whether glutamate-induced CREB phosphorylation in synaptic tissue samples from the amygdala responded differently to the known NR2A antagonist, NVP, compared with similar tissue samples from the PFC/hippocampus. NVP = NVP-AAM077, p-CREB = phosphorylated cAMP Response Element Binding protein; dashed arrows represent hypothesized mechanistic pathway, blue arrows represent experimental assessment.

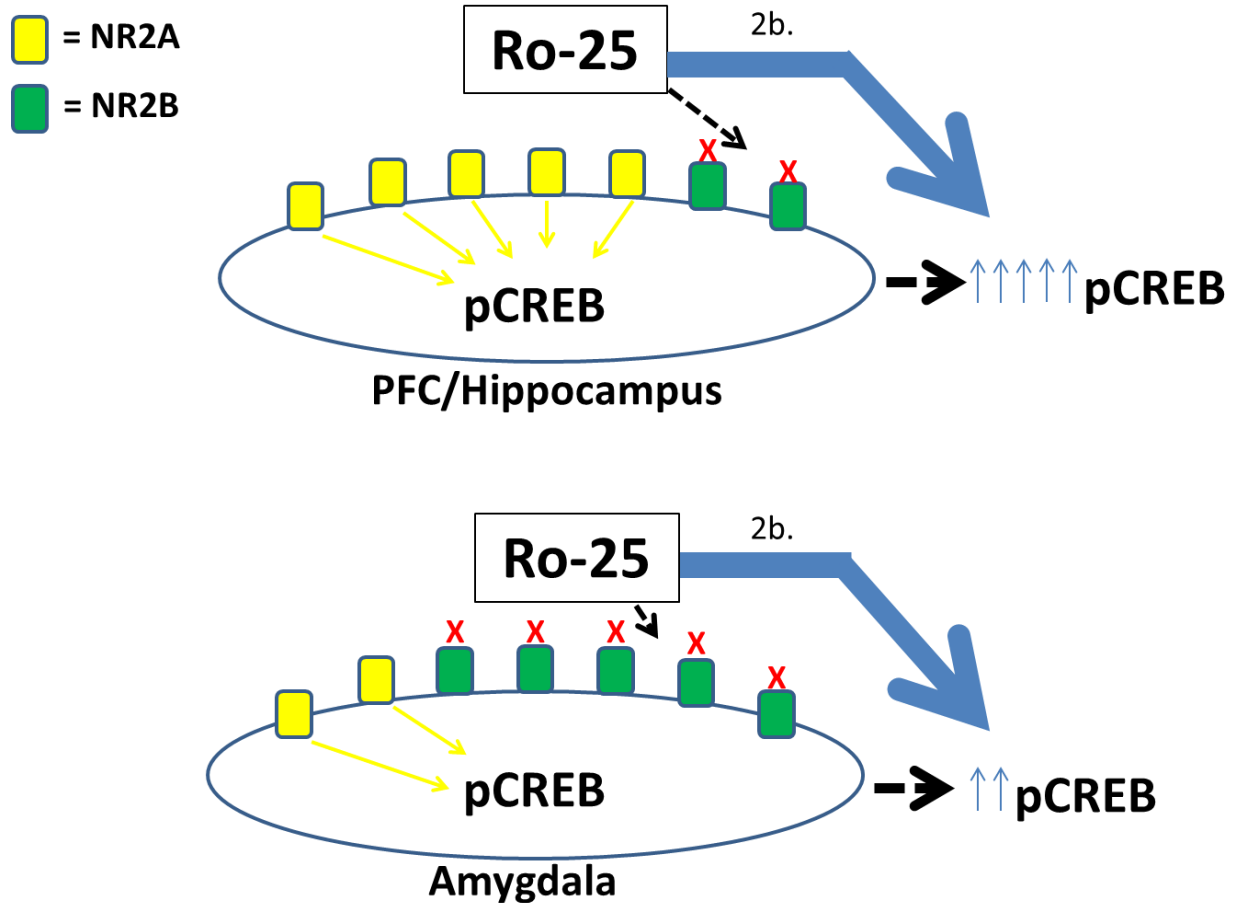


Figure 25B. A hypothetical representation of the effect of NR2B selective inhibition by Ro-25-6981 (Ro-25) on p-CREB expression, assuming previously reported regional differences in NMDA receptor subtype expression. This figure assumes the hippocampus and PFC express more synaptic NR2A receptors (yellow blocks) compared to NR2B receptors (green blocks), with the reverse relationship existing in the amygdala. Under these configurations, administration of Ro-25 would block more synaptic receptors (represented by the red X's) in the amygdala compared to the hippocampus and PFC. This would translate to less CREB phosphorylation following synaptic glutamate release in the amygdala compared to the hippocampus and PFC. Therefore, this assessment represented by the blue arrow labelled "2b" determined whether glutamate-induced CREB phosphorylation in synaptic tissue samples from the amygdala responded differently to the known NR2B antagonist, Ro-25, compared with similar tissue samples from the PFC/hippocampus. Ro-25 = Ro-25-6981, p-CREB = phosphorylated cAMP Response Element Binding protein; dashed arrows represent hypothesized mechanistic pathway, blue arrows represent experimental assessment.

Similarly, Figure 25B shows how p-CREB expression would be affected in the hippocampus/PFC and amygdala by selective NR2B inhibition if our aforementioned assumption regarding NMDA receptor subtype expression is correct. Application of Ro-25-6981 (i.e., Ro-25), that preferentially blocks NR2B

receptors, would leave fewer synaptic NMDA receptors to be activated by glutamate in the amygdala compared to the hippocampus and PFC. This would translate to less post-synaptic CREB phosphorylation following synaptic glutamate release in the amygdala compared to the hippocampus and PFC.

Another assumption of our above noted model, that attempts to account for the differential effects of trauma-induced GCs upon PFC-associated fear extinction memory versus amygdala-associated fear memory, is that SPS does not change the proportions of NMDA receptor subtypes within our brain regions of interest. Therefore, the third assessment of Specific Aim 3, represented in Figure 26, determined 3) whether SPS exposure changed the effect of the known NR2A and NR2B antagonists on glutamate-induced CREB phosphorylation in the PFC, hippocampus and amygdala. Such a change would be expected if trauma exposure altered the representation of a given receptor subtype in one or more of our brain regions of interest.

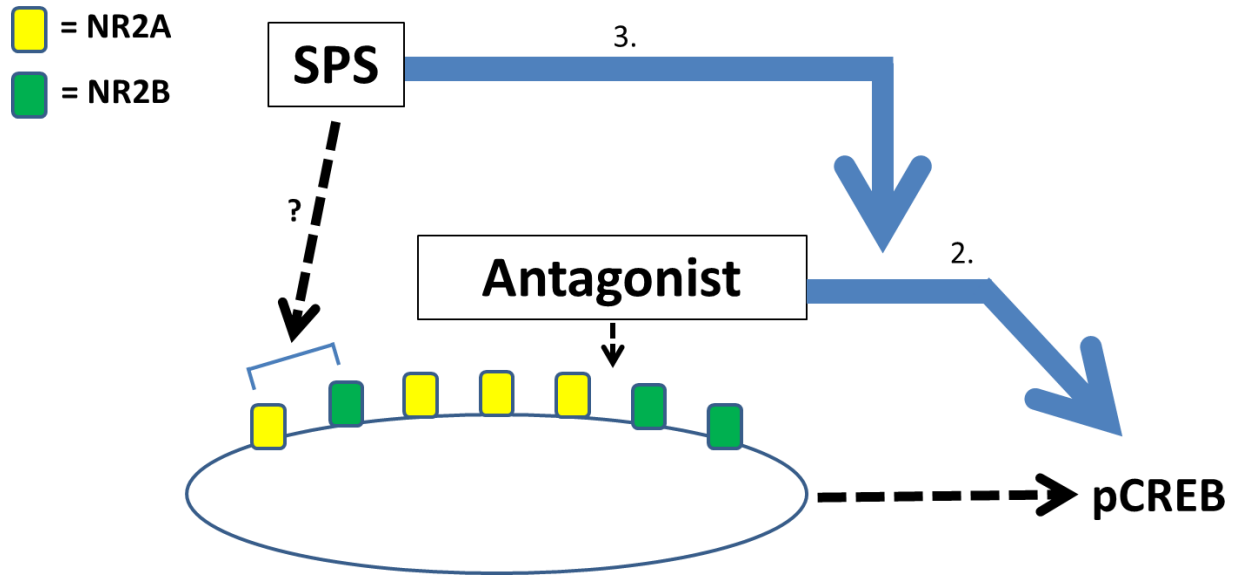


Figure 26. A visual representation of the effect of SPS on p-CREB expression, assuming regional differences in NMDA receptor subtype expression. This figure depicts synaptic NR2A receptors (yellow blocks) and NR2B receptors (green blocks) in any one of the three brain regions analyzed in this study. We predict that SPS will not differentially affect the expression of synaptic NMDA receptor profiles (represented by the dashed black arrow), and will thus not change the outcome of receptor antagonist administration. The third assessment of Specific Aim 3 (represented by the blue arrow labelled with a “3”) aims to determine whether SPS exposure changed the effect of the known NR2A and NR2B antagonists on glutamate-induced CREB phosphorylation in the PFC, hippocampus and amygdala. Ro-25 = Ro-25-6981, p-CREB = phosphorylated cAMP Response Element Binding protein.

**b.i. Comparison of GC-induced inhibition of p-CREB between Brain Regions**

The first goal of Specific Aim 3 was to determine if glucocorticoids produce greater impairment of glutamate-activated CREB phosphorylation in the PFC compared to the amygdala. The results shown in Figure 19 demonstrated that, in tissues from CFC rats, GCs have a general inhibitory effect upon CREB phosphorylation. Given our protocol for this assay, this inhibition occurs within 30 minutes following administration. This is an important finding as it provides some insight into the mechanism behind this action. The classical mechanism associated with GC-induced changes in cellular function depends upon GCs forming a complex with an intracellular, cytoplasmic receptor (Makara and Haller, 2001). This complex then translocates to the nucleus and acts as a transcription factor that alters cellular functions

via alterations in gene transcription and, subsequently, protein synthesis (Makara and Haller, 2001). Because of this dependence on translocation to the nucleus and *de novo* protein synthesis, effects of this classical mechanism can take hours to become evident (Makara and Haller, 2001). Contrary to this, our results showed an acute (i.e., within 30 minutes) effect of GC administration, and suggest that GCs are affecting p-CREB in brain slice preparations through a non-classical mechanism. While we did not investigate the details of this non-classical mechanism further, this finding is consistent with the study done by Xiao et al. (2010) that demonstrated an immediate inhibition of ERK (an upstream mediator of CREB phosphorylation) following GC administration in hippocampal cell culture.

Though we expected GCs to have an inhibitory effect upon p-CREB given previous evidence that GCs can affect downstream synaptic NR2A receptor function, we described above that we anticipated this effect to be most prominent in brain regions that express high proportions of the synaptic NR2A (i.e., the PFC and hippocampus) receptor subtype and not in regions that express high proportions of the synaptic NR2B (i.e., the amygdala) subtype. However, contrary to this expectation, Figure 19 not only shows that GCs significantly decreased p-CREB in all three regions within the CFC group, but also a follow-up analysis revealed that there was no significant difference in the magnitude of this inhibition across the regions.

We have described already how our prediction of the region-specific action of GCs upon CREB phosphorylation was based on 1) the findings of Xiao et al. (2010) that reported GC specificity for suppression of downstream NR2A-associated molecular pathways and 2) the assumption that NMDA receptor subtype profiles differed between the PFC/hippocampus and the amygdala (see Figure 24). Although the significant GC-induced reduction of p-CREB in all 3 regions of interest is not discordant with the inhibitory effect of GCs on the NR2A subtype, our finding that GCs did not exert the predicted regional variation of action implies that both of the above assumptions are unlikely to hold true

together. Alternatively, the findings reported by Xiao et al. (2010) may not have been supported in our study due to a difference in experimental models. For example, Xiao et al. (2010) measured phosphorylation of ERK 1/2, a kinase that is up-stream in the molecular pathway that ultimately phosphorylates CREB, while our study directly measured p-CREB. It is possible that the GC-induced decrease in p-ERK measured by Xiao et al. (2010) does not translate to decreased p-CREB as we expected.

Another factor to consider is that tissue sections taken from adult rats were used in our study while Xiao et al. (2010) utilized hippocampal cell cultures generated from embryonic rat pups. This difference in the nature of the tissue used between the studies could have altered the downstream effect of GC administration upon NMDA dependent synaptic transmission between the two studies. For instance, Xiao et al. (2010) demonstrated that NR2B antagonist-induced inhibition did not alter p-ERK expression in hippocampal cultures, and subsequently concluded that NR2A was the predominant synaptic NMDA receptor subtype. Using this finding from the embryonic rat pup cultures as a basis, they then concluded that the inhibition of p-ERK following GC administration must be occurring via inhibition of NR2A-specific pathways. However, it should also be considered that cellular localization of NMDA receptor subtypes plays an important role in determining these subtypes' ultimate effect on cellular function. For example, activation of NR2B receptors localized at the synapse has been shown to promote the same downstream molecular pathways as synaptic NR2A receptors (e.g., pathways that inhibit apoptosis and promote LTP) (Gardoni et al., 2009), while activation of NR2B receptors localized outside of the synapse induces neuronal apoptosis and LTD (Sinor et al., 2000) via a different pathway. These reports suggest that, in regard to determining which downstream cellular pathways are activated by a given NMDA receptor subtype, membrane localization may also be a significant factor to consider in addition to receptor subtype. Thus, it is possible that in the study by Xiao et al. (2010), using embryonic-derived cultures, GCs were not specifically inhibiting pathways associated with NR2A, but it appeared

that way given the lack of evidence for the presence of the synaptic NR2B subtype in the embryonic derived tissue. In adult-derived tissue, there may be a greater representation of synaptic NR2B subtype compared to embryonic-derived, and GCs may be acting on the downstream pathway this synaptic subtype shares with the synaptic NR2A receptor.

Consequently, it is important to consider that synaptic profiles of receptors can vary greatly between embryonic and adult tissues. For example, expression of particular synaptic NMDA receptor profiles of cultured neurons can vary depending upon the gestational age of pups at the time of neuronal harvesting and how long the neurons have been kept in culture before being tested (Yashiro and Philpot, 2008). Similarly, expression of particular NMDA receptor profiles within a given region of the adult brain can be influenced by differences in levels of neuronal activity that can depend upon environmental factors such as sensory exposure (Carmignoto and Vicini, 1992; Roberts and Ramoa, 1999). This too could prove relevant since our control tissues were collected from rats that had undergone the CFC procedure, which could also have had an effect on NMDA receptor profiles not seen in naïve embryonic rat pups used by Xiao et al. (2010). Taken together, these studies show that a number of variables exist that could explain how synaptic NMDA receptor profiles of neurons from an embryo-derived cell culture might differ from those of adult tissue slices. As such, the discrepancy between the study of Xiao et al. (2010) and this current study may be due to a difference in experimental design where the cultured neurons of Xiao et al. (2010) exhibited mostly NR2A receptors at the synapse, while our adult tissues expressed a heterogeneous mix of synaptic NR2A and NR2B. Conversely, it is also possible that our assumption was incorrect that NMDA receptor subtype profiles differed between the regions analyzed in this study. As stated above, this is an area that is disputed in the literature. In the next section we discuss the results of our experiments that more directly addressed this issue. Overall, our finding that GC administration inhibits p-CREB within 30 minutes indicates that GCs could have a “non-classical”, immediate, negative impact on molecular pathways associated with

long-term memory formation, but does not provide insight into our main goal of determining why there appears to be an apparent difference in long-term retention between fear memories and fear extinction memories. Although our results are not completely discordant with an effect of GCs upon synaptic NR2A downstream molecular mechanisms, they call into question whether the effect is exclusive to this synaptic NMDA receptor subtype.

***b.ii. Relative Representation of Synaptic NR2A and NR2B Receptor Representation Across Amygdala, PFC and Hippocampus***

The next goal of Specific Aim 3 was to determine if NMDA-induced CREB phosphorylation in tissue samples from the amygdala responds differently to NR2A or NR2B antagonism compared with similar tissue samples from the PFC or hippocampus. As depicted in Figure 25, the rationale for the use of these inhibitors was that blockade of a receptor subtype should only significantly reduce p-CREB expression if that receptor subtype provides significant contribution to the synaptic profile of NMDA receptors. This should also address the relative synaptic representation of NR2A and NR2B subtypes across our regions of interest and addresses another potential substrate for the similarity of our GC results upon p-CREB expression across these regions. The results presented in Figures 20 and 21 show that, within CFC rats, NVP (an NR2A subtype inhibitor) and Ro-25 (an NR2B subtype inhibitor) respectively and significantly reduce p-CREB expression in all three regions of the brain tested in this study. Thus, according to our rationale described above, this would suggest that both NR2A and NR2B provide significant contribution to p-CREB expression following synaptic transmission. Furthermore, the results presented in Figure 22 suggest that the proportional representation of these receptor subtypes is similar across the three regions.

These findings are contrary to our prediction that the p-CREB expression in the PFC and hippocampus (presumed to have greater NR2A subtype representation compared to amygdala) would be preferentially decreased by NVP and that the p-CREB expression in the amygdala (presumed to have

greater NR2B representation than the PFC and hippocampus) would be preferentially decreased by Ro-25 (Figure 25). Consequently, these results support the idea that our assumption was not valid that NMDA receptor subtype profiles differ between the hippocampus/PFC and the amygdala.

While the existence of similar synaptic NMDA receptor profiles among the hippocampus, PFC, and amygdala could explain the similar effects across these regions of GCs, NVP, or Ro-25 on p-CREB, it should be noted that these findings are not only contrary to our predictions about comparative subtype profiles, but inconsistent with the results reported by Xiao et al. (2010). The discussion provided in the last section sites the differences between the study design of Xiao et al. (2010) and that of this study (i.e., use of embryo-derived cell cultures versus adult tissue slices, naive animals used versus CFC-exposed animals, measurement of p-ERK versus measurement of p-CREB) that might explain these discrepancies. We have also described above how it is possible that the synaptic profile of the cell cultures used by Xiao et al. (2010) may not translate to adult rat brain tissues. In other words, the synapses of embryo-derived, cultured hippocampal neurons may have a profile that is mostly composed of NR2A receptors, but the synaptic profiles of adult brain slices may have a mix of NR2A and NR2B. Our results support the latter. Considering this and the argument presented in the previous section (b.i.), it may be more appropriate to characterize the effects of GCs on NMDA receptor activity as one that is inhibitory of pathways associated with synaptic activation as opposed to inhibition of pathways associated with a specific synaptic receptor subtype. Ultimately, this interpretation of GC effects on NMDA receptor associated pathways could be another explanation for the apparent inconsistencies between this study and Xiao et al. (2010). Irrespective of whether the effects of GCs on p-CREB are NR2A-preferring or “synaptic-preferring”, our results regarding NR2A/NR2B profiles do not support a receptor profile substrate for a selective effect of GCs on long term memory substrates across our 3 regions of interest.

**b.iii. The Effect of SPS Exposure on Relative Representation of Synaptic NR2A and NR2B Receptor Representation Across the Amygdala, PFC and Hippocampus**

The final goal of Specific Aim 3 was to characterize the effects of SPS on synaptic NR2A and NR2B receptor subtype expression in the amygdala, PFC, and hippocampus. A central concept of this study was the hypothesis that regional variations in synaptic NMDA receptor profiles exist, and that these variations serve as the basis, in part, for explaining why long-term fear extinction memory is impaired by exposure to situational trauma without concurrent disruption of long-term fear memory. As discussed above, we predicted that prior to situational trauma the PFC and hippocampus would express more NR2A receptors than NR2B receptors at the synapse, while the reverse would be true for synapses of the amygdala (Figure 25). We further hypothesized that this configuration would predispose long-term memories formed in the PFC (i.e., fear extinction memories) to be selectively impaired by trauma exposure compared to long-term memories formed in the amygdala (i.e., fear memories) due to a predicted inhibition of NR2A-associated intracellular pathways by GCs released during times of stress (Figures 24 – 25). Since this hypothesis predicted that these profile variations both exist prior to exposure to situational trauma and are instrumental to the manifestation of impaired fear extinction memory recall, we expected that exposure to situational trauma would not alter this synaptic NMDA receptor configuration by the time extinction training takes place. However, contrary to our hypothesis, exposure to SPS did appear to change how some regions responded to receptor subtype inhibitors or GC treatment in regard to p-CREB expression.

As with the tissues of the CFC groups discussed above, we measured the effect of NVP or Ro-25 administration upon p-CREB expression in tissue slices of the amygdala, PFC, and hippocampus, from SPS-exposed rats, to evaluate the contribution of NR2A or NR2B receptors towards synaptic transmission, as well as the consequence of GC exposure for p-CREB expression in SPS-exposed tissues

from these regions. While *ex vivo* experimental results illustrated in Figures 19, 20, and 21 suggested that all three compounds decreased p-CREB expression in all three regions of CFC rats, a different outcome was noted with *ex vivo* studies that used tissues from SPS rats. For example, the data in Figure 19 shows that, following SPS, GCs caused a significant decrease in p-CREB expression within the amygdala, but not within the PFC or the hippocampus. The data in Figure 20 show that, following SPS, NVP (the NR2A subtype inhibitor) caused a significant decrease in p-CREB expression within the hippocampus, but not within the PFC or the amygdala. Finally, the data in Figure 21 show that, following SPS, Ro-25 (the NR2B subtype inhibitor) caused a significant decrease in p-CREB expression within the hippocampus and the amygdala, but not within the PFC. When compared to the results generated from the CFC tissues (i.e., where all three compounds each caused a decrease in p-CREB in all three regions), these findings demonstrate that SPS exposure does actually significantly change the effect of these compounds upon p-CREB expression among the amygdala, PFC, and hippocampus.

One possible explanation for the SPS-induced alteration of the *ex vivo* effect of GCs, NVP, and Ro-25 upon p-CREB expression is that SPS may be causing a change in the function of NMDA receptor subtypes at the synapse. Environmental influences and experiences affect neuroplasticity, and it was discussed above how changes in neuronal activity following sensory stimulation can change the profile of NMDA receptors expressed at the synapse (Carmignoto and Vicini, 1992; Roberts and Ramoa, 1999). More relevant to this study, it has been shown that exposure to different types of stressful experiences can induce variations in synaptic NMDA receptor profiles. For example, Yuen et al. (2009) reported that exposing rats to acute restraint or forced swim increases NR2A and NR2B expression in PFC tissue slices and that this change was associated with an increase in working memory. Conversely, chronic unpredictable stress has been shown to decrease both NR2A and NR2B expression in the frontal cortex and the hippocampus (Feyissa et al., 2009; Lou et al., 2010). Unfortunately, there have been few studies that have evaluated the changes in NMDA receptor expression in the SPS model specifically. Yamamoto

et al. (2008) did show that hippocampal mRNA levels of NMDA receptor subunits were increased during extinction within the hippocampus of SPS-exposed rats compared to controls. However, this increase in NMDA receptor mRNA expression was correlated with a decrease in retention of fear extinction memories, and it was suggested that this increased expression might have been a neuronal compensatory response to an SPS-induced impairment of NMDA receptor function (Yamamoto et al., 2008). While future studies would be needed to verify this theory, the possibility remains that SPS may induce a decrease in NMDA receptor function that is similar to that seen in other models like chronic unpredictable stress.

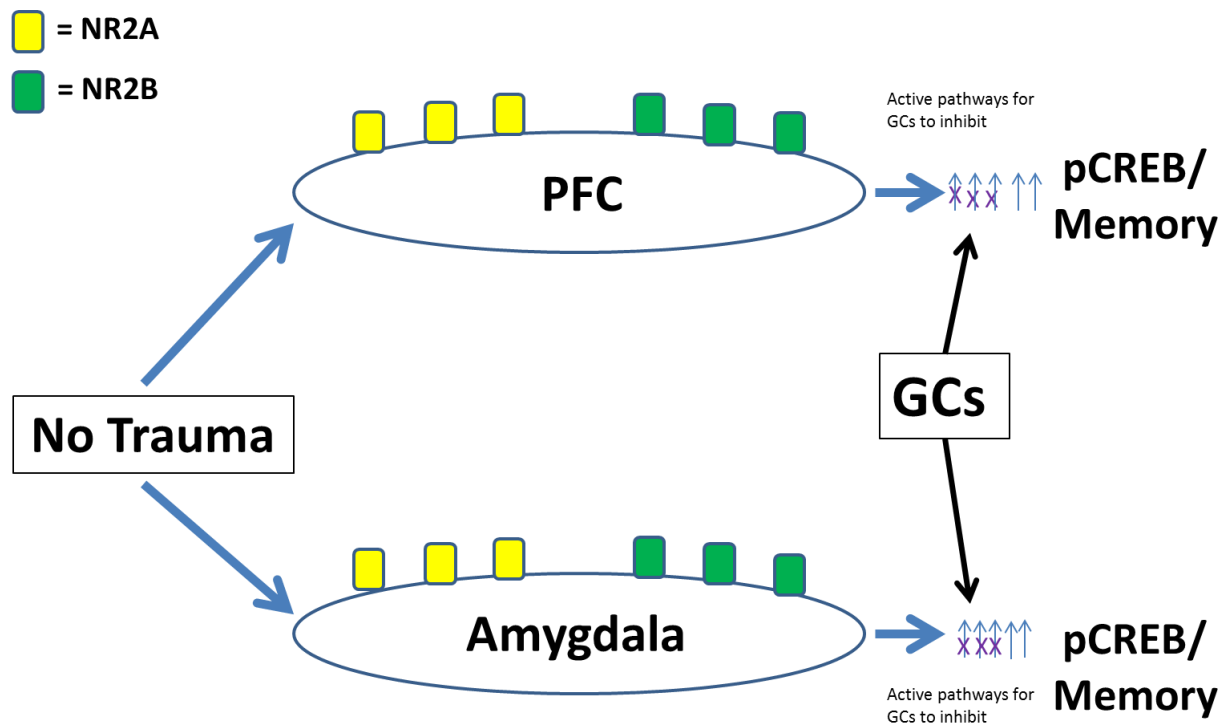


Figure 27. A visual representation of our interpretation, based on our data, of similar synaptic NMDA receptor subtype profiles in the amygdala and PFC, and the effect of GCs on p-CREB production in these regions. Here, GCs have significantly and equivalently inhibited (purple arrows) downstream p-CREB production resulting from activation of all synaptic NMDA receptor subtypes by bicuculline. Small purple arrows represent GC inhibition of p-CREB-producing pathways. GC = glucocorticoids, p-CREB = phosphorylated cAMP Response Element Binding protein.

If SPS is causing a change in synaptic NMDA receptor function or expression in the PFC, amygdala, and hippocampus, the results from this study can provide some insight into the character of this change. As stated earlier, NVP and Ro-25 are preferential inhibitors of the NR2A and NR2B subtypes, respectively, and they should significantly reduce CREB phosphorylation that depends upon activation of their corresponding receptors during synaptic transmission. Conversely, these inhibitors should not significantly reduce CREB phosphorylation that does not depend upon activation of their corresponding receptors during synaptic transmission. Thus, the contribution of NR2A or NR2B NMDA receptor subtypes can be extrapolated from whether or not their corresponding inhibitors cause a significant decreased expression of p-CREB.

For this study, Figure 20 shows that in amygdalar tissue of SPS rats, NVP administration did not significantly reduce p-CREB expression as it did for amygdalar tissues of CFC rats. This would suggest that exposure to SPS may have changed the synaptic NMDA receptor profile such that NR2A receptors no longer provided significant contributions to CREB phosphorylation following synaptic transmission in the amygdala. However, Figure 21 shows that Ro-25 administration significantly reduces p-CREB expression in amygdalar tissue of both SPS and CFC rats. This would suggest that, even after SPS exposure, the synaptic NMDA receptor profile in the amygdala remained one characterized by significant contributions of NR2B to CREB phosphorylation following synaptic transmission. Taken together, these findings suggest that in SPS-exposed tissue there is a loss of significant NR2A synaptic expression seen in CFC tissue, but there is no such loss for synaptic NR2B expression in the amygdala.

For the PFC, SPS appears to have a somewhat different effect. More specifically, Figures 20 and 21 show that in PFC tissue of SPS rats, neither NVP nor Ro-25 administration significantly reduced p-CREB expression as they did for PFC tissues of CFC rats. This would suggest that exposure to SPS may have changed the synaptic NMDA receptor profile such that both NR2A and NR2B receptors no longer

provided significant contributions to CREB phosphorylation following synaptic transmission in the PFC. A subsequent interpretation of these findings might be that in SPS-exposed tissue there is a loss of the significant synaptic expression seen in CFC tissue for both NR2A and NR2B subtypes within the PFC.

Finally, Figures 20 and 21 show that in hippocampal tissue of SPS rats, both NVP and Ro-25 administration significantly reduced p-CREB expression as they did for hippocampal tissues of CFC rats. This would suggest that the synaptic NMDA receptor profile in the hippocampus is characterized by significant contributions of NR2A and NR2B to CREB phosphorylation following synaptic transmission for both SPS and CFC rats. Following the same logic as described above, an interpretation of this could be that SPS does not eliminate the significant synaptic representation of either NR2A or NR2B subtypes seen in CFC tissue in the hippocampus.

In summary, these findings suggest that SPS eliminated significant synaptic representation of both NR2A and NR2B NMDA receptor subtypes in the PFC, did not eliminate synaptic representation of the NR2B subtype in the amygdala, and did not eliminate synaptic representation of either the NR2A or NR2B subtypes in the hippocampus. This might prove relevant when considering the role that the PFC and amygdala play in PTSD pathology. As we have discussed, long-term fear memories are formed in the amygdala while long-term fear extinction memories are formed in the PFC. We discussed above how the data from CFC rats shown in Figures 20 and 21 suggest that the synapses in the PFC and the amygdala utilize both NR2A and NR2B subtypes in synaptic transmission to induce CREB phosphorylation. It is possible that if situational trauma induces a significant decrease in the synaptic representation of both NR2A and NR2B in the PFC, then LTP might be reduced enough that subsequent long-term fear extinction memory formation in the PFC could be impaired (Figure 28, below). Conversely, if situational trauma does not cause a significant decrease in synaptic NR2B subtype expression in the amygdala, there may be enough NR2B expression at the synapse to compensate for a trauma-induced decrease in

synaptic NR2A subtype expression (Figure 28). In this scenario, LTP in the amygdala may be preserved and thus long-term fear memory may persist even in the face of exposure to situational trauma.

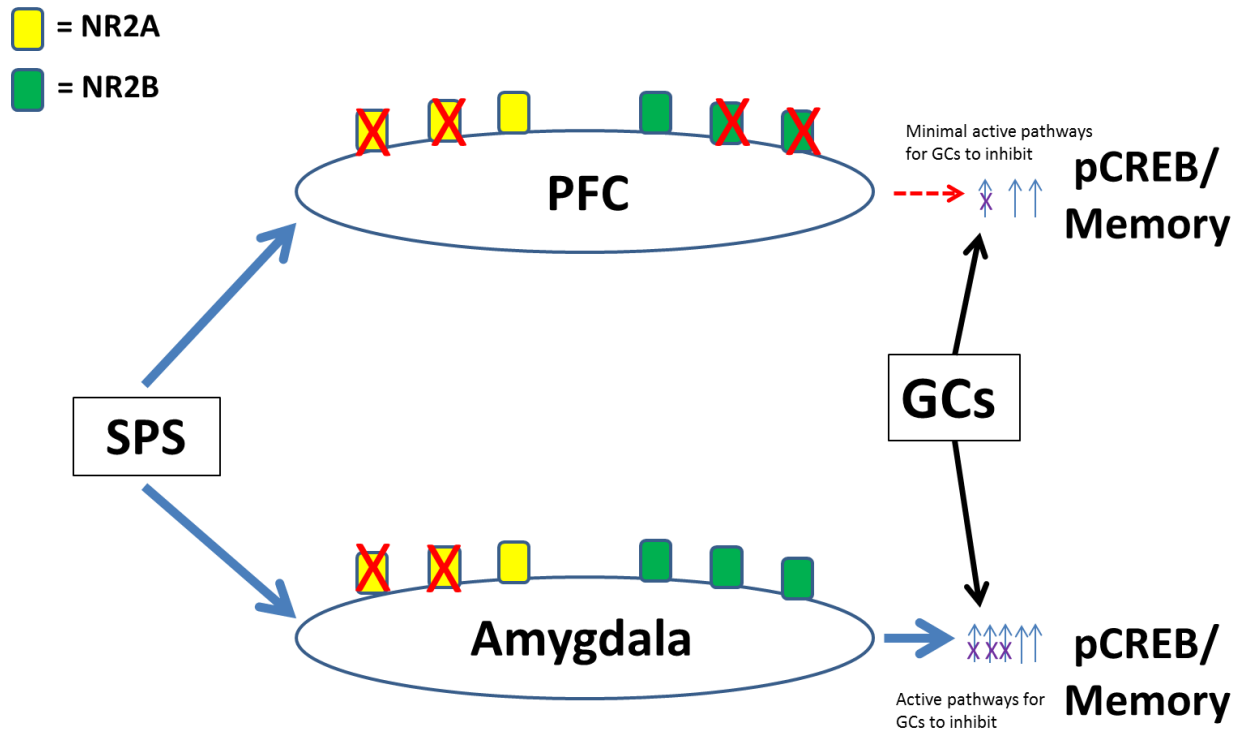


Figure 28. A visual representation of our interpretation, based on our data, of the effect of SPS on NMDA receptor expression and the resulting effect of GCs on p-CREB expression. Our results suggest that SPS could be decreasing the synaptic expression of both NR2A and NR2B (indicated by the red "X") in the PFC, but not NR2B in the amygdala. Upon synaptic activation of NMDA receptors by bicuculline, this may leave very little downstream p-CREB-producing pathway activity to be significantly affected by GCs in the PFC, while there is still a substantial amount of downstream p-CREB-producing pathway activity to be reduced by GCs in the amygdala. Small purple arrows represent GC inhibition of p-CREB-producing pathways. GC = glucocorticoids, p-CREB = phosphorylated cAMP Response Element Binding protein.

The results obtained from NVP and Ro-25 administration may be useful when interpreting the results of GC administration and subsequently attempting to translate those findings to an experience of situational trauma. We discussed above how contrary to the interpretations of Xiao et al. (2010) our

findings of a significant representation of both synaptic NR2A and NR2B subtypes in all regions of CFC rats, combined with the significant GC-induced reduction in p-CREB phosphorylation in all these regions, suggests that GCs could actually inhibit intracellular pathways associated with synaptic NMDA receptor transmission in general, rather than target pathways of one specific NMDA receptor subtype. If true, it would stand to reason that an effect of GC inhibition of p-CREB expression would only be evident when synaptic NMDA receptors are significantly activating these pathways contributing to CREB phosphorylation. We argued above that the data shown in Figures 20 and 21 together suggest that, following SPS, NR2B-dependent synaptic transmission was still significantly activating these pathways in the amygdala. As such, our finding that GCs significantly decreased p-CREB expression in this region is consistent with the above interpretation, as they could be blocking the pathways being activated following synaptic NR2B stimulation. Alternatively, at synapses where the contribution of NMDA receptors is considerably more limited in activating these pathways, the GC inhibition of p-CREB may not be appreciable since there would be limited activation of these pathways in the first place. This may be the case for the PFC considering the data shown in Figures 20 and 21 suggest that, following SPS, both NR2A- and NR2B-dependent synaptic transmission failed to exhibit significant representation. As such, the fact that GCs did not significantly decrease p-CREB expression is consistent with the above interpretation, as there may have been severely limited activation of LTP-inducing pathways available to inhibit.

As such, this potential reduction in both NR2A and NR2B in the PFC of SPS exposed rats, but only NR2B in the amygdala, may contribute to the phenomenon in PTSD where impaired long-term fear extinction memory is exhibited without a similar impairment of long-term fear memory.

## SECTION C: CONCLUSION

Regarding Specific Aim 3, our study failed to show convincing evidence that the interaction of glucocorticoids with differential NR2A/NR2B receptor subtype profiles among the amygdala, PFC and hippocampus is clearly involved in the pathogenesis of PTSD. Though our results showed that GCs could function as “non-classical” inhibitors of p-CREB expression, and thus of long-term memory formation, the inhibition did not differ among the aforementioned regions of interest as we had predicted in our hypothesis. Our use of NR2A and NR2B antagonists suggested that this lack of differential effect of GCs across the amygdala, PFC and hippocampus could be attributable to the absence of differential receptor subtype profiles that we originally assumed would exist from some of the existing literature. Our results for Specific Aim 3 did not allow us to unequivocally distinguish whether GCs can selectively affect downstream elements of synaptic NR2A transmission (see Xiao et al., 2010) or whether they affect downstream elements of synaptic NMDA receptor transmission in general. Use of the NR2A and NR2B antagonists also suggested that, contrary to our original prediction, SPS-exposure altered the synaptic representation of these receptor subtypes in the amygdala and PFC, with a wider-ranging reduction in PFC (both NR2A and NR2B) compared with the reduction in the amygdala (NR2B only). This more extensive impact on elements of glutamatergic synaptic transmission in the PFC, compared with the amygdala, could reflect a potential substrate for the greater impairment of fear extinction memory compared with fear memory previously reported for PTSD. Furthermore, it was shown in the PFC and hippocampus that sensitivity to the immediate inhibitory effects of GCs on p-CREB expression actually diminished in SPS-exposed rats, which further weakens the validity of our prediction that these immediate inhibitory effects on synaptic transmission are a significant contributing factor in the pathogenesis of PTSD.

Our studies were also unable to demonstrate support for our hypotheses suggesting that reductions in astrocytic glutamate transport, followed by glutamate spillover into extrasynaptic locations, are significant components of PTSD pathogenesis. From Specific Aim 1, the comparable effects that DHK (an astrocytic glutamate reuptake inhibitor) and CEF (an astrocytic glutamate reuptake facilitator) had on the SPS-induced fear extinction memory recall deficit contradict the hypothesis that glutamate spill-over is significantly contributing to SPS-induced behavioral and biochemical changes. If spillover were indeed involved, CEF and DHK should have had opposite effects on the extinction memory recall deficit. Furthermore, in Specific Aim 2, our analysis of the effects of SPS-exposure on the GLT-1 astrocytic glutamate reuptake transporter revealed an increase in transporter expression, that was similar in the amygdala and PFC. We originally predicted an SPS-induced decrease in the transporter that would be greater in the PFC than the amygdala, thereby producing greater extrasynaptic spillover in the former. This would produce a greater reduction in p-CREB (a molecular substrate of LTP) in the PFC than amygdala, corresponding to a greater reduction in fear extinction memory processes compared with fear memory processes. Our additional analyses of CREB phosphorylation in Specific Aim 2 also called into question this hypothesized relationship between GLT-1 function, synaptic glutamate spillover and p-CREB levels. The unexpected increase in GLT-1 expression that we observed following SPS-exposure should have reduced any spillover, thereby increasing p-CREB levels. However, no such increase was observed. A further disconnect between changes in GLT-1 function, spillover, and p-CREB levels was observed when we manipulated GLT-1 function with CEF and DHK. As alluded to above, these compounds have been repeatedly shown to have opposite effects on the GLT-1 and therefore should have had opposite effects on spillover, leading to opposite effects on p-CREB. However, CEF and DHK administration either failed to change p-CREB levels or changed them in the same direction. These latter effects also varied with brain region and trauma-exposure. Again, together, our results from Specific

Aims 1 and 2 do not provide convincing support for significant involvement of synaptic glutamate spillover, as we originally conceived it, in the pathogenesis of PTSD.

Instead, our findings point more to a mechanism that involves an SPS-induced decrease in synaptic NMDA receptor function that is more evident in the PFC compared to the amygdala. In fact, for both the *in vivo* study of Specific Aims 1 and 2 as well as in the *ex vivo* study of Specific Aim 3, the data generated from our experiments could be interpreted to support this conclusion. Regarding the *in vivo* study, it was found that both CEF and DHK administration mitigate the effect of SPS on fear extinction memory recall when it was predicted that CEF would mitigate these effects and DHK would exacerbate them. We went on to posit that the unexpected effect of DHK was concordant with an SPS-induced decrease in synaptic NMDA receptor expression, and that that the DHK antagonism of GLT-1 uptake of glutamate might compensate for this by increasing that amount of glutamate at the synapse. Regarding the *ex vivo* study, SPS appeared to induce a decrease in the ability for NR2A and NR2B selective inhibitors to decrease p-CREB expression in the PFC. We argued that this could be another sign of decreased synaptic expression of NR2A and NR2B receptors which could subsequently lead to decreased LTP, possibly explaining the impaired retention of long-term fear extinction memory that characterizes SPS exposure. Considering that the NR2B selective inhibitor still was able to reduce p-CREB expression in the amygdala following SPS, we further made the argument that LTP in the amygdala may still be induced following exposure to SPS, and thus potentially explain why long-term fear memories can persist following situational trauma.

While the results of the CEF and DHK treatments were unexpected and contrary to our hypothesis, they still resulted in relevant effects on SPS-induced behaviors. We just noted above how DHK may mitigate a potential substrate for the fear extinction recall deficit and we discussed earlier how CEF might still exert a mitigating effect upon SPS-induced impaired fear extinction recall through a more

pronounced effect on inhibition of microglial pro-inflammatory activity than on synaptic spillover. Although our findings did not yield a clear mechanism of glial involvement in the behavioral and biochemical changes associated with SPS exposure, the fact that these drugs changed the outcomes of SPS exposure could prove important as it supports the concept of studying glia further as future targets to be employed in novel treatment and therapies for mitigating the devastating effects of PTSD. Furthermore, our finding that SPS produced an increase in expression of the astrocytic reuptake transporter GLT-1 suggests that these glia may be playing a yet to be clarified role in the development of the disorder.

In summary, our findings failed to support our original hypothesis that 1) glutamate spill-over is a contributing factor in the development of PTSD, as well as our hypothesis that 2) the concurrent presence of glucocorticoids and their interaction with differing regional NMDA receptor subtypes underpins the selective impairment of long-term fear extinction memories over long-term fear memories in this disorder. Rather, we conclude from the findings in this study that situational trauma induces a decrease in expression of synaptic NMDA receptor populations, and that this effect varies between the PFC and the amygdala. It is this variation that could actually be a contributing factor to the selective impairment of long-term fear extinction memories over long-term fear memories that is characteristic of PTSD symptomology.

## **SECTION D: FUTURE DIRECTIONS**

This study revealed a number of unexpected results that warrant further investigation in future studies. For example, a key component of the overall hypothesis of this dissertation was the prediction that GLT-1 expression would decrease as a result of exposure to SPS and that glutamate spill-over into extra-synaptic spaces would consequently occur. As discussed above, however, our data showed that the opposite phenomenon occurred where rats exhibited an increase in GLT-1 expression 10 days after exposure to SPS. While we provided possible explanations for this in the Discussion, the experiments conducted in this study do not yield direct evidence for why SPS exposure increased expression in GLT-1. As such, further investigation into this phenomenon is warranted. More specifically, experiments would need to be conducted that measure GLT-1 expression at time-points between SPS exposure and 10-days post exposure. This would better characterize the change in GLT-1 over time following SPS. With a data set that reveals a more complete picture of the changes in GLT-1 expression over time, we would be better able to support or refute the theory we proposed in the Discussion that SPS induces a transient decrease in GLT-1 expression early on that then results in a compensatory increase in GLT-1 expression by Day 10.

Furthermore, it should be noted that just measuring GLT-1 expression does not necessarily give a complete picture of how this transporter may be affecting extracellular glutamate. Under conditions of ischemic injury, movement of glutamate through GLT-1 can reverse directions resulting in glutamate being transported out of astrocytes and into the extracellular space (Rossi et al., 2000). Other studies have shown conditions where GLT-1 can be internalized by astrocytes, and thus prevent the transporter from performing its function at the cell membrane (Sheldon et al., 2008, Susarla et al., 2008). Evaluation of such phenomena was outside of the scope of this experiment, and consequently we have no evidence that would indicate that these specific types of events are occurring. However, it is still important to

consider that there are conditions where expression of GLT-1 may not change, but the effects of the transporter on extracellular concentration of glutamate can be altered significantly. As such, additional studies should be conducted that evaluate the functional capacity of GLT-1. An example of such a study would include an experiment that utilized microdialysis to measure extracellular concentrations of glutamate concentrations. By combining measurement of extracellular glutamate with a more in-depth characterization of the changes in GLT-1 expression over time, understanding of the role of GLT-1 in the pathogenesis of PTSD could be improved beyond the scope of this current study.

The data generated from the *ex vivo* experiment utilizing NVP and Ro-25 also warrants further exploration. As we discussed above, SPS decreased the ability of NVP to inhibit p-CREB expression in the PFC and amygdala, as well as the ability of Ro-25 to inhibit p-CREB expression in the PFC alone. We proposed that these data could be an indirect indication that SPS was inducing a decrease in expression of synaptic NR2A in the amygdala and a decrease in synaptic NR2A and NR2B in the PFC. While designing our experiment in this manner allowed us to isolate the activity of synaptic NMDA receptors from extrasynaptic receptors, there are shortcomings with the design that limit how far we can go with our interpretations. For example, while the inhibitors do preferentially act upon one receptor subtype over the other, studies have reported that NVP and Ro-25 do not entirely selectively inhibit NR2A and NR2B receptors, respectively (Paoletti and Neyton, 2007). To complicate matters further, studies have also shown that triheteromeric receptors, containing both NR2A and NR2B subunits, can exist and that it is unknown exactly how these types of receptors would be affected by either NVP or Ro-25 (Rauner et al., 2011). As such, conducting a follow up experiment that directly measured the protein expression of NR2A, NR2B, and the triheteromeric NR2A/B receptors from synaptosome preparations could provide additional clarity regarding how SPS is specifically changing the synaptic profiles of neurons in the PFC and amygdala.

Yet another unexpected result was the finding that CEF and DHK had similar effects, instead of opposite ones, upon SPS-induced impairment of fear extinction memory retention. In our discussion, we proposed that the effects of CEF administration may have more to do with suppression of microglial pro-inflammatory activity than upregulation of GLT-1 and prevention of glutamate spill-over, as we had originally hypothesized. Pro-inflammatory microglial activity is associated with many neurological diseases/disorders including Alzheimer's disease, neuropathic pain, amyotrophic lateral sclerosis (ALS), traumatic brain and spinal cord injury, and HIV-associated neurocognitive disorder (Burke et al., 2014; Liu et al., 2016; Kumar et al., 2016; Tang et al., 2016; Singh et al., 2015). Of particular note, studies of traumatic spinal injury have shown that inhibiting microglial pro-inflammatory activity results in improved recovery (Kjell and Olson, 2016), and are consistent with the prediction that decreasing the pro-inflammatory state of microglia helps to improve recovery from situational trauma. Though physical trauma to the nervous system is considerably different from psychological trauma, it has been shown that formation of fear memories is associated with microglial production of pro-inflammatory cytokines (e.g., tumor necrosis factor- $\alpha$ ) and suppression of anti-inflammatory markers (e.g., CD209 and CD 206) (Yu et al., 2017). Furthermore, extinction of these fears was correlated with a return to baseline of these anti-inflammatory markers (Yu et al., 2017). As such, it is possible that suppressing the pro-inflammatory activity of microglia following psychological trauma could have a similar protective effect as it does following physical trauma, and might even suppress the formation of fear memories. As such, along with measuring changes in GLT-1 expression immediately following SPS exposure, it may prove valuable to also characterize the microglial responses to situational trauma with particular attention being given to the pro- or anti-inflammatory nature of this response.

In summary, the current study could serve as the starting point for multiple future studies. Firstly, further clarification of the role of GLT-1 and extra-synaptic glutamate spillover in the pathogenesis of PTSD should be conducted that both evaluates the changes in GLT-1 expression and

extracellular glutamate concentrations between exposure to SPS and 10 days post exposure. Secondly, direct measurement of NMDA receptor subtype protein expression should be performed to confirm our indirect findings that SPS induces a change in synaptic NMDA receptor profiles. Finally, investigation into the similar effects of CEF and DHK should be conducted with a more in-depth analysis of the microglial response to situational trauma. If such studies were performed, additional insight into the cellular and molecular mechanisms of PTSD pathogenesis may be achieved that could then lead to new targets for new pharmacological therapies of the disorder.

# CHAPTER VI

## *APPENDICES*

## APPENDIX A: Diagrams

### Key Regions for Fear Expression

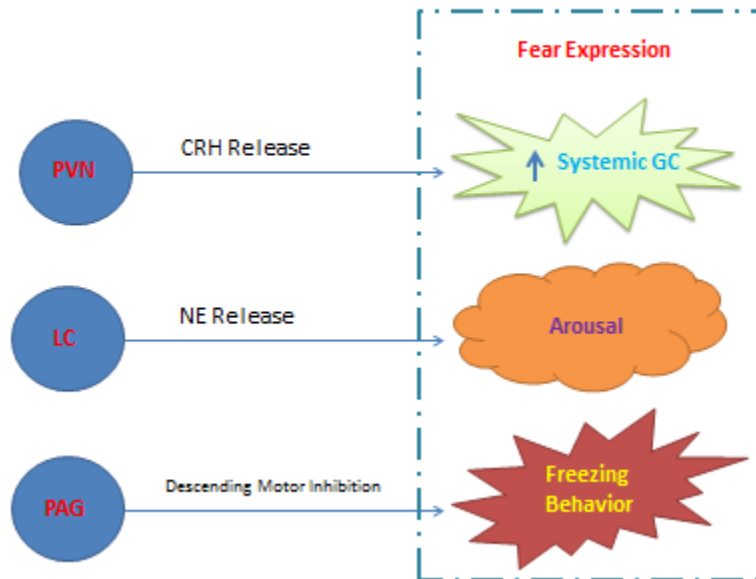


Diagram 1. Studies in rats have shown that fear is a complex behavior that is a composition of hormonal, cognitive, and motor alterations that occur in response to an external stimulus. This figure shows three examples of brain regions involved in these three fear-related alterations. PVN = paraventricular nucleus of hypothalamus; LC = locus coeruleus; PAG = periaqueductal gray matter; NE = norepinephrine; CRH = corticotropin releasing hormone; GC = glucocorticoids.

## How Sensory Stimuli Induce Fear in 4 Steps

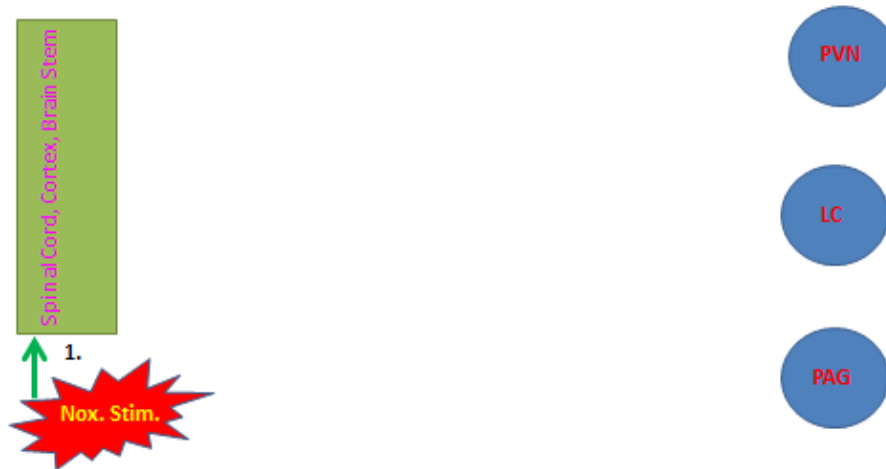


Diagram 2.1. Noxious stimuli (e.g., pain, shocks, predator scent) are detected by the peripheral nervous system, and subsequently transmitted to the CNS (spinal cord, cortex, brainstem) **(1)**.

## How Sensory Stimuli Induce Fear in 4 Steps

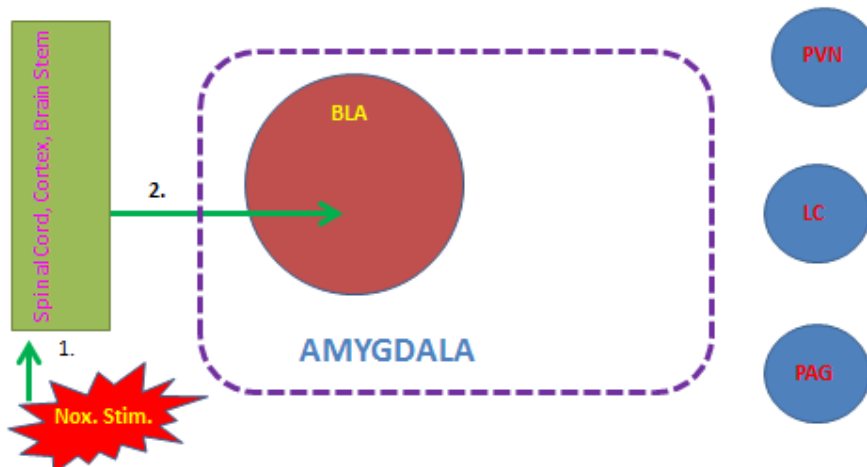


Diagram 2.2. Regions of the CNS carrying the noxious signal send direct glutamatergic (i.e., stimulatory) efferents to the BLA of the amygdala **(2)**. BLA = basolateral complex of the amygdala

## How Sensory Stimuli Induce Fear in 4 Steps

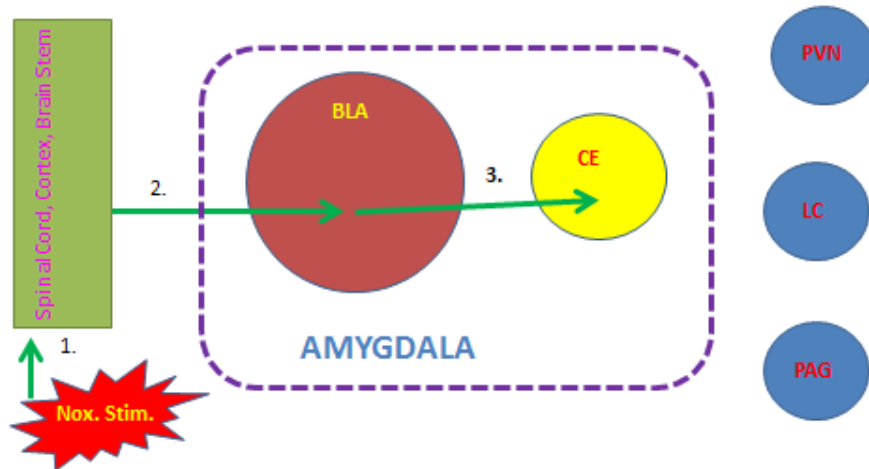


Figure 2.3. The BLA then sends direct glutamatergic efferents to the CE **(3)**. BLA = basolateral complex; CE = central nucleus.

## How Sensory Stimuli Induce Fear in 4 Steps

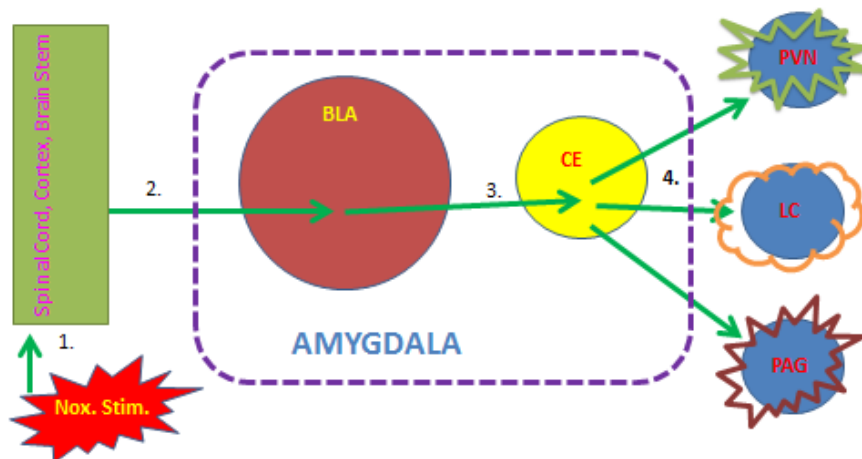


Diagram 2.4. Lastly, the CE sends glutamatergic efferents to the before mentioned regions that cause the fear response. In the case of noxious stimuli, the stimulation of these regions by the CE is strong enough to induce the fear response **(4)**. CE = central nucleus.

### How Neutral Stimuli are Processed in 4 Steps



Diagram 3.1. Neutral stimuli (e.g., soft light, soft tones, neutral odors) are detected by the peripheral nervous system, and subsequently transmitted to the spinal cord, cortex, or brainstem **(1)**.

### How Neutral Stimuli are Processed in 4 Steps

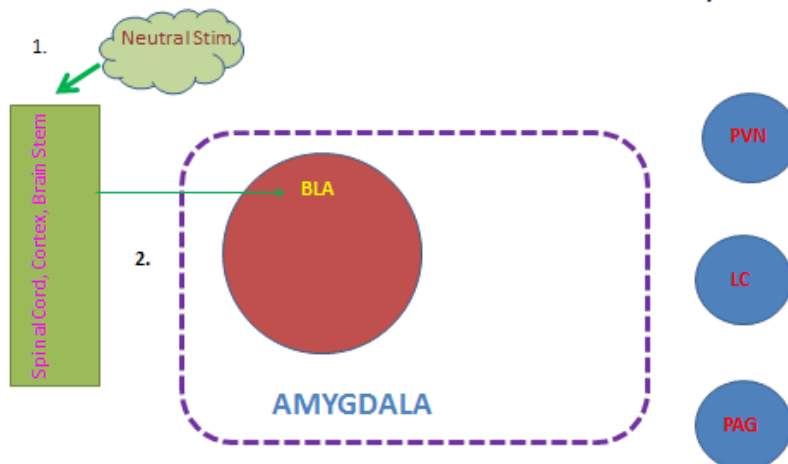


Diagram 3.2. As with noxious stimuli, shown in Figure 2, a signal is sent to the BLA. However, the strength of this signal is weaker compared to noxious signals **(2)**. BLA = basolateral complex.

### How Neutral Stimuli are Processed in 4 Steps

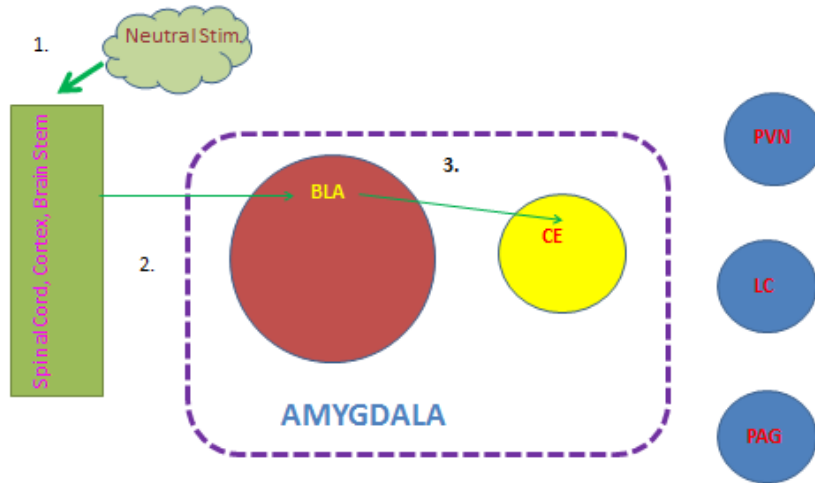


Diagram 3.3. The signal is transmitted to the CE, but again it is weaker compared to the circuit carrying noxious signals shown in Figure 2 **(3)**. CE = central nucleus.

### How Neutral Stimuli are Processed in 4 Steps

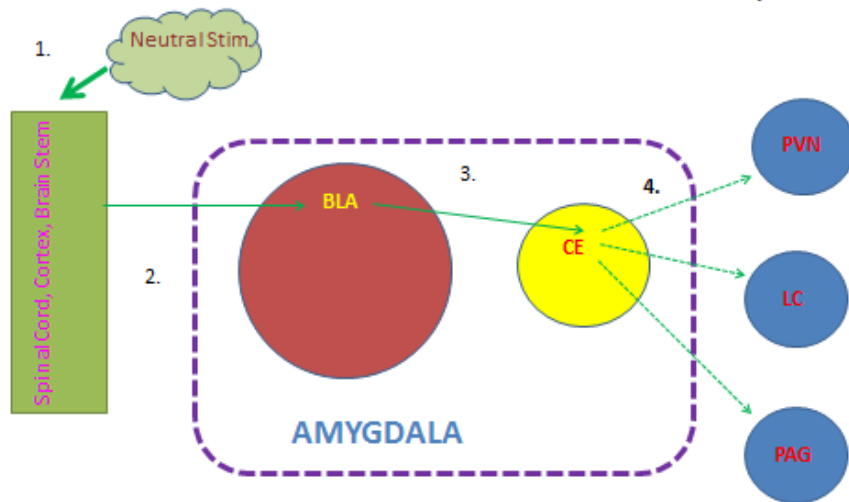


Diagram 3.4. Due to the relative weakness of this circuit, the efferents leaving the CE are not strong enough to stimulate the PVN, LC, and PAG and cause a fear response **(4)**. PVN = paraventricular nucleus of hypothalamus; LC = locus coeruleus; PAG = periaqueductal gray matter; CE = central nucleus.

Modulation of Neutral Stimuli by Noxious Stimuli: Conditioned Fear Memory in 4 Steps



Diagram 4.1. Animals develop a fear of a neutral stimulus when it is temporally paired with a noxious stimulus. When experienced together, the CNS receives signal encoding both the noxious and neutral stimuli (1).

Modulation of Neutral Stimuli by Noxious Stimuli: Conditioned Fear Memory in 4 Steps

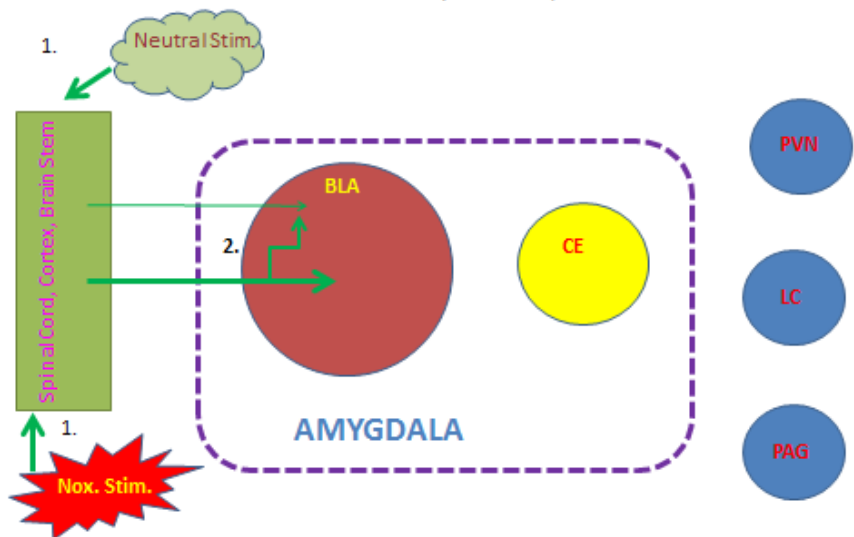


Diagram 4.2. Just as before, both signals are sent to the BLA. However, the presence of an axon collateral extending from the noxious circuit to the neutral one causes potentiation of the neutral circuit synapse in the BLA (2). BLA = basolateral complex.

Modulation of Neutral Stimuli by Noxious Stimuli:  
Conditioned Fear Memory in 4 Steps

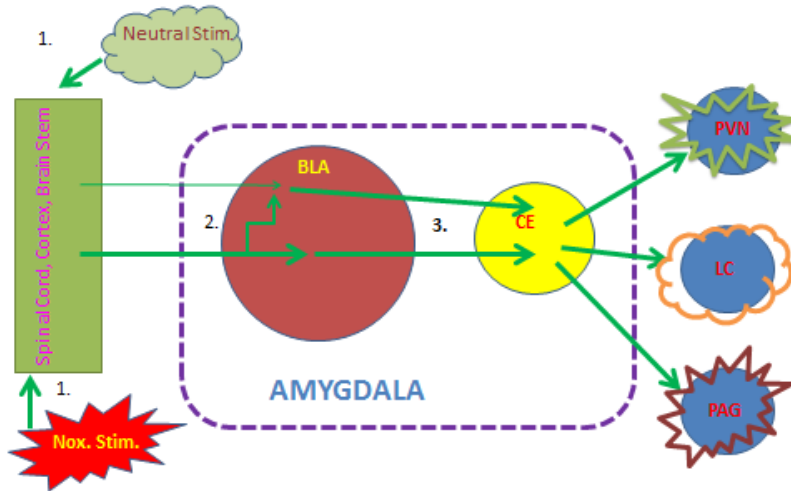


Diagram 4.3. Transmission to the CE and then, subsequently, to the PVN, LC, and PAG proceed as previously mentioned (Diagrams 2 and 3). Due to the strength of these signals, a fear reaction is elicited (3). PVN = paraventricular nucleus of hypothalamus; LC = locus coeruleus; PAG = periaqueductal gray matter; CE = central nucleus.

Modulation of Neutral Stimuli by Noxious Stimuli:  
Conditioned Fear Memory in 4 Steps

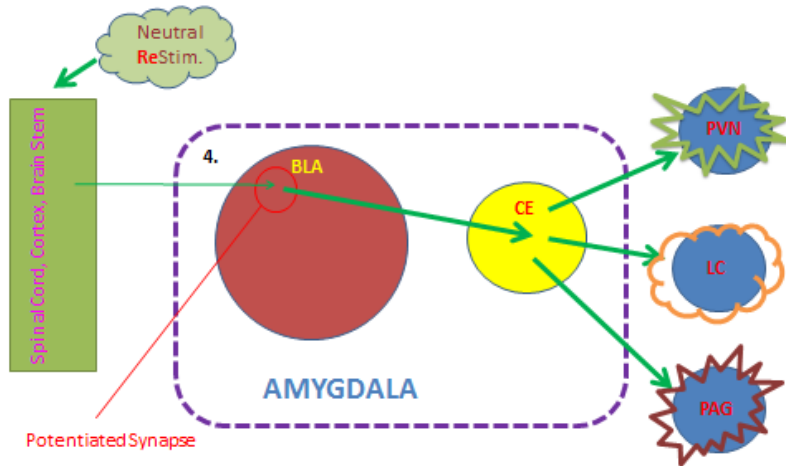


Diagram 4.4. However, following the conditioning process (through which the neutral pathway was potentiated by the noxious pathway), subsequent presentation of the neutral stimulus will be able to elicit a strong enough activation of the PVN, LC, and PAG to cause a fear reaction (even in the absence of the noxious stimulus). Thus, there is now a “**Conditioned Fear Memory**” to the neutral stimulus. PVN = paraventricular nucleus of hypothalamus; LC = locus coeruleus; PAG = periaqueductal gray matter.

## Steps Toward Extinction of a Conditioned Fear Memory

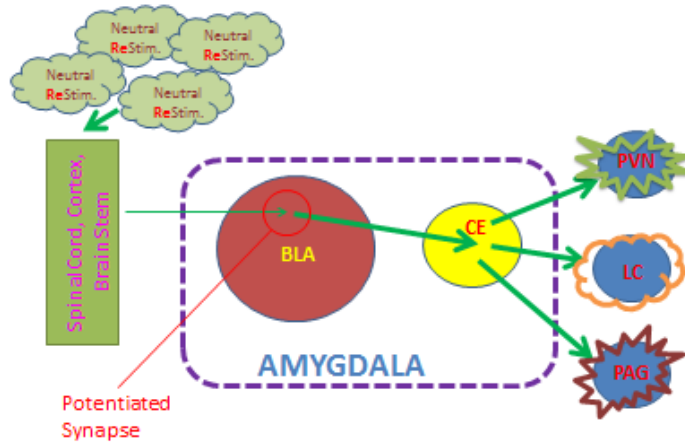


Figure 5.1. Extinction of a conditioned fear memory is the process through which the conditioned fear response to a neutral stimulus is suppressed.

## Steps Toward Extinction of a Conditioned Fear Memory

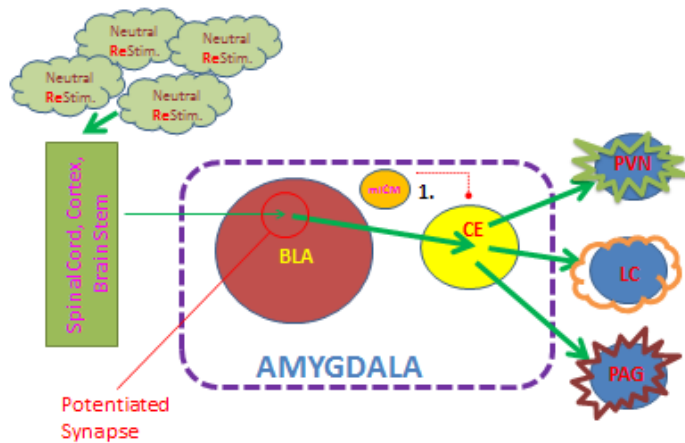


Diagram 5.2. Suppression of amygdala output can be controlled via inhibitory efferents from the medial intercalated cell mass (mICM) to the CE. When stimulated, the mICM will counteract stimulatory signals to the CE neurons from the BLA, thus decreasing the fear response (**1**). BLA = basolateral complex; CE = central nucleus; mICM = medial intercalated cell mass.

### Steps Toward Extinction of a Conditioned Fear Memory

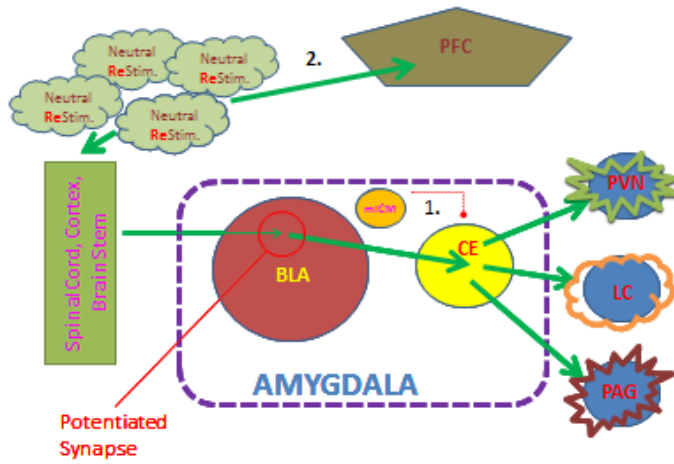


Figure 5.3. Fear extinction is initiated when the conditioned neutral stimulus is presented numerous times without the original noxious stimulus. When this occurs, the PFC is stimulated **(2)**. PFC = prefrontal cortex.

### Steps Toward Extinction of a Conditioned Fear Memory

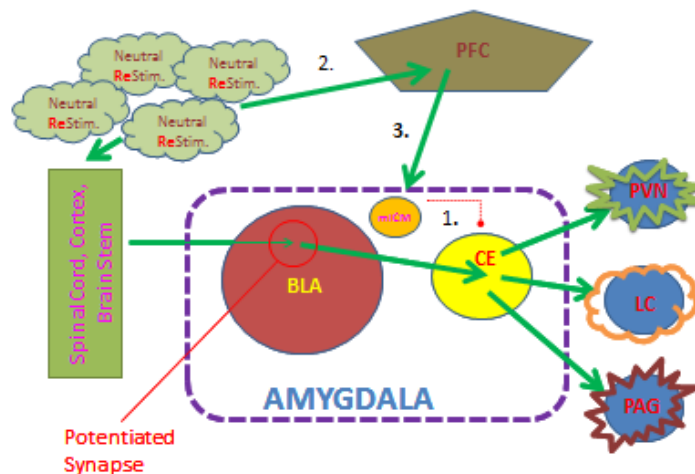


Diagram 5.4. The PFC has direct stimulatory efferents to the mICM. Thus, under these conditions, the strength of the PFC-mICM connection is potentiated **(3)**. PFC = prefrontal cortex; mICM = medial intercalated cell mass.

## Steps Toward Extinction of a Conditioned Fear Memory

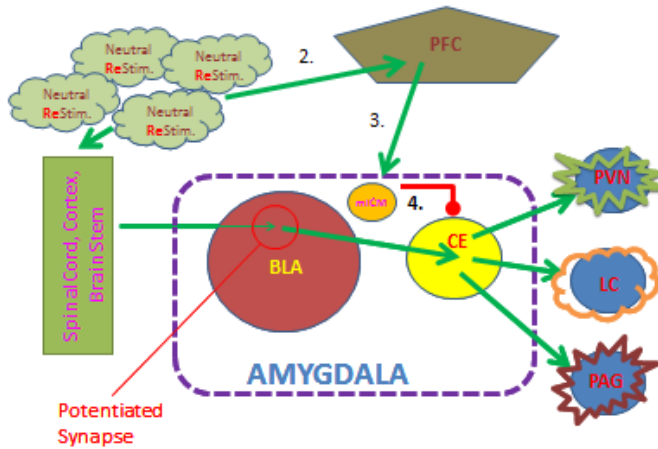


Diagram 5.5. In turn, the inhibitory signal from the mICM to the CE is enhanced (**4**). CE = central nucleus; mICM = medial intercalated cell mass.

## Steps Toward Extinction of a Conditioned Fear Memory

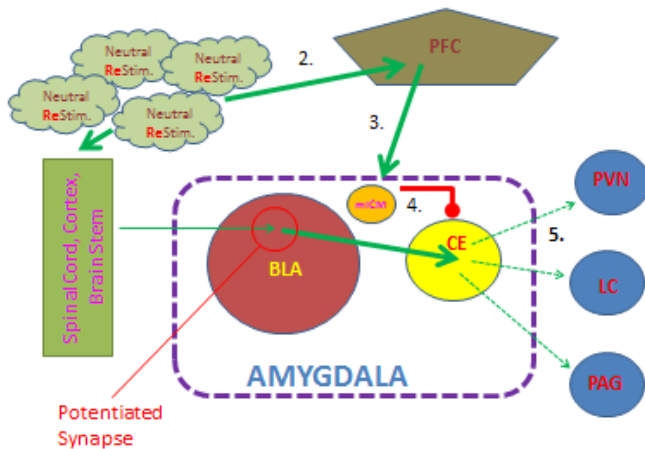


Diagram 5.6. Finally, the enhanced inhibition from the mICM is strong enough to decrease the CE output to the point that the PVN, LC, and PAG are not significantly stimulated (no fear is expressed). The strengthening of this inhibitory circuit is the process of “**Extinction Memory Formation**” (**5**). PVN = paraventricular nucleus of hypothalamus; LC = locus coeruleus; PAG = periaqueductal gray matter; CE = central nucleus; mICM = medial intercalated cell mass.



## Molecular Substrates of Synaptic Strengthening

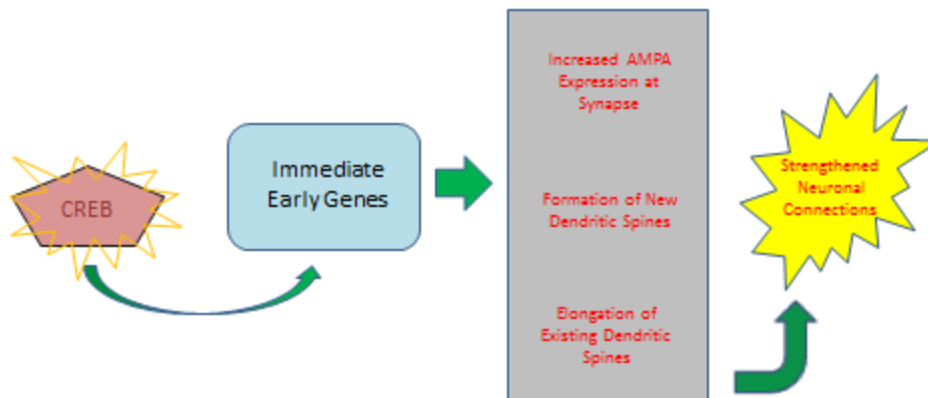


Diagram 6.1. Increasing synaptic strength is known to be a crucial factor in the formation of a new memory. Long-term memory formation requires structural changes to occur in the post-synaptic neuron that depend upon the transcription of several genes and subsequent protein synthesis. When phosphorylated, CREB acts as a transcription factor for many of these genes, and thus plays a pivotal role in long-term memory formation.

## Pathways affecting CREB Phosphorylation

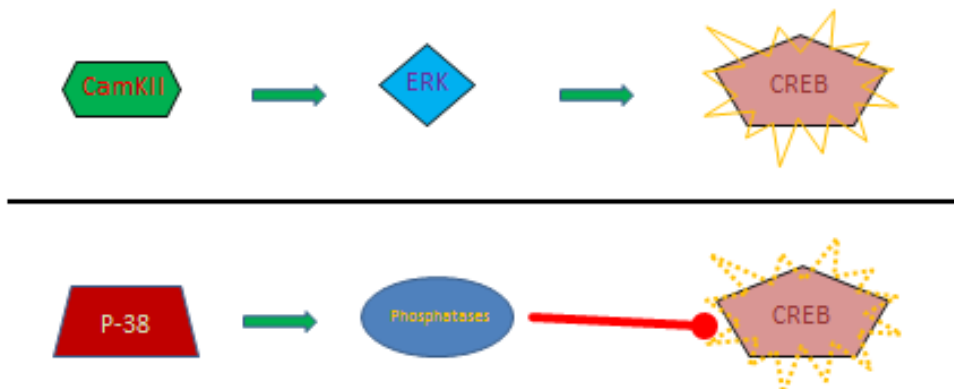


Diagram 6.2. Phosphorylation of CREB in the post-synaptic neuron is regulated by kinases and phosphatases. Phosphorylation of CREB occurs via a CamKII/ERK activation pathway. Conversely, CREB dephosphorylation occurs via a p-38 dependent pathway. At a given time, the balance between these two forms of CREB depends on which pathway predominates. When the CamKII/ERK pathway predominates, long-term memory is promoted. When the p-38 pathway predominates, long-term memory is impaired.

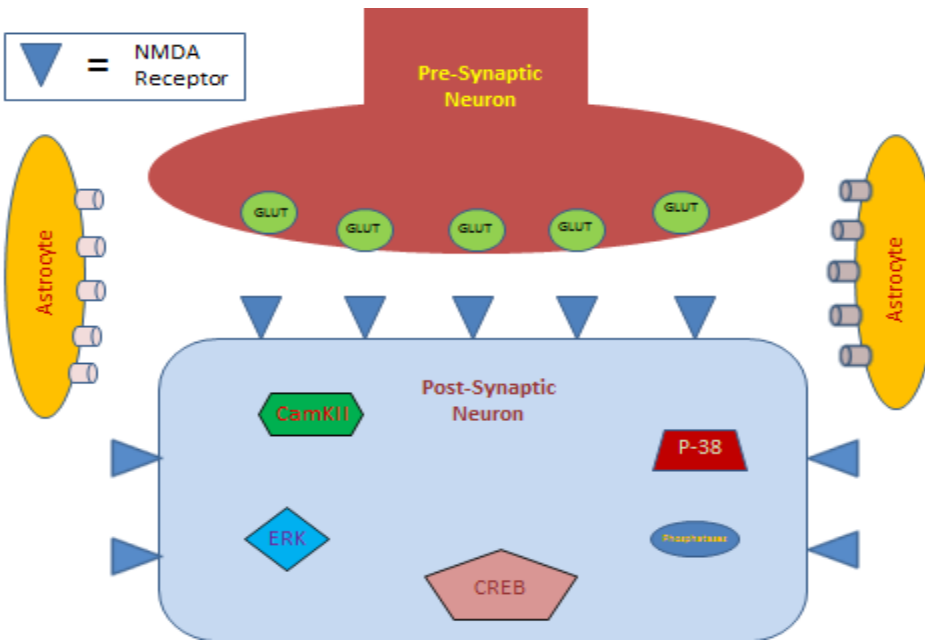


Diagram 7.1. Phosphorylation of CREB during LTP is dependent upon synaptic NMDA receptors.

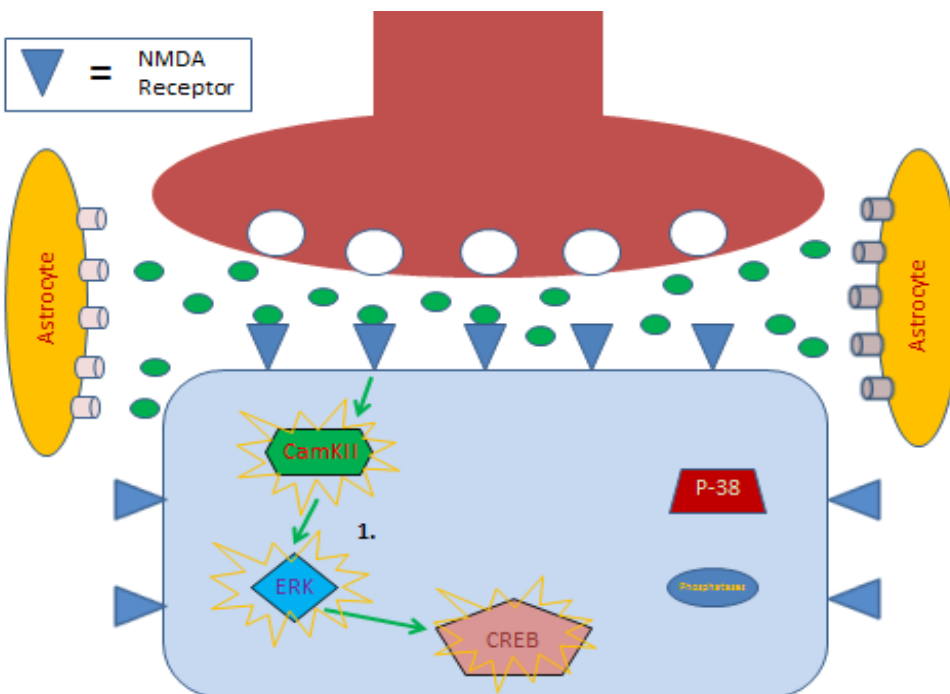


Diagram 7.2. When glutamate is released under physiologically normal conditions, it binds to post-synaptic NMDA receptors at the synapse. This activates the CamKII/ERK pathway that ultimately phosphorylates CREB **(1)**.

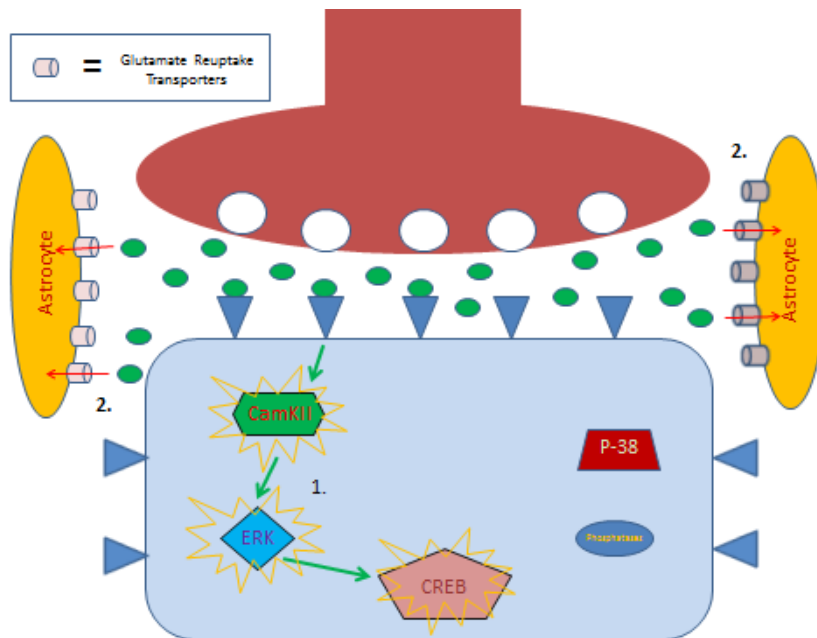


Diagram 7.3. Glutamate is normally restricted to the synapse by astrocyte reuptake transporters (GLAST and GLT-1), preventing activation of NMDA receptors outside of the synapse termed as “extrasynaptic” **(2)**.

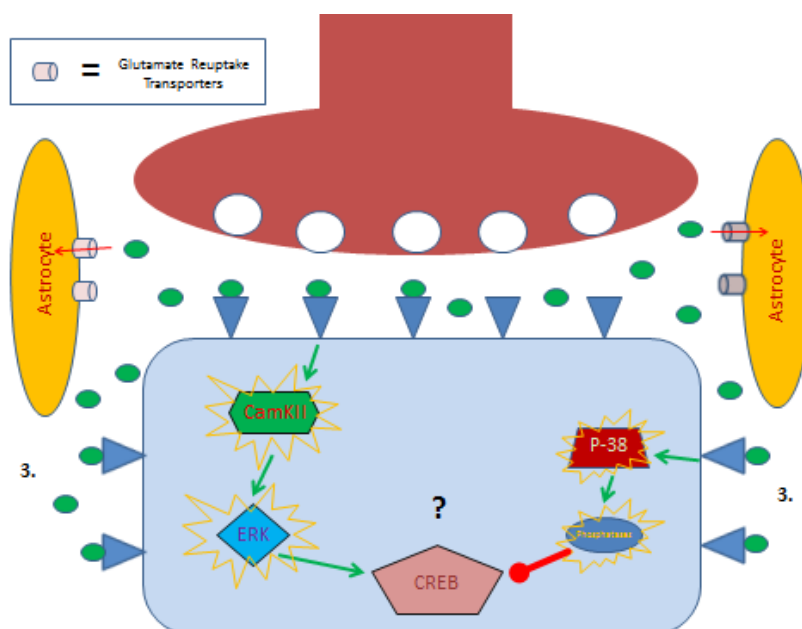


Diagram 7.4. However, if astrocyte glutamate reuptake is impaired (e.g., decreased transporter expression), the astrocytes can no longer restrict glutamate to the synapse, and the neurotransmitter “spills-over” to extrasynaptic locations **(3)**. Under these conditions, glutamate binds to extrasynaptic NMDA receptors and activates the p-38/phosphatase pathway. The level of p-CREB under these conditions will depend upon which pathway is dominant.

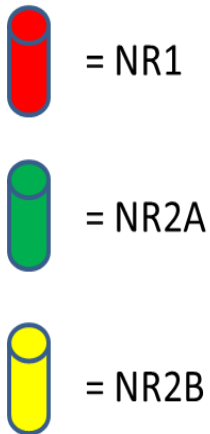
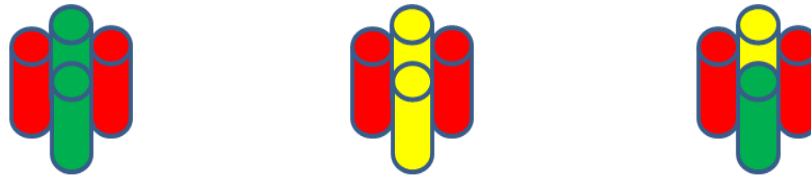


Diagram 8. Not all NMDA receptors are identical. The subunits of NMDA receptors have various subtypes that confer different pharmacological and kinetic properties to the receptor. For example, in the forebrain, the NR2 subunits of the NMDA receptor are most commonly of the NR2A or NR2B subtypes, and differ in their function and cellular localization.

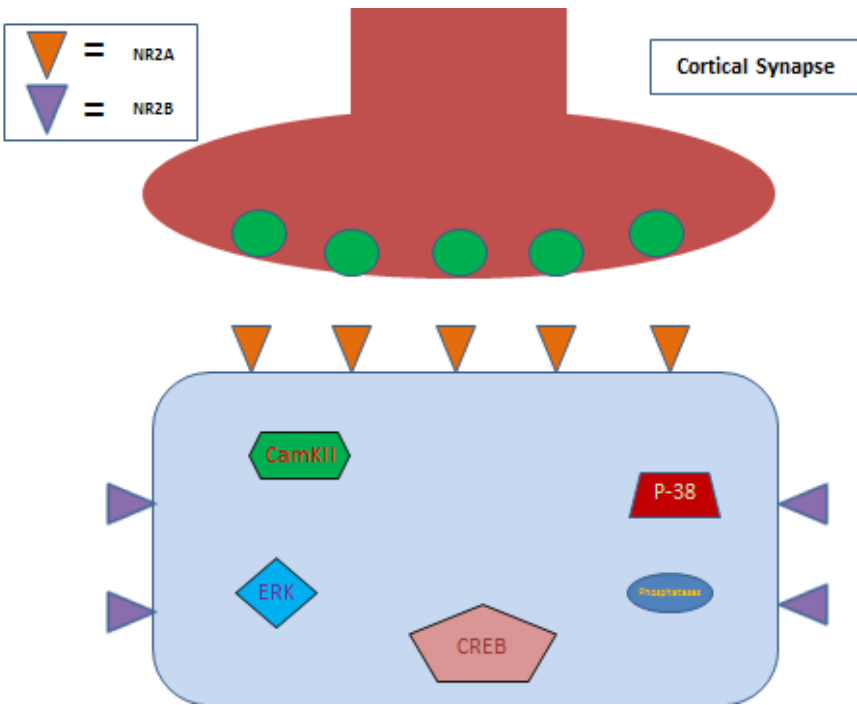


Diagram 9.1. In the cortex, the predominant synaptic NMDA receptor subtype is NR2A, while extrasynaptic receptors tend to be of the NR2B subtype.

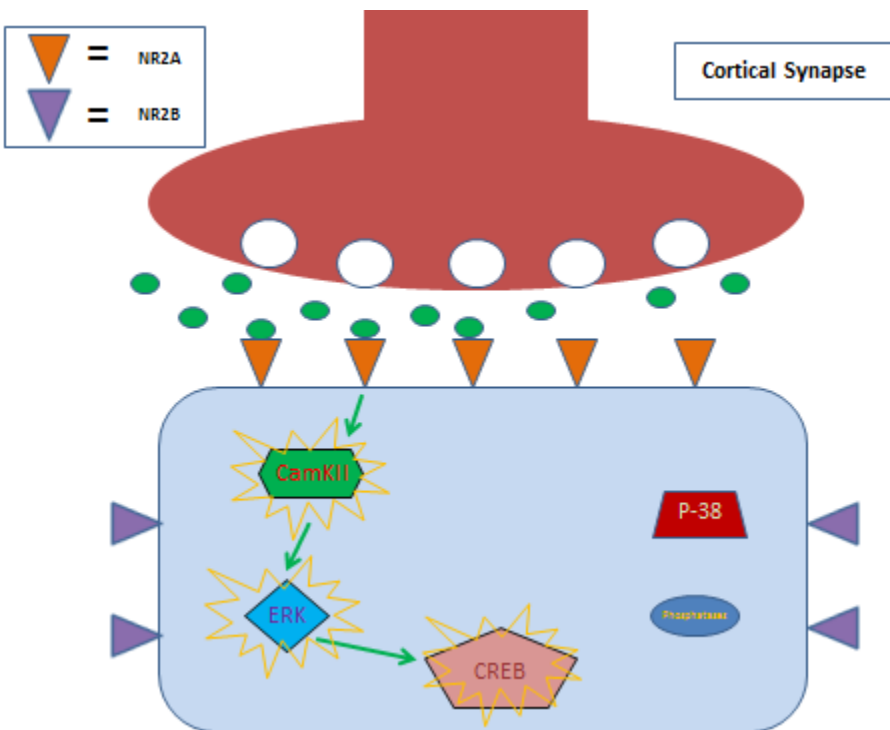


Diagram 9.2. As a result, cortical synapses heavily rely on NR2A to activate the CamKII/ERK pathway. Consequently, late-phase LTP of cortical synapses relies significantly upon NR2A function. LTP = long-term potentiation.

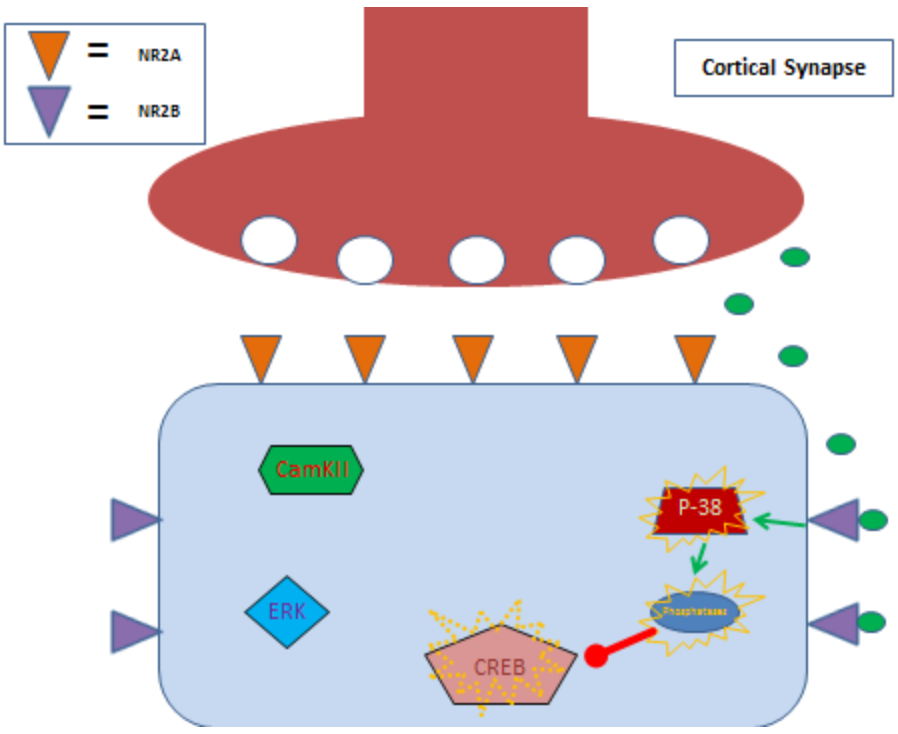


Diagram 9.3. Conversely, extrasynaptic activation of the p-38/phosphatase pathway relies more heavily on NR2B. Consequently, inhibition of late-phase LTP of cortical synapses relies significantly upon NR2B function.

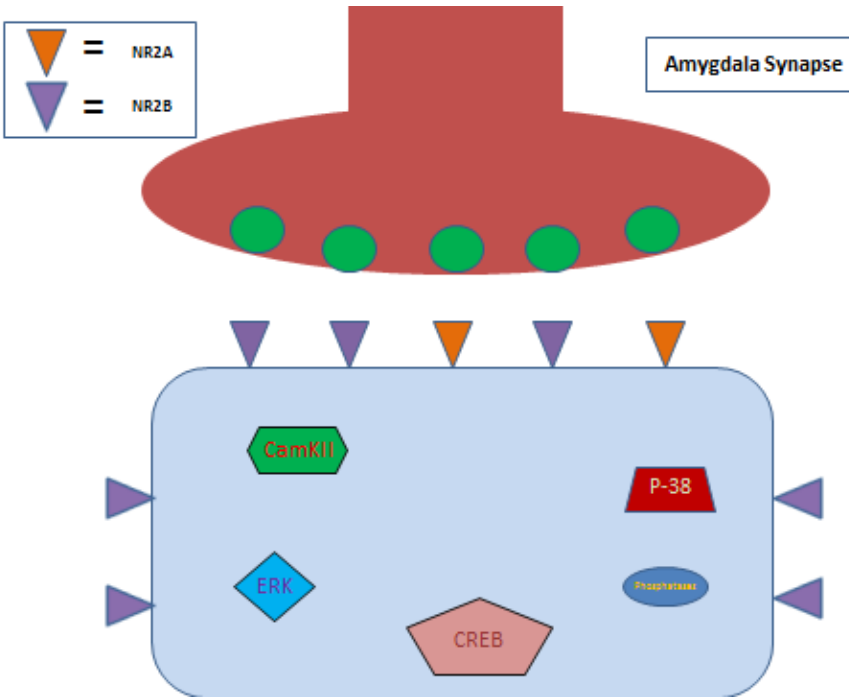


Diagram 10.1. Unlike cortical synapses, amygdala synapses have greater expression of NR2B in the synapse.

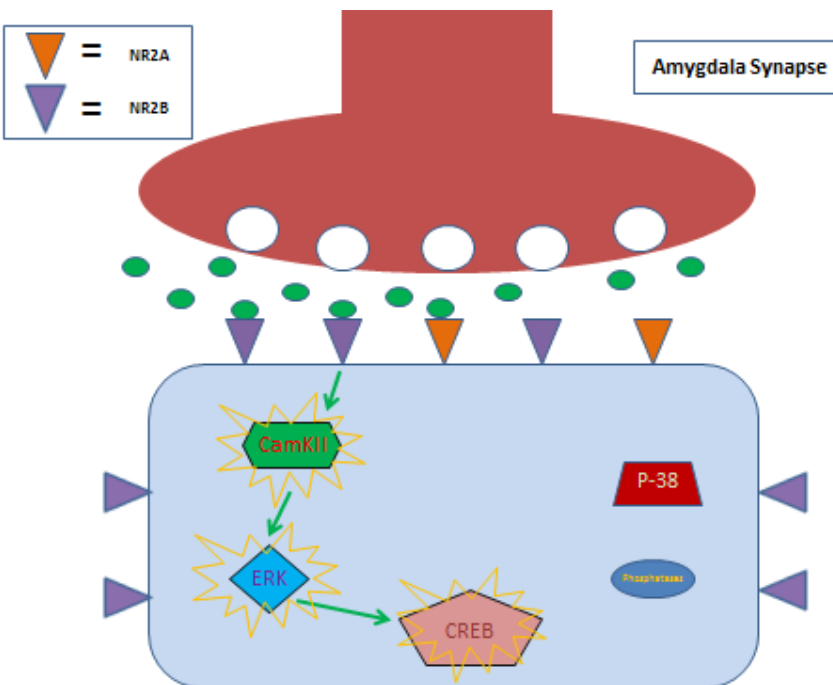


Diagram 10.2. Though extrasynaptic NR2B still leads to activation of the p-38/phosphatase pathway, synaptic NR2B function more like NR2A in the sense that it activates the CamKII/ERK pathway. As a consequence, late-phase LTP can occur in the synapse in a NR2B-dependent manner.

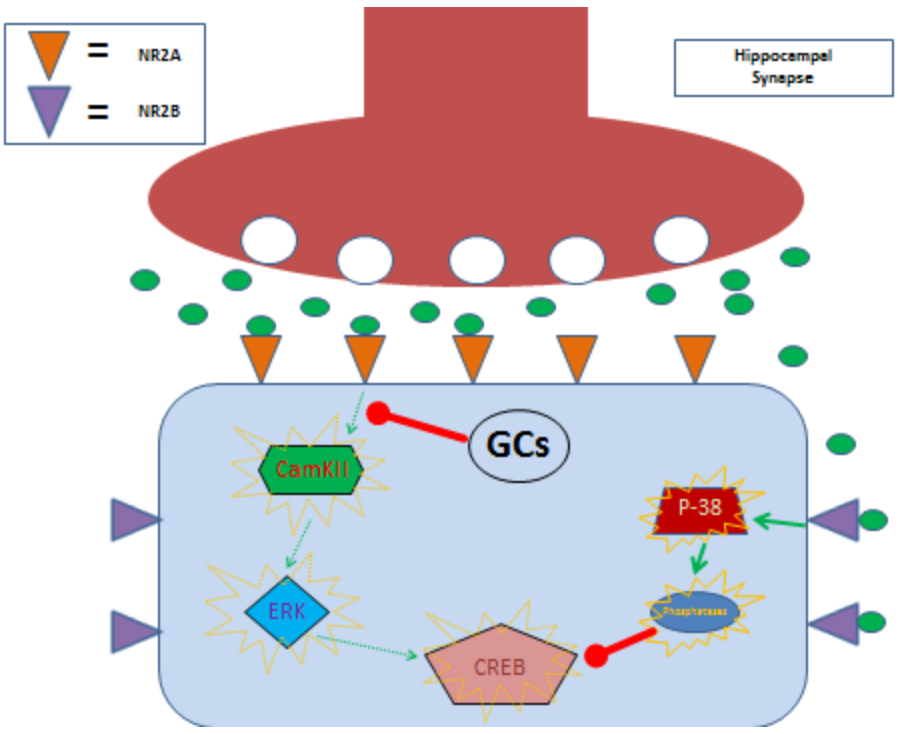


Diagram 11. In hippocampal synapses (which share the NMDA subtype profile of cortical synapses), it was shown that synaptic NR2A activation of the CamKII/ERK pathway could be blocked by GCs, without having a concurrent blockade of extrasynaptic NR2B activation of the p-38/phosphatase pathway. Though CREB phosphorylation was not evaluated, these conditions would most likely result in predominance of CREB dephosphorylation.

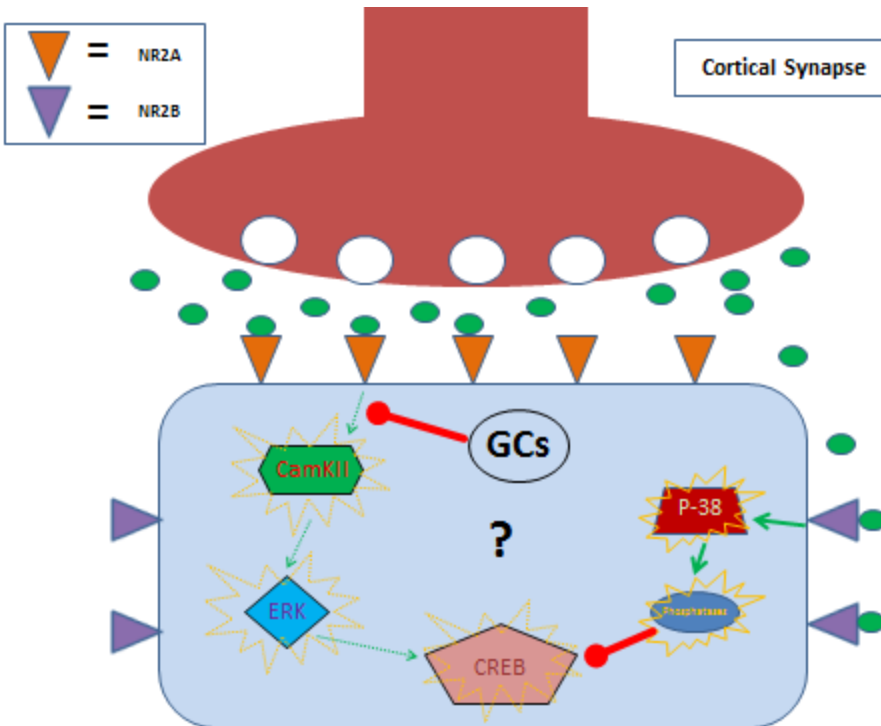


Diagram 12. Due to the similarities between hippocampal and cortical synapses in regard to localization and functional use of NR2 subtypes, there is a reasonable possibility that glucocorticoids would block the CamKII/ERK pathway at cortical synapses while leaving the p-38/phosphatase pathway intact. If so, glucocorticoids could significantly shift the balance of CREB levels to favor the dephosphorylated form when both synaptic and extrasynaptic sites are stimulated (such as when astrocytic glutamate transporters are down-regulated). In this case, simultaneous impairment of glutamate reuptake and CamKII/ERK (by GCs) would result in significant inhibition of late-phase LTP in cortical synapses.

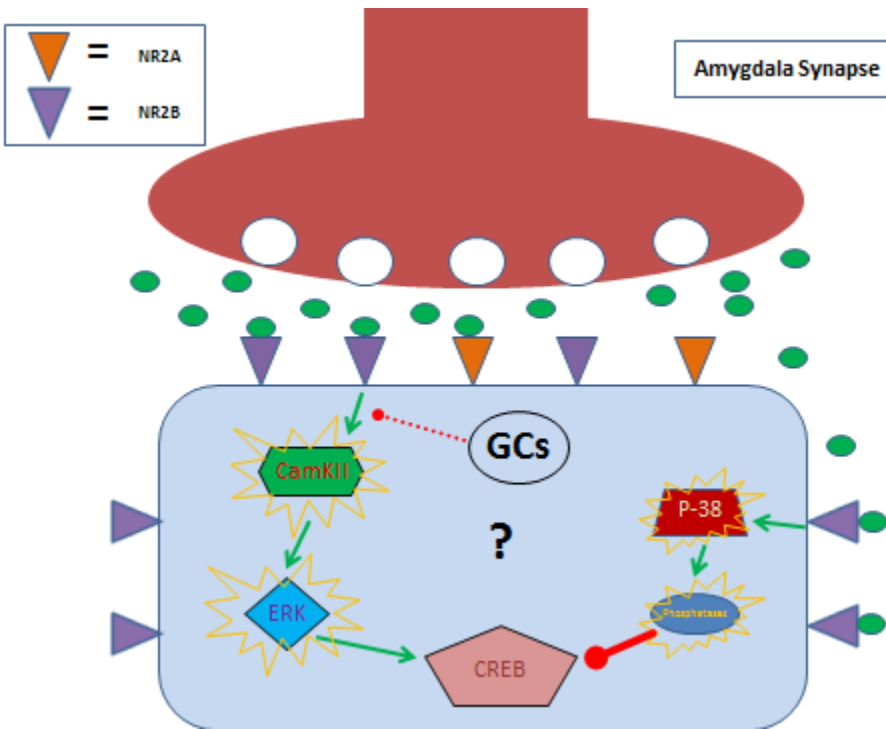
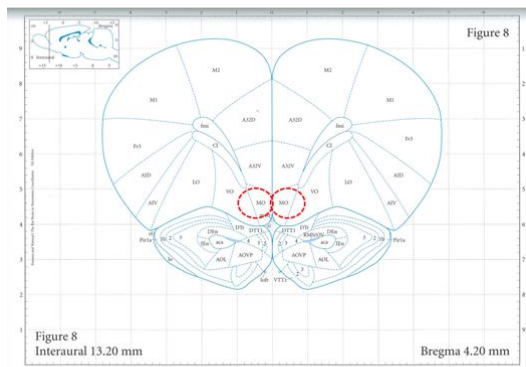
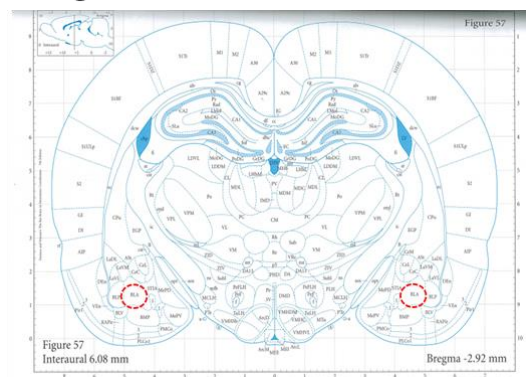


Diagram 13. However, it is unclear if the effects of GCs on the CamKII/ERK pathway are specific to NR2A or synaptic NMDA receptors in general. If the effect of GCs is NR2A specific, there is a possibility that the CamKII/ERK pathway at amygdala synapses would not be impaired in a similar manner due to the presence of NR2B. If so, glucocorticoids would not significantly shift the balance of CREB levels to favor the dephosphorylated form when both synaptic and extrasynaptic sites are stimulated. In this case, simultaneous impairment of glutamate reuptake and CamKII/ERK (by GCs) would result in less inhibition of late-phase LTP in amygdala synapses compared to those in the cortex.

## Diagram 14A



## Diagram 14B



## Diagram 14C

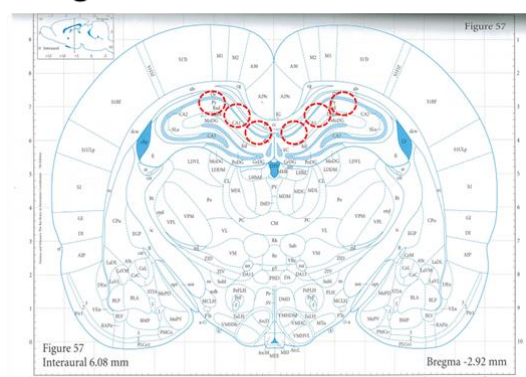
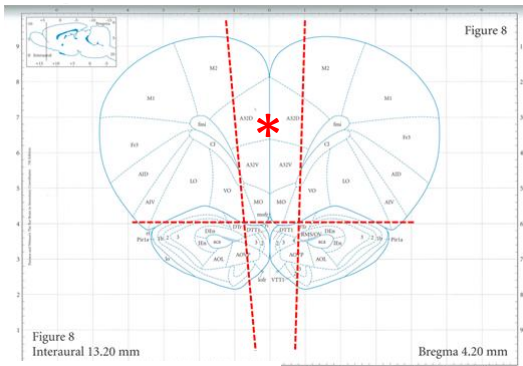
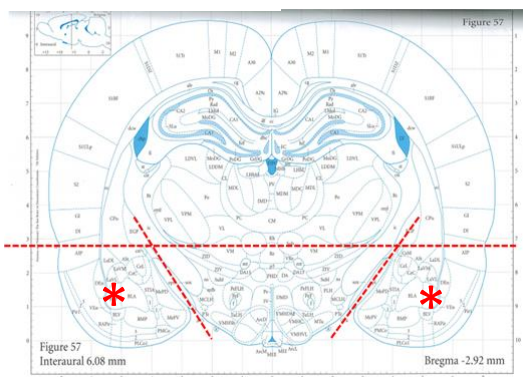


Diagram 14. Representation of areas of the brain from which samples were collected for Specific Aim 2 (red circles represent where the 1-mm microbiopsy punch was placed to collect the samples). Diagram 14A, Diagram 14B, and Diagram 14C show where samples were collected for the prefrontal cortex, basolateral complex of the amygdala, and CA1 region of the hippocampus, respectively.

## Diagram 15A



## Diagram 15B



## Diagram 15C

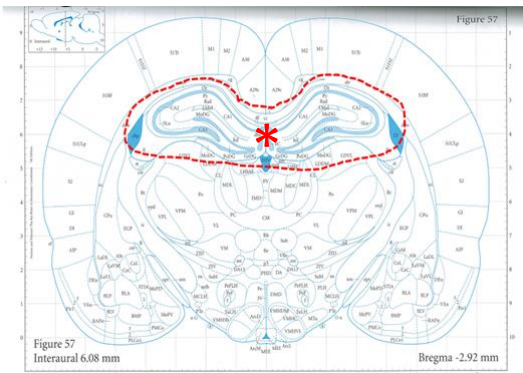


Diagram 15. Representation of areas of the brain from which samples were collected for Specific Aim 3. Red lines represent where cuts were made to remove tissue of interest (indicated by red asterisk). Diagram 15A, Diagram 15B, and Diagram 15C show where samples were collected for the prefrontal cortex, the amygdala, and the hippocampus, respectively.

## APPENDIX B: Figures

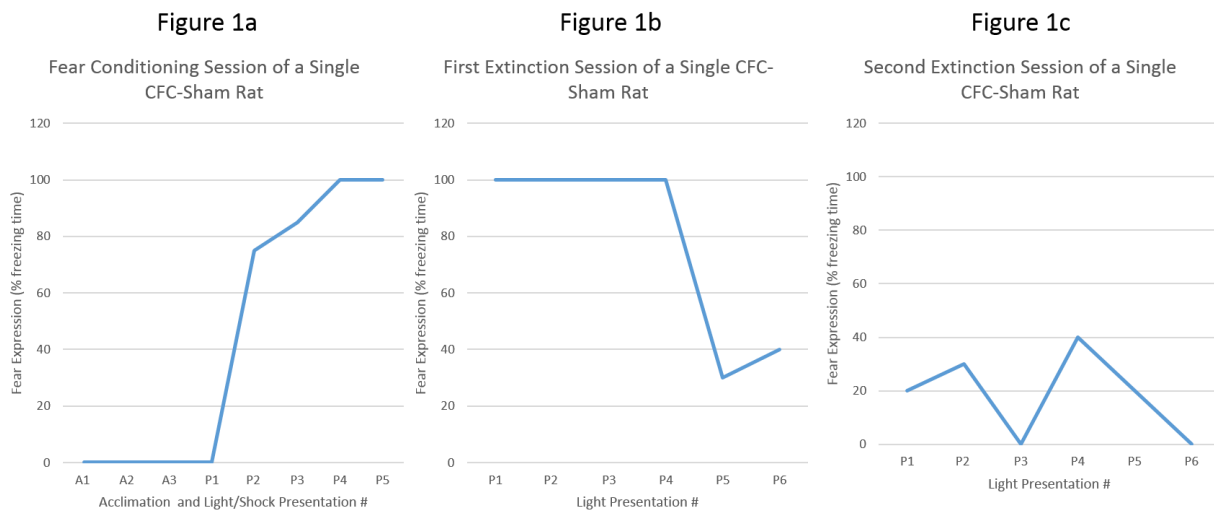


Figure 1. Depiction of fear expression in a single CFC-Sham treated rat during Fear Conditioning (1a), an initial Fear Extinction session 24 hours after fear conditioning (1b), and a second Extinction session 24 hours after the first Extinction session (1c). During Fear Conditioning, the rat was allowed 180 seconds to acclimate to the conditioning chamber (represented by the three 60s intervals designated A1, A2, A3) where no conditioned or unconditioned stimuli were presented (1a). As expected, no fear was exhibited at these acclimation points. However, delivery of a light paired with a shock (represented by the five 10s intervals designated P1, P2, P3, P4, and P5) induced strong fear expression, as seen in the last 5 time points of conditioning (1a). Twenty four hours later, the rat was placed in the chamber again and was exposed to 6 light presentations without concurrent shock (represented by the six 10s intervals designated P1, P2, P3, P4, P5, and P6) (1b). The rat showed high levels of fear expression early in the session, consistent with maintaining a fear memory induced by the conditioning process. Later in the session, however, fear expression diminishes which is consistent with fear extinction learning (1b). Finally, when the Extinction session was repeated 24 hours later, the rat exhibited low levels of fear early in the session that were comparable to the levels exhibited towards the end of the first Extinction session (1c). This is consistent with maintaining the memory of extinction learning from 24 hours earlier. SPS = single prolonged stress; CFC = classical fear conditioning.

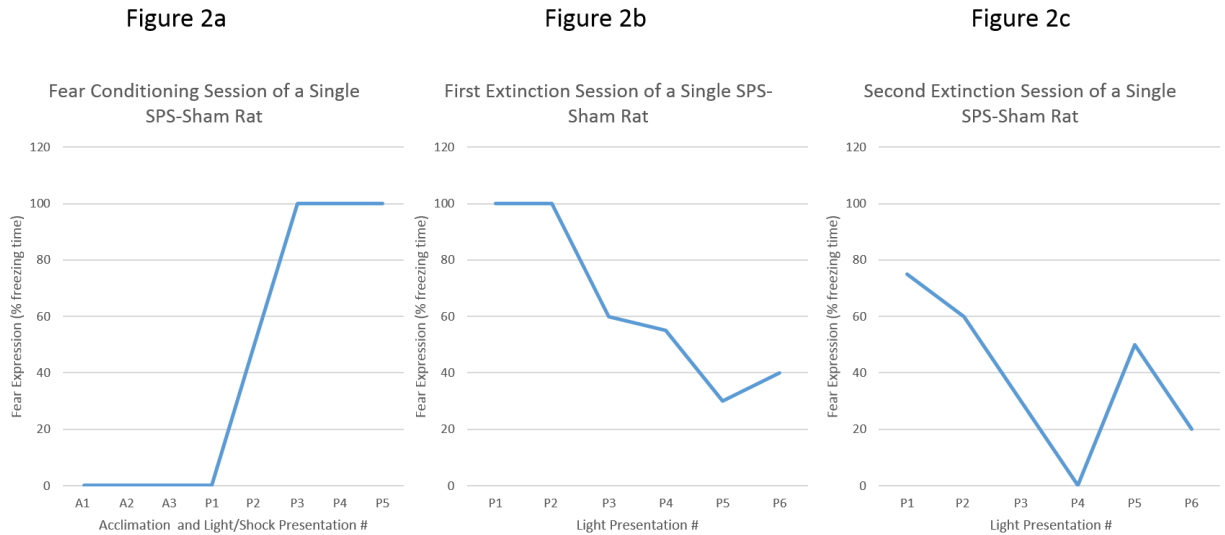


Figure 2. Depiction of fear expression in a single SPS-Sham treated rat during Fear Conditioning (2a), an initial Fear Extinction session 24 hours after fear conditioning (2b), and a second Extinction session 24 hours after the first Extinction session (2c). During Fear Conditioning, the rat was allowed 180 seconds to acclimate to the conditioning chamber (represented by the three 60s intervals designated A1, A2, A3) where no conditioned or unconditioned stimuli were presented (2a). As expected, no fear was exhibited at these points. Delivery of a light paired with a shock (represented by the five 10s intervals designated P1, P2, P3, P4, and P5) induced strong fear expression, as seen in the last 5 time points of conditioning (2a). Twenty four hours later, the rat was placed in the chamber again and was exposed to 6 light presentations without concurrent shock (represented by the six 10s intervals designated P1, P2, P3, P4, P5, and P6) (2b). The rat showed high levels of fear expression early in the trial, consistent with maintaining a fear memory induced by the conditioning process. Later in the trial, however, fear expression diminishes which is consistent with fear extinction learning (2b). Finally, when the Extinction trial was repeated 24 hours later, the rat exhibited higher levels of fear expression in the early part of the session compared to the levels exhibited towards the end of the first Extinction trial (2c). This is consistent with poorer maintenance of the memory of extinction learning from 24 hours earlier, compared with the CFC-Sham rat from Figure 1, and is evidence of impaired fear extinction memory recall relative to the CFC-Sham rat. SPS = single prolonged stress; CFC = classical fear conditioning.

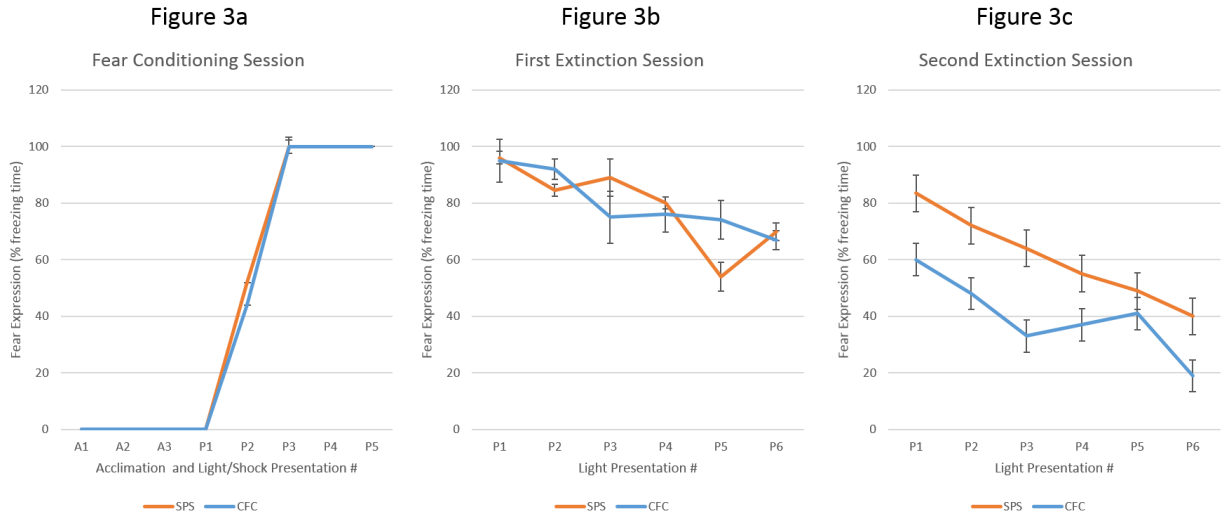


Figure 3. Depiction of mean fear expression across rats, for each time point, for the CFC-Sham and SPS-Sham groups during Fear Conditioning (3a), an initial Fear Extinction session 24 hours after fear conditioning (3b), and a second Extinction session 24 hours after the first Extinction trial (3c). During Fear Conditioning, both groups were allowed 180 seconds to acclimate to the conditioning chambers (represented by the three 60s intervals designated A1, A2, A3) where no conditioned or unconditioned stimuli were presented (3a). As expected, no fear was exhibited at these points in either groups. Delivery of a light paired with a shock (represented by the five 10s intervals designated P1, P2, P3, P4, and P5) induced strong fear expression, as seen in the last 5 time points of conditioning (3a). Twenty four hours later, the rats of both groups were placed in chambers again and exposed to 6 light presentations (represented by the six 10s intervals designated P1, P2, P3, P4, P5, and P6) without concurrent shock (3b). Both groups showed high levels of fear early in the session, consistent with maintaining a fear memory induced by the conditioning process. Later in the session, fear expression diminishes in both groups which is consistent with fear extinction learning (3b). Finally, when the Extinction trial was repeated 24 hours later, the CFC group exhibited levels of fear expression that were comparable to the levels exhibited towards the end of the first Extinction session, while the SPS group exhibited levels of fear expression that were higher compared to the levels exhibited towards the end of the first Extinction session (3c). This suggests that the CFC-Sham rats were more capable of maintaining fear extinction memories than the SPS-Sham rats, indicating that SPS induces an impairment of fear extinction memory recall. SPS = single prolonged stress; CFC = classical fear conditioning. Error bars represent standard deviations.

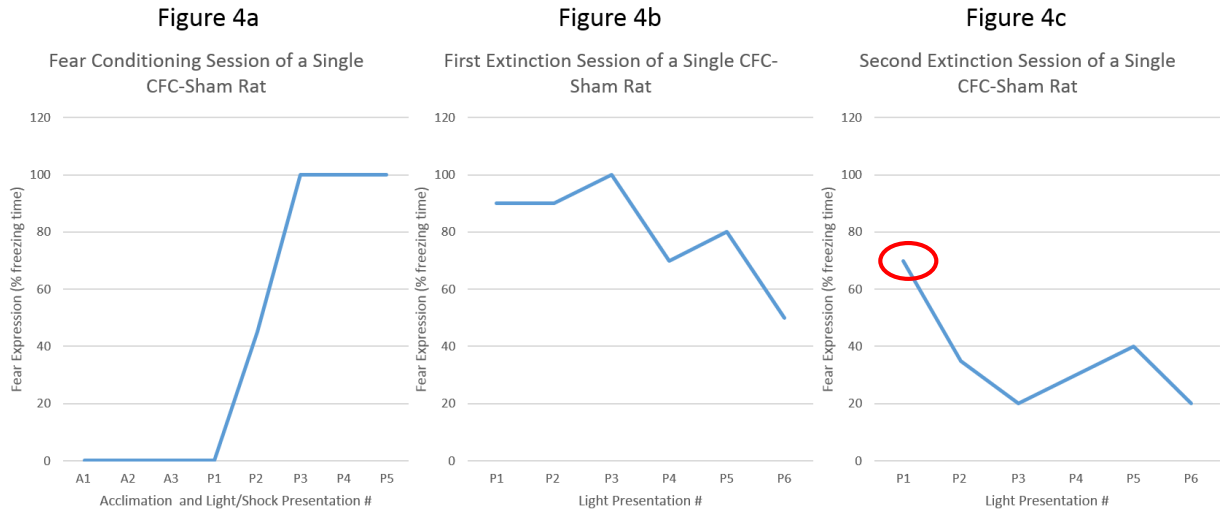


Figure 4. Depiction of fear behavior in another CFC-Sham rat during Fear Conditioning (4a), an initial Fear Extinction session 24 hours after fear conditioning (4b), and a second Extinction session 24 hours after the first Extinction session (4c). Fear expression exhibited a similar pattern to that seen in the case depicted in Figure 1 during Fear Conditioning and the first Extinction session (4a and 4b, respectively). However, the fear expressed during the first presentation in the second Extinction session (circled in red) is considerably higher than that during the remaining presentations (4c). If we were to use the simple approach of comparing behavior of the last presentation of the first Extinction session to that of the first presentation of the second session, it would appear as if this CFC-Sham rat exhibited poorer fear extinction recall than might be the case. SPS = single prolonged stress; CFC = classical fear conditioning; A1, A2, A3 = Acclimation periods 1, 2, 3, respectively.

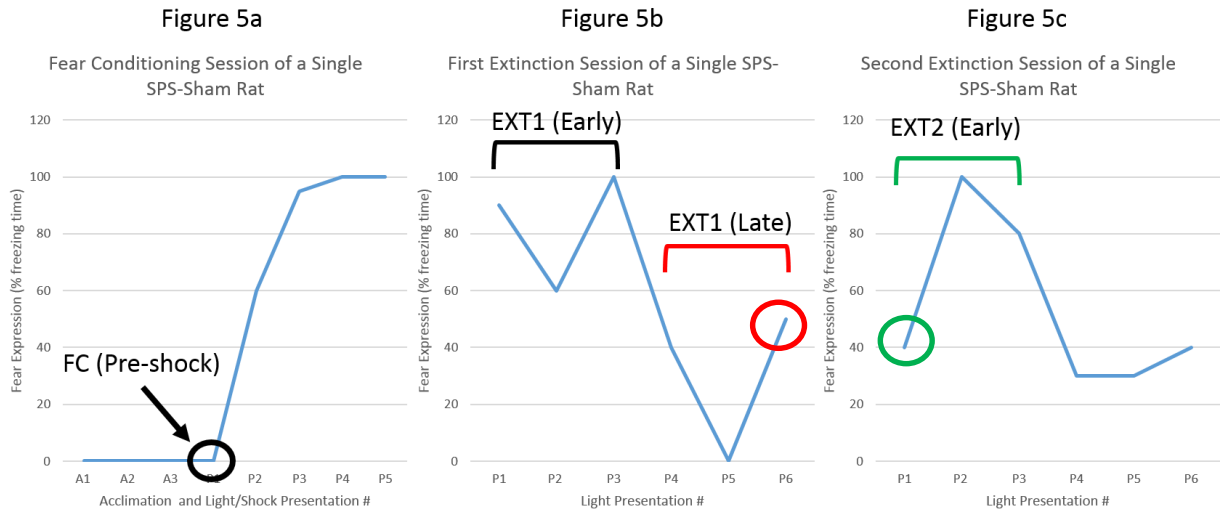


Figure 5. Depiction of fear behavior in a another SPS-Sham rat during Fear Conditioning (5a), an initial Fear Extinction session 24 hours after fear conditioning (5b), and a second Extinction session 24 hours after the first Extinction session (5c). Fear expression exhibited a similar pattern to that seen in the case depicted in Figure 2 during Fear Conditioning. However, there was a large spike in fear expression during the last light presentation of the first Extinction session (Figure 5b, highlighted by the red circle) and a much lower level of fear expression at the beginning of the second Extinction session compared to the subsequent two light-on trials (Figure 5c, highlighted by the green circle). If these data points alone were used to characterize fear extinction recall, it would appear as if fear extinction recall were better than might be the case. Therefore, to better represent fear expression near the end of the first Extinction session and near the beginning of the second Extinction session, we respectively averaged fear expression for the last three light-on trials of the first Extinction session (indicated by the red bracket labeled EXT1(late) in Fig. 5b) and for the first three light-on trials for the second Extinction session (indicated by the green bracket labeled EXT2(early) in Fig. 5c) for each rat. Using a similar rationale, we averaged fear expression for the first three light-on trials of the first Extinction session to characterize the beginning of extinction (indicated by the black bracket labeled EXT1(early) in Fig. 5b). There was only a single trial for each rat that could be used to represent fear expression to the light prior to association with shock (indicated by the black circle labeled FC (pre-shock) in Fig. 5a). SPS = single prolonged stress; CFC = classical fear conditioning; A1, A2, A3 = Acclimation periods 1, 2, 3, respectively.

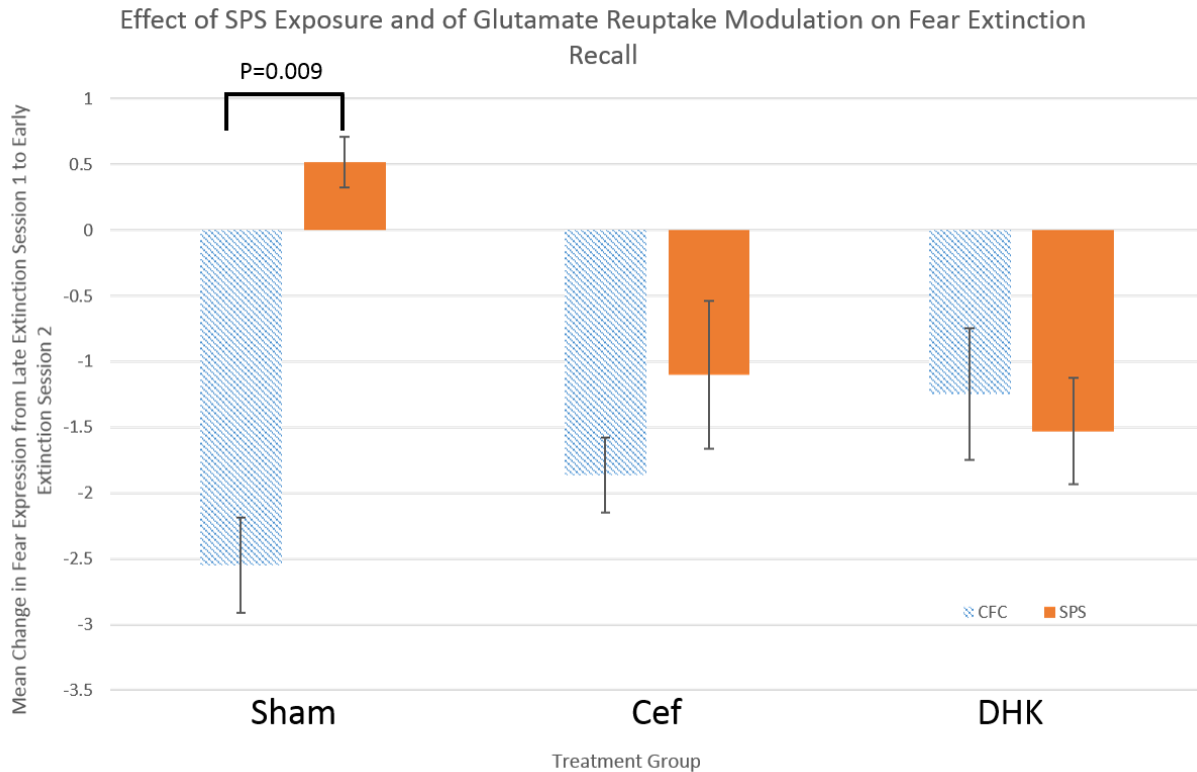


Figure 6. Depiction of the change in fear expression between the end of the first Extinction session and the beginning of the second Extinction session as a result of SPS exposure and drug treatment. For each individual rat within each treatment group, the percentage of time spent freezing during the last three Light-on exposures of the first Extinction session were averaged (EXT1(late)) and subtracted from the average percentage of time spent freezing during the first three Light-on presentations of the second Extinction session (EXT2(early)), resulting in an index representing the change in fear expression between the two (the Fear Extinction Recall Index). A two-way ANOVA was performed that examined the potential interaction of SPS exposure with drug administration, and revealed a significant interaction ( $F(2,53)=4.15$ ,  $p=0.021$ ) of trauma exposure with drug treatment. Post-hoc pairwise analysis revealed that for the sham treated groups, rats exposed to SPS had significantly worse fear extinction recall compared to the CFC-only rats ( $t(53)=-2.73$ ,  $p=0.0085$ ). No other pair wise comparisons between groups revealed significant differences. The results of the SPS-Sham comparison to CFC-Sham show that our SPS model effectively induces impaired fear extinction recall. Additionally, these results show that both Cef and DHK eliminated the statistical significance of SPS-induced impairment of fear extinction recall demonstrated in the sham (saline) treated rats.. SPS = single prolonged stress; CFC = classical fear conditioning; Cef = ceftriaxone; DHK = dihydrokainate. Error bars represent standard errors.

### Change in Fear Expression During Extinction Session 1

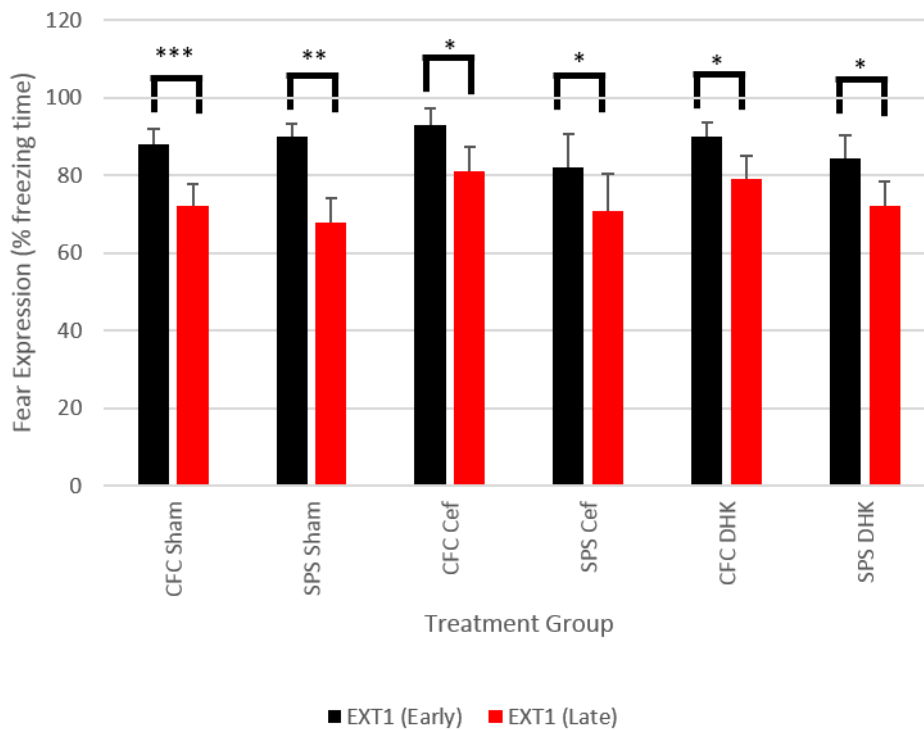


Figure 7. Depiction of fear expression during the first Extinction session, emphasizing the relationship between expression of fear during early and late extinction within each treatment group. To represent fear expression in early Extinction (EXT1(early)), the average % freezing time was calculated during the first three light presentations of the first Extinction session. To represent fear expression during late extinction (EXT1(late)), the average % freezing time was calculated during the last three presentations of the same session. Results of a two way ANOVA revealed that for each treatment group, the fear behavior of EXT1(early) was significantly higher than the fear behavior of EXT1(late) (\* $p < 0.05$ ; \*\* $p < 0.005$ ; \*\*\* $p < 0.0001$ ). This drop in fear expression over the course of the first Extinction session is consistent with the learned disassociation between the conditioned and unconditioned stimuli that is characteristic of fear extinction learning. As such, we concluded from these results that for each group, the parameters of the Extinction session adequately induced fear extinction learning. SPS = single prolonged stress; CFC = classical fear conditioning; Cef = ceftriaxone; DHK = dihydrokainate. Error bars represent standard errors.

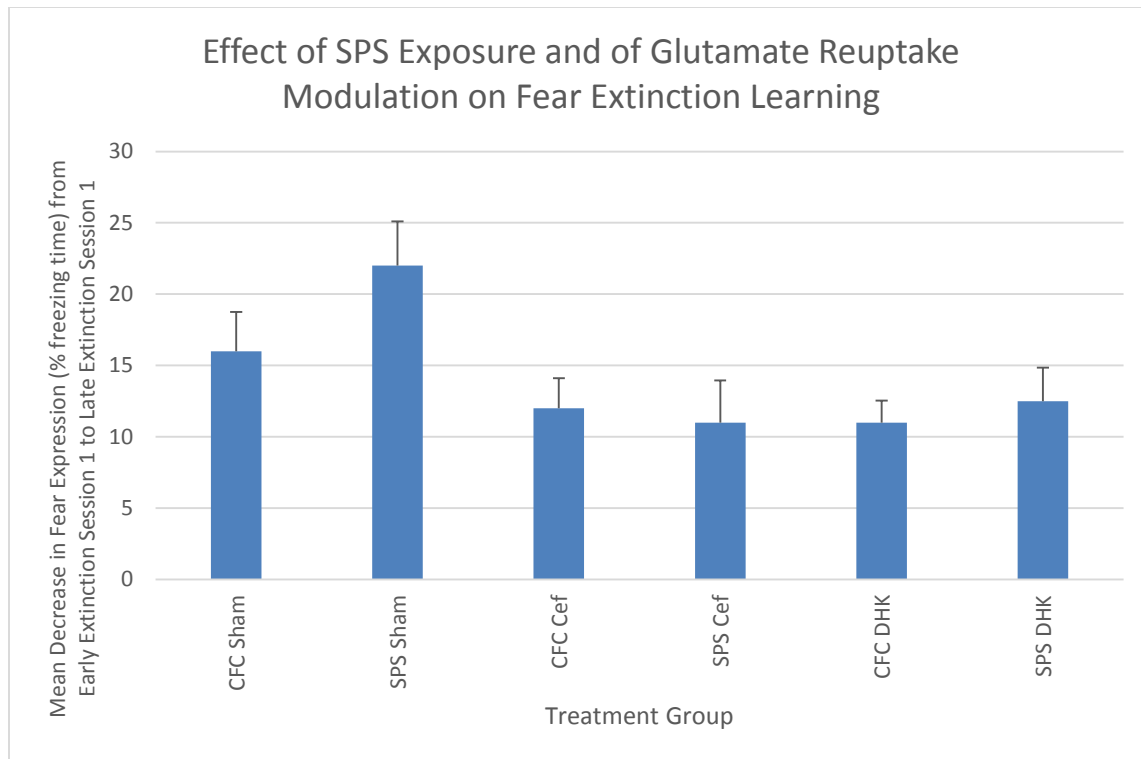


Figure 8. Depiction of the decrease in fear expression from the early part of the first Extinction session to the late part of the same session as a result of SPS exposure and drug treatment. For each individual rat within each treatment group, the percentage of time spent freezing during the last three Light-on exposures of the first Extinction session were averaged (EXT1(late)) and subtracted from the average percentage of time spent freezing during the first three Light-on presentations of the same session (EXT1(early)), resulting in an index representing the decrease in fear expression between the two (the Fear Extinction Learning Index). A nonparametric one-way procedure (Kruskal-Wallis test) revealed no significant differences between the Fear Extinction Learning Indices of any of the groups ( $p$ -value > 0.05). The fact that the indices were not significantly different suggests that the decrease in fear expression for each group was comparable over the course of the first Extinction session. This is consistent with relatively equivalent fear extinction learning, and so we can conclude that the rats in our study were not exhibiting alterations in this respect as a consequence of exposure to SPS or any of the drug treatments. SPS = single prolonged stress; CFC = classical fear conditioning; Cef = ceftriaxone; DHK = dihydrokainate. Error bars represent standard errors.

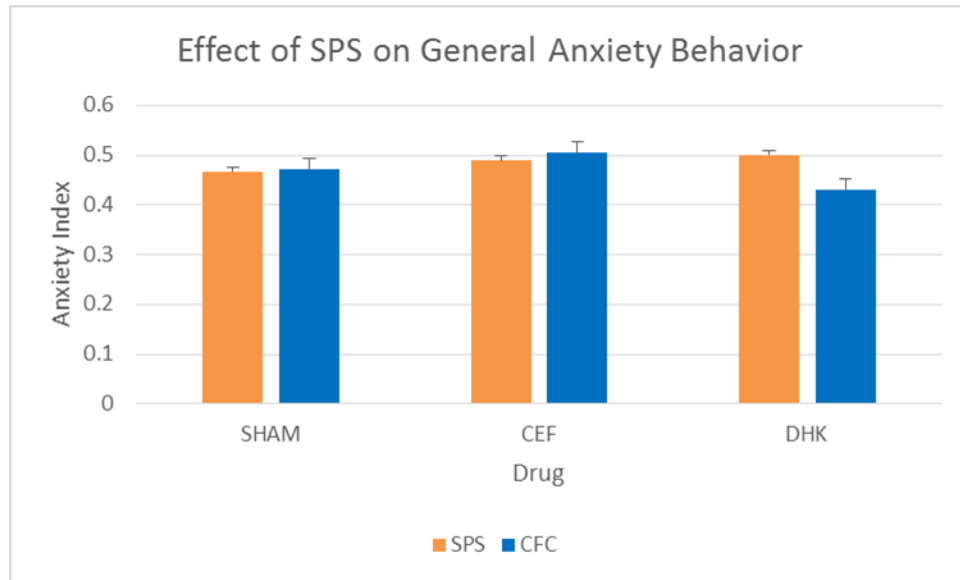


Figure 9. Depiction of the results from the elevated plus maze showing the effect of SPS on general anxiety behavior. A two-way ANOVA (behavior x drug) was performed and failed to reveal a significant interaction ( $F(2,54) = .994$ ,  $p = .377$ ) or main effect of either behavioral treatment ( $F(1,54) = .341$ ,  $p = .562$ ) or drug treatment ( $F(2,54) = .599$ ,  $p = .553$ ). Post-hoc pairwise comparisons showed that within each drug treatment group, SPS exposure did not significantly change the anxiety index. This indicates that the increase in freezing behavior following CFC, as well as the SPS-induced alteration in fear extinction recall (Figure 6), is not likely to be due to an increase in general anxiety behavior. SPS = single prolonged stress; CFC = classical fear conditioning; Cef = ceftriaxone; DHK = dihydrokainate. Error bars represent standard errors.

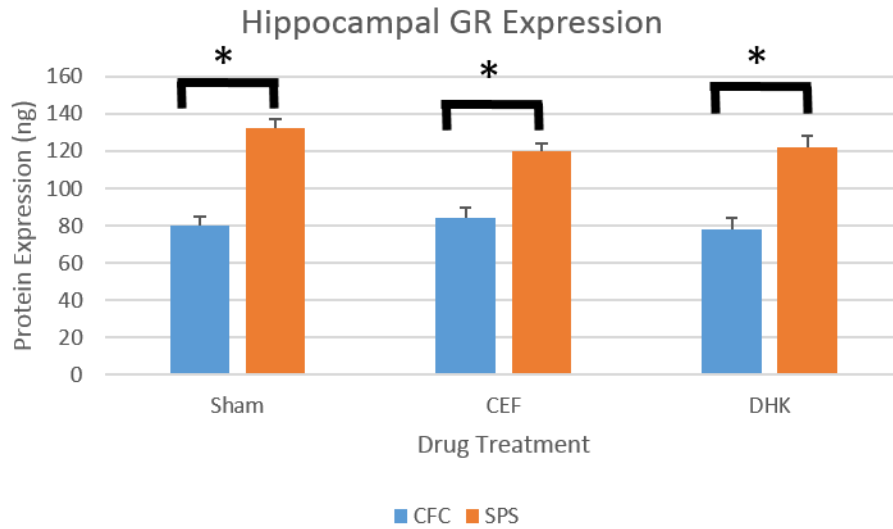


Figure 10. This figure shows GR expression in the hippocampus of each treatment group. Groups connected by brackets were significantly different ( $p < 0.0001$ ). These results demonstrate that hippocampal GR expression was significantly increased in SPS-exposed rats compared to rats not exposed to SPS, regardless of drug treatment. As such, we can conclude that our use of SPS successfully induced biochemical changes characteristic of PTSD, and that our model sufficiently models the disorder. SPS = single prolonged stress; CFC = classical fear conditioning; Cef = ceftriaxone; DHK = dihydrokainate. Error bars represent standard errors. “\*” represent  $p$ -values  $< 0.0001$ .

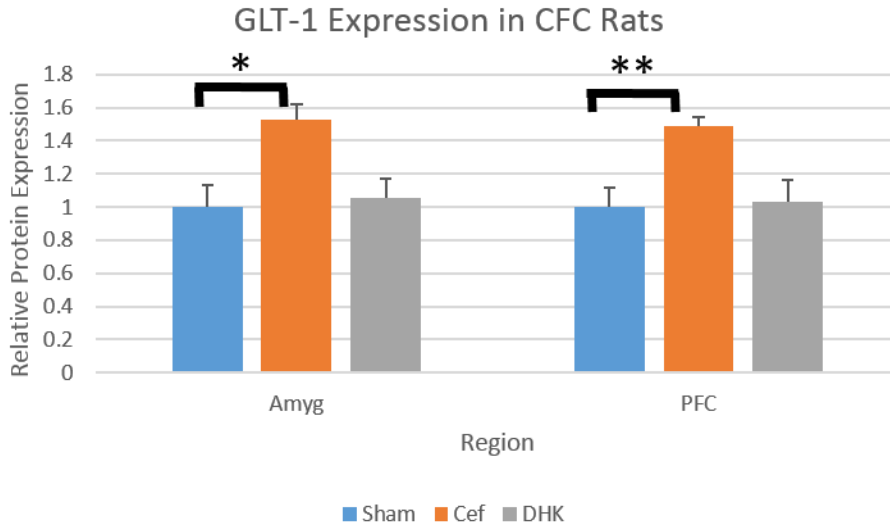


Figure 11. This figure shows GLT-1 expression in the PFC and amygdala of CFC rats only (i.e., rats that were not exposed to SPS) with the purpose of showing the effects of drug treatment on GLT-1 expression. Consistent with our expectations, a significant elevation in GLT-1 expression was found in both the PFC and amygdala of ceftriaxone-treated rats compared to sham-treated controls. Conversely, there was no significant difference found in either region between the DHK-treated group and the sham-treated group. . SPS = single prolonged stress; CFC = classical fear conditioning; Cef = ceftriaxone; DHK = dihydrokainate. Error bars represent standard errors. “\*” represent p-values=0.0006. “\*\*” represent p-values=0.0003.

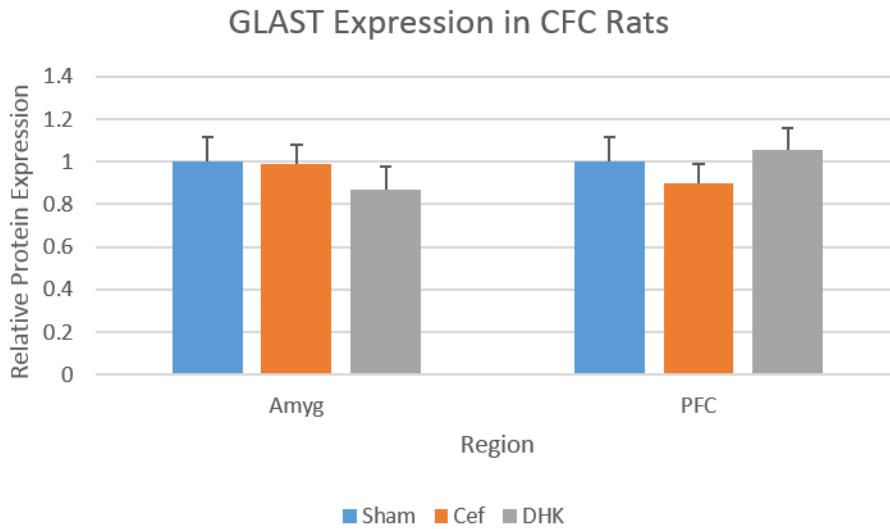


Figure 12. This figure shows GLAST expression in the PFC and amygdala of CFC rats only (i.e., rats that were not exposed to SPS) with the purpose of showing the effects of drug treatment on GLAST expression. Consistent with our expectations, no significant elevation in GLAST expression was found in either the PFC or amygdala of groups treated with ceftriaxone or DHK compared to sham-treated controls. SPS = single prolonged stress; CFC = classical fear conditioning; Cef = ceftriaxone; DHK = dihydrokainate. Error bars represent standard errors.

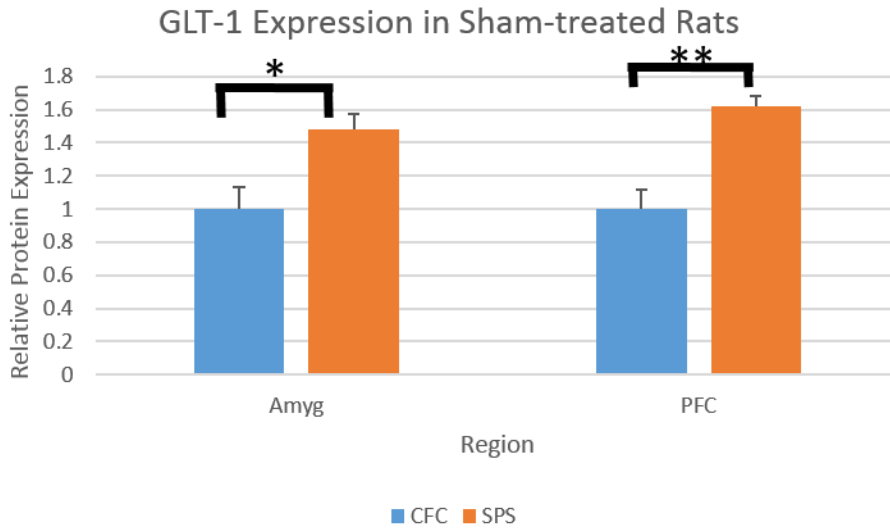


Figure 13. A subset of the results containing only the SPS-Sham and CFC-Sham groups with the purpose of showing how SPS effects GLT-1 expression. The results show that SPS does induce a significant change in GLT-1 expression in both the PFC and the amygdala compared to rats not exposed to SPS. However, contrary to our hypothesis, this SPS-induced change was an increase in expression rather than a decrease. SPS = single prolonged stress; CFC = classical fear conditioning alone; Cef = ceftriaxone; DHK = dihydrokainate. Error bars represent standard errors. “\*” represent p-values=0.0008. “\*\*” represent p-values<0.0001.

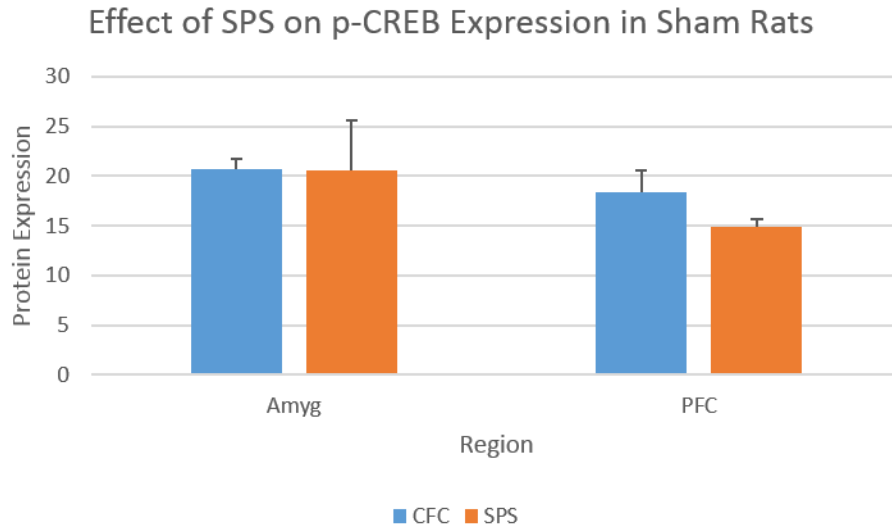


Figure 14. A subset of the results containing only the SPS-Sham and CFC-Sham groups with the purpose of showing how SPS affects p-CREB expression. The results show that SPS does not induce a significant change in p-CREB expression in either the PFC ( $p=0.0635$ ) or the amygdala ( $p=0.9428$ ) compared to rats not exposed to SPS. However, a numerically greater decrease in p-CREB expression was observed in the PFC compared to the amygdala. SPS = single prolonged stress; CFC = classical fear conditioning; Cef = ceftriaxone; DHK = dihydrokainate. Error bars represent standard errors.

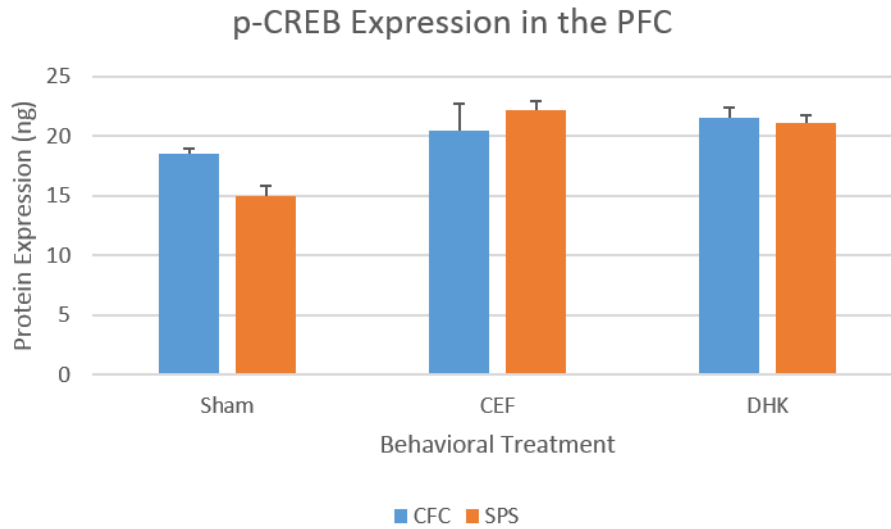


Figure 15. These data represent the results from the comparison of p-CREB expression in the PFC between CFC and SPS rats within each drug treatment group. The results show that no significant difference was found between the CFC and SPS groups treated with the Sham ( $p=0.0635$ ), Cef ( $p=0.3387$ ), or DHK ( $p=0.8436$ ). SPS = single prolonged stress; CFC = classical fear conditioning; Cef = ceftriaxone; DHK = dihydrokainate. Error bars represent standard errors.

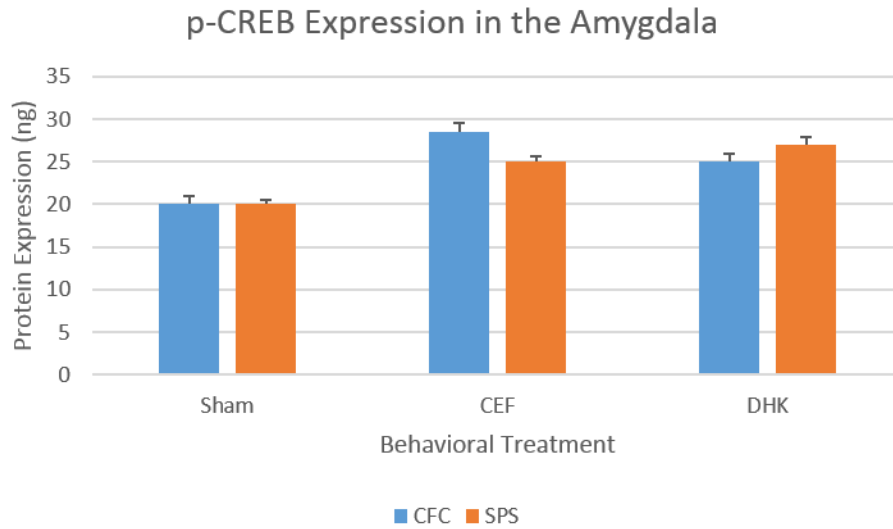


Figure 16. These data represent the results from the comparison of p-CREB expression in the amygdala between CFC and SPS rats within each drug treatment group. The results show that no significant difference was found between the CFC and SPS groups treated with the Sham ( $p=0.9428$ ), Cef ( $p=0.0854$ ), or DHK ( $p=0.3092$ ). SPS = single prolonged stress; CFC = classical fear conditioning; Cef = ceftriaxone; DHK = dihydrokainate. Error bars represent standard errors.

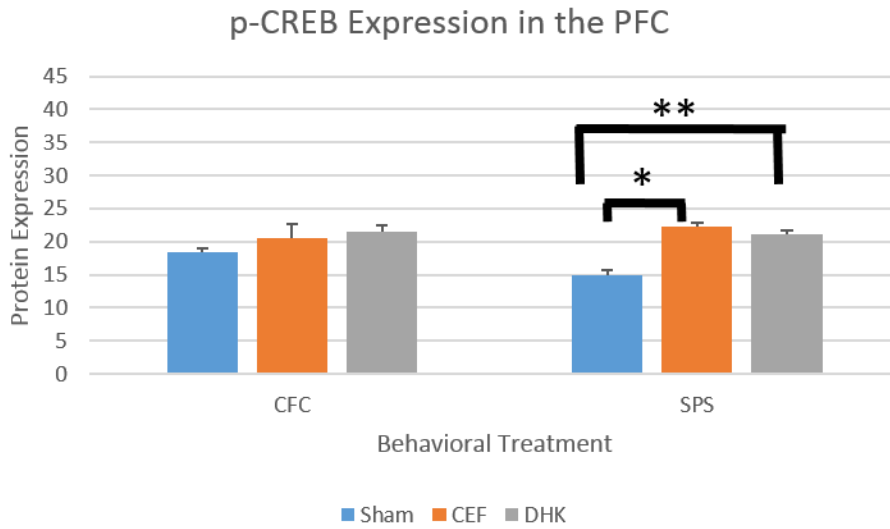


Figure 17. In the PFC of animals not exposed to SPS, there is no significant difference in p-CREB expression between the Sham-treated group and either the ceftriaxone- or DHK-treated groups. Conversely, in the PFC of SPS-exposed rats, both ceftriaxone and DHK administration resulted in a significant elevation of p-CREB expression relative to the Sham-treated group. These data show that the effects of the glutamate reuptake-modulating drugs upon the PFC appear to be dependent upon exposure to trauma. SPS = single prolonged stress; CFC = classical fear conditioning; Cef = ceftriaxone; DHK = dihydrokainate. Error bars represent standard errors. "\*" represent p-values=0.0005. "\*\*\*" represent p-values=0.0032.

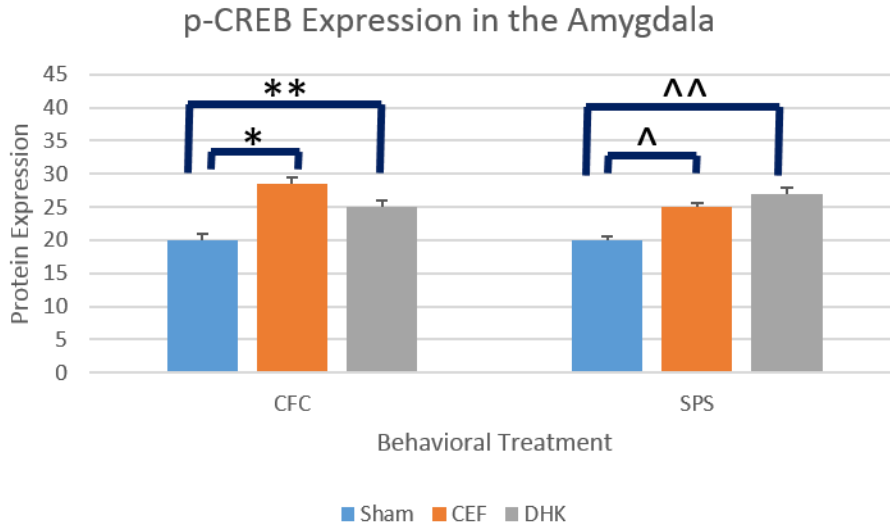


Figure 18. In the amygdala of animals, both exposed and not exposed to SPS, there is a significant difference between the Sham-treated group and both the ceftriaxone- and DHK-treated groups. These findings show that, regardless of trauma exposure, CEF and DHK both increase the level of p-CREB expression in the amygdala. SPS = single prolonged stress; CFC = classical fear conditioning; Cef = ceftriaxone; DHK = dihydrokainate. Error bars represent standard errors. “\*” represent p-values=0.0002. “\*\*” represent p-values=0.0366. “^” represent p-values=0.0118. “^^” represent p-values=0.0007.

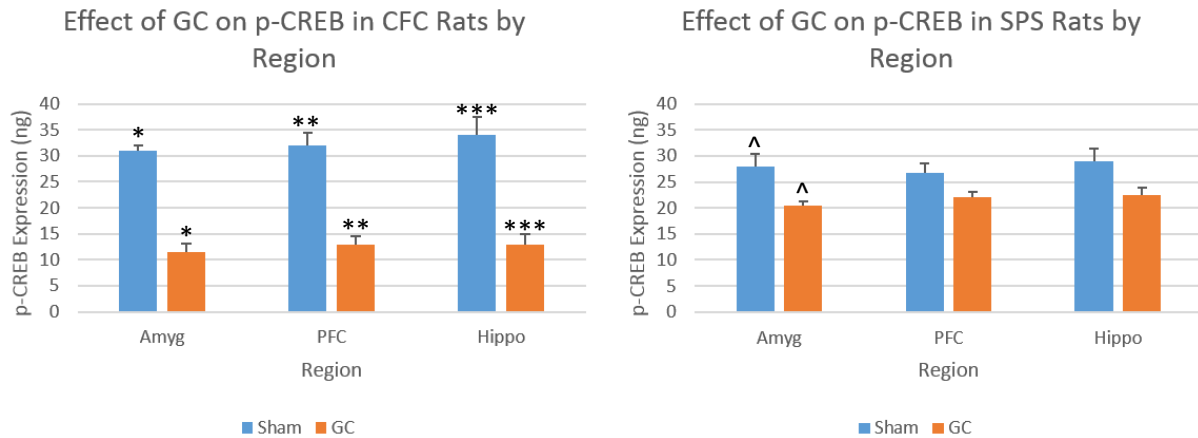


Figure 19. Subset of the results that focus on the effects of GC administration, compared to Sham administration, in the three regions of interest. On the left, GC administration results in significantly lower levels of p-CREB expression in all three regions of CFC rats. On the right, only the amygdala demonstrated a significant decrease in p-CREB expression following GC treatment in SPS rats, suggesting that SPS causes a change in susceptibility to GC-induced p-CREB inhibition in the PFC and hippocampus. SPS = single prolonged stress; CFC = classical fear conditioning only; GC = glucocorticoid. Error bars represent standard errors. Groups sharing the same symbols are significantly different from each other. “\*”, “\*\*\*”, and “\*\*\*\*” represent p-values less than 0.0001, and “^” represent a p-value of 0.028.

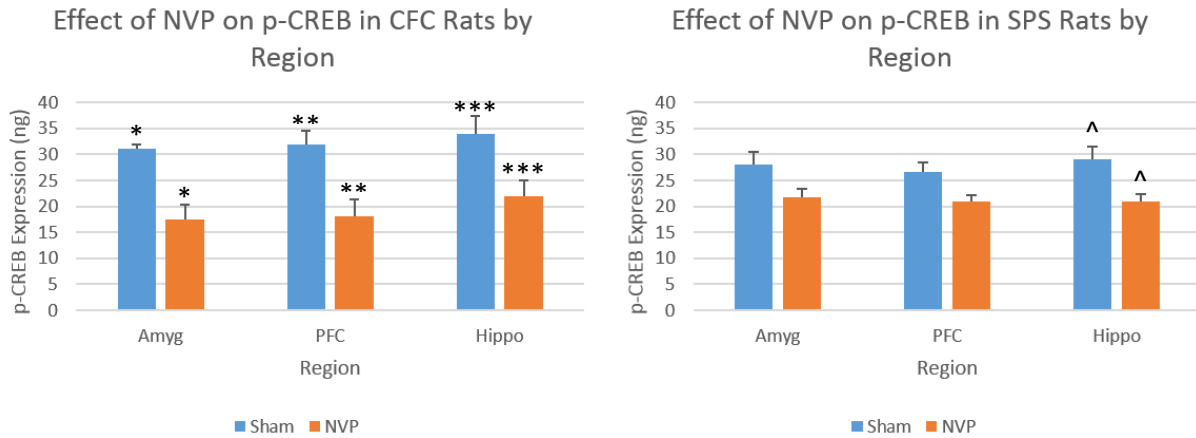


Figure 20. Subset of the results that focuses on the effects of NVP administration (NR2A inhibitor), compared to Sham administration, in the three regions of interest. On the left, NVP administration results in significantly lower levels of p-CREB expression in all three regions of CFC rats. On the right, only the hippocampus demonstrated a significant decrease in p-CREB expression following NVP treatment in SPS rats, suggesting that SPS causes a change in susceptibility to NVP-induced p-CREB inhibition in the PFC and amygdala. This is also suggestive of an SPS-induced change in the synaptic NR2A/NR2B subtype profiles among the three regions. SPS = single prolonged stress; CFC = classical fear conditioning only. Error bars represent standard errors. Groups sharing the same symbols are significantly different from each other. “\*”, “\*\*”, and “\*\*\*” represent p-values of <0.0001, 0.0003, and 0.0002, respectively, and “^” represent a p-value of 0.017.

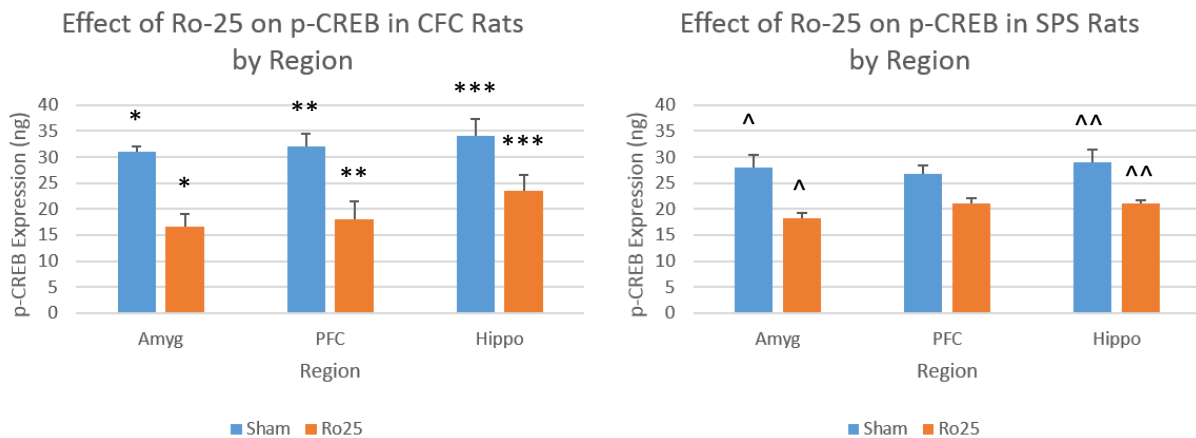
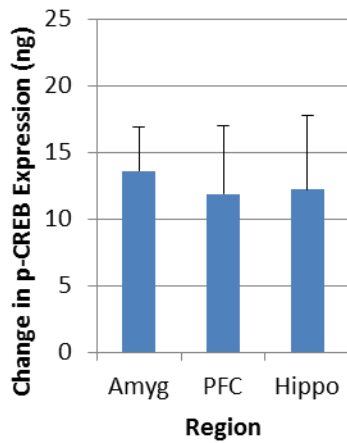


Figure 21. Subset of the results that focuses on the effects of Ro-25 administration (NR2B inhibitor), compared to Sham administration, in the three regions of interest. On the left, Ro-25 administration results in significantly lower levels of p-CREB expression in all three regions of CFC rats. On the right, the amygdala and hippocampus demonstrated a significant decrease in p-CREB expression following Ro-25 treatment in SPS rats, suggesting that SPS causes a change in susceptibility to Ro-25-induced p-CREB inhibition in the PFC. This is also suggestive of an SPS-induced change in the synaptic NR2A/NR2B subtype profiles among the three regions. SPS = single prolonged stress; CFC = classical fear conditioning only. Error bars represent standard errors. Groups sharing the same symbols are significantly different from each other. “\*”, “\*\*”, and “\*\*\*” represent p-values of <0.0001, <0.0001, and 0.0015, respectively, and “^” and “^^” represent p-values of 0.0034 and 0.0188, respectively.

### Magnitude of Change in p-CREB for NVP



### Magnitude of Change in p-CREB for Ro-25

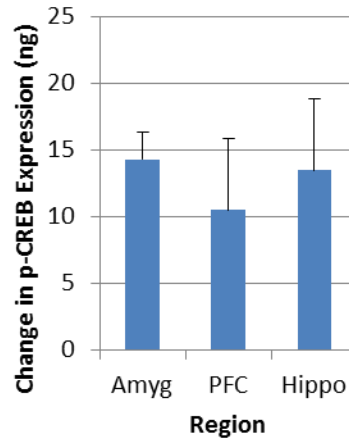


Figure 22. Depiction of the comparison between regions of CFC rats in the magnitudes of change in p-CREB expression induced by NVP (NR2A inhibitor) and Ro-25 (NR2B inhibitor). For each inhibitor, no significant differences were found between any of the regions. These findings suggest that synaptic transmission in these three regions have equivalent dependencies on NR2A and NR2B receptors, which is contrary to our expectation that there would be regional differences in these changes in magnitude. Amyg = amygdala; PFC = prefrontal cortex; Hippo = hippocampus. Error bars represent standard errors.

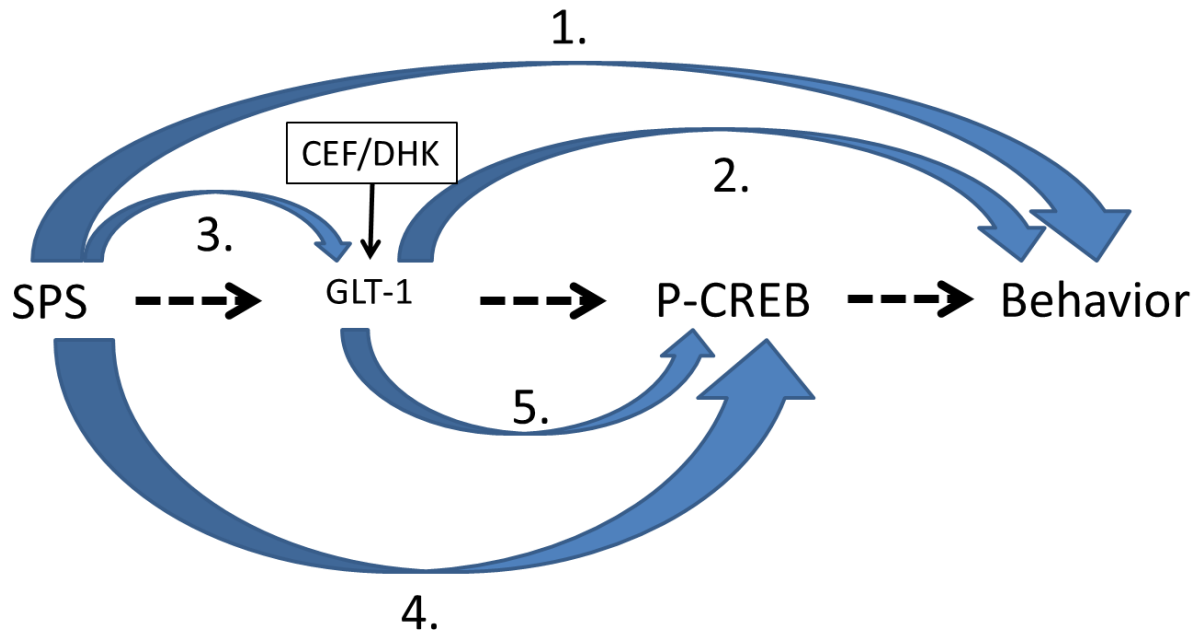


Figure 23. A visual representation of the proposed model representing the first of our two principle hypotheses. The dashed arrows represent the sequence of proposed interactions that potentially links situational trauma, glutamate transporter expression, p-CREB expression, and fear extinction memory recall (denoted as “Behavior”). The curved solid arrows represent assessments that were made to test the validity of the proposed sequence of interactions. These assessments are defined as: 1. Effects of SPS on fear extinction memory recall and GR expression, 2. Effect of GLT-1 modulation upon fear extinction memory recall, 3. Effects of SPS exposure upon GLT-1 expression, 4. Effect of SPS on p-CREB expression, and 5. Effects of GLT-1 on p-CREB expression. Abbreviations: CEF = ceftriaxone, DHK = dihydrokainate, SPS = single prolonged stress; GLT-1 = glutamate transporter 1; p-CREB = phosphorylated cAMP Response Element Binding protein.

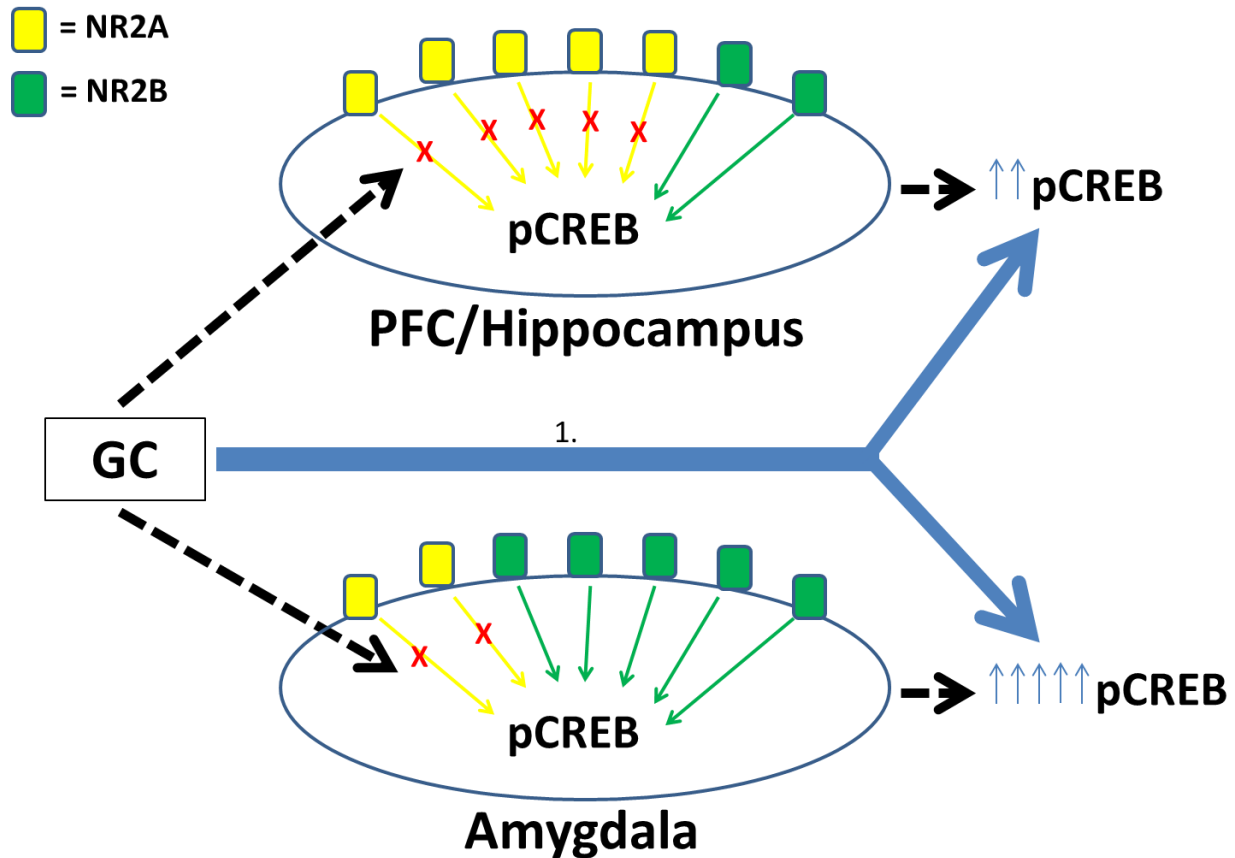


Figure 24. A visual representation of the effect of glucocorticoid (GC) administration on p-CREB expression, assuming regional differences in NMDA receptor subtype expression. This figure depicts the hippocampus and PFC expressing more synaptic NR2A receptors (yellow blocks) compared to NR2B receptors (green blocks). Under these configurations, administration of GCs would cause greater interference with synaptic stimulation by glutamate (represented by the red "X"s covering the arrows connecting the receptors to p-CREB activation) in the hippocampus and PFC compared to the amygdala due to its ability to selectively impair downstream molecular pathway that is associated with NR2A receptors. This would translate to less CREB phosphorylation following synaptic glutamate release in the hippocampus and PFC compared to the amygdala, and would consequently lead to a greater disruption of long-term fear extinction memory compared to long-term fear memory. The first of goal of Specific Aim 3, represented in the figure by the blue arrows labelled with a "1", was to assess whether glucocorticoids produce greater impairment of glutamate-activated CREB phosphorylation in a synaptic tissue preparation from the PFC compared with a similar preparation from the amygdala. GC = glucocorticoids, p-CREB = phosphorylated cAMP Response Element Binding protein.

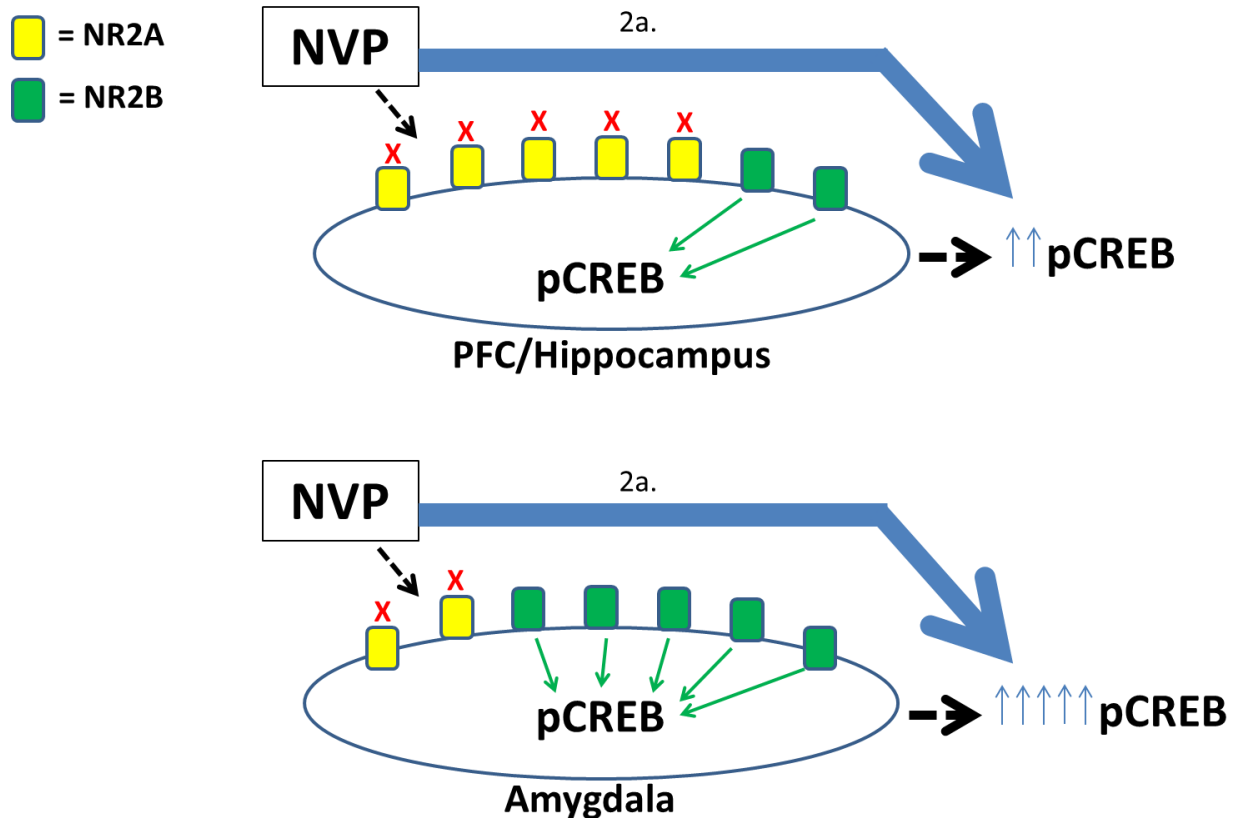


Figure 25A. A visual representation of the effect of NR2A selective inhibition by NVP-AAM077 (NVP) on p-CREB expression, assuming regional differences in NMDA receptor subtype expression. This figure depicts the hippocampus and PFC expressing more synaptic NR2A receptors (yellow blocks) compared to NR2B receptors (green blocks). Under these configurations, administration of NVP would block more synaptic receptors (represented by the red X's) in the hippocampus and PFC compared to the amygdala. This would translate to less CREB phosphorylation following synaptic glutamate release in the PFC and hippocampus compared to the amygdala, and consequently lead to a greater disruption of long-term fear extinction memory compared to long-term fear memory. This assessment (represented by the blue arrow labelled "2a") aims to determine whether glutamate-induced CREB phosphorylation in synaptic tissue samples from the amygdala responds differently to known NVP compared with similar tissue samples from the PFC or hippocampus. NVP = NVP-AAM077, p-CREB = phosphorylated cAMP Response Element Binding protein.

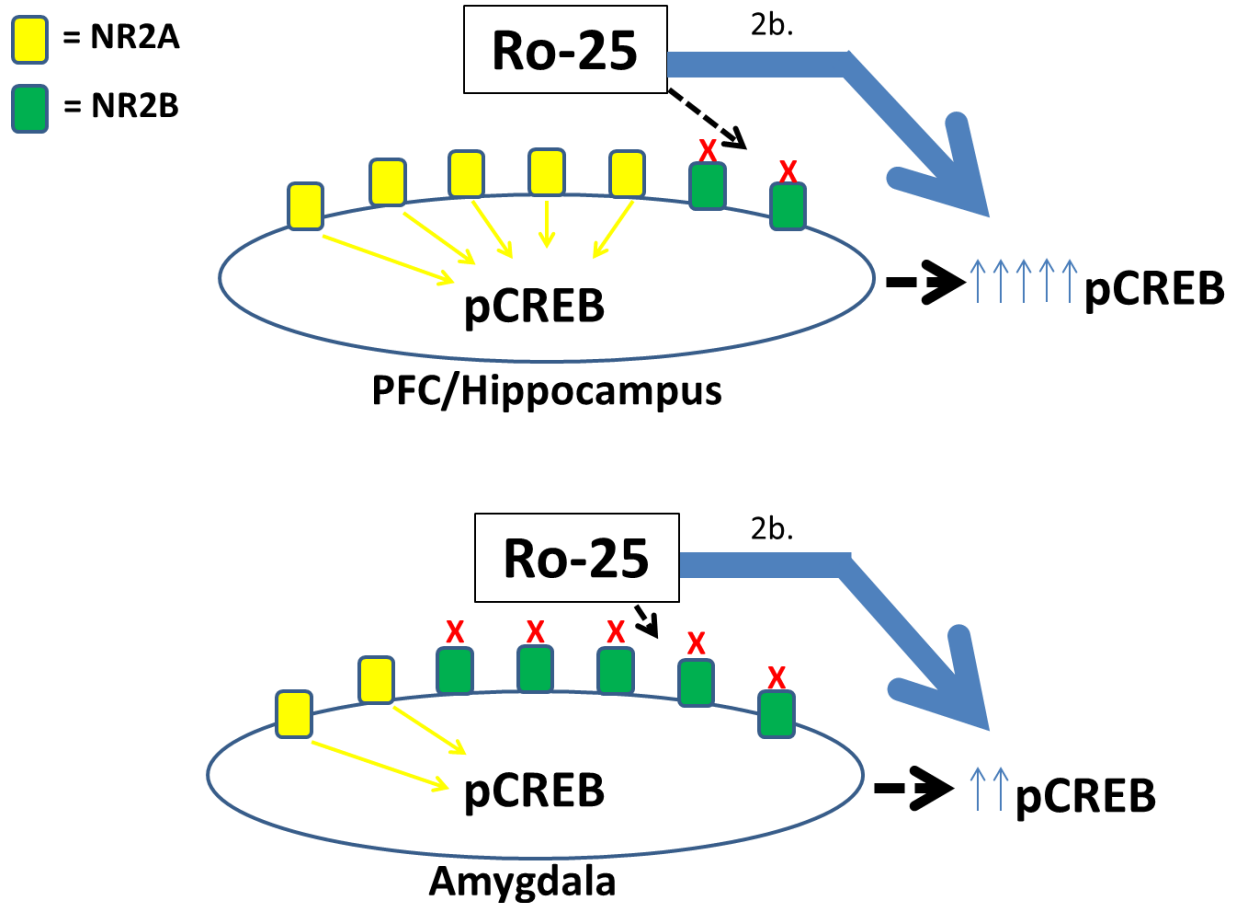


Figure 25B. A visual representation of the effect of NR2B selective inhibition by Ro-25-6981 (Ro-25) on p-CREB expression, assuming regional differences in NMDA receptor subtype expression. This figure depicts the hippocampus and PFC expressing more synaptic NR2A receptors (yellow blocks) compared to NR2B receptors (green blocks). Under these configurations, administration of Ro-25 would block more synaptic receptors (represented by the red X's) in the amygdala compared to the hippocampus and PFC. This would translate to less CREB phosphorylation following synaptic glutamate release in the amygdala compared to the hippocampus and PFC, and consequently lead to a greater disruption of long-term fear memory compared to long-term fear extinction memory. This assessment (represented by the blue arrow labelled "2b") aims to determine whether glutamate-induced CREB phosphorylation in synaptic tissue samples from the amygdala responds differently to known Ro-25 compared with similar tissue samples from the PFC or hippocampus. Ro-25 = Ro-25-6981, p-CREB = phosphorylated cAMP Response Element Binding protein.

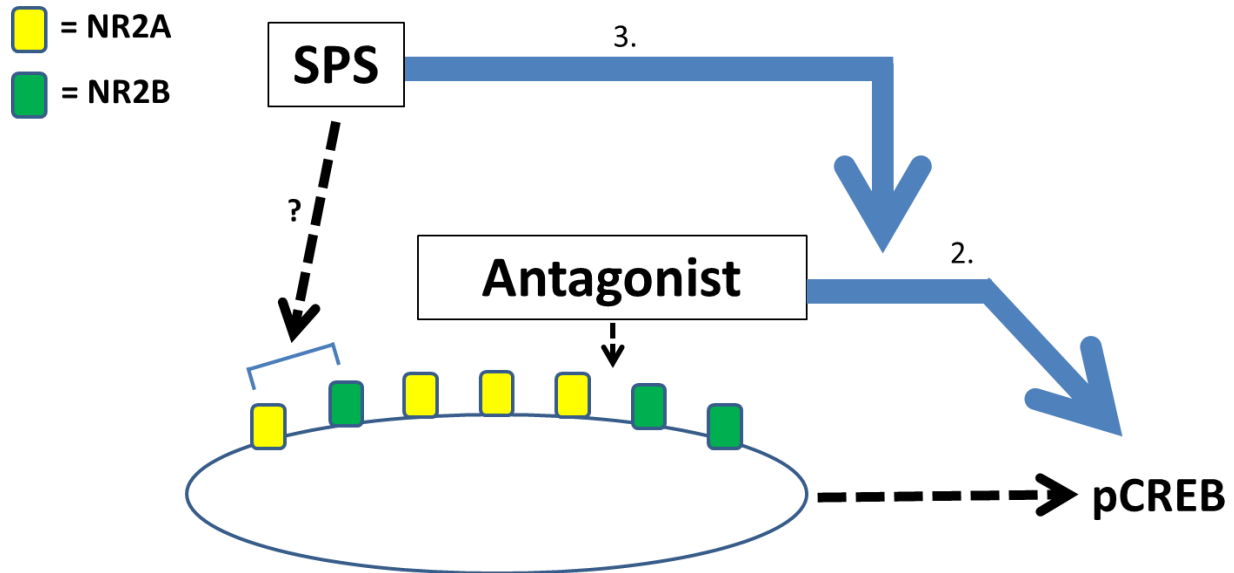


Figure 26. A visual representation of the effect of SPS on p-CREB expression, assuming regional differences in NMDA receptor subtype expression. This figure depicts synaptic NR2A receptors (yellow blocks) and NR2B receptors (green blocks) in any one of the three brain regions analyzed in this study. We predict that SPS will not differentially affect the expression of synaptic NMDA receptor profiles (represented by the dashed black arrow), and will thus not change the outcome of receptor antagonist administration. The third assessment of Specific Aim 3 (represented by the blue arrow labelled with a “3”) aims to determine whether SPS exposure changed the effect of the known NR2A and NR2B antagonists on glutamate-induced CREB phosphorylation in the PFC, hippocampus and amygdala. Ro-25 = Ro-25-6981, p-CREB = phosphorylated cAMP Response Element Binding protein.

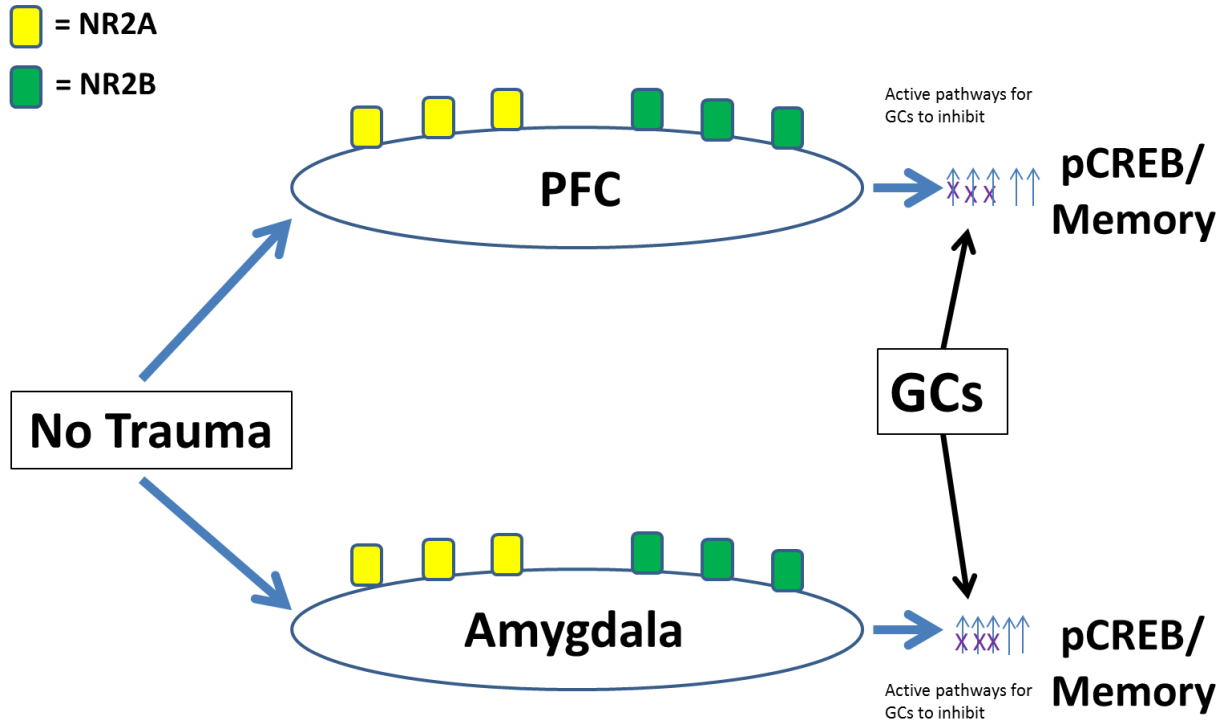


Figure 27. A visual representation of our interpretation, based on our data, of similar synaptic NMDA receptor subtype profiles in the amygdala and PFC, and the effect of GCs on p-CREB production in these regions. Here, GCs have significantly and equivalently inhibited (purple arrows) downstream p-CREB production resulting from activation of all synaptic NMDA receptor subtypes by bicuculline. Small purple arrows represent GC inhibition of p-CREB-producing pathways. GC = glucocorticoids, p-CREB = phosphorylated cAMP Response Element Binding protein.

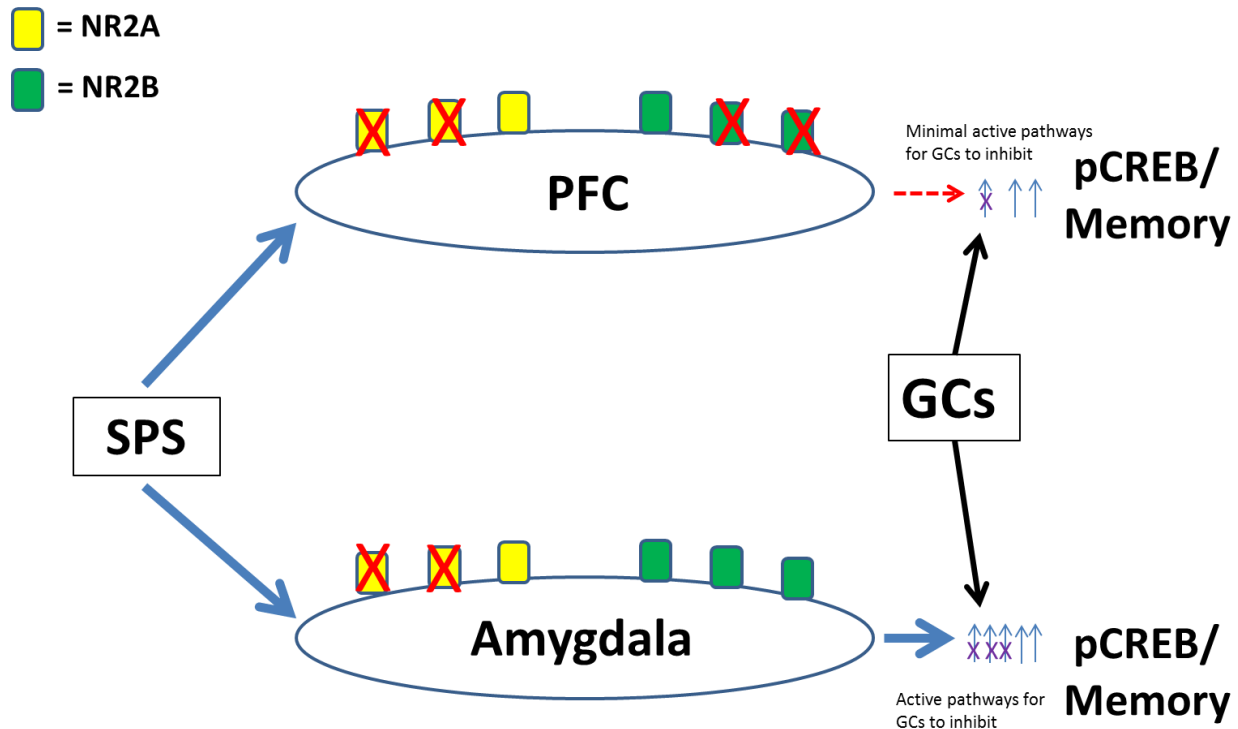


Figure 28. A visual representation of our interpretation, based on our data, of the effect of SPS on NMDA receptor expression and the resulting effect of GCs on p-CREB expression. Our results suggest that SPS could be decreasing the synaptic expression of both NR2A and NR2B (indicated by the red "X") in the PFC, but not NR2B in the amygdala. Upon synaptic activation of NMDA receptors by bicuculline, this may leave very little downstream p-CREB-producing pathway activity to be significantly affected by GCs in the PFC, while there is still a substantial amount of downstream p-CREB-producing pathway activity to be reduced by GCs in the amygdala. Small purple arrows represent GC inhibition of p-CREB-producing pathways. GC = glucocorticoids, p-CREB = phosphorylated cAMP Response Element Binding protein.

## ***APPENDIX C: Glossary***

Extinction: the learning process associated with the formation of extinction memories and the subsequent suppression of associated fear memories.

Extinction Memory: the specific neuronal networks and circuits that are strengthened following Extinction Training that when activated result in the suppression of a fear memory.

Extinction Memory Recall: the retention of long-term fear extinction memories, and the subsequent ability to suppress associated fear memories. For our purposes, loss of retention of extinction memories within 24 hours of extinction training is considered impaired extinction memory recall.

Extinction Training: any procedure or paradigm that results in the suppression of a fear memory.

Extinguished Fear: a fear that is prevented from being expressed following successful fear extinction training. These fears are not permanently lost; rather they are suppressed by the new memories formed during the extinction process.

Extrasynaptic: locations on a neuronal membrane that exclude membrane within synapses. For our purposes here, the term is used to specify the extrasynaptic membrane of dendritic spines, and not extrasynaptic locations elsewhere on the cell.

Fear: a complex physical and mental state that is usually triggered by an animal's perception of endangerment to itself or to something with which it holds a high level of investment. It is characterized by cognitive arousal, negative emotional sensation, and various autonomic

physiological responses. Additionally, stereotypical behavioral responses are commonly exhibited. These behaviors are usually conserved among members of a given species, but are not always conserved across species.

Fear Behavior: a somatic or autonomic reaction of an animal to a fear-inducing stimulus. These are often stereotypical behaviors that are conserved among members of a given species.

Fear Memory: the neuronal networks and circuits that are strengthened following an aversive or noxious experience, and initiate a fear response when activated.

Learning: the process through which a new memory is formed.

Learning Mechanisms: the cellular and/or molecular events that occur within and among neurons that lead to the formation of memory.

Memory: an experience-induced alteration of the existence or the strength of neuronal circuits and/or networks that typically allow for an animal to better adapt to and interact with its environment. These networks can encode any combination of emotional, sensory, episodic, procedural, or reflexive information. Though most of these alterations are to the benefit of the animal, some pathological conditions may result in the formation of maladaptive behaviors.

# CHAPTER VIII

## *REFERENCE LIST*

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