Selective Antagonism of $mGluR_5$ Alters Sleep-Wake and Quantitative EEG and Ameliorates Behavioral Abnormalities in a Rodent Model of Traumatic Stress

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LIST OF ABBREVIATIONS

5-HIAA	5-hydroxyindoleacetic acid
5-HT	5-hydroxytryptamine (aka serotonin)
AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
ANOVA	Analysis of variance
BL	Baseline
BLA	Basolateral amygdala
BNST	Bed nucleus of the stria terminalis
CeA	Central amygdala
CF	Conditioned fear
CFC	Contextual fear conditioning
CSF	Cerebrospinal fluid
CT	Comparative cycle threshold
CTA	Conditioned taste aversion
DNA	Deoxyribonucleic acid
EEG	Electroencephalography
EGR-1	Early growth response protein 1
EMG	Electromyography
ERK	Extracellular receptor kinase
FKBP5	FK506-binding protein 51
fMRI	Functional magnetic resonance imaging
FPS	Fear-potentiated startle
GABA	γ-aminobutyric acid
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GPCR	G protein-coupled receptor
HEK	Human embryonic kidney

HPA	Hypothalamic-pituitary-adrenal axis
HPLC-ECD	High-performance liquid chromatography with electrochemical detection
i.p.	Intraperitoneal
IA	Inhibitory avoidance
IC_{50}	Fifty percent inhibitory concentration
iGluR	Ionotropic glutamate receptor
IL	Infralimbic region of the prefrontal cortex
IP3	Inositol 1,4,5-trisphosphate
kg	Kilogram
LTD	Long term depression
LTP	Long term potentiation
MAPK	Mitogen-activated protein kinase
mg	Milligram
mGluR	Metabotropic glutamate receptor
min	Minutes
MPEP	2-methyl-6-(phenylethynyl)pyridine
mRNA	Messenger ribonucleic acid
mTOR	Mammalian target of rapamycin
NAM	Negative allosteric modulator
ng	Nanogram
NMDA	*N*-Methyl-*D*-aspartate
No.	Number
NPY	Neuropeptide Y
NREM	Non-rapid eye-movement
PAM	Positive allosteric modulator
PET	Positron emission tomography
PFC	Prefrontal cortex

PI3K	Phosphatidyl inositol 3-kinase
PKC	Protein kinase C
PL	Prelimbic region of the prefrontal cortex
PTSD	Post-traumatic stress disorder
qEEG	Quantitative electroencephalography
qRT-PCR	Quantitative real-time polymerase chain reaction
REM	Rapid eye-movement
RIA	Radioimmunoassay
rpm	Rotations per minute
sec	Seconds
SPS	Single prolonged stress
SSRI	Selective serotonin reuptake inhibitor
SWA	Slow wave activity
Temp	Temperature
Veh	Vehicle
VU0409106	3-fluoro-N-(4-methylthiazol-2-yl)-5-(pyrimidin-5-yloxy)benzamide
Y1	Neuropeptide Y receptor subtype 1
Y2	Neuropeptide Y receptor subtype 2

CHAPTER 1

INTRODUCTION

Post-traumatic stress disorder

Clinical features and current therapies

Post-traumatic stress disorder (PTSD) is a psychiatric condition that manifests in a subset of individuals after experiencing a traumatic event(s) (1). PTSD symptoms are categorized into the following four diagnostic criteria: intrusion, including flashbacks and nightmares; avoidance; negative alterations in cognition and mood; and alterations in arousal, such as hypervigilance and poor sleep (1-3). While the lifetime prevalence of PTSD in the general population lies on the order of 6.8% (4, 5) estimates reach as high as 30.9% among subpopulations at particular risk for trauma such as war veterans (6). PTSD symptoms usually begin within one to six months after the trauma, but can last for decades and are associated with significant increases in health care use and costs (7, 8). Current treatments include both psychotherapy, such as exposure therapy and cognitive behavioral therapy (9), and medication (10-12). The selective serotonin reuptake inhibitors (SSRIs) sertraline (Zoloft) and paroxetine (Paxil) represent the front-line pharmacological treatment for PTSD patients, but these drugs are only partially effective and rarely result in full remission of symptoms (10). Sleep disturbances and nightmares are particularly resistant to treatment with currently available therapies, and constitute some of the most prevalent and disruptive symptoms in PTSD patients (2, 11). Thus, there is a critical need to develop novel therapeutic approaches for better management, remission, or prevention of PTSD.

Role of emotional memory

Collectively, PTSD symptoms represent a persistent, inextinguishable stress response initiated by the traumatic event. From an evolutionary perspective, this response can be an adaptive means of enhancing survival when confronted with threatening or fearful stimuli (13, 14). By augmenting associative memory for contextual cues surrounding the threat, activation of the stress response can also compel avoidance or vigilance behaviors that prevent future encounters with the same danger (13, 14). Indeed, multiple studies have demonstrated that memories with an affective component are more vivid and durable than other memories (15). This response becomes maladaptive when the dangers associated with the traumatic event no longer pose a threat. Enhanced implicit and explicit memory for the traumatic event, as well as impaired extinction of this memory and generalization of associated cues, then contribute to the maintenance of chronic symptoms, leading to PTSD (14, 16-20). Thus, PTSD can be conceptualized as a disorder of learning and memory, the proximal cause of which is the acquisition and consolidation of traumatic memory and related negative emotions (14, 16-20).

Mounting empirical evidence demonstrates PTSD-related disruptions in multiple domains of emotional memory that likely contribute to susceptibility for the disorder or the maintenance of symptoms (21-28). These deficits in PTSD patients are linked to structural and functional alterations in various subregions of the prefrontal cortex (PFC), hippocampus, and amygdala, brain areas known to be important for both memory formation and the regulation of emotion (29, 30). Specifically, PTSD symptoms are associated with altered size and function of the hippocampus, impaired activation of the PFC, and a corresponding disinhibition of amygdala activity (29, 30). This pathophysiology may contribute to PTSD symptom development by promoting the formation and maintenance of negative emotional memory during and

immediately after the trauma, or by impairing the successful extinction of these memories once formed (21-25, 31).

Novel treatment strategies

A novel strategy for the treatment of PTSD, therefore, is to modulate emotional memory formation and maintenance in order to attenuate explicit or implicit recall for the traumatic event (14, 16-18, 31, 32). Among neuropsychiatric disorders, PTSD is uniquely well suited to this approach because its diagnosis is contingent upon a requisite environmental component, the traumatic event, which signals the onset of symptoms (1, 13). Pharmacological interventions that promote or inhibit emotional memory formation and maintenance are hypothesized to have therapeutic potential at different points in the timeline of PTSD symptom progression. Based on the learning hypothesis of PTSD, these strategies can be broadly classified into four groups: 1) compounds that inhibit the acquisition or consolidation of emotional memory formation during or immediately after trauma, 2) compounds that inhibit the reconsolidation of emotional memory during subsequent recall of events related to the trauma, 3) compounds that facilitate the extinction of emotional memory associated with the trauma, and 4) compounds that attenuate the general expression of anxious behaviors (Figure 1).

Recent successes with both pharmacotherapeutic and psychotherapeutic interventions have provided clinical proof of concept validation for some of these approaches (14, 18, 31, 32), but the limited availability of pharmacological agents that potently and selectively target molecular mediators of emotional learning and memory represents a hurdle to the clinical advancement of new treatments. Furthermore, the initial discovery and development of pharmacological agents that manipulate emotional memory is performed exclusively in the

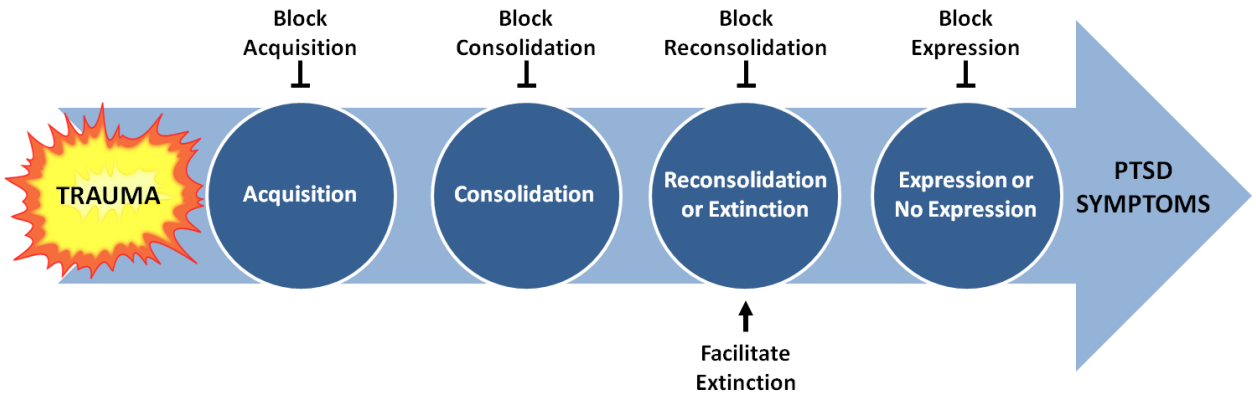

Figure 1. Opportunities for intervention in symptom progression after trauma. These strategies can be broadly classified into four groups: 1) compounds that inhibit the acquisition or consolidation of emotional memory formation during or immediately after trauma, 2) compounds that inhibit the reconsolidation of emotional memory during subsequent recall of events related to the trauma, 3) compounds that facilitate the extinction of emotional memory associated with the trauma, and 4) compounds that attenuate the general expression of anxious behaviors.

preclinical arena, highlighting the need for an animal model of traumatic stress with robust predictive, face, and construct validity.

Rodent behavioral assays of emotional memory

Traditionally, researchers have employed rodent models of aversive associative conditioning, such as auditory cued and contextual conditioned fear (CF), fear-potentiated startle (FPS), inhibitory avoidance (IA), and conditioned taste aversion (CTA), to probe the mechanisms underpinning emotional memory formation and maintenance. Multiple studies employing these and other paradigms have revealed that encoding of emotionally valenced information occurs in distinct phases. Initial acquisition of the memory is followed by consolidation; subsequent recall of the memory can lead to either reconsolidation or extinction depending on the duration of and context surrounding the recall event (33). Each of these steps is

mediated by distinct molecular mechanisms which take place within and between various subregions of the thalamus, PFC, hippocampus, and amygdala (18, 33, 34).

In general, the successful acquisition, consolidation, and expression of threat-based memory requires convergent excitatory signaling in the amygdala which is contextualized by hippocampal input, and gated by PFC afferents to inhibitory interneurons (Figure 2). The induction of long term potentiation (LTP) at thalamic and sensory cortical synapses in the basolateral amygdala (BLA) represents a well-established neurophysiological correlate of emotional memory acquisition (18, 33, 35). This initial excitation of primary BLA neurons during acquisition is followed by the induction of several mechanisms promoting the consolidation of emotional memory, which include the activation of intracellular signaling pathways such as extracellular receptor kinase 1/2 (ERK 1/2) phosphorylation, culminating in transcriptional activation and new protein synthesis (33). For example, activation of early growth response protein 1 (EGR-1) in the amygdala is known to be required for the formation of new threat-based memories (33). EGR-1 is an immediate early gene and transcription factor that lies downstream of ERK1/2 activation, and induces the rapid expression of a host of genes involved in synaptic remodeling (33). Specifically, the protein products of these genes are involved in the maintenance of amygdala LTP and morphological changes such as increased dendritic spine size and density (33, 36, 37). Reactivation of emotional memories through passive recall returns these synapses to a labile state during which reconsolidation or extinction can take place (37-39). The cellular mechanisms that subserve reconsolidation have yet to be fully delineated, but early work suggests that this process shares some of the same molecular mediators with initial consolidation, although reconsolidation may be mediated by post-synaptic rather than pre-synaptic modifications (37-39). Repeated recall of an emotional memory without further reinforcement of

the initial association leads to extinction (37, 39). Extinction is partially mediated by activation of the infralimbic region of the PFC (IL) which attenuates amygdala output through excitation of inhibitory intercalated cells (ITC) and through long term depression (LTD) or depotentation of thalamic and cortical synapses in the BLA that had previously undergone LTP (40-45). Finally, the expression of threat responses related to the emotional memory requires BLA activation of output neurons in the central amygdala (CeA) and the bed nucleus of the stria terminalis (BNST), which is facilitated by persistent activity in the prelimbic region of the PFC (PL) (46-50). Throughout all of these processes, hippocampal activation and long-term plasticity provides context specificity (51).

It is important to note that the neural circuitry involved in emotional memory encoding corresponds to the same brain regions found in functional neuroimaging studies to be altered in PTSD patients (52). Reduced hippocampal volume is a well-established finding in PTSD patients that likely results in poor contextualization of emotional memory, a symptom of PTSD patients that leads to inappropriate stress reactions (52). Reduced activation of the ventromedial PFC, a brain region analogous to the IL in rodents, has also been reported multiple times in PTSD patients, and is associated with enhanced expression and poor extinction of threat-based memories (52). Impaired PFC activation also correlates with disinhibition of the amygdala in PTSD patients, resulting in hyperactivity of this structure and associated behavioral outcomes that include hyperarousal symptoms and sleep disturbances (52).

Using the rodent behavioral assays mentioned above to manipulate the function of these circuits, multiple compounds that target various neurotransmitter systems as well as broad cellular functions such as gene transcription and protein synthesis have been discovered that affect the acquisition, consolidation, reconsolidation, extinction, and/or expression of learned

threat behavior (53). However, there are two important caveats to the interpretation of experimental results gleaned from pharmacological manipulation of the rodent response to aversive associative conditioning. First, it is not known whether the acquisition, consolidation, and recall of a rodent's memory for threatening stimuli fully encompass the complexity of emotional memory formation and maintenance in humans (54). Second, these aversive learning paradigms likely represent normal associative learning after a stressful event, not necessarily the pathological overconsolidation of emotional memory that occurs in PTSD patients who have experienced traumatic stress. While the molecular mechanisms that subserve normal threat learning are likely involved in pathological processes as well, and thus provide valuable insight, the physiological and behavioral consequences of trauma are different from those of mild stress in both rodents and humans. It will be critical, therefore, to test novel pharmacological interventions in rodent models that employ more severe stressors akin to the trauma experienced by an individual who goes on to develop PTSD.

Rodent models of traumatic stress

Putative rodent models of traumatic stress include underwater trauma (55), predator scent stress (56), social conflict (57), learned helplessness (58), and Single Prolonged Stress (SPS) (59). Each of these models recapitulates some of the physiological and behavioral alterations found in PTSD patients, and offers new insight into the pathophysiology of the disorder. Each model has also been used to test novel pharmacological interventions that could prevent or ameliorate these changes (55-59).

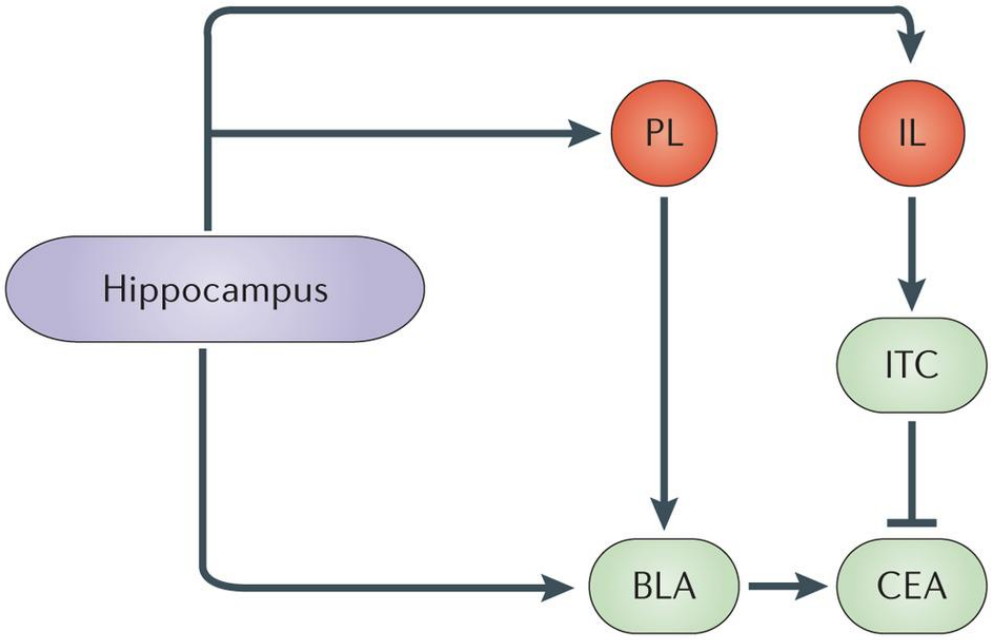

Figure 2. Neural threat circuitry. Adapted from (51). Acquisition, consolidation, and expression of threat-based memory requires convergent excitatory signaling in the amygdala which is contextualized by hippocampal input, and gated by PFC afferents to inhibitory interneurons. PL: prelimbic cortex; IL: infralimbic cortex; BLA: basolateral amygdala; CeA: central amygdala; ITC: intercalated inhibitory cells.

Of these models, SPS exhibits strong face and construct validity (Table 1). SPS is induced by a single presentation of three classically used rodent stressors: two hours of restraint, followed by twenty minutes of forced swim and then exposure to ether inhalation until anesthesia (60). SPS achieves a level of traumatic stress that surmounts the stress that would be induced by each individual stressor alone. In this way, SPS represents a traumatic experience for rodents consisting of an acute physical and psychological insult culminating in simulated death through loss of consciousness. SPS treatment mounts a dramatic stress response in rodents as measured by increases in circulating corticosterone, the rodent analogue of the human stress hormone, cortisol (60). Similar to the pathophysiology of PTSD patients, rats and mice which have undergone SPS subsequently display enhanced negative feedback of the hypothalamic-pituitary-

adrenal (HPA) axis (60), cell death in the hippocampus (61-64), and reduced excitatory tone in the PFC (65). SPS rats also demonstrate increased hippocampal expression of FK506-binding protein 51 (FKBP5), an early stress-responsive gene that acts as a co-chaperone of the glucocorticoid receptor complex (66). Importantly, genetic variability and altered expression of FKBP5 has been associated with PTSD risk, diagnosis, and treatment (67-69).

At the level of behavior, rodents exposed to SPS show increased startle reactivity (70-75), augmented threat responding in aversive associative conditioning assays (70-75), and impaired extinction of threat-related behaviors (76-81), consistent with disruption of normal emotional memory formation and maintenance. The behavioral alterations induced by SPS in rats have been found to be sensitive to SSRI treatment (82), suggesting that this model may also exhibit predictive validity. Recently, it was also found that SPS results in increased cerebrospinal fluid (CSF) levels of the excitatory neurotransmitter, glutamate, caused in part by reduced hippocampal expression of the glutamate transporters GLAST and GLT-1 (83). Together, these findings indicate combined dysfunction of the serotonergic and glutamatergic neurotransmitter systems that could conspire to induce short and long-term PTSD-like behavioral alterations spurred by the persistent expression of traumatic memory. The SPS model, therefore, may be a viable means of discovering novel interventions to prevent or treat PTSD by modulating emotional memory formation and maintenance before, during, or after trauma.

Table 1. Summary of effects of SPS

Effect of SPS	Reference
Increased expression of norepinephrine synthesizing enzyme tyrosine hydroxylase (TH) in locus coeruleus (LC)	Sabban EL, et al. (2015)
Increased CF expression and anxiety-like behavior in open field and EPM	Qiu ZK, et al. (2015)
Altered cocaine intake and reduced dopamine content in the striatum	Enman NM, et al. (2015)
Increased CSF glutamate and decreased glutamate transporter GLAST/GLT-1 expression; increased CF expression and anxiety-like behavior in open field	Feng D, et al. (2015)
Increased neuronal apoptosis in PFC	Li X, et al. (2015)
Increased activity-dependent metabolites in amygdala	Han F, et al. (2015)
Increased CF expression and impaired extinction	Keller SM, et al. (2015)
Impaired reversal learning	George SA, et al. (2015)
Increased glucocorticoid receptor (GR) levels in hippocampus and impaired CF extinction	Keller SM, et al. (2015)
Increased cocaine-mediated hyperlocomotion	Eagle AL, et al. (2015)
Anxiety-like behavior in open field and EPM	Wang HN, et al. (2015)
Anxiety-like behavior in EPM	Sabban EL, et al. (2015)
Anxiety-like behavior in open field, EPM, light-dark box; radial arm maze deficits; increased plasma corticosterone; decreased histone acetylation	Solanki N, et al. (2015)
Increased GR levels and PKC phosphorylation in PFC	Wen L, et al. (2015)
Impaired CF extinction	Eskandarian S, et al. (2015)
Impaired CF extinction; increased GR expression in PFC and hippocampus	George, et al. (2015)
Apoptosis in PFC; impaired Morris Water Maze performance	Yu B, et al. (2014)
Depression-like behavior in FST and anxiety-like behavior in open field and EPM	Ji LL, et al. (2014)
Increased plasma corticosterone, corticotrophin-releasing hormone (CRH) mRNA in mediobasal hypothalamus, and TH and dopamine-β hydroxylase mRNA in LC	Serova LI, et al. (2014)
Increased CRH and FK506-binding protein 5 (FKBP5) mRNA in hippocampus and hypothalamus	Laukova M, et al. (2014)
Decreased body weight; reduced sucrose preference; increased plasma corticosterone; nxiety-like behavior in open field and EPM	Lee B, et al. (2014)
Increased CF expression and anxiety-like behavior in EPM	Miao YL, et al. (2014)
Decreased GR and mineralocorticoid receptor (MR) expression in amygdala	Han F, et al. (2014)
Increased phosphorylation of ERK1/2 in PFC	Qi J, et al. (2014)
Increased CF expression and anxiety-like behavior in EPM; decreased phosphorylation of ERK1/2 in hippocampus	Nie H, et al. (2014)
Depression-like behavior in FST and anxiety-like behavior in open field and EPM	Serova, et al. (2014)
Increased GR expression in PFC and hippocampus	George SA, et al. (2013)
Anxiety-like behavior in open field and EPM	Peng Z, et al. (2013)
Anxiety-like behavior in open field and EPM	Serova LI, et al. (2013)
Apoptosis and reduced phosphorylation of ERK1/2 in hippocampus	Peng Z, et al. (2013)
Increased evoked, reduced spontaneous firing of LC neurons	George SA, et al. (2013)

Increased GR expression and PKB phosphorylation in hippocampus	Eagle AL, et al. (2013)
Increased IA and acoustic startle response	Ganon-Elazar E, et al. (2012)
Decreased Ca^{2+}/calmodulin kinase IIα (CaMKIIα) expression in PFC	Wen Y, et al. (2012)
Altered CaMKIIα expression in dorsal raphe	Xie H, et al. (2012)
Increased 5-HT1A receptor expression in dorsal raphe	Luo FF, et al. (2011)
Altered MR and GR expression in LC	Li M, et al. (2011)
Increased BDNF and tyrosine kinase B (TrkB) receptor expression in hippocampus	Takei S, et al. (2011)
Increased phosphorylation of ERK1/2 and apoptosis in amygdala	Liu H, et al. (2010)
Increased CF expression and impaired extinction; increased GlyT1 mRNA in hippocampus	Yamamoto S, et al. (2010)
Increased CF expression and impaired extinction; increased GlyT1 mRNA in hippocampus	Iwamoto Y, et al. (2007)
Increased CF expression	Takahashi T, et al. (2006)
Enhanced negative feedback of HPA axis	Liberzon I, et al. (1999)

Mechanisms of emotional memory formation and maintenance

Glutamate-mediated emotional memory

Over the past several years, ionotropic and metabotropic glutamate receptors (iGluRs and mGluRs) have emerged as key regulators of the molecular, synaptic, and behavioral correlates of emotional memory formation and maintenance (84). During memory encoding related to stressful stimuli, glutamate acts as the primary excitatory neurotransmitter via activation of postsynaptic α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) and N-Methyl-D-aspartate (NMDA) iGluRs as well as pre and postsynaptic mGluRs of various subtypes (84, 85). NMDA receptor activation, in particular through its induction of synaptic plasticity, is known to be a requisite component of the cellular and behavioral correlates of emotional memory acquisition, consolidation, reconsolidation, extinction, and expression (33, 35).

The facilitation of NMDA receptor-mediated extinction represents a particularly promising approach for the treatment of individuals with PTSD. This approach involves the pairing of a drug treatment with exposure-based psychotherapy to pharmacologically facilitate extinction learning, leading to remission of symptoms (86). Compounds that activate the NMDA receptor result in accelerated extinction of threat responses in several preclinical models of PTSD symptoms, pointing to the therapeutic potential of targeting this receptor in conjunction with exposure therapy. Unfortunately, despite their critical role in every step of emotional memory formation, direct targeting of NMDA receptors has limited clinical utility due to the potential for severe adverse effects (87, 88). NMDA receptor activation can be achieved with therapeutic index, however, using D-cycloserine (DCS), a partial agonist of the strychnine-insensitive glycine binding (GlyB) site on the NMDA receptor. DCS has been shown to facilitate

the extinction of conditioned fear (CF), a rodent behavioral assay analogous to exposure therapy (86). Based on these data, recent clinical experiments were undertaken demonstrating modest efficacy of DCS in augmenting the beneficial effects of exposure therapy in PTSD patients (89). Although these studies offer proof-of-concept validation for this therapeutic approach, other studies employing a similar strategy in PTSD patients found DCS to have no effect or to have a negative effect on recovery (90). These discrepant results are likely due to the fact that DCS, a partial agonist for the GlyB site, can actually act as an antagonist of NMDAR activity in the presence of high synaptic concentrations of the endogenous full agonist, glycine (91).

Selective inhibition of the Glycine Transporter 1 (GlyT1) offers an alternative approach for the indirect modulation of NMDA receptors. Under normal conditions, the GlyB site on the NMDA receptor is not saturated due to the tightly controlled regulation of synaptic glycine levels by GlyT1, expressed in a distribution pattern that closely overlaps with NMDA receptor expression in the cortex and limbic regions of the brain (92). Previous studies have demonstrated that selective inhibitors of GlyT1 can increase synaptic glycine levels sufficiently to produce enhanced NMDA receptor function in preclinical rodent models (93). Recently, we have reported the development and characterization of a novel series of GlyT1 inhibitors, represented by ACPPBII, (2-amino-4-chloro-N-((4-phenyl-1-(propylsulfonyl)piperidin-4-yl)methyl)benzamide), with suitable bioavailability, brain penetration, and physical properties for extensive characterization in vivo (94). Selective inhibition of GlyT1 by ACPPBII may provide a novel target for enhancing the therapeutic effects of exposure therapy in PTSD patients. Studies aimed at addressing this question were undertaken, and are presented in Appendix A. However, due to adverse effects that precluded the interpretation of these data (discussed in Chapter 4), we sought alternative routes to pharmacologically modulate NMDA receptor-dependent emotional memory.

Indirect modulation of NMDA receptor activity and its downstream molecular, synaptic, and behavioral effects can also be achieved through activation or inhibition of certain mGluRs. Recent evidence further supports a role for mGluRs in mediating emotional learning independent of their coupling to NMDA receptor-mediated signaling. Thus, targeting mGluRs as a means of altering traumatic memory formation and maintenance may have the potential to treat or possibly prevent PTSD symptoms.

mGluRs are part of a subfamily of neuromodulatory G protein-coupled receptors (GPCRs) divided into three groups based on sequence homology, synaptic localization, and G protein coupling (for an in-depth review of receptor pharmacology and function see (95)). Group I includes $mGluR_1$ and $mGluR_5$, Group II includes $mGluR_2$ and $mGluR_3$, and Group III includes $mGluR_4$, $mGluR_6$, $mGluR_7$, and $mGluR_8$ (95).

The Group I mGluRs are predominantly postsynaptic (95, 96), and exhibit high expression in PFC, amygdala, and hippocampus (96, 97) (Figure 3). Both $mGluR_1$ and $mGluR_5$ couple to the $G\alpha_{q/11}$ subtype of G proteins, activation of which leads to the induction of classical intracellular signaling pathways including phospholipase Cβ activation, formation of inositol 1,4,5-trisphosphate (IP3), intracellular calcium mobilization, and activation of protein kinase C (PKC) (95). Importantly, $mGluR_1$ and $mGluR_5$ agonism has also been shown to increase activity in the mitogen-activated protein kinase/extracellular receptor kinase (MAPK/ERK) pathway, and the mammalian target of rapamycin (mTOR)/p70 S6 kinase pathway, leading to alterations in gene expression, protein synthesis, and synaptic plasticity particularly relevant to the formation and maintenance of emotional memories (33, 95, 98). In addition, both $mGluR_1$ and $mGluR_5$ have been shown to be structurally and functionally linked to the NMDA receptor (99-104) such that $mGluR_1$ or $mGluR_5$ activation can promote, and its antagonism or genetic deletion can

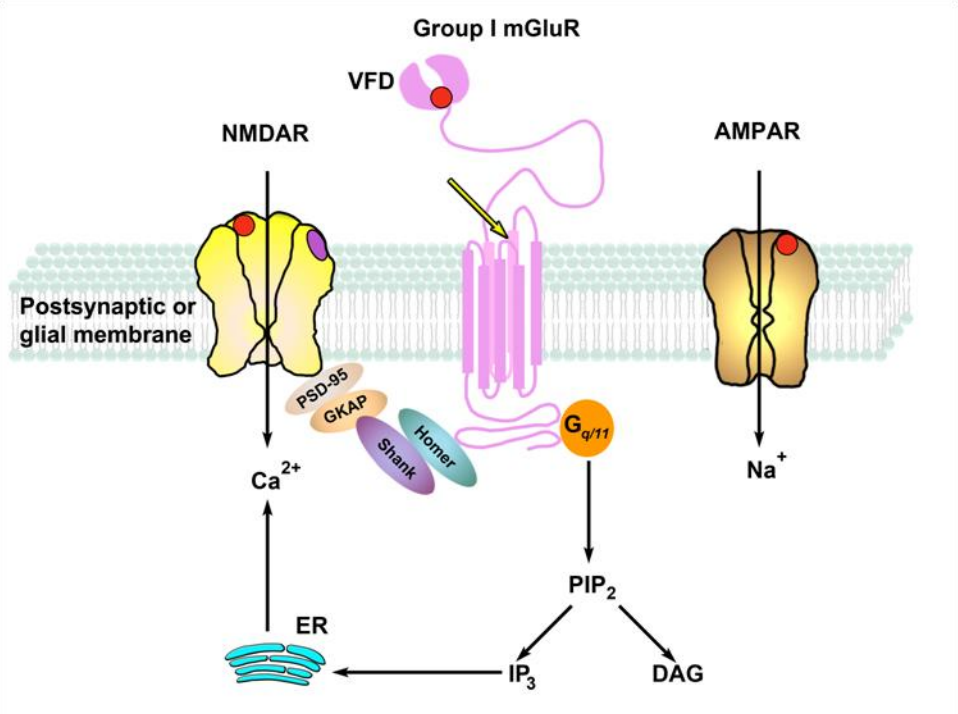

Figure 3. Ionotropic and group I mGluR receptor signaling. Adapted from (105). Group I mGluRs are predominantly postsynaptic and couple to the $G\alpha_{q/11}$ subtype of G proteins, activation of which leads to the induction of classical intracellular signaling pathways including phospholipase Cβ activation, formation of inositol 1,4,5-trisphosphate (IP3), intracellular calcium mobilization, and activation of protein kinase C (PKC). Both $mGluR_1$ and $mGluR_5$ have been shown to be structurally and functionally linked to the NMDA receptor. Arrow indicates a known allosteric binding site within the transmembrane domain.

inhibit, NMDA receptor-dependent LTP and LTD in multiple brain regions (99, 103, 106-110). Based on these initial findings, several studies have gone on to demonstrate that more selective $mGluR_1$ or $mGluR_5$ modulation is sufficient to alter synaptic correlates of emotional memory formation and maintenance with measurable behavioral consequences (42, 111-151).

The Group II mGluRs are generally localized to presynaptic terminals in several brain regions including the PFC, amygdala, and hippocampus (152, 153) where they function as autoreceptors or heteroreceptors to inhibit glutamate and other neurotransmitter release (95). Unlike the Group I mGluRs, $mGluR_2$ and $mGluR_3$ couple to the $G_{i/o}$ subtype of G proteins

leading to inhibition of adenylyl cyclase and liberation of the $G_{\beta\gamma}$ subunit (95). However, both $mGluR_2$ and $mGluR_3$ have also been found to be expressed postsynaptically (154, 155) where their activation causes hyperpolarization of postsynaptic neurons (154). Additionally, $mGluR_{2/3}$ antagonists have been shown to increase mTOR signaling in the PFC, indicating a negative coupling of these receptors to the MAPK/ERK and mTOR/p70 S6 kinase pathways (156). $mGluR_2$ and $mGluR_3$ modulate NMDA receptor function directly (157-159), and indirectly through alteration of presynaptic glutamate release (160). Furthermore, activation of the Group II mGluRs has been shown to enhance LTD and inhibit LTP in both NMDA receptor-dependent and independent manners in multiple brain regions including the cortex, hippocampus, and amygdala (161-175) with relevant effects on behavioral measures of emotional memory (176-196).

Similar to Group II, Group III mGluRs reside primarily in presynaptic terminals, and generally act to inhibit neurotransmitter release through coupling to the $G_{i/o}$ subtype of G proteins (95), although Group III-mediated signaling through the MAPK and phosphatidyl inositol 3-kinase (PI3K) pathways has also been reported (197). Group III mGluRs exhibit diverse expression in the brain. $mGluR_4$ and $mGluR_8$ are both found in relatively low levels in the cortex, hippocampus, and amygdala (198, 199). $mGluR_7$ is expressed throughout the brain, but has a very low affinity for glutamate (199). $mGluR_6$, unlike the other mGluRs in this group, is primarily postsynaptic, and its expression is restricted almost entirely to the retina (199). Consistent with its expression profile, $mGluR_6$ has not yet been shown to be involved in emotional learning and memory. Together, each of the other Group III mGluRs has been shown to be involved in NMDA receptor-dependent and independent plasticity in the hippocampus (199) and amygdala (200), and to affect emotional memory encoding (201-220).

Developing selective ligands represents a major hurdle to delineating the specific role of each mGluR subtype in modulating emotional memory formation and maintenance. Traditional methods of compound development, in which displacement of the endogenous ligand was the primary endpoint, restricted discovery efforts to compounds that bind the orthosteric site of a given receptor (221). In the case of many GPCRs including mGluRs, however, these sites are highly conserved between subtypes, rendering orthosteric ligands largely non-selective (221). Over the past several years, new screening methods and chemical optimization techniques have increasingly allowed for the discovery of small molecules that bind outside the orthosteric site of mGluRs and other GPCRs (221). By binding to less highly conserved regions of the receptor, often in the transmembrane domain, these allosteric ligands generally exhibit greater selectivity for specific GPCR subtypes (221). Allosteric ligands have been discovered that are capable of modulating the GPCR response to orthosteric agonists without having any intrinsic activity themselves; compounds that potentiate receptor response have been named positive allosteric modulators (PAMs) and compounds that inhibit the response have been named negative allosteric modulators (NAMs) (221). To date, multiple PAMs and NAMs have been developed that selectively target specific mGluR subtypes with little or no off-target activity at other GPCRs (95, 221). In addition, many of these selective compounds have been optimized for *in vivo* administration, enabling studies aimed at determining the effect of specifically targeting each mGluR subtype on the formation and maintenance of threat-related behaviors in mice and rats (95, 221).

Due to the significant involvement of $mGluR_5$ in emotional learning and memory, PAMs and NAMs for this receptor may be particularly useful for the treatment of PTSD. As mentioned above, $mGluR_5$ canonically couples to the $G\alpha_{q/11}$ subtype of G proteins; when activated by

stress-induced glutamate release, $mGluR_5$ ultimately leads to the induction of downstream effectors that mediate synaptic remodeling and memory formation/maintenance (Figure 4). Many of these downstream signaling molecules lie also in the pathway that is activated by NMDA receptors, offering an indirect means of targeting the NMDA receptor for therapeutic purposes. This process is also dependent on new protein synthesis, and can be blocked by protein synthesis inhibitors such as anisomycin (33). Importantly, pharmacological inhibition of $mGluR_5$ can also reduce new protein synthesis (222), and as mentioned above, attenuate the neural and behavioral correlates of emotional memory formation.

One of the first studies to specifically implicate $mGluR_5$ in the neural underpinnings of emotional learning and memory demonstrated that both acquisition/consolidation and extinction of combined cued and contextual CF could increase $mGluR_5$ expression in the hippocampus (138). These experiments were quickly followed by several reports that 2-methyl-6-(phenylethynyl)pyridine (MPEP), a selective $mGluR_5$ negative allosteric modulator (NAM), could block, but not reverse, the *ex vivo* induction of thalamo-amygdala LTP (129, 139) and late-LTP (122), a putative neurophysiological correlate of long term threat-based memory (98). In the intact animal, *in vivo* hippocampal LTP was also shown to be susceptible to intracerebroventricular MPEP administration (115). In addition to blockade of $mGluR_5$, recent advances in the development of $mGluR_5$ PAMs have enabled experiments examining the effect of selective potentiation of these receptors on synaptic plasticity in fear circuits. For example, *ex vivo* application of VU-29, a selective $mGluR_5$ PAM, augmented subthreshold electrical induction of hippocampal LTP as well as stimulus or DHPG-induced hippocampal LTD (113). This finding was replicated with a novel $mGluR_5$ PAM, ADX47273, and shown to be absent in

$mGluR_5$ knockout mice (150), suggesting that $mGluR_5$ participates in hippocampal-dependent contextualization of emotional memory.

$mGluR_5$ activation has also been shown to be important for the acquisition of aversive learning in behaving animals. Multiple groups have demonstrated that systemic administration of MPEP blocks the acquisition of FPS (145, 151), cued CF (141), and CTA (144). It has also been found that infusion of MPEP directly into the amygdala is sufficient to inhibit the acquisition of cued and contextual CF (122, 139), and FPS (122), demonstrating that, along with $mGluR_1$ and consistent with the electrophysiological evidence, the amygdala represents an important locus of action for Group I modulation of emotional memory acquisition. MTEP, an analogue of MPEP with similar pharmacological properties, also attenuated the acquisition of FPS (137), and contextual but not cued CF (127). More recently, it was shown that constitutive genetic deletion of mGluR5 in mice also impairs performance in contextual CF (149), although it is not possible in these animals to determine what specific phase of emotional memory formation or maintenance is compromised. The recent development of $mGluR_5$-selective PAMs has enabled studies examining the effect of $mGluR_5$ activation on aversive associative conditioning. Consistent with previous studies demonstrating the importance of $mGluR_5$ signaling in encoding threat-based memory, a recent study found that the novel $mGluR_5$ PAM VU0409551 enhances acquisition of contextual CF (223).

In addition to its role in acquisition of emotional memory, there is an extensive literature demonstrating the importance of $mGluR_5$ activation for the expression of aversive learning. It was first shown in 1997 that S-4C3H-PG, a non-selective Group I mGluR antagonist, increased punished responding in rodents (119), an effect considered to be anxiolytic. Systemic administration of the selective $mGluR_5$ NAMs, MPEP or MTEP, also inhibited the expression of

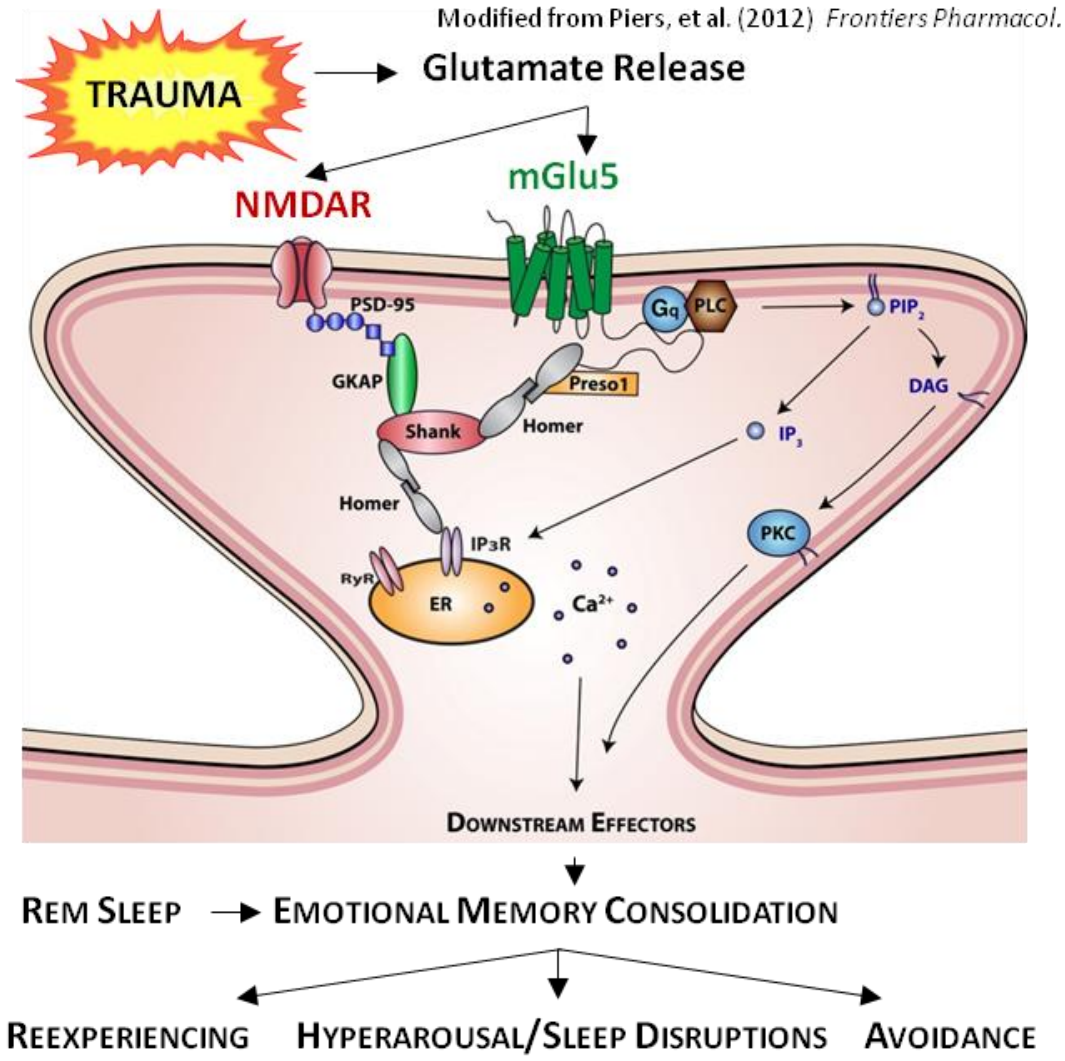

Figure 4. $mGluR_5$ and NMDA receptor involvement in synaptic correlates of emotional memory. Adapted from (224). $mGluR_5$ canonically couples to the $G\alpha_{q/11}$ subtype of G proteins; when activated by stress-induced glutamate release, $mGluR_5$ ultimately leads to the induction of downstream effectors that mediate synaptic remodeling and memory formation/maintenance. Many of these downstream signaling molecules lie in the pathway that is activated by NMDA receptors, offering an indirect means of targeting the NMDA receptor for therapeutic purposes. This process is also dependent on new protein synthesis which allows for synaptic remodeling and long term memory storage that, in a subset of individuals leads to PTSD symptoms after trauma.

fear-based memory as measured by FPS (117, 145), cued or contextual CF (127), punished responding (114, 137, 140, 147), and aversive place conditioning (112). Similar to these prototypical compounds, the novel $mGluR_5$-selective NAM, VU0285683, was shown to increase punished responding (140). Contrary results have been obtained in which there was no effect of MPEP on expression of FPS (122) or cued or contextual CF (122, 139); however, both of these experiments employed direct infusion of the compound into the amygdala, suggesting that the locus of action for $mGluR_5$ antagonism on fear expression may include brain regions outside the amygdala.

Sleep-dependent emotional memory

One mechanism through which the activation or inhibition of $mGluR_5$ and other mGluRs may mediate emotional memory encoding is by altering the amount, timing, or quality of sleep (34, 225, 226). In both rodents and humans, non-rapid eye-movement (NREM) and rapid eye-movement (REM) sleep accrued after learning has been shown to promote the successful consolidation of new memory as evidenced by improved recall or performance on a given task after a delay (34, 225, 226). Mammalian sleep-wake architecture is highly conserved between species (227) such that rodents provide a valid translational model of normal human sleep patterns, as well as aberrant sleep related to various disease states (228, 229). Similar to human sleep studies, rodent sleep is measured using electroencephalography (EEG), in which electrodes are placed in contact with the scalp (in humans) or the dura mater (in rodents), allowing for the detection of electricity generated primarily by cortical neuronal ensembles (227, 228, 230). Based on characteristic changes in the oscillatory electrical activity in the brains of both species, it is possible to detect transitions between arousal states, from wake to NREM sleep to REM

sleep (227, 228, 230) (Figure 5). Although rodents are nocturnal, they cycle between wakefulness, through deepening stages of NREM sleep, and into bouts of REM sleep in the same stereotypical manner as humans (227, 228, 231). Both rodents and humans exhibit longer, more frequent bouts of NREM sleep early in the quiescent phase, transitioning to lighter NREM sleep and more frequent entries into REM sleep as the active phase approaches (227, 228, 231, 232).

Physiological pressure to enter NREM sleep is generated by the gradual accumulation of adenosine, a by-product of activity-dependent use of adenosine triphosphate (ATP) (227). Adenosine acts primarily through the adenosine receptor subtype 2A to activate γ-aminobutyric acid (GABA)-ergic neurons in the ventrolateral preoptic (VLPO) region of the hypothalamus which project to and inhibit wake-stabilizing orexinergic neurons in the lateral hypothalamus, histaminergic neurons in the tuberomammillary nucleus, serotonergic neurons in the dorsal raphe, and noradrenergic neurons in the locus coeruleus (227). During wake, orexinergic and monoaminergic neurons are active, and mediate reciprocal inhibition of the VLPO, ensuring that sleep is not initiated suddenly or unexpectedly (227). Once NREM sleep is initiated, periodic transition into REM sleep is caused by the activation of REM-on glutamatergic and cholinergic cells in the pontine reticular formation (PRF) and the laterodorsal/pedunculopontine tegmental nuclei (LDT/PPT), which project to the cortex and limbic regions of the brain, causing paradoxical excitation of the cortical EEG during REM sleep (227). Firing of monoaminergic neurons during REM sleep approaches zero, however, and the extracellular content of serotonin (5-HT), norepinephrine, and dopamine in the cortex, hippocampus and amygdala is very low, despite functional magnetic resonance imaging (fMRI) studies that reveal significant activation of these regions (227). The serotonergic system, in particular, is a key regulator of both sleep-

wake architecture and emotional memory (233), and represents one of the neurochemical links between these two closely associated physiological and behavioral processes.

While NREM sleep appears to confer a benefit primarily to the consolidation of declarative or episodic memory (34, 225, 226), REM sleep seems to be particularly important for the consolidation and contextualization of emotional memory (34, 225, 226, 234-239), although recent findings suggest involvement of NREM sleep in this process as well (238). The accumulation of REM sleep after emotional learning promotes the consolidation of memory related to the content of the new information as well as its emotional valence (226, 235, 237, 240). This finding appears to be consistent regardless of the phase of emotional memory encoding; REM sleep can improve emotional memory consolidation after initial acquisition (226, 236, 240), reconsolidation (34), or extinction (241-244) in both rodents and humans.

The amount of time spent in each sleep state is not the only factor that determines its contribution to emotional memory formation and maintenance. The spectral composition of the EEG can also change within each sleep-wake state, and these changes are associated with different physiological and behavioral consequences. Although these transitions can be visually distinguished by qualitatively examining the EEG, quantitative EEG (qEEG) methods can also be applied to quantify the relative contribution of different frequencies to the total EEG waveform (Figure 5). These frequencies are grouped into the conventional power bands delta (δ: 0.5-4 Hz), theta (θ: 5-8 Hz), alpha (α: 9-13 Hz), beta (β: 14-30 Hz), low gamma (low γ: 31-50 Hz), and high gamma (high γ: 51-100 Hz). The prominence of each power band in the EEG changes depending on arousal state (227, 228, 230, 231). For example, waking EEG is characterized by power in mixed frequencies, NREM sleep by global increases in delta power, and REM sleep by increases in theta power (227, 228, 230, 231).

The power of each band is not discrete, but can fluctuate continuously within each sleep-wake state (227, 228, 230, 231). Within-state alterations in the relative power of each band are associated with different behavioral outcomes (227, 228, 230, 231), and thus may be useful as biomarkers of disease and/or treatment (228). For example, the low frequency delta oscillations present during NREM sleep, also known as slow wave activity (SWA), are a well-established neurophysiological correlate of the r estorative properties of deep sleep (231, 232), and have been shown to be the component of NREM sleep most important for promoting the consolidation of declarative memory (225, 245). In multiple human studies with both healthy subjects and patients with mental illness associated with cognitive impairments, it has now been shown that this relationship is causative; when SWA is experimentally amplified, recall of prior learning is improved (245-249). For example, using transcranial induction of slow oscillations during NREM sleep, it was shown that declarative memories could be enhanced in healthy subjects (245).

The memory-enhancing function of REM sleep is also dependent on the spectral composition of the EEG. In particular, increases in theta power during REM sleep have been correlated with enhanced emotional memory consolidation in humans and rats (240, 250, 251). Notably, increased REM sleep duration and theta power are not only associated with improved recall after learning, but this enhancement is strongest for negatively valenced information (239, 240), suggesting that REM sleep preferentially promotes the consolidation of negative emotional memory. For example, after memorizing names associated with pictures of faces exhibiting neutral or negative expressions, REM sleep positively correlated with recall of the negative facial expressions, but not the neutral ones (240).

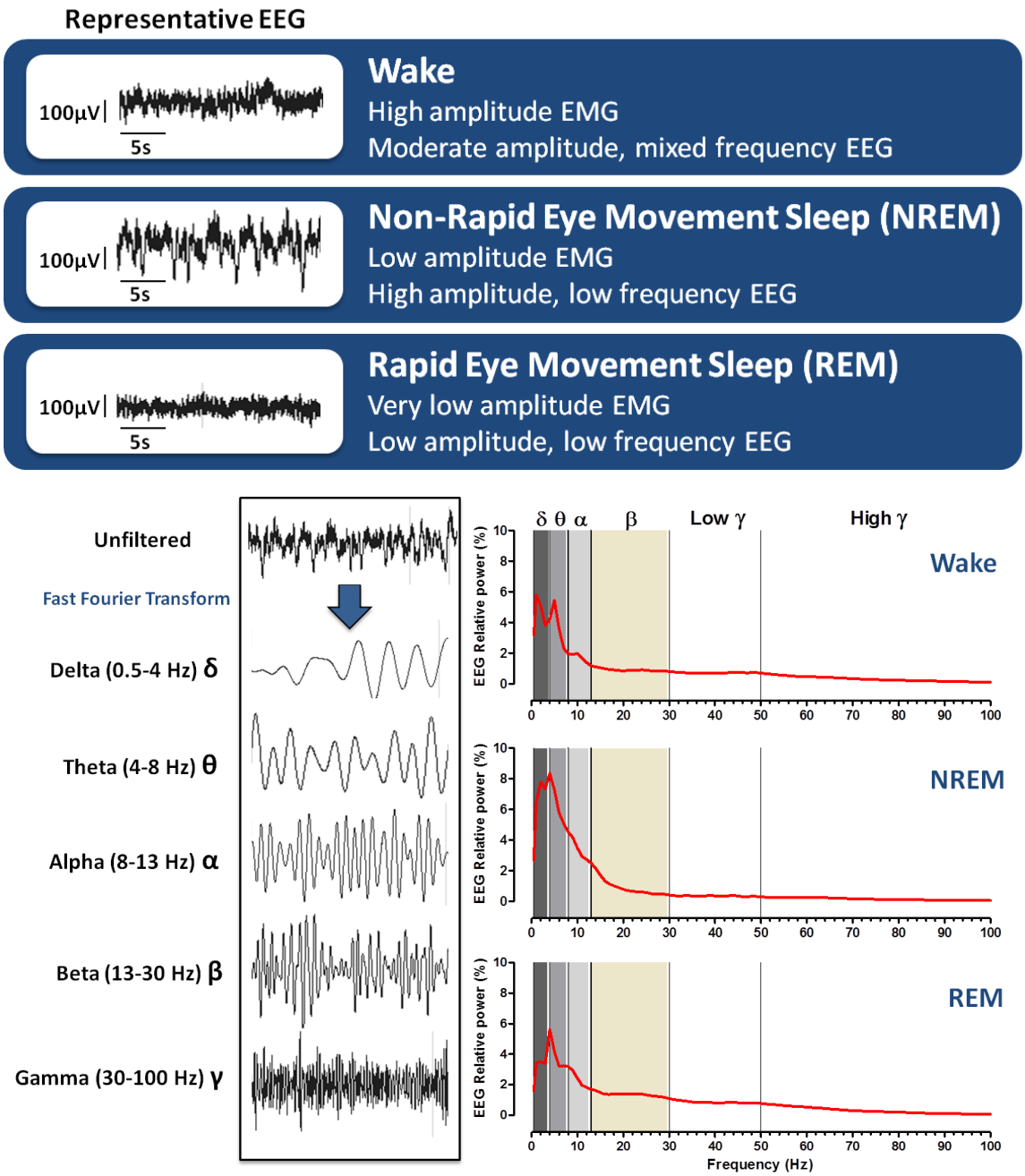

Figure 5. Representative rodent EEG and spectral power analysis characterizing each sleep-wake state. Characteristic changes in the EEG delineate transitions between each sleep-wake state for both rodents and humans. These changes can be visually distinguished in order to stage sleep-wake architecture. qEEG methods can also be applied to decompose the total EEG waveform into its component frequency bands. When power in each band is plotted as a function of frequency, it is clear that the spectral composition of the EEG is distinct between each sleep-wake state. This method also allows for the detection of changes in power spectra within each sleep-wake state that are associated with different behavioral outcomes.

Although these studies have primarily been performed in the experimental setting, it is likely that post-trauma accumulation of REM sleep similarly contributes to the consolidation of traumatic memory and may spur the development of PTSD symptoms (252).

Thus, any pharmacological treatment aimed at therapeutically blocking emotional memory consolidation in the aftermath of traumatic stress should impinge on both wake and sleep-dependent memory encoding processes. As mentioned above, glutamate signaling in cortical and subcortical brain regions is a key mediator of emotional memory formation and maintenance during wakefulness, but it is also critical for the regulation of sleep-wake architecture and state-dependent qEEG spectral power (225). Selective antagonism or genetic deletion of different iGluRs and mGluRs has been found to differentially alter these measures with correlated behavioral consequences in both rodents and humans (253-278). For example, noncompetitive NMDA receptor antagonism with the rapid-acting antidepressant ketamine has been shown to specifically reduce REM sleep and enhance SWA, an effect that correlated with treatment response in depressed patients (263, 277, 278). Ketamine has also been found to increase gamma power in the frontal cortex during wake in rodents and humans, a measure that was successfully used as a translational biomarker of central target engagement (279). These studies highlight the importance of examining sleep-dependent effects of drug treatment, and underscore the potential value of employing qEEG as a biomarker of both target engagement and efficacy. NMDA receptor antagonist-induced suppression of REM sleep could also explain the effectiveness with which this class of compounds impairs emotional memory consolidation in rodents and humans. Although emotional memory consolidation is sensitive to both pharmacological treatment and sleep deprivation (226, 252, 280-282), it is important to note that there may be a critical temporal window for modifying this process (37). Indeed, depending on

the physiological or behavioral endpoint, NMDA receptor blockade and REM sleep deprivation lose the ability to attenuate memory consolidation if performed hours to days after emotional learning (37, 283, 284). Again, although preliminary evidence suggests that this window may be as short as six hours in victims of trauma (31, 37) most of these studies have been performed in experimental settings on healthy human participants, or on rodents subjected to mild aversive associative conditioning paradigms. Thus, it is not clear whether NMDA receptor antagonism after more severe traumatic stress can prevent traumatic memory consolidation and/or PTSD symptom progression, nor is it known exactly what role REM sleep may play in this process.

Unfortunately, to date, no clinical study has objectively measured the effect of trauma on human sleep-wake architecture within hours to days of the traumatic experience (285). The earliest time point at which polysomnographic EEG has been collected in humans is between one week and two months after trauma (286-290). Furthermore, no sleep-dependent pharmacological or behavioral intervention has been attempted in victims of trauma until days after the event (291, 292), likely missing what may be the critical temporal window during which REM sleep suppression would be therapeutic (37, 283, 284). The logistical hurdles inherent to the collection of human EEG data at earlier time points highlight the importance of a valid rodent model of trauma-induced sleep-wake alterations. Such a model would enable studies in which pharmacological manipulation of sleep-wake architecture and state-dependent qEEG spectral power could be tested as a means of attenuating the development of PTSD-like symptoms after trauma. Given the impressive attenuating effect of NMDA receptor antagonism on both REM sleep and emotional memory consolidation, it would be of interest to test the effect of NMDA receptor blockade, and associated REM sleep suppression, on traumatic stress-induced physiological and behavioral alterations. However, as previously mentioned, translation of these

findings to the clinic would be impeded by the known adverse effects and formulation constraints associated with NMDA receptor antagonists such as ketamine (87, 88).

Recently, it was found that the selective $mGluR_5$ NAM 2-methyl-6-(phenylethynyl)pyridine (MPEP) has similar effects to NMDA receptor antagonists, specifically suppressing REM sleep, enhancing SWA, and inducing increased gamma power during wakefulness in rodents (253, 258), possibly with fewer and/or less severe adverse effects (293). These findings are consistent with cellular data pointing to the structural and functional coupling of $mGluR_5$ and NMDA receptors (99-101, 103, 106, 107, 294). They also suggest that both NMDA receptor and $mGluR_5$ activation may promote emotional memory formation in part through increasing time spent in REM sleep after emotional learning. Indeed, antagonism of $mGluR_5$, possibly through downstream inhibition of NDMA receptor function, has been shown to attenuate emotional memory acquisition, consolidation, and extinction at the neural and behavioral level in multiple rodent assays of threat learning (113, 115, 122, 129, 138, 139, 144, 145, 149, 150). These observations suggest that $mGluR_5$ NAMs could be used in a prophylactic approach to block the consolidation of emotional memory immediately after a traumatic event, possibly impeding the development of PTSD symptoms. Despite promising results in rodent behavioral assays of emotional memory (113, 115, 122, 129, 138, 139, 144, 145, 149, 150), however, $mGluR_5$ NAMs have not been tested in a rodent model of traumatic stress such as SPS.

Outline of current studies

Thus, in Chapter 2, we performed studies intended to test whether SPS can be used as a rodent model of traumatic stress-induced physiological and behavioral alterations that would mimic PTSD patients. Specifically, we examined short and long-term alterations in sleep-wake

architecture and state-dependent qEEG spectral power. We also measured corresponding changes in several validated physiologic measures of the rodent stress response, as well as alterations in brain regional serotonergic signaling that may relate to observed disruptions in EEG measures. Then we determined whether these alterations have behavioral consequences, testing the effect of SPS on subsequent threat learning.

Having validated the model, in Chapter 3, we attempted to pharmacologically intervene in the deleterious effects of SPS. First, we tested the effects of systemic administration of 3-fluoro-N-(4-methylthiazol-2-yl)-5-(pyrimidin-5-yloxy)benzamide (VU0409106), a novel, selective, brain penetrant $mGluR_5$ NAM (295) on rat sleep-wake architecture and state-dependent qEEG spectral power to confirm that it behaves similar to reported $mGluR_5$ NAMs in these measures. Then, we tested the effects of VU0409106 on multiple rodent assays of sedation and motor impairments to determine the therapeutic range of this compound, and to aid in selecting a dose for subsequent studies. Finally, we measured the effects of post-trauma administration of VU0409106 on SPS-induced alterations in behavior, sleep-wake architecture, qEEG spectral power, and brain regional serotonin (5-HT) utilization. We hypothesized that VU0409106, in part through acute suppression of REM sleep and impairment of emotional memory consolidation would attenuate the subsequent physiological and behavioral effects of SPS.

CHAPTER 2

TRAUMATIC STRESS INDUCES LASTING SLEEP AND QUANTITATIVE ELECTROENCEPHALOGRAPHIC DISTURBANCES IN RATS

Introduction

SPS represents a valid model of traumatic stress that recapitulates many of the physiological and behavioral alterations present in PTSD patients (59). However, despite the prevalence, severity, and intractability of hyperarousal-associated sleep disturbances associated with PTSD (2, 11), it is not known whether SPS induces alterations in sleep-wake architecture and state-dependent qEEG power spectra similar to those seen in patients.

Polysomnographic studies in chronic PTSD patients as well as recently traumatized individuals have revealed deficits in both NREM and REM sleep, including reduced and fragmented NREM and REM sleep, shortened latency to REM sleep, and increased REM density (2, 288-290, 296). Abnormalities in state-dependent qEEG power spectra indicative of heightened arousal during wakefulness, such as increased high frequency beta power, and inappropriate cortical activation during NREM sleep, such as reduced low frequency delta power (aka SWA) have also been observed in individuals with PTSD (297-301). These abnormalities are correlated with the previously discussed structural and functional alterations in the HPA axis, hippocampus, PFC, and amygdala (29).

As mentioned, SSRIs are the front-line treatment for PTSD (10); this, combined with the finding that PTSD susceptibility and severity is associated with a 5-HT transporter gene polymorphism (302-305) implicates disrupted serotonergic neurotransmission in the

pathophysiology of the disorder. Recent studies suggest that SSRIs may partially exert their therapeutic effects through modulation of neuropeptide Y (NPY) and its Y1 and Y2 receptor subtypes (306). NPY has anxiolytic (307, 308) and sleep-promoting (309, 310) properties, and is significantly decreased in the plasma and cerebrospinal fluid (CSF) of PTSD patients (311, 312). Previous anatomical studies have shown that serotonergic terminals synapse onto NPY-expressing inhibitory interneurons in the amygdala (313), suggesting the possibility that combined disruption of these neurotransmitter systems may contribute to hyperarousal symptoms and sleep-wake disruptions in PTSD patients.

In order to test this hypothesis, and to determine whether SPS induces accompanying alterations in sleep-wake architecture and state-dependent qEEG power spectra, we telemetrically recorded EEG from rats in their home cage. Specifically, we tested whether SPS causes reduced and fragmented NREM and REM sleep that persists beyond the day of traumatic stress, similar to PTSD patients. In addition, we performed qEEG spectral power analysis to evaluate whether SPS induces markers of chronically increased cortical activation during wake and NREM sleep consistent with PTSD-like hyperarousal. To determine whether alterations in sleep-wake architecture coincided with activation of the HPA axis, we assessed changes in several validated physiologic measures of the rodent stress response including hyperthermia, increases in plasma corticosterone (314), and induction of FKBP5, an early stress-responsive gene that acts as a co-chaperone of the glucocorticoid receptor complex (66). Finally, we evaluated the effects of SPS on regional 5-HT utilization, and expression of NPY and its receptors to assess whether disruption of these neurotransmitter systems may be involved in mediating SPS-induced sleep-wake and qEEG spectral power alterations.

Methods

Subjects

All male Sprague-Dawley rats (Harlan, Indianapolis, IN) used in the present studies were housed under a 12 hour light:12 hour dark cycle and given ad libitum access to food and water. All animal experiments were approved by the Vanderbilt University Animal Care and Use Committee and experimental procedures conformed to guidelines established by the National Research Council Guide for the Care and Use of Laboratory Animals. All efforts were made to minimize animal suffering and the number of animals used.

Surgery

Twenty male rats (250-375 grams) were surgically implanted with a telemetry transmitter (4-ET, Data Sciences International, St. Paul, MN) for recording EEG, electromyography (EMG), and body temperature. Under isoflurane anesthesia (3% induction; 1.5-2.5% maintenance) the transmitter was implanted subcutaneously across the back of each rat. Transmitter leads were tunneled subcutaneously to the skull. After holes were drilled in the skull, the exposed wires were placed in contact with the dura and secured in place with dental cement (Butler Schein, Dublin, OH). Three sets of leads were placed bilaterally to record from cortical regions corresponding with the frontal, parietal, and occipital cortices (+2 mm, -2 mm, and -6mm anterior-posterior from Bregma, respectively and +/- 2 mm lateral to the midline). An additional set of leads was placed bilaterally in the nuchal muscles for EMG recording. Rats were individually housed following surgery and allowed to recover and acclimate to the recording room for a minimum of 10 days prior to testing.

Experimental design

After post-operative recovery, each rat was randomized into either the SPS or SHAM group. Continuous 24 hour baseline (BL) recordings were performed for each rat in its home cage to serve as within-subjects comparator for all subsequent sleep-wake, qEEG, and body temperature data. After BL recordings, each rat received either SPS or SHAM treatment. Immediately following treatment, home cage recordings were re-initiated in both groups (Day 0), continued for two days (Days 1 and 2) after which transmitters were turned off, then reactivated on Day 7. Subsequent off-line analysis of sleep-wake and qEEG data was divided into the remaining hours of Day 0, or in 24hr intervals comprising Days 1, 2, and 7 post-SPS or SHAM treatment. Figure 6 depicts the experimental design for the EEG studies (Cohort 1) as well as the time points for tissue collections for the biochemistry and neurochemistry studies (Cohort 2). For all experiments, SPS or SHAM treatment occurred within the first 6 hours of the light phase.

Single Prolonged Stress

SPS was performed according to Liberzon et al. (315). Briefly, rats were restrained for 2 hours, followed by forced swim for 15 minutes in 24 ^{0}C water. Following a 15 minute recovery period, rats were exposed to diethyl ether vapor in a bell jar until anesthesia. The SPS model did not cause mortality. SPS did illicit hallmarks of the rodent stress response such as porphyrin staining of the eyes, and urination and defecation. There were no major individual differences observed in these parameters during each experiment, and no inclusion or exclusion criteria were applied prior to the start of EEG recordings or tissue collection. SHAM treatment consisted of placement in a novel procedure room for 2 hours followed by brief handling. All animals were placed into fresh cages after treatment.

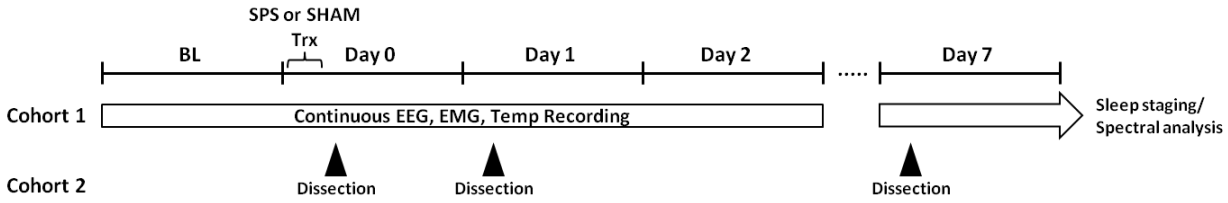

Figure 6. Experimental design for EEG studies and tissue collection. In cohort 1, continuous EEG, EMG, and temperature data were telemetrically recorded from chronically implanted rats throughout successive 24 hour light-dark cycles (ON: 6:00AM; OFF: 6:00PM) before (BL) and several days after (Days 0, 1, 2, and 7) either single prolonged stress (SPS) or SHAM treatment. Both treatments were performed within the first 6 hours of the light phase on Day 0 during which recording was not possible; EEG data from this day was re-initiated when each animal was returned to its home cage. In cohort 2, non-implanted aged-matched rats underwent either SPS or SHAM treatment. SPS rats were sacrificed either one hour (Day 0), one day (Day 1), or seven days (Day 7) later; SHAM rats were sacrificed seven days later.

Tissue collection

For all biochemical and neurochemical endpoints, a group of thirty-six non-implanted rats was randomly assigned to SHAM treatment or one of three SPS groups (Day 0, 1 or 7). Rats were briefly anesthetized with isoflurane, and sacrificed by decapitation either immediately (Day 0), one day (Day 1), or seven days (Day 7) after SPS; SHAM rats were sacrificed immediately after SHAM treatment. Hippocampus, amygdala, and PFC were dissected, rapidly frozen on dry ice, and stored at −80 °C for tissue mRNA and neurochemistry experiments. Trunk blood was collected into heparin-lined tubes, and then centrifuged at 5,000 rpm for 9 minutes at 4 °C to obtain plasma.

Plasma corticosterone

Corticosterone, the rodent analogue of the human glucocorticoid cortisol, was measured using a double antibody radioimmunoassay (RIA) kit (MP Biomedicals, Orangeburg, NY).

Tissue neurochemistry

Tissue concentrations of 5-HT and its metabolite 5-hydroxyindoleacetic acid (5-HIAA) were determined by HPLC-ECD as described previously (316).

Quantitative real-time polymerase chain reaction (qRT-PCR)

Alterations in mRNA expression levels of NPY and its Y1 and Y2 receptor subtypes were measured using Aqueous Micro kits (Life Technologies, Grand Island, NY) for RNA extraction, NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE) for RNA quantification, QuantiTect Reverse Transcription Kit (QIAGEN, Hilden, Germany) for complementary DNA transcription, CFX96 Real-Time PCR Detection System (Bio-Rad, Hercules, CA) using primers from TaqMan Gene Expression Assays (Life Technologies) for qRT-PCR of rat NPY (Rn01410145_m1), Y1 (Rn02769337_s1), and Y2 (Rn00576733_s1). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an internal control; data are presented using the comparative cycle threshold (CT) method normalized to SHAM-treated rats.

Sleep staging

EEG, EMG, and temperature data were collected with Dataquest A.R.T. 4.3 software (DSI, Minneapolis, MN) using a continuous sampling method. Telemetric data were sampled at a rate of 500 Hz and transmitted via a receiver (RPC-2, DSI) placed below the cage of each rat. Each receiver was connected to a data exchange matrix (DSI) which transferred EEG, EMG, and temperature data to a computer for off-line analysis. Two trained observers used Neuroscore 3.0 software to manually stage each 10 second epoch as wake, NREM, or REM sleep based on accepted characteristic EEG and EMG oscillatory patterns (317). All 10 second epochs were

summed into 60 minute bins. For the acute effects of SPS or SHAM treatment on Day 0, 60 minute bins were group averaged to examine the amount of time spent in wake, NREM, or REM sleep. To assess the prolonged effects of SPS or SHAM treatment (Days 1, 2, and 7), 12 hour bins comprising either the light or dark phase of a given day were group averaged.

qEEG spectral power analysis

qEEG relative power spectra from frontal and parietal electrodes were computed for each rat and on each day of recording in 10 second epochs in 1Hz bins from 0.5 to 100 Hz using a Fast Fourier Transform with a Hamming window and overlap ratio of 0.5. Relative power within each 1 Hz increment was calculated as a percent of total power, then binned by stage (wake, NREM, or REM), and averaged across the 12 hour light or dark phase to yield the state-dependent relative power spectrum for each rat. To calculate the percent change from BL the following formula was used:

$$\% \text{ change} = 100 * (\text{relative power (posttreatment day)}) / (\text{relative power (BL)}) - 100$$

where relative power (posttreatment day) is the relative power value of a frequency bin of a rat on Day 0, 1, 2 or 7, and relative power (BL) is the BL value of the same frequency bin for the same rat during the corresponding sleep-wake stage and light-dark phase. The % change values were then group averaged. The qEEG changes are discussed in terms of changes in power bands defined based on convention as delta (0.5-4 Hz), theta (5-8 Hz), alpha (9-13 Hz), beta (14-30 Hz), low gamma (31-50 Hz), and high gamma (51-100 Hz) (227). Slow wave activity (SWA) was defined as relative delta power in the frontal cortex during NREM sleep; a time course of

SWA changes was calculated by normalizing SWA values for each rat, in 2 hour bins, to the same rat's BL SWA value during the first 2 hours of the light phase.

Statistical analysis

For the acute effects of SPS or SHAM on sleep-wake architecture (Day 0) and the effect of SPS or SHAM on qEEG spectral power, a repeated measures two-way analysis of variance (ANOVA) was applied; if significant, a Bonferroni post hoc test was performed with significance defined as $P < 0.05$ for sleep-wake data and $P < 0.01$ for qEEG data. For the prolonged effects of SPS or SHAM (Days 1, 2, and 7), a repeated measures one-way ANOVA followed by a Dunnett's post hoc test was used with significance defined as $P < 0.05$. Two-way ANOVA without repeated measures was used to analyze temperature and SWA changes due to the fact that certain rats did not enter NREM or REM states during various 2 hour epochs resulting in randomly missing values. If significant, Bonferroni post hoc tests were conducted with significance defined as $P < 0.05$. Day 0 sleep-wake, qEEG, and temperature data were analyzed separately from Days 1, 2, and 7 to distinguish between the acute and prolonged effects of SPS, which could differ due to the short-term rebound effects of sleep deprivation. One rat in the SPS group did not enter REM sleep during the light phase of Day 0 and was excluded from spectral and temperature analysis for this period. One rat in the SHAM group was excluded from spectral and temperature analysis on Day 7 due to transmitter failure. For qRT-PCR, tissue neurochemistry, and plasma corticosterone data, analysis was performed by one-way ANOVA followed by Dunnett's post hoc test with significance defined as $P < 0.05$.

Results

SPS induced acute and persistent PTSD-like alterations in sleep-wake architecture.

SPS induced robust acute increases in percent time awake (Figure 7A) (time [$F_{18,162} = 7.99$, $P < 0.0001$], interaction [$F_{18,162} = 12.65$, $P < 0.0001$]) with concurrent reductions in time spent in NREM (Figure 7B) (time [$F_{18,162} = 8.55$, $P < 0.0001$], treatment [$F_{1,9} = 5.35$, $P = 0.04$], interaction [$F_{18,162} = 13.84$, $P < 0.0001$]), and REM sleep (Figure 7C) (time [$F_{18,162} = 5.08$, $P < 0.0001$], interaction [$F_{18,162} = 12.18$, $P < 0.0001$]) during the light (rodent quiescent) phase. The reductions in NREM and REM sleep during the light phase were followed by a rebound in these states during the dark (rodent active) phase. In contrast, SHAM treatment produced minor reductions in percent time awake relative to BL (Figure 7D) (time [$F_{18,162} = 33.98$, $P < 0.0001$], treatment [$F_{1,9} = 51.75$, $P < 0.0001$]), and increased time spent in NREM (Figure 7E) (time [$F_{18,162} = 33.62$, $P < 0.0001$], treatment [$F_{1,9} = 40.15$, $P = 0.0001$]), and REM sleep (Figure 7F) (time [$F_{18,162} = 11.8$, $P < 0.0001$], treatment [$F_{1,9} = 42.3$, $P = 0.0001$]).

We then determined the time spent in wake, NREM, and REM sleep on Days 1, 2, and 7 post-SPS or SHAM treatment to determine whether SPS-induced sleep-wake alterations persisted beyond the day of traumatic stress. Increased wake and decreased NREM and REM sleep during the light phase persisted for at least 2 days post-SPS, but normalized by Day 7 (See Table 2 for statistical analysis). On Day 2, SPS caused reductions in NREM bout length, and increases in NREM bout number, indicative of sleep fragmentation. SHAM treatment produced no sustained effect on sleep-wake architecture (Table 3).

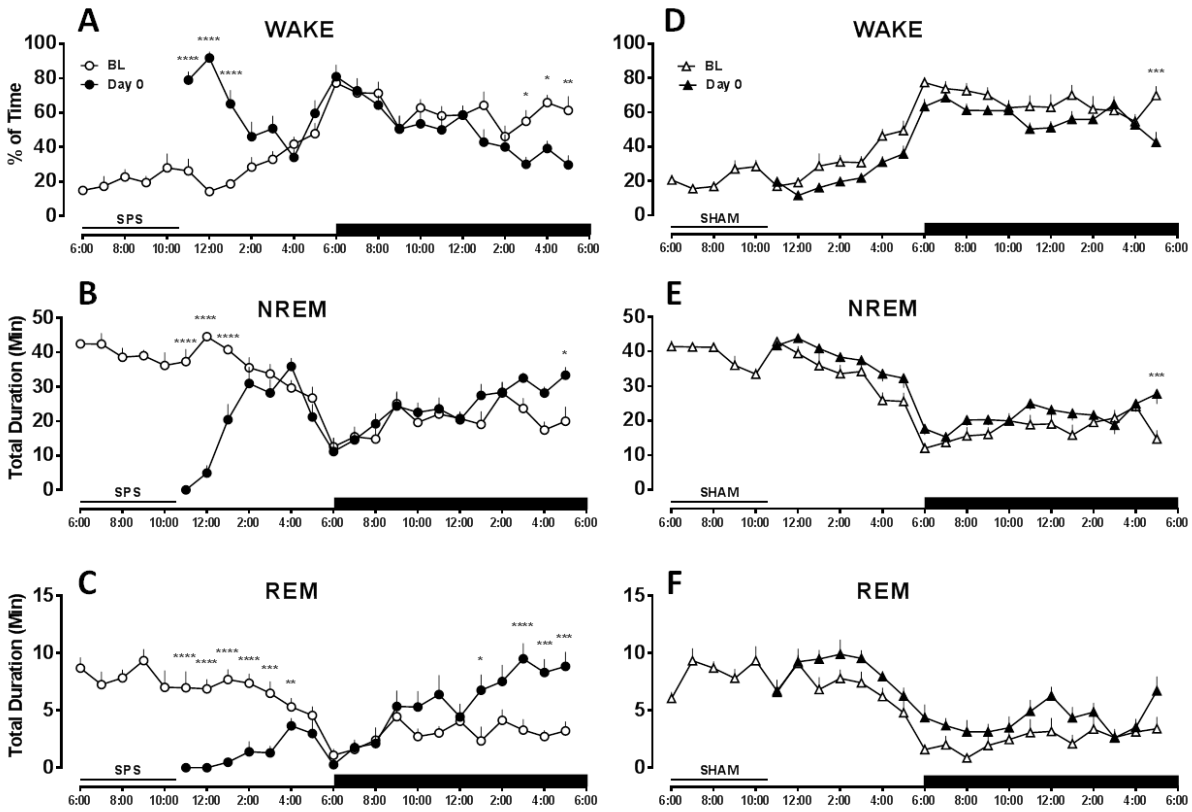

Figure 7. SPS induced acute alterations in sleep-wake architecture the day of treatment. SPS (left panels, *n* = 10) increased (A) % time spent in wake, and suppressed (B) time in NREM, and (C) time in REM sleep during the light phase. Both NREM and REM sleep rebounded during the dark phase. SHAM treatment (right panels, *n* = 10) caused the opposite effect, moderately decreasing (D) % time spent in wake, and increasing (E) time in NREM, and (F) time in REM sleep during the light phase. Black bar indicates dark phase. Missing values occur while the rats were removed from the recording room for treatment. No significant differences detected between SPS BL and SHAM BL. Data are depicted as mean + SEM. Comparison between treatment and BL performed by repeated measures two-way ANOVA. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$ in Bonferroni *post hoc* test compared to BL.

SPS induced acute and sustained PTSD-like alterations in state-dependent qEEG power spectra in the frontal cortex.

We next tested the hypothesis that SPS would disrupt the normal qEEG power spectra within each sleep-wake state in a manner similar to that exhibited by PTSD patients. On Day 0, SPS significantly altered qEEG power spectra in the frontal cortex during light phase wake,

causing an increase in relative theta and high gamma power (black line, Figure 8A) (frequency [$F_{100,900} = 5.91$, $P < 0.0001$], interaction [$F_{100,900} = 5.91$, $P < 0.0001$]). In addition, SPS induced qEEG power spectra changes during dark phase wake, resulting in increased alpha, beta, and low gamma power, and decreased high gamma power (black line, Figure 8D) (frequency [$F_{100,900} = 33$, $P < 0.0001$], interaction [$F_{100,900} = 33$, $P < 0.0001$]).

Table 2. SPS induced persistent disturbances in sleep-wake architecture.

			Light Phase			
	SPS BL	**SPS Day 1**	**SPS Day 2**	**SPS Day 7**	***F***	P
WAKE (min/hr)	15.6 ± 0.7	18.7 ± 0.6***	17.8 ± 0.4*	16.2 ± 0.8	7.80	<.001
NREM (min/hr)	37.2 ± 0.8	35.7 ± 0.5*	35.9 ± 0.5	36.9 ± 0.6	3.11	.043
REM (min/hr)	7.1 ± 0.3	5.6 ± 0.4***	6.1 ± 0.3*	6.9 ± 0.4	10.0	<.001
WAKE bouts/hr	10.8 ± 0.7	11.4 ± 0.8	12.4 ± 0.7	10.9 ± 1.0	2.21	.110
NREM bouts/hr	11.1 ± 0.7	11.8 ± 0.8	13.0 ± 0.6*	11.1 ± 1.0	3.63	.026
REM bouts/hr	4.1 ± 0.2	3.4 ± 0.2	4.1 ± 0.2	4.0 ± 0.4	2.25	.106
WAKE bout (min)	1.3 ± 0.1	1.6 ± 0.1*	1.4 ± 0.1	1.4 ± 0.1	2.88	.054
NREM bout (min)	3.5 ± 0.3	3.1 ± 0.2	2.8 ± 0.2**	3.1 ± 0.2	5.43	.005
REM bout (min)	1.8 ± 0.1	1.6 ± 0.1	1.5 ± 0.1	1.7 ± 0.1	2.62	.071
			Dark Phase			
	SPS BL	**SPS Day 1**	**SPS Day 2**	**SPS Day 7**	***F***	P
WAKE (min/hr)	37.2 ± 1.0	30.6 ± 2.2***	31.6 ± 1.4*	33.6 ± 1.7	7.09	.001
NREM (min/hr)	19.9 ± 0.8	23.8 ± 1.8**	24.1 ± 1.2*	22.8 ± 1.5	6.27	.002
REM (min/hr)	2.9 ± 0.3	5.5 ± 0.7***	4.3 ± 0.3	3.6 ± 0.3	7.34	<.001
WAKE bouts/hr	8.4 ± 0.3	8.8 ± 0.6	8.9 ± 0.9	8.6 ± 0.6	.257	.856
NREM bouts/hr	8.4 ± 0.3	9.0 ± 0.6	9.1 ± 0.9	8.8 ± 0.6	.508	.680
REM bouts/hr	2.5 ± 0.2	3.0 ± 0.2	2.8 ± 0.2	2.7 ± 0.3	1.14	.350
WAKE bout (min)	4.3 ± 0.3	3.5 ± 0.3	4.1 ± 0.5	4.7 ± 0.7	1.86	.160
NREM bout (min)	2.4 ± 0.1	2.8 ± 0.1	2.6 ± 0.2	2.5 ± 0.2	1.80	.172
REM bout (min)	1.2 ± 0.1	1.5 ± 0.1***	1.3 ± 0.0	1.2 ± 0.1	10.7	<.001

Table 3. SHAM treatment had no persistent effect on sleep-wake architecture.

	Light Phase					
	SHAM BL	**SHAM Day 1**	**SHAM Day 2**	**SHAM Day 7**	***F***	P
WAKE (min/hr)	16.8 ± 0.7	16.5 ± 0.7	16.3 ± 0.6	14.8 ± 0.8	2.59	.125
NREM (min/hr)	35.6 ± 0.8	36.2 ± 0.6	36.7 ± 0.6	37.5 ± 0.8	1.76	.181
REM (min/hr)	7.5 ± 0.3	7.4 ± 0.3	7.0 ± 0.3	7.6 ± 0.3	1.06	.385
WAKE bouts/hr	12.8 ± 0.7	12.9 ± 0.7	12.8 ± 1.0	11.6 ± 1.1	1.51	.238
NREM bouts/hr	13.5 ± 0.8	13.4 ± 0.6	13.2 ± 0.9	12.3 ± 0.9	1.39	.270
REM bouts/hr	5.3 ± 0.3	5.0 ± 0.3	5.2 ± 0.4	5.5 ± 0.3	.914	.449
WAKE bout (min)	1.3 ± 0.1	1.3 ± 0.1	1.3 ± 0.1	1.3 ± 0.1	.350	.790
NREM bout (min)	2.8 ± 0.2	2.8 ± 0.2	2.9 ± 0.2	3.2 ± 0.3	2.60	.076
REM bout (min)	1.5 ± 0.1	1.5 ± 0.1	1.4 ± 0.1	1.4 ± 0.1	.603	.620
	Dark Phase					
	SHAM BL	**SHAM Day 1**	**SHAM Day 2**	**SHAM Day 7**	***F***	P
WAKE (min/hr)	39.8 ± 0.9	37.5 ± 1.7	37.9 ± 1.4	37.7 ± 0.7	2.22	.111
NREM (min/hr)	17.6 ± 0.9	19.5 ± 1.5	19.2 ± 1.3	19.69 ± 0.7	2.42	.091
REM (min/hr)	2.6 ± 0.2	3.0 ± 0.3	2.9 ± 0.3	2.6 ± 0.2	1.29	.300
WAKE bouts/hr	9.4 ± 0.4	11.2 ± 0.6	11.1 ± 0.5	10.8 ± 0.8	2.31	.102
NREM bouts/hr	9.3 ± 0.4	11.2 ± 0.6	11.1 ± 0.5	10.9 ± 0.8	2.41	.092
REM bouts/hr	2.6 ± 0.3	2.8 ± 0.3	2.8 ± 0.3	2.8 ± 0.2	.208	.890
WAKE bout (min)	4.4 ± 0.3	3.5 ± 0.3	3.6 ± 0.3	3.7 ± 0.3	2.06	.132
NREM bout (min)	1.9 ± 0.1	1.8 ± 0.1	1.7 ± 0.1	1.9 ± 0.1	.485	.696
REM bout (min)	1.0 ± 0.0	1.1 ± 0.0	1.0 ± 0.0	1.0 ± 0.1	1.14	.353

During light phase NREM sleep on Day 0, SPS increased delta and theta power, and decreased relative power in the higher frequencies (black line, Figure 8B) (frequency [$F_{100,900} = 9.31$, $P < 0.0001$], treatment [$F_{1,9} = 11.82$, $P = 0.0074$], interaction [$F_{100,900} = 9.31$, $P < 0.0001$]), while during dark phase NREM sleep, SPS caused a selective reduction in high gamma (black line, Figure 8E) (frequency [$F_{100,900} = 4.90$, $P < 0.0001$], treatment [$F_{1,9} = 7.61$, $P = 0.0222$], interaction [$F_{100,900} = 4.90$, $P < 0.0001$]). Finally, during light phase REM sleep, SPS increased theta and alpha power on Day 0 (black line, Figure 7C) (frequency [$F_{100,800} = 3.11$, P

< 0.0001], interaction [F100,800 = 3.11, P < 0.0001]), but decreased beta power during the dark phase (black line, Figure 8F) (frequency [F100,900 = 2.52, P < 0.0001], interaction [F100,900 = 2.52, P < 0.0001]).

These alterations in qEEG power spectra were sustained for multiple days after SPS. SPS increased beta and low gamma power, and decreased high gamma power during light phase wake (colored lines, Figure 8A) (frequency [F100,900 = 4.56, P < 0.0001], interaction [F300,2700 = 3.81, P < 0.0001]), and dark phase wake (colored lines, Figure 8D) (frequency [F100,900 = 17.96, P < 0.0001], interaction [F300,2700 = 10.32, P < 0.0001]) over the entire 7 day time course. Delta power was significantly reduced for at least two days post-SPS during light phase NREM sleep, (colored lines, Figure 8B) (frequency [F100,900 = 5.02, P < 0.0001], treatment [F3,27 = 9.07, P = 0.0003], interaction [F300,2700 = 5.46, P < 0.0001]), and more moderately during dark phase NREM sleep (colored lines, Figure 8E) (frequency [F100,900 = 6.05, P < 0.0001], treatment [F3,27 = 3.34, P = 0.0341], interaction [F300,2700 = 4.44, P < 0.0001]). Most alterations in qEEG power spectra during REM sleep normalized by Day 2 with the exception of a sustained decrease in delta during the light phase (colored lines, Figure 8C) (interaction [F300,2700 = 2.16, P < 0.0001]), and dark phase (colored lines, Figure 8F) (interaction [F300,2700 = 1.15, P = 0.0465]).

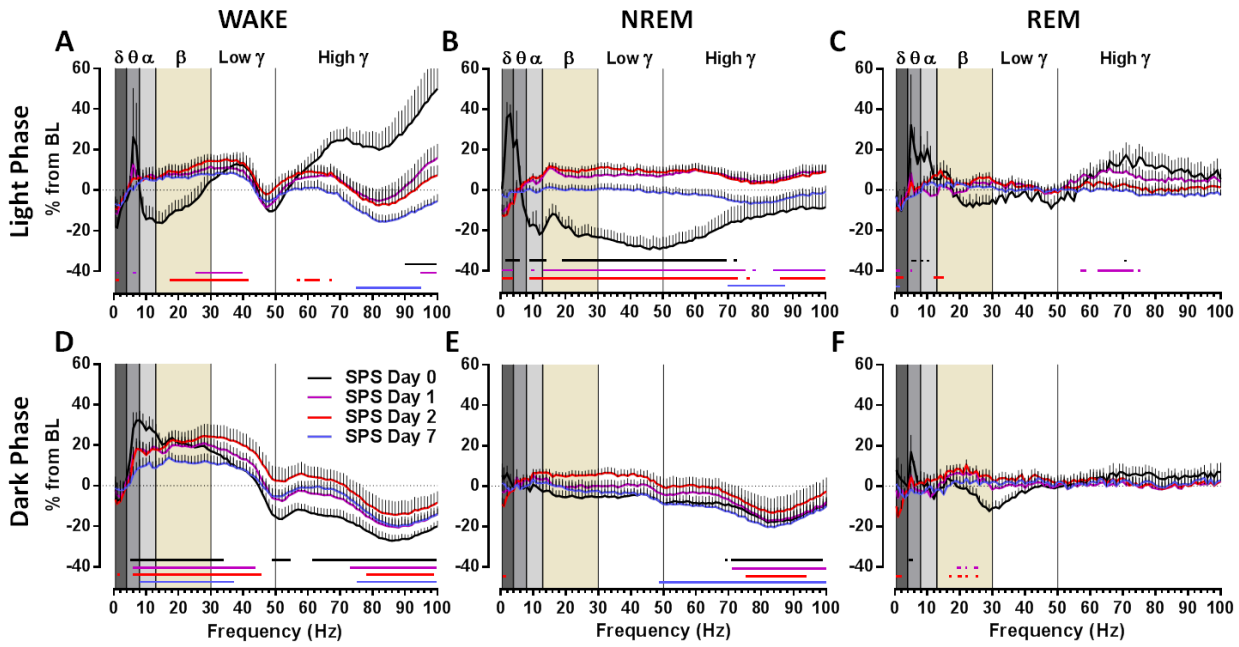

Figure 8. SPS induced acute and sustained alterations in qEEG power spectra in the frontal cortex. In the light phase (top panels), SPS caused (A) a transient increase in high gamma, and a prolonged increase in low gamma during wake; (B) an acute rebound, but a persistent subsequent reduction in delta power during NREM sleep; and (C) a prolonged decrease in delta power during REM sleep. In the dark phase (bottom panels), SPS caused (D) an increase in theta, alpha, and low gamma with a sustained increase in beta, and a sustained decrease in high gamma during wake; (E) a prolonged reduction in high gamma during NREM sleep; and (F) an acute increase in theta during REM sleep. Day 0 only includes values from remaining hours of the light phase immediately after SPS treatment. Data are depicted as mean + SEM (n = 9-10). Background shades delineate power bands delta (δ), theta (θ), alpha (α), beta (β), low and high gamma (γ). Comparison between treatment and BL performed by repeated measures two-way ANOVA. Colored lines below data points correspond to each day and indicate P < 0.01 in Bonferroni *post hoc* test.

SPS induced acute and sustained PTSD-like alterations in state-dependent qEEG power spectra in the parietal cortex.

SPS significantly disrupted the normal qEEG power spectra in the parietal cortex in a manner similar to that observed in the frontal cortex with a few notable differences. On Day 0, SPS significantly altered qEEG power spectra in the parietal cortex during light phase wake (black line, Figure 9A) (frequency [F100,900 = 12.19, P < 0.0001], treatment [F1,9 = 37.86, P <

0.0001] interaction [F100,900 = 12.19, P < 0.0001]) and dark phase wake (black line, Figure 9D) (frequency [F100,900 = 6.7, P < 0.0001], interaction [F100,900 = 6.7, P < 0.0001]) but did not cause reduced high gamma power as in the frontal cortex. During light phase NREM sleep, SPS caused a short-term rebound in delta power (black line, Figure 9B) (frequency [F100,900 = 7.69, P < 0.0001], treatment [F1,9 = 8.99, P = 0.0150], interaction [F100,900 = 7.69, P < 0.0001]), but had no effect during dark phase NREM sleep (black line, Figure 9E). Unlike in the frontal cortex, SPS caused a significant increase in alpha and low beta power during light phase REM sleep, (black line, Figure 9C) (frequency [F100,800 = 3.61, P < 0.0001], interaction [F100,800 = 3.61, P < 0.0001]), and dark phase REM sleep(black line, Figure 9F) (frequency [F100,900 = 8.09, P < 0.0001], interaction [F100,900 = 8.09, P < 0.0001]), but had little or no effect on theta.

Similar to the frontal cortex, SPS-induced qEEG changes were largely sustained in the parietal cortex during light phase wake (colored lines, Figure 9A) (frequency [F100,900 = 4.0, P < 0.0001], treatment [F3,27 = 9.17, P = 0.0002], interaction [F300,2700 = 5.59, P < 0.0001]) and dark phase wake (colored lines, Figure 9D) (frequency [F100,900 = 4.48, P < 0.0001], interaction [F300,2700 = 2.52, P < 0.0001]); light phase NREM, (colored lines, Figure 9B) (frequency [F100,900 = 5.65, P < 0.0001], treatment [F3,27 = 4.65, P = 0.0095], interaction [F300,2700 = 3.88, P < 0.0001]); and light phase REM (colored lines, Figure 9C) (frequency [F100,900 = 1.47, P = 0.0028], interaction [F300,2700 = 1.89, P < 0.0001]), and dark phase REM sleep (colored lines, Figure 9F) (interaction [F300,2700 = 2.13, P < 0.0001]).

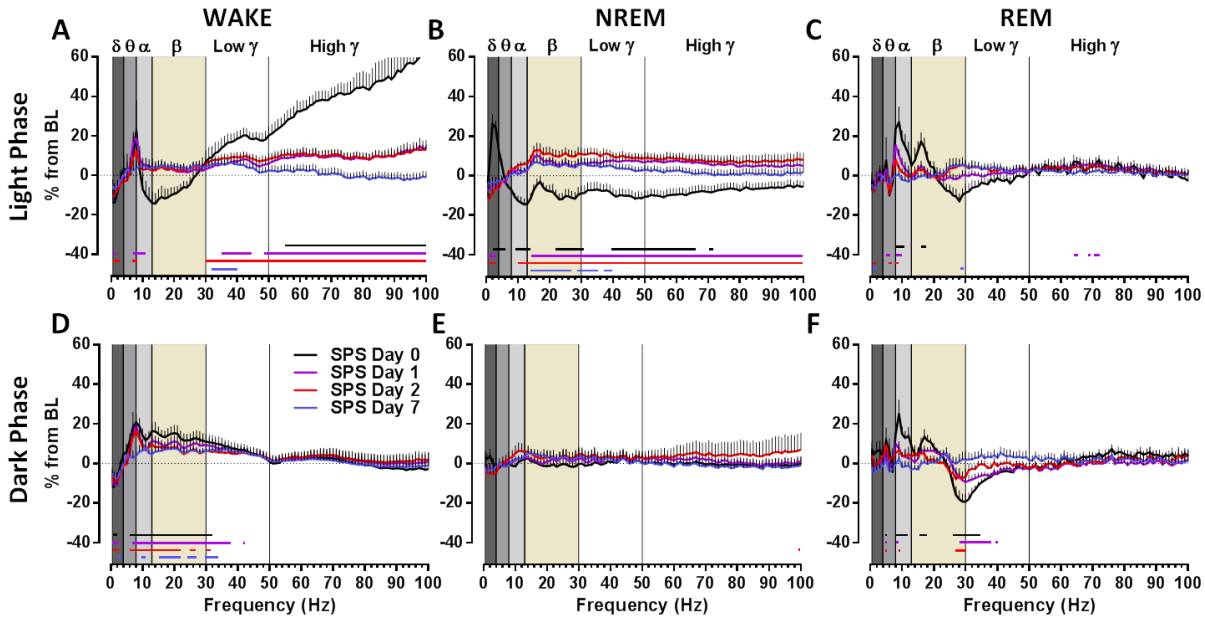

Figure 9. SPS induced acute and sustained alterations in qEEG power spectra in the parietal cortex. In the light phase (top panels), SPS caused (A) a transient increase in high gamma; (B) an acute rebound, but a persistent subsequent reduction in delta power during NREM sleep; and (C) an acute increase in alpha, and a prolonged decrease in delta power during REM sleep. In the dark phase (bottom panels), SPS caused (D) a transient increase in theta, alpha, and low gamma with a sustained increase in beta during wake; (E) no change during NREM sleep; and (F) an acute increase in alpha during REM sleep. Day 0 only includes values from remaining hours of the light phase immediately after SPS treatment. Data are depicted as mean + SEM (n = 9-10). Background shades delineate power bands delta (δ), theta (θ), alpha (α), beta (β), low and high gamma (γ). Comparison between treatment and BL performed by repeated measures two-way ANOVA. Colored lines below data points correspond to each day and indicate $P < 0.01$ in Bonferroni *post hoc* test.

SHAM treatment had minor effects on state-dependent qEEG power spectra in the frontal and parietal cortices.

In contrast to the robust and sustained effects of SPS, SHAM treatment had only minor effects on qEEG relative spectral power in the frontal cortex on Day 0 during light phase wake (Figure 10A) (frequency [F100,900 = 4.95, $P < 0.0001$], treatment [F1,9 = 5.8, $P = 0.04$], interaction [F100,900 = 4.95, $P < 0.0001$]), dark phase wake (Figure 10D) (frequency [F100,900

= 2.57, P < 0.0001], interaction [*F*100,900 = 2.57, P < 0.0001]), light phase NREM (Figure 10B) (frequency [*F*100,900 = 8.41, P < 0.0001], treatment [*F*1,9 = 8.14, P = 0.0190], interaction [*F*100,900 = 8.41, P < 0.0001]), dark phase NREM (Figure 10E) (frequency [*F*100,900 = 2.28, P < 0.0001], interaction [*F*100,900 = 2.28, P < 0.0001]), light phase REM (Figure 10C) (frequency [*F*100,900 = 1.37, P = 0.0134], interaction [*F*100,900 = 1.37, P = 0.0134]), and dark phase REM sleep (Figure 10F) (frequency [*F*100,900 = 1.36, P = 0.0139], interaction [*F*100,900 = 1.36, P = 0.0139]).

On Days 1, 2, and 7, SHAM treatment modestly altered power spectra during light phase wake (Figure 10A) (frequency [*F*100,800 = 3.34, P < 0.0001], interaction [*F*300,2400 = 1.51, P < 0.0001]), dark phase wake (Figure 10D) (frequency [*F*100,800 = 2.73, P < 0.0001], interaction [*F*300,2400 = 1.85, P < 0.0001]), light phase NREM (Figure 10B) (interaction [*F*300,2400 = 1.48, P < 0.0001]), dark phase NREM (Figure 10E) (frequency [*F*100,800 = 3.81, P < 0.0001], interaction [*F*300,2400 = 1.36, P = 0.0001]), light phase REM (Figure 10C) (frequency [*F*100,800 = 2.00, P < 0.0001], interaction [*F*300,2400 = 2.42, P = 0.0001]), and dark phase REM sleep (Figure 10F) (frequency [*F*100,800 = 3.48, P < 0.0001], interaction [*F*300,2400 = 1.98, P = 0.0001]).

In the parietal cortex on Day 0, SHAM treatment modestly altered power spectra during light phase wake (Figure 11A) (frequency [*F*100,900 = 11.79, P < 0.0001], treatment [*F*1,9 = 16.38, P = 0.0029], interaction [*F*100,900 = 11.79, P < 0.0001]), dark phase wake (Figure 11D) (frequency [*F*100,900 = 6.28, P < 0.0001], treatment [*F*1,9 = 10.33, P = 0.0106], interaction [*F*100,900 = 6.28, P < 0.0001]), light phase NREM (Figure 11B) (frequency [*F*100,900 = 4.74, P < 0.0001], treatment [*F*1,9 = 14.54, P = 0.0041], interaction [*F*100,900 = 4.74, P < 0.0001]), dark phase NREM (Figure 11E) (frequency [*F*100,900 = 2.14, P < 0.0001], interaction [*F*100,900 =

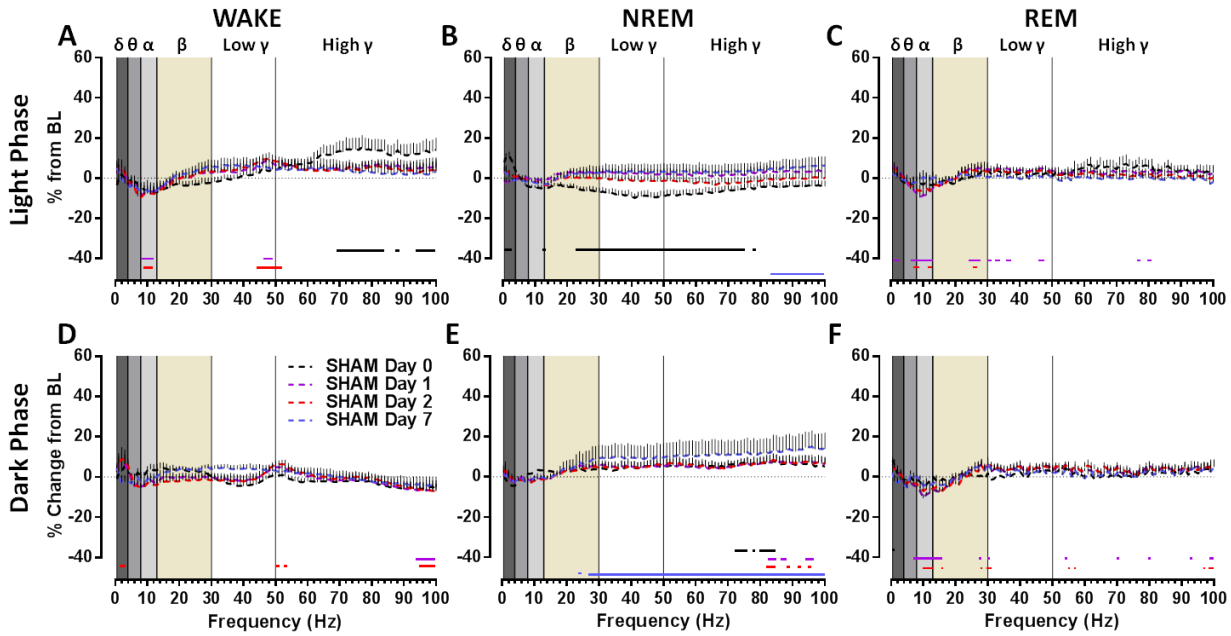

Figure 10. SHAM treatment had minor effects on qEEG power spectra in the frontal cortex. In the light phase (top panels), SHAM treatment had only minor effects during light phase (A) wake, (B) NREM, and (C) REM sleep, and during dark phase (D) wake, (E) NREM, and (F) REM sleep. Day 0 only includes values from remaining hours of the light phase immediately after SHAM treatment. Data are depicted as mean + SEM (n = 9-10). Background shades delineate power bands delta (δ), theta (θ), alpha (α), beta (β), low and high gamma (γ). Comparison between treatment and BL performed by repeated measures two-way ANOVA. Colored lines below data points correspond to each day and indicate P < 0.01 in Bonferroni *post hoc* test.

2.14, P < 0.0001]), and dark phase REM sleep (Figure 11F) (frequency [F100,900 = 1.38, P = 0.0107], treatment [F1,9 = 5.19, P = 0.0488], interaction [F100,900 = 1.38, P = 0.0107]).

On Days 1, 2, and 7, SHAM treatment modestly altered power spectra in the parietal cortex during light phase wake (Figure 11A) (frequency [F100,800 = 3.42, P < 0.0001], interaction [F300,2400 = 1.50, P < 0.0001]), dark phase wake (Figure 11D) (frequency [F100,800 = 4.20, P < 0.0001], interaction [F300,2400 = 1.66, P < 0.0001]), light phase NREM

(Figure 11B) (interaction [F300,2400 = 1.51, P < 0.0001]), and dark phase REM sleep (Figure 11F) (frequency [F100,800 = 1.45, P = 0.0044], interaction [F300,2400 = 1.30, P = 0.0010]).

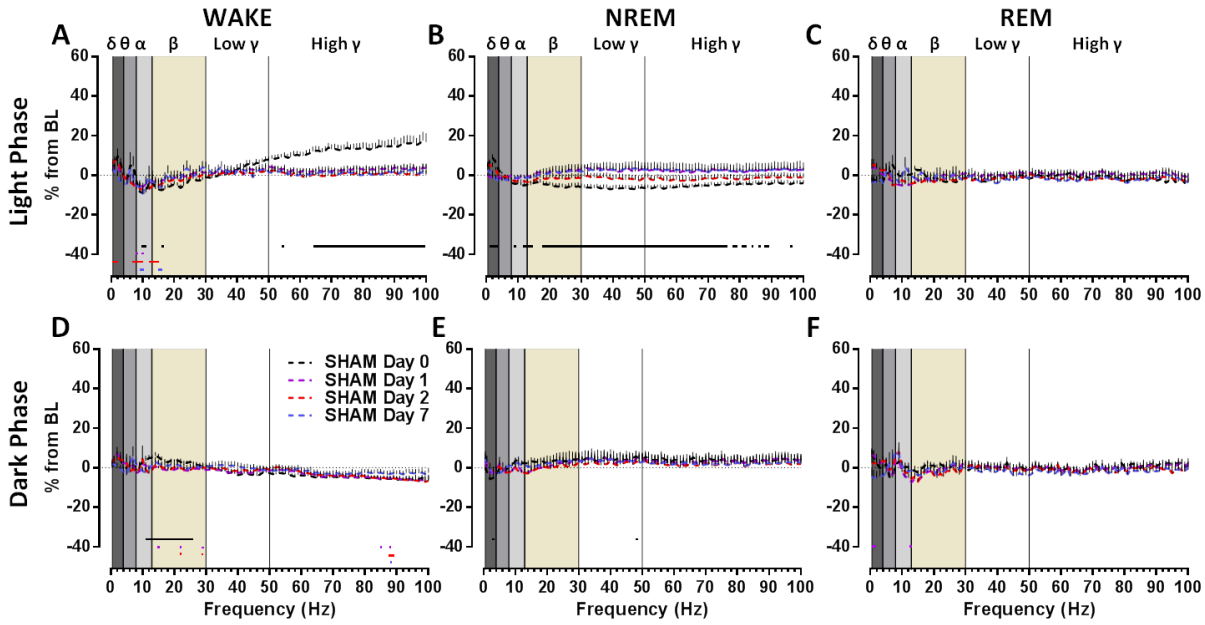

Figure 11. SHAM treatment had minor effects on qEEG power spectra in the parietal cortex. In the light phase (top panels), SHAM treatment had only minor effects during light phase (A) wake, (B) NREM, and (C) REM sleep, and during dark phase (D) wake, (E) NREM, and (F) REM sleep. Day 0 only includes values from remaining hours of the light phase immediately after SHAM treatment. Data are depicted as mean + SEM (n = 9-10). Background shades delineate power bands delta (δ), theta (θ), alpha (α), beta (β), low and high gamma (γ). Comparison between treatment and BL performed by repeated measures two-way ANOVA. Colored lines below data points correspond to each day and indicate P < 0.01 in Bonferroni *post hoc* test.

SPS induced prolonged reductions in SWA

SWA was highest during the early hours of the light phase, and gradually reduced across the quiescent period (Figure 12A), consistent with dissipation of sleep drive (232). Relative to BL, SPS increased SWA on Day 0 (treatment [F1,50 = 42.74, P < 0.0001]) consistent with the

rebound effects of sleep deprivation (232), but produced significantly decreased SWA on Days 1 and 2 post-SPS treatment (Figure 12A) (time [F15,215 = 24.33, P < 0.0001], treatment [F15,215 = 14.19, P < 0.0001]), especially during the early hours of the light phase. By contrast, relative to BL, SHAM increased SWA on Day 0 (time [*F*2,54 = 32.03, P < 0.0001], interaction [*F*2,54 = 5.68, P = 0.0058], treatment [*F*1,54 = 42.23, P < 0.0001]) consistent with the rebound effects of sleep deprivation (232), but produced no prolonged effect on SWA (Figure 12B). No SWA differences were detected between SPS BL and SHAM BL.

SPS induced an acute and persistent physiological stress response.

Given the magnitude and duration of SPS-induced sleep-wake and qEEG disruptions, we measured concomitant changes in several validated measures of the rodent stress response, including hyperthermia, corticosterone release, and brain regional FKBP5 induction (66, 314). SPS induced acute and persistent hyperthermia for several days post-SPS (Figure 13A) during wake (Day 0: hour [F8,158 = 3.2, P = 0.0022], hour [F8,158 = 3.2, P = 0.0022]; Days 1,2,7: interaction [F33,430 = 1.69, P = 0.0116], treatment [F3,430 = 4.67, P < 0.0032], hour [F11,430 = 5.29, P < 0.0001]), NREM (Day 0: hour [F8,155 = 2.13, P = 0.0357], treatment [F1,155 = 4.11, P < 0.0443], interaction [F8,155 = 2.13, P = 0.0357]; Days 1,2,7: interaction [F33,424 = 1.85, P = 0.0035], treatment [F3,424 = 6.5, P < 0.0003], hour [F11,424 = 5.78, P < 0.0001]), and REM

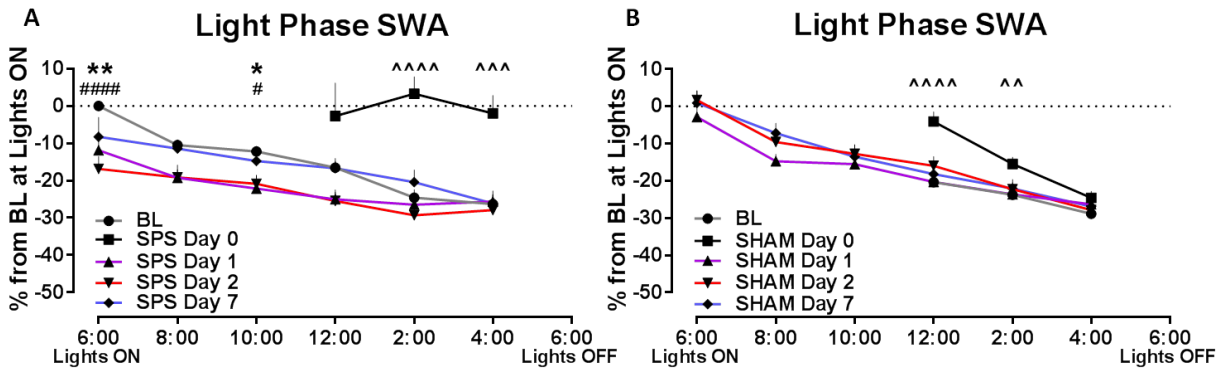

Figure 12. SPS induced prolonged reductions in slow wave activity (SWA). (A) During the light (rodent quiescent) phase, SPS caused an initial rebound in SWA immediately after SPS, but subsequently reduced SWA for up to two days post-SPS. Data are depicted as mean - SEM (n = 9-10). ^^^ P < 0.001, ^^^^ P < 0.0001, Day 0 vs. BL; * P < 0.05, ** P < 0.01, Day 1 vs. BL; # P < 0.05, #### P < 0.0001, Day 2 vs. BL in Bonferroni *post hoc* test. (B) SHAM treatment had no prolonged effect on SWA. During the light (rodent quiescent) phase, SHAM treatment caused an initial rebound in slow wave activity (SWA), but subsequently had no effect. Data are depicted as mean + SEM (n = 9-10). ^^ P < 0.01, ^^^^ P < 0.0001, Day 0 vs. BL in Bonferroni *post hoc* test.

sleep (Day 0: hour [F8,118 = 2.84, P = 0.0064], treatment [F1,118 = 16.82, P < 0.0001], interaction [F8,118 = 2.84, P = 0.0064]; Days 1,2,7: interaction [F33,398 = 1.56, P = 0.0279], treatment [F3,398 = 5.91, P < 0.0006], hour [F11,398 = 6.63, P < 0.0001]), specifically during the light phase. In contrast to SPS, SHAM treatment had only minor effects on body temperature (Figure 13B) during wake (Day 0: hour [F8,162 = 4.07, P = 0.0002], treatment [F1,162 = 23.24, P < 0.0001], interaction [F8,162 = 4.07, P = 0.0002]; Days 1,2,7: hour [F11,418 = 4.2, P < 0.0001], treatment [F3,418 = 4.35, P = 0.005]), NREM (Day 0: hour [F8,162 = 3.42, P = 0.0012], treatment [F1,162 = 15.79, P = 0.0001], interaction [F8,162 = 3.42, P = 0.0012]; Days 1,2,7: hour [F11,420 = 4.00, P < 0.0001], treatment [F3,420 = 4.13, P = 0.0066]), and REM sleep (Days 1,2,7: hour [F11,385 = 2.37, P = 0.0077]).

In parallel with body temperature increases, SPS rats exhibited robust acute HPA axis activation as evidenced by elevated plasma corticosterone (Figure 14A) [$F_{3,29} = 14.67$, $P < 0.0001$]. In addition, there was a concurrent acute induction of FKBP5 mRNA levels in the brain regions that comprise the neural fear circuitry (Figure 14B) including the hippocampus [$F_{3,28} = 40.84$, $P < 0.0001$], PFC [$F_{3,28} = 25.43$, $P < 0.0001$], and amygdala [$F_{3,27} = 36.46$, $P < 0.0001$].

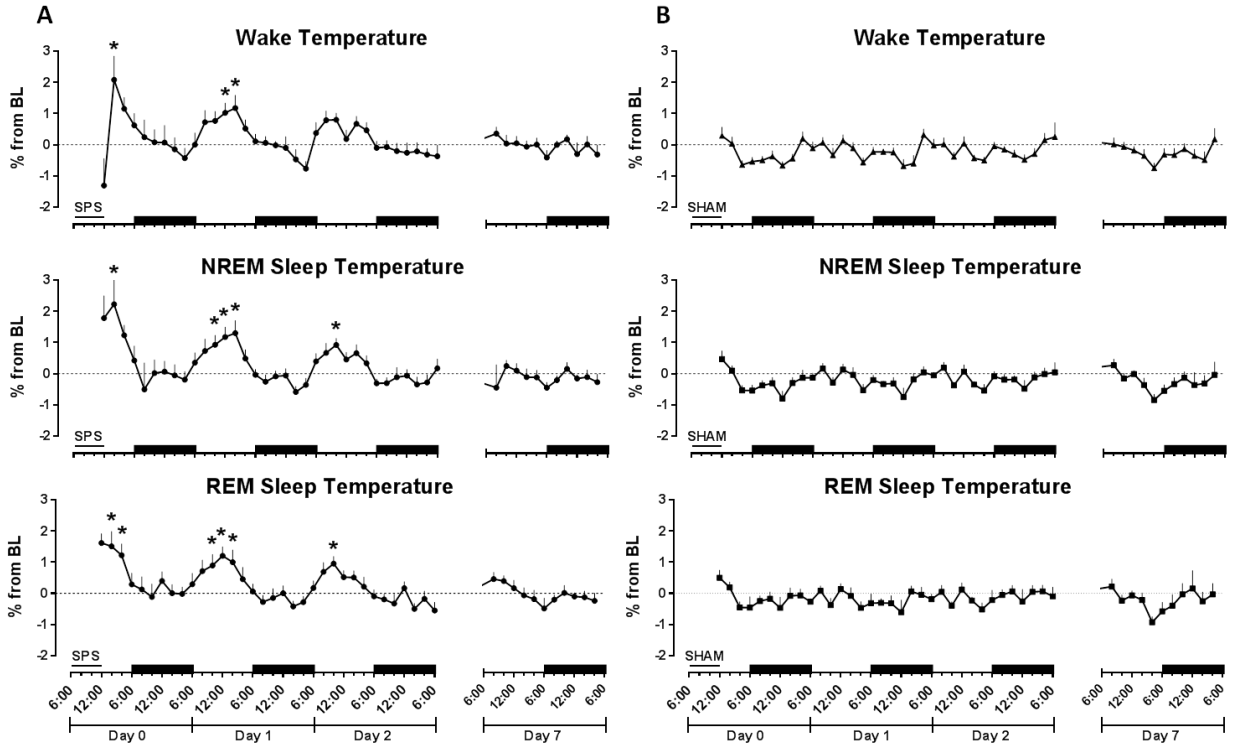

Figure 13. SPS induced acute and persistent hyperthermia. SPS caused (A) increases in body temperature during the light phase of all sleep-wake states that lasted until Day 2. Black bars indicate dark phases. SHAM treatment (B) moderately reduced temperature relative to BL at various time points after treatment. Data are depicted as mean + SEM (n = 9-10). Comparison between treatment and BL performed by two-way ANOVA. * $P < 0.05$ in Bonferroni *post hoc* test.

SPS caused acute and sustained alterations in brain regional 5-HT utilization.

Due to the well-established role of 5-HT in modulating anxiety and sleep-wake architecture (233), we tested whether the observed SPS-induced sleep-wake and qEEG power spectra changes were associated with altered 5-HT signaling. SPS produced acute increases in the levels of the 5-HT metabolite, 5-HIAA, in the PFC (Figure 14A) [$F_{3,32} = 29.31$, $P < 0.0001$] and hippocampus (Figure 15B) [$F_{3,32} = 8.70$, $P = 0.0002$], and prolonged reductions in the amygdala (Figure 15C) [$F_{3,32} = 5.98$, $P = 0.0023$] with no effect on 5-HT levels across the three brain regions (Figure 15D-F).

SPS caused delayed reductions in amygdala expression of NPY.

As previously discussed, NPY signaling in the amygdala plays a critical role in modulating the stress response (307); thus, we hypothesized that SPS would alter expression of NPY and its Y1 and Y2 receptor subtypes specifically in the amygdala. SPS caused NPY mRNA levels to be significantly reduced in the amygdala by Day 7 post-SPS (Figure 16A) [$F_{3,26} = 4.94$, $P = 0.0076$], but had no effect on Y1 or Y2 mRNA levels (Figures 16B,C).

Results summary

SPS produced robust alterations in sleep-wake architecture accompanied by state-dependent changes in qEEG power spectra that resemble PTSD symptomatology. These changes corresponded with time-dependent and brain region-specific alterations in physiological markers of HPA axis activation, 5-HT utilization, and NPY expression, suggesting key alterations in the neural fear circuitry that may potentially underlie PTSD-related hyperarousal and sleep-wake disturbances.

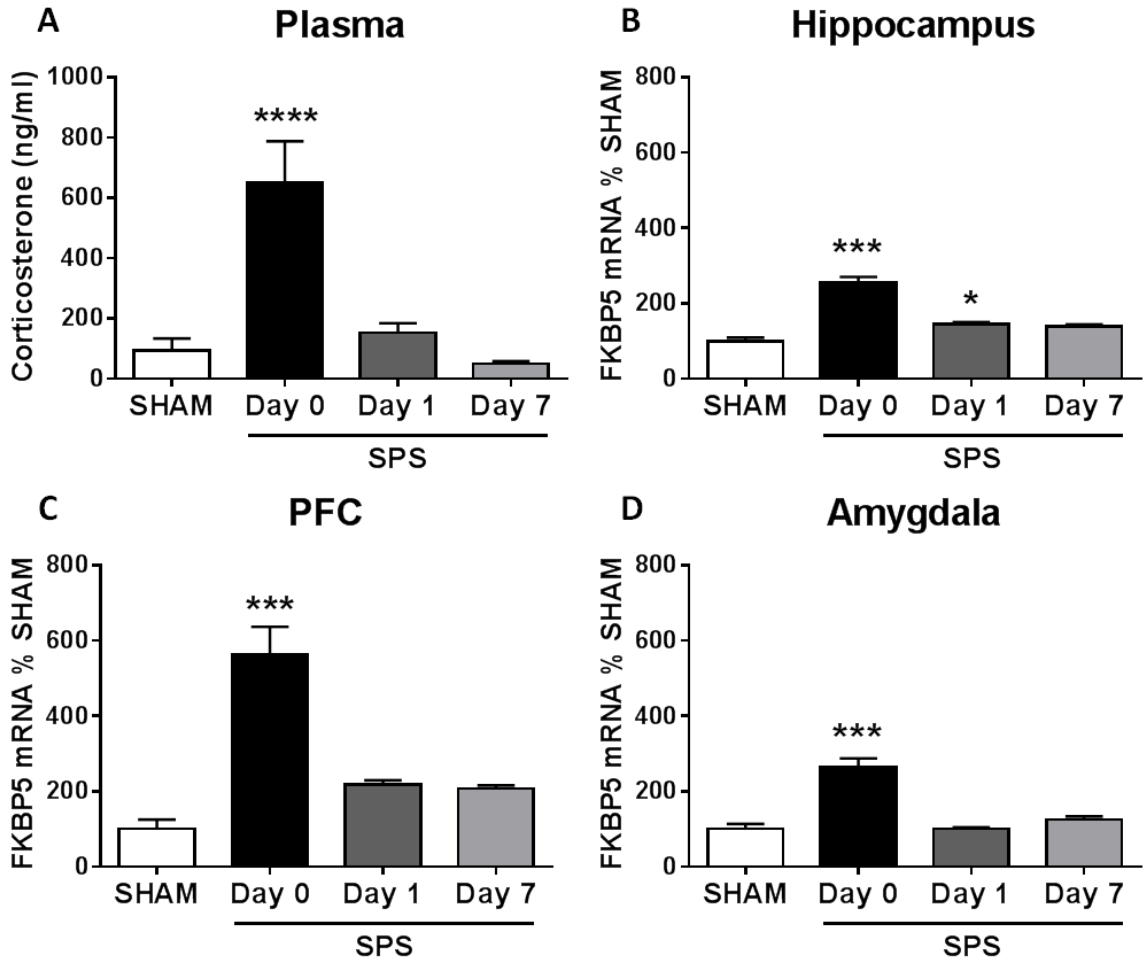

Figure 14. SPS induced an acute and persistent physiological stress response. SPS acutely increased plasma corticosterone concentrations (A), and FKBP5 mRNA levels in the hippocampus (B), PFC (C), and amygdala (D). Data are depicted as mean + SEM (n = 8-9). Comparison between SHAM and SPS Day performed by one-way ANOVA. * $P < 0.05$, *** $P < 0.001$, **** $P < 0.0001$ in Dunnett's *post hoc* test compared to SHAM.

Discussion

Our current findings demonstrate SPS-induced dysregulation and fragmentation of NREM and REM sleep that mirror the abnormal sleep-wake patterns of recently traumatized individuals (287, 288) and patients with chronic PTSD (296). The observed changes in sleep-wake architecture on Day 0 post-SPS likely represent the immediate effects of traumatic stress,

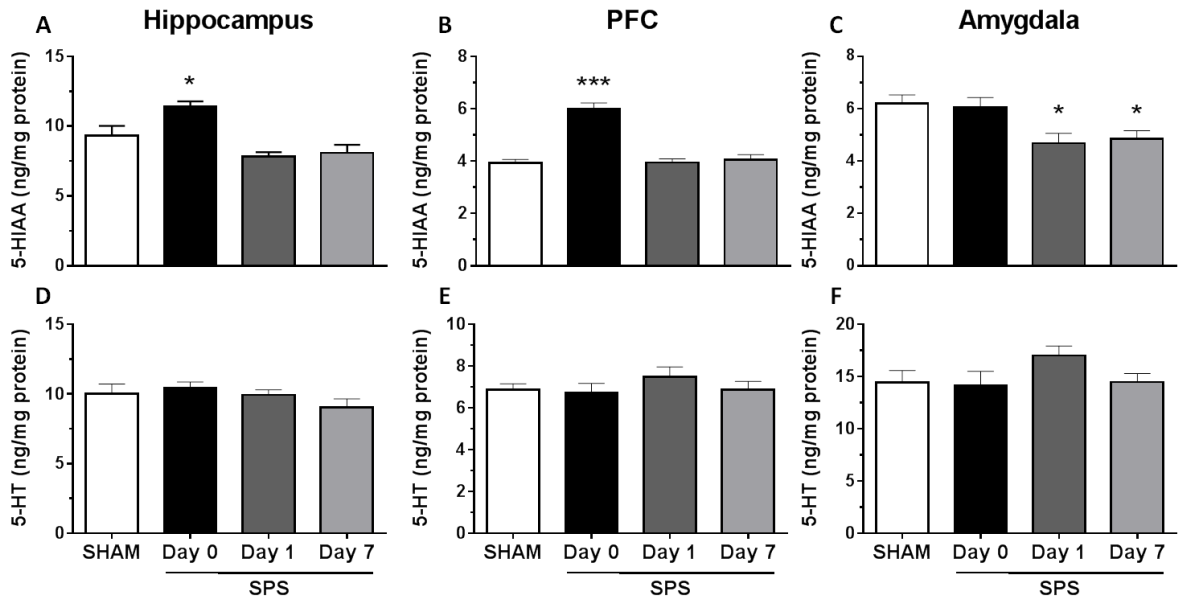

Figure 15. SPS caused acute and sustained alterations in brain regional 5-HT utilization. Concentration of the 5-HT metabolite 5-HIAA was increased by SPS on Day 0 in (A) the PFC and (B) the hippocampus, but decreased on Days 1 and 7 in (C) the amygdala. (D-F) 5-HT levels in these regions were not significantly affected. Data are depicted as mean + SEM (n = 8-9). Comparison between SHAM and SPS Day performed by one-way ANOVA. *P < 0.05, ** P < 0.01 in post-hoc Dunnett's test versus SHAM.

which have not yet been objectively assessed in traumatized clinical populations. As mentioned above, the amount of NREM and especially REM sleep accumulated in healthy subjects immediately following emotional learning imparts strong and lasting benefits to the consolidation and subsequent recall of these memories (235, 236, 240), suggesting that acute SPS-induced reductions in sleep may actually represent a protective response in the hours after traumatic stress on Day 0. In contrast, the significant fragmentation of NREM sleep coupled with increased wake time during the quiescent phases observed on Days 1 and 2 closely resemble documented sleep disruptions in chronic PTSD patients (2, 287, 288, 296, 318). These insomnia-like reductions in NREM and REM sleep on Days 1 and 2, therefore, may be

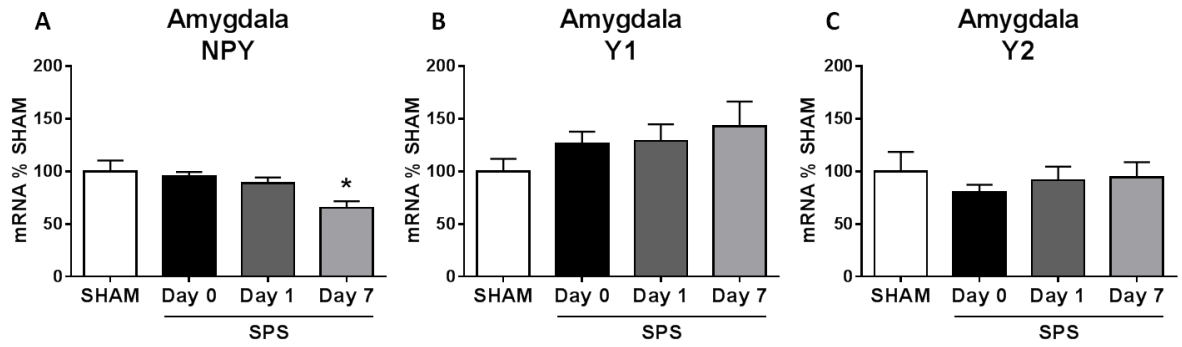

Figure 16. SPS caused acute and sustained alterations in amygdala expression of neuropeptide Y (NPY). mRNA levels of NPY were reduced on Day 7 after SPS, while NPY receptor mRNA levels were not significantly altered in the amygdala. Data are depicted as mean + SEM (*n* = 8-9). Comparison between SHAM and SPS Day performed by one-way ANOVA. ** $P < 0.01$ in post-hoc Dunnett's test versus SHAM.

representative of the sleep symptoms present in patients with established disease, and are unlikely to be part of an early adaptive response to trauma.

The SPS-induced alterations in qEEG power spectra during wake, NREM and REM sleep also recapitulate many of the qEEG abnormalities reported in PTSD patients. Augmentation of beta/low gamma power during wake in the active phase was one of the most enduring effects of SPS, lasting for at least 7 days. In chronic PTSD patient populations, increased waking beta/low gamma power has been reported both at rest (297, 299), and in response to affective stimuli (298), although this finding was absent in one study (319). Reductions in waking high gamma power were also sustained for 7 days post-SPS, a change that was specific to frontal cortical regions as it was absent in recordings from the parietal cortex. A recent fMRI study demonstrated that increases in high gamma power are correlated with activation of corresponding cortical regions (320). The current finding of SPS-induced reductions in this power band specifically in the frontal cortex, therefore, could be indicative of PFC hypoactivity, a commonly reported finding in PTSD patients (29).

During NREM sleep, SPS caused a prolonged, but not acute, increase in beta/gamma power, and a decrease in SWA that mirrors similar deficits in patients with PTSD (300, 301), and likely indicates poor sleep quality (321). Interestingly, despite causing reduced SWA on Days 1 and 2, SPS acutely caused a large increase in SWA on Day 0, again suggesting that rats may exhibit failed protective responses immediately after SPS, prior to the development of PTSD-like symptoms days later. This finding further supports the interpretation that the previously discussed sleep-wake architecture changes present on Days 1 and 2 represent sleep disturbances related to established PTSD. Impaired SWA has also been associated with impairments in sleep-dependent fear extinction memory, a robust PTSD-like behavioral effect of SPS (59). Pharmacologically augmenting SWA, therefore, could represent a therapeutic approach for PTSD patients or recently traumatized individuals that can be tested in this model. In the case of REM sleep, the most significant change in frontal cortical qEEG spectral power was an acute increase in frontal theta power which is thought to promote emotional memory consolidation in rodents (250) and humans (240), perhaps contributing to the subsequent development of PTSD-like symptoms in the SPS model. Future experiments correlating SPS-induced alterations in theta power during REM sleep with subsequent anxiety-like behaviors will be critical for understanding the function of these state-specific oscillations in the processing of traumatic events.

In addition to causing sustained qEEG deficits indicative of hyperarousal, we confirmed that SPS concomitantly and robustly induces markers of HPA axis activation. For example, SPS induced hyperthermia during all sleep-wake states, and caused substantial increases in plasma corticosterone, consistent with previous findings (322). Corticosterone release was accompanied

by induction of FKBP5 expression, a gene that has been associated with PTSD risk, diagnosis, and treatment (67-69), and which may contribute to acute SPS-induced sleep loss (323).

SPS-induced reductions in NREM and REM sleep during the quiescent phase could also be explained by acute increases in 5-HT utilization in the PFC and hippocampus, brain structures implicated in the modulation of sleep-wake architecture (3). This hypothesis is supported by reports that other acute stressors cause 5-HT release in multiple brain regions including the cortex, leading to inhibition of sleep (324). In the amygdala, however, SPS did not increase 5-HT utilization, but rather induced a delayed decrease on Days 1 and 7 which correlated with sustained increases in relative beta/low gamma power during wake. 5-HT exerts a net inhibitory influence on the excitability of lateral amygdala neurons (325, 326) which, when directly stimulated, can induce high frequency EEG oscillations (327) highly comparable to the long-lasting effects of SPS. This finding may help to explain the partial efficacy of SSRIs on hyperarousal symptoms in PTSD patients and their behavioral correlates in SPS-treated rats (82, 328). Similar to 5-HT, SPS caused a delayed reduction in expression of amygdala NPY which also acts to inhibit the firing of projection neurons in the lateral amygdala (329). Importantly, this finding is consistent with previous studies demonstrating the therapeutic efficacy of exogenous NPY administration in SPS-treated rats (330, 331). Moreover, intracerebroventricular infusion of NPY in rats increases low frequency and decreases beta frequency qEEG spectral power (332, 333), in direct opposition to the long-term effects of SPS. The changes in NPY expression reported here were at the level of mRNA, however, and may not translate into reductions in amygdala peptide concentration, or more importantly, peptide release.

Collectively, we have demonstrated that SPS, a rodent model of traumatic stress, leads to alterations in sleep-wake architecture and state-dependent qEEG spectral power that correlate

with regional changes in 5-HT utilization and NPY expression, providing new understanding of possible mechanisms underlying the pathophysiology of PTSD-related hyperarousal and sleep disturbances. Taken together, the observed alterations in sleep-wake architecture also offer novel insight into the acute effects of trauma while simultaneously recapitulating longer term PTSD symptoms, thereby providing an attractive model for testing the efficacy of sleep-dependent prophylactic interventions in the aftermath of a traumatic event.

CHAPTER 3

SELECTIVE ANTAGONISM OF $mGluR_5$ MODULATES SLEEP-WAKE ARCHITECTURE AND AMELIORATES BEHAVIORAL ABNORMALITIES INDUCED BY TRAUMATIC STRESS IN RATS

Introduction

The proximal cause of PTSD is the acquisition and consolidation of a traumatic memory, suggesting that modulation of traumatic memory formation or maintenance may be a viable approach to the prevention or treatment of PTSD symptoms (14, 16, 18, 20). Pharmacological manipulation of glutamatergic signaling before, during, or after trauma offers a promising target for this strategy. Glutamate-mediated activation of NMDA receptors, and ensuing induction of synaptic plasticity in the hippocampus, PFC, and amygdala, is required for the successful acquisition and consolidation of threat-based memories (33, 35). These preclinical findings suggest that systemic NMDA receptor antagonism in the aftermath of trauma could impede the development of PTSD symptoms.

As mentioned above, NMDA receptor activation may promote consolidation of fear-based memory in part through the induction of REM sleep (34, 225, 226, 234-239). NMDA receptor antagonists reduce REM sleep in rats (256, 257, 264, 268) and humans (263, 277, 278), possibly contributing to their blockade of emotional memory consolidation. Unfortunately, the adverse effects associated with currently available NMDA receptor antagonists including drowsiness, hallucinations, and abuse potential limit their clinical utility (87, 88). An alternative strategy to direct blockade of NMDA receptors is to inhibit NMDA receptor-mediated signaling

through antagonism of $mGluR_5$, which is structurally and functionally coupled to NMDA receptors in the hippocampus, PFC, and amygdala (99-101, 103, 106, 107, 294). $mGluR_5$ antagonism with the prototypical $mGluR_5$ NAM MPEP has been shown to attenuate NMDA receptor-mediated signaling and to induce similar physiological and behavioral effects of NMDA receptor antagonists but with fewer adverse effects (101, 334, 335). Importantly, MPEP and other $mGluR_5$ antagonists potently reduce REM sleep similar to NMDA receptor blockers (253, 258).

Recently, our group reported the discovery of VU0409106, a novel, potent and selective $mGluR_5$ NAM with favorable pharmacokinetic properties for *in vivo* testing (223, 295, 336). *In vitro* evaluation of VU0409106 has revealed that this compound competitively binds at the allosteric MPEP binding site, completely attenuates glutamate-mediated calcium mobilization (IC_{50} = 24nM) in HEK293A cells heterologously expressing $mGluR_5$, and displays no significant off-target activity at any of the other seven mGluRs (295). Importantly, positron emission tomography (PET) imaging in rats has revealed that VU0409106 is brain penetrant, occupying about 50% of $mGluR_5$ after 10 mg/kg intraperitoneal (i.p.) dosing, and displays behavioral efficacy in a rat model of anxiety (223).

Thus, VU0409106 provides us with the opportunity to determine whether selective $mGluR_5$ inhibition immediately after traumatic stress can impede the development of PTSD-like symptoms in rats, possibly through attenuation of sleep-dependent emotional memory consolidation. In order to test this hypothesis, we first examined the effects of systemic VU0409106 administration on sleep-wake architecture and state-dependent qEEG power spectra in healthy rats. Then, we took advantage of the findings described in Chapter 2, and tested the effect of post-trauma VU0409106 treatment on SPS-induced alterations in contextual CF, sleep-

wake architecture, qEEG power spectra, body temperature, and amygdala 5-HT utilization, as well as the induction of amygdala early growth response protein 1 (EGR-1), a key molecular mediator of emotional memory consolidation (33).

Methods

Subjects

All male Sprague-Dawley rats (Harlan, Indianapolis, IN) used in the present studies were housed under a 12 hour light:12 hour dark cycle and given ad libitum access to food and water. All animal experiments were approved by the Vanderbilt University Animal Care and Use Committee and experimental procedures conformed to guidelines established by the National Research Council Guide for the Care and Use of Laboratory Animals. All efforts were made to minimize animal suffering and the number of animals used.

Compound

3-fluoro-N-(4-methylthiazol-2-yl)-5-(pyrimidin-5-yloxy)benzamide (VU0409106) was synthesized in house as previously described (295), and dissolved in 10% Tween 80 vehicle, creating a microsuspension prior to intraperitoneal (i.p.) administration at a volume of 2mL/kg.

EEG Surgery

Rats (250-375 grams) were surgically implanted with a telemetric transmitter (4-ET, Data Sciences International, St. Paul, MN) for recording EEG, electromyography (EMG), and body temperature. Under isoflurane anesthesia (3% induction; 1.5-2.5% maintenance) the transmitter

was implanted subcutaneously across the back of each rat. Transmitter leads were tunneled subcutaneously to the skull. After holes were drilled in the skull, the exposed wires were placed in contact with the dura and secured in place with dental cement (Butler Schein, Dublin, OH). Three sets of leads were placed bilaterally to record from cortical regions corresponding to the frontal, parietal, and occipital cortices (+2 mm, -2 mm, and -6mm anterior-posterior from Bregma, respectively and +/- 2 mm lateral to the midline). An additional set of leads was placed bilaterally in the nuchal muscles for EMG recording. Rats were individually housed following surgery and allowed to recover and acclimate to the recording room for a minimum of 10 days prior to testing.

EEG

For experiments testing the effect of VU0409106 on sleep-wake architecture and qEEG spectral power in non-stressed rats, each rat was randomized into vehicle, 3mg/kg VU0409106, 10mg/kg VU0409106, or 30mg/kg VU0409106 dose groups. Baseline recordings were begun at the start of the light phase, then the appropriate compound was administered two hours later, and recordings were allowed to continue for the remainder of the twenty-four hour period. In a partial crossover design, each rat received two different doses or vehicle, allowing for a 5 day washout period between compound administrations.

Spontaneous locomotor activity

Spontaneous locomotor activity was conducted in open-field chambers (27 × 27 × 20 cm) (Hamilton Kinder) equipped with 16 horizontal (x- and y-axes) infrared photobeams. Changes in locomotor activity were measured as the number of photobeam breaks per five minutes, and

were recorded with a Pentium I computer equipped with rat activity monitoring system software (Hamilton Kinder). Rats were pretreated with vehicle or VU0409106, (3, 10, or 30 mg/kg, i.p.) then placed individually into each chamber 30 minutes later. Locomotor activity was assessed for thirty minutes.

Rotarod

The effects of VU0409106 on motor performance were evaluated by using a rotarod (MED Associates, St. Albans, VT). All rats were given an initial training trial of 120 seconds, followed by two additional training trials of 85 seconds, approximately 10 min apart, using a rotarod (7.5 cm in diameter) rotating at a constant speed of 20 revolutions/min. After initial training trials, a baseline trial of 120 s was conducted, and any rats that did not reach the 120 second criteria were excluded from the study. Rats were then treated with vehicle or VU0409106 (3, 10, or 30 mg/kg i.p.), and tested 30 min later. The time each animal remained on the rotarod was recorded, and animals that did not fall off of the rotarod were given a maximal score of 120 seconds.

Experimental design for post-trauma intervention studies

For determining the effects of VU0409106 on SPS-induced alterations in the CF response, rats were randomized to receive either SPS or SHAM treatment followed immediately by administration of vehicle or VU0409106, (3, 10, or 30 mg/kg, i.p.). Fifteen days later, all rats underwent fear conditioning; one day after that, they were tested for their freezing response.

For testing the effect of VU0409106 administration on SPS-induced EEG alterations, each rat was randomized into either SPS/Vehicle or SPS/VU0409106 groups. Two continuous 24

hour baseline (BL) recordings were performed for each rat in its home cage to serve as within-subjects comparator for all subsequent sleep-wake, qEEG, and body temperature data. After BL recordings, each rat received SPS treatment and was administered either 10 mg/kg VU0409106 or vehicle within 30 minutes of SPS completion. Home cage recordings were immediately re-initiated in both groups (Day 0), continued for two days (Days 1 and 2) after which transmitters were turned off, then reactivated on Day 7. Subsequent off-line analysis of sleep-wake and qEEG data was divided into the remaining hours of Day 0, or in 24hr intervals comprising Days 1, 2, and 7 post-SPS treatment.

For testing the effect of VU0409106 administration on SPS-induced alterations in biochemistry and neurochemistry, rats were randomized to receive either SPS or SHAM treatment followed immediately by administration of vehicle or 10 mg/kg VU0409106, i.p. SHAM rats were sacrificed thirty minutes after dosing; SPS rats were sacrificed either thirty minutes, (Day 0), one day (Day 1), or seven days (Day 7) after dosing. On the day of sacrifice, rats were briefly anesthetized with isoflurane and decapitated. Hippocampus, amygdala, and PFC were dissected, rapidly frozen on dry ice, and stored at −80 °C for tissue mRNA and neurochemistry experiments. Trunk blood was collected into heparin-lined tubes, and then centrifuged at 5,000 rpm for 9 minutes at 4 °C to obtain plasma.

Figure 17A depicts the experimental design for behavioral studies (Cohort 1), EEG studies (Cohort 2), and tissue collection studies (Cohort 3). For all experiments, SPS and SHAM treatment occurred within the first 6 hours of the light phase. Figure 17B shows the chemical structure of VU0409106.

Single Prolonged Stress

SPS was performed according to Liberzon et al. (315). Briefly, rats were restrained for 2 hours, followed by forced swim for 15 minutes in 24 ^{0}C water. Following a 15 minute recovery period, rats were exposed to diethyl ether vapor in a bell jar until anesthesia. The SPS model did not cause mortality. SPS did illicit hallmarks of the rodent stress response such as porphyrin staining of the eyes, and urination and defecation. There were no major individual differences observed in these parameters during each experiment, and no inclusion or exclusion criteria were applied prior to the start of EEG recordings or tissue collection. SHAM treatment consisted of brief handling in a novel procedure room. All animals were placed into fresh cages after treatment.

Contextual CF

Fear conditioning and testing were performed in sound attenuating chambers equipped with a stainless steel grid floor for shock delivery and a video camera for recording freezing behavior (MedAssociates, Allentown, NJ). For determining the effects of VU0409106 on SPS-induced alterations in the CF response, rats were randomized to receive either SPS or SHAM treatment followed immediately by i.p. administration of vehicle or VU0409106, (3, 10, or 30 mg/kg, i.p.). Fifteen days later, all rats underwent fear conditioning to the chamber context consisting of a 3 minute habituation period followed by a 4 second, 0.8 mA footshock, and ending with a 1 minute undisturbed period after which each rat was returned to its home cage. One day later, all rats were reexposed to the same chambers, and freezing behavior, defined as motionless posture excluding respiratory movements, was measured in the absence of any shock stimuli for 5 minutes.

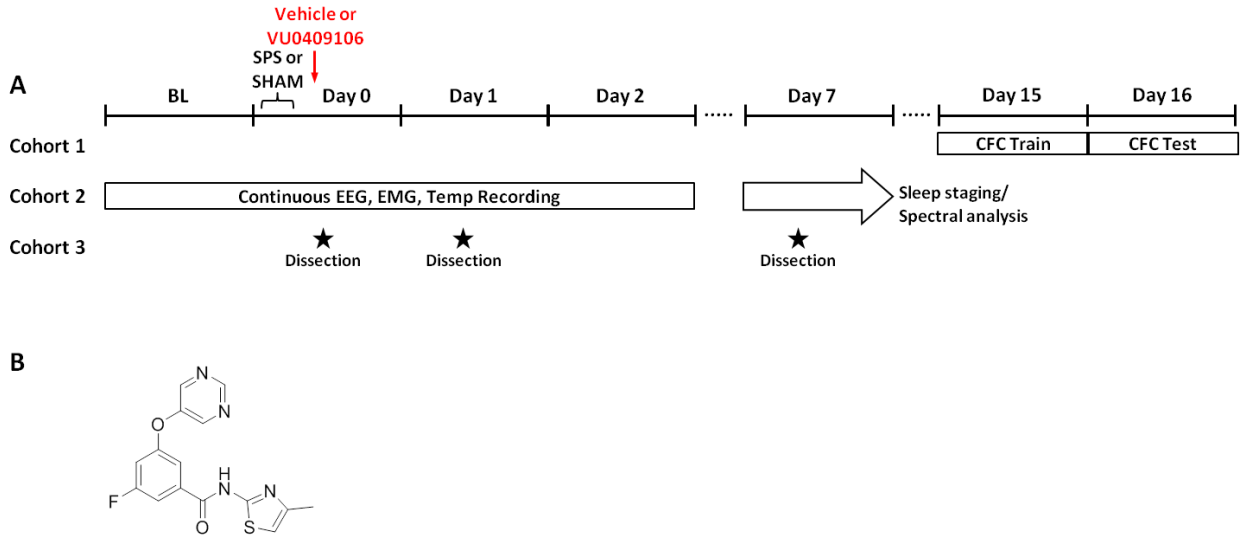

Figure 17. Experimental design for post-trauma intervention studies with VU0409106. (A) In cohort 1, non-implanted rats were exposed to either single prolonged stress (SPS) or SHAM treatment immediately followed by systemic i.p. administration of either vehicle or 10 mg/kg VU0409106. Fifteen days later, all rats underwent contextual fear conditioning (CFC Train); one day later they were reexposed to the context in the absence of shock and freezing response was measured (CFC Test). In cohort 2, continuous EEG, EMG, and temperature data were telemetrically recorded from chronically implanted rats throughout successive 24 hour light-dark cycles (ON: 6:00AM; OFF: 6:00PM) before (BL) and several days after (Days 0, 1, 2, and 7) SPS with systemic i.p. administration of either vehicle or 10 mg/kg VU0409106 immediately after treatment. SPS treatment was performed within the first 6 hours of the light phase on Day 0 during which recording was not possible; EEG data from this day was re-initiated when each animal was returned to its home cage. In cohort 3, non-implanted rats underwent either SPS or SHAM treatment immediately followed by systemic i.p. administration of either vehicle or 10 mg/kg VU0409106. SHAM rats were sacrificed thirty minutes after dosing; SPS rats were sacrificed either thirty minutes, (Day 0), one day (Day 1), or seven days (Day 7) after dosing. (B) Chemical structure of VU0409106.

To determine whether VU0409106 impairs the consolidation of normal fear learning, a separate group of rats having received no prior treatment other than handling underwent fear conditioning to the chamber context consisting of a 1 minute habituation period followed by three 1 second, 0.5 mA footshocks with 1 minute inter-shock intervals, and ending with a 1 minute undisturbed period. Immediately after removal from the chamber, each rat was administered vehicle or VU0409106, (3, 10, or 30 mg/kg, i.p.), after which it was returned to its

home cage. One day later, all rats were reexposed to the same chambers, and freezing behavior, defined as motionless posture excluding respiratory movements, was measured in the absence of any shock stimuli for 5 minutes.

Tissue collection

For biochemical and neurochemical endpoints, a group of non-implanted rats was randomly assigned to SHAM treatment or one of three SPS groups (Day 0, 1 or 7). Rats were briefly anesthetized with isoflurane, and sacrificed by decapitation either immediately (Day 0), one day (Day 1), or seven days (Day 7) after SPS; SHAM rats were sacrificed immediately after SHAM treatment. Hippocampus, PFC, and amygdala were dissected, rapidly frozen on dry ice, and stored at −80 °C for tissue neurochemistry. Trunk blood was collected into heparin-lined tubes, and then centrifuged at 5,000 rpm for 9 minutes at 4 °C to obtain plasma.

Tissue neurochemistry

Tissue concentrations of 5-HT and its metabolite 5-hydroxyindoleacetic acid (5-HIAA) were determined by HPLC-ECD as described previously (316). 5-HT utilization was calculated as 5-HIAA/5-HT in ng/mg protein. 5-HT, 5-HIAA, and 5-HT utilization values were then normalized to the SHAM/Vehicle group average. Values greater than 3 standard deviations from the mean for 5-HIAA and 5-HT utilization were excluded.

Quantitative real-time polymerase chain reaction (qRT-PCR)

Alterations in mRNA expression levels of EGR-1, brain-derived neurotrophic factor (BDNF), NR1, NR2A, and NR2B were measured using Aqueous Micro kits (Life Technologies,

Grand Island, NY) for RNA extraction, NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE) for RNA quantification, QuantiTect Reverse Transcription Kit (QIAGEN, Hilden, Germany) for complementary DNA transcription, CFX96 Real-Time PCR Detection System (Bio-Rad, Hercules, CA) using primers from TaqMan Gene Expression Assays (Life Technologies) for qRT-PCR. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an internal control; data are presented using the comparative cycle threshold (CT) method normalized to SHAM-treated rats.

Plasma corticosterone

Corticosterone, the rodent analogue of the human glucocorticoid cortisol, was measured using a double antibody radioimmunoassay (RIA) kit (MP Biomedicals, Orangeburg, NY).

Sleep staging

EEG, EMG, and temperature data were collected with Dataquest A.R.T. 4.3 software (DSI, Minneapolis, MN) using a continuous sampling method. Telemetric data were sampled at a rate of 500 Hz and transmitted via a receiver (RPC-2, DSI) placed below the cage of each rat. Each receiver was connected to a data exchange matrix (DSI) which transferred EEG, EMG, and temperature data to a computer for off-line analysis. Two trained observers used Neuroscore 3.0 software to manually stage each 10 second epoch as wake, NREM, or REM sleep based on accepted characteristic EEG and EMG oscillatory patterns (317). All 10 second epochs were summed into 2 hour bins. For the acute effects of VU0409106 administration on SPS treatment on Day 0, 2 hour bins were group averaged to examine the amount of time spent in wake, NREM, or REM sleep. To assess the prolonged effects of VU0409106 administration on SPS

treatment (Days 1, 2, and 7), 12 hour bins comprising either the light or dark phase of a given day were group averaged.

qEEG spectral power analysis

qEEG relative power spectra from frontal and parietal electrodes were computed for each rat and on each day of recording in 10 second epochs grouped into conventional power bands (delta δ: 0.5-4.9 Hz, theta θ: 5-8.9 Hz, alpha α: 9-13.9 Hz, beta β: 14-29.9 Hz, low gamma γ: 30-49.9 Hz, and high γ: 50-100 Hz) (227) using a Fast Fourier Transform with a Hamming window and overlap ratio of 0.5. Relative power within each band was calculated as a percent of total power, then binned by stage (wake, NREM, or REM). The percent change from the BL value of the same power band for the same rat during the corresponding sleep-wake stage was then calculated for each individual rat and group averaged. For the acute effects of VU0409106 administration on healthy rats, BL values refer to an average of values collected during the first two hours of the light phase recorded on the day of treatment, in twenty minute bins. For the effects VU0409106 administration on SPS treatment, BL values refer to an average of those gathered during the two BL recording days in twelve hour bins. Slow wave activity (SWA) was defined as relative delta power in the frontal cortex during NREM sleep. Equipment error prevented recording from the frontal lead of one rat in each of the SPS/Veh and SPS/VU0409106 groups, and from the parietal lead of one rat in the SPS/Veh group.

Statistical analysis

For the acute effects of VU0409106 on sleep-wake architecture, qEEG spectral power, temperature, and spontaneous locomotor activity in non-stressed rats, as well as the effects of

post-SPS VU0409106 administration on short and long-term sleep-wake architecture, qEEG spectral power, and temperature, a repeated measures two-way analysis of variance (ANOVA) was applied; if significant, a Bonferroni post hoc test was performed with significance defined as $P < .05$. For the acute effects of VU0409106 on rotarod performance and normal fear learning, and for the effects of SPS on EGR-1 expression in the amygdala, a one-way ANOVA was applied; if significant, a Dunnett's post hoc test was used with significance defined as $P < .05$. For the effects of post-SPS VU0409106 administration on contextual CF, 5-HT utilization, and plasma corticosterone concentration, a two-way ANOVA was applied; if significant, a Bonferroni post hoc test was performed with significance defined as $P < .05$. For the effects of post-SPS VU0409106 administration on sleep latencies, a student's t test was applied with significance defined as $P < .05$.

Results

$mGluR_5$ antagonism preferentially suppressed REM sleep.

VU0409106 modestly increased time spent awake only at the highest dose of 30 mg/kg (Figure 18A) (time [$F_{11,363} = 91.10$, $P < 0.0001$], dose [$F_{3,33} = 11.98$, $P < 0.0001$], interaction [$F_{33,363} = 1.62$, $P = 0.02$]), and there was a main effect of time and dose on NREM sleep (Figure 18B) (time [$F_{11,363} = 91.41$, $P < .0001$], dose [$F_{3,33} = 13.88$, $P < .0001$]), but no time point reached post-hoc significance. The most significant effect of VU0409106 was a dose-dependent suppression of time spent in REM sleep (Figure 18C) (time [$F_{11,363} = 35.38$, $P < .0001$], interaction [$F_{33,363} = 4.88$, $P < .0001$]).

$mGluR_5$ antagonism increased waking high gamma power and NREM sleep delta power in the frontal cortex.

Similar to NMDA receptor antagonists (279), VU0409106 dose-dependently increased high gamma power during wake (Figure 19A) (time [$F_{30,1046} = 6.46$, $P < .0001$], dose [$F_{3,1012} = 32.57$, $P < .0001$], interaction [$F_{90,1012} = 2.25$, $P < .0001$]). Also similar to NMDA receptor antagonists, and consistent with an improvement in sleep quality (263, 277, 278), VU0409106 dose-dependently increased delta power during NREM sleep (aka SWA) (Figure 19B) (time [$F_{30,1012} = 21.34$, $P < .0001$], dose [$F_{3,1012} = 32.57$, $P < .0001$], interaction [$F_{90,1012} = 2.25$, $P < .0001$]).

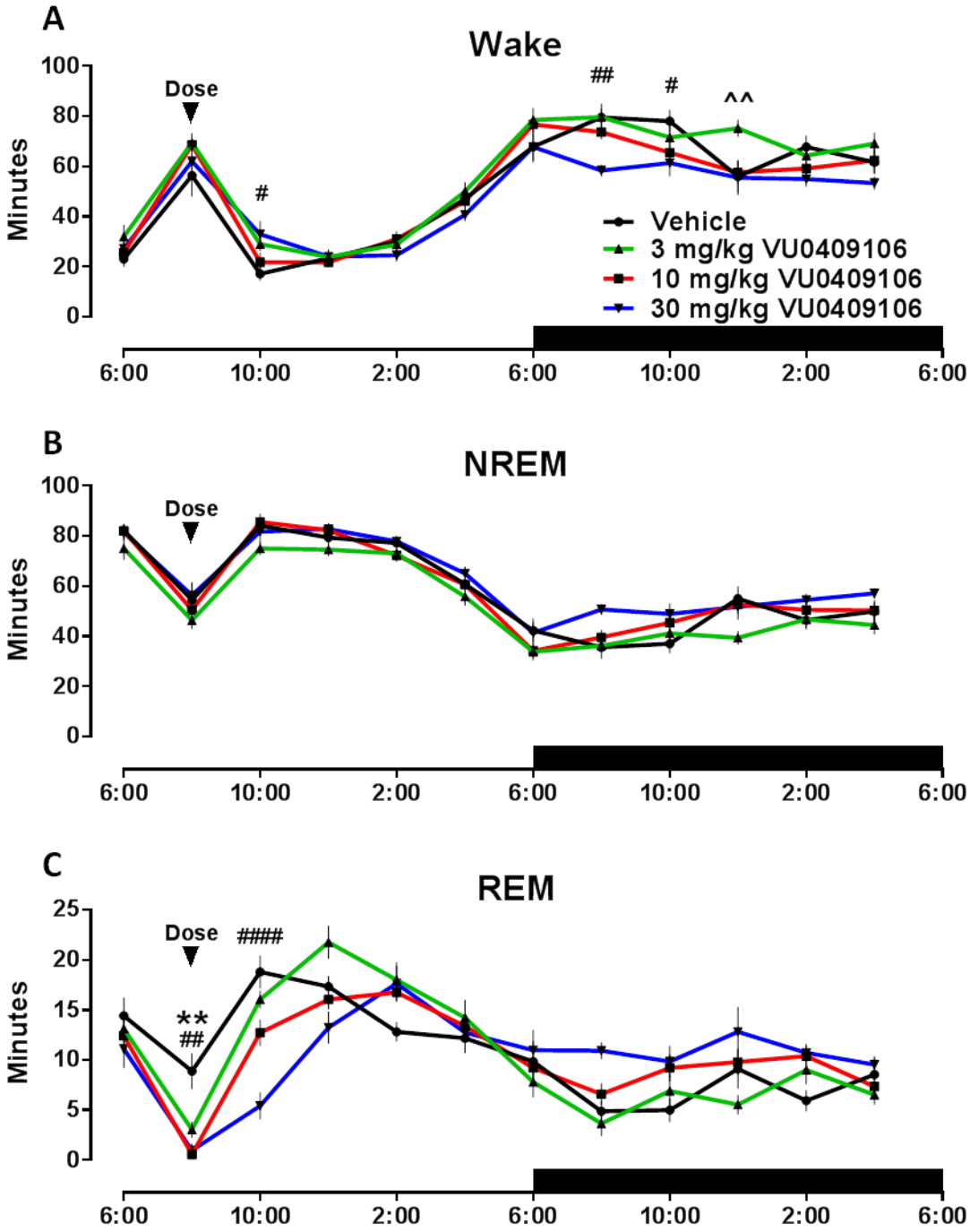

Figure 18. VU0409106 selectively suppressed REM sleep. (A) VU0409106 administration modestly increased time spent in wake at the highest dose of 30 mg/kg, i.p., but (B) had no significant effect on NREM sleep time. (C) By contrast, VU0409106 preferentially and dose-dependently reduced REM sleep time for up to several hours. Black bar indicates dark phase. Data are depicted as mean ± SEM (n = 8-10). Comparison between doses performed by repeated measures two-way ANOVA. ^^ P < 0.01, 3mg/kg vs. Veh; **P < 0.01, 10 mg/kg vs. Veh; #P < 0.05, ##P < .01, ####P < .0001, 30 mg/kg vs. Veh in Bonferroni *post hoc* test.

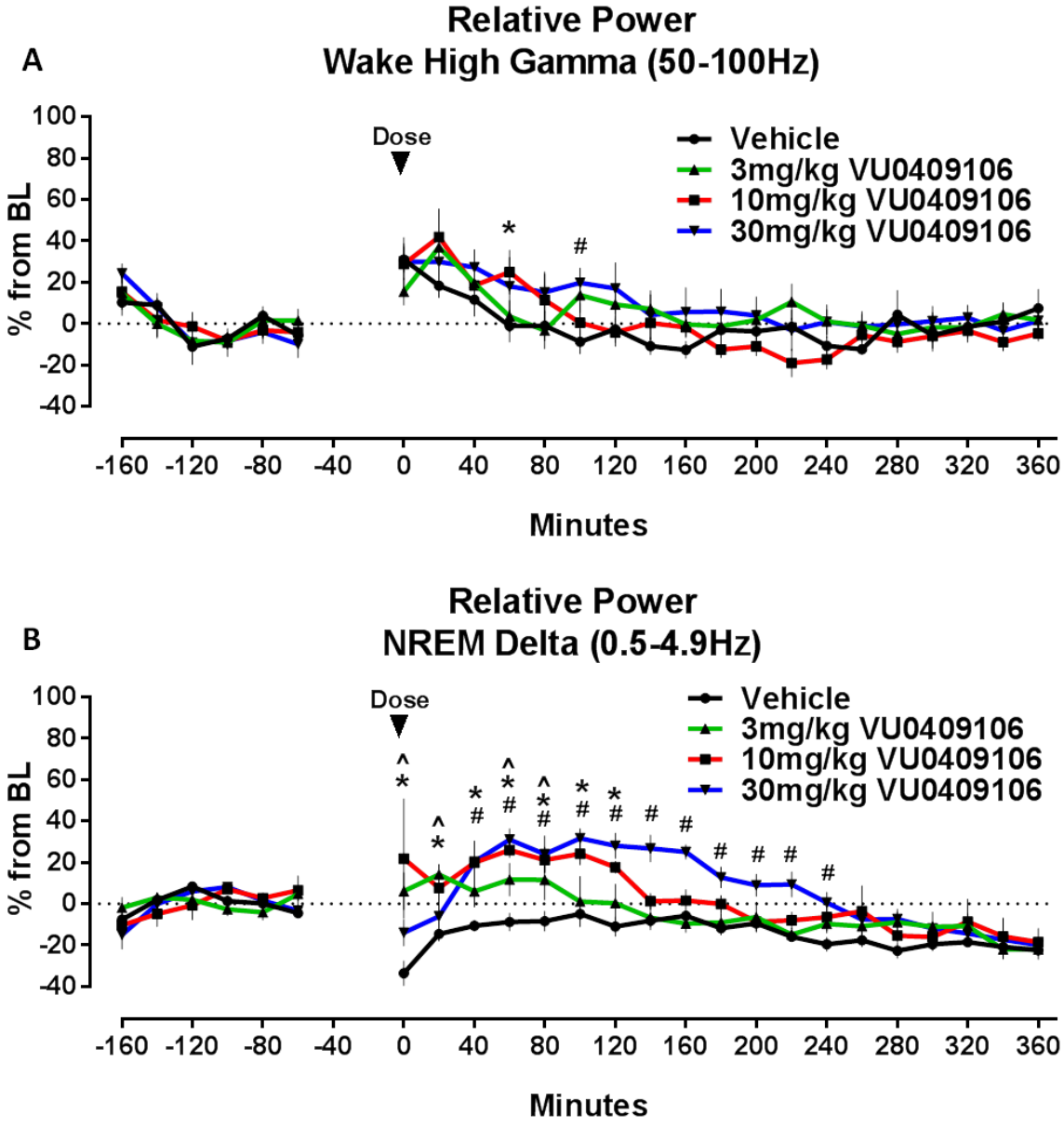

Figure 19. VU0409106 increased waking high gamma power and NREM sleep delta power in the frontal cortex. (A) Relative to baseline, VU0409106 administration dose-dependently increased high gamma power in the frontal cortex during wake, and (B) delta power during NREM sleep, a measure referred to as slow wave activity (SWA). Data are depicted as mean ± SEM (n = 9-10). Comparison between doses performed by repeated measures two-way ANOVA. ^ P < 0.05, 3mg/kg vs. Veh; *P < 0.05, 10 mg/kg vs. Veh; #P < 0.05, 30 mg/kg vs. Veh in Bonferroni *post hoc* test.

$mGluR_5$ antagonism caused dose-dependent reductions in body temperature and sedation.

In order to effectively determine whether post-trauma VU0409106 administration could prevent PTSD-like symptoms in the studies described below, we first attempted to select a dose that did not induce significant adverse effects. Antagonists of both NMDA receptors and $mGluR_5$ have been shown to cause reductions in body temperature, induce sedation, and impair locomotor activity in rodents (337, 338), so we first determined whether VU0409106 also exhibits this liability. Systemic administration of VU0409106 caused acute reductions in body temperature during wake (Figure 20A) (time [$F_{24,792} = 7.24$, $P < .0001$], dose [$F_{3,792} = 15.63$, $P < .0001$], interaction [$F_{72,792} = 3.41$, $P < .0001$]) and NREM sleep (Figure 20B) (time [$24,769 = 5.12$, $P < .0001$], dose [$F_{3,769} = 19.90$, $P < .0001$], interaction [$F_{72,769} = 3.72$, $P < .0001$]). Time spent in REM sleep was so reduced that no temperature data for this state could be gathered during the hours after VU0409106 administration (Figure 20C). VU0409106 also dose-dependently decreased spontaneous locomotor activity (Figure 20D) (time [$F_{5,140} = 75.29$, $P < .0001$], interaction [$F_{15,140} = 3.47$, $P < .0001$]), and reduced the latency to fall in a rotarod test (Figure 20E) ($F_{3,20} = 3.18$, $P = .05$) indicating sedation. Given the insomnia-like effects induced by SPS reported above, however, mild sedation could be an advantageous property for a compound intended to intervene in posttraumatic symptoms. Since 10 mg/kg VU0409106 exhibited modest sedation in the spontaneous locomotor assay, but impaired motor function to a lesser degree than the 30 mg/kg dose in the rotarod assay, this dose was chosen for the post-SPS dosing experiments described below.

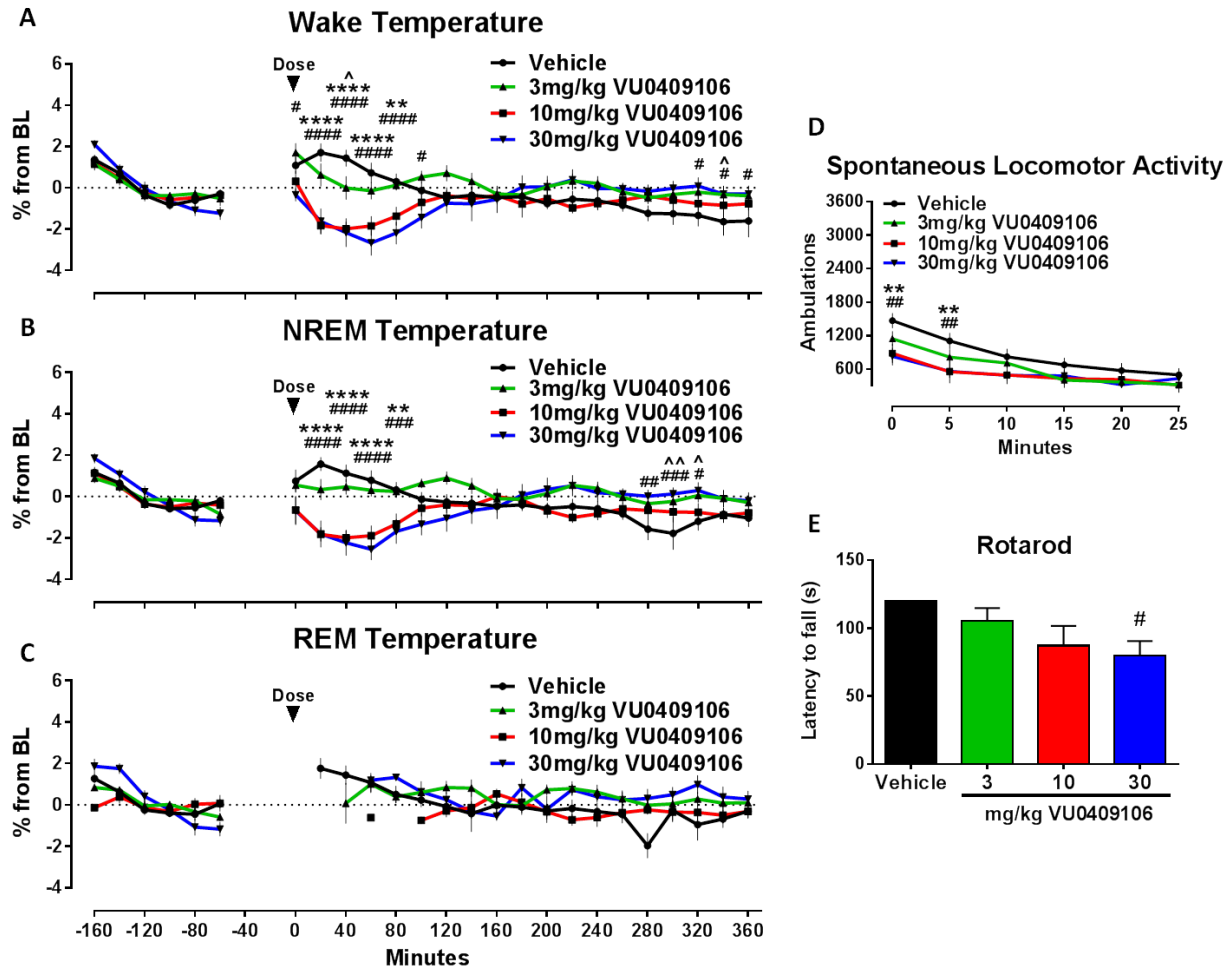

Figure 20. VU0409106 caused dose-dependent reductions in body temperature and sedation. (A) Relative to baseline, VU0409106 acutely decreased body temperature during wake and (B) NREM sleep, but not (C) REM sleep, which was suppressed such that few data points could be collected for temperature. (D) VU0409106 also inhibited spontaneous locomotor activity in an open field, and (E) induced modest motor impairments at the highest dose of 30 mg/kg in the rotarod test. Data are depicted as mean ± SEM (n = 9-10 for A-C, 6-8 for D,E). Comparison between doses performed by repeated measures two-way ANOVA for A-D, and one-way ANOVA for E. ^ P < 0.05, ^^ P < 0.01, 3mg/kg vs. Veh; *P < 0.05, **P < 0.01, ***P < 0.001, 10 mg/kg vs. Veh; #P < 0.05, ##P < .01, ###P < .001, ####P < .0001, 30 mg/kg vs. Veh in Bonferroni *post hoc* test for A-D, and Dunnett's *post hoc* test for E.

Post-trauma $mGluR_5$ antagonism inhibited the development of augmented threat responding without disturbing normal threat learning.

Similar to previous reports (59, 339), SPS induced an augmented threat response as measured by contextual CF (Figure 21A). However, this exaggerated threat response was attenuated in rats that received VU0409106 immediately after SPS (Figure 21A) (treatment [$F_{1,80} = 6.88$, $P = 0.01$], interaction [$F_{3,80} = 2.84$, $P = .04$]), with 10 mg/kg VU0409106 being the most effective dose. VU0409106 administered after SHAM treatment did not affect contextual CF (Figure 21A). In addition to its ameliorative effect on SPS-induced augmentation of threat learning, VU0409106 left intact the consolidation of normal threat learning when administered after contextual CF (Figure 21B) in rats that had not undergone SPS treatment (experimental design depicted in Figure 21C).

Post-trauma $mGluR_5$ antagonism acutely extended REM sleep suppression.

Consistent with our previous findings, SPS significantly increased time spent in wake (Figure 22A) (time [$F_{21,294} = 36.02$, $P < .0001$]), and reduced time spent in NREM sleep (Figure 22B) (time [$F_{21,294} = 50.81$, $P < .0001$]) relative to BL. 10 mg/kg VU0409106 had no significant effect on wake or NREM sleep time, but further reduced time spent in REM sleep (Figure 22C) (time [$F_{21,294} = 27.80$, $P < .0001$], interaction [$F_{21,294} = 2.10$, $P = .0037$]) relative to vehicle-treated rats. In addition, 10 mg/kg VU0409106 significantly reduced the latency to NREM sleep after SPS (Figure 23A) ($t = 2.95$, $p = .01$), and increased the latency to REM sleep after SPS (Figure 23B), and after the onset of NREM sleep (Figure 23C) ($t = 2.96$, $p = .01$).

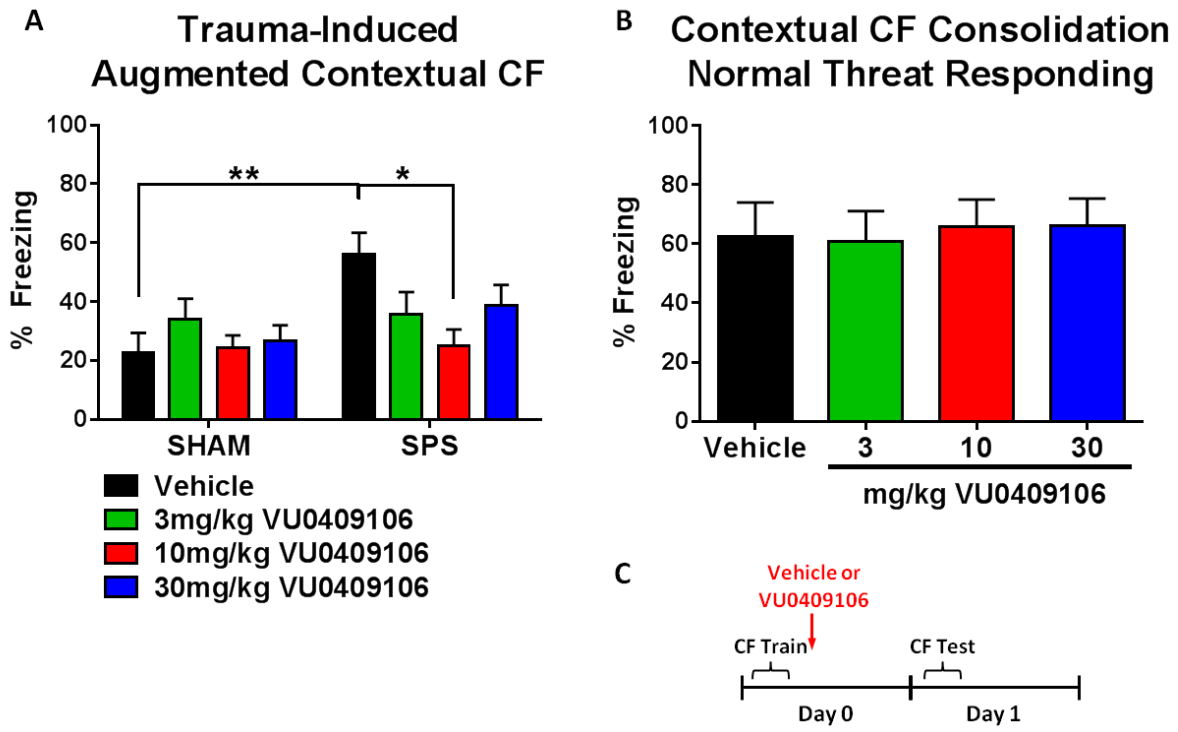

Figure 21. Post-trauma VU0409106 inhibited the development of trauma-induced augmented threat responding without disturbing normal threat learning. (A) SPS caused augmented threat responding in the contextual CF assay relative to SHAM rats fifteen days after treatment; this effect was attenuated if VU0409106 had been administered immediately after SPS. (B) VU0409106 did not induce a general amnesic effect, however, leaving normal threat responding intact (C) when administered immediately after contextual CF training. Data are depicted as mean + SEM (n = 10-11).Comparison between doses performed by two-way ANOVA for A, and one-way ANOVA for B. **P < 0.01, SPS/Veh vs. SHAM/Veh; *P < 0.05, SPS/10 mg/kg VU0409106 vs. SPS/Veh in Bonferroni *post hoc* test.

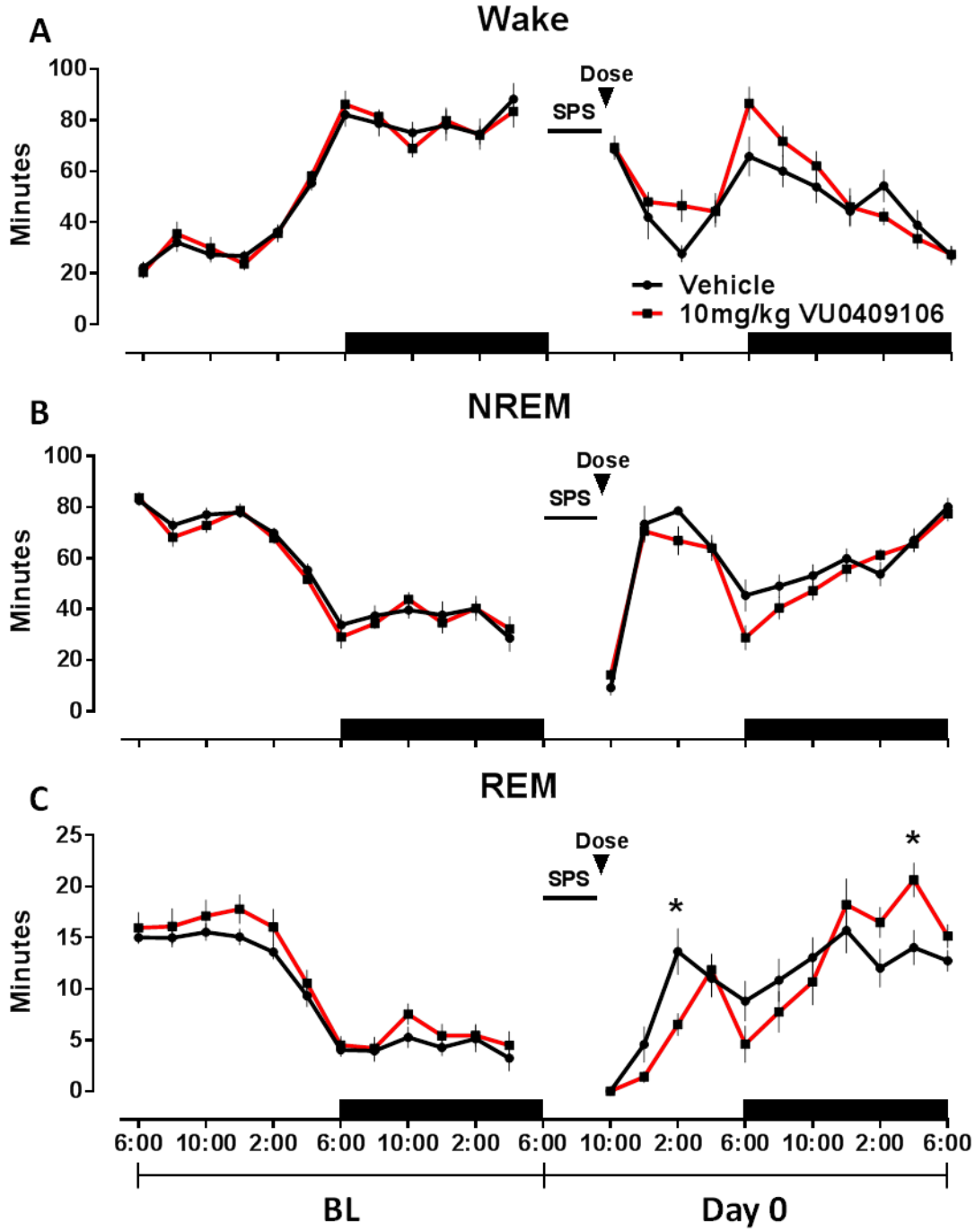

Figure 22. Post-trauma VU0409106 acutely extended REM sleep suppression. (A) VU0409106 had no effect on acute SPS-induced increases in wake or (B) decreases in NREM sleep relative to baseline (BL), but (C) further reduced REM sleep time for up to several hours after SPS relative to vehicle treated rats. Black bars indicate dark phases. Data are depicted as mean ± SEM (n = 8). Comparison between groups performed by repeated measures two-way ANOVA. *P < 0.05, 10 mg/kg vs. Veh in Bonferroni *post hoc* test.

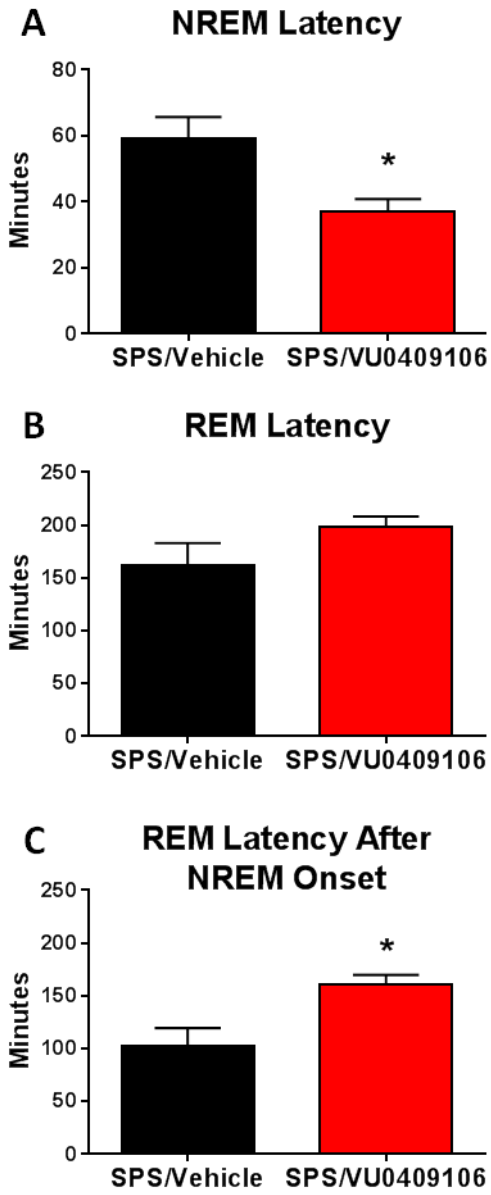

Figure 23. Post-trauma VU0409106 decreased NREM sleep latency and increased REM sleep latency. (A)VU0409106 significantly decreased the latency to first enter NREM sleep after SPS, (B) non-significantly increased the time to enter REM sleep after SPS, and (C) significantly increased the latency to enter REM sleep after first NREM sleep bout. Data are depicted as mean + SEM (n = 8). Comparison between groups performed by t test, *P < 0.05.

Post-trauma mGluR$_5$ antagonism modestly attenuated subsequent sleep reduction and fragmentation.

Consistent with our previous findings, and possibly indicative of an insomnia-like phenotype, SPS caused significant increases in time spent awake during the light phase on Days 1 and 2 (Table 4) (time [$F_{3,42} = 27.93$, $P < .0001$]), culminating in an increased number of bouts (time [$F_{3,42} = 2.44$, $P < .0001$]) and longer average bout lengths (time [$F_{3,42} = 10.08$, $P < .0001$]). These changes in wake were accompanied by reductions in light phase NREM sleep time (time [$F_{3,42} = 16.33$, $P < .0001$]), increased NREM sleep bout number (time [$F_{3,42} = 14.51$, $P < .0001$]), and decreased NREM sleep average bout length (time [$F_{3,42} = 21.85$, $P < .0001$]), as well as decreased REM sleep time (time [$F_{3,42} = 14.9$, $P < .0001$]), and a main effect on REM sleep average bout length (time [$F_{3,42} = 3.84$, $P = .02$]). This fragmentation of NREM and REM sleep during the light phase was modestly attenuated by prior administration of 10 mg/kg VU0409106 post-SPS which decreased the number of wake bouts, and increased their average length (dose [$F_{1,14} = 4.88$, $P = .04$]). Post-trauma 10 mg/kg VU0409106 also attenuated subsequent reductions in light phase REM sleep time (dose [$F_{1,14} = 5.67$, $P = .03$]) relative to rats that had received vehicle after SPS.

During the dark phase, SPS induced significant reductions in wake time on Days 1 and 2 (Table 4) (time [$F_{3,42} = 252.56$, $P < .0001$])), along with increases in bout number (time [$F_{3,42} = 18.21$, $P = .0002$]) and reductions in average bout length (time [$F_{3,42} = 14.13$, $P < .0001$]). SPS also caused persistent increases in NREM sleep time (time [$F_{3,42} = 35.90$, $P < .0001$]) and bout number (time [$F_{3,42} = 8.03$, $P = .0002$]) during the dark phase, as well as increases in REM sleep time (time [$F_{3,42} = 39.02$, $P < .0001$]), bout number (time [$F_{3,42} = 10.84$, $P < .0001$]), and average bout length (time [$F_{3,42} = 7.45$, $P = .0004$]). Rats that received 10 mg/kg VU0409106

Table 4. Post-trauma $mGluR_5$ antagonism modestly attenuated subsequent sleep disturbances.

LIGHT PHASE		Treatment	BL	SPS Day 1	SPS Day 2	SPS Day 7
Time (min)	WAKE	Vehicle	200.3 ± 6.9	238.7 ± 7.2	224.6 ± 9.9	202.2 ± 7.5
		VU0409106	203.5 ± 6.9	249.4 ± 9.9	238.3 ± 8.6	219.2 ± 10.1
	NREM	Vehicle	435.7 ± 5.0	415.0 ± 5.8	423.1 ± 8.6	438.1 ± 6.6
		VU0409106	423.2 ± 7.6	391.2 ± 8.5	406.3 ± 8.7	410.8 ± 8.5
	REM	Vehicle	83.5 ± 2.1	66.2 ± 3.1	72.4 ± 3.5	79.9 ± 3.5
		VU0409106	93.6 ± 4.7	79.5 ± 3.2*	75.2 ± 5.0	89.5 ± 3.9
No. bouts per hour	WAKE	Vehicle	13.8 ± 0.5	15.9 ± 0.7	16.2 ± 0.9	12.3 ± 0.5
		VU0409106	13.7 ± 0.6	14.3 ± 0.6	13.4 ± 0.7*	11.6 ± 0.7
	NREM	Vehicle	14.0 ± 0.4	16.1 ± 0.7	16.2 ± 0.9	12.4 ± 0.5
		VU0409106	14.0 ± 0.6	14.5 ± 0.6	13.4 ± 0.6*	11.7 ± 0.7
	REM	Vehicle	4.1 ± 0.1	3.5 ± 0.2	3.9 ± 0.4	3.7 ± 0.1
		VU0409106	5.3 ± 0.2	3.9 ± 0.1	4.1 ± 0.3	4.4 ± 0.3
Bout length (sec)	WAKE	Vehicle	72.2 ± 3.0	76.1 ± 4.7	71.3 ± 5.3	83.1 ± 4.4
		VU0409106	73.1 ± 3.0	87.2 ± 3.7	90.1 ± 4.6*	98.1 ± 7.1
	NREM	Vehicle	159.2 ± 6.8	131.8 ± 5.5	134.5 ± 7.0	181.2 ± 6.8
		VU0409106	158.8 ± 8.0	137.9 ± 7.2	156.4 ± 9.5	184.1 ± 13.3
	REM	Vehicle	100.2 ± 2.7	95.6 ± 5.5	107.9 ± 2.6	106.4 ± 7.2
		VU0409106	104.3 ± 5.3	104.0 ± 5.4	94.3 ± 6.3	106.4 ± 7.2
DARK PHASE		**Treatment**	**BL**	**SPS Day 1**	**SPS Day 2**	**SPS Day 7**
Time (min)	WAKE	Vehicle	476.5 ± 13.1	407.4 ± 21.6	439.5 ± 18.9	453.9 ± 14.6
		VU0409106	473.7 ± 8.0	384.8 ± 12.8	431.7 ± 8.5	465.8 ± 12.6
	NREM	Vehicle	217.5 ± 10.7	270.8 ± 18.1	247.2 ± 15.2	236.1 ± 12.3
		VU0409106	214.5 ± 8.0	280.6 ± 14.2	243.6 ± 8.7	218.9 ± 11.5
	REM	Vehicle	25.9 ± 3.1	41.7 ± 4.2	33.1 ± 4.2	29.4 ± 3.3
		VU0409106	31.6 ± 2.2	54.4 ± 4.2*	44.6 ± 2.0	35.3 ± 2.5
No. bouts per hour	WAKE	Vehicle	9.9 ± 0.6	10.7 ± 0.7	11.5 ± 0.8	8.9 ± 0.2
		VU0409106	9.9 ± 0.5	11.2 ± 0.4	9.8 ± 0.4	8.8 ± 0.4
	NREM	Vehicle	9.8 ± 0.6	10.6 ± 0.7	11.4 ± 0.8	8.9 ± 0.3
		VU0409106	9.8 ± 0.5	11.2 ± 0.4	9.7 ± 0.4	8.7 ± 0.4
	REM	Vehicle	1.8 ± 0.2	2.3 ± 0.2	2.1 ± 0.2	2.1 ± 0.2
		VU0409106	2.1 ± 0.2	3.0 ± 0.2	2.9 ± 0.1*	2.7 ± 0.2
Bout length (sec)	WAKE	Vehicle	244.9 ± 27.0	205.7 ± 20.6	201.1 ± 20.5	261.2 ± 14.2
		VU0409106	235.3 ± 14.7	177.2 ± 9.8	224.2 ± 6.6	275.7 ± 16.7
	NREM	Vehicle	127.3 ± 7.4	130.5 ± 8.9	113.4 ± 10.3	134.4 ± 5.5
		VU0409106	122.0 ± 8.7	126.6 ± 5.9	126.9 ± 7.5	128.9 ± 8.5
	REM	Vehicle	74.5 ± 7.6	87.0 ± 9.9	86.7 ± 9.9	69.9 ± 3.5
		VU0409106	73.6 ± 4.6	88.8 ± 4.4	78.6 ± 2.7	66.4 ± 4.0

after SPS exhibited further increased time spent in REM sleep (dose [$F_{1,14} = 4.80$, $P = .04$]), and increased REM bouts during the dark phase (dose [$F_{1,14} = 7.02$, $P = .02$]).

Post-trauma $mGluR_5$ antagonism blocked acute increases in frontal theta power during REM sleep and enhanced SWA.

Consistent with our previous findings, SPS induced dramatic acute increases in theta power during REM sleep in the frontal cortex during the light phase on Day 0, an effect that was almost completely attenuated by post-SPS administration of 10 mg/kg VU0409106 (Figure 24A) (time [$F_{4,48} = 13.56$, $P < .0001$], interaction [$F_{4,48} = 5.21$, $P = .0014$]). Also similar to our previous report, SPS caused an acute increase in light phase SWA measured in the frontal cortex on Day 0, but subsequently reduced this measure on Days 1 and 2 (Figure 24B) (time [$F_{4,48} = 62.10$, $P < .0001$]). Rats that received 10 mg/kg VU0409106 after SPS exhibited significantly increased SWA on Day 0 relative to vehicle-treated rats (dose [$F_{1,12} = 9.08$, $P = .01$], interaction [$F_{4,48} = 4.57$, $P = .0033$]), but there was no effect on SWA reductions on Days 1 and 2 (Figure 24B). VU0409106 did not alter REM sleep theta power in the frontal cortex in rats that had not received prior SPS (Figure 25A), nor did it modulate SPS-induced alterations in REM sleep theta power in the parietal cortex (Figure 25B) (time [$F_{4,52} = 7.62$, $P < .0001$]). Additionally, SPS-induced increases in theta power were specific to REM sleep, being absent during wake in the frontal (Figure 25C) and parietal (Figure 25D) cortices.

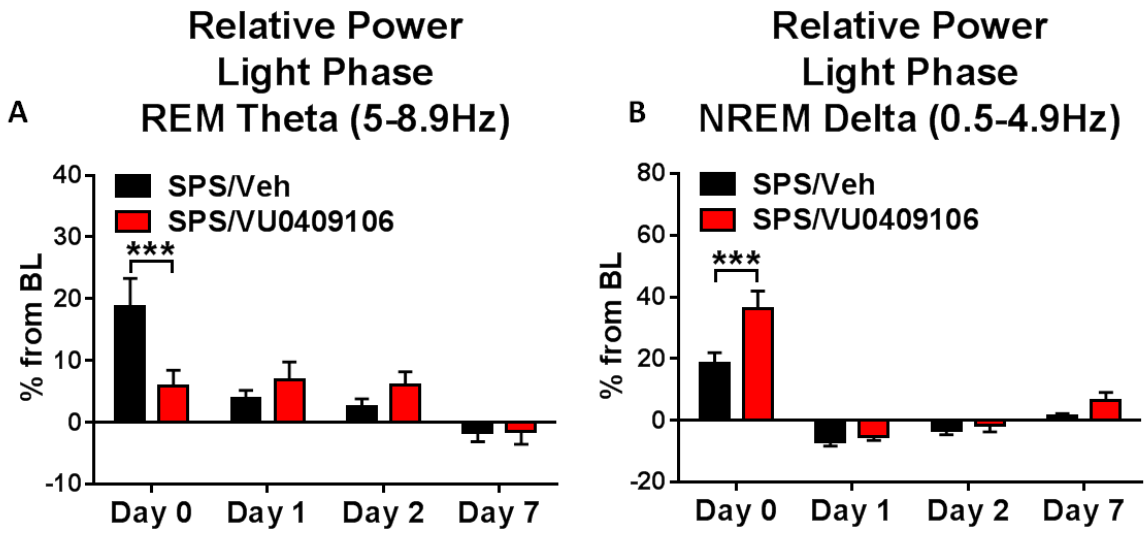

Figure 24. Post-trauma VU0409106 blocked acute increases in frontal theta power during REM sleep and acutely enhanced SWA. (A)SPS treatment acutely increased theta power during REM sleep in the frontal cortex on Day 0; this effect was substantially attenuated by VU0409106 administration. (B) SPS also caused acute increases in delta power during NREM sleep in the frontal cortex (aka SWA); this effect was augmented by VU0409106 administration. SPS also caused modest reductions in SWA on Days 1 and 2, but this change was not reversed by prior VU0409106 administration. Data are depicted as mean + SEM (n = 7). Comparison between groups performed by repeated measures two-way ANOVA. ***P < 0.001, 10 mg/kg vs. Veh in Bonferroni *post hoc* test.

Post-trauma mGluR$_5$ antagonism attenuated reductions in amygdala 5-HT utilization.

SPS caused a delayed reduction in 5-HT utilization in the amygdala beginning on Day 1; 10 mg/kg VU0409106 administered immediately after SPS attenuated this reduction (Figure 26B) (dose [$F_{3,79} = 3.99$, $P = .05$]). Consistent with our findings above, SPS also caused an acute increase in 5-HT utilization in the PFC (Figure 26C) (time [$F_{3,79} = 8.12$, $P < .0001$]) and hippocampus (Figure 26C) (time [$F_{3,80} = 13.60$, $P < .0001$]) on Day 0, but these increases were not affected by VU0409106 administration.

Post-trauma $mGluR_5$ antagonism acutely inhibited SPS-induced hyperthermia with no effect on plasma corticosterone increases.

10mg/kg VU0409106 administered after SPS reduced subcutaneous body temperature, and prevented hyperthermia for several hours during wake (Figure 27A) (time [$F_{57,798} = 30.64$, $P < .0001$], interaction [$F_{57,798} = 2.48$, $P < .0001$]) and NREM sleep (Figure 27B) (time [$F_{57,810} = 12.01$, $P < .0001$], interaction [$F_{57,810} = 2.96$, $P < .0001$]), but not REM sleep (Figure 27C) (time [$F_{56,763} = 7.72$, $P < .0001$]). SPS-induced acute increases in body temperature were accompanied by increased circulating levels of corticosterone on Day 0 (Figure 28) (time [$F_{3,64} = 54.46$, $P < .0001$], dose [$F_{1,64} = 5.01$, $P = .03$], interaction [$F_{3,64} = 3.47$, $P = .02$]).

SPS induces amygdala expression of EGR-1.

In order to confirm that the deleterious effects of SPS are partially mediated by traumatic memory consolidation, we examined the effects of SPS on amygdala expression of EGR-1, a key molecular mediator of emotional memory (33). SPS acutely increased mRNA levels of EGR-1 in the amygdala on Day 0 relative to SHAM-treated rats (Figure 29) ($F_{3,27} = 8.38$, $P = .0004$).

Discussion

In these studies, we demonstrated that the selective $mGluR_5$ NAM VU0409106, when administered immediately after traumatic stress in rats, attenuates subsequent behavioral and physiological changes, possibly through acute suppression of REM-sleep dependent emotional memory consolidation.

We first showed that VU0409106 dose-dependently suppresses REM sleep in healthy rats, while leaving NREM sleep entirely intact. This effect differentiates $mGluR_5$ antagonism from the effects of NMDA receptor antagonists, which at subanesthetic doses can inhibit both REM and NREM sleep (340). However, similar to NMDA receptor antagonists (263, 277-279), VU0409106 substantially and dose-dependently increased high gamma power during wake and delta power during NREM sleep in the frontal cortex. These effects are consistent with the functional coupling of $mGluR_5$ and NMDA receptors (99-101, 103, 106, 107), and suggest that VU0409106 may partially exert its behavioral effects through downstream inhibition of NMDA receptors. Furthermore, NMDA receptor antagonist-induced increases in gamma power during wake and delta power during NREM sleep were recently employed as translational biomarkers of central target engagement and efficacy, respectively (263, 277-279). These exciting advances combined with our current findings suggest that this qEEG approach may also be available for the clinical development of novel $mGluR_5$ NAMs.

We next attempted to determine whether selective $mGluR_5$ antagonism with VU0409016 could inhibit the behavioral consequences of trauma when administered immediately after SPS.

We found that post-SPS VU0409106 administration prevented the development of augmented threat responding, suggesting that $mGluR_5$ inhibition after trauma could impede the progression of PTSD symptoms. We also found that VU0409106 extended SPS-induced REM sleep suppression consistent with the hypothesis that $mGluR_5$ antagonism after trauma may prevent the development of PTSD-like hypervigilance through inhibition of REM sleep-dependent emotional memory consolidation. VU0409106 administration after SPS not only reduced the amount of time spent in REM sleep, but also increased the latency to first enter REM sleep after NREM sleep onset. Importantly, it has been observed that REM sleep latency after

emotional learning can correlate strongly with behavioral measures of memory consolidation (240), possibly due to the existence of a critical temporal window for this process (31, 37). Due to an increased REM sleep rebound during the dark phase of Day 0, rats that were administered VU0409106 after SPS regained much of the REM sleep they had lost during the light phase of that day. This observation underlines the importance of the timing of REM sleep suppression after trauma for therapeutic purposes. It is likely that this REM sleep rebound occurred outside the temporal window during which emotional memory consolidation takes place (31, 37), and therefore did not lead to the subsequent manifestation of PTSD-like behavioral changes. This finding suggests that $mGluR_5$ antagonist-mediated REM sleep suppression would lose its therapeutic effect if introduced several hours or days after trauma. Studies are ongoing to address this question, and to determine how long the temporal window for intervention after SPS might be.

In addition to suppressing the amount of time spent in REM sleep, VU0409106 also attenuated acute SPS-induced increases in theta power in the frontal cortex during REM sleep. Given that increased REM sleep theta power in the frontal cortex is a putative neurophysiological correlate of REM sleep-dependent emotional memory consolidation in both rodents (250) and humans (240), this finding strongly supports the interpretation that $mGluR_5$ inhibition exerts its therapeutic behavioral effect through blockade of traumatic memory consolidation. During post-trauma REM sleep, rhythmic activation of the amygdala at theta frequency likely promotes emotional memory consolidation through the induction of synaptic plasticity in the hippocampus and PFC, target brain structures known to oscillate at theta frequency in phase with the lateral amygdala, and to store long term emotional memory traces (250, 341, 342). Neuronal projections between the hippocampus, PFC, and lateral amygdala are

glutamatergic (341), suggesting that VU0409106 may interfere with reciprocal entrainment of hippocampus, PFC and amygdala at theta frequency via post-synaptic $mGluR_5$ inhibition in these regions, resulting in the observed attenuation of post-SPS increases in theta power during REM sleep, and possibly preventing the induction of synaptic plasticity associated with emotional memory consolidation (250, 341). VU0409106 administration in non-stressed rats did not reduce REM sleep theta power, suggesting that this effect is a specific blockade of trauma-induced alterations. This effect was also specific to the REM sleep state and to the frontal cortex, in support of the hypothesis that traumatic memory consolidation after SPS, and its attenuation by VU0401906, is heavily dependent on REM sleep-specific processes. Future studies are warranted aimed at determining whether SPS induces LTP at cortico-amygdala and hippocampal-amygdala synapses (33, 36, 37), and whether this plasticity is dependent on $mGluR_5$-mediated increases in REM sleep theta power after trauma.

Consistent with the notion that VU0409106 prevents hyperactivation of the amygdala, we also found that this compound attenuated SPS-induced reductions in amygdala 5-HT utilization. As mentioned in Chapter 2, 5-HT exerts a net inhibitory influence on the lateral amygdala (325, 326); the gradual reduction of 5-HT utilization caused by SPS, therefore, is hypothesized to contribute to amygdala hyperexcitability, a common finding in PTSD patients (29). One mechanism through which post-trauma $mGluR_5$ antagonism may spare amygdala 5-HT content

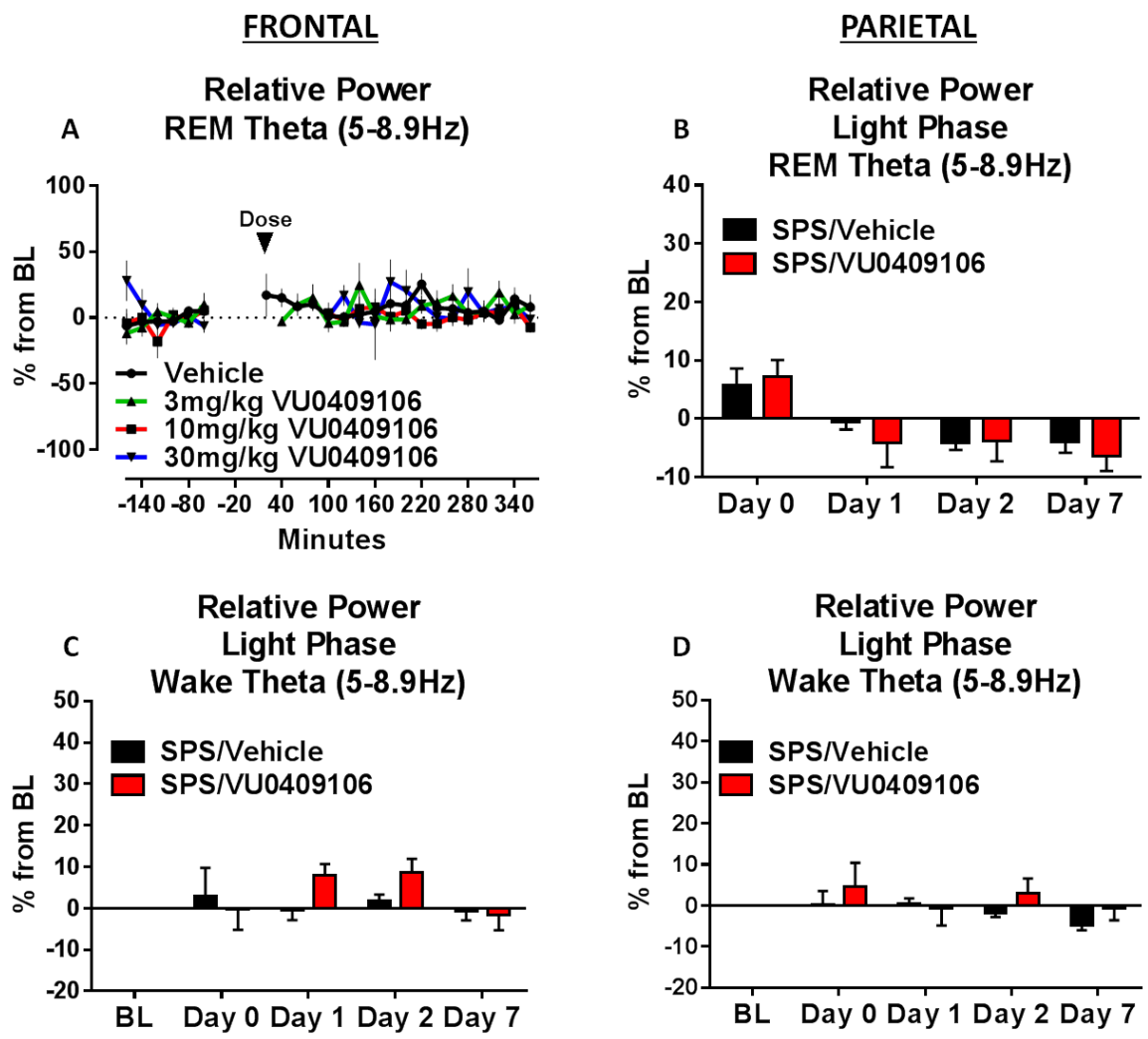

Figure 25. VU0409106-mediated reductions in theta power only occurred after trauma specifically in the frontal cortex during REM sleep. (A) VU0409106 had no effect at any dose on theta power during REM sleep in the frontal cortex in rats that had not received any stress treatment. (B) SPS-induced acute increases in REM sleep theta power were less pronounced in the parietal cortex, and VU0409106 had no effect on this change. (C) SPS also had no significant effect on theta power during wake in the frontal cortex, or (D) in the parietal cortex. Data are depicted as mean ± SEM (n = 7-10). Comparison between groups performed by repeated measures two-way ANOVA.

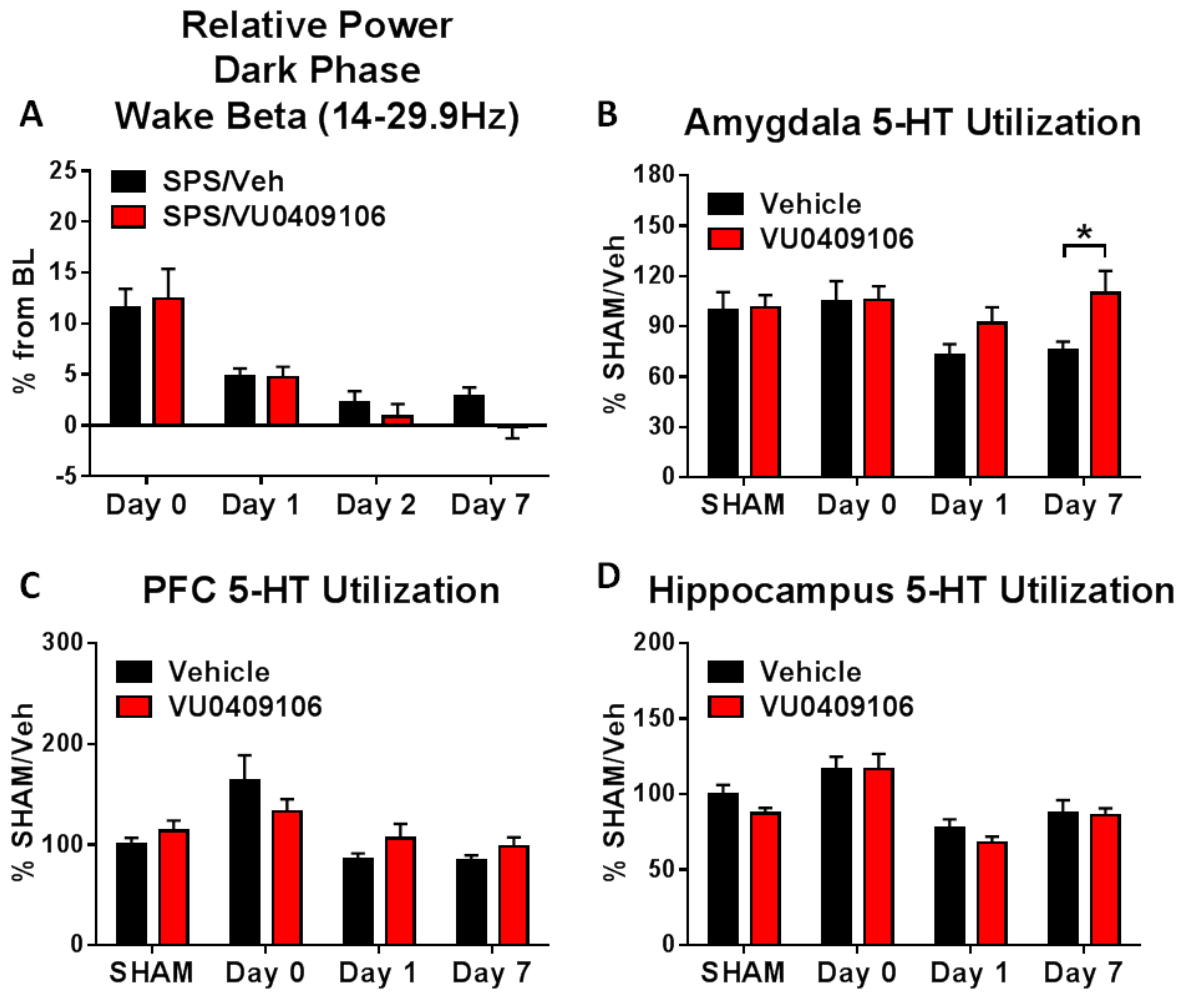

Figure 26. Post-trauma VU0409106 non-significantly accelerated the normalization of beta power during wake while attenuating reductions in amygdala 5-HT utilization. (A) SPS treatment caused acute and persistent increases in beta power during wake in the frontal cortex; VU0409106 administration non-significantly accelerated the normalization of this change (n = 7). (B) SPS treatment caused a concurrent reduction in amygdala 5-HT utilization starting on Day 1; this change was also blocked by VU0409106 administration. (C) VU0409106 administration did not affect SPS-induced acute increases in 5-HT utilization in the PFC or (D) hippocampus (n = 9-13). Data are depicted as mean + SEM. Comparison between groups performed by repeated measures two-way ANOVA. *P < .05 SPS/VU0409106 vs. SPS/Veh in Bonferroni *post hoc* test.

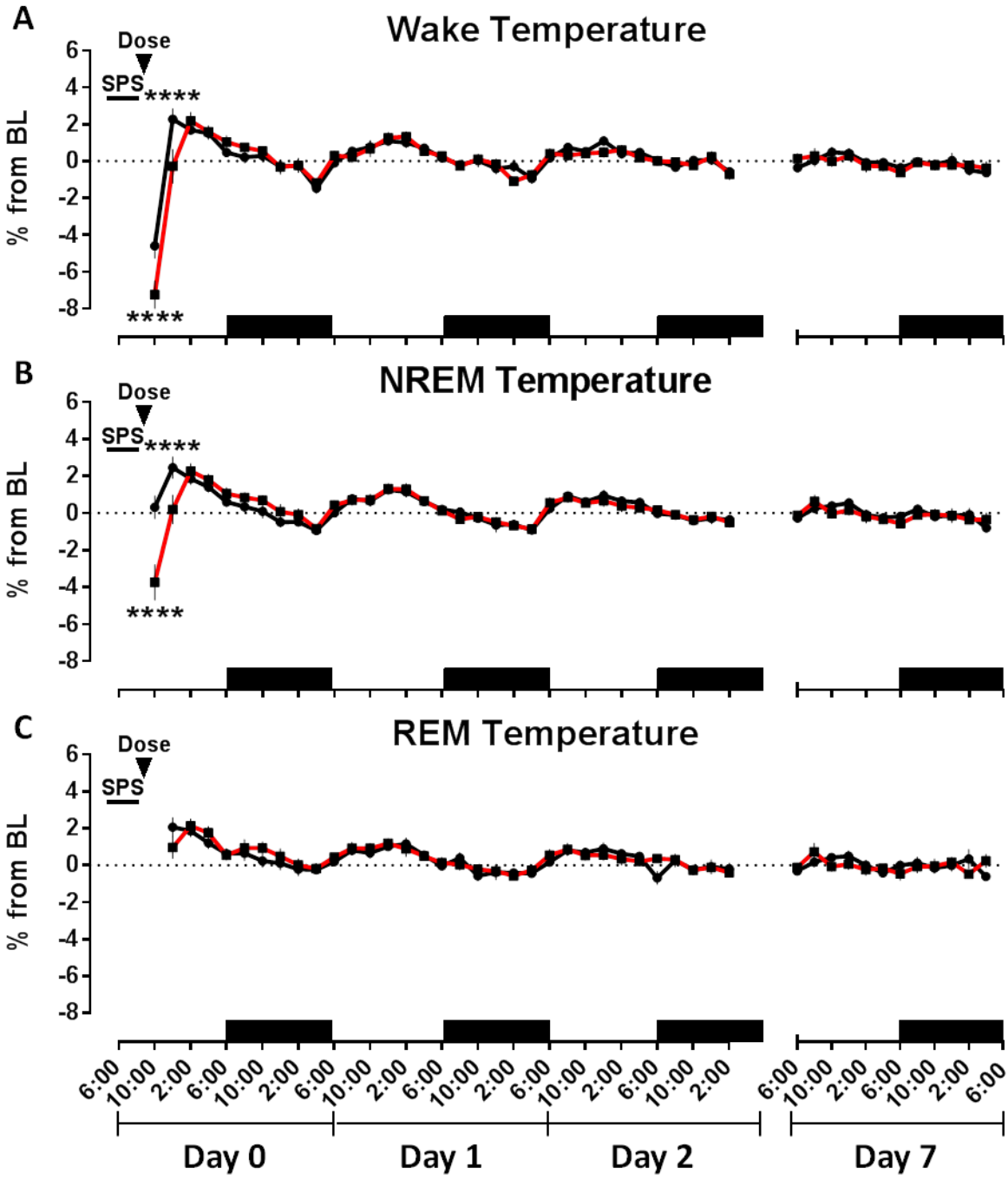

Figure 27. Post-trauma VU0409106 acutely inhibited SPS-induced hyperthermia. (A) SPS caused acute and persistent hyperthermia during wake, (B) NREM sleep, and (C) REM sleep specifically during the light phase; VU0409106 significantly attenuated hyperthermia during wake and NREM sleep for several hours after SPS treatment. Data are depicted as mean ± SEM (n = 8). Comparison between doses performed by repeated measures two-way ANOVA. . ****P < .0001 SPS/VU0409106 vs. SPS/Veh in Bonferroni *post hoc* test.

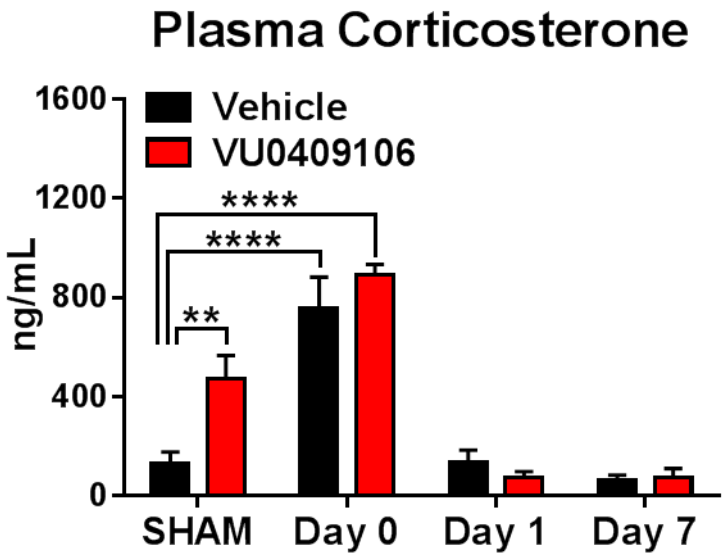

Figure 28. Post-trauma VU0409106 had no effect on plasma corticosterone increases. SPS caused acute increases in circulating corticosterone on Day 0 relative to SHAM treatment. VU0409106 administration increased corticosterone levels in the plasma in SHAM treated rats, but had no effect on SPS-induced corticosterone increases. Data are depicted as mean + SEM (n = 9-10). Comparison between doses performed by repeated measures two-way ANOVA. **P < .01, ****P < .0001 in Bonferroni *post hoc* test.

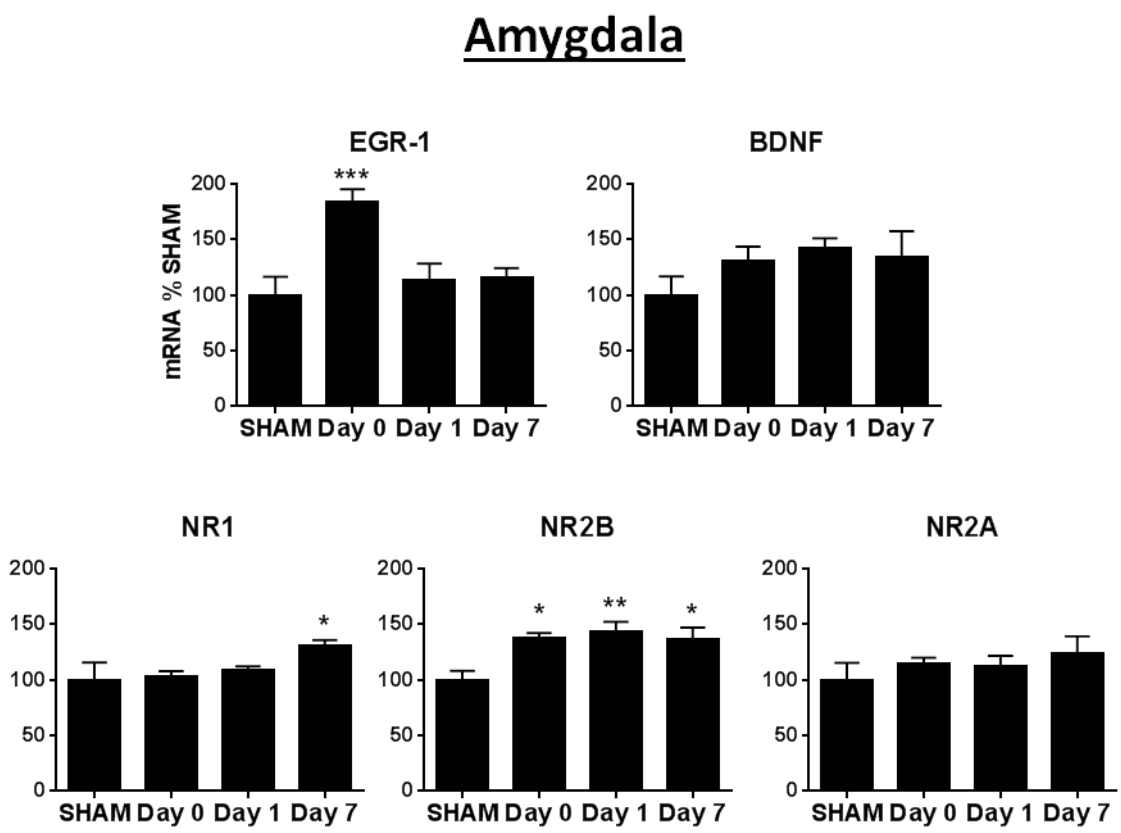

Figure 29. SPS induces amygdala expression of EGR-1 and NMDA receptor subunits. Relative to SHAM-treated rats, SPS caused substantial increases in the mRNA levels of EGR-1 in the amygdala on Day 0. This increase normalized by Day 1. SPS also increased expression of NR1 on Day 7, and NR2B on Days 0, 1 and 7. Data are depicted as mean + SEM (n = 8-9). Comparison between SHAM and SPS Day performed by one-way ANOVA. *P < 0.05, **P < 0.01, *** P < 0.001 in Dunnett's *post hoc* test compared to SHAM.

is by inhibiting the loss of serotonergic neurons in the dorsal raphe nucleus, the anatomical source of 5-HT in the amygdala (326). SPS has previously been shown to cause apoptosis in the dorsal raphe with cell death peaking at Day 7 (343), an observation that is consistent with our finding of delayed reductions in amygdala 5-HT utilization. Cell death in the dorsal raphe could be the result of excitotoxicity spurred by stress-induced release of excessive glutamate in this brain region (85), possibly originating from PFC or amygdala afferents (344, 345). VU0409106 might counteract this process in the dorsal raphe by exerting neuroprotective effects similar to other $mGluR_5$ antagonists (109, 335). It will be important in future studies to determine whether these changes in 5-HT utilization are specific to the lateral amygdala which is the predominant subregional target of serotonergic projections in the amygdala.

Reduced 5-HT utilization in the amygdala, and the resulting disinhibition of this brain structure, could manifest as an increase in high frequency frontal cortical EEG oscillations during wake (327) which have been found to be elevated in PTSD patients and associated with hyperarousal (297-299). While we replicated our previous finding that, similar to these patients, waking beta power is increased up to seven days after SPS, VU0409106-treated rats did not exhibit a significant reduction in this measure. However, there was a trend toward more rapid normalization of waking beta power that coincided with attenuation of the loss of amygdala 5-HT utilization. Replication of these studies is ongoing to determine whether $mGluR_5$ antagonism after SPS can ameliorate this qEEG correlate of hyperarousal.

Another major effect of VU0409106 administration on SPS-induced alterations in qEEG power spectra was a dramatic acute enhancement of SWA. Consistent with our previous results, SPS caused an acute increase in SWA prior to a reduction in this measure on Days 1 and 2 that might reflect similar disturbances in the sleep quality of PTSD patients (300, 301, 321). Rats that

received VU0409106 after SPS displayed a further increase in SWA on Day 0 that coincided with reduced latency to NREM sleep onset after SPS. This finding suggests that the restorative properties of deep NREM sleep may be improved by post-trauma $mGluR_5$ antagonism, an effect that could counteract the sleep disturbances and insomnia reported by recently traumatized individuals (287). Interestingly, NREM sleep and SWA increases are known to preferentially improve recall of declarative memories (225, 239, 245-249), offering the speculative interpretation that augmentation of this measure by $mGluR_5$ inhibition may spare explicit memory for the declarative components of the previously experienced traumatic event while simultaneously inhibiting associated implicit negative emotions. In translating to the clinic, it may be more beneficial to attenuate implicit emotional reactivity to the traumatic memory rather than ablate explicit recall for the memory entirely. This assertion is supported by the observation that trauma victims who report peritraumatic amnesia and related dissociative symptoms may actually be at increased risk for PTSD development (14). With this potential caveat in mind, we demonstrated that multiple doses of VU0409106 administered after contextual CF do not block the consolidation of normal threat learning, lending further support to the interpretation that $mGluR_5$ antagonism after SPS does not induce a general amnesic effect, possibly through sparing SWA. This effect differentiates $mGluR_5$ antagonists from SSRIs and γ-aminobutyric acid A ($GABA_A$) receptor PAMs, both classes of drugs which suppress REM sleep (346, 347), but which also acutely impair SWA (347, 348). $GABA_A$ PAMs such as benzodiazepines are known to be amnesic, and are actually contraindicated for the treatment of acute posttraumatic stress symptoms, possibly for this reason (291, 292, 349).

Preliminarily, we also found that SPS treatment results in the induction of EGR-1 in the amygdala, a molecular correlate of emotional memory consolidation (33). EGR-1 is an

immediate early gene and transcription factor, expression of which is required for initiating many of the cellular changes that are the substrates of memory consolidation after emotional learning (33). One product of EGR-1-mediated transcription is brain-derived neurotrophic factor (BDNF), which can promote increased expression of NMDA receptor subunits NR1, NR2A, and NR2B. We found that SPS had no significant effect on BDNF levels, but that subsequent to EGR-1 expression increases, NR1 and NR2B mRNA levels in the amygdala were also increased, possibly contributing to the hyperactivity of this brain region and concomitant qEEG indices of hyperarousal on these days. EGR-1 expression has also been shown to be specifically increased during REM sleep after aversive associative conditioning (284, 350), suggesting that increased activity of this molecule may represent one mechanism through which traumatic memory is encoded in a sleep-dependent manner. Future studies are warranted aimed at determining whether EGR-1 and NMDA receptor protein levels are also increased in the amygdala, and whether post-trauma $mGluR_5$ antagonism blocks its induction as a means of inhibiting emotional memory consolidation during REM sleep.

Taken together, these data suggest that selective $mGluR_5$ antagonism after trauma may be a safe and effective means of inhibiting or preventing the development of PTSD symptoms. Our data also indicate that the potential therapeutic effects of this approach would be partially sleep-dependent, and possibly grounded in the attenuation of traumatic memory consolidation.

CHAPTER 4

DISCUSSION

In this work, we have demonstrated that SPS offers a viable model of traumatic-stress induced physiological and behavioral changes similar to those exhibited by PTSD patients. In particular, rats which have undergone SPS display acute and persistent alterations in sleep-wake architecture and state-dependent qEEG spectral power indicative of PTSD-like symptoms. This model, therefore, provides the opportunity to test novel pharmacological interventions aimed at preventing these symptoms or treating them once they have already developed. We attempted the former approach by systemically administering the selective $mGluR_5$ NAM VU0409106 immediately after SPS with the hypothesis that this compound would block PTSD-like symptom development through attenuation of REM sleep-dependent traumatic memory consolidation. We found that a single dose of 10 mg/kg VU0409106 prevented the manifestation of augmented threat responding, a correlate of PTSD-related hypervigilance, and that this behavioral change was preceded by acute post-trauma REM sleep inhibition, and accompanied by normalization of 5-HT signaling in the amygdala. These findings add to a growing literature in support of the hypothesis that PTSD symptoms may be prevented by early post-trauma intervention. This work also provides the first evidence that selective $mGluR_5$ NAMs may represent one feasible target for this type of approach, and highlights the need to consider sleep effects in the development of this or other prophylactic drugs.

The pharmacological prevention of PTSD symptoms would represent a major advance in the care of traumatized individuals, and would significantly reduce the societal burden of treating

thousands of chronic PTSD patients (4, 5). Despite the therapeutic potential demonstrated by the preclinical studies outlined above, however, there are significant hurdles that would need to be addressed for the clinical development of $mGluR_5$ NAMs and other compounds aimed at inhibiting PTSD symptom progression after trauma. First, as mentioned, there is likely a restricted temporal window during which emotional memory consolidation could be blocked after experiencing trauma (31, 37, 284). This window may be as short as six hours, significantly limiting the amount of time that professional care givers would have to reach recently traumatized persons in need of prophylactic treatment. This limitation may be less of an impediment in certain populations at high risk for experiencing trauma, such as the military, in which victims of trauma are routinely reached within hours, and regimented care is rapidly initiated. Active members of the military in combat zones would likely be important partners in early proof-of-concept trials to determine whether post-trauma administration of clinically approved $mGluR_5$ NAMs, such as fenobam (351), can prevent PTSD development.

The fact that only about thirty percent of individuals who experience a traumatic event actually develop PTSD (5) represents another limitation to this preventive strategy. Based on this estimate, in the majority of traumatized individuals, prophylactic treatment with an $mGluR_5$ NAM or other compound would be unnecessary. This caveat would be especially important to consider for a drug with a narrow therapeutic index or high adverse effect liability. For care givers, this consideration would affect the cost-benefit analysis of drug treatment in the wake of trauma; it would be less tolerable to administer a drug that has potentially severe adverse effects to individuals at lower risk for PTSD development. Ideally, only those traumatized individuals known to harbor a predisposition for PTSD development would be given an $mGluR_5$ NAM or other preventive drug. This problem could be addressed in two ways. First, it would be

advantageous to develop a drug that has modest adverse effect liability, such that the potential benefits of its administration are not outweighed by its risks. Whether this can be achieved is yet to be determined, but future research should emphasize the elimination of adverse effect liability in compounds, including $mGluR_5$ NAMs, that show promise as inhibitors of emotional memory consolidation. Second, it would be important to determine what genetic and environmental factors contribute to PTSD vulnerability. Although significant resources are being dedicated to answer this question, it is as yet impossible to accurately predict who will get PTSD and who will not (352).

Both of these endeavors could be served by the SPS model of PTSD symptoms. In particular, to determine susceptibility to PTSD, future studies could group rats into vulnerable and resistant subpopulations based on the magnitude or duration of change in one or more of the EEG alterations described above. Subsequent behavioral testing could be employed to determine whether these measures are indeed predictive, and whether there is a differential effect of $mGluR_5$ antagonism on these two groups. For example, it is possible that the degree to which REM sleep theta power is increased in the hours after SPS predicts the likelihood or severity of augmented threat responding two weeks later. These types of studies would offer insight into the mechanisms of resilience while at the same time contributing to the possible discovery of translational methods for the prediction of PTSD susceptibility in humans. In the predator scent stress model, this use of cutoff criteria to define vulnerability and resilience in rats has been employed with interesting results (282, 353-358), and has been successfully used to test multiple pharmacological interventions for the prevention of PTSD-like symptoms (353, 355-358). Interestingly, in one study, post-trauma NMDA receptor antagonism with ketamine did not reduce the number of rats that went on to qualify as high responders in assays of PTSD-like

symptomology (358). This finding begs the question of whether ketamine would be equally ineffective in the SPS rodent model of traumatic stress, despite the efficacy of $mGluR_5$ inhibition as described above. Future studies to test this hypothesis are warranted.

Of note, SPS does not involve the use of ethologically relevant threats as with predator scent stress or social conflict. Nor does SPS incorporate threat from a conspecific during the traumatic experience. As such, despite portraying strong construct (59) and predictive validity (82, 339), the SPS model does not exhibit high levels of face validity as it relates to interpersonal trauma, such as physical or sexual assault. While traumas of this type generally result in higher rates of PTSD, and may cause more severe symptoms (359), it can be argued that traumas encountered during combat, vehicular accident, and natural disaster are not ethologically relevant to humans, and are not necessarily characterized by interpersonal interactions. Thus, SPS may more closely model trauma related to combat or disaster, and its effects may be more relevant to PTSD symptoms resulting from these types of traumatic experiences. As mentioned above, the clinical testing of prophylactic interventions for PTSD would be facilitated by the unique circumstances of military deployment; thus, the effects of SPS as reported here may be the best model for initially examining the benefits and risks of these interventions.

An important unanswered question is whether total sleep deprivation after SPS would have the same ameliorative effects as $mGluR_5$ NAM-mediated REM sleep suppression. We speculate that the unique profile of selective $mGluR_5$ antagonism, which results in REM sleep suppression while simultaneously facilitating SWA during NREM sleep, is necessary to achieve the observed behavioral benefits. Indeed, acute insomnia is a common complaint of recently traumatized individuals, and has actually been correlated with subsequent risk for PTSD development (289). Insomnia is characterized primarily by a loss of NREM sleep enriched in

SWA (301, 321), suggesting that $mGluR_5$ NAMs could counteract this effect while still inhibiting REM sleep-dependent emotional memory consolidation. In this way, the importance of simultaneous REM sleep suppression and NREM sleep facilitation could be explained by the preferential contribution of each of these sleep states to the consolidation of implicit and explicit memory, respectively (252). In experimental settings, recall for implicit and explicit components of a negative emotional memory can be dissociated such that prior stress enhances implicit recall for the affective components of the memory while simultaneously impairing explicit recall for the declarative components of the same memory (360). Victims of trauma can also exhibit enhanced implicit but not explicit memory for trauma-related information (17, 361). This phenomenon is especially striking in PTSD patients with trauma-related amnesia who exhibit the full spectrum of PTSD symptoms but demonstrate no explicit recall for the traumatic memory (14). With few exceptions (238), multiple lines of evidence demonstrate that the accumulation of REM sleep favors the consolidation of implicit memory (34, 225, 226, 234-239), while NREM sleep (and SWA) preferentially improves explicit memory (34, 225, 226, 234-239). This experimental observation, combined with the finding that peritraumatic amnesia predicts PTSD symptom severity (14), suggests that it may be beneficial to selectively suppress REM sleep during the aftermath of trauma in order to specifically inhibit overconsolidation of implicit negative feelings surrounding the traumatic memory, while sparing explicit recall for the event itself. Future studies could compare the effects of $mGluR_5$ NAMs (REM sleep suppressing; NREM sleep sparing; SWA enhancing), SSRIs (REM sleep suppressing; NREM sleep suppressing; SWA impairing) (347), and benzodiazapenes (REM sleep suppressing; NREM sleep sparing; SWA impairing) (348) on SPS-induced behavioral and physiological alterations to determine which sleep-related factors are most important to engender a therapeutic effect. It is of

interest to note that even in the absence of pharmacological intervention, SPS resulted in an acute suppression of REM sleep and an increase in SWA, suggesting that these changes may be part of a failed adaptive response that is facilitated by $mGluR_5$ antagonism.

It could also be informative to determine whether non-pharmacological treatments aimed at specifically reducing REM sleep after SPS, such as the small platform method (362), have any therapeutic effects on the physiological or behavioral consequences of traumatic stress. If so, this finding would suggest that selective targeting of $mGluR_5$ is not necessary, and that the ameliorative effects of inhibiting this receptor come primarily from REM sleep inhibition. Another means of testing this hypothesis would be to perform SPS during the rodent active phase, when there would be no need for sleep deprivation. It is possible that performing SPS at the beginning of the active phase would effectively delay the onset of sleep after trauma, and possibly attenuate subsequent symptoms. Support for this notion comes from the recent finding that this exact parametric manipulation ameliorated the behavioral effects of predator scent stress (282). Similarly, experimental sleep deprivation in healthy human subjects has been shown to preferentially impair consolidation of the implicit components of traumatic memory analogues (252).

These questions arise from the fact that the studies above demonstrate a correlation between $mGluR_5$ NAM-mediated REM sleep suppression and behavioral efficacy in a rodent model of trauma, but they do not show that REM sleep suppression is required for VU0409106 to engender its therapeutic effect. The studies outlined in the previous two paragraphs would go some way toward addressing this question, but in order to demonstrate causality, post-SPS VU0409106 would have to be systemically administered while simultaneously preventing its inhibitory effects on REM sleep time and theta power. This could potentially be achieved by

optogenetically activating REM-on cells in the brainstem (363) while VU0409106 is present in relevant concentrations in the brain. In this study design, if post-trauma $mGluR_5$ antagonism maintains its therapeutic behavioral effects, then REM sleep suppression is not required. However, we hypothesize that elimination of $mGluR_5$ antagonist-mediated REM sleep changes would result in a loss or reduction of therapeutic effect, which would demonstrate a causal relationship between this component of VU0409106 and its benefits.

In addition to suppression of REM sleep, $mGluR_5$ antagonism may block emotional memory consolidation through attenuation of new protein synthesis in the amygdala. New protein synthesis in this brain region is required for the consolidation of emotional memory in rats (364), and selective inhibition of $mGluR_5$ can inhibit new protein synthesis (365). Thus, it is possible that VU0409106 blocks new protein synthesis that would otherwise contribute to the remodeling of synapses in the neural fear circuitry, including the amygdala, preventing traumatic memory consolidation. Future studies aimed at determining whether SPS causes increased protein synthesis in the amygdala, and whether post-trauma administration of protein synthesis inhibitors such as anisomycin can exert similar therapeutic effects as VU0409106 would be informative.

An alternative means of determining the role of $mGluR_5$-mediated sleep alterations in traumatic memory consolidation would be to administer a compound that increases post-trauma REM sleep time or theta power, and determine whether the deleterious effects of SPS are exacerbated. This type of study would lend support to the hypothesis that REM sleep alterations affect the long term response to traumatic stress. To this end, in preliminary experiments, we attempted to positively modulate $mGluR_5$ in an attempt to pharmacologically increase REM sleep in healthy rats, possibly providing an avenue to testing this hypothesis in the SPS model

(Appendix A). We systemically administered the selective $mGluR_5$ PAM VU0409551 (366) to a group of healthy rats implanted with EEG electrodes, and recorded the effects of this compound on sleep-wake architecture. We found that selective $mGluR_5$ activation resulted in dose-dependent increases in time spent awake with concurrent reductions in both NREM and REM sleep. This increase in wake time may have been due to the fact that the neural circuitry that mediates wakefulness substantially overlaps with the brain regions that are activated to generate REM sleep, such as the LDT/PPT which exhibit $mGluR_5$ expression (367). This observation precluded the use of this compound to test whether post-trauma pharmacological increases in REM sleep could exacerbate the effects of SPS. However, VU0409551 has been shown to potentiate $mGluR_5$ signaling *ex vivo* without having any downstream effect on NMDA receptor activity (223). Thus, it is possible that an $mGluR_5$ PAM that does not exhibit this signaling bias could potentiate both NMDA receptor activity and promote entry into REM sleep. This hypothesis is supported by our finding that systemic administration of D-cycloserine (DCS), a partial agonist of the strychnine-insensitive glycine binding (GlyB) site on the NMDAR, was capable of increasing time spent in REM sleep (Appendix A). In addition to being a potentially useful tool for testing the effect of pharmacological increases in REM sleep on traumatic memory consolidation, this finding opens another area of inquiry based on the observation that REM sleep promotes not only the consolidation of emotional memory, but also its extinction (241-244).

Extinction learning represents the behavioral underpinning of exposure-based psychotherapy, and its pharmacological facilitation is a novel target for the treatment of PTSD (16, 242). If successful, this strategy would be capable of addressing the treatment resistant symptoms of PTSD patients who could not be reached quickly enough after experiencing a

traumatic event for prophylactic intervention. This approach would also be practicable without the discovery of predictive biomarkers for PTSD susceptibility because treatment would commence after the disorder has already developed. In both rodents and humans, the amount of REM sleep generated after extinction learning correlates with subsequent reductions in recall or reactivity to the memory (241, 243, 244). Thus, compounds that can increase REM sleep after successful exposure therapy sessions would be predicted to promote extinction of the traumatic memory and accelerate the reduction of associated symptoms. In support of this hypothesis, it was recently found that DCS administration modestly augmented the beneficial effects of exposure therapy in PTSD patients (89, 368). As mentioned in Chapter 1, these studies offer proof-of-concept validation for this therapeutic approach, but must be considered alongside other conflicting studies (90). These discrepant results are likely due to the fact that DCS, a partial agonist for the GlyB site, can actually act as an antagonist of NMDA receptor activity (91), pointing to the need for an alternative means of safely facilitating NMDA receptors.

Selective inhibition of the Glycine Transporter 1 (GlyT1) offers a different approach for the indirect positive modulation of NMDA receptors. Under normal conditions, the GlyB site on the NMDA receptor is not saturated due to the tightly controlled regulation of synaptic glycine levels by GlyT1, expressed in a distribution pattern that closely overlaps with NMDA receptor expression (92). Previous studies have demonstrated that selective inhibitors of GlyT1 can increase synaptic glycine levels sufficiently to produce enhanced NMDA receptor function in preclinical rodent models (93). Recently, we reported the development and characterization of a novel series of GlyT1 inhibitors, represented by ACPPBII, (2-amino-4-chloro-N-((4-phenyl-1-(propylsulfonyl)piperidin-4-yl)methyl)benzamide), with suitable bioavailability, brain penetration, and physical properties for extensive characterization in vivo (94). Selective

inhibition of GlyT1 by ACPPBII may thus provide a novel target for enhancing the therapeutic effects of exposure therapy in PTSD patients.

We performed preliminary experiments aimed at assessing this hypothesis in rats prior to testing ACPPBII in the SPS model. First, we demonstrated the potency and brain penetrance of ACPPBII relative to the prototypical GlyT1 inhibitor NFPS (N-[3-(4'-fluorophenyl)-3-(4'-phenylphenoxy)propyl]sarcosine) (93) (Appendix A). Then, in opposition to the long term effects of SPS and consistent with a therapeutic profile of this compound, we found that ACPPBII increased 5-HT utilization in the amygdala. However, when we tested the effect of this compound on sleep-wake architecture, we found that ACPPBII increased time spent awake, and reduced time spent in NREM and REM sleep. This effect was likely caused by adverse motor effects such as compulsive walking and respiratory depression that manifested in a substantial reduction in spontaneous locomotor activity. These motor side effects are found in other GlyT1 inhibitors, and are likely mediated by increased activation of the strychnine-sensitive inhibitory GlyA site on the glycine receptor which is expressed in the brain stem and mediates autonomic nervous system function (369). This toxicity, and the failure of ACPPBII to enhance REM sleep, likely contributed to our subsequent observation that, unlike the reported effects of DCS, ACPPBII failed to accelerate the acquisition or consolidation of extinction of context-mediated CF. Based on these studies, we determined that the therapeutic index of this compound was not conducive to further testing in the SPS model.

In addition to the glutamatergic system, in preliminary experiments, we identified the histaminergic and orexinergic systems as potential targets for ameliorating the effects of SPS (Appendix B). As mentioned in Chapter 1, histamine and orexin release from hypothalamic neurons promotes transition to and maintenance of wakefulness, suggesting that SPS treatment

may induce an insomnia-like phenotype on Days 1 and 2 partially through augmentation of these neurotransmitter systems. Specifically, we found that SPS induced increased mRNA expression of orexin receptor subtype 1 (OxR1) in the PFC and hippocampus and orexin receptor subtype 2 (OxR2) in the hippocampus in a time course that coincided with the persistent reductions in NREM and REM sleep time. This is consistent with the observation that increased orexin signaling contributes to wake promotion (227), and suggests that orexin antagonists such as the recently clinically approved compound suvorexant, could be an effective treatment for insomnia associated with chronic PTSD (370). Unlike benzodiazepines, suvorexant promotes NREM sleep time without reducing SWA (371), making it an especially attractive option for this application. However, it has been found that levels of the primary orexin signaling peptide, orexin A, are actually lower in the CSF of PTSD patients (372). Histamine also acts as a wake-promoting neurotransmitter (227), so we examined mRNA expression levels of the rate-limiting synthesizing enzyme for this molecule, histidine decarboxylase (HDC) in the hypothalamus. We found HDC expression was substantially decreased after SPS for at least seven days. One possible interpretation of these data is that HDC expression is reduced to compensate for excessive histamine release, which could contribute to insomnia-like symptoms in the SPS model. However, future studies including the measurement of extracellular histamine in the hypothalamus using microdialysis would be required to confirm this hypothesis. Additionally, a cellular mechanism would have to be elucidated through which excessive histamine release is detected resulting in compensatory reductions in HDC expression. Still, this finding suggests that antihistaminergic drugs could be useful as hypnotic agents in addressing PTSD-related insomnia. This hypothesis is supported by the recent observation that the antihistamine hydroxyzine was moderately effective in treating sleep disruptions in PTSD patients (373).

Taken together, our initial validation of the SPS model provides a strong foundation for the examination of this and other alternative interventions aimed at preventing or treating PTSD symptoms. We have also provided the first evidence that post-trauma systemic administration of a selective $mGluR_5$ NAM prevents the development of some PTSD-like symptoms in this model, and may show similar promise in the clinical arena. This therapeutic effect was at least partially mediated by altering the amount and quality of REM sleep in the aftermath of trauma exposure, highlighting the importance of examining sleep-dependent emotional memory consolidation during the discovery and development of novel pharmacotherapeutic strategies for the treatment of PTSD symptoms.

APPENDIX A

POSITIVE MODULATORS OF NMDA RECEPTOR FUNCTION

Methods

Subjects

All male Sprague-Dawley rats (Harlan, Indianapolis, IN) used in the present studies were housed under a 12 hour light:12 hour dark cycle and given ad libitum access to food and water. All animal experiments were approved by the Vanderbilt University Animal Care and Use Committee and experimental procedures conformed to guidelines established by the National Research Council Guide for the Care and Use of Laboratory Animals. All efforts were made to minimize animal suffering and the number of animals used.

Compound

VU0409551 was synthesized in house as previously described (366), and dissolved in 10% Tween 80 vehicle, creating a microsuspension prior to intraperitoneal (i.p.) administration at a volume of 2mL/kg. D-cycloserine (DCS) was purchased from Sigma-Aldrich, and fully dissolved in saline prior to i.p. administration at a volume of 1mL/kg. ACPPBII was synthesized in house as previously described (94), and dissolved in 20% beta-cyclodextrin vehicle, creating a suspension prior to i.p. administration at a volume of 3mL/kg.

EEG Surgery

Rats (250-375 grams) were surgically implanted with a telemetric transmitter (4-ET, Data Sciences International, St. Paul, MN) for recording EEG, electromyography (EMG), and body temperature. Under isoflurane anesthesia (3% induction; 1.5-2.5% maintenance) the transmitter was implanted subcutaneously across the back of each rat. Transmitter leads were tunneled subcutaneously to the skull. After holes were drilled in the skull, the exposed wires were placed in contact with the dura and secured in place with dental cement (Butler Schein, Dublin, OH). Three sets of leads were placed bilaterally to record from cortical regions corresponding to the frontal, parietal, and occipital cortices (+2 mm, -2 mm, and -6mm anterior-posterior from Bregma, respectively and +/- 2 mm lateral to the midline). An additional set of leads was placed bilaterally in the nuchal muscles for EMG recording. Rats were individually housed following surgery and allowed to recover and acclimate to the recording room for a minimum of 10 days prior to testing.

EEG

Eeach rat was randomized into vehicle, VU0409551 (3, 10, 30 mg/kg, i.p.), DCS (3, 10, 30 mg/kg, i.p.), or ACPPBII (1, 3, 10 mg/kg, i.p.) dose groups. Baseline recordings were begun at the start of the light phase, then the appropriate compound was administered two hours later, and recordings were allowed to continue for the remainder of the twenty-four hour period. In a partial crossover design, each rat received two different doses or vehicle, allowing for a 5 day washout period between compound administrations.

Spontaneous locomotor activity

Spontaneous locomotor activity was conducted in open-field chambers (27 × 27 × 20 cm) (Hamilton Kinder) equipped with 16 horizontal (x- and y-axes) infrared photobeams. Changes in locomotor activity were measured as the number of photobeam breaks per five minutes, and were recorded with a Pentium I computer equipped with rat activity monitoring system software (Hamilton Kinder). Rats were pretreated with vehicle or ACPPBII, (1, 3, 10 mg/kg, i.p.) then placed individually into each chamber 30 minutes later. Locomotor activity was assessed for thirty minutes.

***In vitro* glycine uptake**

Human choriocarcinoma (JAR) cells (American Type Culture Collection), endogenously expressing GlyT1 were cultured *in vitro* in complete DMEM supplemented with 10% FBS, 2 mM glutamine, 20 mM HEPES, 0.1 mM nonessential amino acids, 1mM sodiumpyruvate, and antibiotic/antimycotic (Life Technolgies ,Carlsbad, CA) at 37 ºC in 5% CO_2 in a humidified cell incubator. Cells were plated at 20,000 cells per well in 96-well Cytostar-T scintillation microplates (GE Healthcare, Chalfont St. Giles, Buckinghamshire, UK) and cultured for 24 hours. The culture medium was then aspirated and the cells were washed followed by addition of a range of concentrations of ACPPBII, the comparator NFPS, or glycine dissolved in DMSO in combination with 10 ul of [14C]glycine. After two hours, each well was washed to remove radioactivity from the media, and radioactivity counts present inside the cell due to specific glycine uptake were performed using a TopCount (PerkinElmer). Fifty percent inhibitory concentrations (IC_{50}) for each compound was determined after performing non-linear regression analysis in the statistical software Prism (GraphPad).

In vivo pharmacokinetics

15min, 1, 3, 8, 12, and 24 hours after ACPPBII or NFPS administration, rats were deeply anesthetized with isoflurane, rapidly decapitated, and trunk blood and whole brain was collected. Plasma was separated by centrifugation (4000 rcf, 4 °C) and stored at -80 °C until analysis. On the day of analysis, frozen whole-mouse brains were weighed and diluted with 1:3 (w/w) parts of 70:30 isopropanol:water. The mixture was then subjected to mechanical homogenation employing a Mini-Beadbeater™ and 1.0 mm Zirconia/Silica Beads (BioSpec Products) followed by centrifugation. The sample extraction of plasma (20 μL) or brain homogenate (20 μL) was performed by a method based on protein precipitation using three volumes of acetonitrile containing an internal standard (50 ng/mL carbamazepine). The samples were centrifuged (3000 rcf, 5 min) and supernatants transferred and diluted 1:1 (supernatant:water) into a new 96 well plate, which was then sealed in preparation for LC/MS/MS analysis.

LC/MS/MS analysis

Analysis was performed as previously described (374).

Tissue collection

Rats were briefly anesthetized with isoflurane, and sacrificed by decapitation thirty minutes after i.p. administration of vehicle or ACPPBII (1, 3, 10 mg/kg). Hippocampus, amygdala, and PFC were dissected, rapidly frozen on dry ice, and stored at −80 °C for tissue neurochemistry experiments.

Tissue neurochemistry

Tissue concentrations of 5-HT and its metabolite 5-hydroxyindoleacetic acid (5-HIAA) were determined by HPLC-ECD as described previously (316).

Fear extinction

For contextual CF extinction experiments, rats were placed in the CF chambers described above for each component of the experiment. To test the effects of ACPPBII on extinction acquisition, rats were trained with the following protocol: habituation to the chamber for one minute followed by exposure to four 1s, 0.5mA footshocks with intershock intervals of one minute. On each of the subsequent extinction days, rats were returned to the same chamber for seven minutes, and their freezing response was recorded in the absence of footshock. The compound was dosed thirty minutes prior to each extinction session every day.

To test the effects of ACPPBII on extinction consolidation, rats were trained with the following protocol: habituation to the chamber for three minutes followed by two 4s, 0.8 mA footshocks with intershock intervals of 30s and 60s after the last shock. On each of the subsequent extinction days, rats were returned to the same chamber for ten minutes, and their freezing response was recorded in the absence of footshock. The compound was dosed immediately after each extinction session every day.

Statistical analysis

For the acute effects of each compound on sleep-wake architecture, and the effect of ACPPBII on spontaneous locomotor activity a repeated measures two-way analysis of variance (ANOVA) was applied; if significant, a Bonferroni post hoc test was performed with

significance defined as $P < .05$. For the effects of ACPPBII on 5-HT utilization in the amygdala, a one-way ANOVA was applied; if significant, a Dunnett's post hoc test was used with significance defined as $P < .05$.

Results

VU0409551 increased time spent awake (Figure 30) (time [$F_{11,264} = 31.80$, $P < 0.0001$], interaction [$F_{33,264} = 2.02$, $P = 0.0013$]), and decreased NREM sleep (time [$F_{11,264} = 32.98$, $P < .0001$], interaction [$F_{11,264} = 2.06$, $P = .001$]) and REM sleep (time [$F_{11,286} = 17.54$]). DCS had a main effect on time spent in wake (Figure 31) (time [$F_{11,352} = 78.26$, $P < 0.0001$], dose [$F_{3,32} = 3.83$, $P = 0.0188$]) and NREM sleep (time [$F_{11,308} = 70.86$, $P < 0.0001$], dose [$F_{3,28} = 3.77$, $P = 0.0217$]), and acutely increased time spent in REM sleep) (time [$F_{11,308} = 22.21$, $P < 0.0001$]). ACPPBII acutely increased time spent in wake (Figure 32) (time [$F_{11,506} = 117.1$, $P < 0.0001$], interaction [$F_{33,506} = 2.61$, $P < 0.0001$]), and reduced time spent in NREM sleep (time [$F_{11,506} = 115.1$, $P < 0.0001$], interaction [$F_{33,506} = 2.00$, $P = 0.001$]) and REM sleep (time [$F_{11,506} = 26.32$, $P < 0.0001$], dose [$F_{3,46} = 4.05$, $P = 0.0123$], interaction [$F_{33,506} = 2.74$, $P < 0.0001$]).

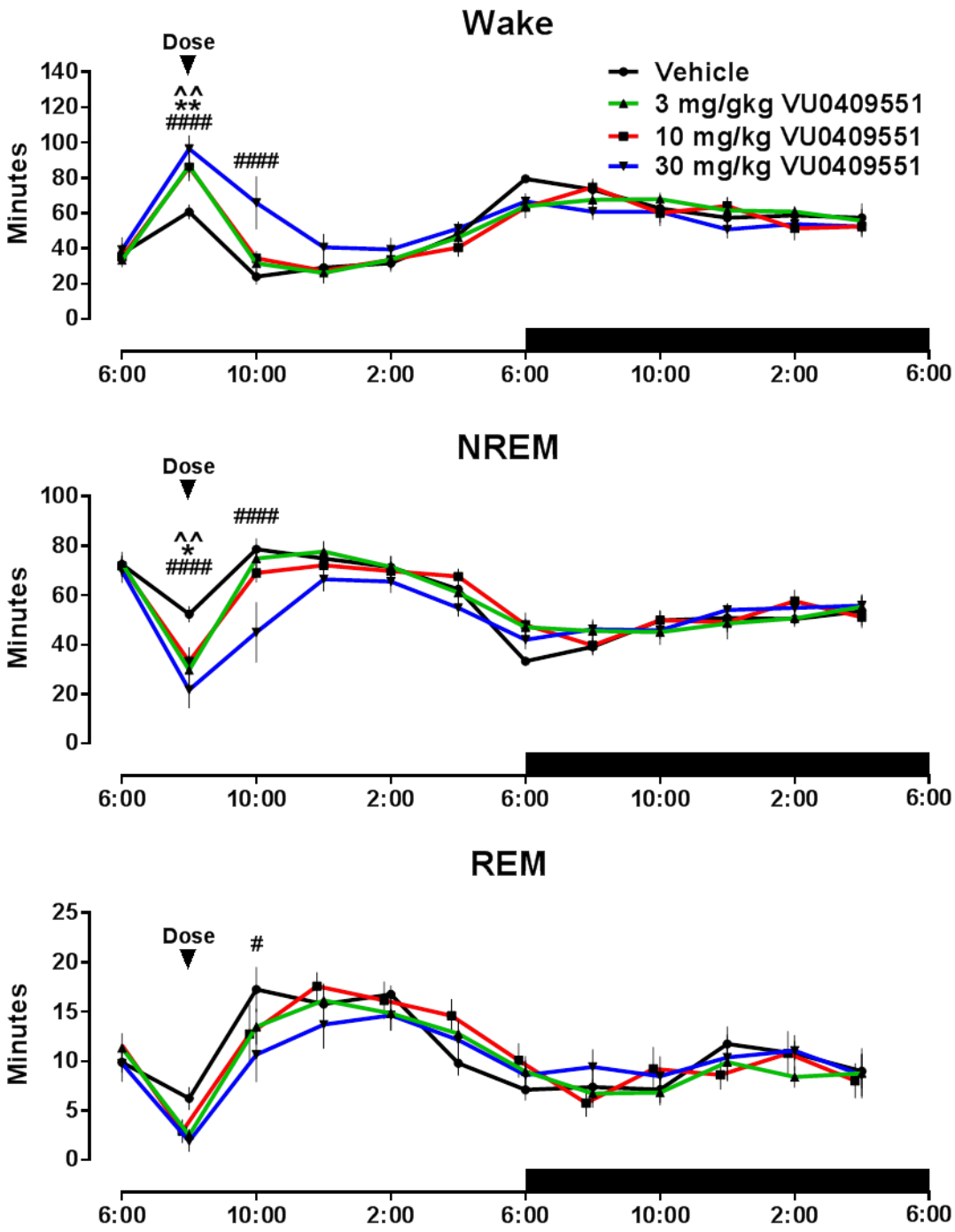

Figure 30. VU0409551 increased time spent in wake. ^^ P < 0.01, 3mg/kg vs. Veh; *P < 0.05, **P < 0.01, 10 mg/kg vs. Veh; #P < 0.05, ####P < .0001, 30 mg/kg vs. Veh in Bonferroni *post hoc* test.

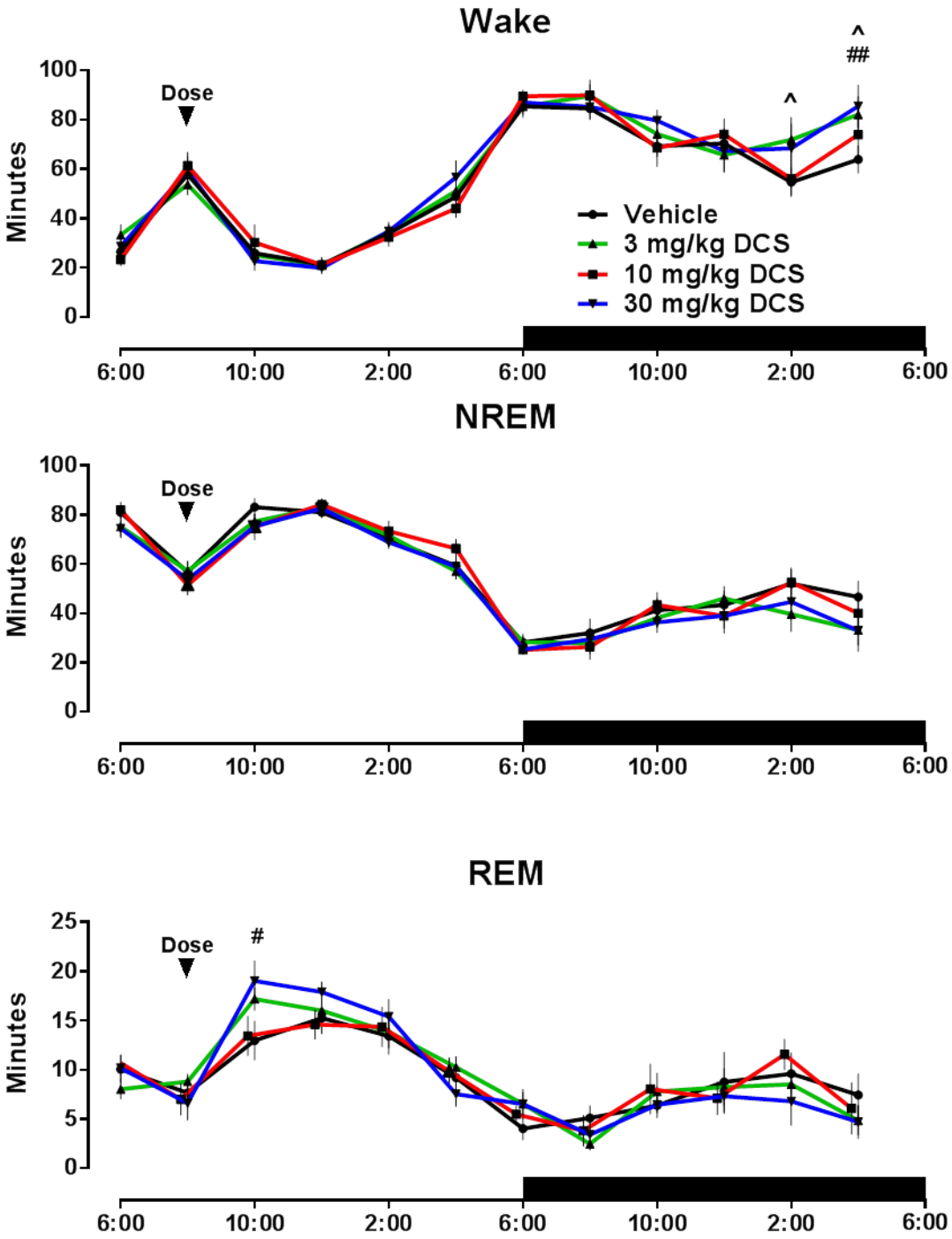

Figure 31. DCS increased time spent in REM sleep. ^ P < 0.05, 3mg/kg vs. Veh; #P < 0.05, ##P < .01, 30 mg/kg vs. Veh in Bonferroni *post hoc* test.

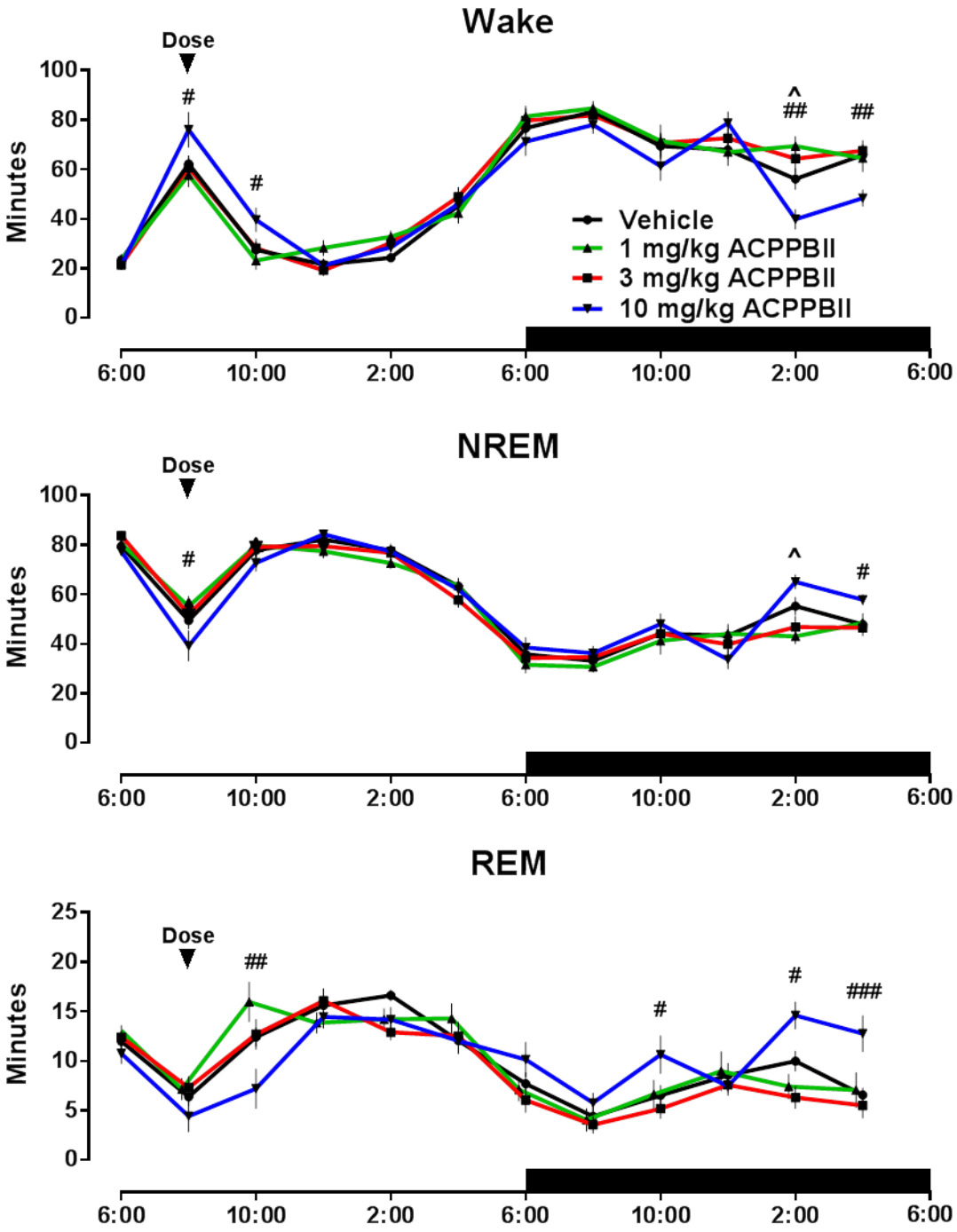

Figure 32. ACPPBII increased time spent in wake. ^ P < 0.05, 1mg/kg vs. Veh; #P < 0.05, ##P < .01, 10 mg/kg, ###P < .001, 30 mg/kg vs. Veh in Bonferroni *post hoc* test.

ACPPBII exhibited an *in vitro* IC_{50} of 45 nanomolar (nM) which was similar in potency to the prototypical GlyT1 inhibitor NFPS (Figure 33) (IC_{50} = 24 nM). ACPPBII was similarly brain penetrant as well, achieving concentrations well above its *in vitro* IC_{50} (Figure 34). ACPPBII also increased 5-HT utilization as evidenced by increased 5-HIAA concentrations in the amygdala (Figure 35) ($F_{3,26} = 1.55$, $P = 0.0392$) with no change in 5-HT concentration. However, ACPPBII also induced adverse motor effects including a reduction in spontaneous locomotor activity (Figure 36) (time [$F_{5,125} = 99.5$, $P < 0.0001$], dose [$F_{3,25} = 5.23$, $P = 0.0061$], interaction [$F_{15,125} = 3.80$, $P < 0.0001$]). Consistent with the observed toxicity of this compound, ACPPBII failed to facilitate the extinction of contextual CF (Figure 37).

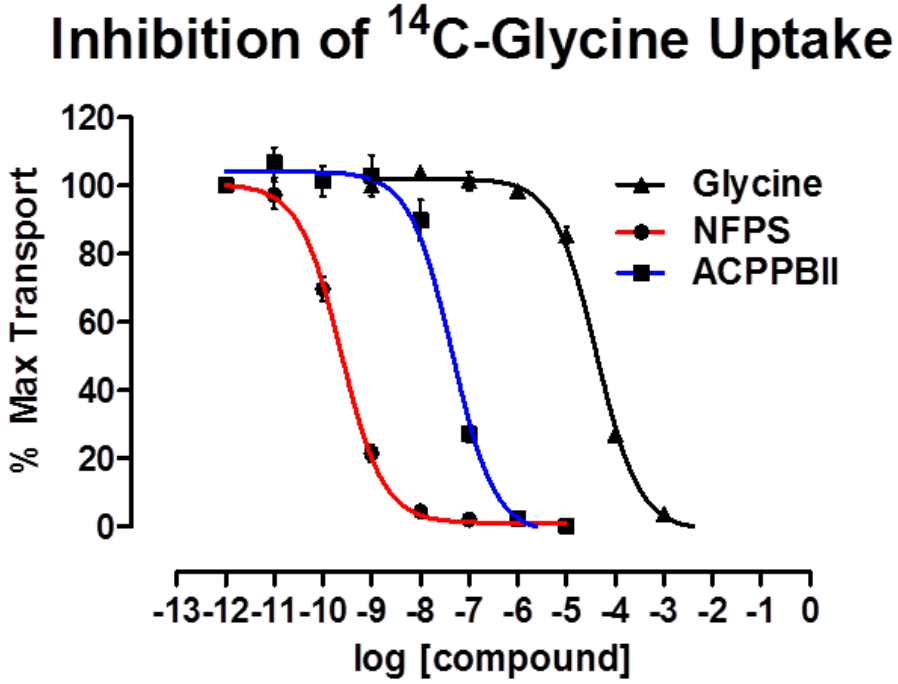

Figure 33. ACPPBII is a potent inhibitor of GlyT1. ACPPBII exhibited a potency of 45nM in inhibiting glycine uptake by GlyT1 in an *in vitro* assay. NFPS exhibited a potency of 24nM.

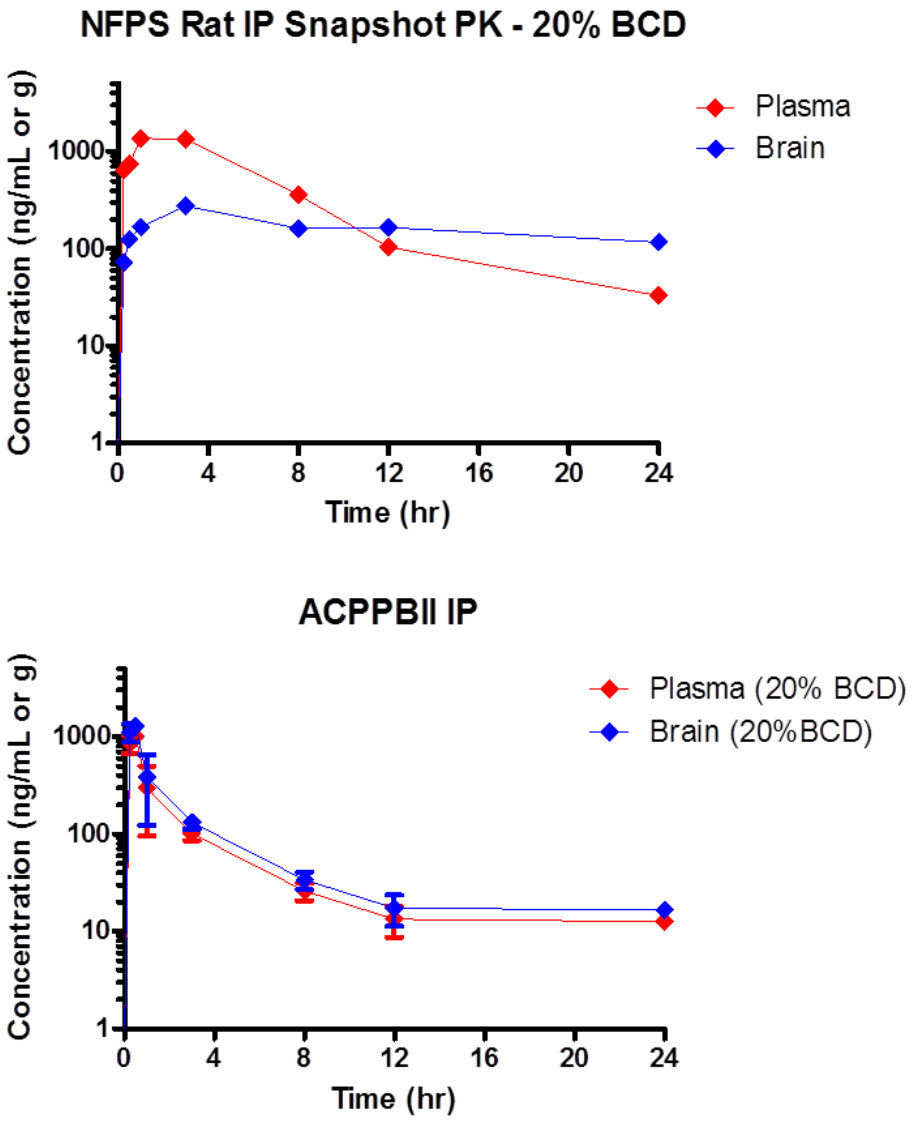

Figure 34. ACPPBII penetrates the central nervous system similar to the prototypical GlyT1 inhibitor NFPS.

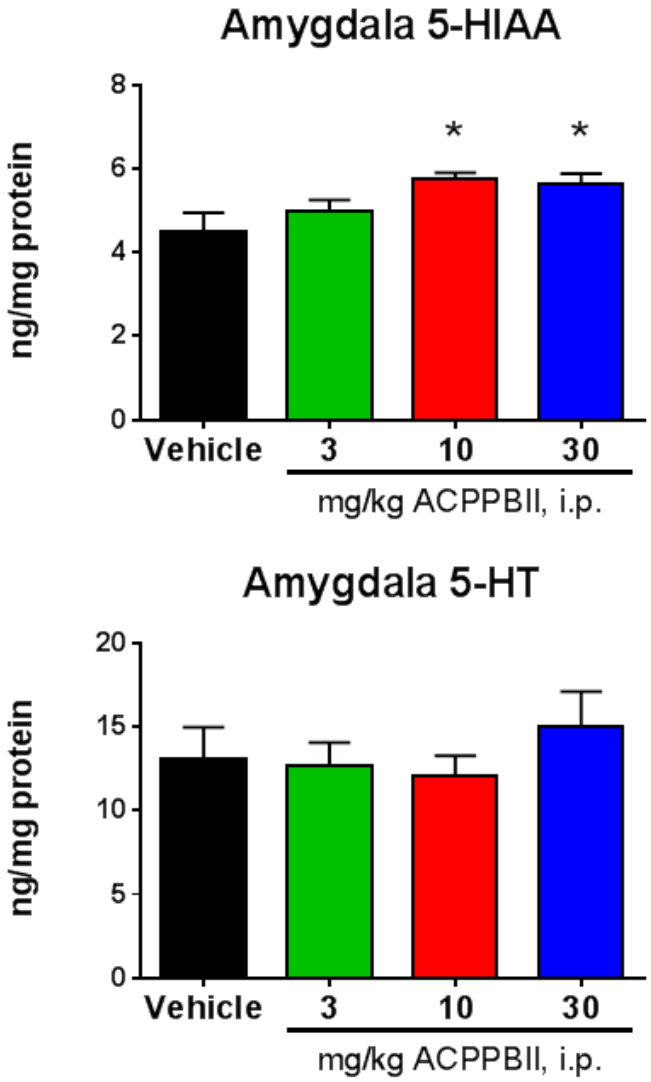

Figure 35. ACPPBII increase 5-HT utilization in the amygdala. *P < 0.05 vs. Veh in Dunnett's post-hoc test.

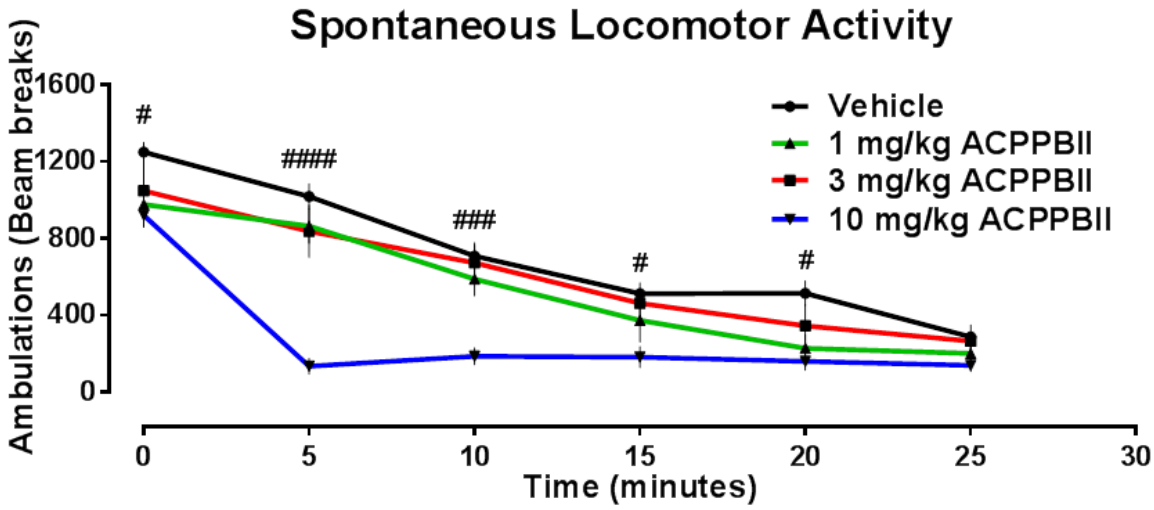

Figure 36. ACPPBII reduces spontaneous locomotor activity. #P < 0.05, ###P < 0.001, ####P < 0.0001 10mg/kg vs. Veh.

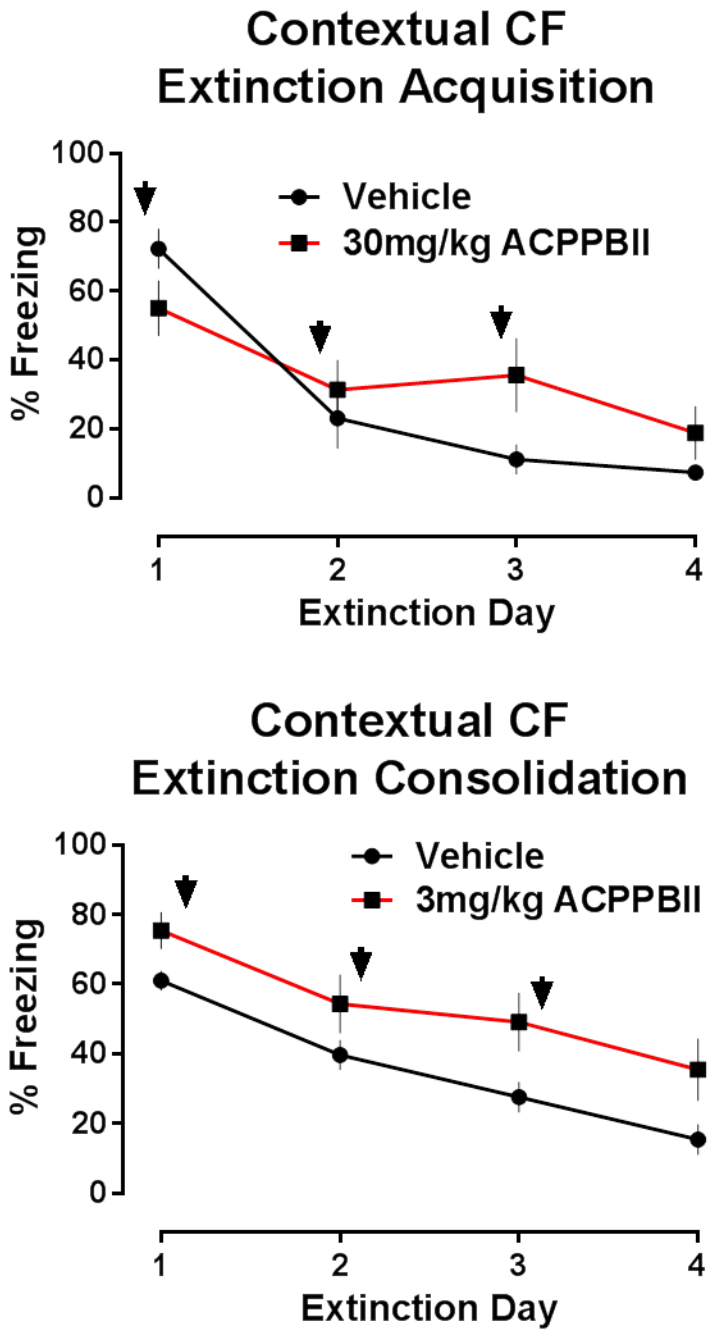

Figure 37. ACPPBII did not enhance extinction learning. ACPPBII failed to promote reductions in the freezing response when administered before or after context-mediated extinction training at 3 or 30mg/kg, i.p. Arrows represent dose times.

APPENDIX B

EFFECTS OF TRAUMATIC STRESS ON OREXINERGIC AND HISTAMINERGIC SYSTEMS

Methods

Subjects

All male Sprague-Dawley rats (Harlan, Indianapolis, IN) used in the present studies were housed under a 12 hour light:12 hour dark cycle and given ad libitum access to food and water. All animal experiments were approved by the Vanderbilt University Animal Care and Use Committee and experimental procedures conformed to guidelines established by the National Research Council Guide for the Care and Use of Laboratory Animals. All efforts were made to minimize animal suffering and the number of animals used.

Single Prolonged Stress

SPS was performed according to Liberzon et al. (315). Briefly, rats were restrained for 2 hours, followed by forced swim for 15 minutes in 24 ^{0}C water. Following a 15 minute recovery period, rats were exposed to diethyl ether vapor in a bell jar until anesthesia. The SPS model did not cause mortality. SPS did illicit hallmarks of the rodent stress response such as porphyrin staining of the eyes, and urination and defecation. There were no major individual differences observed in these parameters during each experiment, and no inclusion or exclusion criteria were applied prior to the start of EEG recordings or tissue collection. SHAM treatment consisted of

placement in a novel procedure room for 2 hours followed by brief handling. All animals were placed into fresh cages after treatment.

Tissue collection

For all biochemical endpoints, rats were randomly assigned to SHAM treatment or one of three SPS groups (Day 0, 1 or 7). Rats were briefly anesthetized with isoflurane, and sacrificed by decapitation either immediately (Day 0), one day (Day 1), or seven days (Day 7) after SPS; SHAM rats were sacrificed immediately after SHAM treatment. Hippocampus, amygdala, and PFC were dissected, rapidly frozen on dry ice, and stored at −80 °C for tissue mRNA experiments.

Quantitative real-time polymerase chain reaction (qRT-PCR)

Alterations in mRNA expression levels were measured using Aqueous Micro kits (Life Technologies, Grand Island, NY) for RNA extraction, NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE) for RNA quantification, QuantiTect Reverse Transcription Kit (QIAGEN, Hilden, Germany) for complementary DNA transcription, CFX96 Real-Time PCR Detection System (Bio-Rad, Hercules, CA) using primers from TaqMan Gene Expression Assays (Life Technologies) for qRT-PCR of rat OxR1, OxR2, and HDC. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an internal control; data are presented using the comparative cycle threshold (CT) method normalized to SHAM-treated rats.

Statistical analysis

For the acute effects of each compound on sleep-wake architecture, and the effect of ACPPBII on spontaneous locomotor activity a repeated measures two-way analysis of variance

(ANOVA) was applied; if significant, a Bonferroni post hoc test was performed with significance defined as $P < .05$. For the effects of ACPPBII on 5-HT utilization in the amygdala, a one-way ANOVA was applied; if significant, a Dunnett's post hoc test was used with significance defined as $P < .05$.

Results

SPS increased mRNA concentration of Ox1R in the PFC on Days 1 and 2 after SPS ($F_{3,28} = 5.52$, $P = 0.0042$), and on Day 2 in the hippocampus ($F_{3,28} = 3.94$, $P = 0.0184$). SPS also increased Ox2R expression in the hippocampus on Day 2 ($F_{3,26} = 3.63$, $P < 0.0259$), but had no significant effect in the hypothalamus or amygdala (Figure 37). SPS also caused a substantial sustained reduction in mRNA concentration of HDC in the hypothalamus (Figure 38) ($F_{3,30} = 4.01$, $P < 0.0163$).

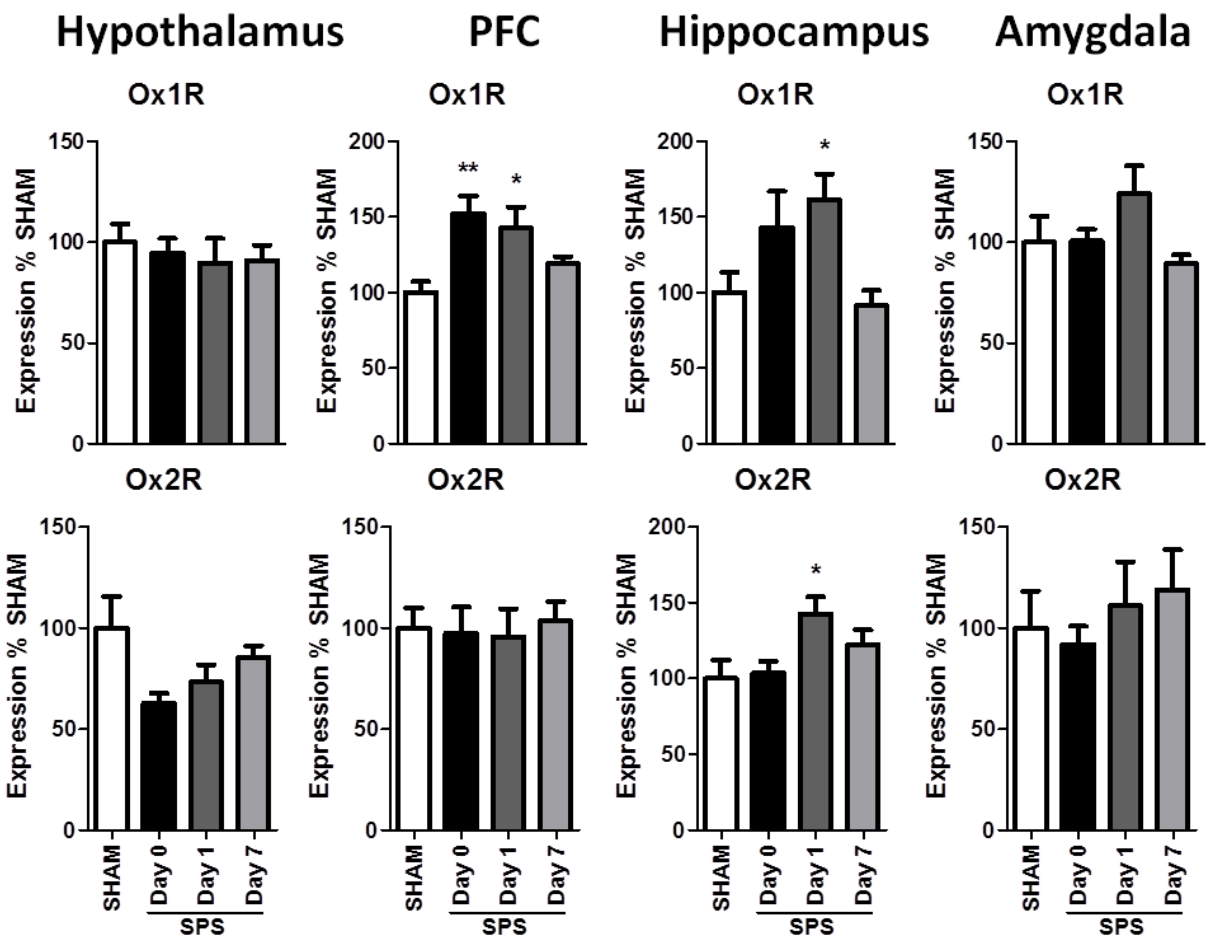

Figure 38. SPS caused an increase in mRNA concentration of orexin receptors in the PFC and hippocampus. SPS increased mRNA concentration of Ox1R in the PFC on Days 1 and 2 after SPS, and on Day 2 in the hippocampus. SPS also increased Ox2R expression in the hippocampus on Day 2. *P < 0.05, **P < 0.01 vs. SHAM in Dunnett's post hoc test.

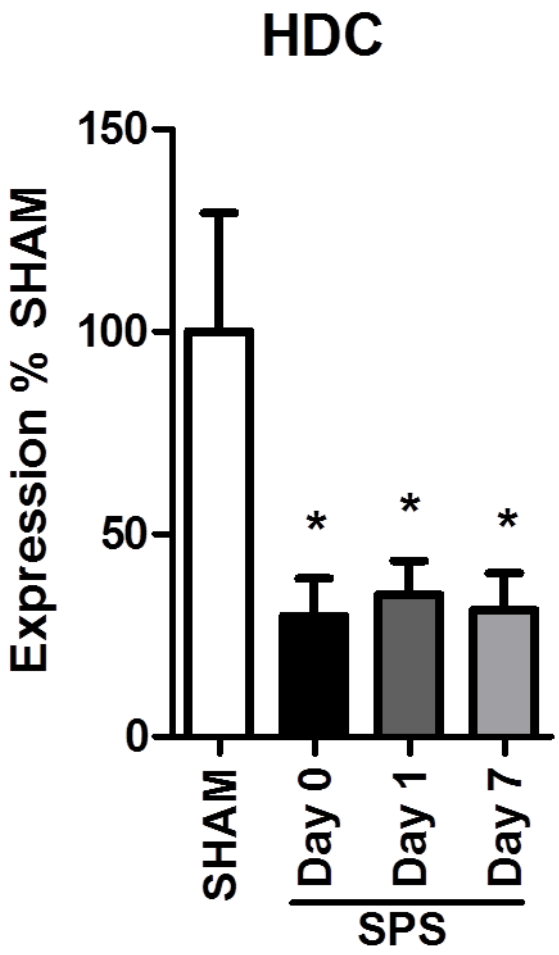

Figure 39. SPS caused a decrease in mRNA concentration of HDC in the hypothalamus. *P < 0.05 vs. SHAM.

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