

THE EFFECT OF MILD BLAST TRAUMATIC BRAIN INJURY ON THE
SEXUALLY DIMORPHIC STRESS AXIS

By

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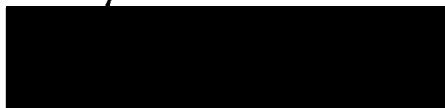
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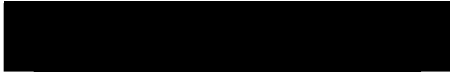
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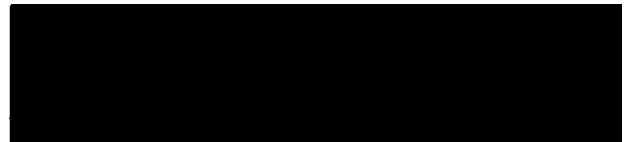
DEDICATION

This dissertation is dedicated to my parents for their relentless love, support and encouragement.

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ABSTRACT

Title of Dissertation: The effect of mild blast traumatic brain injury on the sexually dimorphic stress axis

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Traumatic brain injury (TBI) is a worldwide epidemic, affecting 10 million people every year making it a leading cause of death and disability. Among military populations, a significant portion of TBIs are induced by explosive blast devices. Mild blast TBI (mbTBI) has a unique signature and increases susceptibility to neuropsychological disorders including depression, anxiety and post-traumatic stress disorder. The mechanism by which TBI leads to these disorders is currently unknown. Dysregulation of the hypothalamic-pituitary-adrenal (HPA) axis may bridge the gap between TBI and psychiatric disorders. The HPA axis maintains endocrine, autonomic and behavioral homeostasis after exposure to a stressor. Corticotropin-releasing factor (CRF) located in the paraventricular nucleus of the hypothalamus (PVN) is released into circulation to stimulate the anterior pituitary release of adrenocorticotrophic hormone (ACTH). In turn, ACTH stimulates the release of adrenal glucocorticoids (CORT), which feedback to regulate the HPA axis. In addition to direct feedback on the HPA axis, CORT acts via mineralocorticoid (MR) and glucocorticoid receptor (GR) signaling throughout limbic

structures to modulate the stress response. Extrahypothalamic CRF populations also regulate HPA axis activity by binding to CRF receptor type 1 (CRFR1) and CRF receptor type 2 (CRFR2) to initiate downstream signaling events. Psychiatric disorders are connected to dysregulation at any level of limbic-HPA axis circuitry.

This dissertation focuses on elucidating the effect of mbTBI on the HPA axis to address the gap in mechanism behind the subsequent development of TBI-induced psychiatric disorders. We demonstrate that mbTBI resulted in sex-dependent alterations of the HPA axis after a 7-10 day recovery period. Males exposed to mbTBI had increased restraint-induced CORT, but decreased CRF activation in the PVN. Interestingly, females exhibited the opposite response, with decreased restraint-induced CORT and increased CRF activation in the PVN after mbTBI. mbTBI had no effect on genes involved in the peripheral HPA axis or feedback regulation (MR/GR). We identified an autonomic link to HPA axis dysregulation in females. Additionally, we sought to determine the mbTBI-induced effect on the extrahypothalamic CRF system. mbTBI had no effect on limbic CRFR1 gene expression. We show that mbTBI had a sex dependent effect of limbic CRFR2 gene expression. Males exposed to mbTBI had decreased CRFR2 gene expression in the amygdala and anterior bed nucleus of the stria terminalis, while females had decreased expression in the dorsal hippocampus. Collectively, these findings demonstrate a sex-dependent disruption of stress regulation circuitries after mbTBI. These data suggest that males and females utilize different strategies to cope with mbTBI, highlighting the importance of developing targeted sex dependent treatments.

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LIST OF ABBREVIATIONS

11 β -HSD	11 β -Hydroxysteroid Dehydrogenase
11 β -OHase	11 β -Hydroxylase
2-AG	2-Arachidonoyl Glycerol
aBNST	Anterior Bed Nucleus of the Stria Terminalis
ABS	Advanced Blast Simulator
ACTH	Adrenocorticotrophic Hormone
ANOVA	Analysis of Variance
BNST	Bed Nucleus of the Stria Terminalis
BSA	Bovine Serum Albumin
bTBI	Blast Traumatic Brain Injury
CBG	Corticosteroid Binding Globulin
CCI	Controlled Cortical Impact
cDNA	Complementary Deoxyribonucleic Acid
CHIMERA	Closed Head Impact Model of Engineered Rotational Acceleration
CORT	Glucocorticoids (Cortisol; Corticosterone)
CRF	Corticotropin-Releasing Factor
CRFR1	Corticotropin-Releasing Factor Type 1
CRFR2	Corticotropin-Releasing Factor Type 2
DAI	Diffuse Axonal Injury
ddPCR	Droplet Digital Polymerase Chain Reaction
DEX	Dexamethasone

EPM	Elevated Plus Maze
FG	Fluoro-Gold
FPI	Fluid Percussion Injury
GABA	Gamma Aminobutyric Acid
GCS	Glasgow Coma Scale
GR	Glucocorticoid Receptor
HPA Axis	Hypothalamus-Pituitary-Adrenal Axis
ICV	Intracerebroventricular
IL	Infralimbic Cortex
ir	Immunoreactivity
LOC	Loss of Consciousness
LTD	Long-Term Depression
LTP	Long-Term Potentiation
mbTBI	Mild Blast Traumatic Brain Injury
Mc2R	Melanocortin 2 Receptor
modTBI	Moderate Traumatic Brain Injury
MLC	Myosin Light Chain
mPFC	Medial Prefrontal Cortex
MR	Mineralocorticoid Receptor
mRNA	Messenger Ribonucleic Acid
mTBI	Mild Traumatic Brain Injury
OT	Oxytocin
pBNST	Posterior Bed Nucleus of the Stria Terminalis

PBS	Phosphate Buffered Saline
PFA	Paraformaldehyde
PFC	Prefrontal Cortex
POMC	Pro-opiomelanocortin
pPVN	Parvocellular Subdivision of the Paraventricular Nucleus
PrL	Prelimbic Cortex
PTA	Post-Traumatic Amnesia
PTSD	Post-Traumatic Stress Disorder
PVN	Paraventricular Nucleus of the Hypothalamus
qPCR	Quantitative Polymerase Chain Reaction
ROCK	Rho-Associated Protein Kinase
SCN	Suprachiasmatic Nucleus
sTBI	Severe Traumatic Brain Injury
TBI	Traumatic Brain Injury
UCN	Urocortin
VP	Vasopressin
VEH	Vehicle

CHAPTER 1: Introduction

Traumatic brain injury (TBI) is a significant cause of death and disability worldwide (1). In the United States alone, more than 1.7 million people sustain a TBI annually (128). Within the Department of Defense, nearly 400,000 service members have experienced TBI since 2000 (3). The majority of TBI cases (~80%) are categorized as a mild (m)TBI (3). A mTBI is diagnosed as a loss of consciousness (0-30 minutes), post-traumatic amnesia (less than 24 hours), normal structural imaging, and normal eye, verbal and motor responses (292). Among soldiers, a significant portion of mTBI is blast-induced (180; 286). Blast-induced (b)TBI in soldiers occur within environments of chronic stress and sensory stimulation. Soldiers are also likely to experience multiple bTBIs and the symptoms may be masked by comorbid psychiatric disorders (74). These characteristics make TBI sustained by service members uniquely different from those experienced by the civilian population. The “invisible wounds of war” are one of the major burdens of TBI (386). Increased rates of cognitive and psychiatric disorders such as depression, anxiety, and post-traumatic stress disorder (PTSD) are associated with mbTBI (286; 366). Nearly 30% of patients suffering from TBI also exhibit neuroendocrine dysfunctions (42; 389). A major endocrine axis affected by TBI is the hypothalamus-pituitary-adrenal (HPA) axis (42; 389). Dysregulation of the HPA axis, clinically observed by altered hormone levels, is linked to psychiatric disorders (105; 193; 252; 285; 415).

An organism’s stress response is regulated by the HPA axis (155; 174). A physical or psychological stressor stimulates the release of corticotropin-releasing factor (CRF) from the paraventricular nucleus of the hypothalamus (PVN) into the hypophyseal

portal system via the median eminence (155; 410). CRF acts on corticotrophs on the anterior pituitary to release adrenocorticotrophic hormone (ACTH) (54; 410). ACTH stimulates the synthesis and secretion of glucocorticoids (CORT) from the adrenal cortex (21; 156; 442). CORT provides negative feedback at central and peripheral levels of the HPA axis to regulate hormone secretion (156). In addition, CORT has numerous effects in the cardiovascular, musculoskeletal, immune, respiratory and metabolic systems (261) (Figure 1).

Importantly, the HPA axis does not act in isolation. Rather limbic structures have mechanisms to regulate the neuroendocrine stress response. Mineralocorticoid receptors (MR), glucocorticoid receptors (GR), CRF receptor subtype 1 (CRFR1) and CRF receptor subtype 2 are expressed in the prefrontal cortex (PFC), amygdala, hippocampus, and bed nucleus of the stria terminalis (BNST). CORT binds to MR and GR to dampen the stress response (157). Activation of CRFR1 and CRFR2 modulate anxiety behaviors and the HPA axis (165). Additionally, the HPA axis is partly modulated by direct and indirect projections from these limbic structures (176; 177). Figure 2 illustrates the network of communication between the limbic structures and the HPA axis.

The limbic-HPA response to a stressor is integral for homeostasis and survival (65; 254). Dysregulation of the stress system is observed in psychiatric disorders (105; 415). This is evident in patients with PTSD where cerebrospinal CRF is elevated, but CORT is reduced (105; 329). In patients suffering from anxiety and depression, elevated CORT and, in some cases, hyperactive CRF is observed (22; 254; 415).

Clinically, females are approximately two times more likely than males to be diagnosed with depression, anxiety or PTSD (15; 263). Similarly, although males are

more likely to sustain a TBI, females are at higher risk of developing psychiatric disorders as a result (43; 279; 355). There are inherent sex differences at every level of stress regulation. Rodent models show differences in HPA axis reactivity (141; 155; 418), ligand/receptor distribution and signaling (37; 38), as well as, autonomic response (314; 454). Not only is the response of the HPA axis to TBI unknown, but also sex differences are understudied.

Therefore, the overarching hypothesis is that mbTBI results in the sex-dependent development of psychiatric disorders by disrupting limbic-HPA axis regulation of the stress response. Two aims are proposed to address the neuroendocrine and behavioral responses to mbTBI.

Aim 1: To determine if mbTBI causes long-term changes to the HPA axis stress response. Dysregulation of the HPA axis following mbTBI may be linked with increased risk of psychiatric disorders. Baseline and HPA axis reactivity were assessed 7-10 days after mbTBI in adult male and female mice (Figure 3). C57BL/6J mice were used to examine the effect of mbTBI on CORT release and central and peripheral HPA axis-relevant gene expression. The CRF:tdTomato transgenic mouse line was used to visualize CRF neurons in the PVN (Figure 4). c-FOS, a marker of neuronal activation, was utilized to assess CRF neuron response. Animals received intraperitoneal injections of the retrograde axonal transporter, Fluoro-gold (FG), 3-4 days prior to mbTBI. The presence of FG-immunoreactivity (ir) identified CRF neurons as endocrine projecting. The lack of FG-ir identified CRF neurons as pre-autonomic projecting. mbTBI dysregulated the HPA axis. There were sex-dependent effects of mbTBI on CORT secretion and CRF activation, with a link to autonomic dysregulation in females, but not males.

Aim 2: To determine whether mbTBI alters the limbic-HPA system and associated anxiety-like behaviors. The PFC-amygdala-hippocampus-BNST network is involved in regulating the HPA axis. Dysregulation of these limbic structures may disrupt the circuitry involved in regulation of the HPA axis endocrine, autonomic, and behavioral stress responses. Anxiety-like behaviors were tested in male and female mice using the elevated plus maze (EPM) assay. To determine the integrity of the limbic-HPA CRFR system, CRFR1 and CRFR2 gene expression was measured 7-10 days post mbTBI (Figure 3). mbTBI increased anxiety-like behaviors in males and females but had no effect on CRFR1 gene expression. Overall, there were sex-dependent anatomical differences in CRFR2 gene expression changes after mbTBI.

By understanding the anatomical alterations we can begin to address the mechanisms involved in the development of psychiatric disorders after mbTBI. Successful completion of these experiments demonstrated a sex-dependent dysregulation of the limbic-HPA axis. This study highlighted the importance in developing a targeted treatment to address the different strategies males and females utilize to cope with the consequences of mbTBI.

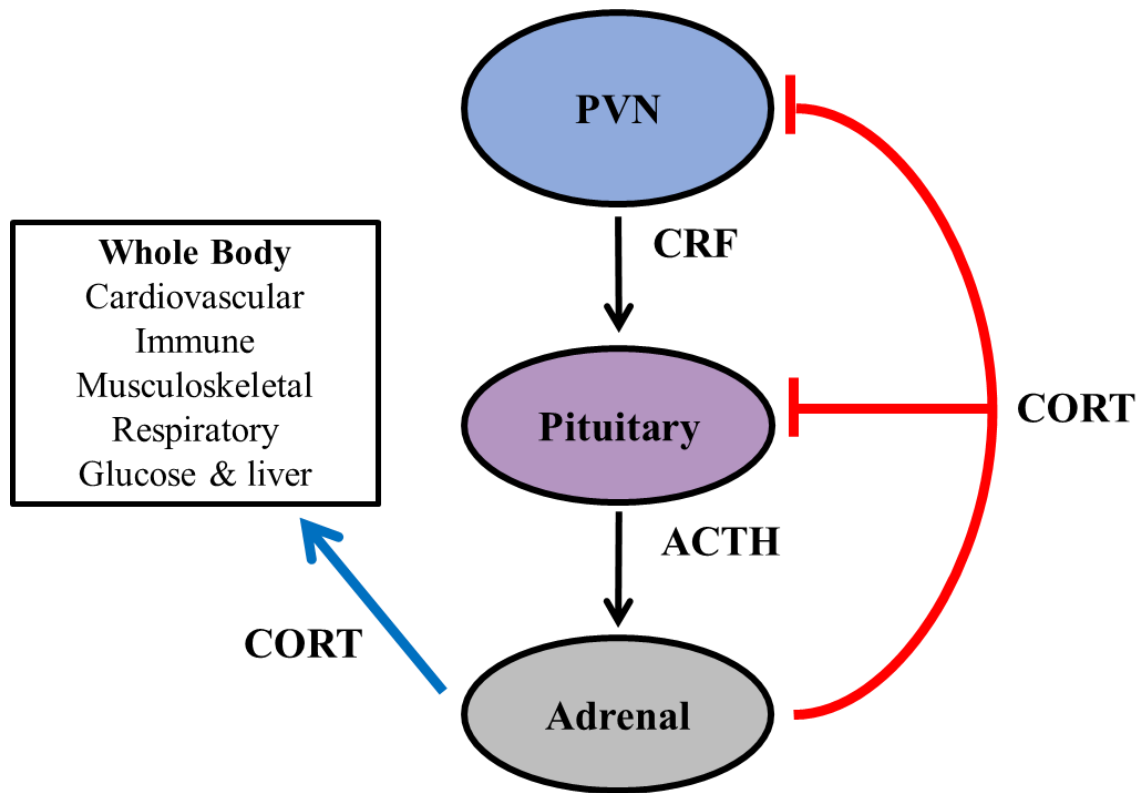


Figure 1. Schematic of the HPA axis.

Corticotropin releasing factor (CRF) is released from the paraventricular nucleus of the hypothalamus (PVN). CRF elicits the release of adrenocorticotrophic hormone (ACTH) from the pituitary to subsequently stimulate glucocorticoid (CORT) release from the adrenal. CORT acts via negative feedback to regulate the HPA axis. CORT also acts throughout other systems in the body.

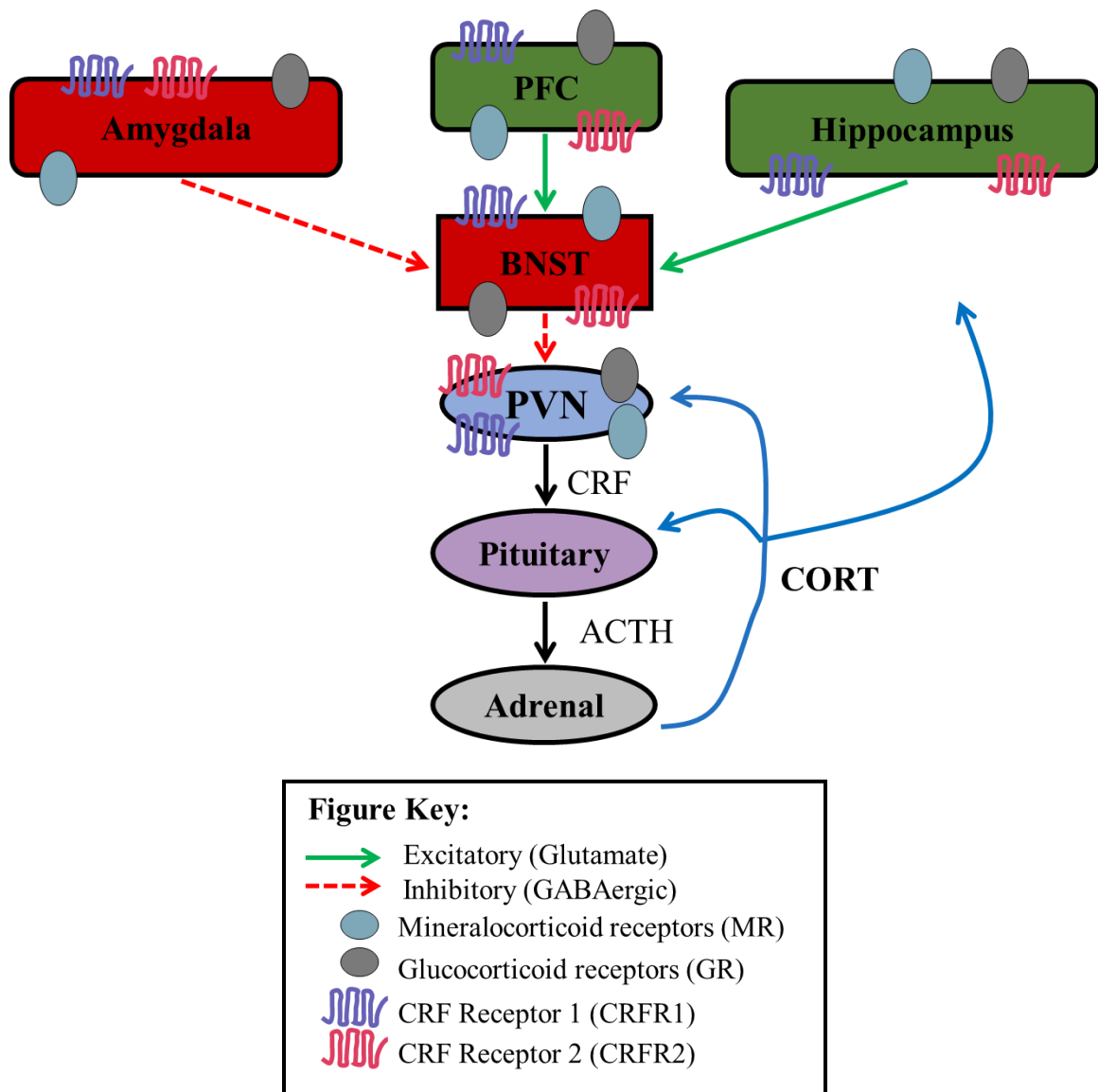


Figure 2. Limbic-HPA axis network.

Three mechanisms by which limbic structures regulate the HPA axis are depicted in this figure. 1) Limbic structures provide excitatory and inhibitory input to the BNST. GABAergic neurons in the BNST then relay these projections to the PVN. 2) Secreted CORT acts on MR and GR located throughout the limbic structures to dampen the HPA axis stress response 3) CRFR1 and CRFR2 are also distributed in the limbic structures and can regulate HPA axis output.

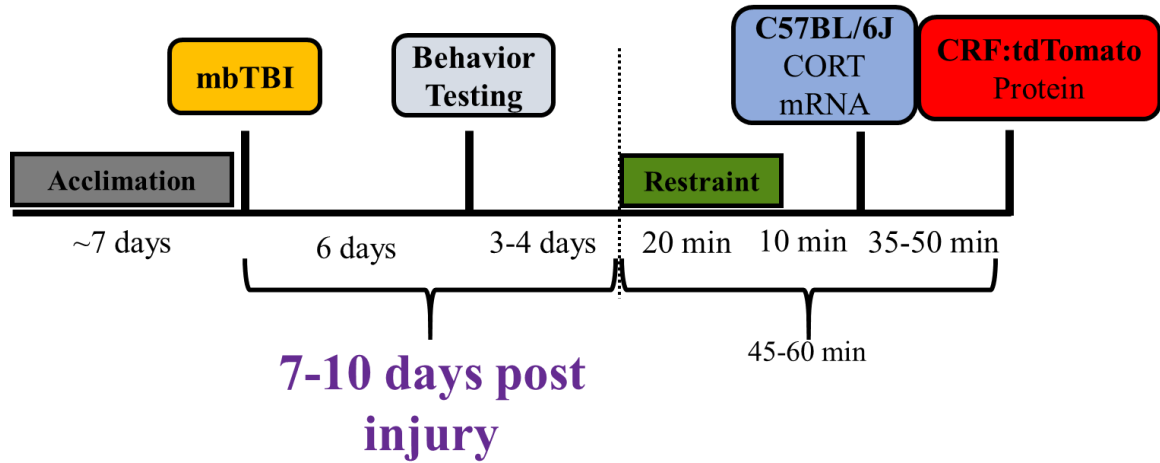


Figure 3. Experimental timeline for Aims 1 and 2.

Behavior testing was conducted 6 days post mbTBI. Animals were exposed to a 20 min psychogenic restraint, 7-10 days after mbTBI. C57BL/6J mice were used for CORT analysis and mRNA expression. CRF:tdTomato mice were utilized for protein expression.

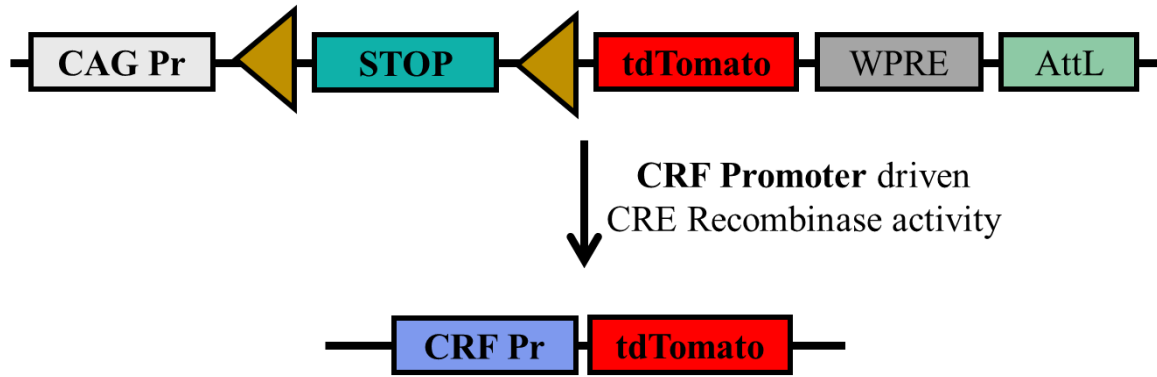


Figure 4. Generation of the CRF:tdTomato transgenic mouse line.

The CRF:tdTomato mouse line allows for visualization of the CRF neurons. To generate this mouse line, male (*CRF-IRES-Cre*) mice were crossed with female (*Ail4*) mice purchased from Jackson Laboratory. After Cre recombinase activity, the CRF promoter will drive expression of tdTomato. Abbreviations and symbols are as follows: Golden triangle = loxP; CAG Pr = CAG Promoter; STOP = Stop codon; WPRE = Woodchuck hepatitis virus Posttranscriptional Regulatory Element; AttL = DNA recombinase site; CRF Pr = CRF Promoter

CHAPTER 2: Literature Review

Introduction

Our understanding of stress stems from the concepts of Walter Cannon and Hans Seyle. Cannon was the first to systematically study an animal's "fight or flight" response and ability to maintain physiological "homeostasis" (64; 65). Hans Seyle later coined the term "stress" under his concept of the general adaptation syndrome (GAS). The GAS consists of 3 stages of stress response (361-363). The first stage, alarm, is the body's fight-or-flight response, consisting of hormone release and energy mobilization. The second stage, resistance, refers to the body's restoration of homeostasis. If a stressor is unrelieved, this pushes the body to the third stage, exhaustion and disease (362-364). This concept of stress has been further refined by Bruce McEwen to incorporate the term "allostasis" (252; 253). Allostasis refers to the ability of the body to maintain homeostasis in lieu of a changing environment (252; 255). An organism's inability to adapt to a changing environment is detrimental to its survival.

The initial response to stress begins in the brain. The brain perceives the threat and elicits adaptive or maladaptive physiological and behavioral responses (256). The stress response is an attempt to maintain homeostasis through the interaction of endocrine, autonomic, immune and behavioral systems (174; 373). Of importance for this review, exposure to a threat mobilizes the neuroendocrine stress system. The coordinated activity of the major neuroendocrine stress regulator, the hypothalamus-pituitary-adrenal (HPA) axis, enables successful stress adaptation (99; 157; 174).

The hypothalamus-pituitary-adrenal (HPA) axis

Function of the HPA axis

The regulation of stress is dependent on communication between the structures of the HPA axis. The HPA axis is comprised of a series of feedback loops between the hypothalamus, anterior pituitary and adrenal cortex. Proper coordination of the HPA axis dampens the physiological response to a stressor, allowing adaptation and a return to homeostasis. The 41 amino acid peptide, corticotropin-releasing factor (CRF), is central to the function of the axis (21; 334).

Neurons that synthesize CRF are primarily located in the paraventricular nucleus of the hypothalamus (PVN) and project to the median eminence. CRF is released into the hypophyseal portal vessels (21; 55; 409; 410). Release of CRF stimulates the synthesis of adrenocorticotrophic hormone (ACTH) and the precursor peptide, pro-opiomelanocortin (POMC), in the anterior pituitary by binding to CRF receptor type 1 (CRFR1) (9; 21; 238; 409). Subsequently, ACTH facilitates the secretion of glucocorticoids (CORT) from the adrenal cortex. ACTH mediates its actions through the melanocortin 2 receptor (Mc2R) (25; 79; 98; 102; 106). In the periphery, CORT is bound to corticosteroid binding globulin (CBG) to abet transport to the target tissue (233; 439; 440). CORT modulates the stress response by feedback regulation of the HPA axis (102; 157). In addition to the contribution of CORT during periods of heightened stress, resting CORT secretion is also critical for homeostasis throughout anticipated circadian cycles (188). CORT levels predictably vary across the cycle of the day. During non-stressed conditions, the suprachiasmatic nucleus (SCN), which is entrained by light-dark cues, synchronizes CORT rhythmicity (84). In rodents, CORT secretion is at its peak during the active

period (lights off) and nadir during the inactive period (lights on). Examination of CORT during the inactive period allows for basal hormone levels. The rhythmicity of CORT abets in synchronizing other systems in the body (84).

CORT can mediate peripheral demands for homeostasis by exerting effects in many system of the body. CORT acts to maintain energy mobilization, metabolism, water balance, as well as, cardiovascular, musculoskeletal, immune and inflammatory systems (261). Behavior and locomotion are also regulated by fluctuations in adrenal hormones (84; 243). Figure 1 in Chapter 1 illustrates the communication between the structures of HPA axis and the physiological roles of CORT.

Feedback regulation of the HPA axis

The physiological actions of CORT are mediated by the mineralocorticoid receptor (MR) and glucocorticoid receptor (GR) (168; 330). These receptors are members of the ligand-dependent transcription factors that, upon binding to glucocorticoid response elements, can positively or negatively regulate gene transcription (290; 429). The MR has a higher affinity for CORT ($K_D=0.5-1\text{nM}$) than the GR ($K_D=2.5-5\text{ nM}$) (330; 381). Therefore, during periods of low circulating CORT, MR are preferentially bound (103). In contrast, GR are bound when circulating CORT levels are high (175).

The downstream action of these receptors is partially due to their location in the brain. Studies by Reul and de Kloet demonstrated that MR and GR have distinct anatomical distribution patterns (330; 331). Autoradiography, immunohistochemistry and in situ hybridization studies have shown that the expression of GR is more widespread than MR. MR is expressed in the septal-hippocampal region, with the highest density in

the CA1, CA2 and dentate gyrus subdivisions of the hippocampus (331; 412). To a lesser extent, MR is also expressed in the cerebral cortex, hypothalamus, thalamus and amygdala (11; 153; 331). On the other hand, GR is highly expressed in the cerebral cortex, amygdala, cerebellum and hypothalamus. In addition, the CA1 and CA2 subdivisions of the hippocampus have high levels of GR (12; 18; 153; 274; 331; 412). Both MR and GR are co-localized on CRF neurons in the PVN that regulate feedback control (70; 76). Interestingly, pre-autonomic projecting neurons in the PVN only express MR (76).

The paraventricular nucleus of the hypothalamus (PVN)

Anatomically, the PVN lies adjacent to the third ventricle in the rostral hypothalamus. The PVN is divided into cytoarchitectural, functional and anatomical subdivisions. Initially, cell size distinguished 2 subdivisions of the PVN. These subdivisions are the parvocellular (small cell) and magnocellular (large cell) neurons (39; 151; 209). Later studies differentiated PVN into 3 functional subdivisions (157). Neurons are divided into neurosecretory parvocellular, neurosecretory magnocellular, or non-neurosecretory long-projecting neurons, dependent on axonal target (23; 157).

CRF neurons are located in the parvocellular PVN. Additionally CRF neurons belong to the neurosecretory parvocellular and non-neurosecretory functional subdivisions. The endocrine response of the HPA axis is controlled by neurosecretory parvocellular CRF neurons. A number of neuropeptides are co-expressed on CRF neurons. For example, vasopressin (VP) and oxytocin (OT) are co-localized and co-released, augmenting the actions of CRF on the anterior pituitary (50; 160; 309; 350; 353;

442). Autonomic functions are mediated by non-neurosecretory neurons that also co-express CRF, VP and OT (269; 352). These neurons project to spinal cord and brainstem structures including the interomediolateral cell column, dorsal vagus complex, nucleus of the solitary tract, periaqueductal gray, dorsal raphe nucleus and rostral ventrolateral medulla (140; 237; 319; 383). CRF is the primary peptide synthesized in the parvocellular neurosecretory and non-neurosecretory divisions. In contrast, the magnocellular neurosecretory division highly expresses VP and OT and sends projections to the posterior pituitary (332; 368; 369).

The PVN is also divided anatomically into anterior, medial, dorsal and lateral subdivisions. These anatomical subdivisions incorporate both cytoarchitectural and functional patterns. The dorsomedial and anterior divisions of the parvocellular PVN project to the median eminence, regulating the endocrine response (10; 385). The magnocellular neurons are located in the anteromedial and dorsolateral subdivisions of the PVN, projecting to the posterior pituitary (10). The lateral, dorsal, and ventromedial subdivisions of the parvocellular PVN contain pre-autonomic neurons projecting to the spinal cord and brain stem structures (10; 383; 385). Figure 1 illustrates the various subdivisions of the PVN and the respective projections.

Limbic regulation of the HPA axis

Projections to the PVN

Limbic structures, including the prefrontal cortex (PFC), amygdala and hippocampus provide feed-forward regulation to the HPA axis (174; 177; 462). There are a sparse number of direct projections to the PVN from these structures (157). Rather, the

PVN is under gamma aminobutyric acid (GABA)ergic tone from the bed nucleus of the stria terminalis (BNST) and the peri-PVN (93; 157; 336). The limbic structures relay to the PVN through these regions (81; 95; 462).

The majority, but not all, of the projections from the BNST are GABAergic. By dividing the BNST into anterior and posterior subdivisions, differential HPA axis responses arise. The anterior (a)BNST activates the HPA axis. Lesions of the aBNST decreased restraint-induced CORT, as well as decreased restraint-induced c-Fos and CRF mRNA expression in the PVN (81; 171). The posterior (p)BNST inhibits the HPA axis. Lesions of the pBNST increased restraint-induced ACTH and CORT. Additionally, pBNST lesions increased restraint-induced c-Fos, CRF, and VP mRNA expression in the PVN (81; 82).

The hippocampus primarily inhibits the HPA axis through relayed projection to the PVN (177). Stimulation of the hippocampus decreased the electrical activity of neurons in the PVN (347). Lesions of the hippocampus increased baseline CORT, as well as increased CRF and VP expression in the PVN (172; 349). The majority of afferent projections sent to the BNST are glutamatergic, ultimately reducing HPA axis reactivity (177; 373). Hippocampus modulation of the HPA axis may be region specific. For example, the ventral hippocampus relays to the PVN through the aBNST (321). Stimulation of CA1 increased CORT whereas stimulation of CA3 and the dentate gyrus decreased CORT (121).

Distinct regions of the PFC may differentially regulate the HPA axis. The rodent medial (m)PFC is divided into 3 subdivisions which include the anterior cingulate, prelimbic (PrL) and infralimbic (IL) cortexes (97). Stimulation of the PrL decreased

restraint-induced CORT, ACTH and neuronal activation in the PVN (194). Glutamate transporter knockdown in the IL cortex enhanced CORT and ACTH (280). Both of these regions inhibit HPA axis reactivity; however, they have different relay projection patterns. The IL cortex projects to the aBNST, amygdala and peri-PVN (417). Conversely, the majority of efferent projections from the PrL cortex are through the peri-PVN (262; 373; 417). This suggests different circuitries may be utilized by the subdivisions of the mPFC to regulate the stress response.

In contrast to the hippocampus and PFC, relayed projections from amygdala activate the HPA axis (177; 462). The majority of efferent projections from the amygdala are GABAergic (176). Stimulation of the amygdala increased glucocorticoids and impaired CORT feedback (373; 432). Lesions of the amygdala decreased stimulation-induced ACTH and CORT (433). Figure 2 in Chapter 1 depicts the limbic-HPA circuitry.

The role of the CRFR system

CRF is widely distributed throughout the central nervous system. CRF immunoreactivity is found in the hippocampus, amygdala, midbrain, cerebellum and spinal cord (400). Parallel to its role in neuroendocrine stress regulation, CRF is involved in the homeostasis of stress behaviors and anxiety. In addition to CRF, members of the CRF-related peptide family include urocortin 1 (UCN1), urocortin 2 (UCN2) and urocortin 3 (UCN3) (414). UCNs differ in tissue distribution (32; 271; 400). UCN1 is predominately located in the Edinger-Westphal nucleus, lateral superior olive, hypothalamus (including PVN), substantia nigra, lateral septum, dorsal raphe and spinal cord (216; 275; 437). UCN2 is distributed in the PVN, supraoptic nucleus, arcuate

nucleus, locus coeruleus and spinal cord (165; 245). UCN3 is located in the medial preoptic nucleus, perifornical area, medial nucleus of the amygdala, BNST, and lateral septal nucleus (41; 165; 224).

Members of the CRF related peptide family bind with differing affinities to CRF receptor subtype 1 (CRFR1) and CRF receptor subtype 2 (CRFR2) to exert effects on the HPA axis and anxiety. CRF and UCN1 preferentially bind to CRFR1, while UCN2 and UCN3 have a higher affinity for CRFR2 (35; 161; 165). These receptors are ~80% homologous with differences in the ligand binding domains (161). Additionally, the distribution of these receptors differs. CRFR1 has widespread distribution and is found throughout the forebrain, midbrain and hindbrain (165; 196; 231; 339). The distribution of CRFR2 is less widespread. CRFR2 is located in the lateral septum, hippocampus, BNST, amygdala, supraoptic nucleus, PVN, raphe nucleus, periaqueductal gray, and the nucleus of the solitary tract (165; 231; 414).

Importantly, CRFR1 and CRFR2 mediate endocrine, and behavioral responses to stress. These receptors are also linked to anxiety behaviors (34; 35; 124; 165; 220). The role of CRFR1 in anxiety is much clearer than that of CRFR2. Activation of CRFR1 predominately increases anxiety-like behaviors (165). CRFR1 knockout mice display decreased anxiety behaviors (32; 33; 90). There are mixed findings on the role of CRFR2 in anxiety. CRFR2 deficient mice displayed increased anxiety behaviors (32; 187), suggesting that CRFR2 may counter the anxiogenic effects of CRFR1. Activation of CRFR2 decreased PTSD-like behaviors (164). However, there is also evidence that chronically activated CRFR2 increased anxiety behaviors (289). Another study showed

that CRFR knockout mice had no differences in anxiety behaviors (92). This suggests that the role of CRFR2 may be more complex and region specific (165; 191).

The role of these receptors in mediating anxiety behaviors may be dependent or independent of the HPA axis. CRFR1 and CRFR2 are located on anxiety-relevant limbic structures. Altered anatomy of function of these regions may result in anxiety behaviors. Conversely, these receptors may mediate anxiety behaviors by a mechanism dependent on the HPA axis. CRFR1 deficient mice have decreased baseline and restraint-induced CORT and ACTH (35; 372), suggesting a decrease in anxiety related hormones. The interaction of CRFR2 and the HPA axis is more complex. Total ACTH and CORT secretion, as well as, CRF mRNA expression in the PVN are unaffected in CRFR2 knockout mice (32; 34). However CRFR2 knockout mice displayed early termination of ACTH release (92). Dependent on length of stressor, studies have shown that CRFR2 knockout mice have increased CORT (32). Optogenetic activation of CRFR2 decreased CORT rise after foot shock stress (164). Although there is conflicting evidence, it is thought that CRFR2 counters the function of CRFR1 and is involved in stress recovery (164).

Overall, the neurocircuitry involved in HPA axis regulation involves a complex series of projections and interactions from limbic structures. In order for the HPA axis to maintain homeostasis, proper coordination at each level of the neurocircuitry is critical.

HPA axis dysregulation

Psychiatric disorders are linked to HPA axis dysregulation. Symptoms of HPA axis hyperactivity such as elevated CORT and enlarged pituitary and adrenal glands are

observed in half of patients suffering from depression (287; 303). Additionally, elevated CORT and ACTH levels are present in many anxiety disorders including generalized anxiety disorder, panic disorder, and social phobia (89; 356). This appears in both adolescent and adult patients (127). In depression and anxiety disorders, CRF concentrations in the cerebrospinal fluid are generally unaffected. However, in patients suffering from PTSD, increased CRF concentration may be observed (333). PTSD also differs from other psychiatric disorders in that CORT is decreased, rather than enhanced (147; 455).

In rodent models, HPA axis dysregulation also coincides with depressive-, anxiety-, and PTSD-like behaviors. Exposure to acute and chronic stressors reliably induces alterations in these behavioral phenotypes in rodents. Acute psychogenic restraint is a mild stressor that reliably activates the HPA axis, demonstrated by increased CORT (169; 419). Unlike internal or external physical stimuli, psychogenic stressors elicit an emotional response such as fear or anxiety (244). Chronically stressed animals exhibit increased CORT and ACTH secretion in response to a subsequent novel acute stress (101). For example, immune challenge and psychological stress increased CORT in animals exposed to prolonged inescapable shock stress (192). Chronic variable stress enhanced CORT to a subsequent novel acute stress 14 days later (130). Six weeks of chronic stress increased depressive-like behaviors and up-regulated CRF, CORT, and ACTH levels (458). Mice exhibiting depressive-like phenotypes have decreased forebrain GR (83; 265) and forebrain GR knockout mice have increased depressive-like behaviors (60). Animals bred for high anxiety have enhanced stress-induced CORT (218).

Administration of MR and GR antagonists increased anxiety- and fear-like behaviors (215). Forebrain overexpression of MR decreased anxiety-like behaviors (342), but GR overexpression increased anxiety (431). Mutated GR decreased anxiety-like behaviors (402). Rodent PTSD models have blunted baseline CORT and enhanced dexamethasone (DEX) suppression of CORT (308; 441). DEX is a synthetic glucocorticoid that has a higher affinity for GR located on the pituitary than CORT. Binding of DEX will initiate a negative feedback mechanism, ultimately suppressing CORT secretion (87). This assay allows for detection of peripheral HPA axis function (436). Single prolonged stress, a model for PTSD-like behaviors, decreased MR and GR expression in the hippocampus and amygdala (153; 460). Inescapable foot shock stress decreased CRF mRNA in the PVN (235).

Although there are conflicts in the literature on how the HPA axis is dysregulated, it is apparent that the HPA axis is linked to many psychiatric disorders. Therefore, any disruption of the HPA axis can have long-term consequences on an organism's well-being.

Traumatic brain injury (TBI)

Overview of TBI

In the United States, 5.3 million people are living with a TBI-related disability (132). The estimated direct and indirect costs of TBI are \$76.5 billion (360). TBI occurs when any external force causes damage to the brain. TBI can lead to emotional, behavioral and cognitive disorders by disrupting the normal anatomy and physiology of the brain. There are two classifications of injury etiologies, primary and secondary.

Primary injury occurs at the initial moment of impact or displacement of the brain. In contrast, secondary injury is the delayed sequence of ongoing cellular and subcellular injury cascades (317; 438).

TBI is classified as mild, moderate or severe. The 15-point Glasgow Coma Scale (GCS) grades eye, verbal and motor responses. In addition, loss of consciousness (LOC), post-traumatic amnesia (PTA), and neuroimaging are determinants of injury severity. Mild (m)TBI is defined as a GCS score of 13-15, LOC for less than 30 minutes, PTA for less than 24 hours and normal neuroimaging. Moderate (mod)TBI is defined as a GCS score of 9-12, LOC for 30 minutes to 1 day, PTA for 1 to 7 days and normal or abnormal neuroimaging. Severe (s)TBI is defined as a GCS score of 3-8, LOC for more than 1 day, PTA for more than 7 days and abnormal imaging (74; 246; 397).

The recovery period after TBI can be divided into 3 crudely defined time points. These include acute (hours to days), sub-acute (days to weeks) and chronic (weeks to months) recovery periods post TBI. It is hypothesized that the injury cascade occurs during the acute and sub-acute periods, while the injury recovery phase occurs during the chronic period (424). Hallmarks of the acute phase after TBI include cytokine production and release, necrosis, ion channel activation, hyper-excitability and microglial activation (222; 223; 421). Microglial activation persists into the sub-acute phase of recovery (250; 453). Additionally components of the sub-acute injury cascade include neuronal death, astrocyte activation, monocyte infiltration, reactive oxygen species production and neurogenesis (250; 324; 421). Neurogenesis continues into the chronic recovery phase (288). Cognitive and emotional impairments typically appear in the acute and sub-acute

phase and reside over time. However, these impairments may be long-lasting and emerge in the chronic recovery periods.

Experimental TBI models

Treatment of TBI has proven difficult because of the differences among individuals in injury mechanism, severity and symptomology. Every TBI poses a unique set of anatomical, cellular and behaviors cascades. Various animal models have been developed. Each experimental model attempts to replicate a specific injury mechanism and the subsequent pathophysiology of clinical TBI. To address the many types of TBI some widely used models include controlled cortical impact (CCI), fluid percussion (FPI), weight drop, acceleration impact and blast-induced overpressure.

CCI uses a piston to deliver a controlled force to the intact dura, leading to deformation of the cortex (337; 450). These injuries can range from mild to severe depending on the depth and velocity of the impact (337). CCI can result in diffuse axonal injury (DAI), glial infiltration, blood brain barrier breakdown, excitotoxicity, apoptosis and vascular damage (77; 88; 134; 230; 301; 371). Fine and gross motor deficits, as well as cognitive impairments in memory and learning, occur after CCI (134; 152; 371; 393; 450).

Similar to CCI, FPI causes deformation of the neural tissue. However, FPI uses a pendulum to strike a piston filled with fluid to deliver a controlled and reproducible impact on the intact dura (16; 450). FPI induces glial proliferation and scar formation, neuronal death, hemorrhage, vascular damage and axonal injury (16; 91; 197; 240).

Additionally, FPI causes motor, cognitive and emotional deficits. FPI affects learning and memory (66; 379). Anxiety and fear impairments are also observed after FPI (195; 229).

The weight drop model involves the controlled release of a weight from a determined height onto a closed or open skull (273; 450). The cellular and behavioral effects are highly variable dependent on site and force of impact. Weight drop can result in DAI, necrosis, apoptosis, hemorrhage, blood brain barrier breakdown and inflammation (14; 46; 133; 198; 207; 450). Injury severity determines the presence and magnitude of motor and cognitive impairments (181; 198; 207; 451).

Experimental closed head models have incorporated acceleration-deceleration head movements to better replicate clinical TBI. The impact acceleration model and the closed head impact model of engineered rotational acceleration (CHIMERA) induce DAI, inflammatory response and tau phosphorylation (163; 283; 284). CHIMERA results in motor deficits, memory impairments, and anxiety-like behaviors (283; 284).

Blast induced (b)TBI is a closed head injury model that exposes an animal to a blast overpressure wave. Mild bTBI is the most common (~67%) form of TBI suffered by soldiers (459). bTBI is unique from other forms of TBI. Additionally, the environment in which this injury primarily occurs contributes to the uniqueness of bTBI compared to other forms of forms that occur in isolated events. Typically at the time of injury, soldiers are exposed to prolonged stressful environments, such as those during wartime (74; 338). This exposure may predispose soldiers to psychiatric disorders. Additionally, military personnel are likely to experience repetitive, undiagnosed bTBIs during training and wartime (74), complicating the isolation of the mechanism of an individual injury. These hallmarks of bTBI give this injury a unique signature and potentially make military

personnel more prone to developing psychiatric disorders. The exposure to bTBI consists of four mechanisms of injury: 1) the primary shock overpressurization wave 2) release of shrapnel from the site of explosion 3) displacement of the body and 4) exposure to toxins and burns. Many experimental bTBI models expose animals only to the primary shock wave. The high-energy blast wave is able to penetrate the skull and have a diffuse impact throughout the brain (150). Preclinical studies have shown that bTBI can cause microglial activation, blood brain barrier breakdown, cell death, tau phosphorylation and upregulation of cytokines (71; 139; 142; 307; 318). DAI is common after bTBI (123; 428). bTBI induces inflammation and apoptotic cascades, resulting in neurodegeneration (382; 411). bTBI results in increased risk of lasting psychiatric disorders, potentially due to the cascade of physiological and molecular events that occur after impact. Common psychiatric disorders after bTBI include motor deficits, memory and learning impairments, diminished concentration and increased anxiety, PTSD, and depression (71; 122; 307; 318; 345). In a repetitive bTBI rat model, anxiety and fear-related behaviors may persist for many months post injury (122).

These models all provide a unique mechanism to better understand the complexity and uniqueness of clinical TBI. The depth of tissue deformation, site of impact and recovery period prior to testing all yield different results. Each model addresses different effects of TBI on anatomy, physiology and behavior. Table 1 synthesizes the effect of various experimental models on the HPA axis.

TBI and HPA axis dysregulation

Psychiatric disorders following TBI are common. After TBI, there is a high incidence of depression (14-77%), anxiety disorders (3-28%), panic disorder (4-17%), substance abuse (5-28%) and PTSD (3-27%) (110; 357; 456). Additionally, women have poorer outcomes after TBI (43). Subsequent development of neuropsychiatric disorders after TBI may be a comorbid diagnosis or a direct consequence of the injury. Although there is a strong association between TBI and psychiatric disorders, the mechanism linking these is unknown. As previously described, psychiatric disorders are connected to the HPA axis. Therefore, TBI induced dysregulation of the HPA axis may provide the missing link to the development of psychiatric disorders.

The neuroendocrine system is prone to TBI-induced dysfunction. This dysfunction is observed in clinical and preclinical settings (6; 228; 393). Symptoms include sleep disturbances, impaired attention and memory, and emotional disturbances (387; 461). All of these can be associated with dysregulation of the HPA axis. Up to 1/3 of TBI patients will experience a neuroendocrine dysfunction that persists for more than 3 months (158; 388). Acute (less than 3 days), sub-acute (weeks), and chronic (months to years) anterior hypopituitarisms are observed after TBI (6; 42; 359; 389). ACTH and growth hormone are among the primary pituitary hormones altered after TBI (6; 8; 390). TBI-induced dysregulation of the HPA axis occurs in approximately 15-20% of patients (7; 45). Pituitary dysfunction is more frequent after sTBI; however, cases are observed in all severities of injury. Deficiencies in basal (morning) CORT and stimulated CORT are present in the acute, sub-acute, chronic phases after sTBI (7; 13; 228; 294; 296). Similarly, CORT deficiencies are observed during all periods of modTBI recovery (5; 7;

85; 294). Serum levels of CORT can be elevated or decreased in the acute and sub-acute periods post mTBI (104; 210; 374). In the chronic recovery phase post mTBI, ACTH and CORT insufficiencies are observed (374; 391).

Three additional observations arise in the clinical data that are worth noting. First, deficiencies can occur in more than one neuroendocrine axis at a time (313).

Additionally, many hormone deficiencies improve over the first 12-month recovery period (390; 391). Lastly, in some patients, acute and sub-acute hormone levels appear normal, but become deficient chronically (354). The difference between acute and delayed hypopituitarism is unknown. However, any change in HPA axis hormone levels may have long-term consequences.

Altered pituitary hormone levels are also observed in experimental models of TBI. The presence and directionality of HPA axis dysregulation depends on injury mechanism, severity and recovery period. Moderate CCI enhanced acute (4 hours post TBI) baseline CORT (143) and sub-acute (7 days post TBI) restraint-induced CORT (393). During the chronic phase post moderate CCI, restraint decreased CORT secretion (393; 394; 396). Forced swim test stress also decreased CORT chronically (21 and 54 days) post moderate CCI (393). Mild CCI has the opposite effect on CORT. Seven days post mild CCI, restraint decreased CORT, while in the chronic recovery period restraint increased CORT (393). Forced swim test stress decreased CORT 21 days post injury, but increased CORT 54 days post injury (393). Using a mild/moderate FPI model, acute, sub-acute and chronic baseline CORT is decreased, while baseline ACTH is increased at 1 and 7 days post injury (145; 341). Sub-acutely (7 days post TBI), restraint-induced CORT was increased. One week post FPI restraint increased ACTH, but forced exercise

decreased ACTH (145; 146). In the closed head weight drop model, CORT and ACTH were acutely elevated (up to 8 hours post injury) (367). Using a bTBI model, our lab has shown that restraint increased CORT in males, but decreased CORT in females 7-10 days post injury (Chapter 3, Figure 3).

Few studies have investigated HPA axis dysregulation beyond the hormone response. One day post CCI, GR mRNA expression was increased in the contralateral hippocampus and decreased in the ipsilateral hippocampus. CCI had no effect of MR mRNA expression (251). In the chronic phase post moderate CCI there was no change in GR protein levels in the PFC, amygdala or hippocampus (396). Using an acceleration TBI model, hippocampal GR protein levels gradually decreased over 6 days (137). In our bTBI model, we observed no change in MR or GR mRNA expression throughout the brain 7-10 days post injury (Chapter 3, Tables 3 and 4).

Using a FPI model, CRF gene expression is upregulated 2 hours after injury in the PVN and amygdala (335). FPI increased dendritic branching and complexity in the PVN 57-59 days post injury (341). Although alterations of the HPA axis are observed after injury, the data is inconsistent. Additionally, although there are inherent sex differences in HPA axis reactivity (94; 155; 418), male and female responses to TBI are understudied.

Summary

For survival, it is necessary for the HPA axis to properly coordinate physiological responses to adapt to a changing environment. The communication between limbic structures and the HPA axis is crucial for maintaining homeostasis. Dysregulation of this

circuitry is strongly linked to psychiatric disorders. In addition, TBI is also linked to the development of psychiatric disorders, however the mechanism is unknown. The HPA axis may provide the missing piece by which TBI-induced psychiatric disorders can occur (Figure 3).

TBI Model	Type of Injury	HPA Axis Response			
		Acute		Sub-Acute	Chronic
CCI	Open skull	Mild		Decreased restraint-induced CORT (Rat; left parietal; 1.5 mm)(393)	Increased restraint-induced CORT (Rat; left parietal; 1.5 mm) (393; 394; 396)
		Mod.	Increased baseline ACTH (Rat; left parietal; 1.5 mm) (143)	Increased restraint-induced CORT (Rat; left parietal; 2.0 mm) (393)	Decreased restraint-induced CORT (Rat; left parietal; 2.0 mm) (393; 394; 396)
FPI	Open skull	Mild-Mod.	Decreased baseline ACTH (Rat; left parietal; 1.5 atm) (146)	Increased restraint-induced CORT and ACTH Decreased exercise-induced ACTH (Rat; left parietal; 1.5 atm) (145; 146)	Increased baseline CORT (Rat; midline; 2.1 mm – Mod.) (341)
Weight drop	Closed or open skull	Not def.	Increased baseline ACTH and CORT (Rat; left cortex)(367)		
Acceleration models	Closed skull	Uninvestigated			
Blast induced	Closed skull	Mild		Increased restraint-induced CORT (male) Decreased restraint-induced CORT (female; mouse 15.5 Psi) (Chapter 3, Figure 3)	

Table 1. The effect of various experimental TBI models on the HPA axis.

HPA axis response is dependent on severity of injury (mild or moderate), site on injury (lateral or midline), depth of injury (mm), species, and sex. Mild TBI is defined as a GCS score of 13-15, LOC <30 min, PTA <24 h. Moderate TBI is defined as GCS score of 9-12, LOC for 30 min to 1 day, PTA for 1 – 7 days. Acute, sub-acute, and chronic are defined as <3 day, weeks and months-years, respectively. CCI = controlled cortical impact; FPI = fluid percussion injury; mod = moderate; CORT = glucocorticoids; ACTH = adrenocorticotrophic hormone.

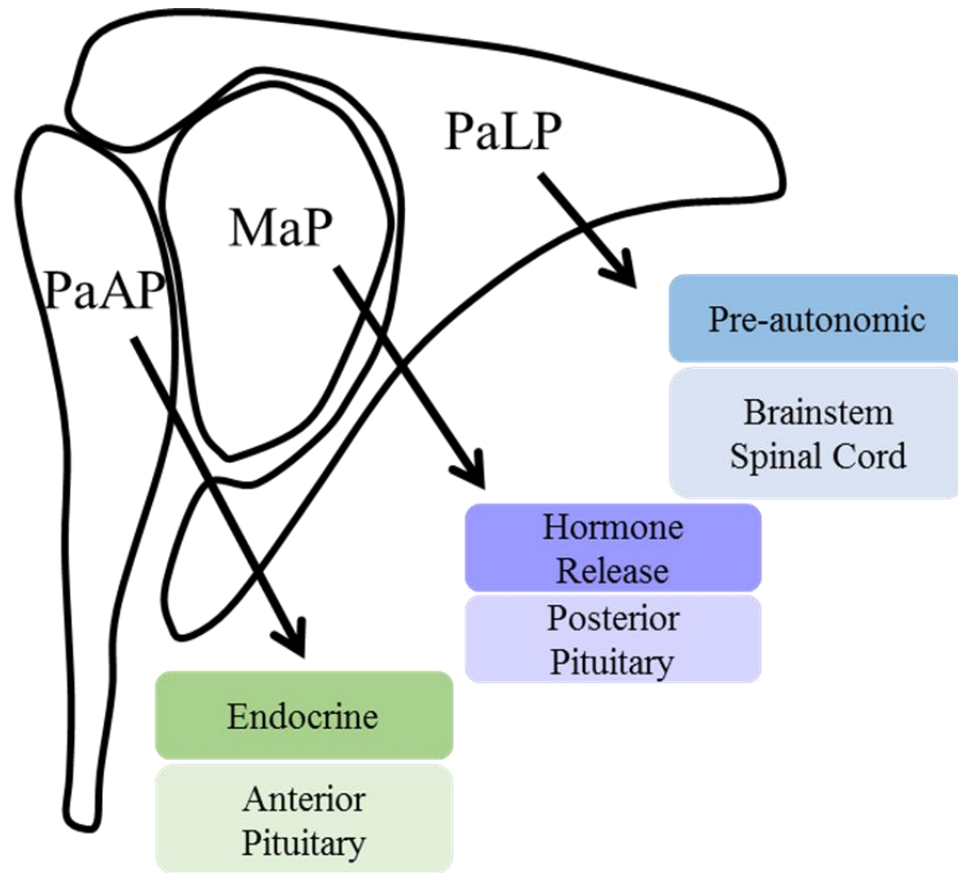


Figure 1. The PVN has diverse projections to regulate multiple systems. The PVN lies adjacent to the 3rd ventricle (3V). Neurons that synthesize CRF are located in the anterior parvocellular PVN (PaAP) and the lateral parvocellular PVN (PaLP). The PaAP neurons project to the anterior pituitary through the median eminence to regulate endocrine response. The PaLP neurons are pre-autonomic, projecting the brainstem and spinal cord. The magnocellular PVN (MaP) is primarily composed of neurons that synthesize oxytocin and vasopressin. These project to the posterior pituitary.

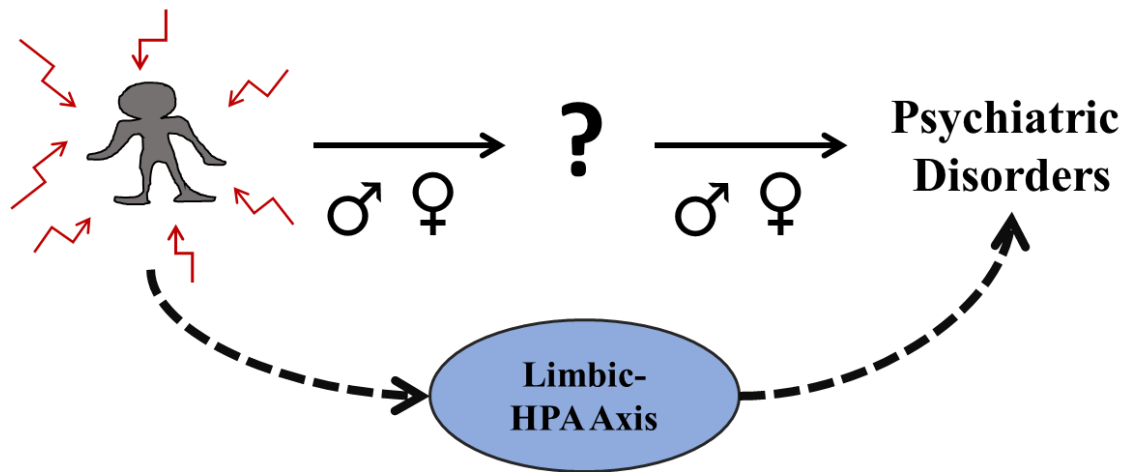


Figure 2. The mechanism by which mbTBI results in psychiatric disorders is unknown. The limbic-HPA axis may provide the missing link connecting these. Dysregulation of the limbic-HPA axis is highly connected to the symptoms of psychiatric disorders.

CHAPTER 3: Manuscript 1

Title: Differential responses of the HPA axis to mild blast traumatic brain injury in males and females

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Conflict of Interest

Any opinions, findings and conclusions contained herein are those of the authors and are not to be construed as reflecting the views of the funding agencies or author affiliations. The authors have no conflicts of interest to disclose.

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ABSTRACT

Traumatic brain injury (TBI) affects 10 million people world-wide, annually. TBI is a leading cause of death and disability and is linked to increased risk of psychiatric disorders. TBI induced by explosive devices has a unique and unknown phenotype. Over 1/3 of people who are exposed to blast-induced (b)TBI have prolonged neuroendocrine deficits, shown by anterior pituitary dysfunction. Dysregulation of the hypothalamus-pituitary-adrenal (HPA) axis is linked to increased risk for psychiatric disorders such as anxiety, depression and PTSD. Not only is there limited information on how the HPA axis responds to mild (m)bTBI, sex differences are understudied. Therefore, we examined central and peripheral HPA axis reactivity 7-10 days after mbTBI in male and female mice. Males exposed to mbTBI had increased restraint-induced serum corticosterone (CORT), but attenuated restraint-induced corticotropin-releasing factor (CRF)/c-FOS-immunoreactivity (ir) in the paraventricular nucleus of the hypothalamus (PVN). Interestingly, females displayed an opposite response to mbTBI, with attenuated restraint-induced CORT and enhanced restraint-induced PVN CRF/c-FOS-ir. We examined potential mechanisms underlying this dysregulation and found that mbTBI did not affect pituitary (POMC, CRFR1) or adrenal (11 β -OHase, 11 β -HSD1 and Mc2R) gene expression. Likewise, mbTBI did not alter MR or GR gene expression in the PVN or relevant limbic structures. However, in females, but not males, mbTBI decreased c-FOS ir in non-neuroendocrine (FG⁺, presumably pre-autonomic) CRF neurons in the PVN. While we demonstrated a sex-dependent link to stress dysregulation of pre-autonomic neurons in females, we hypothesize that mbTBI may disrupt limbic pathways involved in HPA axis coordination in males. Overall, mbTBI altered the HPA axis in a sex-dependent

manner, highlighting the importance of developing therapies to target the individual strategies that males and females utilize to cope with mbTBI.

Précis

The HPA axis responds differently in males and females after mild blast traumatic brain injury.

INTRODUCTION

Blast-induced traumatic brain injuries (bTBI), such as those from improvised explosive devices, account for ~80% of TBIs among service members (227). Mild (m)TBI accounts for ~75% of sustained injuries (1). mTBI is defined as a loss of consciousness (<30 min), post-traumatic amnesia (<24 h) and minimal deficits in motor, verbal and eye responses (203). Symptoms of mTBI may go unnoticed, but result in long-term behavioral, cognitive and emotional impairments. There is increased prevalence of psychiatric disorders after mbTBI (241). Moreover, hypothalamus-pituitary-adrenal (HPA) axis hormonal dysregulation is one of the primary symptoms of neuropsychiatric disorders such as anxiety, depression and post-traumatic stress disorder (PTSD) (105; 415). TBI induced dysregulation of the HPA axis has been observed in both clinical and preclinical studies (6; 228; 341). Therefore, HPA axis dysregulation may increase the risk for stress-related disorders after mbTBI.

Physical and psychological stressors activate corticotropin-releasing factor (CRF) neurons in the paraventricular nucleus of the hypothalamus (PVN) (21; 442). CRF is released into the hypothalamo-hypophyseal portal vasculature to activate anterior pituitary corticotrophs and stimulate the release of adrenocorticotrophic hormone (ACTH). ACTH subsequently elicits the release of glucocorticoids (CORT; cortisol in humans, corticosterone in rats and mice) from the adrenal cortex (102). CORT regulates the HPA axis through the activation of neuronal mineralocorticoid (MR) and glucocorticoid (GR) receptors in a number of brain areas. These receptors are widely distributed throughout the brain including the following limbic structures that are involved in HPA axis

feedback: prelimbic (PrL) and infralimbic (IL) cortices (prefrontal cortex; PFC), bed nucleus of the stria terminalis (BNST), amygdala, and hippocampus (12; 177; 262; 330).

Proper coordination of the HPA axis is required for stress adaptation. In rodents, TBI is thought to cause acute and long-term HPA axis dysregulation, although the literature is somewhat confused. For example, TBI has been shown to increase plasma ACTH and CORT acutely (143; 251). Different reports suggest that TBI can increase or decrease restraint-induced CORT chronically (7 or more days). Mild controlled cortical impact injury (CCI) attenuated CORT 7 days post injury, but heightened restraint-induced CORT 34 and 70 days post TBI (393). Fluid percussion injury decreased CORT 54 days post TBI (341). This inconsistency may be due to differences in injury mechanism, severity and time of recovery.

Women have an increased prevalence of anxiety disorders and an increased diagnosis of psychiatric disorders after TBI (43; 263). In rodents, the inherent sex differences of the HPA axis are well established. Females have enhanced resting and stress-induced HPA axis activation (94; 155; 159; 418). However, there is limited information on the sex dependent differences in response to mbTBI.

Therefore, to address the sex difference in HPA axis response to injury, adult male and female mice were exposed to mbTBI. Central and peripheral HPA axis reactivity was examined 7-10 days post injury. Serum CORT, stress-relevant gene expression and CRF neuronal activation, using c-Fos as a marker, were determined. Our data showed that the HPA axis of males and females responds differently to mbTBI, suggesting the utilization of different compensatory strategies.

MATERIALS AND METHODS

Animals

C57BL/6J. Adult male (N=60) and female (N=72) *C57BL/6J* mice were purchased at 7-9 weeks of age from the Jackson Laboratory (RRID:IMSR_JAX:000664; stock number 000664; Jackson Laboratory, Bay Harbor, ME). Upon arrival, *C57BL/6J* mice were acclimated to the facility for at least one week before experiments.

CRF:tdTomato. Male (N=72) and female (N=80) *CRF:tdTomato* mice were generated by crossing *B6(Cg)-Crh^{tm(cre)Zjh}/J* (*CRF-IRES-Cre*; RRID:IMSR_JAX:012704; stock number 012704, Jackson Laboratory) mice and *B6.Cg-Gt(ROSA)26Sor^{tm14}(CAG-tdTomato)^{Hze}/J* (*Ail14*; RRID:IMSR_JAX:007914; stock number 007914, Jackson Laboratory) mice. *CRF:tdTomato* mice are from the *C57BL/6J* background (78; 298; 427).

Housing conditions. Housing conditions were maintained at 22-25 °C, 50% humidity and on a 12:12 light:dark cycle (lights on at 0100 h). Animals were provided *ad libitum* access to food and water. Animals were same sex housed, 2-3 per cage.

All handling and care of animals was conducted in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals. Procedures were approved by the Institutional Animal Care and Use Committee at the Uniformed Services University of the Health Sciences, Bethesda, MD.

Experimental design

There were 4 experiments in this study. For all experiments, HPA axis reactivity was assessed 7-10 post injury. Unless otherwise stated, experiments were conducted between 0700 – 1100 h (lights off at 1300 h).

Experiment A: C57BL/6J mice were randomly assigned to one of 4 treatment groups. The effect of injury (sham v. mbTBI) and restraint (no restraint v. restraint) on serum CORT, mRNA expression was examined in males and females.

Experiment B: This experiment was designed as a 2 x 2 factorial design to examine the effect of mbTBI (sham v. mbTBI) on CORT secretion after the dexamethasone (DEX) suppression test (VEH v. DEX). Testing was conducted between 1230 – 1300 h (immediately before lights on) in male and female C57BL/6J mice.

Experiment C: CRF:tdTomato mice were randomly assigned to one of 4 treatment groups. The effect of injury (sham v. mbTBI) and restraint (no restraint v. restraint) on CRF neuron activation as determined by c-Fos –ir induction in male and female mice was examined.

Experiment D: CRF:tdTomato mice were assigned to one of 2 experimental groups to examine the effect of mbTBI on activated Fluoro-Gold (FG)⁺ and FG⁻ CRF populations in male and females. All animals were exposed to restraint to induce the expression of c-Fos in stress activated neurons.

Mild blast traumatic brain injury (mbTBI)

Mice were exposed to mbTBI using the Advanced Blast Simulator (ABS; ORA Inc., Fredericksburg, VA; Figure 1). The ABS consisted of three chambers: the driver chamber, the transition section and the test chamber. The driver chamber was sealed with 3 acetate sheets (0.010” thick, 18” x 30”; Industrial Division Sales-Grafix, Inc) and 2 pet screen mesh (14 x 10 grids/in², wire diameter 0.025”; New York Wire). Increased pressure (by compressed air) in the driver chamber ruptured the acetate/mesh seal, allowing the blast wave to travel via the transition section to the test chamber (Figure 1A,

B). A pencil probe pressure gauge (Model Number: 137B23A; PCB Piezotronics, Depew, NY) recorded pressure at the level of the animal located in the test chamber. The mean pressure of the blast wave was not different between males (15.76 ± 0.09 Psi) and females (15.74 ± 0.10 Psi; Table 1; Figure 1C).

Mice were anesthetized by isoflurane inhalation (Isoflurane, USP, Baxter, Deerfield, IL), secured and mounted prone in the test chamber. Sham animals were anesthetized but were not exposed to the blast injury. There were no differences in time of anesthesia exposure between males and females. Anesthetic exposure time was less in sham females than mbTBI females (Table 1). Immediately, after sham or injury procedures, mice were observed for righting reflex and returned to the home cage for recovery.

Righting reflex

Righting reflex was measured on the day of injury to assess loss of consciousness. After removal from anesthesia, the animals were placed on their backs in the home cage. The time it took for the animal to return to an upright position (forepaws touching the ground) was recorded as the time to right (326).

Dexamethasone (DEX) suppression test

To test for peripheral HPA axis function, C57BL/6J male and female mice were injected subcutaneously 7-10 days after mbTBI with DEX (Sigma, St. Louis, MO), at a dose of 30 μ g/kg at 0600 h, following previously established protocols (436). Animals were between 1230 – 1300 h (lights off at 1300).

Retrograde Fluoro-Gold (FG) injection

To identify neuroendocrine versus pre-autonomic projecting CRF populations, male and female CRF:tdTomato mice were intraperitoneally injected with FG (Fluorochrome, LLC, Denver, CO) dissolved in 0.9% saline at a dose of 15 mg/kg (266). Animals were injected 4-6 days prior to mbTBI. All animals that received FG injections were exposed to restraint 7-10 days after mbTBI.

Restraint

Animals were individually restrained for 20 min in clear Plexiglas tubes (3.81 x 10.16 cm; Stoelting Company, Wood Dale IL), 7-10 days after sham or mbTBI procedures. Mild restraint activates the HPA axis. Mice were anesthetized for rapid decapitation (C57BL/6J) or intracardiac perfusion (CRF:tdTomato) at 10 min or 45-60 min after the conclusion of restraint, respectively.

Tissue collection and processing

For *Experiments A* and *B*, C57BL/6J mice were deeply anesthetized with carbon dioxide inhalation and subsequently killed by decapitation. Pituitary, adrenals, liver and brain were collected and immediately frozen. Blood was collected and immediately placed on ice, centrifuged and serum was harvested. Brains were removed and flash frozen in 2-methylbutane (-40 °C). All were stored at -80 °C until analyzed.

Brains were sectioned at 250 μ m (HM525 Cryostat, Thermo Fisher Scientific, Waltham, MA). Either 1 mm (Integra York PA, Inc., York, PA) or 0.5 mm diameter (Harris Uni-Core, Sigma Aldrich, St. Louis, MO) tissue punches were taken to isolate specific regions. The following regions were collected (respective bregma coordinates based on the Franklin and Paxinos Mouse Brain Atlas): PrL (1.98 to 1.34 mm), IL (1.98

to 1.34 mm), anterior (a)BNST (0.62 to 0.02 mm), posterior (p)BNST (-0.10 to -0.58 mm), PVN (-0.22 to -1.22 mm), amygdala (-0.70 to -2.30 mm), dorsal hippocampus (-1.34 to -2.80 mm) and ventral hippocampus (-2.70 to -3.80 mm).

For *Experiments C and D*, CRF:tdTomato mice were deeply anesthetized with carbon dioxide inhalation and sequentially intracardially perfused with 0.9% saline followed by 100 mL of 4% paraformaldehyde buffered by a 0.1 M phosphate solution (PFA; pH 7.4). Brains were collected and post-fixed in PFA overnight. Subsequently, brains were incubated in an increasing graded buffered sucrose gradient (12%, 15%, 18% sucrose in 0.1 M phosphate buffered saline, respectively). Brains were sectioned at 40 μ m (HM525 Cryostat, Thermo Fisher Scientific). Sections were stored in 0.1 M phosphate buffered saline (PBS) containing 0.05% sodium azide (Sigma) until use.

CORT assay

Serum CORT was measured using a commercially available ELISA (Catalog # K014; Arbor Assays, Ann Arbor, MI), following manufacturer's instructions. Samples were diluted to a final concentration of 1:150 and analyzed in duplicate. Subsequent to dilution, samples were treated with the dissociation buffer and heated to 60 °C. Changes in binding were measured using a plate reader (VICTOR V3; PerkinElmer, Waltham, MA) which read absorbance at a wavelength of 450 nm. Values were obtained by comparison to a standard curve ranging from 78.125 pg/ml to 10000 pg/ml. Intra assay coefficient of variance was 7.5% and the minimum detectable CORT concentration was 16.9 pg/ml.

Gene expression

RNA extraction. Total RNA was extracted from pituitary, adrenals, liver and brain regions of C57BL/6J mice using a commercially available RNA prep kit (Direct-zol RNA MiniPrep Kit; Catalog # 11-331; Zymo Research, Tustin, CA) following manufacturer's protocol. Briefly, tissue was homogenized in 1 ml (pituitary, adrenal, liver) or 500 μ l (brain) RiboZol (Thermo Fisher Scientific) prior to column extraction. The total RNA concentration was determined by a spectrophotometer (NanoDrop Lite; Thermo Fisher) measuring absorbance at 260 and 280 nm, and the purity was determined from the 260/280 ratio.

cDNA synthesis. cDNA was synthesized from purified RNA using reverse transcriptase (Thermo Scientific Maxima Reverse Transcriptase kit; Catalog # K1671; Thermo Fisher Scientific).

Quantitative real-time PCR (qPCR). Pituitary, adrenals, and liver mRNA expression were measured by qPCR following previously established protocols (80). Briefly, 2 ng of cDNA template was amplified using iQ SYBR Green Supermix (Bio-Rad, Hercules, CA). Primer sequences for pituitary (POMC, CRFR1), adrenal (11 β -OHase, 11 β -HSD1, Mc2r), and liver (CBG) were designed to have a common annealing temperature of 60 °C using the NCBI Primer blast (Supplemental Table 1; (2)). Each run consisted of an initial denaturation (3 min at 95 °C) followed by 40 cycles that included denaturation (10 sec at 95 °C), annealing (30 sec at 60 °C) and extension (30 sec at 72 °C) using the CFX Connect Real-Time System (Bio-Rad). Melt curve analysis was performed after each run to ensure a single amplicon. All data was normalized to *TBP*

and expressed relative to the control group. The delta delta Ct ($2^{-\Delta\Delta CT}$) method was used for analysis. All samples were analyzed in duplicate.

Droplet digital PCR (ddPCR). Absolute copy number of *MR* and *GR* was quantified utilizing ddPCR in the following brain regions: PrL, IL, PVN, aBNST, pBNST, amygdala, and dorsal/ventral hippocampus. Primer sequences were generated from NCBI Primer blast (Table 2 (2)). Each 20 μ L ddPCR reaction contained EvaGreen supermix (1X solution; Bio-Rad), forward and reverse primers (200 nM each), and cDNA template (2 ng). Droplets were generated using the Automated Droplet Generator (Bio-Rad). Thermal cycling conditions were: enzyme activation (5 min at 95 °C), followed by 40 cycles of denaturation (30 sec at 95 °C) and annealing/extension (1 min at 60 °C) and 1 cycle of signal stabilization (5 min at 4 °C followed by 5 min at 90 °C). Droplets were read using the QX200 Droplet Reader and QuantaSoft software (Bio-Rad). Number of copies per ng cDNA template was used for analysis.

Immunohistochemistry

c-FOS. 40 μ m sections were processed for c-Fos ir following previously established protocols (448). Sections were washed in 0.1 M PBS containing 0.5% Triton X-100 (PBS-T). Tissue was then blocked in PBS containing 5% normal goat serum, 1% bovine serum albumin (BSA) for 1 h at room temperature. Sections were incubated in anti-c-Fos antibody (1:1500; RRID:AB_2631318; EMD Millipore, Billerica, MA) overnight at 4 °C, with gentle agitation. Tissue was washed with PBS-T and incubated with goat anti-rabbit IgG coupled to Alexa Fluor 488 antibody (1:1000; RRID:AB_2338052; Jackson Immuno Research Laboratories, West Grove, PA) for 2 h at

room temperature. Tissue was washed, mounted and cover slipped with Prolong Gold antifade reagent (Invitrogen, Eugene, OR).

Fluoro-Gold and c-FOS. Sections were washed and blocked following above procedure. Sections were incubated in anti-FG (1:50,000; RRID: AB_2314408; Fluorochrome) diluted in company recommended diluent (50 nM KPBS, 0.4% Triton, 1% BSA and 1% normal goat serum) overnight at 4 °C. Tissue was washed with PBS-T and incubated with an Alexa Fluor 647 AffiniPure Fab Fragment Goat anti-rabbit, F(ab')₂ fragment specific (1:700; RRID:AB_2338058; Jackson Immuno Research Laboratories) for 2 h at room temperature. Sections were then placed in anti-c-FOS (1:1500) following the above protocol. For labeling determination, counts were conducted on nuclear c-FOS and cytoplasmic FG labeling. Antibody information is found in Supplemental Table 2.

Microscopy. All images were acquired with a Zeiss 700 Confocal Laser Scanning Microscope using a 20x objective. Number of CRF positive, c-Fos positive, and where applicable, FG positive cells were manually counted from z-stack images compiled using Zeiss Zen 2 software (RRID:SCR_013672). All images were coded and counted by two independent counters using the ImageJ software (RRID: SCR_003070). Any count above a 15% difference between independent counters was recounted by a third counter and values >15% difference were excluded. The data was compiled and counts were averaged.

Statistical analysis

Data was analyzed separately in males and females using two-way analysis of variance (ANOVA; GraphPad, La Jolla, CA). Fisher's least significant difference test

was used for *post hoc* analysis when a significant effect was present. $p < 0.05$ was considered significant.

The DEX suppression test (conducted between 1230 - 1300 h) and FG retrograde labeling (conducted in restraint animals) were used to examine the effect of mbTBI only. These data sets were analyzed using unpaired t-tests in male and female (GraphPad). $p < 0.05$ was considered significant.

RESULTS

Effect of mbTBI on righting reflex

Righting reflex was used to measure return to consciousness (Figure 2A). Two way analysis of variance showed that males and females exposed to mbTBI had increased time to right compared to the respective sham group ($p < 0.05$).

Effect of mbTBI on body weight change

Body weight was used to assess health and well-being after injury (Figure 2B). Animals were weighed every other day before and after injury. There was no difference in baseline weights (data not shown). mbTBI did not alter percent body weight change at any point during the 7-10 day recovery period in males or females ($p > 0.10$).

Effect of mbTBI on serum CORT

Serum CORT. Serum CORT was assayed to measure baseline and restraint-induced HPA axis reactivity (Figure 3). Two-way ANOVA showed that in males, there was a main effect of injury ($p < 0.05$), a main effect of restraint ($p < 0.05$) and no significant interaction. Post hoc analysis showed that restraint increased CORT, regardless of injury ($p < 0.05$). mbTBI had no effect on baseline CORT but enhanced restraint-induced CORT compared to sham males ($p < 0.05$; Figure 3A).

In females, a two-way ANOVA revealed a significant interaction of injury and restraint ($p < 0.05$) and a main effect of restraint ($p < 0.05$). Post hoc analysis showed that restraint increased CORT regardless of injury ($p < 0.05$). mbTBI did not alter baseline CORT, but attenuated restraint-induced CORT compared to sham females ($p < 0.05$; Figure 3B).

DEX suppression test. To test for peripheral HPA axis feedback, animals were administered a DEX suppression test and CORT was measured at its diurnal peak (1230 - 1300 h; Figure 4). In both males and females, there was a main effect of DEX treatment ($p < 0.05$). DEX administration attenuated CORT in males ($p < 0.05$) and females ($p < 0.05$) compared to respective VEH treated animals. In males, mbTBI had no effect on VEH or DEX treatment ($p > 0.10$; Figure 4A). In females, mbTBI had no effect on DEX suppression of CORT ($p > 0.10$). However, mbTBI increased the diurnal CORT peak in VEH treated animals ($p < 0.05$; Figure 4B). Overall, mbTBI did not affect CORT suppression to the DEX suppression test in males or females.

Effect of mbTBI on gene expression in the peripheral HPA axis

HPA-relevant gene expression was examined in the pituitary, adrenals, and liver (Table 2). There was no effect of either mbTBI or restraint on pituitary (POMC, CRFR1) or adrenal (11β -OHase, 11β -HSD1 and Mc2r) genes in males or females ($p > 0.10$). A two-way ANOVA revealed a main effect of injury on liver CBG mRNA expression in males ($p < 0.05$). mbTBI increased restraint-induced CBG mRNA expression compared to sham animals ($p < 0.05$).

Effect of mbTBI on MR and GR mRNA expression

The response of MR and GR mRNA expression to mbTBI was analyzed in the limbic-HPA axis 7-10 days post injury.

PVN. A two-way ANOVA showed that there were no main effects of injury or restraint on male (Figure 5A) or female (Figure 5B) MR mRNA expression in the PVN ($p > 0.10$). Likewise, there were no main effects of injury or restraint on male (Figure 5C) or female (Figure 5D) GR mRNA expression in the PVN ($p > 0.10$).

MR mRNA expression in limbic structures. In males, there was no effect of injury or restraint on MR mRNA expression in the PrL, IL, aBNST, pBNST, amygdala, dorsal hippocampus or ventral hippocampus ($p>0.10$; Table 3). Likewise, in females, neither injury nor restraint had an effect on MR mRNA expression in the aforementioned structures ($p>0.10$; Table 3).

GR mRNA expression in limbic structures. There was no effect of mbTBI or restraint on GR mRNA expression in any of the above-mentioned limbic structures in males or females ($p>0.10$; Table 4).

MR:GR mRNA expression ratio in limbic structures. The ratio of MR:GR mRNA expression was also analyzed to determine changes in relative expression (Table 5). There was no effect of injury on MR:GR ratio in the PrL, IL, aBNST, pBNST, amygdala or ventral hippocampus in males or females ($p>0.10$). In males, there was an effect of restraint in the dorsal hippocampus. In shams, restraint decreased the MR:GR ratio ($p<0.05$).

Effect of mbTBI on CRF neuron activation in the PVN

CRF neuron activation in the PVN was quantified by visualization of c-Fos immunoreactivity (Figure 6). There was a main effect of restraint in males and females. In both sham and injured males and females, restraint increased CRF activation compared to the respective no restraint group ($p<0.05$). In males, mbTBI attenuated the restraint-induced increase in CRF activation ($p<0.05$; Figure 6C). In contrast, mbTBI increased restraint-induced CRF activation in females ($p<0.05$; Figure 6D).

Effect of mbTBI on activation of FG⁺ and FG⁻ CRF populations

To determine the ratio of activated CRF neurons (c-FOS⁺) in the PVN, animals received the retrograde tracer, FG to label neuroendocrine populations (Figure 7). All animals received restraint. In males, mbTBI did not alter the percentage of activated FG⁺ CRF neurons ($p>0.10$; Figure 7B) or activated FG⁻ CRF neurons ($p>0.10$; Figure 7C). However, in females, we observed an effect of mbTBI on the percentage of activated CRF neurons. mbTBI decreased the percent of activated FG⁻ CRF (non-neuroendocrine) populations ($p<0.05$; Figure 7C). There was also a non-significant trend to an increase of activated FG⁺ CRF neurons ($p=0.057$; Figure 7B).

DISCUSSION

The results of the present study showed that mbTBI dysregulated CORT and CRF neuron activation in males and females, but in an opposing fashion. In males, mbTBI increased restraint-induced CORT, but decreased restraint-induced CRF neuron activation. Conversely, in females, mbTBI decreased restraint-induced CORT, but increased restraint-induced CRF neuron activation. These results suggested that mbTBI impacts the HPA axis, but may alter parallel limbic and brainstem circuitries mediating endocrine and autonomic stress responses in males and females. Previous studies showed that TBI could disrupt neural circuitry and physiology systems to contribute to the development of psychiatric disorders (71; 110; 272; 394). Our current studies supported this link.

Males and females had opposite CORT responses to restraint after mbTBI.

In males, mbTBI increased restraint-induced CORT levels, but attenuated restraint-induced CORT release in females. To determine if the dysregulation occurred at the level of the peripheral HPA axis we measured expression of genes involved in ACTH and CORT synthesis and secretion. Circulating CRF activates the G-protein coupled receptor, CRFR1, on pituitary corticotrophs (9). In corticotrophs, ACTH is cleaved from its precursor protein, POMC, and following secretion, binds to Mc2r located on the adrenal (268; 370). In the adrenal, 11 β -OHase is the final enzyme involved in the synthesis of CORT and 11 β -HSD1 converts inactive cortisone to the active form CORT (57; 59). Our present results showed that there are no changes in gene expression of the pituitary (CRFR1, POMC) or adrenal (11 β -OHase, 11 β -HSD1, Mc2r). Because peripheral genes are unaffected by mbTBI, this suggests that the injury-induced

dysregulation is not here. Animals were administered the DEX suppression test after mbTBI to examine peripheral feedback. DEX suppressed the diurnal peak in CORT suggesting intact feedback. Clinically, there is increased prevalence of acute and chronic hypopituitarism (6; 389) and hypoadrenalism (316), specifically after multiple mbTBIs or single moderate-severe bTBI (42; 443). While we did not measure ACTH release from the pituitary, gene expression analysis allowed for an alternate route of investigation. With no apparent peripheral gene alteration, CORT dysregulation after mbTBI may be due to a central disruption in feedback.

mbTBI did not alter MR or GR mRNA expression.

mbTBI did not alter MR or GR mRNA expression in the PVN or stress-relevant limbic structures. HPA axis feedback is governed by CORT binding to MR and GR. Feedback regulation occurs at the level of the PVN (351) or via PVN projecting limbic structures (100; 157). MRs have a higher affinity for CORT and are involved in resting HPA axis regulation (413). GRs are preferentially bound during periods of high CORT secretion (177; 330; 331). mbTBI did not affect baseline CORT, therefore we did not anticipate changes in MR. Altered restraint-induced CORT suggested a change in GR mRNA expression. Chronic, but not acute, stress down-regulates GR protein levels (109; 348), potentially explaining the lack of effect we observed. Our results also coincide with previous studies (57+ days post CCI), which revealed no change in GR protein levels in injured animals (396). In the acute phase post CCI, hippocampal GR levels were increased (251). Our data were restricted to gene expression at 7-10 days post mbTBI and acute responses of MR and GR profiles were not measured. The lack of MR/GR change

suggested that the expression of limbic receptors involved in feedback regulation of the PVN are intact after mbTBI.

mbTBI had opposing effects on CRF neuron activation in males and females.

CRF colocalization with c-FOS immunoreactivity measured neuronal activation in the central regulator of the HPA axis: the PVN. Acute restraint increased CRF neuron activation in the PVN of males and females, as previously shown (179; 418). mbTBI attenuated restraint-induced CRF neuron activation in males but enhanced CRF neuron activation in females. This discrepancy to CORT response to restraint in these same animals may arise from a sex-dependent dysregulation at the level of CRF neurons in the PVN. The PVN regulates neuroendocrine, autonomic and behavioral responses to stress (384; 405). Our anatomical understanding of the mouse PVN is extrapolated from rat studies (52). CRF neurons in the PVN are divided into two categories: parvocellular neuroendocrine neurons and non-neurosecretory (presumably pre-autonomic) neurons (70; 209; 352; 383). Anterior and medial subdivisions of the parvocellular (p)PVN project to the median eminence (23; 211) to regulate endocrine response. Projections from the dorsomedial cap, ventral and posterior subdivisions of the rat pPVN are sent to sympathetic neurons of the intermediolateral nucleus of the spinal cord and pre-sympathetic neurons of the rostroventrolateral medulla (26; 140; 237; 319; 383), regulating autonomic function. We observed the highest density of activated CRF neurons in the anterior and medial subdivisions of the pPVN in both males and females (not quantified). This suggested the primary output of activated CRF neurons is involved in neuroendocrine regulation. However, it is important to examine mbTBI's impact on

the activation of neuroendocrine and non-neuroendocrine CRF populations to begin to address projection integrity after injury.

Peripheral FG injection labels hypophysiotrophic neurons based on their projections outside the blood brain barrier to allow secretion into the bloodstream. Therefore, subsequent cytoplasmic labeling in CRF neurons, distinguished neuroendocrine from non-neuroendocrine CRF populations. mbTBI decreased the percentage of activated non-neuroendocrine (presumably pre-autonomic projecting; FG⁺) CRF neurons in females, but not males. This suggested a decreased utilization of the pre-autonomic system to cope with a stressor after mbTBI in females. Previous studies showed enhanced autonomic responses to acute stress in females. Restraint, confinement and re-housing induced greater hyperthermic responses in females than males (260; 399). Foot shock increased circulating levels of catecholamines (dihydroxyphenylalanine, dihydroxyphenylalanine, norepinephrine and epinephrine) in females, but not males (434). In our sham animals, females had increased restraint-induced activation of non-neuroendocrine (pre-autonomic) CRF neurons compared to males, paralleling evidence for an enhanced role of the autonomic nervous system in females. mbTBI reduced non-neuroendocrine (pre-autonomic) CRF activation in females, potentially disrupting this circuitry. Pre-autonomic PVN neurons are under constant inhibition by peri-PVN GABAergic interneurons (336). Injection of the GABA antagonist, bicuculline, into the PVN increased heart rate and arterial pressure (226; 247). Glutamate administration into the PVN increased blood pressure, heart rate and renal sympathetic nerve activity (226). Therefore, integrity of the GABA-glutamate circuitry is crucial for proper coordination of PVN outputs. Experimental TBI models (CCI, FPI and penetrating injury) dysregulate

glutamate and GABA, as well as their respective receptors throughout the HPA axis-relevant limbic structures (148; 396). For example, CCI decreased hippocampal and amygdalar inhibitory neurons and bicuculline reversed the TBI-induced CORT attenuation response (396). It is unclear if mbTBI mimics these effects in the same fashion as other forms of TBI. However, if mbTBI disrupted the inhibitory tone on the PVN, this may interfere with sympathetic and pre-sympathetic CRF projections to brainstem structures in females.

In addition to the role in sympathetic reactivity, limbic GABA-glutamate projections to the PVN are also pivotal for proper coordination of the HPA axis. Neuroendocrine projecting CRF neurons are surrounded by a shell of GABAergic neurons involved in neuronal inhibition (336). Limbic structures project indirectly to the PVN via two primarily GABAergic regions: the BNST and the peri-PVN (119; 177). The HPA axis is mainly inhibited by indirect hippocampal and PFC projections and activated by amygdala projections (177). Therefore, a disruption in limbic GABA and glutamate projections after mbTBI may also manifest through HPA axis dysregulation. Further studies are needed to elucidate the integrity of these peptides after mbTBI.

Females had an enhanced peak in diurnal CORT after mbTBI.

In females, mbTBI enhanced the diurnal CORT peak. More than 70% of TBI patients experience a sleep disturbance after injury (422). In a fluid percussion (FPI) TBI model, the circadian timing of clock genes is dysregulated. After FPI, the clock gene, *Cry1*, was increased in the suprachiasmatic nucleus (SCN), but decreased in the hippocampus (56). The SCN is entrained by the light-dark cycle to coordinate circadian rhythms. The SCN controls the diurnal rhythm of CORT, while SCN lesions disrupt the

rhythmicity of CORT secretion (4; 343). In rodents, peak CORT secretion is observed during the active period (dark phase, (380). In humans, a second spike of CORT observed just after the time of awakening is referred to as the cortisol awakening response.

Dysregulation of natural CORT rhythmicity can disrupt homeostasis, and is linked to depression and anxiety (111; 257; 267). Additionally, circadian disruptions are linked to cardiovascular disorders and PTSD (447). There are inherent sex differences in SCN morphology, gonadal steroid receptor distribution, electrical activity and efferent projections (31; 452). These differences may attribute to the sex dependent disruptions of CORT rhythmicity observed after mbTBI. More detailed studies are needed to investigate if this enhanced diurnal peak in CORT is linked to an underlying mechanism of HPA axis dysregulation.

Different experimental TBI models may elicit different HPA axis responses.

Few studies have examined mbTBI, but rather they have utilized other animal models of TBI such as CCI and FPI. To our knowledge we are the first to show differential HPA axis responses to mbTBI in males and females. Previous studies have showed altered CORT in acute and chronic phases post-injury, however, most have only focused on responses in males. In previous studies, male rats have been shown to have a heightened baseline CORT level 4 hours post-TBI (143), but an attenuated CORT level 54 days post FPI (341). Dependent on injury mechanism, acute psychogenic restraint can increase (145) or decrease (393) CORT throughout the chronic recovery period (7 or more days after injury). Twenty-one days after injury, forced swim stress attenuated CORT chronically (393). At 28 days post injury, CCI-induced suppression of baseline CORT levels in male and ovariectomized female rats (395). Our data conflict with

previous literature potentially due to inherent differences in our blast-induced injury model. Different experimental models of TBI may alter different neuroanatomical pathways. The complex mechanisms of the physical wave and the diffuse nature of the injury make mbTBI distinct from other forms of closed head injury (286). Among military populations, bTBI is a primary cause of trauma (227), making it a “signature wound of the war on terror” (51). PTSD, depression and/or anxiety are observed in more than one third of veterans (180; 416). Due to the risk of developing psychiatric disorders after bTBI (338), it is important to understand the underlying physiological response to this form of injury.

Conclusions

In summary, present findings demonstrated that mbTBI altered the HPA axis and that this may stem from a disruption at the level of the central stress regulator: the PVN. The HPA axis is susceptible to TBI in both males and females, however, the profile of reactivity differed. We conclude that males and females utilize different strategies to cope with mbTBI induced HPA axis dysregulation.

<i>Condition</i>	<i>Anesthesia length (sec)</i>	<i>Blast pressure (Psi)</i>
Male Sham	340.23 ± 7.28	N/A
Male Blast	345.98 ± 7.18	15.76 ± 0.09
Female Sham	325.20 ± 6.17	N/A
Female Blast	348.15 ± 7.76*	15.74 ± 0.10

Table 1. Parameters of sham and injury procedures in male and female mice.

Table presents length of anesthetic (sec) and the pressure of the blast wave (Psi), where applicable. Data represent mean ± SEM. *p<0.05 comparing sham v. blast in respective sex.

Male				
Gene	<i>Sham</i>		<i>Blast</i>	
	<i>No Restraint</i>	<i>Restraint</i>	<i>No Restraint</i>	<i>Restraint</i>
POMC	1.0 ± 0.1	1.0 ± 0.1	1.1 ± 0.2	1.0 ± 0.1
CRFR1	1.0 ± 0.1	1.0 ± 0.4	1.0 ± 0.4	1.0 ± 0.4
11β-OHase	1.0 ± 0.1	1.0 ± 0.1	1.1 ± 0.2	1.1 ± 0.1
11β-HSD1	1.0 ± 0.1	0.9 ± 0.1	1.0 ± 0.1	0.8 ± 0.1
Mc2r	1.0 ± 0.1	1.1 ± 0.1	0.9 ± 0.1	1.0 ± 0.0
CBG	1.0 ± 0.2	0.8 ± 0.1	1.2 ± 0.1	1.3 ± 0.1*
Female				
Gene	<i>Sham</i>		<i>Blast</i>	
	<i>No Restraint</i>	<i>Restraint</i>	<i>No Restraint</i>	<i>Restraint</i>
POMC	1.0 ± 0.2	1.1 ± 0.2	1.0 ± 0.1	1.1 ± 0.2
CRFR1	1.0 ± 0.1	1.0 ± 0.1	1.0 ± 0.1	1.0 ± 0.1
11β-OHase	1.0 ± 0.1	0.9 ± 0.3	1.1 ± 0.1	1.0 ± 0.1
11β-HSD1	1.0 ± 0.0	1.1 ± 0.1	1.0 ± 0.1	1.0 ± 0.0
Mc2r	1.0 ± 0.1	1.0 ± 0.1	1.1 ± 0.1	1.0 ± 0.1
CBG	1.0 ± 0.1	1.0 ± 0.1	1.0 ± 0.1	0.9 ± 0.1

Table 2. Relative expression of peripheral HPA axis genes in the pituitary (POMC, CRFR1), adrenal (11β-OHase, 11β-HSD1 and Mc2r) and liver (CBG). Neither mbTBI nor restraint had an effect on gene expression in male or female mice. Data represent mean ± SEM. *p<0.05 comparing blast restraint v. sham restraint.

	Male				Female			
	<i>Sham</i>		<i>Blast</i>		<i>Sham</i>		<i>Blast</i>	
	<i>No Restraint</i>	<i>Restraint</i>	<i>No Restraint</i>	<i>Restraint</i>	<i>No Restraint</i>	<i>Restraint</i>	<i>No Restraint</i>	<i>Restraint</i>
<i>PrL</i>	826.25 ± 131.55	895.58 ± 57.46	886.00 ± 72.33	870.90 ± 40.29	770.58 ± 152.29	719.63 ± 116.71	960.38 ± 47.07	802.00 ± 125.29
<i>IL</i>	1118.00 ± 216.01	1123.79 ± 161.20	1282.93 ± 203.63	1597.00 ± 211.21	1106.57 ± 148.87	953.00 ± 99.69	1095.30 ± 162.49	997.58 ± 142.73
<i>aBNST</i>	1456.06 ± 235.31	1583.75 ± 132.45	1512.82 ± 211.86	1422.88 ± 141.48	1799.27 ± 184.13	1720.93 ± 307.25	1559.06 ± 265.41	1938.33 ± 316.99
<i>pBNST</i>	1320.44 ± 137.11	1075.79 ± 159.08	1239.57 ± 304.35	1397.43 ± 122.36	1368.38 ± 161.57	1333.50 ± 262.19	1535.83 ± 150.12	1164.42 ± 76.32
<i>AMG</i>	2041.05 ± 365.86	1990.18 ± 290.21	1679.27 ± 177.76	1988.39 ± 315.78	1571.21 ± 165.63	1224.33 ± 70.46	1493.96 ± 206.30	1653.83 ± 270.99
<i>dHIPP</i>	7155.50 ± 771.51	6076.67 ± 825.87	6220.00 ± 737.09	6662.81 ± 643.13	5859.50 ± 667.61	7824.00 ± 191.54	5476.39 ± 529.22	6636.11 ± 676.93
<i>vHIPP</i>	5545.56 ± 560.45	4491.44 ± 857.41	5493.18 ± 598.68	5009.17 ± 825.03	4852.00 ± 586.94	4586.67 ± 187.05	5648.18 ± 955.04	4687.50 ± 511.03

Table 3. There was no effect of mbTBI on MR expression in limbic structures of male and female mice.

PrL = prelimbic cortex, IL = infralimbic cortex, aBNST = anterior bed nucleus of the stria terminalis, pBNST = posterior bed nucleus of the stria terminalis, AMG = amygdala, dHIPP = dorsal hippocampus, vHIPP = ventral hippocampus. Data represent mean ± SEM.

	Male				Female			
	<i>Sham</i>		<i>Blast</i>		<i>Sham</i>		<i>Blast</i>	
	<i>No Restraint</i>	<i>Restraint</i>	<i>No Restraint</i>	<i>Restraint</i>	<i>No Restraint</i>	<i>Restraint</i>	<i>No Restraint</i>	<i>Restraint</i>
<i>PrL</i>	2570.00 ± 390.32	2560.71 ± 353.56	3013.57 ± 200.27	2676.00 ± 312.62	2342.50 ± 475.79	2632.50 ± 420.13	3330.00 ± 70.39	2799.17 ± 360.29
<i>IL</i>	2500.83 ± 371.88	2546.79 ± 352.67	2861.43 ± 164.25	3206.00 ± 180.83	2405.00 ± 178.59	2795.00 ± 14.90	2760.00 ± 344.28	2758.00 ± 356.54
<i>aBNST</i>	2393.00 ± 382.99	2403.89 ± 192.48	2229.55 ± 210.76	2665.71 ± 76.57	2865.46 ± 201.18	2665.71 ± 465.32	2400.72 ± 344.28	2758.00 ± 356.54
<i>pBNST</i>	2494.44 ± 298.18	2154.38 ± 333.02	1986.93 ± 361.38	2530.86 ± 121.06	2595.00 ± 247.44	2235.33 ± 461.23	2487.50 ± 286.75	2306.29 ± 463.12
<i>AMG</i>	2426.00 ± 223.57	2288.18 ± 241.51	2319.09 ± 318.52	2479.00 ± 343.43	2443.33 ± 212.67	1989.17 ± 129.17	2253.18 ± 165.55	2332.22 ± 151.30
<i>dHIPP</i>	2430.00 ± 312.15	2917.78 ± 223.58	2595.50 ± 31.50	2848.13 ± 228.45	3030.56 ± 168.52	3047.50 ± 519.46	2162.22 ± 330.70	2976.11 ± 171.39
<i>vHIPP</i>	2111.11 ± 197.36	2175.94 ± 240.60	2523.64 ± 192.08	1970.50 ± 328.45	1846.50 ± 190.75	1971.11 ± 140.84	1712.41 ± 132.20	1765.70 ± 148.98

Table 4. There was no effect of mbTBI or restraint on GR expression in limbic structures of male or female mice.
Data represent mean ± SEM.

	Male				Female			
	<i>Sham</i>		<i>Blast</i>		<i>Sham</i>		<i>Blast</i>	
	<i>No Restraint</i>	<i>Restraint</i>	<i>No Restraint</i>	<i>Restraint</i>	<i>No Restraint</i>	<i>Restraint</i>	<i>No Restraint</i>	<i>Restraint</i>
<i>PrL</i>	0.32 ± 0.01	0.31 ± 0.01	0.29 ± 0.01	0.34 ± 0.03	0.34 ± 0.03	0.28 ± 0.02	0.29 ± 0.02	0.28 ± 0.02
<i>IL</i>	0.45 ± 0.06	0.45 ± 0.04	0.44 ± 0.05	0.50 ± 0.06	0.46 ± 0.05	0.33 ± 0.04	0.39 ± 0.04	0.42 ± 0.08
<i>aBNST</i>	0.67 ± 0.04	0.83 ± 0.14	0.66 ± 0.04	0.59 ± 0.04	0.62 ± 0.03	0.65 ± 0.02	0.65 ± 0.05	0.66 ± 0.06
<i>pBNST</i>	0.68 ± 0.13	0.63 ± 0.08	0.59 ± 0.05	0.55 ± 0.05	0.52 ± 0.03	0.66 ± 0.06	0.63 ± 0.04	0.55 ± 0.10
<i>AMG</i>	0.82 ± 0.12	0.93 ± 0.18	0.87 ± 0.13	0.86 ± 0.16	1.67 ± 0.15	1.77 ± 0.10	1.76 ± 0.24	1.80 ± 0.41
<i>dHIP</i>	3.15 ± 0.41	2.09 ± 0.24*	2.48 ± 0.18	2.33 ± 0.14	2.17 ± 0.91	2.40 ± 0.26	2.76 ± 0.32	2.19 ± 0.16
<i>vHIP</i>	2.65 ± 0.13	2.07 ± 0.39	2.20 ± 0.18	2.36 ± 0.50	2.58 ± 0.17	2.42 ± 0.17	2.82 ± 0.31	2.64 ± 0.14

Table 5. The was no effect of mbTBI on MR:GR ratio throughout the limbic structures. In males, restraint decreased the MR:GR ratio in the dorsal hippocampus. Data represent mean ± SEM. *p<0.05 comparing sham restraint versus sham no restraint.

Gene	Accession No.	Primer Sequence	Amplicon Size (bp)
TBP	NM_013684.3	(F) 5'-CCTATCACTCCTGCCACACC-3'	161
		(R) 5'-ATGACTGCAGCAAATCGCTTG-3'	
MR	NM_001083906.1	(F) 5'-GATGGGTACCCGGTCCTAGA-3'	195
		(R) 5'-GTTGTGTTGTCCTTCCACGG-3'	
GR	NM_008173.3	(F) 5'-AGTGGAAGGACAGCACAATTA-3'	171
		(F) 5'-TACTCCGTTTGCTTGGGGAC-3'	
CRFR1	NM_007762.5	(F) 5'-AGGATCAGCAGTGTGAGAGC-3'	174
		(R) 5'-AGCCATTGTTTGTGGTGTGTAG-3'	
11 β -OHase	NM_001033229.3	(F) 5'-TTTTTCAGGCACAGTGTAGGGA-3'	199
		(R) 5'-AGCTGCAGTCGGTTGAAGTA-3'	
11 β -HSD1	NM_008288.2	(F) 5'-AGCCGCACTTATCTGAAGCC-3'	154
		(R) 5'-TTCCCTGGAGCATTTCTGGTC-3'	
Mc2r	NM_008560.3	(F) 5'-TCAGAGCTGAAGCCAGCAAG-3'	193
		(R) 5'-GTGGTGTTTGCCGTTGACTT-3'	
POMC	NM_001278581.1	(F) 5'-CGACAGGCAGGAGACTGAAC-3'	127
		(R) 5'-AGATGCGCAGAGAAACGAGG-3'	
CBG	NM_007618.3	(F) 5'-AGGTGGGGCAAGCTTATGAT-3'	178
		(R) 5'-GGAGTACCGTTAATGTCAAGGGA-3'	

Supplemental Table 1. Primer sequences used for gene expression analysis.

<i>Target</i>	<i>Specificity</i>	<i>Source</i>	<i>Working Dilution</i>
c-Fos	N-Terminus	EMD Millipore (ABE457): Rabbit polyclonal	1:1500
Fluoro-Gold		Fluorochrome: Rabbit polyclonal	1:50000

Supplemental Table 2. Antibody information.

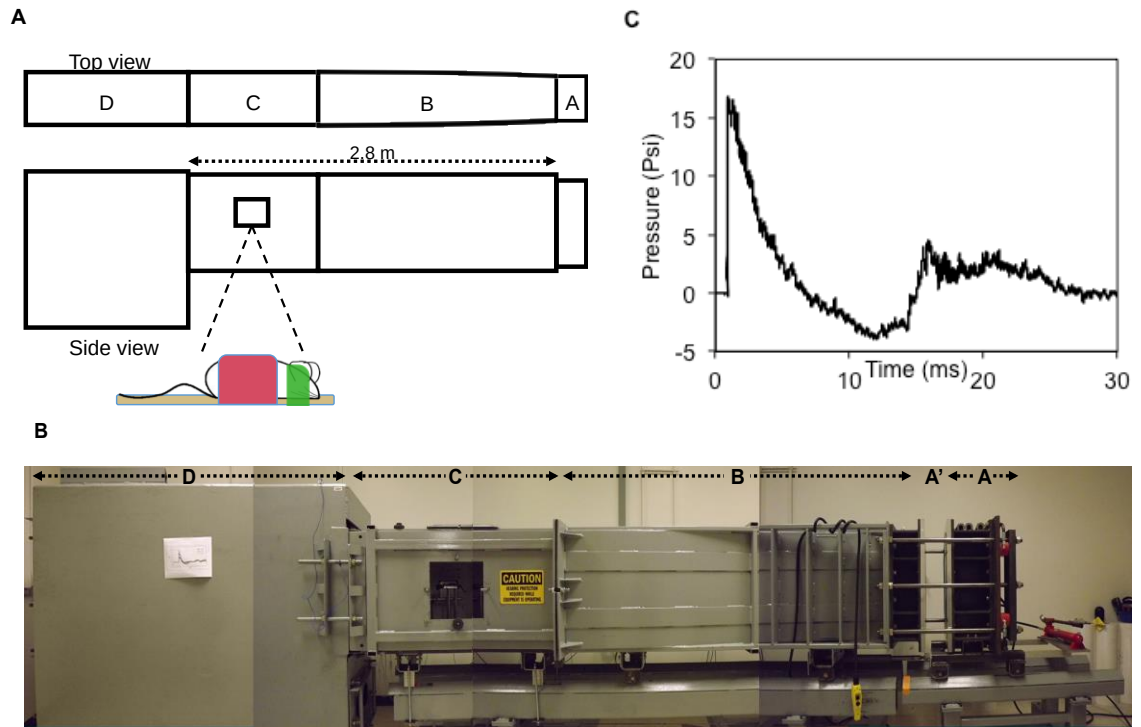


Figure 1. Schematic depiction of the Advanced Blast Simulator (ABS). Schematic view from the top and side (Figure not drawn to scale; Modified from figure courtesy of Dr. Yeonho Kim). The ABS consists of a) a high pressure driver a') membrane b) a transition section c) a test section and d) an end wave eliminator/muffler. B) Montage of photographs of the ABS. C) Representative profile of a blast over pressurization wave from the present study (courtesy of Dr. Yeonho Kim).

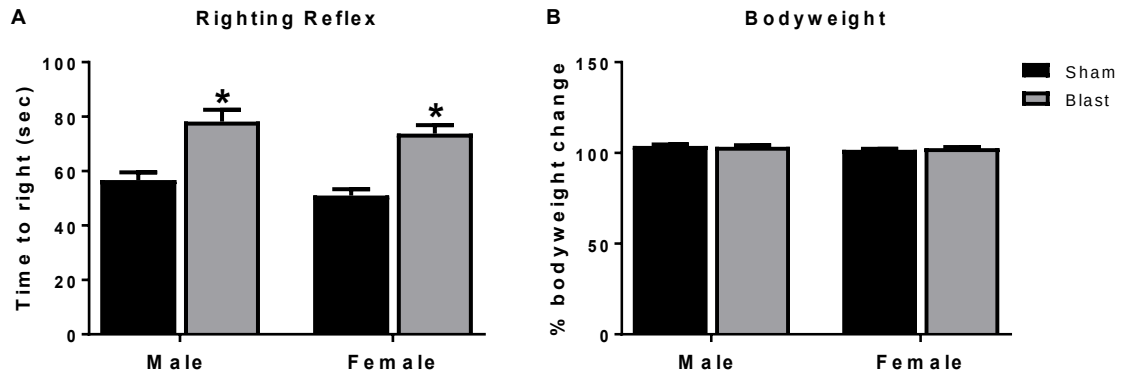


Figure 2. Effect of mbTBI on righting reflex and body weight change.

A) Males and females exposed to mbTBI had an increased time to right compared to the respective sham group B) mbTBI had no effect on percent body weight change 7-10 days post injury in males or females. Data represent mean $\pm$ SEM.

*p<0.05.

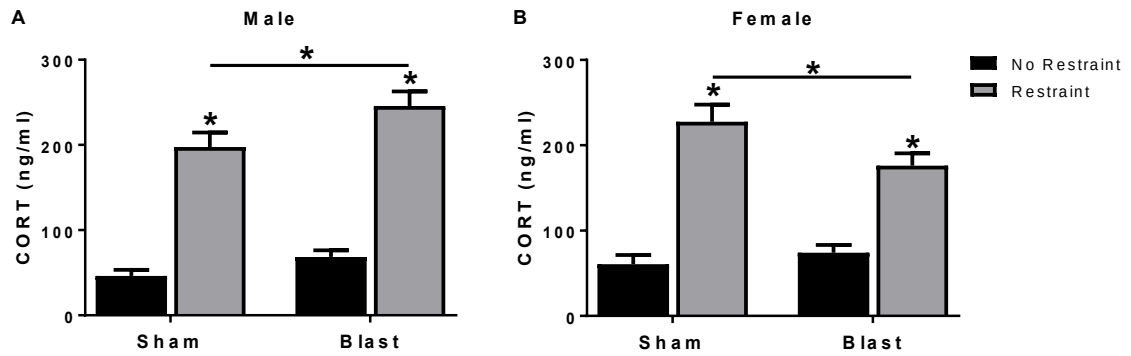


Figure 3. Effect of mbTBI on baseline and restraint-induced serum CORT. A,B) Restraint increased CORT in males and females, regardless of injury. There was no effect of mbTBI on baseline CORT in males or females. A) In males, mbTBI increased restraint-induced CORT. B) In females, mbTBI attenuated restraint-induced CORT. Data represent mean $\pm$ SEM. * $p < 0.05$.

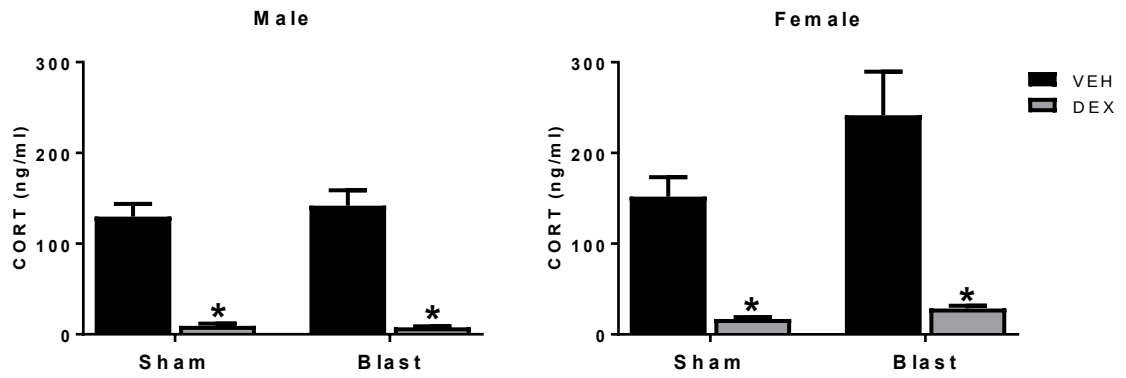


Figure 4. Effect of mbTBI on the DEX suppression test.

A,B) DEX attenuated diurnal CORT regardless of injury. A) mbTBI had no effect on the DEX suppression in males. B) In females, mbTBI increased the diurnal CORT peak but had no effect on DEX suppression. Data represent mean $\pm$ SEM. * $p < 0.05$.

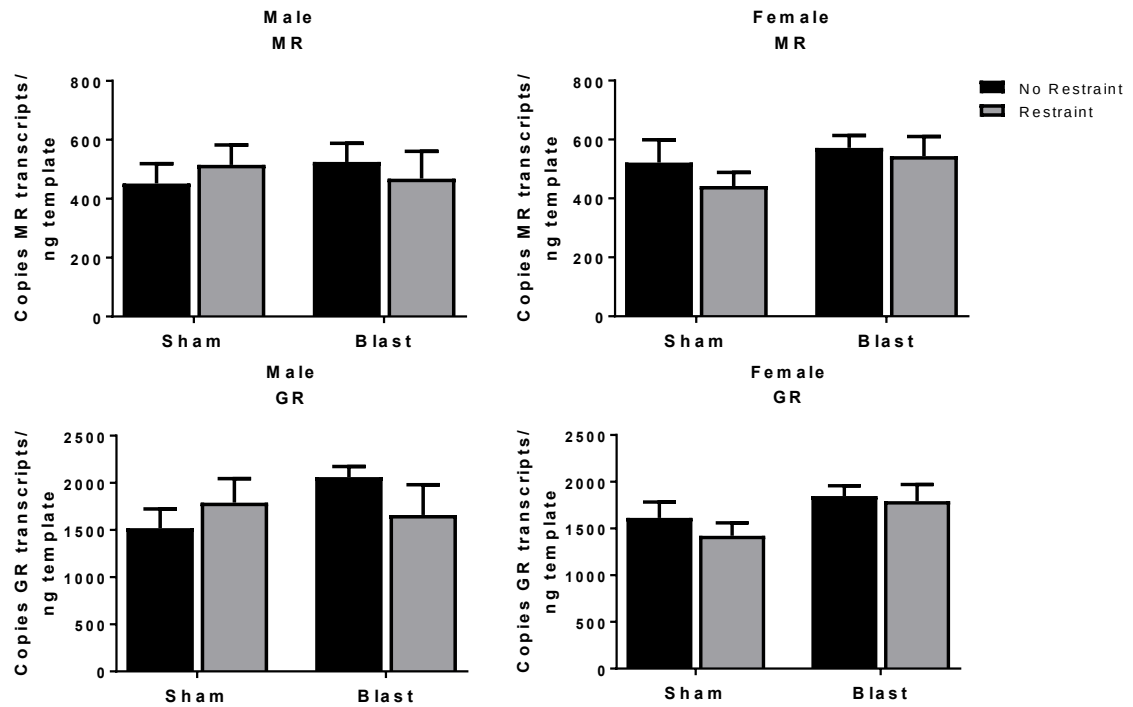


Figure 5. Effect of mbTBI on MR and GR mRNA expression in the PVN. mbTBI had no effect on MR mRNA expression in either A) males or B) females. Injury also had no effect on GR mRNA expression in C) males or D) females. Data represent mean $\pm$ SEM.

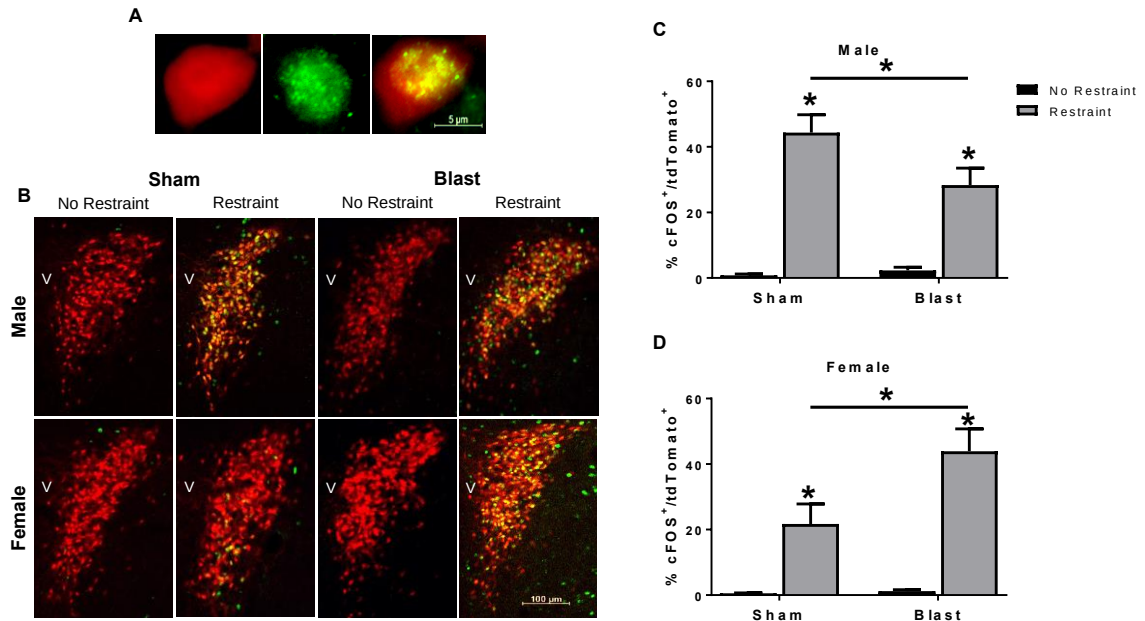


Figure 6. Effect of mbTBI on CRF activation in the PVN.

Photomicrographs showing confocal images at a) 63X and b) 10X. A) Example of CRF (red) and c-Fos (green) colocalization. B) Representative images of CRF and c-FOS in the PVN of males (top row) and females (bottom row). A,B) Restraint increased CRF activation regardless of injury. A) In males, mbTBI attenuated restraint-induced CRF activation. B) In females, mbTBI increased restraint-induced CRF activation. Data represent mean $\pm$ SEM. * $p < 0.05$.

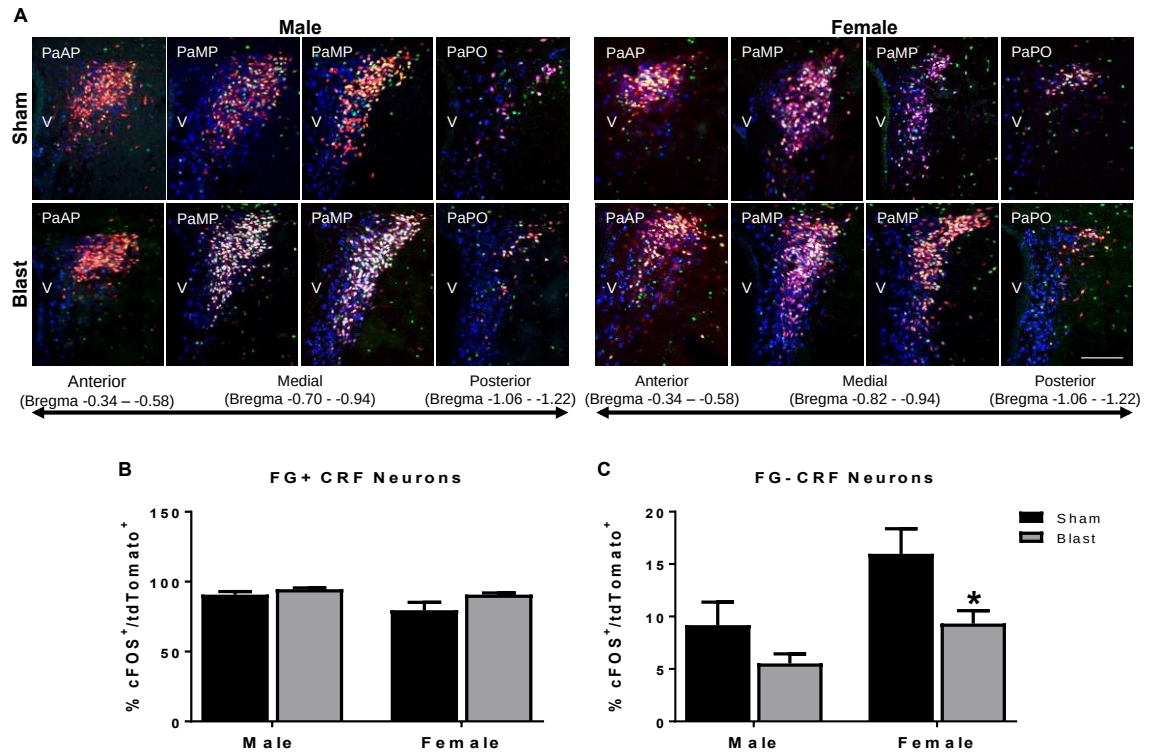


Figure 7. Effect of mbTBI on activated FG⁺ and FG⁻ CRF populations.

A) Photomicrographs of confocal images at 20X throughout the rostral to caudal span of the PVN. Images represent colocalization patterns of CRF (red), c-FOS (green) and FG (blue) in the following subnuclei of the PVN: anterior parvocellular PVN (PaAP), medial parvocellular PVN (PaMP) and posterior parvocellular PVN (PaPO). B) mbTBI had no effect on activated FG⁺ or FG⁻ CRF neurons in males. C) In females, mbTBI decreased activated FG⁻ CRF (pre-autonomic) neurons. Data represent mean $\pm$ SEM. * $p < 0.05$.

CHAPTER 4: Manuscript 2

Title: Mild blast traumatic brain injury effect on CRFRs:
Potential link to anxiety-like behaviors

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ABSTRACT

Traumatic brain injury (TBI) affects 1.7 million people in the United States every year, resulting in increased risk of death and disabilities. A significant portion of TBIs experienced by military personnel are induced by explosive blast devices. Active duty military personnel are especially vulnerable to mild (m)TBI and the associated long-term effects, such as anxiety disorders. The mechanism by which mbTBI results in anxiety disorders is unknown. Corticotropin releasing factor (CRF) is a brain signaling system that is linked to anxiety. CRF and its family of related peptides modulate anxiety-related behaviors by binding to CRF receptors subtypes 1 and 2 (CRFR1, CRFR2, respectively). These receptors are distributed throughout limbic structures that control behaviors related to emotion, memory, and arousal. Therefore we examined the effect of mbTBI (~15.5 Psi) on the limbic CRFR system in male and female mice. Males and females displayed increased anxiety-like behaviors 6 days after mbTBI ($p < 0.05$). Surprisingly, mbTBI did not alter CRFR1 gene expression in males or females. However, mbTBI disrupted CRFR2 gene expression in different limbic structures in males and females. In males, mbTBI increased baseline CRFR2 gene expression in the ventral hippocampus ($p < 0.05$). mbTBI decreased restraint-induced CRFR2 gene expression in the aBNST and amygdala of males ($p < 0.05$). In females, mbTBI decreased restraint-induced CRFR2 gene expression in the dorsal hippocampus ($p < 0.05$). Decreased CRFR2 gene expression may contribute to increased anxiety-like behaviors. Differentiating the sex-dependent circuitry may provide a means to combat anxiety symptoms by targeting CRFR2.

Keywords: anxiety, traumatic brain injury, corticotropin releasing factor, receptor,
sex differences

INTRODUCTION

Traumatic brain injury (TBI) affects approximately 1.7 million people annually and is a leading cause of death and disability in the United States (128). The majority (~75%) of TBIs are diagnosed as mild (3). Mild (m)TBI is defined as a transient loss of consciousness (<30 min), transient post-traumatic amnesia (<24 h) and limited impairment in verbal, motor and eye responses as rated on the Glasgow Coma Scale (292). mTBI can result in acute or prolonged physical, cognitive, emotional and behavioral symptoms. With the current means of warfare, exposure to mild blast (mb)TBI by bombs and improvised explosive devices is increasingly common in military populations (180). The physics of the blast wave, number of exposures, and sequelae of events that follow make bTBI uniquely different from other forms of non-bTBI (74). bTBI patients are prone to anxiety, impaired memory, irritability, sleep disturbances and post-traumatic stress disorder (PTSD) (286). Females are more susceptible to developing neuropsychiatric disorders after TBI than males (355).

CRF is widely distributed throughout the central nervous system (232). CRF is classically known for its role in the hypothalamus-pituitary-adrenal (HPA) axis stress response (410). At the level of the paraventricular nucleus of the hypothalamus (PVN), CRF initiates a series of downstream events leading to the eventual release of glucocorticoids (CORT) from the adrenals (157). Extrahypothalamic CRF family members are thought to mediate the behavioral responses to stress by activating G-protein coupled receptors (GPCRs): CRF receptor subtype 1 (CRFR1) and subtype 2 (CRFR2) (333). CRFR1 has widespread distribution throughout the forebrain, midbrain, diencephalon, brainstem and spinal cord (165). CRFR2 expression is more limited, found

in the lateral septal nuclei, bed nucleus of the stria terminalis (BNST), hypothalamus, amygdala, and hippocampus (165). Specifically, the hippocampus, amygdala and prefrontal cortex (PFC) and BNST are key structures involved in the memory, interpretation and processing of a stressor. These structures communicate with each other and also the HPA axis to coordinate behavioral and endocrine homeostasis. Any disruption in anatomical or functional integrity of these structures may result in anxiety.

Psychiatric disorders, such as depression, anxiety, and PTSD, are linked to the brain CRFR system (333). CRFR1 knockout models have decreased anxiety-like behaviors (372; 400). Clinically, hyperstimulated CRFR1 by CRF is present in stress-related disorders (204). Blocking CRFR1 in patients suffering from anxiety and depression reduces psychiatric symptoms (186). Although less known and more complex, CRFR2 is believed to counter CRFR1 by dampening the stress response. There is evidence that CRFR2 mediates anxiolytic behaviors. CRFR2 deficient mice have increased anxiety-like behaviors (33). Genetic studies in patients with psychiatric disorders show mixed correlation between CRFR2 and psychiatric symptoms in women but not men (446). Dependent on the CRFR2 single nucleotide polymorphism, women can have either increased or decreased risk of PTSD (446), introducing sex differences in these receptors.

There are inherent sex differences in the expression and function of the CRFRs. For example, male voles have higher CRFR2 mRNA expression in the BNST (231). There are also sex differences in CRFR1 signaling and trafficking. In the locus coeruleus, a region involved in the physiological stress response, unstressed females have greater G-protein coupling. Stress increased CRFR1 internalization in males, but not females (37).

These sex differences may influence stress sensitivity and the prevalence of psychiatric disorders after TBI.

Six days after mbTBI, male and female mice were tested for anxiety-like behaviors in the elevated plus maze (EPM). Additionally, due to the link of the sexually dimorphic CRFR system to anxiety, we examined the expression of CRFR1 and CRFR2 after mbTBI.

MATERIALS AND METHODS

Animals

Male and female C57BL/6J mice at 7-9 weeks of age were purchased from the Jackson Laboratory (Stock number 000664; Jackson Laboratory, Bay Harbor, ME). Animals were provided food and water *ad libitum* and pair housed in a temperature (22-25 °C), humidity (50%) and light (12:12 light:dark cycle; lights on at 0100 h) controlled facility.

All animal protocols were approved by the Institutional Animal Care and Use Committee at the Uniformed Services University of the Health Sciences, Bethesda, MD and conducted in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

Experimental Design

To examine CRFR gene expression after mbTBI, animals were randomly assigned to one of four groups (males n=13/group; females n=19/group). This study was conducted as a 2 x 2 x 2 factorial design examining the effect of injury (sham v. mbTBI), restraint (no restraint v. restraint) and sex (male v. female). Animals received either sham or mbTBI and were allowed 7-10 days for recovery post injury prior to restraint and subsequent tissue collection.

Mild blast traumatic brain injury (mbTBI)

All procedures were conducted between 0700 – 1100 h (lights on). Animals acclimated to the room for one hour prior to procedures. All animals were anesthetized by isoflurane inhalation (Isoflurane, USP, Baxter, Deerfield, IL). Sham animals received anesthesia only.

Advanced Blast Simulator (ABS)

The ABS (ORA Inc., Fredericksburg, VA) was used to induce mbTBI. An acetate/mesh seal separated the driver chamber from the transition and test chambers (3 acetate sheets, 0.010" thick, 18" x 30", Industrial Division Sales-Grafix, Inc.; 2 mesh sheets, 14 x 10 grids/in², wire diameter 0.25", New York Wire). Increased air pressure in the driver section ruptured the seal allowing the blast wave to travel to the test chamber via the transition chamber. In the test chamber, anesthetized animals were mounted in a prone position to receive the blast wave nose-on. Blast pressure was measured with a pencil probe pressure gauge (Model Number: 137B23A; PCB Piezotronics, Depew, NY) located at the level of the animal.

Behavioral tests

All behavioral procedures were conducted between 0700 – 1100 h (lights on).

Righting reflex

Time to right was recorded immediately following anesthetic removal on day of sham/injury procedures. Animals were removed from anesthesia (sham) or test chamber (injured) and placed dorsally in the home cage. Righting reflex was measured as the time until the forepaws touched the ground.

Open field

A cohort of animals was tested in the open field assay 6 days after mbTBI. The open field chamber was 40 cm x 40 cm (Stoelting Company, Wood Dale, IL) with opaque walls. Animals were individually placed in the center of the apparatus and allowed to freely move for 30 min. Behaviors were video recorded and tracked with ANY-maze software (Stoelting Company).

Elevated plus maze (EPM)

A cohort of animals was tested in the EPM 6 days after mbTBI. The EPM consisted of 2 open and 2 closed arms (5 cm x 35 cm). The closed arms were enclosed with dark plastic walls (16 cm high). Animals were placed in the center square (5 cm) at the intersection of the open and closed arms. Mice were allowed to freely explore for 5 min during which behavior was video recorded and tracked with ANY-maze software (Stoelting Company).

Restraint

Seven-10 days post mbTBI animals were individually restrained in clear Plexiglas tubes (3.81 x 10.16 cm; Stoelting Company) between 0700 – 1100 (lights on). Animals were restrained for 20 min. Ten min after the end of restraint animals were deeply anesthetized by carbon dioxide over-inhalation and rapidly decapitated.

Dissection of brain nuclei

Immediately after decapitation, brains were removed and flash frozen in 2-methylbutane (-40 °C) and stored at -80 °C until use. The following regions were isolated from 250 µm brain sections: prelimbic cortex (PrL; 1.98 to 1.34 m), infralimbic cortex (IL; 1.98 to 1.34 mm), anterior (a)BNST (0.62 to 0.02 mm), posterior (p)BNST (-0.10 to -0.58 mm), amygdala (-0.70 to -2.30 mm), dorsal hippocampus (-1.34 to -2.80 mm) and ventral hippocampus (-2.70 to -3.80 mm). All regions were isolated using a 1 mm biopsy punch except for the PVN, in which a 0.5 mm biopsy punch was used.

Gene expression

RNA extraction

Punched tissue was immediately placed in 500 µl RiboZol (Thermo Fisher Scientific, Waltham, MA) and homogenized. Brain RNA was extracted using the Direct-zol RNA MiniPrep Kit (Zymo Research, Tustin, CA) following manufacturer's protocol. The RNA concentration and quality was determined using a NanoDrop Lite spectrometer (Thermo Fisher Scientific). Purified RNA was used for cDNA synthesis using the Maxima Reverse Transcriptase kit (Thermo Scientific) following manufacturer's protocol.

Droplet Digital polymerase chain reaction (ddPCR)

ddPCR reaction mixes were prepared as follows: 20 µl 1X EvaGreen supermix (Bio-Rad, Pleasanton, CA), 200nM forward and reverse primers and 2 ng cDNA template. The following primer sequences were used: CRFR1 (for) 5'-AGGATCAGCAGTGTGAGAGC-3'; CRFR1 (rev) 5'-AGCCATTGTTTGTGGTGTGTAG-3'; CRFR2 (for) 5'-CCCCTGTGGACACTTTTGGA-3'; CRFR2 (rev) 5'-CTCTGTAGGCATTCCGGGTC-3'. The Automated Droplet Generator, the QX200 Droplet Reader and QuantaSoft software (Bio-Rad) were used for droplet generation, reading and analysis, respectively. Manufacturer's protocols were followed. Thermal cycling conditions were: 5 min enzyme activation at 95 °C, followed by 40 cycles of 30 sec denaturation at 95 °C and 1 min annealing/extension at 60 °C, then signal stabilization for 5 min at 4 °C followed by 5 min at 90 °C. Analysis was conducted on number of copies per ng of cDNA template per reaction.

Statistical analysis

Data were analyzed using IBM SPSS Statistics software (Armonk, NY). Graphs were generated with GraphPad Prism 7 (La Jolla, CA). Behavioral data (righting reflex, open field and EPM) were analyzed using an unpaired t-test in males and females, separately. mRNA expression data was analyzed using a three-way analysis of variance (ANOVA), followed by a pairwise comparison when a significant effect was present. Sex difference data is not shown on graphs, but described in results and figure legends. In cases where there was no effect of sex on mRNA expression, data was analyzed by separate two-way ANOVAs in males and females. This was followed by a Fisher's least significant difference post hoc analysis when an effect was present. $p < 0.05$ was considered significant.

RESULTS

Effect of mbTBI on behavior

mbTBI increased time to right.

mbTBI increased time to right in males and females ($p < 0.05$) (data not shown). mbTBI increased time to right by 128% in males (sham = 50.0 ± 2.5 sec; mbTBI = 64.1 ± 2.1 sec) and 138% in females (sham = 46.0 ± 1.4 sec; mbTBI = 63.6 ± 1.3 sec).

mbTBI had no effect on open field behaviors.

mbTBI had no effect on any parameter in the open field assay including: distance traveled, rearing episodes, mobile, immobile, center, or peripheral times (Table 1).

mbTBI increased anxiety-like behaviors in the EPM.

Males and females had increased anxiety-like behaviors 6 days after mbTBI (Figure 1). Males exposed to mbTBI had decreased open arm time and increased closed arm time compared to sham ($p < 0.05$). Females exposed to mbTBI had a trending decrease in open arm time ($p = 0.058$) and increased closed arm time compared to sham ($p < 0.05$).

mbTBI had no effect of CRFR1 mRNA expression in males or females.

There was no effect on CRFR1 mRNA expression in any limbic structure including: PrL, IL, aBNST, pBNST, amygdala, PVN, dorsal hippocampus or ventral hippocampus (Table 2; Table 3).

In males, mbTBI increased basal CRFR2 gene expression in the ventral hippocampus.

A three-way ANOVA showed an interaction of injury and sex and an interaction of restraint and sex ($p < 0.05$; Figure 2G). In males, mbTBI increased baseline CRFR2 mRNA expression in the ventral hippocampus ($p < 0.05$). In males exposed to mbTBI, restraint decreased CRFR2 mRNA expression ($p < 0.05$). In sham females, restraint increased CRFR2 mRNA expression ($p < 0.05$).

In males, mbTBI decreased restraint-induced CRFR2 gene expression in the aBNST and amygdala.

aBNST. A three-way ANOVA showed an interaction of injury and restraint ($p < 0.05$) and an interaction of injury, restraint and sex ($p < 0.05$; Figure 2C). In sham males, restraint increased CRFR2 mRNA expression ($p < 0.05$). mbTBI ablated this restraint-induced increase ($p < 0.05$). Additionally, in males exposed to mbTBI, restraint decreased CRFR2 mRNA expression ($p < 0.05$). There was no effect of mbTBI on CRFR2 mRNA expression in females.

Amygdala. A two-way ANOVA showed an effect of injury in males ($p < 0.05$; Figure 2F). mbTBI decreased restraint-induced CRFR2 mRNA expression in the amygdala compared to sham males ($p < 0.05$). There was no effect of injury or restraint in females.

In females, mbTBI decreased restraint-induced CRFR2 gene expression in the dorsal hippocampus.

A three-way ANOVA showed an interaction of injury and restraint ($p < 0.05$) and a trending interaction of injury and sex ($p = 0.05$; Figure 2H) in the dorsal hippocampus. In

sham females, restraint increased CRFR2 mRNA expression in the dorsal hippocampus ($p < 0.05$). mbTBI blunted this restraint-induced increase ($p < 0.05$). There was no effect of injury or restraint on CRFR2 mRNA expression in males.

mbTBI had no effect on CRFR2 gene expression in any other limbic structure.

There was no effect of injury or restraint on CRFR2 mRNA expression in the PrL, IL, pBNST and PVN in males or females (Figure 2A, B, D, E). Table 3 depicts a summary of the CRFR2 gene expression data, comparing differences between males and females in response to mbTBI.

There were inherent sex differences in CRFR gene expression.

aBNST. Females had higher CRFR1 mRNA expression in the aBNST than males ($p < 0.05$; Table 2). After mbTBI, females had higher baseline CRFR1 mRNA expression ($p < 0.05$). Males had higher restraint-induced CRFR2 mRNA expression in the aBNST than females ($p < 0.05$; Figure 2A).

Amygdala. Females had higher CRFR2 mRNA expression in the amygdala than males ($p < 0.05$; Figure 2F). After mbTBI, females had increased baseline CRFR2 mRNA expression ($p < 0.05$).

Dorsal hippocampus. In general, females had greater CRFR1 mRNA expression in the dorsal hippocampus than males ($p < 0.05$; Table 2). A pairwise comparison revealed no differences among groups. Males had higher expression of CRFR2 mRNA expression in the dorsal hippocampus than females ($p < 0.05$; Figure 2H).

Ventral hippocampus. There was an effect of sex on CRFR2 mRNA expression in the ventral hippocampus ($p < 0.05$; Figure 2G). In sham conditions, females had higher

restraint-induced CRFR2 mRNA expression than males ($p<0.05$). In animals exposed to mbTBI, males had higher baseline CRFR2 mRNA expression ($p<0.05$).

DISCUSSION

The results of the present study show that mbTBI increased anxiety-like behaviors in male and female mice. This is consistent with clinical observations (206) and previous studies in rodent TBI models (71; 122). Most of the previous studies in experimental TBI models were conducted in males only. To account for inherent sex differences, we extended these findings to females. Women are almost two times more likely than men to develop anxiety disorders (355). After mTBI, women have poorer outcomes and more symptoms of post concussive syndrome, including headaches, impaired cognitive abilities, and memory deficits (355). In rodents, females have higher baseline and stress-induced glucocorticoid secretion than males (157), suggesting sex differences in HPA axis reactivity. In addition to anxiety-like behaviors, we also showed sex dependent alterations in gene expression of CRFR2, but not CRFR1, after mbTBI. It is hypothesized that the limbic CRFR system may be associated with anxiety-like behaviors (165), making this network a potential target for TBI-induced pathophysiology.

We show that mbTBI altered CRFR2 gene expression, but surprisingly had no effect on CRFR1 gene expression. The role of CRFR1 in anxiety is clearer than that of CRFR2. CRFR1 activation induced anxiety-like phenotypes (165). CRFR1 antagonists decreased anxiety-like behaviors (35). Clinically, CRFR1 hyperactivity is thought to be associated with increased risk for psychiatric disorders in patients (204). CRFR1 antagonists have entered into clinical trials but have had mixed success in treatment of psychiatric disorders with adverse side effects (464). With the current breadth of experimental and clinical studies, we anticipated that mbTBI would alter CRFR1 gene expression. However, mbTBI had no effect on CRFR1 gene expression, but rather altered

CRFR2. There is evidence that CRFR2 may be involved in anxiety-like phenotypes. There are two hypotheses on how this can happen. First, CRFR2 may counter the activity of CRFR1 to reduce anxiety-like behaviors (165). Therefore, the down regulation we observed may reduce the ability of CRFR2 to compensate for the anxiogenic function of CRFR1, increasing anxiety-like behaviors after mbTBI. This hypothesis coincides with previous studies showing decreased CRFR2 mRNA expression in fear susceptible animals (124). CRFR2 knockout mice have increased anxiety-like behaviors (32). Alternatively CRFR2 may act independently to mediate anxiety. For example, CRFR2 knockdown in the posterior medial (pm)BNST decreased fear susceptibility in PTSD-like mouse model (220). Therefore, the function of CRFR2 in stress and behavior may be region and cell type specific. Although limited studies have examined neuron specific knockdown of CRFR2, we can extrapolate from CRFR1 data that this may hold true for CRFR2 as well. For example, conditional CRFR1 knockout in forebrain glutamatergic neurons decreased anxiety-like behaviors, while CRFR1 knockout in forebrain GABAergic neurons had no effect on anxiety (327). CRFR1 knockout in dopaminergic midbrain neurons increased anxiety-like behaviors (327). The role of CRFR2 throughout the limbic structures is not characterized. Our study, however, demonstrated that CRFR2 gene expression is differentially altered in various anxiety-relevant limbic structures in response to mbTBI.

We showed that mbTBI affected CRFR2 gene expression in different limbic structures of males and females. In males, mbTBI increased basal CRFR2 in the ventral hippocampus and decreased restraint-induced CRFR2 in the aBNST and amygdala. Conversely, in females mbTBI decreased restraint-induced CRFR2 expression in the

dorsal hippocampus. This suggests that there are inherent sex differences in pathways used to cope with stressors after mbTBI. Symptoms of anxiety disorders are linked to an imbalance of activity of these anatomically and functionally interconnected limbic structures.

In the ventral hippocampus, mbTBI increased basal CRFR2 gene expression in males. The ventral hippocampus mediates emotional processing, social and anxiety-related behaviors. To regulate behavior, glutamatergic projections from the ventral hippocampus are sent to numerous structures including the nucleus accumbens, PFC, amygdala and BNST (24; 30; 177). Previous work showed that activation of ventral hippocampus projections to the nucleus accumbens increased susceptibility to depressive-like behaviors in mice (30) while inhibition of the ventral hippocampus-PFC pathway decreased anxiety-like behaviors (300). To our knowledge, there are no studies specifically examining the relationship between ventral hippocampal CRFR2 populations and anxiety. However, optogenetic activation of CRFR2 in the functionally connected lateral septum, increased anxiety behaviors. Administration of CRFR2 agonists also increased anxiety-like behaviors (167). In our model, increased CRFR2 in the ventral hippocampus may result in increased excitatory output to target structures and increased anxiety-like behaviors.

In the amygdala, mbTBI decreased restraint-induced CRFR2 gene expression in males. The amygdala is defined by its well-established role in fear and anxiety processing. The principle output from the amygdala stems from inhibitory neurons in the central and medial nuclei (CeA and MeA, respectively) (190). Activation of GABA receptors by agonists decreased anxiety, while receptor antagonists increased fear and

anxiety behaviors (291). Glutamatergic projections from the basolateral amygdala regulate the inhibitory output from the CeA. Overexpression of glutamate receptors in the amygdala increased anxiety and fear behaviors (425). Ligand stimulation of CRFR2 in the amygdala increased anxiety-like behaviors (344). Acute and chronic noise stress decreased CRFR2 in the amygdala (125). CRFR2 immunoreactivity is found on both GABAergic and glutamatergic neurons in the amygdala (165), however no studies have been conducted examining the function of CRFR2 on these specific neuronal populations. In our study, altered function or disrupted signaling of the amygdala by mbTBI, as shown by decreased CRFR2 gene expression, may increase anxiety-like behaviors in males.

In sham males, restraint increased CRFR2 gene expression in the aBNST. mbTBI ablated this restraint-induced increase. The BNST is a relay structure involved in the autonomic, endocrine stress responses, as well as, anxiety behaviors. Specifically, activation of the aBNST decreased anxiety-like behaviors (208). The primary output BNST is GABAergic (221). Additionally, CRFR2 immunoreactivity is expressed on GABAergic neurons in the BNST (165). Overexpression of CRFR2 in the BNST decreased fear behaviors (124). CRFR2 knockdown in the BNST increased arousal and anxiety (220; 221). In the BNST, CRFR2 overexpression attenuated fear-like behaviors (124) and optogenetic activation of CRFR2 decreased anxiety-like behaviors (164). In our current study, the mbTBI-induced down-regulation of CRFR2 in the aBNST of males may contribute directly to the observed increase in anxiety behaviors.

The BNST also has dense projections to midbrain and brainstem structures regulating multiple facets of the stress response. A major projection of the BNST and also the amygdala is to the locus coeruleus (LC) (221), a region involved in the

physiological responses to anxiety. CRFR2 is expressed on norepinephrine (NE) neurons in the LC (165). NE deficiency is associated with anxiety. CRFR2 agonists up-regulated NE transporter expression (183). Activation of NE neurons in the LC is involved in stress-induced anxiety (249). Therefore, in our study, decreased output of the aBNST or amygdala may indirectly result in anxiety-like behaviors by decreasing the efficiency of the LC-NE system.

In contrast to males, in females, mbTBI decreased CRFR2 gene expression in the dorsal hippocampus. The dorsal hippocampus is highly involved in spatial memory, contextual fear learning and anxiety behaviors (205; 457). To mediate these behaviors, the dorsal hippocampus projects to the medial PFC, the lateral septum and the BNST. CRFR2 is expressed on glutamatergic neurons, which are the primary output of the dorsal hippocampus (165). In males, CRFR2 agonists and antagonists had no effect of glutamate release (27). Additionally, blocking hippocampal CRFR2 in males had no effect of freezing behaviors (322). To date, most studies have focused on males, however we are introducing a female-specific effect of mbTBI on CRFR2 gene expression. We show that within the dorsal hippocampus, sham females had higher CRFR1 gene expression than males, while males had higher CRFR2 gene expression than females. Assuming the role of CRFR2 is to counter CRFR1, a greater CRFR1:CRFR2 ratio in the dorsal hippocampus of females may increase female susceptibility to anxiety.

In addition to the studies after mbTBI, we observed inherent sex differences in receptor expression in sham animals. Sham females had higher CRFR1 mRNA expression in the aBNST and dorsal hippocampus, while males had higher CRFR2 mRNA expression in these regions. Sham females had higher CRFR2 mRNA expression

in the amygdala. To date, no sex differences in rodent receptor distribution have been reported; however, in voles, males have higher mRNA expression of CRFR2 than females in the BNST (231). The sex differences we observed may be attributed to the presence of circulating gonadal hormones. CRFRs have gonadal hormone response elements located on the promoter region (69; 435). Androgen increased CRFR2 mRNA expression and binding capacity in the lateral septum, hypothalamus and hippocampus of males (435). The role of estrogen and progesterone on CRFR2 mRNA expression in the brain has yet to be determined. Interestingly, there are sex differences in CRFR1 signaling and trafficking. Females have increased coupling of the G-protein subunit to CRFR1 and a lack of receptor internalization following acute swim stress (36; 37). We observed no differences in gene expression based on estrous cycle (data not shown). These data suggest an organizational effect on the CRFR system, where there are inherent sex differences resulting from neural differentiation. Our current data do not suggest an activational role of steroid hormones. The organizational effect may later determine the individual mechanisms that males and females utilize to cope with mbTBI.

Although not examined in the current study, members of the CRF-related peptide family, including CRF, urocortin 1 (UCN1), UCN2 and UCN3 regulate anxiety-like behaviors (165; 275; 306). Intracerebroventricular (ICV) administration of the CRFR1 agonists, CRF and UCN1, produce anxiogenic effects (212). The roles of the CRFR2 agonists, UCN2 and UCN3 are more ambiguous. Previous studies have shown that ICV administration of UCN2 can increase (306) or decrease (408) anxiety-like behaviors. Additionally, mice that overexpress UCN3 display increased anxiety behaviors (217). To

fully understand the impact of mbTBI on the CRFR system, future studies are needed to explore tissue specific expression and binding profiles of these ligands.

Although males and females developed anxiety-like phenotypes after mbTBI, they have different anatomical patterns of altered CRFR2 gene expression. This suggested that region specific gene alterations after mbTBI may contribute to sex dependent sensitivities to anxiety disorders. In addition to the behavioral impact, mbTBI may also affect different physiological stress responses, such as the autonomic and endocrine systems. In summary, although we showed that males and females utilize different pathways to cope with mbTBI, CRFR2 may provide a potentially universal target to treat anxiety symptoms after injury.

	Male		Female	
	<i>Sham</i>	<i>Blast</i>	<i>Sham</i>	<i>Blast</i>
Distance (m)	75.63 ± 5.45	82.73 ± 11.87	95.66 ± 4.12	96.72 ± 6.36
Mobile time (sec)	1259.13 ± 39.59	1277.65 ± 62.31	1375.98 ± 63.70	1407.63 ± 92.46
Immobile time (sec)	540.95 ± 39.57	522.35 ± 62.31	424.03 ± 63.70	392.38 ± 92.46
Center time (sec)	184.10 ± 15.68	158.38 ± 18.67	128.63 ± 19.28	149.23 ± 54.25
Peripheral time (sec)	1476.05 ± 40.64	1528.23 ± 38.49	1560.88 ± 28.46	1543.98 ± 82.95
Rearing episodes (no)	14.25 ± 3.20	19.50 ± 7.79	6.25 ± 1.38	14.25 ± 7.57

Table 1. Open field activity 6 days after mbTBI.

There was no effect of injury on any behaviors in male or female mice.

CRFR1								
	Male				Female			
Regions	Sham		Blast		Sham		Blast	
	No Res	Res	No Res	Res	No Res	Res	No Res	Res
PrL	704.97 ± 234.30	603.07 ± 126.26	598.14 ± 87.12	544.10 ± 41.94	515.60 ± 176.27	493.50 ± 73.64	798.38 ± 44.83	621.67 ± 146.64
IL	440.75 ± 140.59	273.80 ± 21.96	272.10 ± 30.20	332.38 ± 16.59	334.25 ± 31.42	466.40 ± 96.82	337.75 ± 88.93	412.10 ± 109.67
aBNST	98.79 ± 21.19	100.83 ± 20.25	108.722 ± 17.43	127.67 ± 25.34	150.50 ± 24.74	160.31 ± 30.01	218.55 ± 40.48	164.75 ± 33.82
pBNST	142.28 ± 21.31	182.68 ± 31.28	96.56 ±14.81	137.14 ± 30.41	149.89 ± 26.09	172.82 ± 31.96	183.46 ± 42.59	175.72 ± 39.52
PVN	71.75 ± 5.13	90.88 ± 1.32	106.00 ± 5.70	94.56 ± 5.22	88.90 ± 3.20	104.06 ± 3.56	123.55 ± 6.15	118.07 ± 4.36
AMG	277.87 ± 47.87	251.68 ± 54.03	247.82 ± 44.24	224.56 ± 40.34	279.73 ± 39.58	323.06 ± 39.89	290.96 ± 31.64	317.85 ± 43.10
dHIPP	140.14 ± 27.97	155.88 ± 25.70	166.77 ± 27.76	161.17 ± 20.54	202.42 ± 18.12	176.41 ± 32.26	201.32 ± 45.92	242.22 ± 32.11
vHIPP	199.06 ± 29.38	189.50 ± 37.11	207.19 ± 30.24	172.56 ± 24.63	248.50 ± 46.78	185.06 ± 17.60	218.77 ± 27.55	232.25 ± 30.22

Table 2. Neither mbTBI nor restraint had an effect on CRFR1 expression in any limbic structure.

Females had higher CRFR1 expression in the aBNST and dorsal hippocampus (dHIPP) than males. (No Res = No Restraint; Res = Restraint)

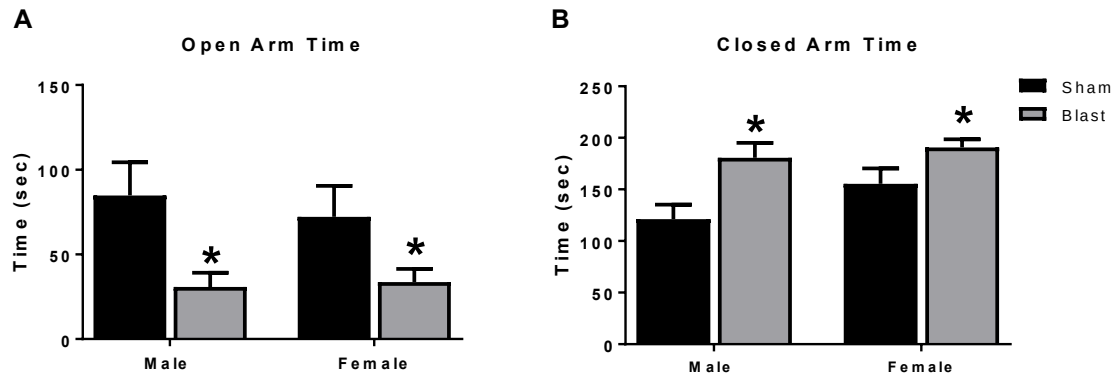


Figure 1. Effect of mbTBI on anxiety-like behaviors in the elevated plus maze. mbTBI increased anxiety-like behaviors in males and females 6 days post injury. A) mbTBI decreased open arm time and B) increased closed arm time in males and females. * $p < 0.05$.

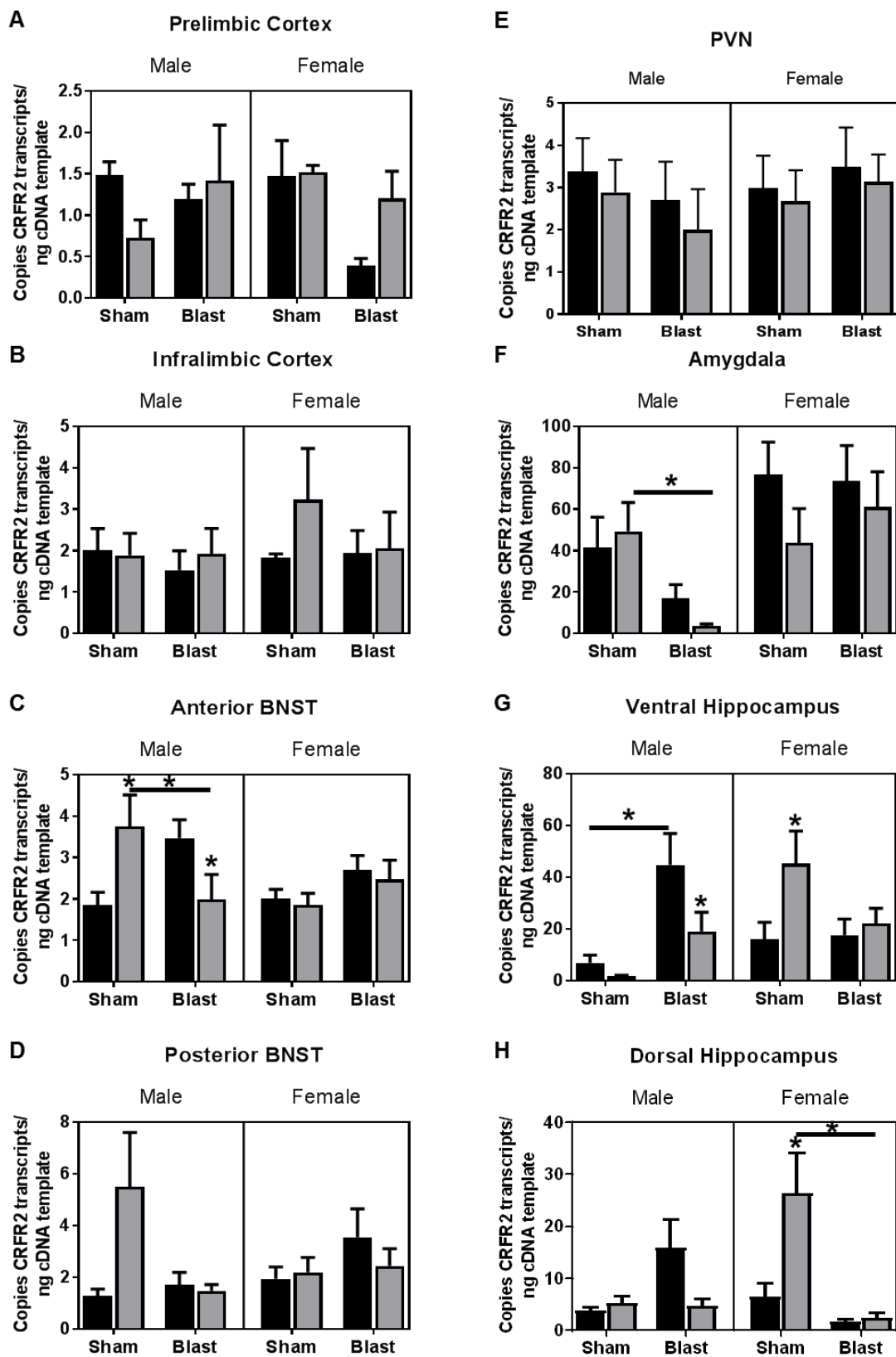


Figure 2. Effect of mbTBI on CRFR2 mRNA expression in the limbic structures in males and females.

A) mbTBI had no effect on CRFR2 mRNA expression in the A) PrL or B) IL. C) In the aBNST, restraint increased CRFR2 mRNA expression in males. mbTBI blunted this effect. mbTBI had no effect on CRFR2 mRNA expression in the aBNST of females. D) There was no effect of injury or restraint on CRFR2 mRNA expression in the pBNST of males or females. E) mbTBI had no effect on mRNA expression in the PVN F) In males, mbTBI decreased restraint-induced CRFR2 mRNA expression in the amygdala. G) In sham females, restraint increased CRFR2 mRNA expression. mbTBI decreased restraint-induced CRFR2 mRNA expression in the dorsal hippocampus. H) In the ventral hippocampus, mbTBI increased baseline CRFR2 mRNA expression in males. Males exposed to mbTBI had lower CRFR2 mRNA expression after restraint. In sham females, restraint increased CRFR2 mRNA expression in the ventral hippocampus. * $p < 0.05$. Bars represent mean $\pm$ SEM. Black bars = no restraint; Gray bars = restraint

CHAPTER 5: Summary

TBI increases the risk of developing subsequent psychiatric disorders; however, the link connecting these is unknown. Our studies demonstrate that the limbic-HPA axis is prone to TBI-induced dysregulation. Moreover, we elucidate that disturbance of this system occurs in a sex dependent manner. The limbic-HPA circuitry is critical for homeostasis and adaptation to stress (157; 173; 190; 256). Physiological alterations of this system are present in psychiatric disorders (35; 38; 166). Increased and/or decreased adrenal hormones are observed in depression, anxiety, and PTSD. Therefore, dysregulation of the HPA axis may bridge the gap between TBI and psychiatric disorders.

Using the ABS as a model for mbTBI, we found that injury resulted in sex dependent HPA axis dysregulation. Restraint, 7-10 days after mbTBI, increased CORT secretion in males, but reduced CORT secretion in females. To determine where in the circuitry this dysregulation was stemming from we examined peripheral and central components of the HPA axis. Peripheral HPA axis genes were not altered. mbTBI had no effect on CRFR1 or POMC gene expression in the pituitary. Additionally, mbTBI had no effect on 11 β -HSD, 11 β -OHase or Mc2R gene expression in the adrenal. Central feedback genes were also not altered. mbTBI had no effect on MR or GR gene expression in the PrL, IL, aBNST, pBNST, amygdala, PVN, dorsal or ventral hippocampus. We observed an effect of mbTBI on CRF activation in the PVN. mbTBI decreased restraint-induced CRF activation in males, but enhanced restraint-induced CRF activation in females. Using retrograde tracing techniques, we show that mbTBI decreased the percentage of activated non-neuroendocrine (pre-autonomic) projecting CRF neurons in

the PVN of females only. This is a novel finding in that we have identified a sex-dependent autonomic link to mbTBI-induced dysregulation in females. Our studies point to involvement of the PVN-brainstem connections in coping with mbTBI-induced mbTBI pathologies. The impact of mbTBI on the PVN-brainstem circuitry may be an underlying result of HPA dysregulation. In females, although mbTBI decreased restraint-induced CORT secretion (Chapter 3, Figure 3), it increased restraint-induced CRF activation (Chapter 3, Figure 6). Decreased autonomic output from the PVN may act to compensate for the increased neural activity observed in CRF neurons after mbTBI. However, this does not rule out that mbTBI may directly impact the axonal projections to autonomic brainstem structures. More work is needed to understand how mbTBI disrupts autonomic pathways. Additionally, while we introduce this idea of autonomic dysregulation, future work will need to address the underlying neurocircuitry involved.

CRF neurons are not only located in the PVN, but throughout relevant limbic structures (55; 275; 297). In addition, actions of CRF are mediated by CRFR1 and CRFR2 (35; 165). Anatomical and functional studies of the CRF system suggest its importance in behavioral, endocrine, and autonomic responses to stress (11; 12; 35; 96; 124; 204; 213; 315). Therefore, we sought to examine the effect of mbTBI on the limbic CRF system as a mechanism of HPA axis dysregulation. Previous studies have shown that CRFR1 increases anxiety-like behaviors and HPA axis reactivity (35; 186; 370; 400). Interestingly, we demonstrated that mbTBI had no effect on CRFR1 gene expression. In our model, mbTBI decreased restraint-induced CRFR2 in the amygdala and aBNST of males and the dorsal hippocampus of females. Previous studies have shown that CRFR2 mediates anxiety-like behaviors and decreases HPA axis reactivity (32; 124; 164; 165;

220; 446). However, the role of the CRFR2 receptor is ambiguous and may be neuron, region, and sex specific (191). In congruence with CRFR2 reduction, mbTBI increased anxiety-like behaviors in males and females. We propose that CRFR2 may be involved in the pathophysiology of TBI-induced HPA axis dysregulation and subsequent development of psychiatric disorders.

In Aim 1, we discovered that mbTBI dysregulated the HPA axis. In a functioning HPA axis, increased CRF activation would reflect through increased CORT secretion. In our study, we found that after mbTBI, this was not true. Males exposed to mbTBI had increased restraint-induced CORT secretion, but decreased restraint-induced CRF activation. On the other hand, females exposed to mbTBI had decreased restraint-induced CORT secretion and enhanced restraint-induced CRF activation. We found no evidence of dysregulation within components of the HPA axis. However, we observed mbTBI-induced disruption in limbic structures that project to and modulate the HPA axis.

After mbTBI, CRFR2 gene expression in limbic structures is altered. Figure 1 depicts the communication between the limbic structures and the PVN. GABAergic neurons in the BNST receive input from the limbic structures. Inhibitory projections are relayed from the BNST to the PVN, inhibiting the HPA axis. The amygdala primarily sends inhibitory projections to the BNST, reducing activation of the BNST. This projection disinhibits (activates) the HPA axis. On the other hand, the PFC and hippocampus primarily send excitatory projections to the BNST, activating the BNST. Activation of the BNST will inhibit the HPA axis. Therefore, activation of the PFC and hippocampus inhibits the PVN.

In males, we showed that mbTBI decreased CRFR2 gene expression in the amygdala. This may suggest decreased function of the amygdala as a result of mbTBI. Activation of the amygdala would disinhibit the PVN by relayed projections through GABAergic neurons in the BNST. Decreased activation of the amygdala may decrease the inhibition on the BNST, activating the BNST. Activation of the BNST would increase the inhibitory tone of the PVN. In males, the decreased CRF activation (Aim 1) may be attributed to increased inhibition on the HPA axis after mbTBI-induced amygdala dysfunction. The hypothesized alteration in circuitry is depicted in Figure 2.

In males, mbTBI also decreased CRFR2 gene expression in the aBNST. The primary projections from the BNST to the PVN are GABAergic. Therefore, activation of the BNST inhibits the HPA axis. Decreased function of the BNST may decrease the inhibitory tone on the HPA axis, activating the stress response. This is reflected in males by the increased restraint-induced CORT observed in Aim 1. Figure 3 illustrates the potential alterations in BNST pathways after mbTBI. By examining the CRFR2 system in relevant limbic structures, we are able to provide a mechanism by which the HPA axis may be dysregulated after mbTBI in males.

In females, mbTBI decreased CRFR2 gene expression in the dorsal hippocampus. The output of the hippocampus is primarily glutamatergic. The majority of the projections from the hippocampus are relayed through the GABAergic neurons in the BNST to ultimately inhibit the HPA axis. mbTBI-induced decreased function in the hippocampus may decrease the excitatory input to the BNST. This would ultimately result in decreased inhibition of the PVN. In reference to Aim 1, decreased inhibition of the PVN may result in increased restraint-induced CRF activation in females after

mbTBI. Therefore, disruption of the hippocampus-BNST-PVN circuitry may explain one component of the HPA axis dysregulation after mbTBI in females. Figure 4 illustrates the potential means by which mbTBI alters hippocampal projections, disrupting the HPA axis. The decreased restraint-induced CORT secretion after mbTBI (Aim 1) may be attributed to the autonomic system response to TBI; however, this needs further investigation.

In summary, we show that mbTBI differentially dysregulates not only the HPA axis, but also the limbic structures that modulate stress reactivity. Figure 5 illustrates the proposed mbTBI-induced disruption of the limbic-HPA axis in males and females.

Future work will need to further address the effect of mbTBI on limbic structure activation and projection integrity. Immunohistological studies in the PFC, amygdala, BNST, PVN and hippocampus will be needed to determine overall neuronal activation and cell death after mbTBI. Additionally, co-examination of these markers with predominant neuropeptides (GABA, glutamate, dopamine, serotonin) will allow the investigation of distinct neuronal populations affected by mbTBI. Tract tracing studies are also needed to assess axonal integrity after mbTBI. This will help to identify limbic projections to neuroendocrine and pre-autonomic CRF neurons in the PVN to further elucidate the mechanism of the proposed model. These experiments will extend beyond the described limbic-HPA axis to incorporate brainstem structures involved in autonomic and emotional responses to stress.

A thorough analysis of CRFR2 expression and function after mbTBI will need to be completed. Our current data suggest a CRFR2-dependent mechanism HPA dysregulation and associated anxiety-like behaviors after mbTBI. Previous studies have

shown that animals expressing PTSD-like symptoms have downregulation of CRFR2 in the pBNST. Overexpression of CRFR2 reduces the PTSD susceptibility (124). Activation of CRFR2 in the pBNST decreased anxiety, promoted stress recovery and decreased trauma-induced PTSD-like symptoms (164). Additionally, CRFR2 deficient mice have increased restraint-induced CORT and ACTH (32), and a lack of increase in cardiovascular function after UCN administration (92). This suggests that CRFR2 in the BNST and the associated ligands may play a role in mediating TBI-induced behaviors. Currently, there are no CRFR2 agonists or antagonists commercially available or in clinical trials. In experimental settings, the selective CRFR2 agonists, UCN2 and UCN3, have yielded mixed dose- and sex- dependent results with no effect, stimulatory or inhibitory actions on the HPA axis (28; 75; 189; 225; 226). Many studies report decreased anxiety- and depressive-like behaviors associated with UCN2 and UCN3 (29; 35; 75). Targeting CRFR2 populations with the specific ligands UCN2 and UCN3 may reduce behavioral symptoms observed after mbTBI. To fully elucidate the effect of these agonists, region specific screening needs to be conducted.

It is evident that there is a link between TBI and psychiatric disorders. The mechanism bridging the connection between mbTBI and these disorders is unknown. Proper coordination of the HPA is necessary for homeostasis and well-being. Disruption at any level of the HPA axis, including altered hormone release or CRF neuronal populations, is linked to psychiatric disorders. Therefore, due to the involvement of the limbic-HPA axis in psychiatric disorders, this circuitry provides a promising target to address the gap. Our current studies implicate that dysregulation of the HPA axis and associated limbic CRFR2 system may contribute to anxiety-like behaviors after mbTBI.

These experiments suggest that there may be sex dependent anatomical, and presumably functional, differences in response to mbTBI that may warrant different strategies to cope with the injury-induced stress dysregulation.

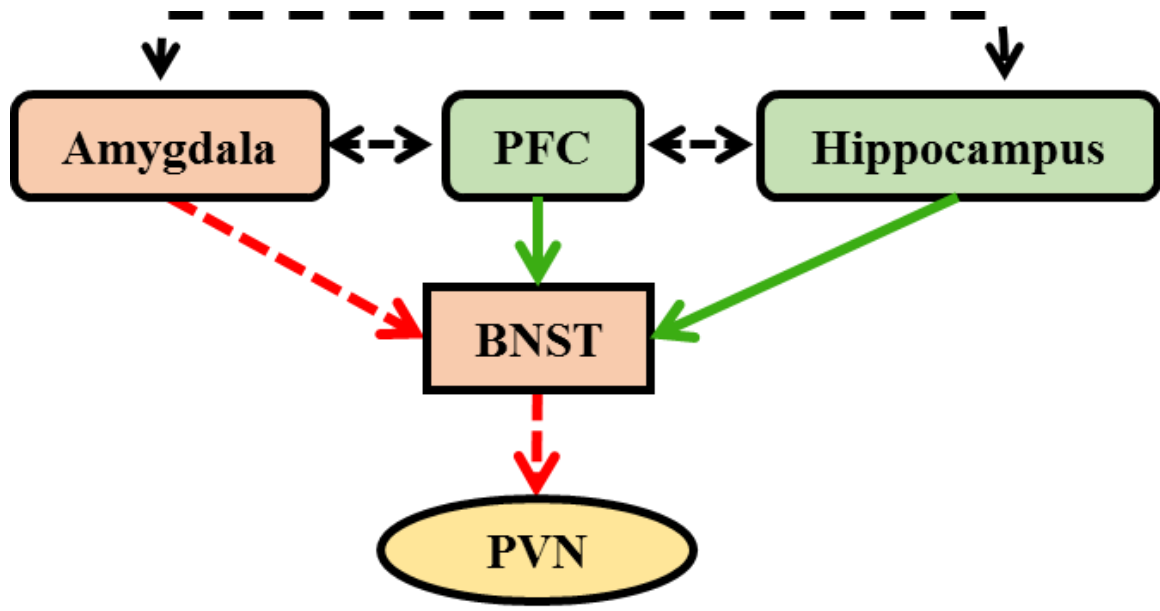
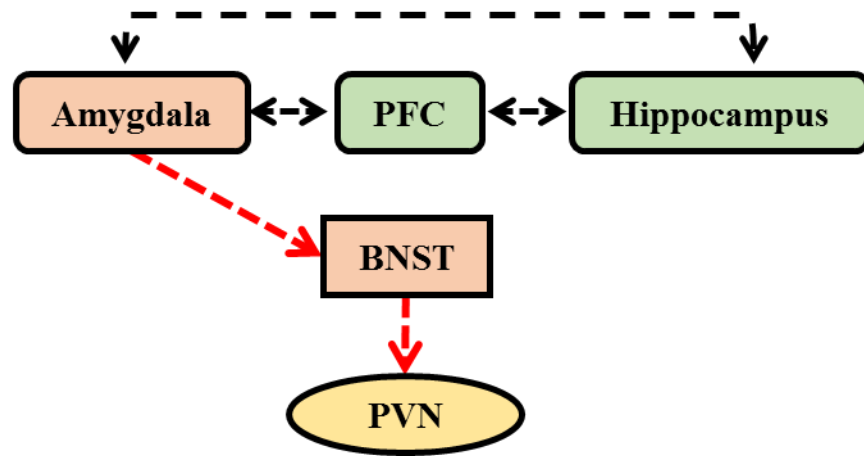


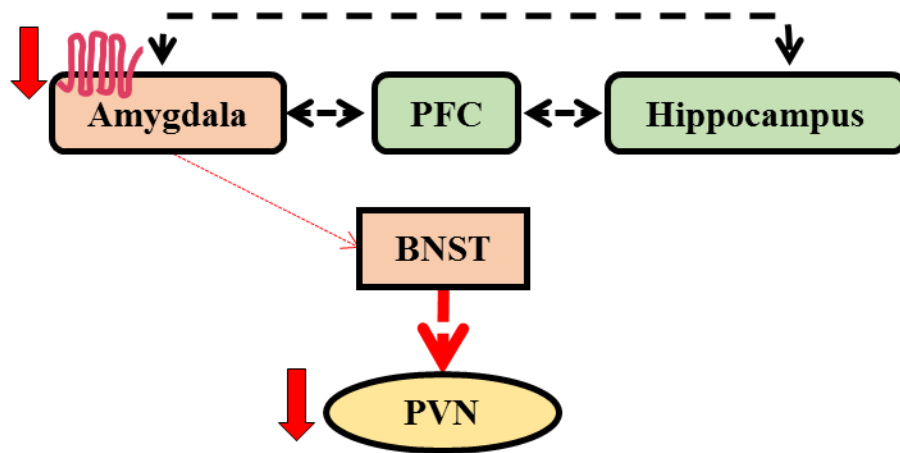
Figure 1. Illustration of limbic-PVN circuitry.

The amygdala, PFC and hippocampus communicate with one another. Additionally, these structures modulate the PVN by relayed projections through the BNST. The majority of the projections from the amygdala are GABAergic, while the majority of projections from the PFC and hippocampus and glutamatergic. These projections converge on GABAergic neurons in the BNST. The PVN is under inhibitory tone from the BNST. (Red dashed arrow = GABAergic/inhibitory projections; Green solid arrow = glutamatergic/excitatory projections)

A



B



Male CRF Activation

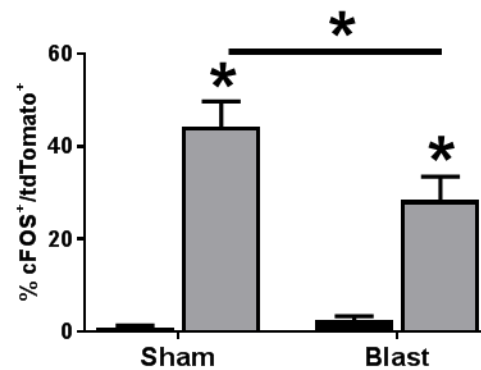
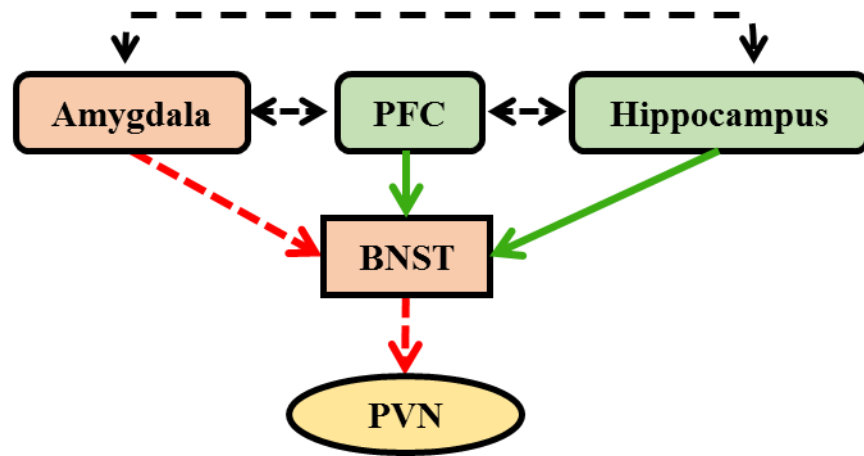


Figure 2. In males, mbTBI decreased CRFR2 gene expression in the amygdala, ultimately increasing CRF activation.

A) In a functioning circuitry, the amygdala sends GABAergic projections to the BNST to disinhibit (activate) the PVN. B) In males, mbTBI decreased CRFR2 gene expression in the amygdala. This may decrease GABAergic projections sent to the BNST, removing the disinhibition, ultimately reducing PVN activation. This increased PVN inhibition may be linked to the decreased restraint-induced CRF activation. (Red dashed arrow = GABAergic/inhibitory projection; Black bar = no restraint; Grey bar = restraint).

A



B

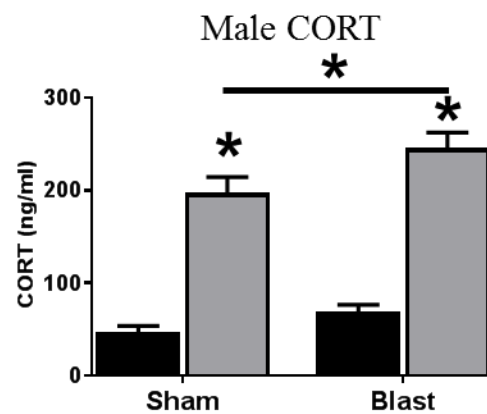
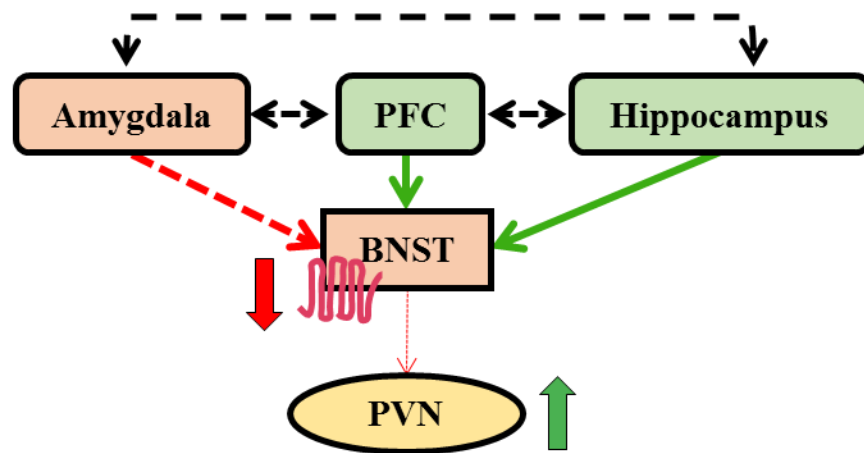
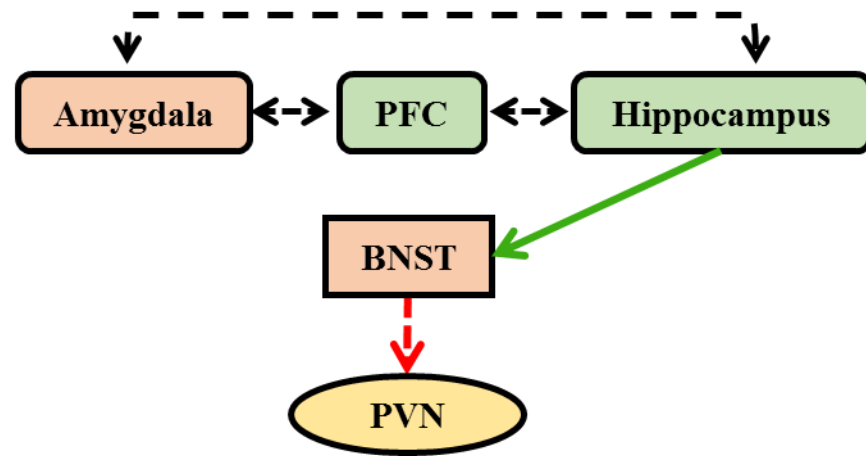


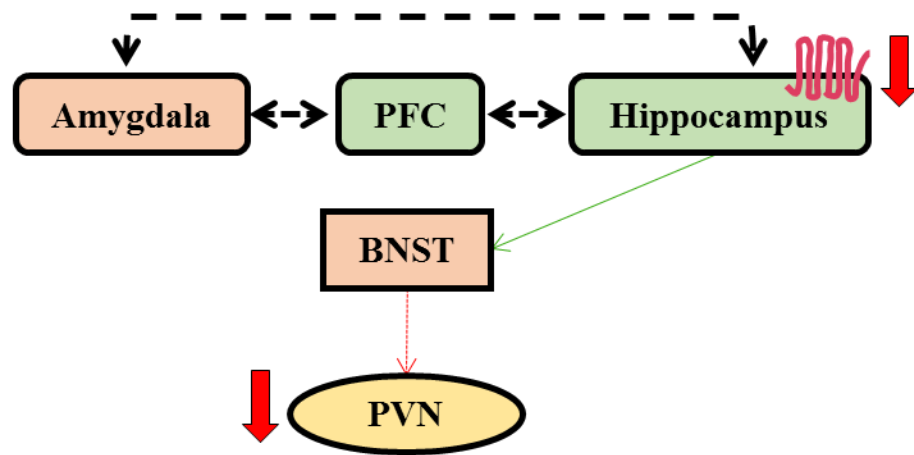
Figure 3. In males, mbTBI decreased CRFR2 gene expression in the aBNST, ultimately reducing CORT secretion.

A) In a functional circuitry, the BNST receives input from the amygdala, PFC and hippocampus. The BNST sends GABAergic projections to the PVN, inhibiting the HPA axis. B) In males, mbTBI decreased CRFR2 gene expression in the aBNST. This may decrease the inhibitory tone of the PVN, activating the HPA axis. This increased activation of the HPA axis may be linked to the increased restraint-induced CORT secretion after mbTBI. (Red dashed arrow = GABAergic/inhibitory projection; Green solid arrow = glutamatergic/excitatory; Black bar = no restraint; Grey bar = restraint).

A



B



Female CRF Activation

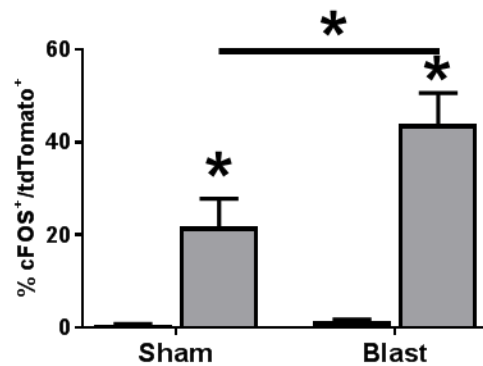


Figure 4. In females, mbTBI decreased CRFR2 gene expression in the dorsal hippocampus, ultimately increasing CRF activation.

A) In a functional network, the majority of the projections from the hippocampus are glutamatergic. These excitatory projections are relayed through GABAergic neurons in the BNST, ultimately to inhibit the HPA axis.

B) In females, mbTBI decreased CRFR2 gene expression in the dorsal hippocampus. Decreased function of the hippocampus may decrease glutamatergic input to the BNST, decreasing the inhibitory tone on the PVN. Decreased inhibition of the PVN may manifest as increased restraint-induced CRF activation after mbTBI. (Red dashed arrow = GABAergic/inhibitory projection; Green solid arrow = glutamatergic/excitatory; Black bar = no restraint; Grey bar = restraint).

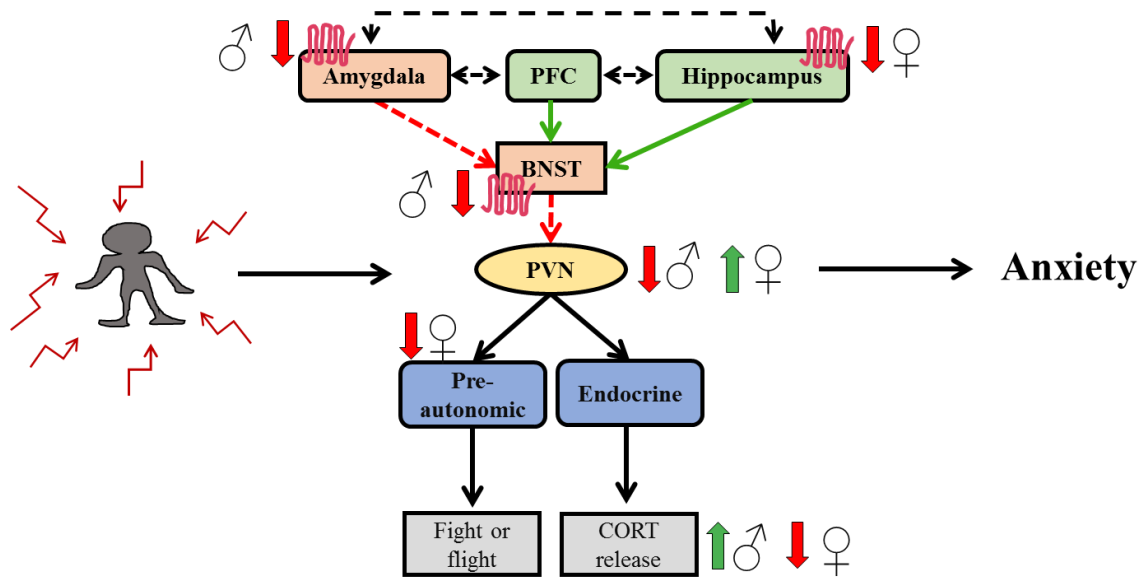


Figure 5. Proposed model of how mbTBI dysregulates the limbic-HPA axis to results in anxiety behaviors.

In males, mbTBI increased restraint-induced CORT release but decreased restraint-induced CR activation. Additionally, mbTBI decreased CRFR2 gene expression in the amygdala and aBNST, regions that regulate the HPA axis output. In females, mbTBI decreased restraint-induced CORT release, but increased CRF activation. Additionally, mbTBI decreased activation of pre-autonomic CRF projections and decreased CRFR2 gene expression in the hippocampus. The combination of these two phenomena may result in the mbTBI-induced dysregulation of the HPA axis. Disruption of the limbic-HPA network may result in anxiety symptoms after mbTBI. (Red dashed arrow = GABAergic/inhibitory projection; Green solid arrow = glutamatergic/excitatory).

SUPPLEMENTAL CHAPTER 1: Literature Review

Title: Factors promoting vulnerability to long-lasting changes in stress reactivity and stress-related disease: a brief review.

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Abbreviated Title: Factors mediating stress vulnerability

Key Words: stress, oxytocin, inflammation, glucocorticoids, depression, anxiety, TBI

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ABSTRACT

Effective coordination of the biological stress response is integral for the behavioral well-being of an organism. Stress reactivity is coordinated by an interplay of the neuroendocrine system and the sympathetic nervous system. The hypothalamus-pituitary-adrenal (HPA) axis plays a key role in orchestrating bodily responses to stress, and activity of the axis can be modified by a wide range of experiential events. This review will focus on several experiential factors that substantially influence subsequent HPA axis reactivity to later stress challenges, including early life adversity, chronic stress, and traumatic brain injury. The central premise is that each of these experiences serve as general vulnerability factors that accelerate future HPA axis reactivity in ways that make individuals more sensitive to stress challenges imposed later in life, and therefore feed forward into exacerbation of ongoing, or greater susceptibility toward, future stress-related disease states.

Highlights

The stress response is regulated by a complex central and peripheral network that relies on effective coordination among systems.

Environmental disruptors, such as chronic stress, early life adversity and traumatic brain injury alter stress reactivity.

Dysregulated stress reactivity may manifest in the development of neuropsychiatric illnesses and/or an inflammatory diseases.

INTRODUCTION

The ability to successfully adapt to environmental challenges, including stressful circumstances, is central to organism survival and has been a key topic in biomedical research for decades. Indeed, Walter Cannon first introduced the concept of “fight or flight” to describe an organism’s response to acute stress challenges that threaten physiological function (64). In doing so, he coined the term “homeostasis” (65) to extend Claude Bernard’s concept of “milieu intérieur”, which generally refers to the tendency to maintain constancy of the interior environment (48). Thus, Cannon defined homeostasis as “The coordinated physiological processes which maintain most of the steady states in the organism...”(65). In contrast, Hans Selye expanded the definition of stress by introducing the concept of the general adaption syndrome as the organism’s pathology in response to a chronic stressor (363). These pioneers of the stress field built the conceptual framework for how most researchers view stress challenges, how an organism adapts physiologically to those threats, and best practices for understanding the diverse nature of threats to organism health. This work also highlighted the need to further understand mechanisms underlying the stress response (256), revealing the complexity of communication between the autonomic, immune, nervous and endocrine systems. Perturbations in the homeostasis of the stress network may hinder psychological and behavioral well-being. This review discusses various ways in which an organism’s stress response may be altered including early-life stress, chronic stress, and brain injury.

The generalized stress response

The generalized physiological response to stress, whether psychogenic or systemic in nature, consists of activation of the sympathetic motor system and the hypothalamus-pituitary-adrenal (HPA) axis. Both systems are controlled centrally from the hypothalamus, the master command center for autonomic and neuroendocrine systems. The stress response writ large consists of engaging multiple cellular and physiological processes, marshaling metabolic resources to support the mobilization of those processes, and restoring the different systems to baseline levels upon termination of the stress exposure. The stress activation of sympathetic motor and HPA neuroendocrine outputs is coordinated at the level of the hypothalamus to engineer these different components of the stress response within prescribed time points following the stress presentation. Thus, the sympathetic system is activated initially to assign salience and to effectuate fight or flight by increasing arousal, augmenting blood flow, increasing blood oxygenation, and mobilizing cellular energy processes, with obvious acute selective benefits for survival. The HPA response is slower in onset and longer in duration due to its neuroendocrine nature, its protracted kinetics being timed for maintenance of tissue fitness following sympathetic activation and for termination of the response upon cessation of the stress exposure (Figure 1). Glucocorticoids are an end secretory product of the HPA axis activation, and are a main effectuator of the slow HPA component of the stress response.

The hypothalamus-pituitary-adrenal (HPA) axis

Among the many stress-responsive systems, the HPA axis remains one of the most studied indexes of stress reactivity and adaptation. This cascade is initiated by the release of neuropeptides and hormones from the parvocellular paraventricular nucleus of the hypothalamus (pPVN). Afferent stress signals converge in space and time to produce a neuroendocrine output in the form of corticotropin-releasing factor (CRF) secretion into the portal circulation of the anterior pituitary (21; 334; 442) (Figure 2). CRF stimulates corticotrophic cells within the anterior pituitary to release adrenocorticotrophic hormone (ACTH). ACTH travels through systemic circulation and binds to receptors within the adrenal gland where it elicits glucocorticoid (CORT; cortisol in humans, corticosterone in rodents) synthesis and secretion (102). Stress activation of the HPA axis, therefore, leads ultimately to a surge of corticosteroids into the systemic bloodstream. Glucocorticoids have a plurality of cellular consequences that act throughout the body by binding to mineralocorticoid (MR) and glucocorticoid receptors (GR). In addition to influencing most physiological systems, MR and GR are both widely distributed in key brain regions that regulate the stress response, and participate in negative feedback regulation, which ultimately curtails further activity of the axis (330; 331). The neuroendocrine nature of the HPA axis, which involves a multi-stage sequence of hormone secretion, blood transport, receptor activation, and corticosteroid synthesis and secretion (Figure 2), makes this a relatively slow and protracted process. The HPA component of the stress response, therefore, lags behind the sympathetic activation, and is phased to complement in function and in timing the acute activational properties of the sympathetic component of the response.

Although this canonical description of HPA axis reactivity creates the impression of a simplistic response to stress, each level of the axis can be modified by numerous other factors that are influenced by recent (or distal) stress history. The interaction and communication of many systems coordinates the propensity for homeostasis. In addition to glucocorticoid feedback, HPA axis-relevant limbic structures are activated in response to stress and, in turn, provide excitatory and inhibitory projections to the PVN, regulating the stress response (177). Various neuropeptides may accompany activation of the HPA axis. For example arginine vasopressin (AVP), acts synergistically with CRF to activate downstream HPA axis responses (358). Basal and stress induced HPA axis reactivity are also altered by oxytocin (OT) that is synthesized and released from the PVN, where OT attenuates activity within the axis. Ultimately, output of the HPA axis has a profound influence on the immune system, autonomic regulation and behaviorally-relevant limbic regions (185). Thus, the HPA axis is a highly dynamic, stress responsive system that is both causally activated by stressful circumstances and crucial for orchestration of organism-wide responses to stress.

Importantly, experiential factors have the ability to disrupt many levels of this circuitry, thereby creating vulnerabilities to subsequent expression of stress-related disorders. To better understand vulnerability factors that promote HPA axis dysregulation and its relation to stress-related disorders, a session was formed at the International Workshop in Neuroendocrinology in August 2017. Thus, the present review article was prepared to highlight three distinct experiential events that serve as vulnerability factors for later changes in stress reactivity, and the fundamental neurobiological mechanisms that subserve them. As this review will highlight, the adult stress response is sensitive to

disruptors such as early life stress, chronic stress in adulthood and traumatic brain injury (TBI). These environmental threats have feed-forward influences on subsequent physiological outcomes of the stress response. Dysregulation of stress reactivity can result in the development of neuropsychiatric illnesses, inflammation and other stress-related disease states (Figure 3). In this way, the following review will utilize an exemplar-driven approach to better understand mechanisms that might be common to diverse threats (early life stress, chronic stress in adulthood, and mild TBI) that contribute to later stress dysregulation.

CELLULAR MECHANISMS OF RAPID GLUCOCORTICOID NEGATIVE FEEDBACK

Glucocorticoid receptors

Glucocorticoids are steroid hormones whose actions canonically follow the rules of nuclear signals by binding to intracellular receptors and regulating gene transcriptional activity. The classical nuclear glucocorticoid receptor belongs to the nuclear receptor superfamily of ligand-dependent transcription factors. However, while transcriptional regulation by glucocorticoids may influence long-term expression patterns and signaling capacity of glucocorticoid-sensitive target cells, transcriptional regulation is unlikely to play a defining role in the immediate glucocorticoid regulation of target tissues engaged in the acute stress response. Transcriptional glucocorticoid signaling is implicated in repeated or chronic stress exposure, but rapid, non-canonical glucocorticoid signaling mechanisms are required for glucocorticoids to mediate the acute HPA response to stress. A growing body of research has focused recently on rapid glucocorticoid actions that are mediated by the activation of one or more membrane-associated glucocorticoid receptors and downstream signal transduction mechanisms.

Crucial to the homeostatic function of the generalized stress response is the termination of the HPA response and the return to baseline of the HPA signals following cessation of the stress exposure. A critical role for glucocorticoids, among others, is to mediate feedback inhibition of HPA axis activation. Negative glucocorticoid feedback can occur at multiple levels of the HPA neuroendocrine axis, including at the CRF neurons (126) and the pituitary corticotrophs (426) as well as at upstream structures integrated into the broader limbic stress circuitry (e.g., hippocampus (170) and prefrontal cortex (118)) (Figure 2). While intuitively obvious from the rapid rate of glucocorticoid

suppression of HPA activation, it was not until relatively recently that the rapid negative feedback effects of glucocorticoids on the HPA axis were understood to be mediated by non-transcriptional mechanisms via the activation of membrane-associated receptors (114; 126).

Intra-cerebral administration of glucocorticoids in the bilateral PVN rapidly suppresses stress-induced HPA activation via a membrane-associated receptor and endocannabinoid-dependent mechanism (126). Glucocorticoids stimulate the synthesis of the endocannabinoid 2-arachidonoylglycerol (2-AG) in PVN CRF neurons via the activation of a membrane-associated receptor (114-116) and a cAMP signaling pathway (116; 242). The glucocorticoid-induced 2-AG is released at excitatory synapses onto the CRF neurons and is transmitted retrogradely to presynaptic glutamatergic axon terminals, where it suppresses glutamate release by binding to CB1 receptors (114; 117) (Figure 4). This endocannabinoid-induced suppression of synaptic excitation in the CRF neurons dampens the excitatory drive to the CRF neurons, which provides a circuit mechanism for the rapid negative feedback regulation of the HPA axis by glucocorticoids. The rapid glucocorticoid-induced, endocannabinoid-mediated suppression of synaptic excitation has also been found in magnocellular neuroendocrine cells (115; 116) and in preautonomic neurons of the PVN (58). The spatial restriction of the glucocorticoid-induced, as well as activity-dependent, endocannabinoid actions to excitatory synapses in PVN neuroendocrine cells is controlled by astroglial regulation of extracellular 2-AG trafficking (117).

The membrane-associated glucocorticoid receptor in neurons has not been identified. Several pharmacological studies have indicated that intracellular signaling by

the receptor is G protein- and protein kinase-dependent (116; 242), and that its activity is inhibited by intracellular immunoneutralization of the Gs, but not Gq, subtype of G protein (116). This points to the possibility that the membrane glucocorticoid receptor is a G protein-coupled receptor. However, electron microscopic studies have revealed the nuclear glucocorticoid receptor located in membranes of hypothalamic neurons (234), neurons of the BLA (193), hippocampal neurons, medial PFC neurons. Biochemical data (281) suggest that its membrane localization may be regulated in pituitary cells by glucocorticoids (112). Additionally, conditional genetic knockdown of the nuclear glucocorticoid receptor in PVN neurons blocks the rapid glucocorticoid modulation of excitatory synaptic transmission in PVN CRF and magnocellular neuroendocrine cells (112) and the glucocorticoid negative feedback regulation of the HPA axis (375), indicating that the rapid glucocorticoid effects on the CRF neurons are dependent on the nuclear glucocorticoid receptor. These findings together suggest the possibility that the membrane glucocorticoid receptor may be the nuclear glucocorticoid receptor localized to the membrane. However, because the nuclear glucocorticoid receptor regulates thousands of genes (see (293)), the loss of the rapid glucocorticoid effect in CRF neurons following the genetic knockdown of the nuclear receptor does not mean per se that the membrane receptor is the nuclear receptor, because the loss of the rapid glucocorticoid effect may be due to the loss of a signal in the membrane receptor signaling pathway that depends on transcriptional regulation by the nuclear glucocorticoid receptor. Therefore, the existing data do not indicate conclusively whether the membrane glucocorticoid receptor in the CRF neurons and magnocellular neuroendocrine cells of the PVN is the nuclear glucocorticoid receptor located at the membrane, or a distinct G protein-coupled

receptor, or a membrane transport protein that shuttles the glucocorticoid into the cell and triggers a G protein signaling cascade in the process.

Rapid glucocorticoid regulation of pre-hypothalamic limbic structures

Glucocorticoid-induced retrograde endocannabinoid suppression of synaptic transmission has also been found to exert rapid feedback synaptic regulation in the hippocampus, prefrontal cortex (PFC), and amygdala. In the hippocampus, stress exposure *in vivo* and corticosterone application to *in-vitro* hippocampal slices causes an increase in hippocampal 2-AG levels and enhances the endocannabinoid-dependent suppression of synaptic inhibition induced by depolarization or cholinergic activation in CA1 pyramidal neurons (430). The hippocampus/subiculum exerts an inhibitory influence on the HPA axis, relayed via an inhibitory relay in the bed nucleus of the stria terminalis (321). The glucocorticoid-induced suppression of inhibition in the hippocampus, therefore, is thought to activate this pathway to contribute to the glucocorticoid suppression of the HPA axis. However, rapid glucocorticoid facilitation of inhibitory synaptic inputs to CA1 pyramidal neurons via a nitric oxide-dependent activation of local inhibitory interneurons has also been reported (182; 398). The glucocorticoid disinhibition of the CA1 neurons via endocannabinoid suppression of GABA release would be expected to oppose the glucocorticoid-induced, nitric oxide-dependent excitation of local inhibitory circuits, rendering the net effect of rapid glucocorticoid modulation of inhibitory synaptic inputs to CA1 pyramidal neurons unclear. However, corticosterone has also been shown to rapidly increase excitatory synaptic inputs to hippocampal pyramidal cells via activation of presynaptic MR (200),

suggesting that the overall rapid glucocorticoid action in hippocampal pyramidal neurons is likely to be excitatory, and may contribute to the rapid feedback inhibitory effect of glucocorticoids on the HPA axis.

Stress exposure also causes a glucocorticoid receptor-dependent increase in 2-AG content in the medial PFC *in vivo* (178). Corticosterone pretreatment of PFC slices leads to the suppression of GABAergic inhibitory synaptic inputs to layer V pyramidal neurons, which is dependent on 2-AG activation of CB1 receptors. Like the hippocampus, the PFC exerts an inhibitory influence on HPA activation via an inhibitory relay in the bed nucleus of the stria terminalis (320; 321), such that disinhibition of the PFC output neurons by stress-induced glucocorticoids would also be expected to contribute to the negative feedback regulation of the HPA axis. This may be a delayed effect compared to the direct negative glucocorticoid feedback occurring at the PVN, and may serve to prolong or sustain the feedback inhibition of the HPA axis. It should be noted, however, that glucocorticoids also rapidly modulate inhibitory synaptic inputs to pyramidal neurons in layer II/III of the medial PFC, but rather than suppressing GABA release through retrograde presynaptic endocannabinoid signaling, glucocorticoids rapidly increase inhibitory synaptic inputs by activating upstream local inhibitory interneurons (398). How the rapid glucocorticoid-induced increase in inhibition of layer II/III neurons and decrease in inhibition of layer V neurons integrate to create a net PFC output to the HPA axis is not known.

In the basolateral amygdala (BLA), stress-induced glucocorticoids also modulate inhibitory synaptic transmission via an endocannabinoid-dependent mechanism (113). Glucocorticoids stimulate the synthesis and retrograde release of 2-AG at GABA

synapses onto principal cells in the BLA, and this stress-induced rise in intra-BLA endocannabinoid contributes to the anxiety-like behavior caused by acute stress. Excitatory synapses in the BLA also respond to glucocorticoids with an endocannabinoid-induced suppression of glutamate release, although this is seen only following *in-vivo* priming with a prior stress exposure or *in-vitro* priming of the BLA excitatory synapses with prior corticosterone treatment (199), suggesting that this endocannabinoid mechanism at excitatory synapses may play a role in chronic stress adaptation or sensitization. The endocannabinoid-dependent suppression of synaptic excitation may be purposed to restrain the increase in excitatory synaptic inputs to the BLA principal cells caused by acute stress and rapid corticosteroid actions at MR (199; 201).

PERINATAL ENVIRONMENTAL INTERVENTIONS HAVE LONG-TERM CONSEQUENCES ON STRESS REACTIVITY

The mechanisms by which the nervous system can perceive, conduct, process, and memorize; as well as the subsequent motor and/or endocrine responses, have been extensively studied. Although many studies have focused on adult animals, the pioneering work of Levine and others (365) has shown that even an apparently non-noxious environmental intervention on a newborn can induce long-lasting behavioral and neuroendocrine dysregulation. Epigenetic studies highlight the importance of environment of development and adult stress reactivity (73; 264). Developing animals and humans are surrounded by all kinds of stimuli, with different nature, intensity, frequency, timing, pattern, and most importantly salience to the animal. Early developmental experiences, during the perinatal age (before or shortly after the time of birth) have the ability to produce life-long changes in organism health, underscoring the importance of early life adversity (40). At least two important variables come into play in developing animals: the animal's capacity to process the stimulus; and the specific stimulus salience to the animal at that moment in life. An animal is vulnerable to long-lasting disturbances during this "critical period" of heightened plasticity.

A variety of approaches have been used to study the impact of perinatal environment and maternal behavior on the OT system and the subsequent development of social behaviors in rodents. These include rodent OT knockout (OTKO) models and examination of the natural OT amino acid sequence changes after maternal and paternal offspring interaction. Here, we summarize results from the following mild environmental interventions used in laboratory rodents: (1) the impact of the neonatal handling

procedure; (2) partial handling (split litter); (3) naturally occurring variations in maternal care; (4) litter overlapping; (5) prenatal stress. Although other approaches have been implemented (e.g. maternal separation and maternal deprivation), we will focus on mild environmental interventions and the interplay of neuro-hormones on the development of social behaviors and stress reactivity.

The interplay of the neuroendocrine system and the OT system are related to social and stress coping behaviors. For example, the OT system regulates the HPA stress response at baseline and during threat exposure. OT can act at the level of the hypothalamus, or throughout the limbic circuitry, providing direct and indirect modulation of stress and coping behaviors (184; 295). OT has also been vastly associated with social behaviors, especially those related to bonding (mother-infant; pair bonding, monogamy). Environmental events early in life can change the OT system and thus be the basis for changes in adult social behaviors (63; 129).

The early life mild interventions, such as neonatal rearing experience and social environment can have a profound impact on neuroendocrine and socially-relevant processes later in life. The effects of the neonatal handling procedure, which consists of brief, repeated manipulation of the whole litter have been previously reviewed (see (325)). Briefly, early life mild intervention reduced non-aggressive social interactions, in pups (reduction in maternal odor preference), juvenile (reduction in play behavior) and adulthood (reduction in sexual behavior and increased aggressiveness). Neonatal exposure to brief, repeated litter splitting, reduces sexual behaviors in aged (90-100 day old) males (47). In addition to neonatal behaviors, natural variations in maternal care (high and low licking mothers) affect sexual receptiveness in female rats. Adult females

reared by high licking mothers show reduced lordosis (406). Similarly, the litter overlapping procedure (pups+2 juveniles from a previous pregnancy) reduced sexual receptiveness in female rats (407).

To this end, OT has been implicated in the expression of social processes, aggression and sexual behaviors as a result of prenatal and neonatal manipulations in rodent models. For instance, neonatal handling reduced the number of OT-positive cells in the pPVN in adult male and female rats (401). Adult male rats stressed during gestation and reared by stressed mothers show a reduction in hypothalamic OT and AVP cells. Separately, the interventions (gestational stress and cross fostering) did not change OT expression (108). The involvement of the OT system in social and stress coping behaviors has also benefited from the use of OTKO that allow for examination of effects arising from reduced OT early in life. OTKO males showed reduced social behaviors, but no change in sexual activity (219). OTKO females have a reduction in sexual receptiveness and ovulation were shown (44; 463). OTKO females show reduced OT receptors and AVP receptors (AVPR) in the hippocampus. V1aR are also reduced in the hypothalamus. Moreover, OTKO reduced estrogen receptor alpha gene expression in the PFC (463). Similar effects have been observed in genetic variants of OT. For instance, the OT variant (Pro in position 8) in monkeys is related to males displaying paternal behavior. In male rats, the administration of OT variants (Val³Pro⁸OXT and Pro⁸OXT) tended to increase pup-related behaviors. Thus, OT appears to play an important role in programming later expression of social interactions.

Commensurate with this, exposure of the developing organism to active stress challenges such as gestational stress (restraint) plus cross-fostering induced changes in

the social behaviors of the offspring. Adult male rats stressed during gestation and reared by mothers that were also stressed show reduction in social non-aggressive interaction (but increased aggressive behaviors). Male pups stressed during gestation but reared by non-stressed mothers showed no dysregulation of later social behaviors (108) (females not studied). Overall, there is a complex interplay between mild early life interventions and exposures and long-lasting influences on the adult stress reactivity.

Overall, mild early life environmental interventions can affect several systems, resulting in long-lasting behavioral effects and stress-relevant outcomes. The neonatal neuroendocrine system seems to be “open” to those interventions; however, the “messenger(s)” between the environment and the effector systems remains to be established. OT could exert that role. The challenge that remains is to understand the processes and mechanisms involved in the development of social behaviors during that early period. What would be the salience of a stimulus to an animal that encounters it for the first time? Since there was no previous adaptation and other central processing of the stimulus, how would it be perceived by the neonatal? Would the stimuli during infancy induce stable memories? How would they “build” future sociability later in life? Considering the evolutionary importance of social behaviors and the role of neuro-hormones, such as OT, those questions are crucial for understanding adult stress circuitry.

CHRONIC STRESS ALTERS LIMBIC SIGNALING REGULATING THE HPA AXIS

The adult brain also displays a high degree of plasticity, a property that is essential for functional adaptation to a constantly changing environment. Brain areas involved in the stress response include the hypothalamus, hippocampus (learning and memory), the PFC (executive function) and the amygdala (threat appraisal) (258). These structures play a key role in integrating physiological and behavioral responses during stress and coping with subsequent stressful events (214).

On the other hand, repeated exposure to stressors negatively influences the physiology of organ systems. Repeated stress sensitizes the stress system to the development of complex disorders such as autoimmune and cardiovascular diseases, cancer, obesity and mental diseases such as anxiety and depressive disorder (86). Interestingly, epidemiological studies have revealed a strong comorbidity among these diseases (248). Recent molecular analysis identified a common gene expression signature, providing a potential shared mechanism for these stress-related pathologies (149).

Chronic stress causes dysfunction of several brain structures, resulting in a feed-forward impact on stress reactivity and the associated behavioral adaptation. Several stress paradigms in rodents have been used to model the structural, functional and neuroendocrine (enhanced HPA axis activity) abnormalities in the brain of depressed patients. These models use chronic exposure to a single type of stressor (mild or strong) (445) or unpredictable and variable stressors (202). Chronic restraint in male rats reduced the responsiveness to reward (sucrose consumption) (62; 68), analogous to anhedonia, which is a core symptom of depressive disorders (444). Chronic stress also promotes an

incapacity to cope with a stressful stimuli, as demonstrated by reduced escape-oriented behaviors in the forced swim test (swimming and struggling) (62; 68; 138; 236). In mice, selective inhibition of dopaminergic neurons in the ventral tegmental area triggered depressive-like behaviors in chronically stressed animals (reduced sucrose consumption and tail suspension struggling behavior) (403). This suggests that activity of dopaminergic nuclei may be inhibited by chronic stress.

The influence of chronic stress is not restricted to alteration of hedonic processes, but also extends to cognitive function. Chronically stressed animals displayed reduced spatial learning, suggestive of disruption in hippocampal functioning (276). Furthermore, postmortem studies have revealed a reduction in the volume of the hippocampus and PFC in depressed patients (120). Postmortem studies have also suggested that depressed patients present poor glutamate clearance, suggesting increased activation of glutamate receptors (346), an effect also observed in chronically stressed rodents (270; 312). Thus, changes in levels of glutamate may affect the balance of excitatory and inhibitory cues, resulting in alterations of stress reactivity.

Glutamate activates both metabotropic and ionotropic (AMPA and NMDA) receptors. Variation in cell surface exposure of these receptors determines changes in excitatory synaptic efficacy, such as long-term depression (LTD) or long-term potentiation (LTP). Chronic stress reduced LTP in CA1 neurons and dentate gyrus of the hippocampus (19; 304). Studies have examined the expression of glutamate receptors to understand this variation in LTP. In the dorsal hippocampus, chronic stress reduced NMDA receptor 1 (NR1; obligatory subunit) and resulted in a negative shift in NMDA receptor 2A/NMDA receptor 2B (NR2A/NR2B) (299). Given that the subunit

configuration of NMDA receptors influences deactivation kinetics of the channel and the affinity for glutamate (420), these variations may explain the reduction in LTP in chronically stressed animals (305). Collectively, these studies suggest that chronic stress alters the strength of hippocampal excitatory synapses, probably through the variation in synaptic contact, a mechanism that may be important for stress-induced disruptions in cognitive function.

In addition to changes in GABA-glutamate signaling, chronic stress also produces morphological changes in the central nervous system (CNS), including dendritic atrophy and reduced spine density in neurons of hippocampus and PFC (68; 138; 259; 310; 423). These findings suggest that processes implicated in the formation and stabilization of synaptic connections can be altered under chronic stress and depressive disorder. Some studies have found that the stress-induced reduction in spine density is associated with a reduction of hippocampal N-cadherin levels (68), an adhesion molecule required for spine enlargement and LTP at mature CA1 synapses of the hippocampus (61). Morphological changes of axonal growth, dendritic arborization and dendritic spines during development and in the adult brain are driven by fast modifications of F-actin dynamics. These modifications are under the control of Rho-family small GTPases (144; 239; 282; 392). For instance, active RhoA diminishes spine length and density in cultured neurons (239; 282). A downstream effector of RhoA is the Rho kinase (ROCK) (282; 392), which in non-muscular cells, stimulates the myosin-dependent contractibility of F-actin by inactivating myosin light chain (MLC) phosphatase through the phosphorylation of its regulator MYPT1, an exclusive target of ROCK (20). Interestingly, a recent study revealed that chronic stress triggered a rise in phospho-MYPT1 levels in the

hippocampus, suggesting activation of the RhoA-ROCK pathway, which may explain the reduction in spine density in hippocampal CA1 neurons (67). More recently, it has been described that systemic administration of a ROCK inhibitor (Fasudil) prevented both the stress-induced increase in phospho-MYPT1 and the reduction in spine density of CA1 hippocampal neurons (138). Interestingly, the administration of the ROCK inhibitor prevented stress-induced immobility observed in the forced swimming test (138), similar to the effects of antidepressant drugs (62; 404). Structural changes in neurons within the hippocampus and the PFC are associated with the pathophysiology of depression (311). A reduction of spine densities in dendrites of CA1 neurons has been associated with depression-like behaviors in several animal models of chronic stress, suggesting altered glutamatergic excitatory neurotransmission. Studies have shown that antidepressant drugs with different pharmacological targets reverse the stress-induced behavioral alterations and spine loss in hippocampal neurons (49). Furthermore, recent evidences suggest that the disruption of the Rho-ROCK pathway and/or inhibition of other kinases by Fasudil seem to exert antidepressant-like actions and prevent hippocampal spine loss. Further studies are necessary to evaluate whether chronic stress alters Rho-ROCK pathways, to determine mechanisms for pharmacological tools to ameliorate depressive symptomology. Altered stress reactivity may result in depressive behaviors, however, the mechanisms by which chronic stress and the associated synaptic and dendritic changes result in this need to be examined.

TRAUMATIC BRAIN INJURY (TBI) INDUCES LONG-LASTING ALTERATION IN STRESS RESPONSE CIRCUITRY

TBI refers to an external force impacting the brain and resulting in functional dysregulation and/or impairment. Different forms of TBI include concussion, closed head injury, diffuse axonal injury, blast over pressurization, penetrating wound, laceration, hemorrhage, contusion and anoxia. TBI is categorized into one of three clinically defined categories: mild, moderate or severe based on the prevalence and persistence of post-injury symptoms. Mild TBI (mTBI) is the most prevalent (~80%) level of TBI sustained (3), and symptoms of mTBI may be transient or even go unnoticed.

TBI results in detrimental physical, cognitive, emotional, social and stress coping behaviors. Immediately following mTBI, symptoms may appear mild; however, persistent symptoms may manifest as life-long impairments. Approximately 15% of people diagnosed with mTBI experience persistent neurobehavioral problems (17). TBI exhibits a high comorbidity diagnosis with psychiatric disorders such as depression, anxiety disorders panic disorder, substance abuse, and post-traumatic stress disorder. The subsequent development of neuropsychiatric disorders after TBI may be a result of secondary injury mechanisms in brain structures that play a role in behavioral coordination, but there is increasing recognition that mTBI may also exacerbate emotional reactivity to circumstances, reflecting greater vulnerability to stress challenges later in life.

Experimental evidence connects TBI and the subsequent development of detrimental anxiety and fear reactivity behaviors, potentially mediated by altered stress reactivity. Acute and chronic increases of anxiety and PTSD-like behaviors were

observed in various rodent TBI models. For instance, in the open field test and elevated plus maze, both of which are used to measure anxiety-like behaviors in rodents, increased levels of anxiety related behaviors persisted months after injury (302). Similarly, TBI induced hyper-responsivity and enhanced fear retention in the acoustic startle and Pavlovian fear conditioning tests, respectively (162; 328). Importantly, these outcomes in rodent models mimic symptoms observed in PTSD patients, underscoring the translational value of rodent models in delineating neurobiological mechanisms by which mTBI influence later stress reactivity.

Most endocrine axes are prone to TBI-induced dysfunction. Clinically, chronic hypopituitarism is observed in a large population of TBI patients (131). Clinical and experimental evidence shows that the HPA axis was vulnerable to dysregulation as a result of injury (228; 393). ACTH and CORT insufficiencies were observed in the acute phase (less than 2 weeks) post injury (388). Although HPA axis disruption recovered post injury, neuropsychiatric symptoms persisted as much as 6 months later (374). Preclinical data also provides supporting evidence of TBI-induced HPA axis dysregulation. Increased plasma ACTH and CORT (143) levels were present in the acute phase of injury. CORT remained dysregulated up to 70 days post injury (393). mTBI decreased CORT in the sub-acute phase (up to 21 days post injury) after restraint and forced swim stressors. Conversely, in the chronic phase of injury, stress-induced CORT levels are elevated (393).

Alteration in central regulation by the PVN and/or feedback mechanisms (MR and GR) results in HPA axis disruption. After diffuse TBI, cell death was not evident in the PVN; however neuronal plasticity was observed. In pPVN neurons, TBI resulted in

increased neuronal process branch point number and complexity, potentially as a result of secondary injury mechanisms (341). A second outcome of TBI was a transient effect on MR and GR levels. TBI increased GR levels (up to 3 days post injury) and decreased MR (1 day post injury) levels in the hippocampus (146; 251). MR and GR levels returned to baseline at 7 days post injury (146). Administration GR antagonists were shown to decrease TBI-induced hippocampal cell death (251) and anxiety-like behaviors (135).

There is also evidence that TBI can alter neural regulation of HPA axis activity by affecting CNS sites that project to the PVN. Clinically, alterations in the PFC, amygdala and hippocampus were apparent after TBI (53; 154). These limbic structures provide direct and indirect projections to CRF neurons in the pPVN to regulate the behavioral stress response (177; 190). This communication is either excitatory (glutamatergic) from the amygdala or inhibitory (GABAergic) from the PFC, hippocampus and BNST. In experimental models, cell death also occurs throughout these limbic structures (323; 340). Changes in glutamate and GABA are also observed after TBI (148). For example, a loss of inhibitory neurons (measured by GAD-67 and parvalbumin immunohistochemistry) was observed in the amygdala and hippocampus after TBI (396).

Taken together, these findings suggest that TBI can have a profound influence on subsequent activity of the HPA axis when stress challenges are encountered post-injury. In this way, individuals with a history of acute or repeated TBI may be more sensitive to the adverse consequences of stress later in life, an effect that may persist for months to years post-injury. Thus, ongoing studies are required to better understand the neurobiological mechanisms by which TBI influences later stress reactivity later in life.

INTERPLAY OF THE STRESS AND IMMUNE AXES

As noted above, a variety of insults including early life adversity, chronic stress exposure, and TBI can all have a profound influence on cognitive function, with many of these effects being mediated by CORT. Indeed, the PFC and hippocampus are highly sensitive to the deleterious effects of CORT (72; 107) and endogenous CORT can have detrimental effects on behavior. However, the downstream mechanisms by which CORT exerts its effects on brain function remain an active area of study. It can be noted, however, that both stress and CORT have powerful influences through downstream modulation of inflammatory processes. Glucocorticoids, in general, typically have anti-inflammatory properties, since they act by inhibiting the gene expression of pro-inflammatory mediators (cytokines and reactive oxygen and nitrogen species) in the periphery and CNS. These effects are particularly evident with use of exogenous, pharmacological concentrations of glucocorticoids or in cases in which high stress levels of endogenous glucocorticoids have been abrogated. Despite these well-documented anti-inflammatory actions of glucocorticoids, there is increasing evidence suggesting that glucocorticoids are not solely anti-inflammatory and in some cases, may even increase certain aspects of the inflammatory response, mainly in the CNS (377).

The ability of glucocorticoids to exert either pro-inflammatory or anti-inflammatory effects is dependent on timing, concentration and duration of glucocorticoid exposure. The specifics of the inflammatory actions of glucocorticoids are not well understood. Chronic unpredictable stress increases circulating CORT (277; 278). In the PFC and hippocampus, elevated CORT increased the expression of pro-inflammatory genes (iNOS, IL-1 β , TNF- α). Additionally, elevated CORT decreased anti-

inflammatory genes including IL-1ra, IL-10, and MKP-1 in these limbic structures (277; 278). Pre-treatment with the GR antagonist, RU486, reduced this pro-inflammatory effect, suggesting that this is indeed GR mediated (277; 278).

The PFC and hippocampus are prone to detrimental effects by glucocorticoids, potentially mediated by the inflammatory roles of microglial and astrocytes. The pro-inflammatory role of glucocorticoids after kainic acid insult, increased cell death (378). Blocking microglial activation reduces the glucocorticoid-induced worsening of neuron survival (378). Administration of high levels of glucocorticoids decreased anti-inflammatory gene expression in the hippocampus, but increased the pro-inflammatory gene, IL-1 β (378). After injury (kainic acid or focal ischemia), glucocorticoids decreased neuronal survival in GR knockout animals (376). To this end, glucocorticoids may activate microglia, putting them in a “primed” state (136).

In immune disorders, such as multiple sclerosis (MS), synthetic glucocorticoids, such as methylprednisolone (MP) and dexamethasone (DEX; high affinity for GR) are the standard treatment for acute disease symptomatology. In an experimental model of MS, the experimental autoimmune encephalomyelitis (EAE), Wurst et al (449) showed that MP administration prior to immunization, resulted in increased leukocyte infiltration into the CNS. A worsening of the symptoms was also observed, adding more evidence in a disease model, that glucocorticoids are not uniformly anti-inflammatory.

Overall, this suggests a bidirectional communication between stress reactivity and inflammation. The emerging, pro-inflammatory influence of stress challenges broadly, and CORT specifically, might help explain the general paradox that inflammatory disease states are typically exacerbated (rather than ameliorated) during times of chronic stress.

This is particularly important given the large number of disorders that involve inflammation, and that are known to be exacerbated by stress, including obesity, asthma, diabetes, cardiovascular and neurodegenerative diseases, and cancer to name a few. Thus, a major path for the future is to better understand the mechanistic relationship between classic stress-responsive systems such as the HPA axis, inflammation, and subsequent disease states.

OVERALL CONCLUSIONS

The over-arching goal of this brief review was to identify how a range of common, yet disparate, experiential events influences activity of the HPA axis to stress challenges that occur later in life (Figure 3), as discussed at the International Workshop in Neuroendocrinology in Concon, Chile in August, 2017. Early life adversity, chronic stress during adulthood and TBI were all discussed as established or emergent experiences that alter stress responses, and all of which tended to produce feed-forward activity within the axis. Although the mechanisms underlying responses to each of these experiences are likely to be unique, the long-lasting influence on the stress system (HPA axis and sympathetic nervous system) certainly influences later stress reactivity, an effect that may magnify the psychological and physiological consequences of future stress challenges. Vulnerability of the stress system can make an organism susceptible to maladaptation and poor stress coping behaviors. Dysregulation can result in a sequela of outcomes such as the development of neuropsychiatric disorders, inflammation and exacerbation of other disease states. These outcomes, however, may also further perturb the stress response, setting the stage for feed-forward effects on maladaptive consequences of stress.

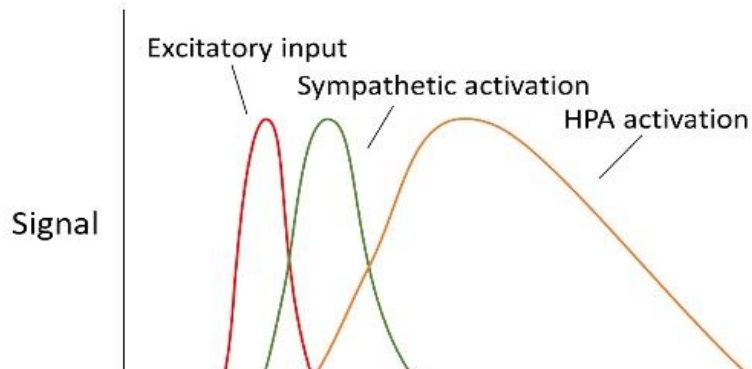


Figure 1. Relative time courses of major components of the generalized stress response
Stress exposure stimulates excitatory input to the hypothalamus, which triggers a rapid activation of the sympathetic motor system, followed by a delayed activation of the HPA axis.

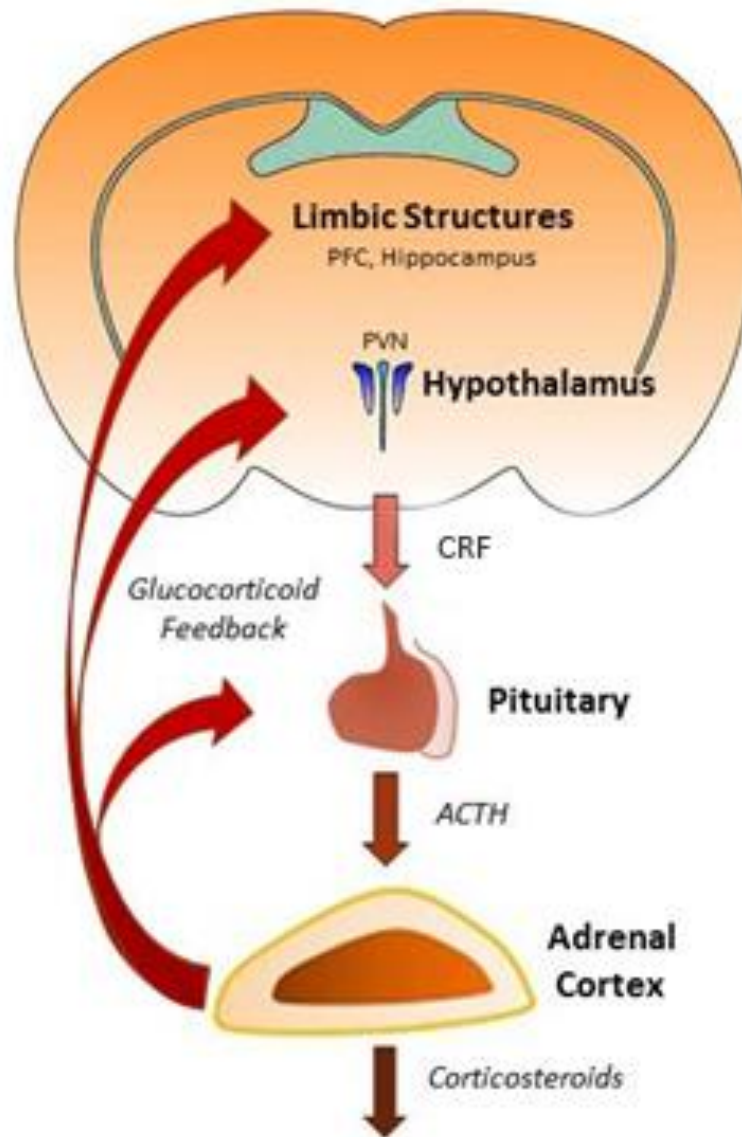


Figure 2. HPA axis.

HPA axis activation consists of CRF release from the hypothalamus, which stimulates ACTH secretion from the anterior pituitary and corticosteroid secretion from the adrenal cortex. Glucocorticoids feed back at multiple levels of the HPA axis and limbic system.

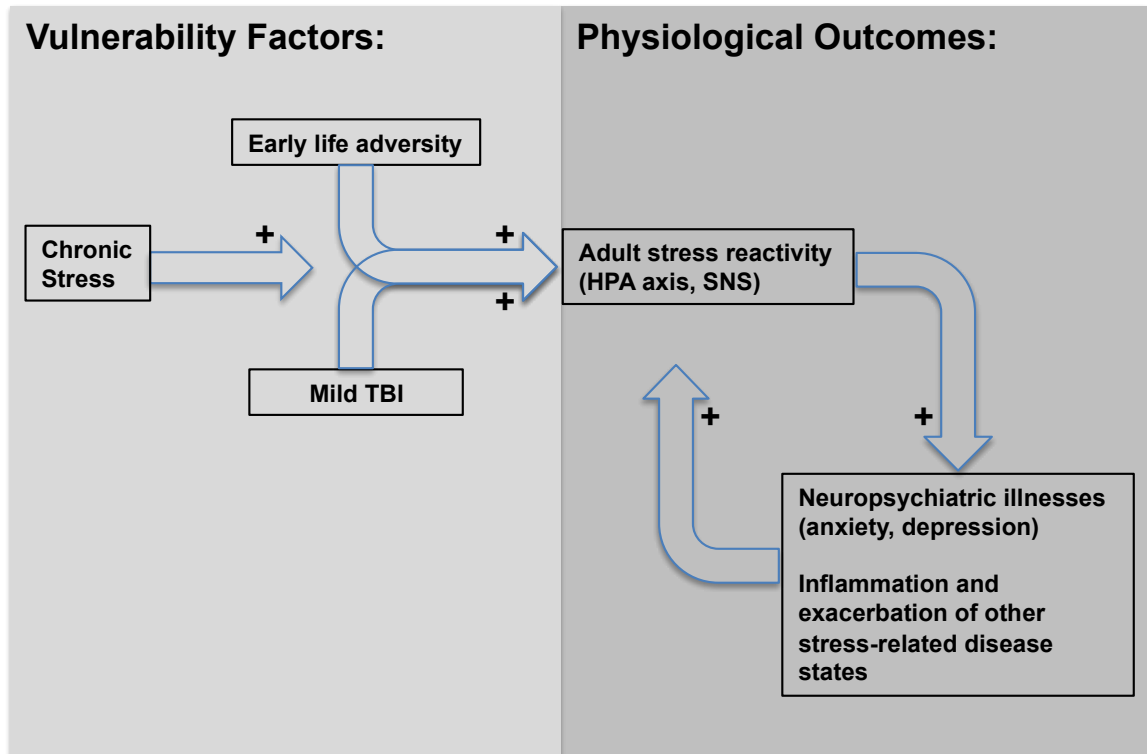


Figure 3. Schematic summary of feed-forward influences of external threats on subsequent stress reactivity.

(TBI = traumatic brain injury, HPA = hypothalamus-pituitary-adrenal axis, SNS = sympathetic nervous system).

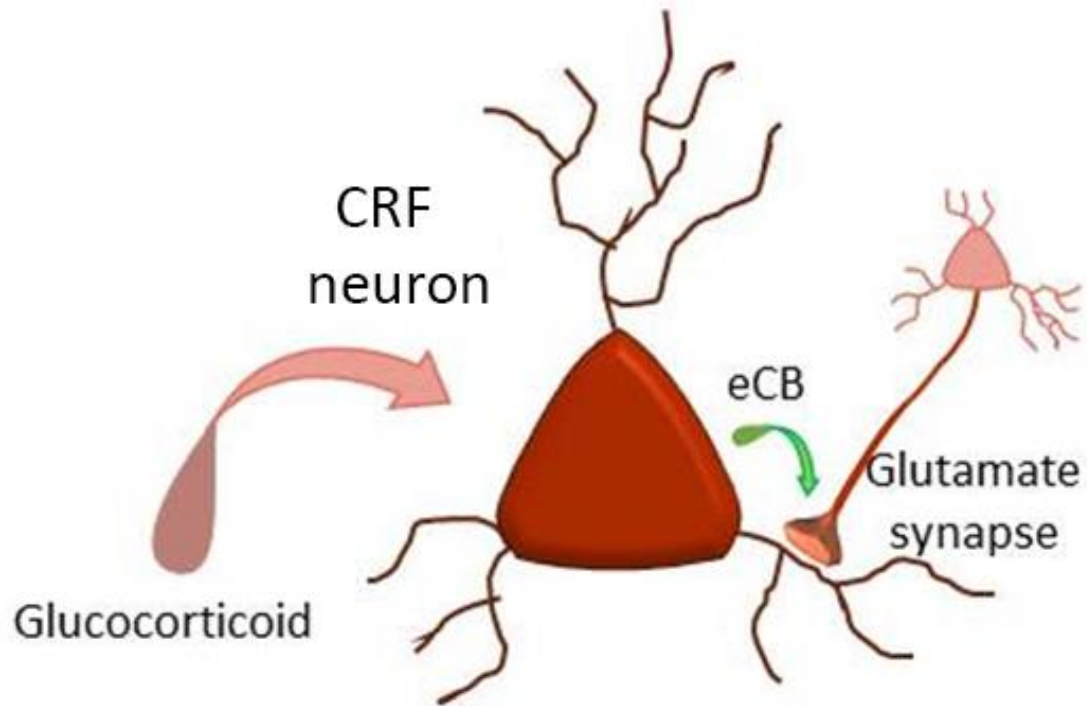
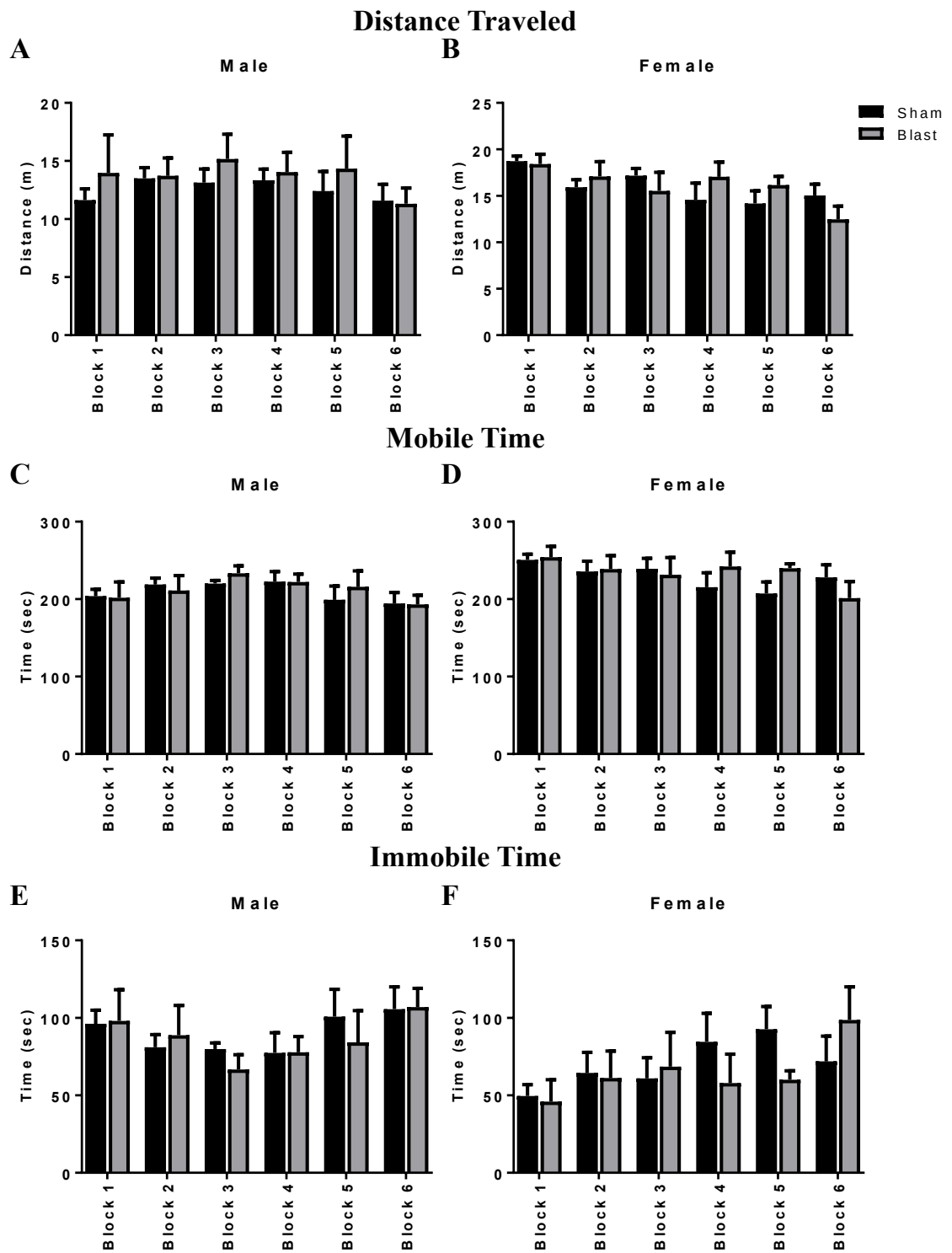
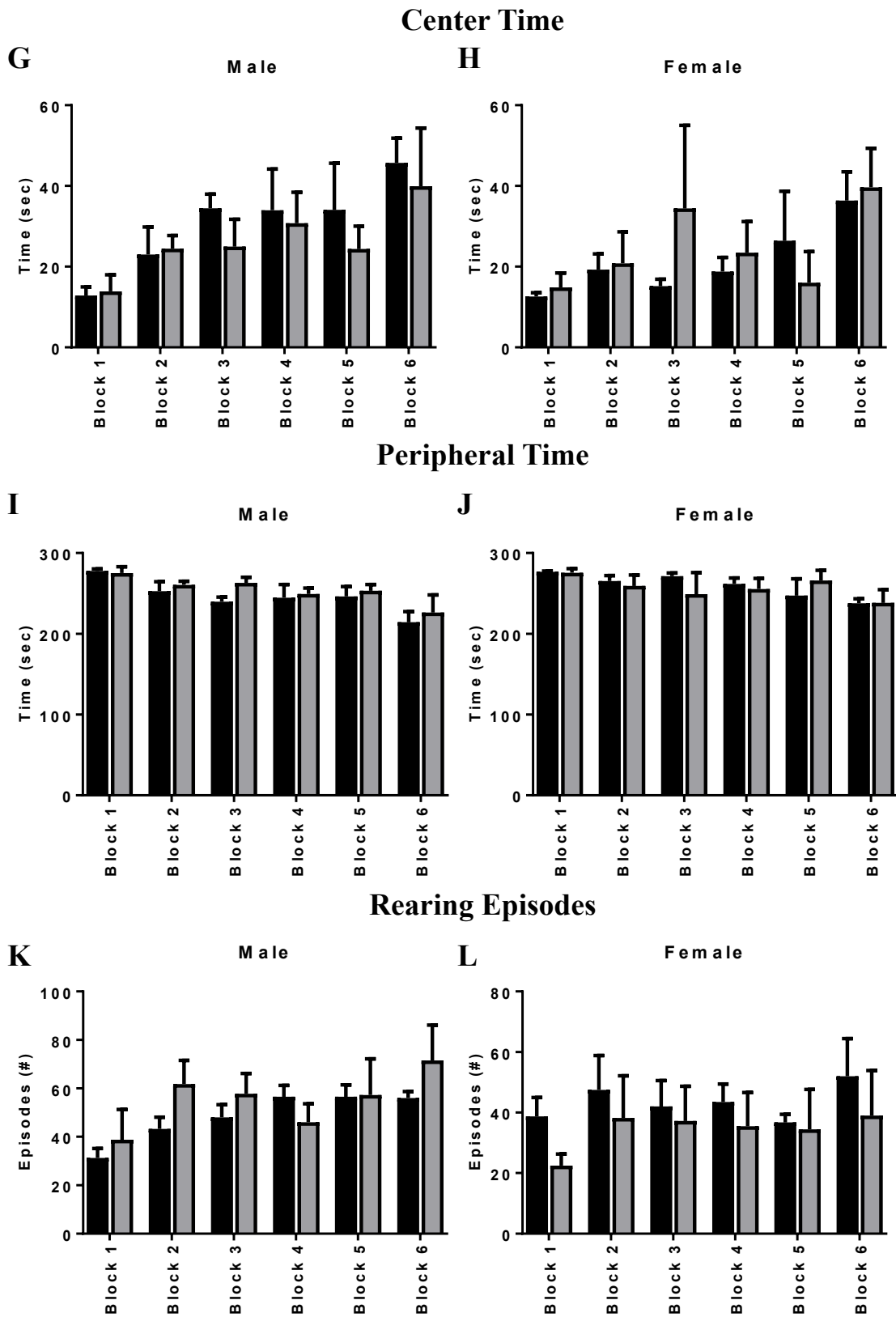


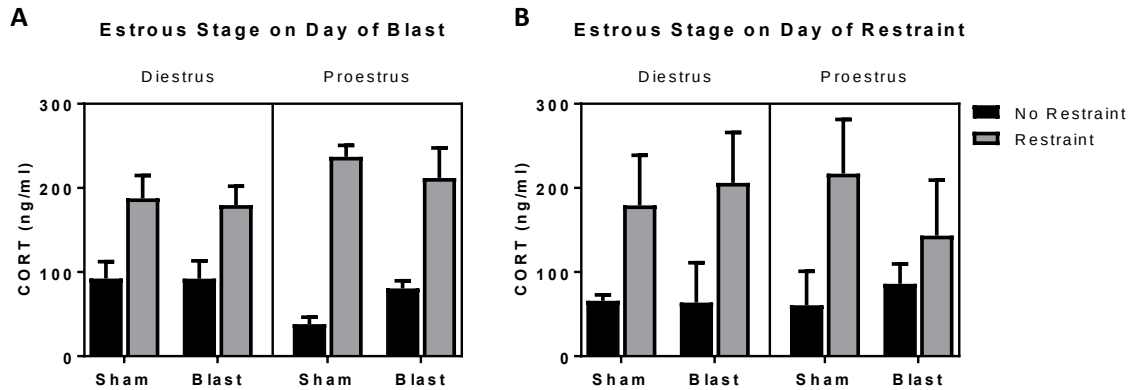
Figure 4. Glucocorticoid-induced suppression of synaptic excitation. Glucocorticoids stimulate the synthesis of the endocannabinoid 2-AG (eCB), which acts as a retrograde messenger to inhibit glutamate release from excitatory synapses via presynaptic CB1 receptor activation.

SUPPLEMENTAL CHAPTER 2: Additional Figures



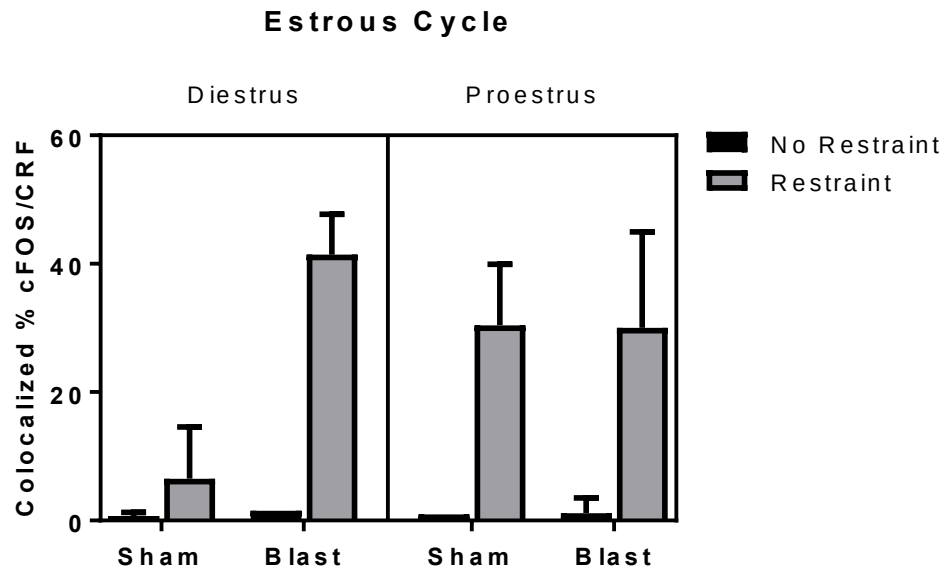


Supplemental Figure 1. mbTBI had no effect on open field behaviors throughout the test. Six days after mbTBI, male and female mice were tested in the open field assay for 30 min. Analyzed parameters of the open field include: A,B) Distance traveled C,D) Mobile time, E,F) Immobile time G,H) Center time I,J) Peripheral time and K,L) Rearing episodes. The 30 min testing session was divided into 5 min blocks (to total 6 blocks) for analysis. The data from Aim 2 was analyzed in this manner to determine if there were differences at any time point throughout the open field assay. There was no effect of mbTBI on any 5 min blocks of the open field behaviors analyzed in males or females. (Black bar = sham; Grey bar = mbTBI) (Block 1 = 0-5 min; Block 2 = 5-10 min, Block 3 = 10-15 min, Block 4 = 15-20 min, Block 5 = 20-25 min, Block 6 = 25-30 min).



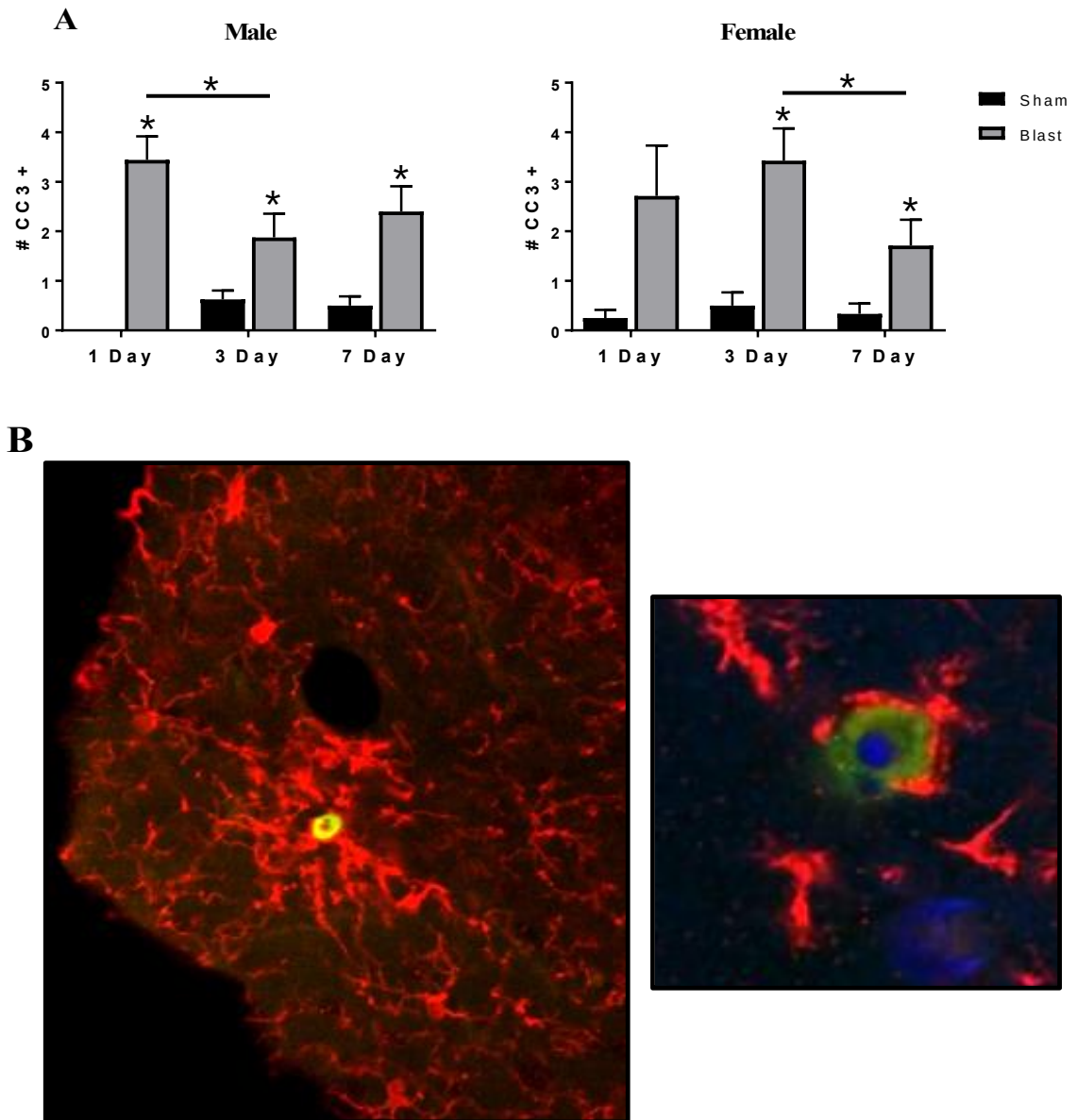
Supplemental Figure 2. Estrous cycle had no effect on CORT secretion after mbTBI.

The goal of this experiment was to determine if circulating gonadal steroid hormones had an impact on HPA axis response after mbTBI. Throughout the entirety of the study female C57BL/6J mice were tested for estrous cycle stage. Estrous cycle was tested two ways. Throughout the experimental timeline, females received vaginal lavage every day and cytology was determined to stage the estrous cycle. To confirm estrous cycle stage of the day of restraint, ovaries were collected. Ovaries were stored in 4% PFA until processing. Ovaries were embedded in paraffin and subsequently sectioned at 10 μ m and stained with hematoxylin and eosin. The presence of follicles or a corpus luteum determine proestrus and diestrus stages, respectively. A) On day of blast, the stage of estrous cycle was determined to examine if this had any effect on later response to restraint stress. There were no differences in CORT secretion 7-10 days after mbTBI between females that received mbTBI while in proestrus (high estrogen) or diestrus (low estrogen). B) On the day of restraint, the stage of estrous cycle was also determined. Stage of estrous cycle had no effect of restraint-induced CORT release after mbTBI.



Supplemental Figure 3. The stage of estrous cycle had no effect on CRF activation after mbTBI.

The goal of this experiment was to determine if the stage of estrous cycle on the day of restraint had an effect on the percentage of CRF neurons that were activated. To visualize CRF neurons, the CRF:tdTomato mouse line was used. Immunohistochemical labeling of cFOS was performed in order to measure neuronal activation. Estrous cycle stage was determined on the day of restraint by collecting the ovaries, following the methods in Supplemental Figure 2. Estrous cycle, on day of restraint, had no effect ($p > 0.10$) on CRF activation after mbTBI. There were no differences in CRF activation between proestrus (high estrogen) and diestrus (low estrogen) females.



Supplemental Figure 4. Mild blast TBI increases CC3-immunoreactivity in the hippocampus.

In order to examine if mbTBI results in apoptosis, we measured cleaved caspase 3 (CC3) immunoreactivity at different time points post-mbTBI. C57Bl/6J male and female mice were exposed to mbTBI and brains were collected either 1 day, 3 days, or 7 days post injury. Brains were placed in 4% PFA overnight and processed through a sucrose gradient in PBS. Brains were sectioned at 40 μ m and immunohistochemically stained for CC3 (green). Sections were mounted and cover-slipped with Prolong Gold Antifade with DAPI (blue). Additionally, we sought to determine if mbTBI increased microglial infiltration. In order to visualize microglial infiltration, we immunohistochemically stained tissue for iba1, a microglia marker (red).

A) Exposure to mbTBI increases CC3-ir in the hippocampus at 1 day, 3 days and 7 days post injury in both males and females. In males, the highest amount of CC3-ir was present 1 day after mbTBI. In females, CC3-ir remained high up to 3 days post mbTBI. By 7 days post mbTBI, CC3-ir reactivity decreased. Our preliminary data suggest that CC3-ir is only present in the hippocampus. B) Preliminary immunohistochemistry stains suggest microglia infiltration after mbTBI in the hippocampus (Red = iba1; Green = CC3; Blue = DAPI)

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