

Neuro-Behavioral Effects of Blast Injury on Neurohypophysial Hormone Producing
Neurons in Sprague Dawley Rats: Impact on Allostasis

by

Susan Galloway

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DEDICATION

This dissertation is dedicated to the memory of my parents, Kenneth and Theresa Jenkins, whose love and example led me to where I am.

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Susan Galloway

June 29, 2015

ABSTRACT

Title of Thesis: Neuro-Behavioral Effects of Blast Injury on Neurohypophysial Hormone Producing Neurons in the Sprague-Dawley Rat: Impact on Allostasis

Author: Susan J. Galloway, Doctor of Philosophy, 2015

Thesis directed by: Christine E. Kasper, Ph.D., Professor
Graduate School of Nursing

Service members and civilians are exposed to mild blast-induced neurotrauma through combat, terrorist, and other man-made events. Mild blast traumatic brain injury (mbTBI) can cause neurodegeneration with cognitive and behavioral changes that negatively impact health and quality of life. Magnocellular and parvocellular neurons in the paraventricular nucleus (PVN) and supraoptic nucleus (SON) produce oxytocin and vasopressin. These neuropeptides act as allostatic mediators to influence the stress response and learning and memory that underlie social behaviors. Following mbTBI the anti-inflammatory effect of minocycline can mitigate damage to neuronal tissue, and housing in an enriched environment (EEN) may also aid in neuronal tissue recovery. This study used immunohistochemistry to visualize the PVN and SON of Sprague-Dawley rats (N=23) to determine the effect of an mbTBI (20±3 p.s.i.) on oxytocin and vasopressin producing neurons. We then compared the effect of EEN and minocycline therapy on oxytocin and vasopressin neurons following mbTBI. We also compared the mbTBI effect on these neurons to behavioral test results of the Elevated Plus Maze (EPM), Barnes Maze (BM), and Open Field Test (OFT). Our results showed that mbTBI

resulted in complete absence of oxytocin and vasopressin immunostaining in the PVN and SON. Compared to controls, rats that were environmentally enriched had more oxytocin neurons in the PVN. Treatment with minocycline both immediately and at 35-38 days after injury, reversed the effect of mbTBI on expression of oxytocin and vasopressin neurons. We did not find that negatively affected neurons were related to spatial learning and memory scores. Furthermore, decreased number of oxytocin and vasopressin neurons did not reflect increased anxiety, but locomotor activity did decrease over time possibly due to the stress of repetitive testing. We can conclude that mbTBI resulted in significant loss of expression of oxytocin and vasopressin. This loss did not reflect poorer behavioral scores compared to controls. Minocycline and EEN did result in an increase in oxytocin and vasopressin neurons in the injured animals.

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Chapter 1: Introduction

Blast injury accounts for at least 75% of U.S. military casualties with 84.2 % of the greater than 300,000 known traumatic brain injuries between the years 2000-2014 classified as mild traumatic brain injury (mTBI). The authors of a recent report by the Institute of Medicine (157) concerning the effects of blast injury concluded that there is sufficient evidence demonstrating an association between mild blast TBI (mbTBI) and posttraumatic stress disorder (PTSD), post-concussive symptoms and headache. Based on studies of human subjects, the authors of this same report could not definitely conclude whether symptoms and disorder were a manifestation of the psychological impact of the experience, or if the injury resulted in the symptoms and disorders. However, when blast injury damages structures that mediate memory, learning and stress response, it is possible that PTSD and other neurobehavioral deficits can develop following mild blast-induced TBI (54).

The neuropeptides oxytocin and vasopressin influence certain behavioral characteristics in mammals and humans including fear, affiliation, social cognition, maternal behavior and anger in mammals and humans (41; 103; 121; 128; 140; 166; 197). The neurons which produce these peptides are located in central brain regions including the hypothalamus which may be affected by blast injury (154; 182). Most of the behaviors mentioned above are associated with learning, memory and stress response, thus giving rise to the search for a link between oxytocin, vasopressin and limbic structures including the medial prefrontal cortex, amygdala and hippocampus (16; 44; 58; 119; 238). This project sought to determine the effect of mild blast injury on neurons in

the PVN and SON of the hypothalamus and if a detrimental effect on these neurons correlated to behavior, learning, spatial memory outcomes and stress response.

BACKGROUND

Limbic hypothalamic pituitary adrenal (LHPA) structures in the brain are involved in memory, learning, emotion and stress response (58; 91; 105; 150; 198) . While a definitive description of the blast injury effect on neuronal structure and neurocircuitry has not been determined, studies using a rat model of blast-induced TBI have shown that components of limbic structures and the hypothalamic pituitary adrenal (HPA) axis can be compromised by primary blast wave (55; 114; 217; 225). Axonal and cell body damage can be a consequence of even mild TBI (38; 125; 212). Furthermore, it is recognized that many of the hormones produced and/or stored in the pituitary can be affected by TBI (216) and that oxytocin and vasopressin serum levels may be decreased suggesting a compromise somewhere in the oxytocinergic and vasopressinergic systems (225).

Role of oxytocin and vasopressin in learning and memory

Behavioral responses are associated with learning and memory processes that span multiple neuronal networks (59). Information to be learned and remembered is transferred between neurons via neurotransmitters and neuromodulators. Oxytocin and vasopressin produced in magnocellular and parvocellular neurons in the hypothalamus contribute to behavior, learning and memory by acting as both neurotransmitters (fast-acting chemicals that influence postsynaptic potential) and neuromodulators (chemicals that cause long-term changes by slowing or mediating synaptic effectiveness) (59; 81).

Nearly 50 years ago David De Wied and colleagues discovered through rodent models of passive and active avoidance behavior, that vasopressin facilitates consolidation and retrieval of acquired information while oxytocin tends to attenuate the same processes related to memory and learning (45). This discovery prompted hundreds of research projects by scientists seeking to confirm the effects of oxytocin and vasopressin and understand the underlying mechanisms of the effects. Research has continuously led to further questions about the roles and mechanisms of action of neuropeptides. Reviews of this ongoing body of research confirm oxytocin and vasopressin take part in behavior, learning and memory through interaction with numerous chemicals, limbic structures and neuronal pathways (37; 58; 147).

Role of oxytocin and vasopressin in stress and anxiety modulation

Conscious and unconscious threats to well-being activate the hypothalamic pituitary-adrenal (HPA) axis to produce glucocorticoid (GC) hormones. GC positively aid in response to threat through activating immune and metabolic processes in order to maintain homeostasis (105). After an extensive review of the literature, Engelmann et al. (58) concluded that oxytocin, vasopressin and corticotropin releasing hormone (CRH) all influence the production and release of adrenocorticotropin hormone (ACTH) and thus GC by acting on the anterior pituitary of the HPA axis. In response to stressors, oxytocin and vasopressin produced in the PVN reach the anterior pituitary through axon release in the median eminence, the posterior pituitary and vascular pathways (58).

Oxytocin, vasopressin and CRH also activate and mediate the stress response through central processes as well as by the HPA axis. Using microdialysis, Bulbul et al. (24) demonstrated that CRH released from the amygdala and PVN in response to acute

restraint stress was diminished with intracerebroventricular (i.c.v.) administration of oxytocin. This effect was accompanied by CRH mRNA decrease in conjunction with an increase in PVN oxytocin cells. When oxytocin release was blocked by an antagonist, CRH gene expression was upregulated during acute stress suggesting oxytocin has a central effect on attenuating stress.

In an emotional stress model based on an intruder male rat, Wotjak et al. (230) used microdialysis and plasma samples to show that a reaction of social defeat was accompanied by a significant increase in vasopressin released from the PVN. Administration of vasopressin and CRH antagonists via microdialysis into the PVN resulted in a significant increase in plasma ACTH. The researchers concluded that information could be relayed to the PVN by central structures to increase local vasopressin release from magnocellular neurons. This surplus of vasopressin then inhibits ACTH secretion at the level of the HPA. To demonstrate the effect of oxytocin in response to loss of vasopressin, researchers used a forced swim model and vasopressin knockout mice. Results showed a greater increase in oxytocin gene expression in the PVN with corresponding oxytocin plasma levels compared to controls (237).

Together these and many other research results confirm the hypothesis that neuropeptides mediate the stress response through central actions. To date, the general consensus is that oxytocin and vasopressin are vital to behavior in contextual, time, gender and even species dependent conditions. This variability of neuropeptide influence makes it difficult to make wide generalizations about their action across conditions and populations (45; 59; 81; 122).

Alleviating Blast-Induced Traumatic Brain Injury

With TBI as a major cause of physical and psychological disabilities, there is an urgency to find ways of treating TBI that mitigates or reverses the consequences of the resulting damage. The anti-inflammatory properties of minocycline have proven to decrease neural damage in rodent models of TBI making it a prospective pharmaceutical treatment for mbTBI (124; 192). Early classical studies have shown that rats raised in an EEN demonstrate greater cognitive abilities including problem solving (85). Recent studies have shown that rats treated with EEN after mbTBI have better cognitive outcomes than controls (123; 206). Neuroprotective qualities of EEN after TBI include increased hippocampal neurons and decreased lesion size compared to controls (159). Few studies have examined the effect of either minocycline or enriched environment (EEN) treatment modalities on neuropeptides after mbTBI. Here, in separate experiments, we tested the effects of minocycline and EEN on the neuropeptides and corresponding behavioral effects of mbTBI.

Effects of minocycline treatment on TBI outcomes

The inflammatory response following TBI involves cytokines known to be involved in a cascade of events that can lead to cell death (125; 179; 192). In a rodent model of TBI, cell death, injury lesion size and motor dysfunction were decreased with administration of minocycline after injury (124; 178; 192). Researchers have sought to understand if minocycline therapy improves anxiety and memory deficits as a result of its positive effects on alleviating inflammatory consequences. Several studies have shown improved memory function in a rat model of mild and moderate TBI when minocycline is given immediately after injury and over a period of several days. These same studies did not reveal a definite decrease in anxiety with the use of minocycline (124; 202).

Effects of Enriched Environment on TBI outcomes

In the laboratory setting an enriched environment (EEN) for a rodent generally consists of group housing and a cage large enough to hold a variety of objects aimed at stimulating curiosity and exploration. Following the work of Hebb (85), researchers sought to determine the structural and cellular effects underlying the improved cognitive effects of EEN. Increased depth of the cerebral cortex with corresponding decrease in neurons, glia and blood vessels was an early discovery of the positive effect of EEN following traumatic brain injury (47). Application of EEN as a therapeutic agent following TBI has improved motor function (142) and spatial memory in rodents (123; 142) as soon as one week after injury. Environmental enrichment significantly decreased markers of axon degeneration (TAU) in limbic structures compared to normal housed rats after TBI (123).

Social interaction through group housing seems to be a major factor in the EEN that contributes to significantly decreased anxiety, increased motor function and prevention of depression-like symptoms (133). Oxytocin neurons have been found to be significantly increased in the PVN, glucocorticoid receptors upregulated in the HC and serum corticosterone decreased in rats subjected to repeated restraint and then treated with group housing (133). This is an important finding since high levels of oxytocin in the PVN has been shown to increase efficiency of the HPA axis and simultaneously attenuate memory consolidation in a predator scent stress test (41). Together these findings provide support for further investigation of the effect of mbTBI on neuropeptides and therapies which may restore their functionality in case of their damage or destruction.

KNOWLEDGE GAP AND SIGNIFICANCE

A relationship between exposure to blasts and neuropsychological disturbances has been documented during the Napoleonic Wars, the American Civil War, World War I and II and to current conflicts in Iraq and Afghanistan (110; 180; 199). “Wind contusion”, “nervous disease”, “shell shock” or “post-concussive syndromes” are different names in different eras for traumatic combat-related injury with negative neurobehavioral consequences. Current military conflicts have resulted in an estimated 59% of military members experiencing TBI (8) and 63% of that number is due to blast effects (12). The cost of TBI to an individual and society is staggering. Human disorders associated with low-level blast injury may include headache, sleep disturbances, fatigue, depression, memory and attention deficits, anxiety and hypotension (14; 46; 187; 200). Approximately half of mild TBI patients have symptoms that persist indefinitely and can affect quality of life including difficulty with personal relationships and diminished ability to maintain employment that requires social skills, memory and concentration (201). In a recent epidemiologic study of service members with mild TBI, 41.3% reported a negative change in their overall health post-deployment compared to those with no TBI (16.5%) (86). Treatment for mild TBI is approximately \$32,000.00 per year per patient and care for combat-related depression and PTSD which may result from TBI was estimated at over \$6.0 billion dollars during a two year period (187).

Despite the known negative results of mbTBI on neuronal tissue and behaviors, few studies have examined the effect of mbTBI on neurons producing oxytocin and vasopressin. We also know that EEN and minocycline can mitigate mbTBI insult on neurons and behavior, but we do not know the effect of these treatments on oxytocin and vasopressin expression. To address this gap a rat model of mbTBI will be used to

examine expression of oxytocin and vasopressin with and without EEN and minocycline treatment. We also compare expression of oxytocin and vasopressin to behavioral test results to determine an effect between behavior and quantity of expression.

HYPOTHESES AND SPECIFIC AIMS

Hypotheses

The hypotheses of this dissertation were:

1. Mild blast TBI will decrease the number of oxytocin and vasopressin producing neurons in the PVN and SON of the hypothalamus.
2. Following mbTBI, minocycline and/or EEN will decrease loss of oxytocin and vasopressin producing neurons in the PVN and SON of the hypothalamus.
3. Loss of oxytocin and vasopressin producing neurons will result in a) increased anxiety b) decreased learning and c) decrease spatial memory.

Specific Aims

Using a rodent model of mbTBI, the specific aims of this project were to:

1. Determine the quantity of oxytocin and vasopressin producing cells after mbTBI and after treatment with a) minocycline or b) enriched environment.
2. Determine if there is an association between quantity of oxytocin and vasopressin neurons in the PVN and SON following mbTBI to behaviors known to reflect anxiety, learning and spatial memory

CONCEPTUAL FRAMEWORK

The conceptual framework guiding the logic underlying the aims and hypotheses of this study rests on the process of allostasis as the means of anticipating and adapting to

stress and change. Allostasis, allostatic load and allostatic overload will be discussed in detail in chapter two, but the aim is to determine if blast trauma results in damage to structures within the brain that are intended to help interpret, compensate and adjust to stressors. Brain damage involving mediators of allostasis may contribute to allostatic load. Untreated, allostatic load becomes allostatic overload which can manifest as disease.

Oxytocin and Vasopressin as Mediators in Allostasis

The neuropeptides oxytocin and vasopressin play a role in modulating neurobehavioral phenomena related to stress and behavior via central and HPA axis circuitry by acting as both hormones and neurotransmitters (45; 138; 211; 228). While stress stimuli activate the HPA axis, oxytocinergic systems mediate limbic hypothalamo-pituitary-adrenal (LHPA) activity to produce an anxiolytic effect demonstrated by decreased glucocorticoid levels and adaptive behavior in both animals and humans (128; 163; 203). Vasopressin on the other hand, may have an opposing effect on social behaviors dependent upon species and area of the brain releasing the hormone (45; 218). Vasopressin and oxytocin are two of the neuro-endocrine system mediators involved in allostasis that contribute to adaptation and stability (108; 195). Because vasopressin and oxytocin may be important mediators of allostasis, traumatic injury to brain areas that synthesize the hormones may result in increased anxiety and difficulty with spatial learning and memory. Oxytocin and vasopressin are vital chemicals associated with a number of sophisticated, but basic social behaviors. It is well established that oxytocin and vasopressin-like hormones exist across species including the most primitive vertebrates and invertebrates (4; 57; 96; 148; 160). The ancestral gene encoding oxytocin

and vasopressin neuropeptides has existed across the animal kingdom for at least 700 million years (4). Oxytocin and vasopressin neurons have evolved from single and bipolar neurons to multi-dendritic neurons with axon communication far outside the HPA axis to include limbic and forebrain regions (119; 120). That these peptides have been conserved and evolved for eons across species speaks to their importance in the well-being of organisms as part of systems that strive to achieve “stability through change” or allostasis (207).

SUMMARY

Mild bTBI may damage hypothalamic neurons that contribute to homeostasis through allostatic processes affecting social and emotional well-being. Behavioral and cognitive consequences of mbTBI involve brain regions which are influenced by oxytocin and vasopressin. This study was designed to address the hypotheses that mbTBI has a significant negative effect on magnocellular neurons, which results in a reduction in oxytocin and vasopressin with subsequent detrimental behavioral consequences. We also hypothesize that use of minocycline and/or enriched environment will mitigate the biologic negative effects of mbTBI by rescuing oxytocin and vasopressin neurons. The significance of this study is based on the assumption that oxytocin and vasopressin are mediators involved in allostasis and that a loss of these neuropeptides compromises physical and psychological stability. Considering the costs of TBI, it is important to know if neurons that produce important neuropeptides are affected. This new knowledge can then serve to prompt new research into how damage occurs and how it may be alleviated.

Chapter 2: Review of Literature

This chapter reviews the relevant literature supporting the specific aims and hypotheses of the study. A review of terms and theory related to stress reveals how allostasis and allostatic load provide a conceptual model of how organisms interact with, and respond to, environmental cues in order to anticipate and adapt to change. To explain the role of oxytocin and vasopressin in allostasis we review the literature surrounding the origins, evolutionary history and current knowledge of these hormone systems in man and rat. The importance of oxytocin and vasopressin as allostatic

mediators in the HPA axis will also be discussed in relation to mechanisms and consequences of mbTBI, minocycline and enriched environment.

THE CONCEPT OF STRESS

Humor and Greek medicine

All living things face a daily bombardment of challenges to the body. Life is dependent on the execution of appropriate adaptations to stimuli that challenge the balance between illness and health. The physical and psychological reaction to these challenges may have good or bad effects depending on many factors. Most of these challenges and the body's response to them go unnoticed or minimally so by the conscious mind.

The concept of balance in terms of health and illness has roots in antiquity. The 5th century Greek philosopher and medic, Alcmaeon described the need for equality between contrasting powers such as wet, dry, cold, hot, bitter and sweet in order for health to be present and disease kept at bay (97). The great late 5th century physician, Hippocrates built his theory of health and illness on the ideas of symmetry and equilibrium laid out by the Greek philosopher and mathematician Pythagoras. Hippocrates' school of medicine describes four humors or fluids, which were related to the elements of water, earth, fire and air. These humors, black and yellow bile, phlegm and blood were in constant motion in a healthy body and regulated by forces of nature. Similar to modern day thoughts regarding holistic medicine, Hippocrates emphasized the need to thoroughly examine the whole person including his background and lifestyle in order to discover the cause of the imbalance in the humors (23).

Stress

In the 19th century, French physiologist Claude Bernard hypothesized that it was the organism's ability to regulate fluids surrounding cells in tissues and organs of the body that was essential to life and health. American physiologist Walter B. Cannon extended Bernard's work by identifying internal and external factors that influence the physiologic regulation of an organism. Cannon coined the term "homeostasis" to describe the body's attempts to maintain physiologic balance (27). Somewhat in accord with Bernard's meaning of internal milieu, Cannon proposed that health begins at the cellular level. Importantly, Cannon recognized external influences as being both outside the body and outside the cell. Temperature, pH, hunger and thirst prompt adjustments in glucose, water, salts, proteins, oxygen and fat in order to maintain homeostasis (27). Cannon also proposed additional and yet to be discovered homeostatic regulating systems were involved in homeostasis. Today systems such as neuropeptides are recognized as central mediators of allostasis, but at the time of Cannon's writing he did not believe additional processes were as valuable as chemical regulation in homeostasis (27).

In 1956, Hungarian endocrinologist Hans Selye published "The Stress of Life" which brought his scientific work and concepts related to stress into contact with an audience outside scientific circles. Selye adopted the term 'stress' from physics and engineering to describe forces acting upon the body. According to Selye, the body reacts to stress by exhibiting a non-specific response through common elements and systems to restore homeostasis (77). Basic scientific studies using animal models led Selye to describe three stages of stress response which he termed the "General Adaptation Syndrome". Upon initial stress body reacts in alarm which is followed by adaptation. If adaptation is not achieved the organism will face exhaustion and death (77). Obviously,

adaptation to stress must be common considering the challenges and stresses faced by all living things on a daily basis.

CURRENT CONCEPTS RELATED TO STRESS

Blast injury incurs stress to the body that disrupts homeostasis. This disruption sets processes in motion to restore balance. These processes may not be effective or efficient if the mechanisms needed in the adaptive response have themselves been damaged by the physical stressor of blast. The result of the insult may be objectively witnessed and measured through immunohistochemical studies and behavioral responses.

Definition of terms

Stress

As previously pointed out, the term stress has its origins in the physical sciences, but in the health sciences and with the public in general, stress has come to be used to describe the many responses or conditions resulting from a force acting on the mind and body. Use of the term can be associated with speech as in “she stressed the word ‘maybe’”; as a physical pushing or pulling on an object; in reference to a type of injury e.g. “stress fracture”; or a state of psychological being such as “stressed out”. Here we will use the term *stressor* to mean an event or events that are perceived by the brain as threatening or disruptive to homeostasis. This threat sets in motion physiologic and behavioral processes and systems to maintain or restore homeostasis (153) which is commonly known as the stress response. *Stress*, then, is the psycho-neuro-biologic interpretation of possible adverse external or internal stimuli. In this study we use a systems concept of stress to explain how blast injury damages oxytocin and vasopressin neurons and how that damage can negatively affect allostasis.

Allostasis, allostatic load and allostatic overload

The brain is the interpretive center of incoming external environmental and internal signals and stimuli. Stimuli are received, processed and interpreted based on prior knowledge of the stimulus (193). If the stimulus is a stressor the response is to activate systems for adaptation. These systems include the HPA axis, the autonomic nervous system, immune and metabolic systems (149; 153). The activation of any of these systems to predict demand and adapt through change or make adjustments to tissues, organs and systems in the body is known as *allostasis* (78; 149) . Allostasis can be highly individualized since interpretation, environment, developmental history and genetics all impact perception of stressors (153). The dominant facilitators of allostasis are known as *mediators* and include the hormones of the HPA axis, catecholamines and cytokines (153). Although adrenal GC are recognized as the primary mediators in the HPA axis, additional hormones also mediate allostasis (108). Considering the influence of oxytocin and vasopressin on central and peripheral systems associated with social, psychological and physical health, it seems reasonable to place oxytocin and vasopressin also in the category of mediators important to allostasis.

Allostasis initiates a cascade of responses that result in a change in the usual time limit and/or quantity of mediators. The daily demand for these internal adjustments puts a strain on the systems that make the physiologic adjustments. This strain due to stress response is referred to as *allostatic load* (152). Allostasis is normal and some adjustments such as blood pressure, temperature and cortisol are expected in diurnal patterns. However, frequent stress, failure to shut down allostatic response, or inadequate allostatic response may become *allostatic overload* with consequences

including disease or psychopathology (40; 152; 153). We believe mbTBI contributes to allostatic overload as a result of damage to oxytocin and vasopressin producing neurons. Damage to these neurons results in an inadequate allostatic response to stress.

REGULATION OF THE HPA AXIS TO STRESS

Appropriate response to stressors is required to achieve homeostasis and avoid allostatic overload. Control of the stress response is also required for the same reasons. Parvocellular neurons (PCN) in the PVN release vasopressin and CRH to activate the HPA axis, but magnocellular neurons (MCN) in the PVN and SON also influence the HPA axis via oxytocin and vasopressin. The neurohypophysial peptide system which influences the HPA axis stems from magnocellular regions of the PVN and supraoptic nucleus (SON). Vasopressin and oxytocin are produced in magnocellular neurons found in both the PVN and SON. The neuropeptides are released from soma, dendrites and axon terminals to act as neurotransmitters and neuromodulators. When the neuropeptides are secreted into systemic circulation, they act as hormones (89; 127).

Hypothalamic-Pituitary-Adrenocortical Axis

Homeostasis is mediated through allostatic mechanisms, which include the autonomic nervous system (ANS) and the HPA axis. The ANS provides the classic “fight or flight” response to threatening stressors described by W.B. Cannon (27). Preganglionic neurons in the thoracic and lumbar regions of the spine are part of the sympathetic division of the ANS and serve to aid in emergency response to threat through innervation of the adrenal glands causing release of adrenaline. Opposing actions to sympathetic innervation is accomplished through the parasympathetic division of the ANS. Preganglionic neurons of the ANS direct information from the brainstem and

sacral sections of the spinal cord. Both sympathetic and parasympathetic divisions of the ANS must be integrated in order to avoid chaos and maintain or restore homeostasis (91; 105). The relatively slower neuroendocrine response to stress with the end result of GC release is accomplished through the HPA axis which includes the PVN, the median eminence, the pituitary gland and adrenal cortex. The paraventricular nucleus of the hypothalamus (PVN) is the epicenter of the stress response via three main effector pathways; the HPA axis, neurohypophysial peptide signals and autonomic regulation (88; 89; 93). Oxytocin and vasopressin are produced in the PVN and each play a role in these effector pathways. These peptides are also produced in the SON with axons projecting to the posterior pituitary (25) as well as somato-dendritic release into the SON (58; 165). See Figure 1 for a description of ANS and HPA axis influences on the adrenal organ.

Figure 1. HPA Axis and Autonomic Nervous System Responses To Stress.

Exposure to a stressor activates preganglionic sympathetic neuron in the thoracolumbar region of the spinal cord and innervates the adrenal cortex via intermediary neurons to produce adrenalin (blue circles). Opposing forces via the parasympathetic system (red circles) are also activated. The HPA axis (right side of figure) is activated by a stressor to secrete hormones in the paraventricular nucleus of the hypothalamus. Corticotropin and vasopressin are delivered to the pituitary to produce glucocorticoids. Other hormones enter systemic circulation from the pituitary gland. Adapted by permission from Macmillan Publishers Ltd: Nature Reviews Neuroscience ADDIN EN.CITE <EndNote><Cite><Author>Ulrich-Lai</Author><Year>2009</Year><RecNum>242</RecNum><DisplayText>(215)</DisplayText><record><rec-number>242</rec-number><foreign-keys><key app="EN" db-id="r2eda92rr55zfee50rcxt9zzr0ez0fsa95fw" timestamp="1377350450">242</key></foreign-keys><ref-type name="Journal Article">17</ref-type><contributors><authors><author>Ulrich-Lai, Y. M.</author><author>Herman, J. P.</author></authors></contributors><auth-address>Department of Psychiatry, University of Cincinnati, Ohio 45237-0506, USA.</auth-address><titles><title>Neural regulation of endocrine and autonomic stress responses</title><secondary-title>Nature reviews.Neuroscience</secondary-title></titles><periodical><full-title>Nature reviews.Neuroscience</full-title></periodical><pages>397-409</pages><volume>10</volume><number>6</number><keywords><keyword>Animals</keyword><keyword>Autonomic Nervous System/physiology</keyword><keyword>Brain/anatomy & histology/physiology</keyword><keyword>Endocrine System/physiology</keyword><keyword>Humans</keyword><keyword>Hypothalamo-Hypophyseal System/physiology</keyword><keyword>Neural Pathways/physiology</keyword><keyword>Pituitary-Adrenal System/physiology</keyword><keyword>Stress, Physiological/physiology</keyword></keywords><dates><year>2009</year></dates><pub-location>England</pub-location><isbn>1471-0048; 1471-003X</isbn><urls></urls><electronic-resource-

Anatomy of the PVN and SON

In the rat the PVN is a wing shaped structure that lies on both sides of the third ventricle in the hypothalamus. The neurons which compose the PVN are classified by function as 1) parvocellular secretory with axons projecting to the median eminence 2) magnocellular secretory with axons communicating with capillaries of the posterior pituitary and 3) long-projecting with axons extending to the brain stem and spinal cord

(83). Large magnocellular neurons that express oxytocin are mainly located in the lateral posterior magnocellular region. This area lies adjacent to a medial magnocellular site expressing vasopressin neurons. The smaller parvocellular neurons are found in the anterior and ventral medial parvocellular areas of the PVN. Both parvocellular regions send axons to the median eminence as well as extend to extrahypothalamic limbic areas

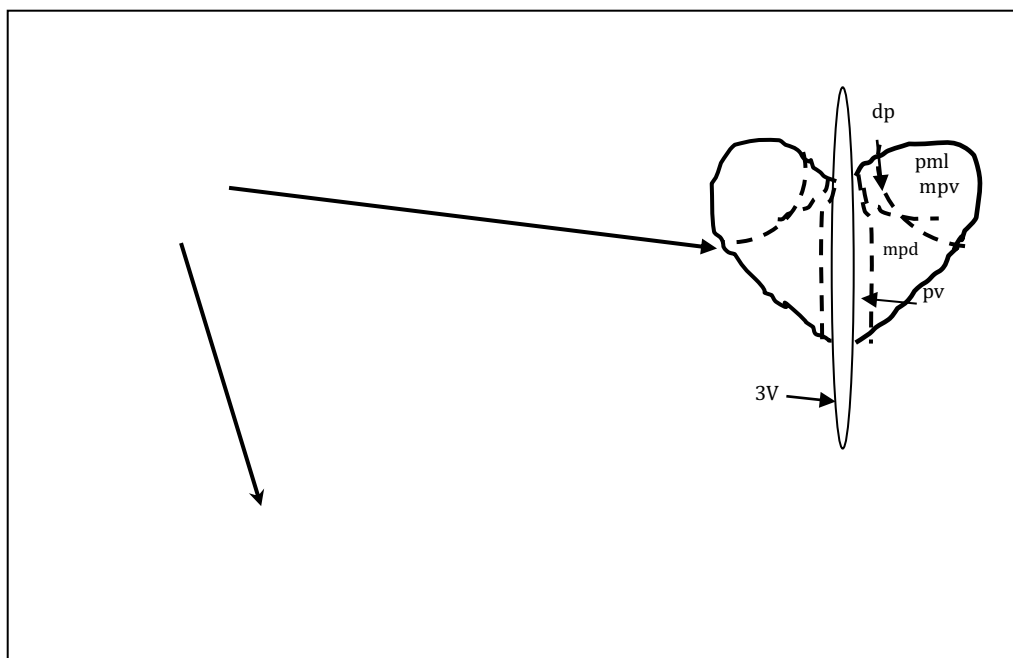
(83). In addition to resident neurons that give structure to the PVN, interneurons are also found in the PVN and aid in communication to extrahypothalamic and limbic regions

(83). The SON extends from the dorsolateral area of the optic chiasm (OC) rostral to the organum vasculosum of the lamina terminalis and caudally into the posterior hypothalamus. It is an oblong structure 1.5mm in length and 400 μ m at its widest point

(49) (see Figure 2).

Figure 2. PVN and SON of Hypothalamus.

Magnocellular neurons are mainly found in the lateral posterior magnocellular (pml) and ventral medial parvocellular (mpv) and smaller parvocellular neurons are located in the dorsal parvocellular (dp), dorsal medial parvocellular (mpd) and anterior parvocellular (pv) regions. The third ventricle (3V) divides the PVN into mirror sections of itself. OC; optic chiasm, Small dots in SON represent oxytocin and vasopressin magnocellular neurons.



The SON in the hypothalamus has been the site of choice for studying the gene expression of vasopressin and oxytocin due to almost exclusive production of those two products in that nucleus (72; 127; 205). It is generally believed that two phenotypes for magnocellular (MCN) and parvocellular (PVC) neurons exist in the PVN (52; 72). Neuropeptide expressing neurons in the SON are almost exclusively MCN that synthesize both oxytocin and vasopressin (21). Both MCN and PCN neurons are fairly large with the MCN neuron being the larger of the two and both having two to three distinct dendrites. One or the other neuropeptide is produced per neuron in approximately 97% of the population. There is evidence that at least some of the neurons produce both vasopressin and oxytocin in the same neuron (72). We found numerous neurons producing both neuropeptides in both the PVN and SON (see Figure 3). Magnocellular dendrites, as well as the soma, are capable of synthesizing the neuropeptides under investigation in this study (65; 127; 162).

Figure 3. Oxytocin and Vasopressin Co-Expression. Oxytocin (A), vasopressin (B) and merged (C) in same MCN in PVN. Oxytocin (D), vasopressin (E) merged (F) in same MCN in SON.

Vasopressin and corticotropin releasing hormone (CRH) expressing neurons are co-located in the parvocellular areas of the PVN and synergistically promote the release of adrenocorticotropin hormone (ACTH) in response to threats at basal and heightened stress periods (89; 194; 241). Oxytocin has also been found to influence the HPA axis response by attenuating release of ACTH in a stress-specific manner, and in some situations to instead increase ACTH (75; 167). In a study by Zheng et al., 2010 (239), CRH rose sharply and was accompanied by gastric dysfunction after exposure to an acute stress in a rat model. Gastric dysfunction was restored to normal emptying after i.c.v. administration of oxytocin. This restoration was reversed following i.c.v. administration

of an oxytocin antagonist. In addition, oxytocin mRNA and cells were significantly increased suggesting that oxytocin helps to dampen the stress response. There is also evidence of magnocellular system involvement in the immune response to stress.

Inflammatory response markers activate magnocellular neurons in the PVN and SON which then activate IL-6 neurons and stimulate release of oxytocin and vasopressin under both psychogenic and physical stress (22; 106; 172).

Activation of the PVN

The PVN is activated in response to acute homeostatic threats such as hypernatremia, hypovolemia and hypoxia via neurons projecting from the brainstem and spinal cord, but the PVN also responds in anticipation of events or stress (88; 89; 215). This anticipatory response must be mobilized through instinct or memory involving brain regions responsible for integration and interpretation of stressors of external origin. Central regions of the nervous system interpret internal and external signals and influence neuroendocrine systems to bring about an orchestrated response to threat that promotes allostasis. These central regions mainly involve limbic structures including the amygdala, medial prefrontal cortex (mPFC) and the hippocampus (HC) which are part of the effector system in relation to the PVN (88; 105; 215; 241). There is little direct innervation of the PVN by these limbic regions that influence the HPA axis, but there is evidence that they indirectly communicate with the PVN through intermediary neurons (119; 120; 164). Immunohistochemistry studies show that intermediary glutaminergic and gamma aminobutyric acid (GABA) carrying fibers project from limbic sites to glutaminergic and GABAergic projecting neurons in the PVN as a means of carrying

information to the PVN (68; 90; 161) . In the rat brain there are approximately 600 axon synapses to the magnocellular neurons in the SON and over 2800 in the PVN (52; 118).

In comparison to psychogenic stressors, following administration of a systemic inflammatory stressor, lesions of the infralimbic (IL) mPFC also result in increased ACTH secretion and PVN c-fos activation (66; 106).

The amygdala is key to emotional perception, fear response, anxiety and aggression. Lesions to the medial amygdala (MeA) result in HPA axis down regulation to stressors such as restraint, foot shock and noise indicating this area is responsible for reacting to psychogenic stressors (105; 215). The central amygdala (CeA) seems to be more reactive to systemic stressors than psychogenic stress (91).

The hippocampus (HC) is vital to memory function. Lesion studies to this limbic area result in enhanced HPA axis activity indicating the HC is primarily inhibitory in nature (87; 91; 105; 215). The HC is rich in glucocorticoid receptors (GR) making it a vital part of the HPA axis negative feedback loop (104; 170).

In summary, stressors are interpreted by limbic areas that forward the information to the PVN. The PVN activates the HPA axis neuropeptide responses to act as mediators and effectors in the allostatic system. Dysregulation of oxytocin and vasopressin systems may contribute to dysregulation of the HPA axis and allostatic overload.

OXYTOCIN AND VASOPRESSIN SYSTEMS: ROLES IN HEALTH, SOCIAL WELL-BEING AND RELATION TO ALLOSTASIS

Nearly all vertebrates and non-vertebrates have some form of the oxytocin-vasopressin peptide family in their genetic make-up. Arginine vasotocin is present in all non-vertebrates and is the ancient neuropeptide of vertebrates. Genetic changes to

vasotocin resulted in the two similar, but functionally different peptides now known as vasopressin and oxytocin (3; 4).

It is remarkable that the vasopressin gene has been conserved for at least 700 million years which attests to its essential role in biology. Although the basic structure of both vasopressin and oxytocin is highly conserved across mammalian species, oxytocin and vasopressin receptor distribution varies widely among species. The wide spread distribution of oxytocin and vasopressin receptors in central and peripheral tissue probably influences the varied and often opposing behavioral effects of these hormones (79).

While the basic chemical structure of oxytocin and vasopressin has been conserved over eons, the neurons that produce them have undergone transformation in both morphology and location (79; 120). Extrahypothalamic influence of these neuropeptides may occur by specialized sub-groups of neurons within the PVN that project to the central amygdala to attenuate fear response (117). In addition to direct synaptic contact with other neurons, somatic and dendritic release of the neuropeptide allows for diffusion into extracellular fluid to reach receptors outside the PVN and SON (120).

Physiologic influences of oxytocin and vasopressin

Oxytocin is well known for its contractile effect on the uterus during delivery and for milk production following suckling stimulus of the nipple. In males it is synthesized in testis, the epididymis and prostate glands (84; 128). Vasopressin's main physiologic effect is to act as an antidiuretic by increasing urine osmolality and decreasing water excretion. The antidiuretic effects of vasopressin are mobilized when serum osmolality

reaches 282 mOsm/kg (in humans) causing vasopressin to be released from the pituitary gland (211). Vasopressin is also a potent vasoconstrictor in response to hypovolemia by its influence on constriction of splenic, hepatic and renal vessels to conserve volume (25; 211).

Central influence of oxytocin and vasopressin

As previously discussed, a dynamic relationship exists between an organism's genetic make-up and its environment. This relationship impacts allostasis and subsequently physical and psychological health. Events which result in allostatic overload may be manifested by anxiety, depression and other stress-related disorders (40; 78; 151). Despite considerable progress in understanding the neurobiologic relationship between stress and behavior, current treatment for affective disorders do not usually result in a cure of the disorder. A more complete understanding of key components and pathways in the neurobiology of the disorders is needed to find optimal treatments (164). Because oxytocin and vasopressin are synthesized and released in response to social, stressful, and anxiety situations, they have become targets for research as therapeutic agents to combat anxiety and other affective disorders (28; 130; 158; 174).

Quantification of peptide response to stressors is usually measured with saliva, plasma and/or urine samples. Variation in quantity between sample source results suggests a difference between central and peripheral effect of stress on the neuropeptides (58; 127; 223). It can be difficult to conclude that low plasma levels of oxytocin and vasopressin reflect central response and impact since extrahypothalamic secretion of oxytocin and vasopressin may influence limbic areas without being reflected in plasma levels (112; 162; 164). The source of oxytocin and vasopressin levels is important in

interpretation of study results when trying to make an association between neuropeptide expression and behavior. The current method of choice is to measure microdialysates of oxytocin and vasopressin from i.c.v. and plasma neuropeptide levels (162; 231). Figure 4 depicts the effect of nasal application of oxytocin on brain areas responsible for learning, memory and stress response.

Figure 4. Brain Oxytocin System. Neuronal projections, release, receptor-mediated effects, and transport and effect of application of nasal oxytocin. Endogenous and exogenous oxytocin is released in extracellular fluid and binds to receptors in the PVN and limbic areas. Oxytocin in the PVN stimulates the HPA axis and is released into circulation. Little oxytocin passes the blood brain barrier, but it is easily transmitted to cerebral spinal fluid via nasal mucous membranes. The brain vasopressin system is comparable for synthesis, central release, peripheral secretion, and external application. Abbreviation: AN, accessory magnocellular nuclei. Reprinted from Trends in Neuroscience, 35(11), I.D. Neumann and R. Landgraf, Balance of oxytocin and vasopressin: Implications for anxiety, depression and social behaviors, p 650, 2012, with permission from Elsevier Ltd.

Oxytocin and vasopressin relationship to anxiety, stress and fear

Generally, anxiety is considered a negative emotion. We encounter events on a daily basis that we anticipate will be unpleasant. This negative emotional anticipation can produce feelings of anxiety. Acting in usually opposite directions, oxytocin and vasopressin aid in regulating anxiety (71; 126; 164). Oxytocin can be anxiolytic in response to anxiety producing stressors (128; 167; 226; 235). Oxytocin receptor antagonists are often used to determine the effects of oxytocin and vasopressin on behavior. Other methods used to determine oxytocin effect have included lesions to the PVN, limbic areas and HPA axis, comparisons between high anxiety behavior (HAB) and low anxiety behavior (LAB) rats, and gene knockout breeds of rats. (107; 144; 224).

Oxytocin administered via microdialysis in or near the PVN while subjecting the animal to a behavioral test has had varying results. Variability may be due to location of the microdialysis tubes, subject gender and/or estrogen cycle (167; 230). Studies that controlled for estrogen by ovariectomy and estrogen therapy prior to exposure to stressor and i.c.v. oxytocin, demonstrated an anxiolytic effect of oxytocin. Elevated plus maze results significantly showed the oxytocin treated animals spent a greater amount of time in, and entries to, the open arms than control groups suggesting oxytocin lessened anxiety (221; 226).

In regards to influence on the HPA axis response, oxytocin appears to decrease corticosterone production by attenuating CRF expression in the PVN (24; 75; 144; 239).

Infusion of i.c.v. oxytocin and real time polymerase chain reaction analysis has shown significantly decreased amounts of CRF in the PVN following acute and chronic stress (239). Immunohistochemistry studies of oxytocin in the PVN shows a significant increase in oxytocin expressing cells after all stress and especially after acute stress. Cohen et al. (41) demonstrated infusion of oxytocin into the HC results in increased stress resistance as demonstrated by a significant decrease in stress responses measured by acoustic startle and elevated plus maze tests. Researchers proposed that oxytocin may disrupt consolidation of short term memory to long term memory in the HC resulting in the anxiolytic effect (41).

Vasopressin tends to have the opposite effect on behavior compared to oxytocin. Methods similar to those used in determining oxytocin effects have been employed with vasopressin. A number of studies have shown that vasopressin is anxiogenic (19; 20; 98). It is interesting to note that experiments with several different vasopressin V1b receptor antagonists have proven to result in increased anxiety (98; 230). Similar to oxytocin, but in a different direction, vasopressin appears to enhance HC memory processes that may contribute to anxiety (6).

Oxytocin, vasopressin and social behaviors

According to social brain hypothesis, human brain size developed in adaptation to an increasingly complex social environment that required sophisticated processing and interpretation of complex social cues (51). In support of the hypothesis is size of the neocortex in comparison to the medulla. The neocortex where higher order cognitive processes such as learning, memory, emotions and consciousness are processed, is over one hundred times the size of the medulla (50). While location of oxytocin and

vasopressin neurons and circuitry to the neurohypophysis is well conserved across species, there seems to be significant plasticity for expansion of these peptide circuits to central brain regions that involve social behaviors (51; 79).

Table 1. Behavioral Effects of Oxytocin and Vasopressin.

As shown in Table 1, oxytocin and vasopressin are associated with a number of social and emotional behaviors. Social memory and cognition are key to recognizing significant others and to the creation of bonds that contribute to meaningful relationships, a sense of belonging and satisfaction (128). This social bonding, or affiliation, is important in helping to curb allostasis from becoming allostatic overload by mitigating stress and anxiety (77; 149). The affiliative behaviors of mating and pair bonding are facilitated by oxytocin and vasopressin as demonstrated in the prairie vole which tends to be monogamous (228). Experiments showed that both oxytocin and vasopressin antagonists blocked partner preference after mating compared to control groups which sought out their mated partner over other females (228). In addition, vasopressin antagonist blocked aggression following mating in prairie voles without previously established aggressive behaviors (102; 228). Bonding in humans requires trust and

insight into other's feelings and emotions. Studies using intranasal oxytocin tend to show an increase in trust and emotional intelligence in subjects treated with oxytocin (128).

A fascinating randomized control study by Kosfeld et al. (121) included a trust game involving an investor and trustee in an exchange of real money. The experimental group of investors received intranasal oxytocin prior to deciding how much money to give the trustee. The trustee would receive three times the initial investment from the experimenter and had the option to return none to all of the money to the investor at the end of the game. The amount of money the investor was willing to trust to the trustee was statistically significantly higher in the experimental group versus the placebo group ($p = .02$). To understand if oxytocin overcame a general aversion to risk or contributed to trust, the same experiment was run using a random mechanism for decisions made by the trustee (example, spin the dial or coin toss). Knowing the risk was up to chance, the investor control and experimental groups did not differ in the amount of money willing to invest, which was a substantially lower amount than when the human trust element was involved.

Parental behavior is a strong determinant of offspring health with parental affiliation to newborns having long-term and even trans-generational effects (33; 69). Rat pups that were handled in the first days of life developed increased number of glucocorticoid receptors (GR) in the HC. The increase in GR enhanced the negative feedback response associated with the HPA axis resulting in a decrease in circulating glucocorticoids (156). Positive maternal behavior in the rat includes nest building, pup carrying, licking and grooming and arched-back nursing (176). Licking and grooming

and arched-back nursing have been correlated to neural and cognitive development (34). Pups cared for by mothers with high levels of maternal behaviors developed greater numbers of GR that persisted into adulthood (132). The increased GR contributes to efficiency of the glucocorticoid negative feedback system. Decreased circulating GC means less wear and tear on the body over the course of a lifetime. In human females, oxytocin predicts maternal behaviors. Mothers with higher oxytocin levels demonstrate high levels of maternal behaviors including gazing at the infant, affectionate touch and vocalizations to the infant (63).

In summary, magnocellular and parvocellular neurons in the PVN produce oxytocin and vasopressin which play a role in allostasis through mediation of the HPA axis. In addition, these hormones activate, and are acted upon by limbic areas that influence behavior and affiliation. Figure 5 depicts a proposed system of oxytocin and vasopressin as mediators related to the HPA response to stress.

Figure 5. Role of Oxytocin and Vasopressin as Allostatic Mediators.

The HPA is the monitored variable (corticosterone (in the rat) and ACTH). Interpretation of information surrounding the stressor is processed in the brain as Afferent Information. Information relayed to the PVN activates mediators including oxytocin and vasopressin to change output according to predicted allostatic need. Oxytocin and vasopressin exchange information with the limbic region to further influence interpretation and reaction to stimuli.

BLAST TRAUMATIC BRAIN INJURY AND EFFECT ON ALLOSTASIS

Traumatic brain injury is a fairly common injury in the U. S. with around 1.4 million people per year seen in hospitals and clinics following injury (210). Traumatic brain injury is also common in military populations with over 300,000 such injuries and 82% of these being mTBI between the years 2000 and 2014 (29). While blast TBI is uncommon in most civilian populations, it is a significant mechanism of injury among military personnel due to explosive devices used in combat situations (187). Symptoms of survivors of blast TBI include memory impairment, anxiety, attention deficits, depression, headache, neuroendocrine deficits and problem solving deficits (31; 38; 187; 191) all of which are associated with mTBI (14). In addition to similar human cognitive deficits, studies of blast TBI in rodent models have revealed proteomic, genetic, endocrine and cellular changes (38; 114; 115; 125; 182). Considering the physical and psychological effects of blast TBI, disruption of allostatic and homeostatic mechanisms should be evaluated in survivors. Oxytocin and vasopressin have been shown to influence many of the behaviors that are disrupted or manifested by mbTBI. The aim of this study was to uncover evidence that blast injury could result in damage to oxytocin and vasopressin neurons and that this damage correlated to behaviors typically associated with survivors of mbTBI.

Mechanisms of blast-induced brain injury

The physical disturbances to the brain caused by explosions are complex and being studied with the use of forensics, animal models and computational modeling. (209). The consequences of blast generated forces to the body depend on five main factors peak pressure of the initial blast wave, duration of the overpressure exerted on the body, materials used to cause the explosion, one's distance from the detonation of the explosion, and the amount of focus to the body by the blast wave (30; 208).

The ensuing physical damage can be found to result in four main categories of injury that include 1) primary blast injury 2) secondary injury caused by debris that is hurled at the body by the force of the blast wave 3) tertiary injury from the acceleration of the body being thrown into objects or the ground causing blunt trauma 4) burns from the heat generated by the explosion (30; 56; 209). Figure 6 illustrates primary, secondary and tertiary injury mechanisms generated by an explosive blast wave.

Figure 6. Primary, Secondary and Tertiary Effects of Blast Waves.

Primary blast wave is

generated by the explosion. Blast waves travel rapidly away from the explosion causing

objects to be hurled through air. When objects strike the body, secondary injury may ensue.

Tertiary injury occurs when the body is propelled into objects including walls and ground.

The mechanism of blast injury in this project is produced by a primary blast wave. The initial wave of air caused by an explosion travels outward from the epicenter of the explosion causing a rise in pressure that generates a high velocity wind (229). The space left by the blast is the blast over-pressure (BOP) which is the amount of pressure over the normal atmospheric pressure (56). Research concerning the pathologic effects of primary blast waves has been challenging because the effects of blast in an open environment differs from those in an enclosed environment which can give rise to multiple waves (35; 185; 229). The threshold for detectable primary blast injury in a lab setting has generally been 17-24 pounds per square inch (psi); pressures which would require close proximity (10-20 m) to the explosion (35). In a real world setting, this closeness to the explosion would probably cause death due to secondary and tertiary mechanisms (35).

In the controlled environment of the research laboratory, injuries resulting from primary blast waves alone are better determined than in the natural setting. Minus secondary or tertiary effects, the etiology of bTBI is currently thought to be due to . degree of skull flexibility (17), transmission of increased thoracic pressure to the brain (ripple effect) (32), head acceleration (80), and/or direct entry of blast wave to the cranium (43; 114).

Determination of blast psi threshold for bTBI has generated a great deal of research over the past several decades. A study by Vandevord et al. (217) demonstrated astrocytosis, cell death, neurodegeneration, and learning and memory deficits at blast levels between 117 kPa (16.9 psi) and 153 kPa (22.1 psi) with the most significant histologic and behavioral changes found at 117 kPa. Similar findings in a study by Kamnaksh et al. (115) found evidence of astrocytosis and cell death in the hippocampus following a single blast injury with an average BOP of 138 kPa (20.01 psi). In this same study, no significant behavioral differences attributable to injury were found. PSI between 7.10-11.2 have also produced astrocyte death and increased amyloid precursor protein (APP) (143). APP is involved in the generation of amyloid plaques found in Alzheimer's Disease and is thought to increase risk of developing the disease following TBI (2). Another study found oxytocin and vasopressin gene alterations to be among the 676 gene expression alterations discovered following mbTBI (182).

Altogether, these research findings help to establish thresholds for low level blast injury that results in detectable molecular, cellular, and behavioral changes. The blast levels used on animals that this study is based on averaged 20.6 ± 3 psi (142.06 kPa). Thus, the BOP referred to in this study was equivalent to similar studies of low-level blast and injury results at those pressure levels.

INTERVENTIONS TO MITIGATE EFFECTS OF BLAST-INDUCED MILD TBI

Minocycline

Minocycline is a second generation tetracycline that has been safely used for decades to treat gram positive and gram negative bacteria (73). Minocycline is accessible to cells in the central nervous system and cerebral spinal fluid due to its ability to cross

the blood brain barrier (73). With the discovery of minocycline's neuroprotective effects in an animal model of ischemia (73), the door was opened to research activities that showed the drug to also be effective against numerous neurologic disorders including multiple sclerosis, spinal cord injuries, Parkinson's disease, Huntington's disease (36), Alzheimer's disease (39), and amyotrophic lateral sclerosis (36; 39; 73; 234).

Important to this study is minocycline's non-antibiotic effect on mbTBI. Studies that demonstrated minocycline ability to decrease microglia activity (61; 134) and its anti-inflammatory and neuroprotective effects (192; 234) have prompted researchers to investigate the effect of minocycline on TBI. As previously discussed, TBI is known to induce cell death and inflammatory response (124; 179; 204). An animal model of TBI using minocycline treatment demonstrated significantly decreased inflammatory markers compared to a control group of sham-injured saline treated rats (124). There may be an endocannabinoid connection with minocycline that promotes neuroprotection. In a closed head injury experiment, mice treated with minocycline had significantly decreased brain edema, diffuse axonal injury (DAI) and decreased neurological deficits compared to a control group. These positive effects of minocycline were absent in the group treated with endocannabinoid receptor antagonists, suggesting a role for endocannabinoid in the beneficial effects of minocycline (137). Several studies have shown that minocycline can significantly reduce the size of TBI induced lesions (95; 192; 220). Caspase 1 and 3 known to be involved in apoptosis after TBI, is repressed with the use of minocycline (190; 217) and protein markers NSE, NF-H, Tau, S100 β are decreased following treatment with minocycline (124). Inflammatory and protein markers have been found to be significantly elevated in the amygdala, prefrontal cortex, dorsal hippocampus (DHC)

and ventral hippocampus (VHC) of injured vehicle treated animals, but these markers were significantly less in minocycline treated groups (124). Similar results for markers of neuron injury were found in all four brain regions with the exception of GFAP which was not significant in the amygdala. Minocycline treated rats had significantly decreased levels of these same injury markers in the VHC.

Altogether, the results demonstrate the efficacy of minocycline in reducing neuronal injury and inflammation related to mbTBI. Decreased injury and inflammation results in decreased lesion size. Behavioral test results in the study by Kovesdi et al. (124) also revealed significant differences between minocycline and saline treated animals with respect to locomotor activity, anxiety and memory. Injured vehicle treated animals had worse scores in behavioral activity than the minocycline group.

Neuroprotective effect of oxytocin

There may also be a role for oxytocin in neuroprotection following mbTBI. Cells incubated with oxytocin and then exposed to neurotoxin 6 hydroxydopamine (6-OHDA) mimic effects similar to Parkinson's disease. The cells incubated with oxytocin had a significantly higher survival rate over cells not provided oxytocin (10). This same study also demonstrated oxytocin as a significant mediator for proliferation of neuroblastoma cells, suggesting that oxytocin may have an anabolic role in the cell cycle. Similar oxytocin mediated cell proliferative effect was seen when oxytocin was administered following a cold water swim stress test. Under this stress GC is activated with a decrease in neurogenesis in the HC. The rats treated with oxytocin continued to show neurogenesis compared to saline treated animals suggesting a neuroprotective effect as well as proliferative effect when oxytocin administration was sustained (129).

Normal physiologic processes produce oxygen metabolites that are harmful to cells and cellular processes (236). In a healthy body, mechanisms exist to maintain a balance between production and elimination of oxygen metabolites. An imbalance between production and elimination of oxygen metabolites is known as oxidative stress and is responsible for tissue destruction and disease (236). Markers of oxidative stress have been found as a result of mbTBI (1; 184). Vascular damage to brain tissue including the blood-brain-barrier (BBB) (1) and neuronal degeneration in the HC are consequences of oxidative stress secondary to mbTBI (190). In combination with a social environment, oxytocin has been shown to decrease markers of oxidative stress, inflammatory response and infarct size in an ischemic injury model (116).

Social factors influence injury and disease outcomes (123; 129) and oxytocin has neuroprotective and cell proliferation effects following injury (116; 129). Due to their anti-inflammatory, antioxidant and neuroprotective effects, minocycline and oxytocin together may be promising therapeutic agents in treating the neurobehavioral effects of mbTBI.

Enriched Environment

In animal models (typically rodent), enriched environment (EEN) is the application of a housing environment that includes toys, running wheels, tunnels, ramps and other objects in a large living area (18). Animals are usually also housed in groups to provide social stimulus (145). This type of living arrangement has been shown to replicate a more complex physical and cognitive environment that stimulates the brain (18; 168). Opposed to EEN, standard (normal) rodent housing allows only one to two

same sex animals to co-habit a typical 17 square inch laboratory cage. Standard housing usually accommodates for basic sustenance only (7; 206).

Enriched environment began to be used as an investigative variable in the 1960's to identify reasons for increased brain weight of rats exposed to environmental complexity and testing (ECT) (18; 47; 48). The early EEN researchers found significant histology changes in the brains of the ECT rats which they proposed was due to increased dendritic branching with increased glial cells to support the dendrites (47; 48). Once EEN was firmly established as a means of influencing changes in the brain, understanding the mechanisms responsible for those changes became the focus of many studies (168). Relevant to this study, Kovesdi et al. (123) examined the effect of minocycline on behavior, molecular and cellular responses to mbTBI. Using a shock compression driven tube to deliver a mbTBI, researchers found that rats housed in an EEN scored significantly better than normal housed rats on the Barnes Maze test at various test periods between days 16-71 after injury. These rats also scored slightly better than sham injured rats housed under normal conditions suggesting that EEN positively influenced learning and memory. The same study did not find a significant difference in anxiety scores on the elevated plus maze, but molecular markers for axon degeneration (TAU) and inflammatory response were significantly less in the injured EEN group versus the injured normally housed rat. Together, findings indicate that EEN aids in recovery of neural structures following mbTBI (123).

Animal models for studying human disease have been in use since the time of the early Greek physicians. Following a period of disuse in the Middle Ages, there has been a sustained resurgence in animal models of disease since the Renaissance (70). In the

twentieth century an appreciation for the standardization of subjects and processes led to an increase in reliability of test results for translation to clinical application (60; 70; 171). Rodents emerged as a cost-effective subject malleable through genetics and environment of mimicking many human maladies. More than thirty-four studies between 1996-2013 have used a rodent animal model to examine the effect of EEN to TBI (18).

Oxytocin, Vasopressin and EEN

Group housing of animals is usually a feature of EEN based on positive social consequences to stressful experimental conditions such as restraint (133; 189). In a study by Ruscio et al. (189), rats housed in isolation were found to have significantly higher plasma corticosterone levels than rats housed with a sibling or even a stranger. The researchers also found that vasopressin cells of isolated rats were significantly decreased in the PVN compared to group housed rats. There was no difference in the number of oxytocin cells. A study by Liu et al. (133) looked at the effect of housing type on anxiety and depression-like responses to chronic restraint stress. Interestingly, there was a significant increase in oxytocin neurons in the PVN of the group housed mice as opposed to the single housed animals. This increase in neurons was found in both restraint and non-restrained groups in EEN housing. The increase in oxytocin neurons also correlated to decreased behaviors of anxiety and depression as measured by the elevated plus maze, forced swim test and open field test. Another study which used an animal stroke model found that mice housed in groups had decreased infarct size, neuroinflammatory response, and oxidative stress. The beneficial effects of grouped housing were eliminated when oxytocin antagonist was applied (116).

Altogether, these studies encompassing brain damage and behavioral results of stress models have demonstrated that oxytocin and vasopressin play a role in the neuroprotection that is enhanced by the social element of EEN. To date, few studies have examined the effect of EEN following mbTBI on oxytocin and vasopressin neurons in the PVN and SON. This study hypothesizes that treatment with EEN after mbTBI results in a significant increase in saved oxytocin and vasopressin cells as compared to animals housed under conventional means.

SUMMARY

Stress and its effects on the human body and mind has been attempted to be understood since ancient times. Modern pharmacologic and laboratory tools as well as animal models, have helped to identify psycho-neuro-biologic processes involved in stress. Stress is a system response to internal and external stimuli that prompt the PVN in the hypothalamus to make allostatic adjustments to achieve biologic stability. Oxytocin and vasopressin are products of neurons in the PVN that may be used as mediators in the allostatic system. As mediators they may directly influence the HPA axis and stress response through secretory actions, but may also interact with limbic regions by direct (axon and dendritic diffusion) and indirect (intermediary neurons) means to influence interpretation of stimuli. Low level blast injury is known to inflict brain injury including cell death, neuron degeneration and increased levels of inflammatory markers (115; 125; 182). In addition, learning and memory deficits and behavioral changes have been noted following mbTBI. Oxytocin and vasopressin gene alterations have been noted at low level BOP. Minocycline and EEN have proven to mitigate BOP damage to the brain. Several studies have shown oxytocin to have neuroprotective effects in trauma and

disease. This doctoral dissertation was conducted to further the knowledge concerning the role of oxytocin and vasopressin as mediators in the PVN stress response and their possible role in neuro-behavioral responses to injury following mild blast TBI.

CHAPTER 3: METHODS

In this chapter a review of the experiments which yielded the donated brains including a) mild bTBI due to blast overpressure b) minocycline treatment at various time periods after injury c) enriched environment treatment after injury and d) behavioral tests will be described. The experiments conducted for the current study include a) specificity controls b) IHC c) imaging techniques and d) quantification of neurons method. A description of the statistical methods with the rationale used will be given at the end this chapter.

OVERVIEW OF EXPERIMENTAL DESIGN

Rat brains (N=23) were obtained from those banked during the conduct of Veterans Administration (VA) Grant B5044R to Dr. C. E. Kasper. The experimental design of these VA Grant experiments were all randomized pretest-posttest with control and experimental groups. Results of the three VA studies on these animals contributed to formation of the questions, specific aims and hypotheses that are proposed in the current study. Experiments conducted directly by the author of this dissertation research included tests for primary antibody specificity, immunohistochemistry (IHC) and microscopy for visualization and quantification of hypothalamic neurons in the laboratory

by the candidate. Results of these experiments were compared to behavioral test outcomes.

Hypothesis 1

The hypothesis that mbTBI would decrease oxytocin and vasopressin neurons in the PVN and SON was tested through an experimental posttest-only comparison group design. The dependent variables were quantity of oxytocin and quantity of vasopressin. Independent variable was injury type; blast (n=7) or sham (n=6).

Hypothesis 2

We hypothesized that housing the injured rats in an EEN or treating them with minocycline after injury would decrease loss of oxytocin and vasopressin neurons in the PVN and SON. Testing and analysis of effect was done in two separate experiments.

In the effect of housing experiment, after injury rats (N=8) were assigned to the following groups: sham injured – normal housed (SI/NH; n=2), sham injured – enriched environment (SI/EEN; n=2) which served as control groups for; injured – normal housed (I/NH, n=2) and injured – enriched environment (I/EEN; n=2).

In the pharmaceutical type experiment, rats (N=14) were assigned to groups: sham injured – saline – normal housed (SI/V/NH; n=2), sham injured – acute minocycline – normal housed (SI/AM/NM; n=2) and sham injured – delayed minocycline – normal housed (SI/DM/NH; n=2) which served as control groups for; injured – acute minocycline – normal housed (I/AM/NH; n=2), injured – saline – normal housed (I/V/NH; n=5) and injured – delayed minocycline – normal housed (I/DM/NH; n=1).

Variables

The dependent variable in both experiments of hypothesis 2 is number of oxytocin and vasopressin expressing neurons in the PVN and SON. Experiment 1 independent variables are housing type (normal or EEN) and injury type (blast or sham). Experiment 2 independent variables are injury type (blast or sham) and pharmaceutical treatment type. Pharmaceutical treatment type included saline, acute phase minocycline or delayed phase minocycline.

Hypothesis 3

An experimental pretest-posttest with comparison group was used to test if loss of oxytocin and vasopressin neurons increased anxiety, decreased learning and/or decreased memory. Only rats that had not received minocycline or EEN treatment were included in the hypothesis testing. The number and timeframe of behavioral tests varied between the three different VA experiments from which we used banked brains for this study. Also, testing prior to injury was not conducted for every behavioral subtest. Only behavioral subtests with available pre-injury and post-injury test scores were used for analysis in this study. Table 2 describes the behavioral subtests used in this study.

Table 2. Behavioral Test Periods and Sample Size. Pre and posttests conducted on injured rats that received minocycline or EEN before or after blast injury. BM, Barnes Maze; EPM, elevated plus maze; OF, open field; TOA, time in open arms; TDT, total distance traveled; TCA, time in closed arms; HA

BM	yes	3-14	21-51	-	7
EPM-TOA	yes	2-9	43-45	-	5
EPM-TDT	yes	2-9	43-45	-	2
EPM-TCA	yes	2-9	43-45	-	2
OF-HA	yes	1	8-28	45-63	5
OF-TDT	yes	1	8-28	45-63	5
OF-VA	yes	1	8-28	45-63	5
OF-RT	yes	1	8-28	45-63	5

Variables

The independent variable in hypothesis 3 is time with up to 4 levels (pretest and posttest periods). Dependent variables include behavioral test scores on the Barnes Maze test, EPM subtests; time in open arms (TOA), total distance travelled (TDT), time in closed arms (TCA) and OF subtests; horizontal activity (HA), TDT, vertical activity (VA) and rest time (RT). TOA, TCA, RT, Barnes Maze scores are measured in seconds. TDT is measured in meters. HA and VA is measured by the number of light beam breaks.

SUBJECTS

All animals in the experiments were handled according to the protocols approved by the Institutional Animal Care and Use Committee (IACUC) at the Uniformed Services University (USU).

For these experiments brains were harvested from 23 male Sprague-Dawley rats (Charles River Laboratories, Wilmington, Massachusetts) weighing 245-265 kg. Only males were studied to avoid variability caused by hormonal fluctuation during the estrous cycle in females. A reverse light-dark cycle with 12 hours each period was used to allow for testing during the naturally nocturnal rat's active period (181). Prior to the beginning of experiments the animals were allowed to habituate for seven days. Next, animals underwent behavioral testing to establish randomized group assignment. There was no

statistically significant difference in EPM horizontal activity behavior between groups which was the deciding factor in determining normalcy between animals (125).

BLAST OVERPRESSURE MODEL

In this study we used rat brains exposed to a blast overpressure (BOP) that replicated a mild primary blast that humans may be exposed to in battlefield or terrorist activities. The compression driven shock tube used to produce the BOP is kept at the Walter Reed Army Institute of Research in Silver Spring, Maryland. The overall length of the tube is 17.5 feet and one foot in diameter. The 2.5 foot compression chamber is filled by an air compressor to the point of rupturing a predetermined number of Mylar sheets that result in the down-tube BOP of force prescribed by the experiment (136). In this case the BOP generated an average of 20.6 psi at the terminal end of the tube. This amount of pressure is within a range determined to result in a mbTBI to the animal stationed at the end of the tube (114; 123-125; 136; 217).

Animal Preparation

All subjects were anesthetized using 4% isoflurane while in a plastic chamber prior to blast injury in order to minimize pain and distress. While it is an ethical and IACUC requirement to decrease pain and distress, care must be taken in choosing the concentration, type and delivery of anesthesia in order to avoid effects which may distort or influence outcomes following blast injury (233). Compared to fentanyl, isoflurane at the above dose resulted in rats that performed better on motor and cognitive activities and had less loss of HC neurons following experimentation and application of anesthesia (188).

After being anesthetized, the rats were outfitted with a protective vest before being placed in the holder. Long et al. (136) found the thoracic Kevlar protective vest saved all rats from death following injury by BOP between 126 kPa (18.27 psi) and 147 kPa (21.32 psi). Following anesthesia and protective vest placement, rats were placed in a transverse prone position and secured in the holder by tourniquets at the head, mid-section and over hind legs (233). Both sham and injured animals underwent identical preparation, but only the injured animals received a mild BOP. After injury, rats were placed back in their home cages and returned to the animal facility at the Uniformed Services University of the Health Sciences (USUHS).

HOUSING

A total of four animals (two sham-injured and two injured) were placed in an EEN following injury. The cage used for enriched housing was a four-tiered metal enclosure measuring 36”L x 25”W x 62” H. Interactive objects included toys such as wheels, tunnels, balls and igloos. Toys were replaced weekly with new ones to avoid habituation (123). Because EEN is known to improve certain neurologic disease and rats are naturally social creatures, it was hypothesized that EEN would attenuate effects of mbTBI by rescue of neurons involved in prosocial behaviors. (123; 133; 142; 168). All other rats (control) in the EEN and minocycline related experiments maintained habitation of two animals per standard laboratory cage with wood chip bedding and free access to food and water for the duration of the experiments (days 1-69) (123; 124).

MINOCYCLINE TREATMENT

All subjects in the minocycline experiment lived in standard (normal) housing. Minocycline treated subjects received 50mg/kg of body weight of clinical grade

minocycline (Minocin®) (Triax Pharmaceuticals, Italy). All rats in the minocycline experiments received minocycline or saline for four days beginning four hours after the injury (or sham injury). Rats in the delayed phase minocycline did not receive minocycline or saline until days 35-38 after injury or sham injury. Vehicle dose of saline was 0.25ml/100 gm of body weight which was approximately 0.9ml for a 350 gram rat. Minocycline and vehicle were administered intraperitoneally (i.p.) (115). The minocycline dose and treatment regime were based on similar experiments with rodent models. (124). Weight was monitored daily to ensure health and safety of animals. Based on the animal's weight the range of minocycline administered was 13.5mg-16.5mg which equaled a volume of 0.56ml - 0.8ml per dose. The animals whose brains are included in the current study received the full regiment of minocycline without disruption or significant weight loss (113).

BEHAVIORAL TESTS

In order to test hypothesis 3, all rats underwent evaluation of locomotor activity (open field activity), anxiety (elevated plus test) and spatial learning and memory (Barnes Maze test). Baseline behavioral testing prior to experimental injury intervention (with the exception of VA experiments originally conducted using EEN) was conducted to determine normality. No significant differences were found between rats after baseline data was evaluated. This assured the researchers that prior to injury, all groups were similar since all rats were similar (123; 124). Post-intervention evaluations ranged from 24 hours to 63 days after BOP injury.

Open Field Activity

The open field (OF) activity tests for possible locomotor deficits. The test has also been used to assess anxiety when subtests related to vertical activity, time spent in the margins and time spent in the center of the field are measured (125). We did not measure time spent in the margins or time in the center of the OF device. OF tests were performed using the AccuScan Open Field model (Omnitech, Electronics, Inc.) to measure vertical activity (index of depression and exploration), horizontal activity (general health and locomotion) and total distance traveled (locomotion) (115; 123; 124).

The AccuScan instrument measures 16.5Lx 16.5W x 13H inches and is made of clear Plexiglas with perforations in the lid. Light monitor consists of light beam arrays in the horizontal X and Y axes. The hardware detects beams broken by the animal so that the investigator can use computer software to determine the location of the rodent within the cage. The animal is tracked in the horizontal plane to record travel and rearing behavior and data is tracked and recorded with the manufacturer's software (Fusion 3.4) (169). To avoid confounding anxiety due to separation and novel environment, cage mates were tested concurrently for 60 minutes per session. After being placed in the AccuScan, animals were left undisturbed while activity was recorded (115). Test periods ranged from 24 hours to 63 days after injury. Test result scores used to calculate responses in this study are the average group scores for horizontal activity, total distance traveled, rest time and vertical activity at each of the three testing time points.

Elevated Plus Maze

The elevated plus maze (EPM) is an exploration-based model widely used to study ethological responses to threats that may be found in nature (26) and that reflect anxiety (124). Rats have a natural tendency to explore new spaces and things, but at the

same time want to avoid unprotected, bright and elevated places which makes the EPM a valid model for comparing motor activity to anxiety (26). Therefore, EPM was used to measure anxiety (time in the open and closed arms) and general locomotor activity (total distance traveled) (123; 124). The EPM has four intersecting arms that are 19.5L x 4W x 15.5H inches. Two of the arms have walls on three sides (closed arms) and two of them are open (open arms). The structure is positioned just over 39 inches above ground with a 90 lux light source over the center of the maze (123; 124). The light is quite low at this level and it was demonstrated by Pellow (177) that preference for the closed arms did not appear to be based on lighting levels. Animals were placed in center of the maze individually and allowed to roam for five minutes per test session. Activity was video-taped for later analysis using ANY-maze software version 4.2 by Stoelting Company, Wood Dale, Illinois. All rats included in this data analysis underwent baseline data testing. Post-injury data obtained was combined from various experimental periods to create two post-injury periods that spanned 2-9 days and 43-45 days after injury. Each rat was tested for one 5 minute period during each pre-injury and post-injury session. The maze was cleaned with 30% ethanol solution between subjects to avoid confounding influence of conspecific odor on activity level (123; 124).

Barnes Maze

The Barnes Maze is a less stressful test than the Morris Water Maze to assess learning and spatial memory in a rodent exposed to TBI (125; 141; 155). The rat is required to learn the position of a hole in the maze that leads to a darkened escape box. The animal should be motivated to escape from the open and lit surface of the maze to find the dark escape box. An improvement in ability to find the escape box is an

indication of learning and memory (115; 186). The Barnes Maze equipment is a circular platform with a 1.2 meter diameter. There are 18 holes around the periphery with one hole leading to the escape box. The escape box cannot be seen from the surface of the maze, but visual cues are set up to direct the rat to the correct box. The position of the escape box relative to the other holes did not change during the test periods.

Prior to injury each rat was trained to locate the escape box. Training involved first placing the rat in the escape box for 30 seconds before putting the box in the center of the maze. The rat was then allowed to explore the maze for a short period of time before being returned to its home cage. On each of two subsequent training days the rat was placed in the center of the maze and allowed to roam until it found the escape box or exceeded 240 seconds without finding the box. If the box was not found during the training period the rat was placed in the escape box for one minute. Training continued until latency time to find the box averaged 10 seconds indicating that spatial learning had occurred (123; 124). During test sessions, each rat was tested twice a day for five days at each time period. Video-taping was used to record the event and measure latency to find the escape box. While testing, if greater than 240 seconds elapsed without finding the box, as during training the rat was placed in the escape box for one minute before being returned to the home cage. Testing periods were compiled to include periods between 3-14 days and 21-51 days after BOP injury.

TISSUE COLLECTION

At the end of all tests animals were sacrificed and tissue was collected. Animals were placed singly in an induction chamber saturated with isoflurane and deeply anesthetized until there was no reaction to tail pinch. To preserve the tissue for histology,

the rats were transcardially perfused with cold phosphate-buffered saline (PBS) followed by 4% buffered paraformaldehyde solution. The rats were decapitated, brains removed and stored overnight in 4% buffered paraformaldehyde solution before immersion in cold 15% and 30% sucrose solution for cryoprotection. Brains were then embedded in Optimal Cutting Temperature (O.C.T.) solution in a cyromold before being moved to a freezer and stored at -80°C until sections were obtained (123; 124). Each of the 23 brains were whole-brain sectioned coronally at a 20 μ m thickness using a microtome inside a cryostat and mounted on positive charged slide plates with two sections per slide. Each slide was labeled with the subject number and numbered sequentially from rostral to caudal ends. Slices were then stored at -80°C in airtight slide containers until immunohistochemistry was performed on the sections containing the PVN and SON.

IMMUNOHISTOCHEMISTRY

Immunohistochemistry was used to detect antigens through the use of oxytocin and vasopressin antibodies. The antibodies were made visible by use of a secondary antibody that was conjugated to a fluorescent molecule. Sections from the PVN and SON were identified using coordinates and morphology described by Paxinos and Watson Rat Brain Atlas (175). Sixty four brain sections per animal (total 1,280 μ m brain area) were available to determine the sequence of slides that would encompass the paraventricular nucleus (PVN) and supraoptic nucleus (SON). Using frames 42-46 in Paxinos and Watson (175) and the prominent mammillary region of the hypothalamus and third ventricle as landmarks, eight sequential sections were determined to best represent the location of the PVN and SON. Slides 21-28 from each of the 23 subject brains were used for immunohistochemistry to visualize oxytocin and vasopressin.

A sequential double-labeling immunohistochemistry (IHC) procedure to identify oxytocin and vasopressin neurons was conducted according to Abcam, Inc. (Cambridge, Massachusetts) protocol recommendations and the Kasper laboratory. For quality control we worked with no greater than 16 slides per immunostaining test. Each immunostaining test was performed as follows; slides were removed from the freezer and allowed to thaw for 15 minutes before being rehydrated with PBS for 15 minutes. To decrease incidence of autofluorescence that could interfere with visualization of specific fluorescent tagged antibodies, ammonium chloride (50 mM in PBS) was applied to each slide for one hour at room temperature after thawing and rehydration followed by washing the slides in PBS for 5 minutes per wash. The ammonium chloride interferes with the fixative residues (formaldehyde) that tend to bind to proteins creating a product that emits light (11; 219). To facilitate availability of the antigens of interest to the antibodies, a membrane permeabilization solution of 200µl 3% Triton-X 100 in 800 µl PBS was applied to the samples for one hour. This detergent-based solution breaks protein links formed in the fixation period that hides the antigen and its epitope thus improving antibody penetration of the cell. A blocking solution of 200 µl 3% Triton-X 100 in PBS plus 500 µl 5% normal goat serum (NGS) plus 300 µl PBS to equal a 1 ml solution was applied to the slides and allowed to rest for one hour. The blocking solution allows the NGS to bind to non-specific antigens. Binding non-specific antigens decreases the possibility that the antibody of interest binds to extraneous antigens which would confound the test results with unwanted signal. The same solution minus the NGS was used to dilute the antibodies. Primary antibodies included mouse anti-oxytocin (ab39363) and rabbit anti-vasopressin (ab78364). All antibodies were purchased from

abcam®, (Cambridge, Massachusetts). The primary antibody solution was of 1 ml blocking solution with 1 µl oxytocin antibody (1:1000) and 4 µl vasopressin (1:250). Slides were covered with primary antibody solution in a tray, covered and incubated overnight at 4°C. The next morning the slides were washed with PBS three times for ten minutes then incubated for two hours with the secondary antibody (1:200). Secondary antibodies included Alexa® Fluor 594 donkey anti-mouse IgG and Alexa® Fluor donkey anti-rabbit IgG (abcam®, Cambridge, Massachusetts). Secondary antibody preparation and application was done in a darkened room to reduce photo bleaching. At the end of this period slides were washed with PBS one time for 15 minutes. Counterstaining was achieved using a DAPI solution of 10 µl DAPI plus 15 ml PBS. DAPI solution was prepared and applied one tray at a time for quality control that minimized exposure to light. Slides were incubated for 3 minutes and then washed with PBS for 5 minutes. A permanent mounting medium (CC/Mount, Sigma-Aldrich®, St. Louis, MO) used to preserve the fluorescent stained specimens was applied to each slide. Slide trays were covered and allowed to dry for several days before microscopy.

Controls

From an uninjured untreated rat, a 1,280 µm (64 brain sections at 20 µm/section) section of tissue containing the hypothalamus was used to identify the area of interest and as a positive control. Using a rat brain atlas and magnifying glass, landmarks indicating the approximate location of the hypothalamus were identified. Thirty two slides (2 sections/slide) were stained with oxytocin and vasopressin antibodies based on standard laboratory protocols and those of abcam®. The optimal oxytocin and vasopressin antibody ratios were established as 1:1000 for oxytocin and 1:250 for vasopressin. From

this testing the optimal sequential range of slides for further IHC were also identified. The same methodology was utilized to determine the negative control in an injured untreated rat.

Specificity

To avoid misinterpretation of staining results, a preabsorption test was used to confirm that the primary antibodies were binding specifically to the epitopes of interest in the tissue under investigation. For each primary antibody, control and blocking tubes were created that contained the concentration of antibody to blocking solution found to produce optimum staining (oxytocin 1:1000 and vasopressin 1:250). Using two times the concentration for optimal staining, 2 μ g Abcam® oxytocin peptide (#ab120186) and 8 μ g Abcam® vasopressin (#ab120175) was added to each tube labeled “blocked”. The second tube labeled “control” had an equivalent amount of PBS added (2 ml) minus the antibodies. The four tubes were incubated overnight with agitation at 4°C. Our standard IHC staining protocol was then performed on adjacent slides in the areas of interest using the blocked antibody for one slide and the control for the other. Slides were observed for absence of oxytocin and vasopressin staining.

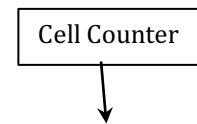
Microscopy

All sections were analyzed using a Nikon Eclipse 80i digital microscope with appropriate filters (FITC and TRITC) to detect the immunofluorescent staining. Images were captured on computer disc for cell counting and to preserve immunostaining results. Software used was Nikon Imaging Software AR version 4.1.

Quantification of Neurons

Cell counting for quantification of neurons was done using ImageJ software downloaded from <http://imagej.nih.gov/ij/download.html>. ImageJ is freeware developed at National Institutes of Health. A cell counter plug-in from ImageJ was added to the software download. After opening the neuron photomicrograph on file and manually clicking on the cell, the ImageJ cell counter leaves a mark on the cell so that the cell is not inadvertently recounted. An automatic counter keeps track of the number of cells marked. Figure 7 is an example of a marked photo of oxytocin neurons counted. The average number of oxytocin and vasopressin-stained neurons was calculated from the total number of neurons counted divided by the number of photomicrographs used per rat. The average number of neurons per rat was entered to data analysis software for computing statistical analysis. A comparison of cell counts between researchers showed minimal variation in results confirming reliable results of manual and automatic cell count.

Figure 7. Example of use of ImageJ cell counter. The neurons (oxytocin) in the photo that have been manually counted are marked (arrow) and the tally of counted neurons is seen in the cell counter box.



DATA ANALYSIS STRATEGY

SPSS Statistics 22, IBM, Armonk, New York was used for all data analysis.

Hypothesis #1

To test if mbTBI resulted in a decrease in oxytocin and vasopressin neurons an independent samples t-test to compare the mean score of neurons in the mbTBI subjects to the sham injured subjects was planned. The sample populations are mutually exclusive to injury type and were randomly selected to sham injury or injury groups. The

dependent variable, number of oxytocin or vasopressin neurons, was measured on an interval scale and the independent variable, injury type is nominal level data. However, using IHC we noted a complete absence of neurons in the injured animals. Because we observed complete absence of oxytocin and vasopressin staining we were unable to test the hypothesis using standard statistical measures.

Hypothesis #2

Data analysis related to EEN

It was believed that housing in an EEN would decrease the loss of neurons in the injured animal compared to number of neurons lost in an injured animal housed under normal or standard conditions. To test for interactions between factors (variables) where there is one dependent variable (quantity of oxytocin neurons or vasopressin neurons) and two independent variables (injury type with two levels and housing type with two levels) a 2x2 factorial design (Table 3) was used to analyze results. Two-way between subjects analysis of variance (ANOVA) allowed for testing combinations of injury type to housing type and interactions between injury and housing type. The same 2x2 design was used for testing number of oxytocin and vasopressin in separate tests. Table 3, depicts the 2 x 2 factorial design. Numbers of oxytocin and vasopressin neurons were compared in both the PVN and SON for a total of 4 statistical tests.

Table 3. Hypothesis 2 EEN. 2x2 factorial design for interaction between injury and housing with dependent variable of quantity of oxytocin or vasopressin neurons. EEN-enriched environment, NH- normal housing, mbTBI- mild blast traumatic brain injury.

Assumptions to allow for comparing groups were satisfied for correct level of measurement and random sampling of subjects into groups. The data points are independent of each other (presence or not of neurons in particular brain areas does not affect presence in another area). Histograms tend to be skewed to the left due to zero neurons in the injured animals, but numbers in sham and EEN groups somewhat compensate to move the curve towards normalcy. Homogeneity of variance could not be generated in any of the statistical tests, but the cell sizes are similar to allow for continued ANOVA testing. Significance level in all tests is <.05.

Data analysis related to minocycline

We hypothesized that minocycline treatment following blast injury would decrease loss of neurons in the injured animal compared to the number of neurons in an injured animal not treated with minocycline. A 2x3 factorial design (Table 4) was used to describe testing plan for interactions between factors. The dependent variable was

quantity of oxytocin or vasopressin neurons. The independent variables were 1) injury type with two levels (mbTBI and sham injured) and 2) treatment type with three levels (acute phase minocycline, delayed phase minocycline and vehicle). Two-way between subjects analysis of variance (ANOVA) allowed for testing combinations of injury type to treatment type and interactions between injury and treatment type. The same 2x3 design was used for testing number of oxytocin and vasopressin in separate tests. Numbers of oxytocin and vasopressin neurons were compared in both the PVN and SON for a total of four statistical tests Significance level in all tests is $<.05$.

Table 4. Hypothesis 2 Pharmaceutical Treatment. 2x3 factorial design for interaction between injury and treatment with dependent variable of quantity of oxytocin or vasopressin neurons. Mino-minocycline (acute phase), Mino-Delayed[phase], Vehicle(saline), mbTBI- mild blast traumatic brain injury.

Hypothesis 3

This hypothesis tested the impact of loss of neurons on behavioral test scores (BM, EPM and OF) measured before and after blast injury. Paired samples t-test was used to test for a difference between pretest and post injury scores in injured subjects untreated with minocycline or EEN. Wilcoxon Signed Rank Test was used for samples with less than 5 subjects per group (TDT and TCA). Anxiety was reflected by EPM scores for subtests TOA, TCA and TDT and OF subtest scores for VA. Spatial memory

(Barnes Maze test) was attributed by a score of latency (seconds) to locate the escape box and general locomotion was reflected by OF test scores of TDT, HA and RT. Statistical significance for all measurements was determined by $p < 0.05$.

Chapter 4: Results

The results are presented according to hypothesis and dependent variable. Only significant ($p \leq .05$, two-tailed analysis) and interesting trends or results are included in the narrative. Relevant statistical tables are located in Appendix A and figures in Appendix B.

SPECIFICITY OF PRIMARY ANTIBODIES

Slides stained with blocked antibodies did not reveal oxytocin or vasopressin proteins. Those stained with unblocked antibodies did show oxytocin and vasopressin indicating the primary antibodies were specific for both oxytocin and vasopressin. See Figure 8 for micrographs showing negative and positive oxytocin and vasopressin results of preabsorption test.

Figure 8. Specificity Test for Oxytocin Antibody in PVN. A- vasopressin blocked antibody (4x); B-control (20x); C-oxytocin blocked antibody (10x); D-control (10x). III-Third Ventricle. Scale bar = 100 μ m.

HYPOTHESIS 1

There is a significant difference in quantity of oxytocin and vasopressin neurons in the hypothalamus (PVN and SON) following mbTBI compared to sham-injured animals. N=7 and 16 brain sections were examined for oxytocin and vasopressin

immunostained neurons per subject. This equaled a 320 μ m section of the hypothalamus that included the PVN and SON.

Quantity of Oxytocin and Vasopressin Neurons in Hypothalamus

Unexpectedly, zero proteins for either oxytocin or vasopressin were found through antibody staining in the PVN and SON of any of the injured subjects (Figure 9). Oxytocin and vasopressin were found in abundance in both the SON and PVN of control animals (Table 5). One control subject had zero vasopressin in the SON or PVN. Since pre-absorption tests confirmed vasopressin antibody specificity and that other control subjects did have vasopressin in those nuclei, it is assumed there was an issue in that particular staining process that resulted in finding no vasopressin in that subject. A summary for the presence of oxytocin and vasopressin immunostaining in the PVN and SON is found in Table 6. Figure 10 is representative for microscopic presentation of oxytocin and vasopressin in control animals (normal housed and sham injured). Other micrographs of oxytocin and vasopressin in control animals are located in Appendix B, Figures 22-30.

Table 5. Neuropeptides in Control Rats. Range and mean numbers of oxytocin and vasopressin found in uninjured and untreated rats. Oxt, oxytocin; VP, vasopressin; PVN, paraventricular nucleus; SON, supraoptic nucleus; Tx, treatment.

Table 6. Summary of Hypothesis 1. Neurons found with positive oxytocin and vasopressin immunostaining.

Figure 9. Absence of Oxytocin and Vasopressin. There is an absence of oxytocin and vasopressin in the PVN of injured normal housing subjects. Oxytocin (red), vasopressin (green), DAPI (blue). A) oxytocin, vasopressin, DAPI merged, magnified 4x B) vasopressin, 10x, C) oxytocin and DAPI merged, 10x D) cartoon of hypothalamus with PVN, SON and third ventricle (3V); red circles represent usual locations of neuropeptides.

Figure 10. Oxytocin and vasopressin in sham injured animals.
(A) Oxytocin (red) merged with DAPI (blue) in PVN magnified 4X., (B) oxytocin in PVN magnified 10X, (C) merged vasopressin (green) DAPI and oxytocin in PVN magnified 20X (C) and (D) vasopressin in SON magnified 20X.

HYPOTHESIS 2

Results Related to Housing and Injury

Housing in an EEN will decrease the loss of oxytocin and vasopressin neurons in the hypothalamus of an injured animal compared to an injured animal housed in normal/standard conditions. Rats were grouped by injury type (sham, n= 4 or blast, n=4) and housing type (normal, n=4 or EEN, n=4). Because two separate areas of the hypothalamus were inspected for effect of injury and housing, testing was done to examine oxytocin in the PVN, oxytocin in the SON, vasopressin in the PVN and vasopressin in the SON. No post hoc tests were conducted for this hypothesis since there were only two levels in each independent variable.

Number of oxytocin neurons in the PVN

A 2 x 2 factorial between subjects ANOVA was conducted on number of oxytocin neurons in the PVN, with housing type (NH or EEN) and injury type (blast or sham) as factors. A significant main effect of injury type on number of oxytocin neurons in the PVN was found, $[F(1,4)=9.04, p=0.04]$ with a large effect size (partial Eta squared=.84). There was no main effect for housing on number of neurons $[F(1,4)=1.5, p=0.28]$ and the housing type by injury type interaction was not significant, $[F(1,4)=1.5, p=0.28]$. As shown in Table 7, the data suggests blast injury, but not housing type affected the number of oxytocin neurons found in the PVN. The mean number of neurons in the injured EEN group = 16.65; the mean of the injured normal housing group = 0.0. The sample size may have been too small to reveal a significant effect of housing.

Number of oxytocin neurons in the SON

A 2 x 2 factorial between subjects ANOVA was conducted on number of oxytocin neurons in the SON, with housing type (NH or EEN) and injury type (blast or sham) as factors. A significant main effect for injury on number of oxytocin neurons in

the SON was found, $[F(1,4)=68.85, p=.001]$ with a large effect size (partial Eta squared=.95). There was no significant main effect for housing on number of neurons $[F(1,4)=.29, p=.61]$. The interaction of housing type by injury type was not significant, $[F(1,4)=.29, p=.61]$. There were no oxytocin neurons in the SON of the injured EEN type animals. The data suggests injury, but not housing affected the number of oxytocin neurons found in the PVN.

Number of vasopressin neurons in the PVN

A 2 x 2 factorial between subjects ANOVA was conducted on number of vasopressin neurons in the PVN, with housing type (NH or EEN) and injury type (blast or sham) as factors. No main effect of housing type on number of vasopressin neurons in the PVN was found, $[F(1,4)=0.0, p=0.99]$. There was no main effect of injury type on number of vasopressin neurons $[F(1,4)=4.39, p=0.10]$. The interaction of housing type by injury type on number of neurons was not significant $[F(1,4)=0.0, p=0.99]$. It is important to note that there was no vasopressin stained neurons in the injured EEN housed animals.

Number of vasopressin neurons in the SON

A 2 x 2 factorial between subjects ANOVA was conducted on number of vasopressin neurons in the SON, with housing type (NH or EEN) and injury type (blast or sham) as factors. A significant main effect of injury type on number of vasopressin neurons in the SON was found, $[F(1,4)=10.0, p=.03]$ with a large effect size (partial Eta squared=.71). There was no main effect of housing type on number of neurons, $[F(1,4)=1.82, p=.24]$ and there was no housing type by injury type interaction, $[F(1,4)=1.82, p=.24]$. As shown in Table 7, the data suggests blast injury, but not housing type affected

the number of vasopressin neurons found in the SON. There were no vasopressin neurons found in the injured animal treated with EEN.

Table 7. Mean and Standard Deviation of Number of Neurons in PVN and SON. 2 x 2 two-way ANOVA results found significant main effect of injury type on number of neurons. Mean differences suggests blast injury resulted in decreased neurons found PVN and SON. No significant main effect of housing type on number of neurons was found, but oxytocin neurons were found in the I-EEN group (indicated in red).

Below is a comparison of quantity of oxytocin neurons in the PVN of a sham injured normal housing subject and an injured subject treated with enriched environment (Figure 11). There are clearly less neurons present in the injured animal treated with enriched environment than the control subject. However, there are zero neurons in the injured untreated animals.

Figure 11. Oxytocin in PVN and EEN. Micrograph of oxytocin (red) found in EEN sham injured subjects (A) compared to injured subjects treated with EEN (B). Magnification 20X.

Results Related to Pharmaceutical Treatment and Injury

We hypothesized that treatment with minocycline would decrease the loss of oxytocin and vasopressin neurons in the hypothalamus of an injured animal compared to

an injured animal treated with saline (vehicle). Rats were grouped by injury type (sham, n= 6 or blast, n=8) and pharmaceutical type (saline, n=7 or minocycline, n=7). Because two separate areas of the hypothalamus were inspected for effect of injury and housing, testing was done to examine oxytocin in the PVN, oxytocin in the SON, vasopressin in the PVN and vasopressin in the SON for a total of four statistical tests .

Number of oxytocin cells in PVN

A 2 x 3 factorial between subjects ANOVA was conducted on number of oxytocin neurons in the PVN, with injury type (blast or sham) and pharmaceutical type (saline, acute phase minocycline and delayed minocycline) as factors. The injury type and pharmaceutical treatment type interaction was significant [$F(2,8)=6.20$, $p=0.02$] with a large effect size (0.60). The interaction effect did not qualify the main effect of injury type [$F(1,8)=69.67$, $p=0.000$] or pharmaceutical treatment type [$F(2,8)=19.21$, $p=0.000$]. When a pharmaceutical treatment was given to an injured rat, the number of oxytocin neurons in the PVN was always less than the control groups though both acute minocycline and delayed minocycline did improve quantity of neurons compared to saline treatment (Table 8 and Figure 12). By splitting the file from the two way between groups ANOVA analysis, two (2) one way ANOVA were conducted to investigate which group most influenced number of neurons. Sham injured group result was not significant [$F(2,3)=6.54$, $p=0.08$], blast injured group was significant, [$F(2,5)=21.20$, $p=0.004$]. Post hoc tests were not computed due to less than two cases in the delayed minocycline group (n=1). We can conclude that acute minocycline group differed significantly from the saline treated group.

Table 8. Mean Number of Oxytocin in PVN of Injury Type and Pharmaceutical Types. A 2 x 3 Between Subjects ANOVA shows Mean number of oxytocin neurons in injured rats treated with pharmaceuticals to always have less oxytocin in the PVN than controls.

Figure 12. Mean Number of Oxytocin in PVN by Injury and Pharmaceutical Treatment Type. 2 x 3 Factorial ANOVA demonstrated the effect of blast injury treated with acute minocycline results in significantly more oxytocin stained neurons ($p < .05$).

Number of Oxytocin Cells in SON

A 2 x 3 factorial between subjects ANOVA was conducted on number of oxytocin neurons in the SON, with injury type (blast or sham) and pharmaceutical type (saline, acute phase minocycline and delayed minocycline) as factors. The injury type and pharmaceutical treatment type interaction was significant [$F(2,8)=6.35$, $p=0.02$] with a large effect size (0.61). There was no significant main effect for injury type [$F(1,8)=.97$, $p=.35$] and no significant main effect for pharmaceutical treatment type [$F(2,8)=2.24$, $p=.16$] were discovered. By splitting the file from the two way between groups ANOVA analysis, two (2) one way ANOVA were conducted to investigate which group effect was significant. Sham injured group result was not significant [$F(2,3)=1.88$, $p=0.29$], blast injured group was significant, [$F(2,5)=83.15$, $p=0.000$]. Post hoc tests were not computed due to less than two cases in the delayed minocycline group ($n=1$). Table 9 and Figure 13 show mean numbers of oxytocin in the SON according to injury and treatment type. We can conclude that injured acute minocycline group differed significantly from the injured saline treated group.

Table 9. Mean Number of Oxytocin in SON of Injury Type and Pharmaceutical Types. No oxytocin stained neurons were found in the rats treated with saline after blast injury. Minocycline treated rats always had greater number of neurons than sham injured rats, but results are not significant.

Figure 13. Mean Number of Oxytocin Neurons in SON After Injury and Pharmaceutical

Treatment. A 2 x 3 Between Subjects ANOVA demonstrated the effect of blast injury treated with acute minocycline significantly increased the number of oxytocin stained neurons ($p < 0.05$).

Vasopressin in PVN

Vasopressin was only found in the PVN of sham injured saline treated rats ($M = 42.75$). No statistical tests were conducted.

Vasopressin in the SON

A 2 x 3 factorial between subjects ANOVA was conducted on number of vasopressin neurons in the SON, with injury type (blast or sham) and pharmaceutical type (saline, acute phase minocycline and delayed minocycline) as factors. The injury type and pharmaceutical treatment type interaction was significant [$F(2,8) = 23.14$, $p = 0.000$] with a large effect size (0.85). There was no significant main effect for injury type [$F(1,8) = 0.03$, $p = 0.95$], and no significant main effect for pharmaceutical treatment type [$F(2,8) = 2.67$, $p = 0.12$] was discovered. By splitting the file following two way between subjects ANOVA, two (2) one way ANOVA were tested to determine which group

affected the number of vasopressin neurons. Sham injured group was not significant [$F(2,3)=7.64$, $p=0.06$], blast injured group was significant, [$F(2,5)=33.20$, $p=0.001$].

Table 10 and Figure 14 show mean numbers of vasopressin in SON according to injury and treatment type. We can conclude injured acute minocycline group differed significantly from the injured saline group. As in the case of vasopressin in the PVN, vasopressin was not found in the SON of sham injured rats in the acute phase minocycline treatment, but it was present in all other groups in the SON.

Table 10. Mean Number of Vasopressin in SON of Injury Type and Pharmaceutical Types.

No vasopressin stained neurons were found in the blast injured or in the sham injured acute minocycline groups.

Figure 14. Vasopressin in SON, Comparison of Means. A 2 x 3 Between Subjects ANOVA demonstrated the effect of blast injury treated with acute minocycline significantly increased the number of neurons ($p < 0.05$).

Summary of Hypothesis 2

Statistical analysis revealed blast injury treated with acute phase minocycline to affect a significant increase in oxytocin stained neurons compared to injured saline treated groups in the PVN. Interestingly, vasopressin was not found in the PVN of the injured animal when treated with either acute or delayed phase minocycline. Vasopressin was found in the SON of injured and sham injured groups and it significantly increased the number of neurons in the injured group treated with acute phase minocycline. Figures 15-18 show oxytocin and vasopressin neurons in sham injured and injured treated animals in the PVN and SON related to hypothesis number two.

Figure 15. Oxytocin in PVN of Minocycline Treated Injured Subjects. (A) Micrograph of oxytocin in NH-SI rodent treated with acute phase minocycline. (B) is oxytocin in I-NH rodent after treatment with acute phase minocycline. Minocycline treated injured rodent has fewer oxytocin than the NH-SI, but significantly more than the NH-I rodent that has zero oxytocin. Both micrographs are magnified 20X.

Figure 16. Oxytocin and Vasopressin in SON of Minocycline Treated Injured Subjects. Micrographs show oxytocin (red) vasopressin (green) in the SON of NH-SI (A, B) and I-NH rodent (C,D) treated with acute phase minocycline. Magnification 20X.

Figure 17. Oxytocin Neurons in PVN of Delayed Phase Minocycline Treated Injured

Rodent. Oxytocin (red) in PVN of SI-NH delayed phase minocycline treated rodent (A,B) and in PVN of I-NH delayed phase minocycline treated rodent (C, D). Magnified 10X (A) and 20X (B,C,D).

Figure 18. Oxytocin and vasopressin neurons in SON of delayed phase minocycline treated subjects. Micrograph of oxytocin (red), vasopressin (green) and DAPI (blue) in injured delayed phase minocycline, magnification 20X.

HYPOTHESIS 3

Paired samples t-test analyses were performed on Barnes Maze, EPM and OF activity behavioral tests. Wilcoxon Signed Rank test was used to measure change in TDT and TCA of EPM for groups with less than 5 subjects. The rodent sample for this hypothesis is animals with loss of both OXT and VP neurons in the hypothalamus (N=7). We hypothesize that loss of neurons increases anxiety and decreases spatial memory and affects ability to learn. The behavioral test scores for Barnes Maze, OF and EPM are the dependent variables and time (pre and post injury) is the independent variable. Only statistically significant results and interesting trends are presented in the narrative. Assumptions underlying use of paired samples t-tests including correct level of measurement, random sampling (rats were randomly assigned to groups prior to testing), independence of observations, normal distribution and homogeneity of variance were conducted. Normal distribution and homogeneity were not always met possibly due to small sample size, but consultation with Dr. Cara Olsen (USUHS statistician) determined paired sample t-test to be a usable statistical technique except where samples sizes were less than five.

Barnes Maze Results

A paired samples t-test was conducted to determine the effect of loss of oxytocin and vasopressin neurons on Barnes Maze (spatial memory and learning) test scores. The independent variable was time (pre and post blast injury) and the dependent variable was score on the Barnes Maze test measured in seconds to find the darkened escape box. Pairs included pre-injury and post-injury one (n=7) and pre-injury and post-injury two (n=7). Post-injury one time period included five sequential days of testing between a

period of 3-14 days after blast injury. Post-injury two period included five sequential days of testing between a period of 21-51 days after injury.

There was a significant decrease in time to find the escape box between pre-injury ($M=44.99$, $SD=21.02$) and post-injury test period one [$M=23.99$, $SD=20.40$, $t(6) = 3.11$, $p = .02$]. The eta squared statistic (.61) indicated a large effect size. There was also a significant decrease between pre-injury ($M=44.99$, $SD=21.02$) and post-injury test period two [$M=26.42$, $SD=21.17$, $t(6)=3.30$, $p=0.01$]. Eta squared statistic (0.64) indicated a large effect size. There was a significant decrease in time to find the escape box after injury compared to the pretest score (Figure 19). There was a very slight increase in time to find the box between pre-injury time and post-injury time two compared to post-injury time one, was not significant from post-injury time one ($M=21.0$, $SD=16.96$) to post-injury time two ($M=18.57$, $SD=10.81$), [$M=2.42$, $SD=10.55$, $t(6)=8.60$, $p=0.56$].

Figure 19. Pre-Injury and Post-Injury Barnes Maze Results for Injured No

Treatment Group. There was a significant decrease in time to find the escape box between pre-injury and post-injury test periods.

Elevated Plus Maze Results

Data was collected on injured non-treated subjects (no oxytocin or vasopressin proteins). Pre-injury behavioral data was not obtained for all the experiments making the number of subjects small ($n=2$) for subtests TDT and TCA. Non-parametric test, Wilcoxon Signed Rank was used to test these subtests. Paired samples t-tests were conducted to evaluate the impact of injury on the scores for TOA ($n=5$).

Total Distance Traveled

Pre-injury score did not significantly differ from post-injury one ($p=0.18$) or post-injury time two ($p=0.18$). The alpha level was set at 0.05. A paired samples t-test was performed between post-injury time one and post-injury time two ($n=5$). Paired samples t-test revealed a significant difference [$M=3.67$, $SD=1.67$, $t(4)$, $p=0.01$], $n=5$.

Time in Closed Arms

There was no significant difference between pre-injury scores and post-injury time one scores ($p=0.18$) or post-injury time two ($p=0.18$). Alpha was set at 0.05. Paired samples t-test did not reveal a significant difference between post-injury time one and post-injury time two ($n=5$).

Time in Open Arms

There was no significant difference between pre-injury scores and post-injury time one scores [$M=10.36$, $SD=10.25$, $t(4)$, $p=0.08$], $n=5$. There was no significant difference between pre-injury scores and post-injury time two scores [$M=22.86$, $SD=93.60$, $t(4)$, $p=0.61$], $n=5$ (Figure 20). Alpha was set at 0.05.

Figure 20. Elevated Plus Maze Behavior Test Scores. No significant differences between pre-injury and post-injury scores for subtests TCA, TOA (A) and TDT (B). There was a significant difference between post-injury time one and two in the subtest TDT (n=5).

Open Field Activity

There were no significant findings in any of the OF sub-tests (Figure 21).

Figure 21. Open Field Behavior Test Scores. Paired samples t-test revealed no significant changes between pre-injury and post-injury scores in any of the subtests.

SUMMARY

Immunohistochemistry (IHC) results showed a complete absence of oxytocin and vasopressin in the PVN and SON. A pre-absorption test confirmed specificity of oxytocin and vasopressin antibodies for binding to oxytocin and vasopressin antigens. Oxytocin neurons were found in the PVN after injury and housing in EEN, but it was not significantly different. There were no oxytocin or vasopressin neurons in the SON nor

were vasopressin neurons in the PVN of injured subjects treated with EEN. In examining the effect of minocycline on number of oxytocin and vasopressin neurons, we found several significant interaction effects. Further analysis by 1-way ANOVA revealed that minocycline given immediately after injury significantly increased the number of oxytocin neurons in the PVN and SON and vasopressin neurons in the SON. The comparison between zero oxytocin and vasopressin neurons to behavioral test scores did not show a deficit between pretest and posttest scores on any of the tests performed. There was a significant decrease in TDT between post-injury test time one and time two where $n=5$. There were significant decreases in time to find the escape box in the Barnes Maze test.

Chapter 5: Discussion

Blast injury has been called the signature injury of recent conflicts involving U.S. military members (94). The residual effects of mbTBI can be sustained over time and disruptive to quality of life (53; 86; 135). The purpose of this project was twofold; to increase knowledge surrounding the effect of mbTBI on neurons that produce oxytocin and vasopressin and determine if treatment with minocycline or EEN attenuates neuronal damage after mbTBI. The present project was designed to examine the histological effects of mild blast injury on neurons in the hypothalamus that produce the neuropeptides oxytocin and vasopressin. Both neuropeptides are associated with mammalian reproductive and social behaviors (116; 128; 139; 163). Following this line of thought, we examined the effects of mbTBI on oxytocin and vasopressin expressing neurons to behaviors associated with anxiety, learning and spatial memory . We also sought to determine if treatment with enriched environment or minocycline might mitigate negative effects of blast injury on the neurons. It was hypothesized that: 1) mbTBI will decrease the number of oxytocin and vasopressin neurons (13; 31; 182); 2) housing in an enriched environment or treatment with minocycline will attenuate negative effects of mild blast TBI (18; 47; 109; 123) by helping to preserve or restore oxytocin and vasopressin neurons; 3) loss of oxytocin and vasopressin neurons will a) increase anxiety b) decrease learning and c) decrease spatial memory as measured by pretest and posttest scores on the EPM, Barnes Maze and Open Field tests.

MILD BLAST-INDUCED TBI EFFECTS

Blast injury, including mild blast overpressure is known to affect neurochemistry and structure that includes neuronal loss (30; 32; 38; 115). At least some of the neurobehavioral pathology that accompanies mbTBI must stem from the neural damage that occurs in the days and months following the trauma. Our findings from immunohistochemistry showed that rats exposed to an mbTBI had no oxytocin or vasopressin expressed in the PVN or SON. All rats in the control groups related to housing type, minocycline treatment and sham injury did show oxytocin and vasopressin in the same brain areas. This is a new finding that adds to the understanding of effect of blast overpressure at a range of 20 ± 3 psi. These findings also add to the evidence from previous studies that showed significant loss of other neuron types after mild blast overpressure (38; 125; 190). Despite few studies that have examined the effect of mbTBI on neuronal structures in the PVN and SON, our results are substantiated by previous researchers who found that central oxytocin and vasopressin gene expression is significantly altered by low level blast injury (182). This is important evidence helping to confirm our findings since the PVN and SON are the main sites of oxytocin and vasopressin expression. Although hypothesis one predicted that neurons would be lost by blast overpressure, it was disturbing to find a complete absence of expression of oxytocin and vasopressin in all of the injured animals.

Validation of Immunohistochemistry Protocol

From an uninjured animal, a $1,280\mu\text{m}$ (64 brain sections at $20\mu\text{m}/\text{section}$) section of tissue containing the hypothalamus was used to identify the brain area of interest and as a positive control. Using a rat brain atlas and magnifying glass, landmarks indicating the approximate location of the hypothalamus were identified. Thirty-two slides (2

sections/slide) were stained with oxytocin and vasopressin antibodies (see Methods). The optimal oxytocin and vasopressin antibody ratios were established for subsequent staining and the sequential range of slides for further IHC were also identified. The same methodology was utilized to determine the negative control in an injured untreated subject. All primary and secondary antibodies came from the same supplier (abcam®) and were kept refrigerated and none exceeded expiration dates. The protocol used was strictly adhered to at all times without variation after the optimal antibody ratios were established with the positive control. In addition, a preabsorption test to confirm specificity of primary antibodies to bind to epitopes of oxytocin and vasopressin antigens in the PVN and SON was carried out with positive results. Definite confidence in the IHC protocol was established, which supports that IHC results are trustworthy.

Housing Effects

Because in cases of TBI, EEN is known to decrease loss of brain tissue (141), reduce cortical lesion size (206), reduce cell loss (206) and reduce markers of cellular injury (123) it was expected that EEN would decrease loss of oxytocin and vasopressin neurons. Indeed, oxytocin neurons were found in the PVN of injured rats treated with EEN, although they were not found in the SON. Vasopressin was also not found in the PVN or SON of the EEN treated group. It would not appear that immunohistochemistry method or materials were faulty since vasopressin and oxytocin were found in abundance in both regions of non-injured subjects. Although we did not see a significant increase in vasopressin neurons in the PVN or SON or of oxytocin in the SON with EEN, the fact that oxytocin was found in the PVN suggests that EEN may sometimes decrease the processes involved in cellular damage. The number of oxytocin cells found in the PVN

of the injured EEN (M=16.6, N=2) did not reach a statistically significant difference from sham injured normally housed subjects (M=50.5, N=2). The difference is visually dramatic and suggests EEN did not substantially support preventing loss of neurons. The small sample size may account for failure to reach a significant value.

Minocycline Effects

Again in both experiments on effects of 1) acute and 2) delayed minocycline treatment for mbTBI, injured saline (vehicle) treated rats were found to have a complete absence of oxytocin and vasopressin immunostaining in the PVN and SON. With the exception of vasopressin in the PVN, injured subjects treated with both acute and delayed minocycline tested positive for oxytocin and vasopressin neurons. However, differences between non-treated injured and treated minocycline groups did not reach significance. Previous work by Kovesdi et al. (124) demonstrated significant elevated inflammatory markers CRP and MCP-1 in mbTBI injured rats. Treatment with 50mg/kg. minocycline immediately after blast injury resulted in normal levels of these inflammatory markers. This was the same dose of minocycline given to the subjects receiving acute phase minocycline in this experiment. Mild blast overpressure is known to initiate an inflammatory response in brain tissue that results in neuronal loss (1; 38; 124). Our findings suggest this inflammatory response was involved in the loss of oxytocin and vasopressin neurons found in our work, but that minocycline had a neuroprotective effect that was evidenced by presence of these neurons in injured animals. The neuroprotective effects of minocycline after TBI have been shown to be effective when administered up to five weeks after injury (95). A single dose of mild blast overpressure can result in elevated inflammatory markers p38, MMP8 and FPR1 in injured rats compared to naïve

non-injured rats (5). Microglial cells become activated after TBI causing the release of pro-inflammatory chemicals and neurotoxic agents such as reactive oxygen species that cause oxidative stress. Microglia are part of the pro-inflammatory response that can lead to neuronal death. This microglial activation can persist for as long as 17 years after injury (92). Minocycline has been shown to decrease microglial activation caused by status epilepticus (222), schizophrenia (240) and in closed head injury of mice (95). We found preservation of oxytocin and vasopressin cells treated with minocycline within several hours post-injury to as long as 35-38 days after injury, suggesting that the inflammatory response which can cause neuronal death related to microglia was attenuated even with delayed treatment.

Behavioral Effects

Under normal conditions, behavior is dependent upon many factors including motivation, emotion, learning and memory (45; 59; 147). Oxytocin and vasopressin are known to affect behavior through neuromodulation of all these factors (45; 59; 62). The influence of oxytocin and vasopressinergic systems is related to the type of learning, the stage of learning and memory acquisition (45; 59), and neuronal systems involved in that type of learning (59). Mild bTBI has been shown to affect spatial learning and memory (9; 74; 123), stress response (54; 125; 173) and general locomotor activity, all of which may impact behavioral response (123; 173). We hypothesized that a decrease in oxytocin neurons with subsequent decrease in availability of these neuropeptides would be reflected in decreased learning and memory. In addition, we thought anxiety would be increased as a result of loss of oxytocin which is known to be anxiolytic (41; 167; 203).

Barnes Maze

The Barnes Maze is a type of passive avoidance test used to evaluate the ability of the subject to learn and remember the path to the preferred (dark and confined) environment in order to escape the undesirable (open area and bright light) environment. Here we did not find that lack of oxytocin or vasopressin affected time to find the escape box. This finding would suggest that loss of these neuropeptides did not negatively influence learning and memory. One possible explanation for the findings in this study may be related to an amnesic effect of oxytocin on memory. It has generally been thought that oxytocin has opposite learning, memory and other behavioral effects from those of vasopressin (45; 59). There can be increased latency to find the escape box after i.c.v. injection of oxytocin into the hippocampal region of the rat brain (122; 232). The loss of oxytocin neurons in our findings may mimic the effect of oxytocin as an amnesic which would help explain the improved spatial learning task results.

Another possibility is that oxytocin and vasopressin play a greater role in social memory than they do in spatial memory and learning. The effects of oxytocin and vasopressin on social behavior are well established and have been discussed extensively in this document. Spatial learning and memory may encompass different neural pathways and structures from those involved in social recognition memory (81). Ability to recognize individuals underlies development of personal relationships and appropriate social behavior (82). In many mammals social recognition is mediated by the olfactory system (82; 99; 100). The olfactory system innervates the medial amygdala (MeA) where oxytocin mediates long-term social recognition memory (82). This evidence is supported by a lack of social recognition memory in oxytocin knockout mice except when oxytocin is injected directly into the MeA (64). Future studies examining the

effect of loss of oxytocin and vasopressin neurons after mbTBI should incorporate social recognition tests which may demonstrate an effect of loss of oxytocin and vasopressin expressing neurons.

Elevated Plus Maze

The EPM was used to evaluate anxiety (TOA and TCA) and locomotor activity (TDT). Similar to the outcomes from previous work (123), our findings showed a significant decrease in locomotion over time. The study by Kovesdi et al.(123), included subjects also used in this study and showed control and injured groups treated with EEN with decreased locomotion over time. Kovesdi et al. (123) hypothesized that this tendency to decrease exploring may have been due to habituation of the maze. We suspect habituation may have contributed to our findings as well.

Another possible explanation for the TDT results is that repeated EPM testing is itself stressful and causes the rats to decrease exploratory behavior. Repeated EPM tests have been shown to result in significantly decreased exploratory behavior, increased number of defecations, and increased self-grooming which are indicators of increased anxiety (15). Oxytocin has been proven to have anxiolytic effects in various mammals and humans. Anxiety manifested by progressively decreasing time exploring in the EPM could be a result of the loss of oxytocin neurons.

In the studies by Kovesdi et al. (123; 124), time spent in the open arms of the EPM by the injured rats gradually increased over time. This was interpreted as a gradual decrease in anxiety over time. Our study did not find a significant difference between pretest scores and any posttest scores on TOA or TCA of injured subjects. These results would indicate neutral behavior and no behavioral deficit due to injury. Future studies

should also record the other indicators of anxiety during the EPM such as number of defecations and self-grooming activity.

Open Field Test

The OF test was used to measure general locomotor activity, but has also been used as an indicator of anxiety (111; 196). Eight days after injury (post-injury test session one), our subject's Horizontal Activity (HA) increased and Rest Time (RT) decreased. In the second post-injury test session, HA decreased and RT increased. The activity difference was not significantly different from pretest scores in either session. In the study by Kovesdi et al. (124), all groups including controls exhibited declining HA and increasing RT. The decline in activity was speculated to be related to either habituation of the environment or the more mature age of the rat. Our behavioral results could indicate progressing detrimental effects of the blast-induced neurotrauma. The studies by Kovesdi et al. (123; 124), demonstrated significantly elevated markers of axon injury (Tau) and inflammation (CRP, MCP-1 and TLR9) upon completion of their studies. Our brains came from the same cohorts which suggests decreased activity could be secondary to progressive neuron injury.

Summary – Hypotheses Revisited

Hypothesis one cannot be confirmed. Although no oxytocin or vasopressin expression was found in the PVN or SON of any of the injured non-treated rats, further tests need to be conducted that would test for neuron death. However, the loss of expression of oxytocin and vasopressin is a new finding of the effects of blast overpressure of 20 ± 3.0 PSI. This is a range normally considered to result in mild injury.

Hypothesis two was found to be true under limited conditions. Injured subjects treated with an EEN after blast injury were found to have oxytocin neurons preserved in the PVN, but not the SON. Vasopressin was not found in the SON or PVN of injured animals treated with EEN. We also found that minocycline appears to have a neuroprotective effect when administered immediately and at delayed times after injury. Oxytocin and vasopressin were found in the SON after injury when the subjects were treated with minocycline. The exception to this finding was that no vasopressin was found expressed in the PVN of injured animals.

Hypothesis three did not reveal significant behavioral effects as indicated by the Barnes Maze, EPM or OF tests. Interestingly, considering the complete absence of oxytocin in the injured animals, the negative anxiety results could possibly be explained by an amnesic effect of oxytocin which has been demonstrated by oxytocin antagonists in other work. The decrease in locomotion over time in both the EPM and OF test could be an indicator of anxiety versus motor deficits, habituation or age if repeated exposure to these tests are themselves stressful to the subjects. Decreased locomotion could also be an effect of progressing neuronal tissue damage.

STUDY LIMITATIONS

The most significant limitation of this study was the sample size. When first undertaking the study, 23 brains seemed like an adequate number, but it was not until well into the project and analysis of the behavioral data that it became apparent that there were significant inequalities in group sizes. Based on similar experiments, a sample size of 8 subjects per group would have provided a better opportunity to conduct more powerful and meaningful statistical outcomes. Fortunately, there were 7 injured non-

treated subjects, which provided the most exciting and important data showing loss of oxytocin and vasopressin expression.

Another limitation was the lack of chemistry and serum from the subjects which would have made further analysis of neuron damage possible. Publications from studies which involved the animals from showed increased markers of neuronal and glial loss, inflammation and structural damage (123; 124). Although those results are from limbic areas, they do help validate our findings of neuronal compromise found in this study. Given the opportunity, this study could be designed to treat subjects with BRDU to determine cell death. The animals should be sacrificed at various time points after injury to determine temporal loss of neurons.

FUTURE DIRECTIONS

Oxytocin, vasopressin and the PVN which produces these neuropeptides, has been called a veritable Rosetta Stone for future studies involving stress and behavior. The finding that mbTBI can lead to a complete loss of expression of oxytocin and vasopressin should lead to studies that seek to identify and understand the neurochemical, molecular and temporal variables and processes involved in the loss. This study should be replicated with a design that would sacrifice the animals at various time points following injury in order to learn when death is most likely to occur. Because oxytocin and vasopressin are so vital to social behaviors, future studies should include females. Females who suffer traumatic brain injury may be at risk for alterations in maternal behavior. Ideally, revised the study of the effect of mbTBI on oxytocin and vasopressin would examine behaviors that these chemicals are most associated with such as pair bonding, aggression, fear extinction, anxiety and parental behavior. Tests that target

learning and memory underlying social behaviors such as the Social Recognition Test should be included in future studies.

SUMMARY AND CONCLUSION

Oxytocin and vasopressin are neuropeptides that have existed for millions of years. The neurons expressing these neuropeptides have evolved from simple single polar cells to complex cells that influence behavior, learning and memory. Oxytocin and vasopressin receptors and axons are located in, and communicate to, limbic regions suggesting that these neuropeptides are involved in neuronal processes related to those areas (120). The progressive development of oxytocin and vasopressin as complex systems over time and their presence in all mammalian life forms suggests a vital importance in the chemistry of life. The PVN is the epicenter of the stress response and oxytocin and vasopressin are two of the major products of the PVN. It would seem to follow that these chemicals must have a role as mediators in allostatic processes to initiate and respond to internal and external stimuli that help maintain allostasis.

In this study we demonstrated that mbTBI is associated with the loss of these important mediators of health. Considering the number of people who experience brain injury, the impact of loss of these neuropeptides should be further explored. . The consequence of diabetes insipidus has been associated with loss of vasopressin after TBI for many years. Typically however, vasopressin is not monitored unless the trauma has been severe. Further study of the effects of mbTBI effect on oxytocin and vasopressin may justify teaching clinicians to include vasopressin and oxytocin as part of the chemistry tested when evaluating trauma patients. It is hoped that the results of this study may increase awareness and prompt curiosity to better understand the effects mbTBI on

expression of oxytocin which seems to be as one scientist said “the great facilitator of life” (128).

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Appendix A: Tables

Table 11. ANOVA – Number of oxytocin neurons in injured subjects treated with EEN (PVN).

Table 12. ANOVA – Number of oxytocin neurons in injured subjects treated with EEN (SON).

Table 13. ANOVA – Number of vasopressin neurons in PVN of injured subject treated with EEN.

Table 14. ANOVA – Number of vasopressin neurons in SON of injured subject treated with EEN.

Table 15. One Way ANOVA – Number of oxytocin neurons in PVN of injured animal treated with minocycline.

Table 16. Post hoc test for number of oxytocin neurons in SON.

Table 17. Number of vasopressin neurons in PVN of injured subject treated with minocycline.

Table 18. ANOVA – Number of vasopressin neurons in injured subjects treated with minocycline.

Table 19. Paired Samples t-test of Barnes Maze test comparing injured non-treated subjects to control.

Table 20. Paired Samples t-test. OF Horizontal Activity comparing injured non-treated subjects to control at various time points.

Table 21. Paired Samples t-test OF Rest Time comparing injured non-treated subjects to controls at various time points.

Table 22. Paired Samples t-test OF Total Distance Traveled comparing injured non-treated subjects to controls at various time points.

Table 23. Paired Samples t-test OF Vertical Activity comparing injured non-treated subjects to controls at various time points.

Table 24. Paired Samples t-test OF Center Time comparing injured non-treated subjects to controls at various time points.

**Table 25. Paired Samples t-test EPM Time in Open Arms
comparing injured non
-treated subjects to controls.**

Appendix B: Figures

Figure 22. Magnocellular and parvocellular neurons in PVN of control subjects. Normal housing, sham-injured. Oxytocin (red) A-C. Oxytocin (red), vasopressin (green), DAPI (blue) merged, D. A,C, D magnified 20X, B 10X.

Figure 23. Magnocellular neurons in SON. Red (oxytocin), green (vasopressin), blue (DAPI). 20X.

Figure 24. Magnocellular and parvocellular neurons in PVN. Oxytocin (red) in
PVN of sham injured/EEN treated animals (A, B) and injured/EEN treated subjects (C, D). A,B, D magnification 10X. C 20X.

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Figure 25. Magnocellular neurons in SON. Oxytocin (red), vasopressin (green), and DAPI (blue) in SON of sham-injured/EEN treated subjects. Magnification 20X.

Figure 26. Magnocellular and parvocellular neurons in PVN.
Oxytocin (red) in
PVN of injured, minocycline (acute phase) treated subject (A, B)
and oxytocin in
sham-injured, vehicle treated subject (C, D). A,B,C magnified
20X. D 10X.

Figure 27. Magnocellular neurons in SON. Oxytocin (red), vasopressin (green) and DAPI (blue) in SON of injured, minocycline (acute phase) subjects (A, B) and sham injured, minocycline (acute phase) subjects (C, D). Magnification 20X.

Figure 28. Oxytocin (red) in PVN of sham-injured subject treated with minocycline (acute phase). A, 4X. B, 20X. C, 20X, SON.

Figure 29. Oxytocin (red) in PVN of injured, minocycline (delayed phase) subjects (A, B) and sham-injured, vehicle treated subjects (C, D). C, 10X, D, 20X oxytocin merged with DAPI (blue).

Figure 30. Oxytocin (red), vasopressin (green) and DAPI (blue) in SON of injured, minocycline (delayed phase) subject (A).).

Appendix C: Abbreviations

ACTH	Adrenocorticotropin hormone
AMY	Amygdala
ANOVA	Analysis of variance
ANS	Autonomic nervous system
APP	Amyloid precursor proteins
BNST	Bed nucleus of stria terminalis
BOP	Blast overpressure
bTBI	blast traumatic brain injury
CeA	Central amygdala
CRF	Corticotropin releasing factor
CRH	Corticotropin releasing hormone
CT	Center time
CVO	Circumventricular organs
DAI	Diffuse axonal injury
DHC	Dorsal hippocampus
ECT	Environmental complexity testing
EEN	Enriched environment
EPM	Elevated plus maze
GABA	Gamma aminobutyric acid
GC	Glucocorticoid
GR	Glucocorticoid receptor
HA	Horizontal activity
HAB	High anxiety behavior
HC	Hippocampus
HPA	Hypothalamic pituitary adrenal
I.E.D.	Improvised explosive device
IACUC	Institutional Animal Care and Use Committee
ICV	Intracerebral venous
IHC	Immunohistochemistry
IL	Infralimbic
kPa	Kilo Pascals

LAB	Low anxiety behavior
LHPA	Limbic hypothalamic pituitary adrenal
mbTBI	Mild blast traumatic brain injury
MCN	Magnocellular neuron
MeA	Medial amygdala
mPFC	Medial prefrontal cortex
mRNA	Messenger ribonucleic acid
NA	Nucleus acumbus
NGS	Normal goat serum
O.C.T.	Optimal Cutting Temperature
OF	Open field
OXT	Oxytocin
PBS	phosphate-buffered saline
PL	Prelimbic
psi	pounds per square inch
PTSD	Post-traumatic stress disorder
PVC	Parvocellular neuron
PVN	Paraventricular nucleus
RT	Rest time
SON	Supraoptic nucleus
TBI	Traumatic brain injury
TCA	Time in closed arms
TDT	Total distance traveled
TOA	Time in open arms
USU	Uniformed Services University
	Uniformed Services University of the Health
USUHS	Sciences
VA	Veterans Affairs
VA	Vertical activity
VHC	Ventral hippocampus
VP	Vasopressin
WRAIR	Walter Reed Army Institute of Research

