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ABSTRACT

MICROGLIA INDUCED NEUROINFLAMMATION THROUGH THE NLRP3 INFLAMMASOME FOLLOWING BLAST TRAUMATIC BRAIN INJURY

by
Daniel Younger

The incidence of traumatic brain injury (TBI) among military personnel have been steadily increasing with modern conflicts. A recent RAND report estimated 320,000 service members, totaling 20% of deployed forces, suffer from TBI. However, of this population roughly 60% have not seen a medical professional specifically for TBI. Unlike the civilian population, the primary cause of TBI for active-duty military personnel is blast exposure. Blasts now account for over 70% of all US military casualties in operation Iraqi Freedom (OIF) and Operation enduring freedom (OEF) and are the major cause of TBI. Among many pathological mechanisms associated with blast TBI, disruption of Blood Brain Barrier (BBB) and subsequent leakage of blood-borne macromolecules into brain parenchyma is reported to be the earliest event, which could trigger sustained neuroinflammation in blast TBI (bTBI). Accordingly, several studies have implicated neuroinflammation in the pathology of blast induced TBI. However, the role of the central nervous system's innate immune response, specifically the involvement of resident microglia and the pathways through which microglia contribute to neuroinflammation, has not been thoroughly investigated. Hence, the temporal and spatial evolution of microglia activation and specific mechanisms operative during the course of microglial action are critically warranted in blast TBI. It is hypothesized that microglia contribute to chronic neuroinflammation in bTBI which plays a pivotal role in the neuropathological and neurobehavioral changes in bTBI. This study is divided into 3 aims: Aim 1 identifies temporal distribution of microglia's four active phenotypes and a morphological description of each phenotype in hippocampus and thalamus. Cell surface markers

specific to all four active phenotypes were used to identify the microglia cells and a manual analysis done to describe their morphology. These studies were carried out at mild/moderate overpressure of 180 kPa and at six time points: 4 hours, 24 hours, 3 days, 7 days, 15 days, and 30 days. The rationale for choosing the hippocampus and thalamus is due to previous studies from this lab that reported that these regions were most vulnerable to BBB disruption and oxidative stress, and due to their critical involvement in neurocognitive and neurobehavioral outcomes. Aim 2 investigates the role of the NLRP3 inflammasome pathway and its involvement in the production of the pro-inflammatory cytokine IL-1β. Through a series of immunostains the cellular source of the inflammasome and its components are identified. Aim 3 investigates how the inhibition of NLRP3 inflammasome formation through the use of a specific inhibitor MCC950 will impact on the cognitive outcomes in rats following blast TBI.

Results indicated that microglia become activated acutely (4h) and such activation persists up to 30 days after bTBI. Such microglial activation is more pronounced in the vicinity of vascular rupture (BBB disruption) compared to areas away from the site of BBB leakage. Further, levels of proinflammatory cytokine IL-1β shows a sustained increase in both hippocampus and thalamus and such raise in IL-1β is comparable to a parallel increase in NLRP3 inflammasome complex. Ultimately, inhibiting NLRP3 inflammasome by the administration of specific inhibitor MCC950 displays a significant improvement in motor function, anxiety/depression as well as improves short-term memory in animals exposed to blast injury. This project therefore addresses the key role of resident microglia in the evolution of chronic neuroinflammation via the activation of NLRP3 inflammasome and proinflammatory cytokine production, key events contributing to neurobehavioral deficits in bTBI. Therefore, targeting CNS innate immune system (microglia) response by NLRP3 inflammasome activation may have a therapeutic potential counteract neurobehavioral deficits in bTBI.

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by
Daniel Younger

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APPROVAL PAGE

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Dedicated to my mother Hedy Younger.

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CHAPTER 1

INTRODUCTION

1.1 Introduction

Traumatic brain injury (TBI) is one of the leading causes of mortality and morbidity around the world. In 2013, in the US alone there were about 2.8 million emergency room visits, among which there were 282,000 hospitalizations, and 56,000 deaths. The three leading causes of TBI related hospitalizations include falls (47%), impact by striking objects (15%), and automobile accidents (14%) [1]. TBI accounts for approximately 30.5% of all injury-related deaths in the US. Over the past decade, there has been a sharp increase in incidents of TBI resulting from combat related injuries as well as insurgent activities on civilian population [2]. In 2010, the direct medical costs of TBI was 76.5 billion dollars [3]. Although there has been a lot of effort focusing on treatment modalities for TBI, not much success was achieved in developing a therapeutic strategy to treat TBI-associated deficits. Accordingly, over 30 stage III clinical trials failed to show significant improvement in TBI patients [4]. This failure is probably due to lack of clear understanding of the primary and secondary mechanisms in the evolution of injury pathology and due to highly heterogeneous nature of TBI phenotype[4]. This tantalizing problem of TBI has motivated academics and physicians to find novel mechanisms of TBI pathology in order to delay and prevent the evolution of TBI induced neuropathological events and to prevent behavioral deficits.

TBI is classified based on the mechanical insult that causes the injury. TBI can be classified as either ballistic, blunt, or blast. For ballistic injury, an object penetrates the skull and enters the brain parenchyma. The number of penetration injury has been significantly reduced through the use of composite body armor. Blunt injury occurs

when the victims head collides with a stationary or moving object. During injury the head experiences localized force near the point of impact. Peak force, contact area, and the duration of the impact determine the severity of injury. Typical blunt injuries include epidural/subdural hematoma, subarachnoid hematoma and contusion and severe hemorrhages with concomitant increase in intracranial pressure(ICP)[5].

Blast injury is considered the most complex of the injury types because it includes aspects of both Ballistic and blunt injury. Blast injury is divided into four major categories (1) Primary (direct effects of overpressure (2) secondary (effects of projectiles/shrapnel) (3) tertiary (effects from fall and blast winds), and (4) quaternary (Burns, asphyxia, and exposure to toxic inhalants). Depending on the environment, size of explosive device, and distance from blast a combination of these injuries will be experienced [6].

With the rise of global terrorism and the war in Iraq and the war in Afghanistan many TBI cases are induced by blast exposure cause by improvised explosive device (IED) [7, 8, 9]. IED accounted for nearly 80% of all casualties reported to the Joint Theater Trauma Registry (JTTR) between October 2001 to January 2008 [10]. A US army review of total casualties in Afghanistan and Iraq found that from 2001 to 2007 explosives were associated with 63% of all TBI diagnoses [11]. A similar study conducted by the US Navy and Marine Corps found the explosives were associated with 52% of all TBI cases.

After detonation of the explosives, a steep rise in atmospheric pressure occurs forming the shock front. Following the shock front is an expansion wave which slowly depletes the overpressure wave as it travels. The combination of these two waves form the positive phase of the blast wave which is responsible for primary blast injury. Moderate and mild TBI is conjectured to be caused by primary blast wave due to the non-fatal nature of injury and lack of skull fracture or open wounds [12, 13, 14].

Primary blast injury can further be divided in primary injury, or the mechanical insult causing the injury, and secondary injury comprising the biochemical sequelae following mechanical insult. Several mechanisms of primary injury have been proposed. (1) Thoracic mechanism in which energy from the shockwave is transferred from the thorax through the vasculature to increase brain pressure [15, 16] (2) Translational and rotational head acceleration [16] (3) Direct shock wave transmission through the skull [17, 18, 19] (4) Skull flexure [5, 20] (5) Cavitation [21, 22]. This primary injury leads to skull fractures, brain contusions, axonal injury, rupturing of blood vessels, and intracranial hemorrhages [23]. Primary injury is thus thought to be irreversible, forms immediately at the time of injury and plays a pivotal role in propagating secondary injuries during the evolution of bTBI pathology.

Secondary injury on the other hand evolves over time, and is characterized by a complex of biochemical events the lead to blood brain barrier (BBB) disruption, neuroinflammation, elevated intracranial pressure, brain edema, cerebral hypoxia, ischemia, and delayed neurodegeneration [24, 25, 26]. Subsequent biochemical events generate a large amount of toxic and pro-inflammatory molecules such as reactive oxygen species, reactive nitrogen species, and pro-inflammatory cytokines. These molecules further lead to BBB disruption, edema, cell death via apoptosis or necroptosis, lipid peroxidation, and local hypoxia. These secondary injury mechanisms are considered to be distinct events that contribute to chronic pathologicaland neurobehavioral deficits, but recent studies show that primary and secondary events are highly interactive and often occur in parallel [27].

Many neurocognitive deficits have been reported in returning soldiers with bTBI include an array of deficits that are grouped into five nosological clusters. The first being cognitive dysfunction which consists of difficulties in executive function, language, attention, memory, sensory motor integration, visuospatial cognition, and affect recognition. These symptoms are associated with damage to the neocortex [28].

The second cluster of symptoms is neurobehavioral disorders. These consist of affect, anxiety, mood, posttraumatic stress disorders, as well as agitation, loss of libido, and sleep problems. These symptoms are associated with damage to the cortex, limbic system, and/or brain stem monoaminergic projections [28]. The third cluster of symptoms are somatosensory disruptions. This can include impaired equilibrium, hearing, smell, somatosensory perception, taste, and vision. These symptoms are believe to be caused by trauma to the sensory organs themselves or through their projection passing through the brain stem to their respective processing centers [28]. The fourth nosological cluster of symptoms is somatic symptoms that consist of chronic pain and headaches. The fifth and final symptom cluster is substance dependence.

After suffering mild TBI from blast exposure, many soldiers are unable to recognize they have been injured and thus will not seek medical attention [29]. The first indication that the soldier may have been injured include signs such as short term memory loss, vertigo, headaches, and difficulty multitasking or concentrating [30, 31, 32]. Blast even at a low intensity is known to produce set of neurological symptoms known as "shell shock" or "blast concussion". Like other forms of TBI symptoms of blast TBI include somatic, behavioral, psychological and cognitive symptoms. Collectively these symptoms are referred to as post concussive syndrome (PCS) and can include compromised executive function, confusion, retrograde amnesia, headache, mood disturbances, anxiety, altered sleep patterns, and difficulty concentrating [33]. An increase in the occurrence post-traumatic stress disorder (PTSD) symptoms [34, 35] is also observed following blast. This claim is difficult in that TBI and PTSD are often comorbid [36, 37, 38]. The symptoms observed may be due to TBI, PTSD, or a combination of the two [33]. The fact that symptoms of explosive blast mTBI and PTSD can occur in the same individual [31, 39, 40] is not

surprising given the inherently violent nature of an explosive blast. Because of the similarities of these two diseases, there is the potential for misdiagnosis [29].

The American congress of rehabilitation medicine (ARCM) [41] defines mild TBI as possessing are least one of the four following criteria. (1) Any period of loss of consciousness (2) Any loss of memory for events immediately before or after the accident, (3) any alteration in mental state at the time of the accident (e.g., feeling dazed, disoriented, or confused), and (4) focal neurological deficits that may or may not be transient. The patient must not exceed (1) loss of consciousness of 30 minutes or less, (2) after 30 minutes an initial GCS of 13-15, and (3) post traumatic amnesia (PTA) not greater than 24 hours [41].

For a psychiatric diagnosis to be made, a patient must report the symptoms that meet the published diagnosis criteria. These patients must present with a minimum number of elements of the diagnosis criteria [42]. These diagnostic methods do not take into account the underlying pathological changes caused by blast exposure. Due to overlap of symptoms between TBI and PTSD, there is no rational scientific basis to attribute these symptoms to one, the other, or both of these two disorders [43].

The diagnosis of TBI following exposure to an explosive device is complicated by several factors. These explosions produced typically cause injury to more than just the central nervous system. Due to secondary, tertiary, and quaternary effects of blast injury, the injury is typically a combination of blast, blunt, penetrating injury. The complexity of these multiple injury makes diagnosis of blast TBI difficult [43]. For example, when patients did not exhibit acute presenting signs experienced physicians failed to diagnose 36% of primary blast TBI cases [44]. This bias in diagnosis may be contributed to by the historical belief that loss of consciousness is necessary for TBI [44].

Second, TBI patients report a wide range of problems following injury. These can include some or all of neurological problems, emotional difficulties (anxiety or

depression), or cognitive changes (impaired memory, and concentration) [45, 46, 47]. Impaired concentration, increase irritability, angry outbursts, insomnia, and decreases interest in usual activities are diagnosis criteria for both TBI and PTSD [48]. This overlap makes it difficult to attribute the patients neurological and psychological symptoms to TBI or a combination of TBI and PTSD [49].

The final complicating factor is that battlefield reports of blast TBI are missing information about the injury event. This information is critical for understanding the epidemiology of the injury. It is, therefore, not possible to create accurate statistical associations between injury severity and presentation of clinical symptoms [43].

Determining the pathology associated with the persistent cognitive deficits associated with mild bTBI is problematic due to no demonstrable abnormalities appear on standard MRI scans [50]. Recent studies using more powerful 7 tesla MRI have found soldier suffering from mild blast TBI have significant metabolic injury (significant reductions in the ratio of N-acetyl-acetatem (NAA) to choline (Ch) and NAA/Cr) to the anterior hippocampus (close to amygdala) while soldiers suffering from non bTBI related PTSD suffer metabolic injury to the posterior hippocampus [51, 52]. TBI or PCS may be suspected when the predominant symptoms are cognitive, physical, and somatic symptoms, where PTSD will be suspected when the psychological symptoms are the primary concern [37].

1.1.1 Animal Models of Blast TBI

Blast animals models have been established in both rodents [53, 54, 55, 56, 57] and swine [58, 59]. These models have used either live explosives [55, 58] or recreate the shockwave using compressed air [56, 60, 61]. The models were developed to study the pathology of bTBI and develop pharmacological agents to help with the healing process.

Animal models play an important role in TBI research in that they provide tools to study the cellular, molecular, and biomechanical events of TBI which can't be addressed in the clinical setting due to lack of data [62].

As noted in the previous paragraphs, both primary and secondary pathological mechanisms contribute to bTBI and that these events further lead to neurocognitive deficits that negatively impact on the quality of life in veterans returned from recent combats.

1.2 Motivation for Dissertation Research

While some information related to primary and secondary pathology in bTBI is available, a puzzling question still remains as how all these events coherently contribute to ultimate neurocognitive and neurobehavioral deficits that the veterans currently experience. A fundamental gap in the knowledge for the lack of sufficient progress is that precise mechanisms that contribute to sequential progression of pathological events are clearly not defined. As noted earlier, the vast majority of soldiers experience a mild form of bTBI that leads to both neurocognitive and neuropsychological dysfunctions immediately following injury as well as develop years after the conflict. Therefore, to formulate effective treatments to either prevent or mitigate the cognitive decline associated with bTBI, an understanding of the exact pathology of the injury is the crucial first step. We were therefore motivated to focus our work understanding how acute and chronic neuroinflammation evolves in bTBI and what are the major mechanisms responsible for such neuroinflammatory events. We have also attempted to integrate neuroinflammatory factor(s) with behavioral and cognitive deficits in bTBI by using specific inhibitor of NLRP3 inflammasome, a major mechanism that produces chronic neuroinflammation in bTBI.

1.3 Hypothesis and Specific Aims

Based on the need to characterize the secondary injury specifically the immune response to primary injury caused by BBB disruption [63] produced by exposure to shockwave, we hypothesize that blast induced TBI exerts chronic neuroinflammation mediated by activated microglia through the stimulation of the NLRP3 inflammasome. A corollary to this is inhibition of NLRP3 inflammasome activation will mitigate the cognitive decline associated with bTBI.

In order to test this hypothesis, we have identified three specific aims each with their own sub hypothesis.

1. Evaluate microglia activation process as a function of time post- mild bTBI
    - Hypothesis: Microglia activate almost immediately following blast and will remain activated for at least 30 days
2. Explore the temporal activation of the NLRP3 inflammasome in two vulnerable brain regions
    - Hypothesis: A primary mechanism responsible for chronic microglia activation is stimulation of NLRP3 inflammasome and activation of their downstream products (IL-1β and active caspase-1)
3. Examine the effects of NLRP3 inflammasome inhibition on neurocognitive outcome
    - Hypothesis: Inhibition of NLRP3 inflammasome activation (and probable reduction in IL-1β) will mitigate the neurocognitive decline observed in bTBI

The novelty in this work lies in the specific targeting of the NLRP3 inflammasome as the source of the neuroinflammation and associated cognitive decline found after mild bTBI. This work will allow for opening new therapeutic avenues that will be effective in the treatment of bTBI.

1.4 Organization of Dissertation

Here in Chapter 1, we outlined the background of bTBI and the diagnosis of the associated cognitive decline, the motivation for the work as well as my hypothesis and specific aims. In Chapter 2, we will introduce microglia role in the secondary injury following TBI. Here we will introduce the methodology to investigate microglia activation, and how it can be measured. In this chapter, we will also introduce the different TBI animal injury models. In Chapter 3, aim 1 will be explored. In this aim, we will identify the activation profile of microglia found in the hippocampus and thalamus over 30 days following blast. At the conclusion of the chapter, we will have identified the distribution of activated microglia that will be involved in secondary injury following blast. In Chapter 4, aim 2 will be explored. In this aim, the activation of NLRP3 inflammasome and its downstream products of activation will be assessed as well as their cellular origin. The results from this study will implicate NLRP3 as the mediator of the inflammatory response as well as give insight into the activating stimuli for the inflammasome. In Chapter 5, aim 3 will be addressed. In this aim, the inhibition of the NLRP3 inflammasome via low dose MCC950 treatment effect on the cognitive decline associated with bTBI. In the final Chapter 6, we summarize the findings, outline the scientific contributions and along with future direction of research.

CHAPTER 2

ROLE OF MICROGLIA IN THE PATHOGENESIS OF TBI

2.1 Microglia Response to Brain Injury

Microglia constantly survey the CNS microenvironment for any changes in homeostasis using their highly motile processes. In their resting state, microglia possess a rod shaped soma with processes extending out symmetrically in all direction [64]. Processes are motile with an average extension and retraction rate of 1.47 μm per minute and ranging from 0.4 – 3.8 μm per minute, respectively. Upon microglia activation a series of characteristic morphological changes occurs. Usually, upon injury the processes motility changes from undirected to targeted movement towards the injury site [64]. In this instance, the processes begin to retract and the soma enlarges and become spherical in shape [65]. Finally, microglia cells begin to migrate to the site of injury at a rate of 1-2 μm per hour [64].

Borrowing from the macrophage literature, Boche et al proposed a similar system for classifying microglia activation [66]. Classical microglial activation was categorized as M1 (proinflammatory) state or M2 (anti-inflammatory) state [66, 67]. The M1 state is initiated by events such as TBI, wherein, the microglia synthesize and release excess superoxide, nitric oxide, proinflammatory cytokines and chemokines. Although, the secretion of these compounds by microglia are primarily for host defense, often an exaggerated response follows an insult resulting in by-stander injury of the surrounding tissue [68]. When in M2 state, microglia produces anti-inflammatory cytokines (IL-4, IL-10, IL-13, IL-18) which promotes matrix remodeling, angiogenesis, tissue repair and regeneration among other functions [69]. Recent studies have shown that activated microglia can exist in both states

simultaneously where individual cells produce danger associated molecular patterns (DAMPS) or pathogen associated molecular patterns [70].

The application of the nomenclature of macrophages to microglia from the study of peripheral macrophages can be considered ambiguous in defining the precise role of resident microglia [71]. Fist, the M1/M2 definition largely derived from exposing isolated cells to purified stimuli in vitro. These conditions are rarely found in vivo. Second, the cardinal regulators of macrophages bias are never found in vivo in isolation. Third, M1 and M2 states fail to emerge as isolated pure phenomena in vivo. Fourth and possibly most important, macrophage polarization was developed using monocyte or bone-marrow-derived macrophages, that invade infected, traumatized or neoplastic tissues but not a physiological characteristic of resident microglia in the CNS, which are resident tissue macrophages, originate from extra-embryonic yolk sac [72], that are distinct from the circulating hematopoietic system [73] and are highly adapted to the CNS environment [74].

A more modern description of activated microglia phenotypes is that they fall along a spectra as opposed to two binary states [75]. Depending on the stimulus present in the microglia local microenvironment, the microglia will be polarized to either the M1 or M2 like states. Ransohoff suggests that a new classification should be created based on, but not limited to transcriptomic and proteomic profiles, regional heterogeneity, sexual dimorphism, function in healthy nervous system, and pattern of response to changes caused by trauma, infection, systemic inflammation, tumor, ischemia, and neurodegeneration [71].

Similar to human microglia, rodent resident microglia display four main phenotypes in grey matter; resting, primed, activated, and amoeboid [76, 77, 78]. Resting microglia possess a small spherical cells body with several highly branched processes radiating in all directions. Although these cells are called resting, these cells are highly active constantly surveying their microenvironment for any changes

to homeostasis. Primed microglia possess a larger more oblong cell body but present with similar number of primary and higher order processes to resting microglia. The primed microglia act as an intermediate between resting microglia and activated microglia. These cells have detected a priming stimulus the induces the production of proinflammatory precursor molecules. Activated microglia possess an amoeboid cell body with fewer, shorter, and less branched processes as compared to resting and primed microglia. These cells have received a full activating stimulus and at this point capable or phagocytosis, antigen presenting, and the release of proinflammatory or anti-inflammatory molecules. Amoeboid microglia possess an amoeboid cell body and can possess up to two unramified processes roughly equal in length to the cell body [76, 77, 78, 79] (Figure 3.3). These cells have dedicated themselves to phagocytic activity but are still able produce inflammatory modulators.

Majority of literature of microglia activation in TBI used four major animals models used include weight drop injury (WD), control cortical impact injury, (CCI), fluid percussion injury (FPI), and blast/diffuse brain injury [80]. The WD model mimics cerebral contusion found in TBI. It is performed by dropping a weight from a predetermined height onto the skull or exposed dura. In a modified version of WD model, also known as the closed head injury model, a metal plate is placed above the skull to distribute weight over a larger area and prevent skull fracture. WD model in mice showed signs of diffuse neuronal loss, neuroinflammation, markers of apoptosis, and short and long term cognitive impairments [81]. Similar to the WD model, CCI injury uses a solid impactor to damage exposed dura. To increase the reproducibility of the injury, a pneumatic or electrochemical device is used. This mechanism also reduces rebound injury produced by gravity driven devices [82]. By adjusting location, shape of impactor, velocity, and the depth of brain deformation, different types of injuries can be produced. CCI injuries are typically manifest as cortical tissue loss, axonal injury, BBB dysfunction, and subdural hematoma [83, 84, 85, 86].

In the FPI injury model, a craniotomy is performed to expose a portion of the dura. The injury is inflicted by directing a fluid pulse, produced by a pendulum striking a piston of fluid reservoir, against the exposed dural surface [82]. The injury model is characterized by a diffuse injury producing vascular and axonal damage. The model produces amalgamation of cortical contusion and diffuse subcortical neuronal injury [80]. Studies have varied the location of injury site, but most commonly, the injury is performed lateral to the sagittal suture (LFPI) or medially (CFPI) [82]. The, blast/diffuse injury, attempts to replicate the shockwaves from explosive devices to cause a diffuse brain injury. To recreate the Friedlander waveform produced in the explosion, either live explosives or a gas driven shock tube are used. Blast/diffuse injury is characterized by diffuse cerebral brain edema, extreme hyperemia, a delayed vasospasm, and diffuse axonal injury [2, 87].

Microglia activation follows a different temporal pattern depending upon the type and severity of brain injury CCI and FPI are the most studied of the different injury models where microglia activity state have been thoroughly investigated. Using markers such arginase (Arg-1) [88], CD206 [89, 90], and YM-1 [90, 91] to signify M2 activated microglia and CD16 [90], CD86 [89, 90] for M1 activated microglia. In blast and weight drop models only the time profile of microglia activation has been studied. For instance, following CCI injury M2 like microglia increase in the first week following injury. The number of M2 microglia peak at 5 days post injury but decrease rapidly in number immediately afterwards [89, 92]. M1 microglia begin to increase in the cortex, striatum and corpus callosum at 1 week [92], peaking at 4 weeks following injury [89]. M2 type microglia predominate in the acute phase of injury while the M1 phase of microglia remain during the chronic phase after focal injury produced by CCI. Similar to focal injury, diffuse injury produced by FPI induced transient activation of M2 type of microglia that is resolved within 7 days after injury. M1

type microglia remained in their activated amoeboid morphology for up to 30 days following injury [92].

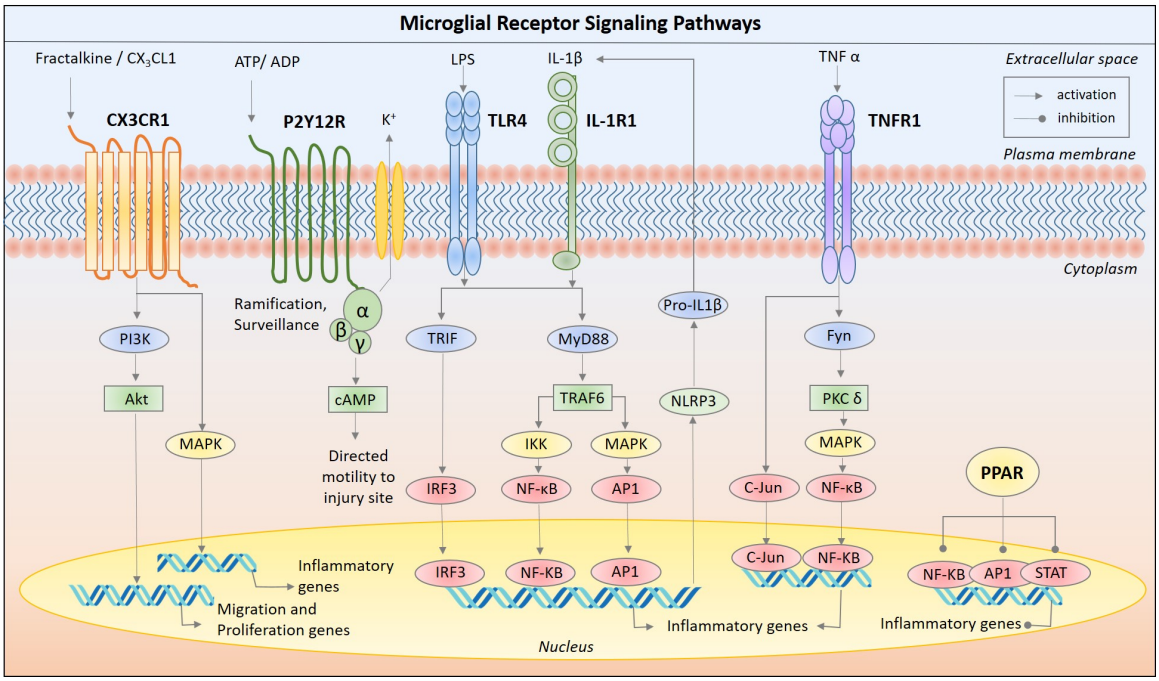

Figure 2.1 Possible receptor activation in microglia following blast.

It is interesting to note that microglia activation patterns differ not only temporally, but also spatially following brain injury. The spatial variability in microglia response to injury may depend upon regional differences in mechanical loading and/or the intrinsic property of the tissue. Despite the limited studies on mechanical signaling in microglia, a recent study showed cultured microglia cells were susceptible to mechanical changes [93]. In line with this, another study showed that mechanical loading was capable of modulating microglia proliferation, activation and chemotaxis [94]. The response of microglia to injury may also depend on the intrinsic property of the tissue such as microglia/blood vessel distribution and neuronal vulnerability within the region. There is a lower occurrence of microglia in grey matter areas compared to the white matter. Regionally, the lowest of occurrence is the cerebellum (0.3%), frontal (4.7%), parietal (3.6%) and occipital (2.9%) lobes of

the cerebrum with highest expression levels in the white matter track of the medulla oblongata (16.9%) [95]. A recent study showed that microglia activation paralleled the pattern of neuronal loss in a mouse model of CCI injury [96]. This in line with another study that observed prominent occurrence of activated microglia in regions of neuronal loss including the ipsilateral cortex, hippocampus, and thalamus after injury [97]. In a blast model of brain injury, microglia activation was found to be closely associated around the blood vessels suggest that the difference in the distribution of microglia and blood vessels may underlie some of the regional vulnerabilities to injury [65].

2.2 Microglia Activation Contribute to Secondary Complications in TBI

Depending on the type of TBI, the secondary injury may involve a wide array of mechanisms including oxidative stress, neuroinflammation, BBB disruption, cells death, mitochondrial dysfunction, and neurotransmitter release [98, 99]. Studies conducted thus far, have reported the involvement of microglia receptor activation in not only altering microglial morphology and motility, but also in neurotransmitter release, modulating neuronal-glial synaptic transmission, secretion of cytokines, generation of reactive oxygen species, and production of nitric oxide. Here, we summarize the microglia mediated secondary mechanisms following brain injury.

2.2.1 Aberrant Neurotransmitter Release

Activated microglia contribute to neuronal excitotoxicity by releasing neurotransmitters in response to several external stimuli. The known neurotransmitters released by microglia include glutamate and ATP. Glutamate release by microglia can be triggered by activation through multiple receptor systems. For instance, microglia release of glutamate may be induced by secreted amyloid precursor protein (APP) [100] or Amyloid β [101]. Similarly, TNF-α induced the release of glutamate by

upregulating glutaminase. Microglia are capable of releasing sufficient amount of glutamate to contribute to neural degeneration [102]. Such excess release of glutamate has been reported following ischemic brain injury [103]. However, there is no direct evidence to show microglia release of glutamate in TBI models. The excess glutamate may activate glutamate receptors in neurons leading to excitotoxicity. Alternatively, they can activate microglia glutamate receptors including AMPA receptor [104, 105, 106], metabotropic glutamate receptors [107, 108, 109, 110], and NMDAR [111, 112, 113] resulting in a cascade of secondary events.

Adenosine triphosphate (ATP) is the other commonly released neurotransmitter by microglia. ATP release from microglia cells can be induced by bacterial endotoxin lipopolysaccharide (LPS) [114, 115]. Lysophosphatidic acid (LPA) induced the release of ATP via activation of the LPA3 receptor [116]. High intracellular calcium levels have been shown to induce ATP release in microglia [117]. Extracellular ATP was shown to enhance radiation-induced brain injury through microglial activation and paracrine signaling via P2X7 receptor [118]. Microglia abundantly express ATP-mediated purinergic receptors. Although the microglia release of ATP in brain injury has not been shown, the microglia response via purinergic signaling has been well-studied in brain injury models [119]. The purinergic receptors in microglia are abundant and are implicated in important functions such as cytokine production (P2X receptors), motility (P2Y12 receptor) and in phagocytosis (P2Y6 receptors) [120, 121, 122]. The expression and function of these receptors in neuronal/glial cells in neuropathologies have been reviewed previously [123]. However, little is known of their role in brain injury and is worthy of investigation.

2.2.2 Oxidative Stress

Under normal physiological conditions, there is a delicate balance between reactive oxygen species (ROS) and their removal via antioxidants. Following TBI, the balance

can be disrupted leading to an excess buildup of ROS [124, 125]. Accumulation of ROS/RNS is known to mediate cellular damage via lipid peroxidation, protein modification, and/or DNA strand breaks [126]. Activated microglia produce superoxide by the enzymatic activity of NADPH oxidase (NOX), a multi-subunit enzyme that catalyzes the production of superoxide from oxygen [127]. Microglia, like all phagocytic cells express NOX2 [128]. NOX2 generates superoxide molecules which help in the neutralization of foreign pathogens [129]. It was shown that NOX2 mediated ROS production was strongly upregulated in M1 but not M2 polarized microglia in CCI injury. Inhibiting NOX2, by using selective peptide inhibitor gp91ds-tat or NOX2 knockout mice, reduced markers for M1 activated microglia, limited tissue loss, and improved motor recovery. Inhibition of NOX2 also promoted M2 like activation in microglia [130]. In FPI injury, inhibition of NOX with apocyanin had no effect on neuromotor function but reduced the release the proinflammatory cytokines IL-1β, and TNF-α at 3h and 24h after injury [131]. Recently, it was shown that NOX2 expression was increased at 24h after moderate blast injury (180 kPa) and this directly correlated with elevated ROS production [132]. Activation of cytokine receptors such as IL-2R [133, 134], IL-15R [135], TNFR1 [136], INF-γR and thrombin receptor [137] in microglia have also been implicated in ROS production. ROS production by microglia may also be mediated by Notch-1 receptor [138, 139], dopamine receptor D1, D2 [140, 141], and Angiotensin II receptor [142].

2.2.3 BBB Disruption

The central nervous system vasculature differs from the rest of the vasculature in that it possesses the BBB. Microglia, together with endothelial cells (ECs), pericytes and astrocyte end feet, form the functional BBB, a specialized structure that selectively separates the brain parenchyma from the peripheral blood. Studies on the interaction between microglia and BBB in both physiological and pathological conditions is

limited and has recently gained more attention [143, 144]. The perivascular microglia communicate with the ECs and survey the influx of blood-borne components into the CNS. Hence, any disruption of the BBB as reported following brain injury, can prime and attract microglia [65].

Microglia activation dependent alterations to BBB after TBI is thought to be predominantly mediated by neuroinflammation and oxidative stress [145, 146, 147]. Therefore, most TBI studies that investigate both microglia activation and BBB disruption focus on shifting microglia from their proinflammatory M1 state to their anti-inflammatory M2 state. For instance, following CCI injury binding of 2 arachidonylglycerol (2-AG) (agonist) to the cannabinoid receptor in microglia shifted microglia at 3 and 7 days from M1 to M2 activated phenotypes. This shift in activation was coupled with the reduction of BBB permeability at the same time points [148]. Reduction in the release of pro-inflammatory cytokines and reduction of inducible ROS production prevented further degradation BBB [148]. Pretreatment with apocynin (NOX inhibitor) prevented BBB disruption following injury produced by WD [149] , as well as, blast injury model [150]. Microglia as the major source of NOX mediated ROS production are implicated as the cause for the increase permeability of the BBB following injury [149]. In a mouse model of mild blast injury, microglia activation was restricted to regions close to the blood vessel microdomains, as evidenced by rapid microglial process retraction and increased amoeboid morphology [65]. Taken together, these TBI studies confirm that there is a close association between microglia activation and BBB disruption following injury.

2.2.4 Neuroinflammation

Neuroinflammation is one of the key mediators of secondary injuries following brain injury. The acute response includes secretion of pro-inflammatory cytokines within minutes following injury. The activation of resident microglial cells, alongside

the infiltration of peripheral macrophages, are key mediators of neuroinflammatory responses after TBI. Several studies have attempted to discriminate the differential roles of resident microglia and infiltrated monocytes after brain/spinal cord injury [151, 152, 153]. In this dissertation, we highlight the contribution of microglia to inflammation, however, the role of infiltrating monocytes/macrophages in neuroinflammation and overall TBI pathology cannot be overlooked.

Upon foreign ligand detection, microglia can release a wide range of cytokines and chemokines. These molecules guide the expression of adhesion molecules, signal peripheral immune cells to infiltrate the injury site, and further release of pro-inflammatory mediators and growth factors that regulate neuronal death or regeneration [154]. Many studies have investigated cytokines and chemokines expression following TBI. These studies have focused on levels of cytokines found in homogenized tissue section or CSF. These studies attribute the increased expression of cytokine to the activity of activated microglia. This assertion may be correct, but microglia's contribution to the inflammatory environment is unlikely to be exclusive [155].

Following CCI injury, levels of IL-1β and IL-18 increased in brain homogenates surrounding the contusion site. Levels of IL-1β peaked at 6h post injury and decreased to control level by 7 days. IL-18 levels gradually increased over the 7d observation period. Microglia specific release was confirmed by colocalization of NLRP3 inflammasome and its associated components (ASC and caspase1) in microglia cells but not with astrocytes or neurons [156]. Chio et al found that administration of Etanercept, a TNF-α receptor antagonist, attenuated the release of TNF-α following FPI leading to reduced motor and neurological deficits as compared to the control and saline treated groups. At 72 hours after injury, TNF-α secreted by microglia increased in ischemic cortex, white matter, hippocampus, and hypothalamus. Double immunostaining confirmed that neuronal and astrocytic

TNF-α levels were not significantly different between control, saline and etanercept treated groups. Leading to the conclusion that microglia was the prominent source of TNF-α production following diffuse TBI [157].

Bachstetter et al showed that p38α (MAPK14) protein kinase plays a role in the production of TNF-α and IL-1β by cultured microglia cells [158]. Using midline FPI model they investigated whether myeloid specific deletion of p38α influenced microglia cytokine production following injury. Unexpectedly they found that during the acute phase (0-12 h), release of IL-1β, IL-6, and TNF-α was greater in the p38α knockout (KO) compared to the wild type injured animal. This increase could not be accounted for by the increase in infiltrating immune cells (macrophages and neutrophils) or by astrogliosis, as cell number were decreased in the KO animals relative to the WT. In the chronic phase (7d), levels of IL-1β were significantly reduced in the p38α KO animals as compared to the WT injured animals. These findings suggest that microglia may not contribute significantly to inflammation in the acute phase but is responsible for the pro-inflammatory environment found in the chronic phase of injury [159].

Microglia release proinflammatory cytokines such as IL-1β upon ligand binding to the NMDAR [113], angiotensin II receptor [142], and IFN-γR [154]. Release of TNF-α has been reported upon actation of AMPA receptor [104, 105], NMDAR [113], kainate receptor [106, 112], metabotropic glutamate receptor [108, 110, 160, 161] α1a, α2a, β1 adrenergic receptor [162], IFN-γR [154], TLR2 [163], TLR3 [164], TLR9 [165], CD-14 [166], and thrombin receptors [166]. Release of IL-6 upon GABAR [167] α1a, α2a, β1adrenergic receptor [162], TNFR1 [168], IFN-γ [154], IL-1R1 [169], TLR2 [163], TLR3 [164], TLR5 [165], TLR9 [165], CD-14 [166], Thrombin receptor [137] activation. Release of IL-12 was noted upon activation of GABAR [167], IFNγR [170], TLR3 [164], TLR9 [165], CD-14 [166], and thrombin receptors [137].

Microglia are also capable of releasing chemokines, such as release of CCL2 upon IFN-γR [170] TLR2 [163] activation. Release of CCL3 upon IL-13R [171, 172] CD-14 activation. Release of CCL5 upon IL-3R [171, 172] activation. Release of CXCL-10 upon TLR3 [164] and TLR4 [164] activation. Release of CXCL2 upon CD-14 [166] activation. Once released these factors affect adjacent cells such as astrocytes and/or neurons in a paracrine manner since these cells possess receptors for these factors. Additionally, these factors could also affect microglia in an autocrine manner. In both scenarios, the net effect exacted by these factors include lipid peroxidation, immune cell recruitment, BBB disruption, and the development of cerebral edema[27, 173, 174].

2.3 NLRP3 Inflammasome

The innate immune response of the central nervous system plays an essential role in the pathology following injury. The activation is initiated by pattern recognition receptors (PRR), such as Toll like receptors (TLR) cell surface receptors responding to pathogen associated molecular patterns (PAMPS) and DAMPS, NOD-like receptors (NLR) cytosolic receptor for PAMPS the enter the cell through phagocytosis or pores, and RIG-I like receptors response (RLR) cytosolic receptors the respond to viral DNA and RNA. The receptors are responsible for initiating the production of proinflammatory cytokines such as IL-1β, IL-18 tumor necrosis factor, and type 1 interferons that activate the adaptive immune system[175].

Our interest focuses on the NLRP3 inflammasome largely due to its known expression in microglia cells [176, 177, 178]. NLRP3 the most studied inflammasome is typically found in innate immune cells and is found to be activated by a wide range of stimuli such as viral, bacterial, and fungal components components[179, 180] endogenous danger signals such as extracellular ATP [181, 182, 183], amyloidβ [176]

and uric acid crystals [176, 183, 184], and environmental micro particles such as silica crystals [185].

The activation of NLRP3 is a two-step process that requires two activating stimuli[186]. The first signal usually through TLR receptors primes the cell to transcribe and translate pro-IL-1β and in some cases, NLRP3 expression [176, 183, 187]. The second signal typically ATP or oxidative stress, induces the formation of the inflammasome complex which cleaves the pro-IL-1β into its active form and is subsequently released from the cell.

The fact that NLRP3 activation is induced by various stimuli suggests that the NLRP3 inflammasome acts as a general sensor of cellular damage or stress [186]. It has been shown to be activated by multiple secondary injury mechanisms including ion fluxes induced by extracellular ATP [188, 189], endosomal rupture [178, 182, 185, 190], production of reactive oxygen species (ROS) [181, 191, 192, 193, 194], and mitochondrial dysfunction [195, 196, 197] have been repeatedly shown to trigger the activation of the NLRP3 inflammasome[186].

Originally thought to be expressed exclusively in microglia, NLRP3 is now found to be expressed in neurons. The time course for expression following TBI differ among the two cell types. Where early (acute) expression is found in neurons, and later (subacute) expression to be found in microglia [79].

CHAPTER 3

EVALUATE MICROGLIA ACTIVATION AS A FUNCTION OF TIME POST MILD TBI

The first part of specific aim 1 is to identify the microglia activation profile following exposure to mild 180 kPa blast in the hippocampus and thalamus over 30 days. The rationale behind this work is th glean the information in understanding neuroimmune response to blast injury. Next, the distribution of activated microglia in response to the site of vascular damage (BBB disruption) was investigated to get more insight into the response of microglia to the localized microenvironment. These studies will lay the foundation to answer in aim 2 and 3 whether the neuroimmune response (neuroinflammation) could be a driving factor in the development of cognitive decline observed in returning veterans exposed to blast. This study will also give insight into specific vulnerabilities of different brain regions to primary blast injury by using microglia activation as a marker for injury severity.

3.1 Materials and Methods

3.1.1 Animal Preparation

Adult 10-week-old male Sprague-Dawley (Charles River Laboratories) rats weighing 300–350 g were used in this study. The animals were housed with free access to food and water in a 12h dark-light cycle at 22 °C with 40% humidity. All procedures followed the guidelines established in the Guide for the Care and Use of Laboratory Animals and were approved by Rutgers University Institutional Animal Care and Use Committee. Rats were divided into two groups (sham controls and animals exposed to a mild blast of 180 kPa). A total number of 35 rats was used in this study as follows: immunofluorescence studies (five controls and 30 blast-injured n=5 per time point); For immunofluorescence studies, each brain tissue was processed to obtain several coronal sections (= 20μm) of the hippocampus and thalamus. Each of those

sections were used for identification of microglia and their morphological states in the hippocampus and thalamus near and far from the vasculature.

3.1.2 Primary Blast Wave Exposure of Animals

Using the 9-inch by 9-inch cross section compressed gas driven shock tube at the Center of Injury Biomechanics, Materials, and Medicine (CIBM3) (New Jersey institute of technology, Newark) we determined the effects single mild 180 kPa blast exposure on 10-week old male Sprague Dawley rats [13, 63, 150, 198, 199, 200]. The primary shockwave generated in this shock tube was validated against the pressure-time profiles measured experimentally in the live-fire explosion experiments [201] and against theoretical pressure-time profiles associated with the detonation of C4 explosive [199, 202].

Before exposing to blast, rats were anesthetized with isofluorane. Sham controls were placed next to the shocktube and only exposed to noise without shockwave exposure. Animals for blast exposure were mounted inside the test section located 2.80 meters from the breach and 3.05 meters from the exit with their head oriented to the direction of the shockwave (Figure 3.1). The rats were strapped securely to the bed with cotton cloth restraints to eliminate head motion. This is done to minimize the contributions of secondary blast injury to total head injury. The animals were exposed to single blast at 180 kPa in a prone position.

Immediately after blast exposure, animals were monitored for any signs of apnea and their neurological status was assessed using modified neurological severity score as reported earlier [199].

3.1.3 Sample Collection

Both sham control and animals exposed to bTBI were sacrificed 4h, 24h, 3d, 7d, 15d, and 30d after blast exposure. Sham animals sacrificed at the different time points were

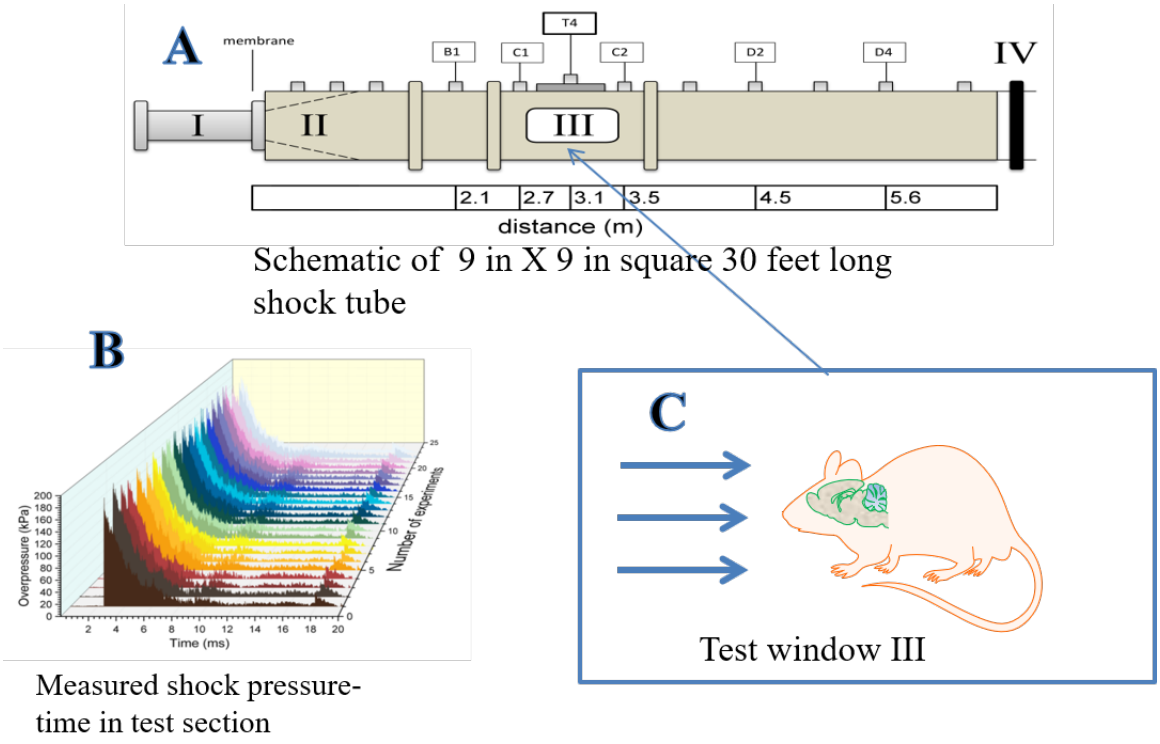

Figure 3.1 (A) Schematic of shocktube with section I-Breech with high pressure helium gas separated from section II by different thickness of mylar sheets that generate pure shock wave in section III where the specimens are located. Section IV is past the section and is a design requirement; the pressure-time cycle is identical to live fire tests with actual C-4 (or TNT equivalent) explosives at specified stand-off distance. (B) Composite of actual experimental profiles that generate 180 kPa with only about 5 kPa variation in peak pressure and less than a millisecond in duration. The front of the pressure rise indicates shock wave conditions. (C) Schematic of rodent model in prone facing the shock front. The shock travels in the caudal-rostral direction traversing pre-frontal cortex, striatum, hippocampus, thalamus, visual cortex and cerebellum within a period of a millisecond with no attenuation of pressure loading.

combined into one group as their biochemical values did not differ at any time points. Animals were anesthetized with a mixture of ketamine and xylazine (10 mg/kg) at 10:1 ratio administered via intraperitoneal injection mixture. For immunofluorescence analysis, rats were transcardially perfused first with phosphate buffered saline (PBS, pH 7.0) (PBS) followed by 4% paraformaldehyde (PFA).

3.1.4 Immunofluorescence and Microscopy

At 4h, 24h, 3d, 7d, 15, and 30d post-injury after perfusion, the brains were removed from cranial vaults and incubated in 4% PFA for an additional 48h and cryoprotected by immersing in 30% sucrose. Coronal brain tissue sections (20 μm thickness) were prepared using the Leica VT1000S vibratome taken from a single location along the longitudinal axis of the brain interaural 6.72 mm Bregma -2.28 mm and immunofluorescence was performed. Tissue sections were mounted on glass slides prepared from five individual animals in each group and were washed with 10 mM PBS, fixed in ice-cold methanol (100%) solution for 10 min at -20 °C. The tissue sections were blocked with 10% donkey serum at room temperature for 1h in 1X PBS containing 0.03% Triton X-100. Fixed tissues were incubated overnight at 4 °C with respective primary antibodies to Iba1 (Pa5-18039 1:250) and RECA-1 in 2% donkey serum. A second 1-hour incubation with Biotin-SP affiniPure Donkey Anti-rabbit IgG to increase the sensitivity of Iba-1 primary antibody.

Double immunofluorescence was performed using Streptavidin Alexafluor 594 conjugate (S32356) for RECA-1, donkey-anti goat Alexafluor 488 for Iba1. The specificity of each antibody staining was validated by excluding each primary antibody (negative controls) and visualized for any nonspecific fluorescence. The primary antibody specificity, however, was not validated independently by blocking the binding to tissue with the corresponding antigen. Slides containing different brain regions were digitized (20x magnification) using Leica Aperio Versa 200 fluorescent

microscope and slide scanner. Fluorescence intensities in each region were quantitated using AreaQuant software (Leica Biosystems) and expressed as average fluorescence intensity*unit area.

3.1.5 Cell Counting/Morphological Analysis

Four separate regions of interest in the hippocampus 500 x 500 μm in size were used for cell counting. For the hippocampus, the different regions of interest comprised of images from CA1, CA3, and the Dentate gyrus for each rat. Five separate regions of interest in the thalamus 500 x 500 μm in size was used for cell counting. For cell counting near the vasculature, a 250 μm diameter circle centered at the center of the vessel of interest was used. A second 250 μm diameter circle adjacent to the vascular area in the same region of the hippocampus or thalamus not containing any vasculature was used for counting microglia away from the vasculature. Total number of microglia within the specified brain regions, determined by the presence of a defined soma and confirmed with presence of nuclei using DAPI stain, was counted manually and expressed in terms of cells per mm^2 (Figure 3.2).

Similar to human microglia, rodent resident microglia display four main phenotypes in grey matter, resting, primed, activated, and amoeboid [76, 77, 78]. Resting microglia possess a small spherical cells body with several highly branched processes radiating in all directions. Primed microglia possess a larger more oblong cell body but present with similar number of primary and higher order processes to resting microglia. The primed microglia act as an intermediate between resting microglia and activated microglia. Activated microglia possess an amoeboid cell body with fewer, shorter, and less branched processes as compared to resting and primed microglia. Amoeboid microglia possess an amoeboid cell body and can possess up to two unramified processes roughly equal in length to the cell body [76, 77, 78, 79]

(Figure 3.4). Using these morphological criteria were assessed for the activation state and expressed as a percent of total microglia.

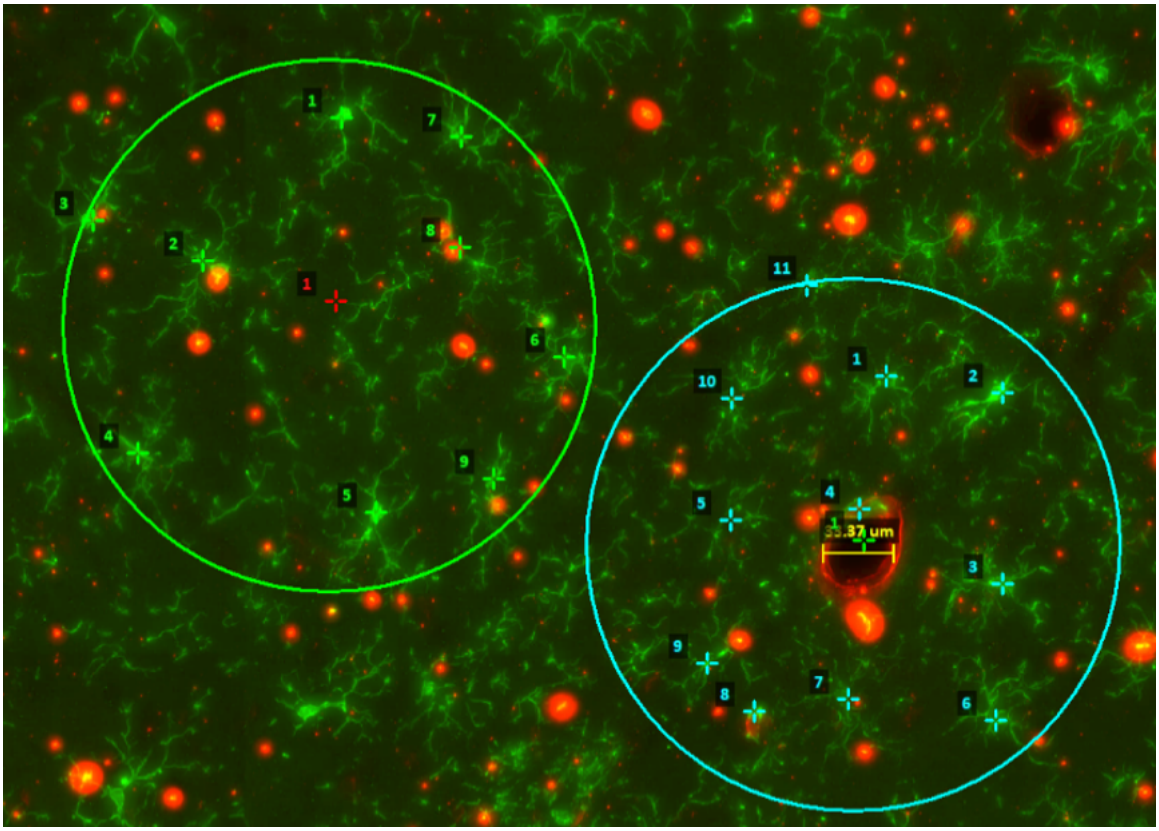

Figure 3.2 Representative examples of the regions in the hippocampus and thalamus used for counting of microglia.

3.1.6 Data Analysis

Statistical analysis on immunofluorescence was expressed as the mean +/- SEM. Statistical analysis of the data was performed using SPSS version 25. Comparison between sham and blast exposed samples was performed by multi-factorial ANOVA. For activation near vessel an Independent sample t-test was used. Differences will be considered significant at $p < 0.05$. If significance is found, a post hoc Dunnett's test will be conducted for main effect of time following primary blast injury. Boxblot analysis was performed to identify outliers and Shapiro-Wilk and Levene's tests was

performed to assess normality of data distribution and homogeneity of variances, respectively.

3.2 Results

3.2.1 Microglia Activate Following bTBI and Remain Active Chronically

To assess the level of microglia activation following blast we first quantified the number of microglia in both the hippocampus and thalamus following blast. The number of microglia significantly increased in both the hippocampus and thalamus starting as early as 4 hours post injury with a 26.36% and 39.47% increase, respectively (Figure 3.3 C,D). In the hippocampus, the number of microglia was significantly elevated at all time points except 3 days ($p=0.055$), with a peak number at 7 days (28.87% increase) (Figure 3.3 C). In the thalamus, the number of microglia was significantly increased at all time points with a peak value at 15d (33.4% increase) post injury (Figure 3.3 D).

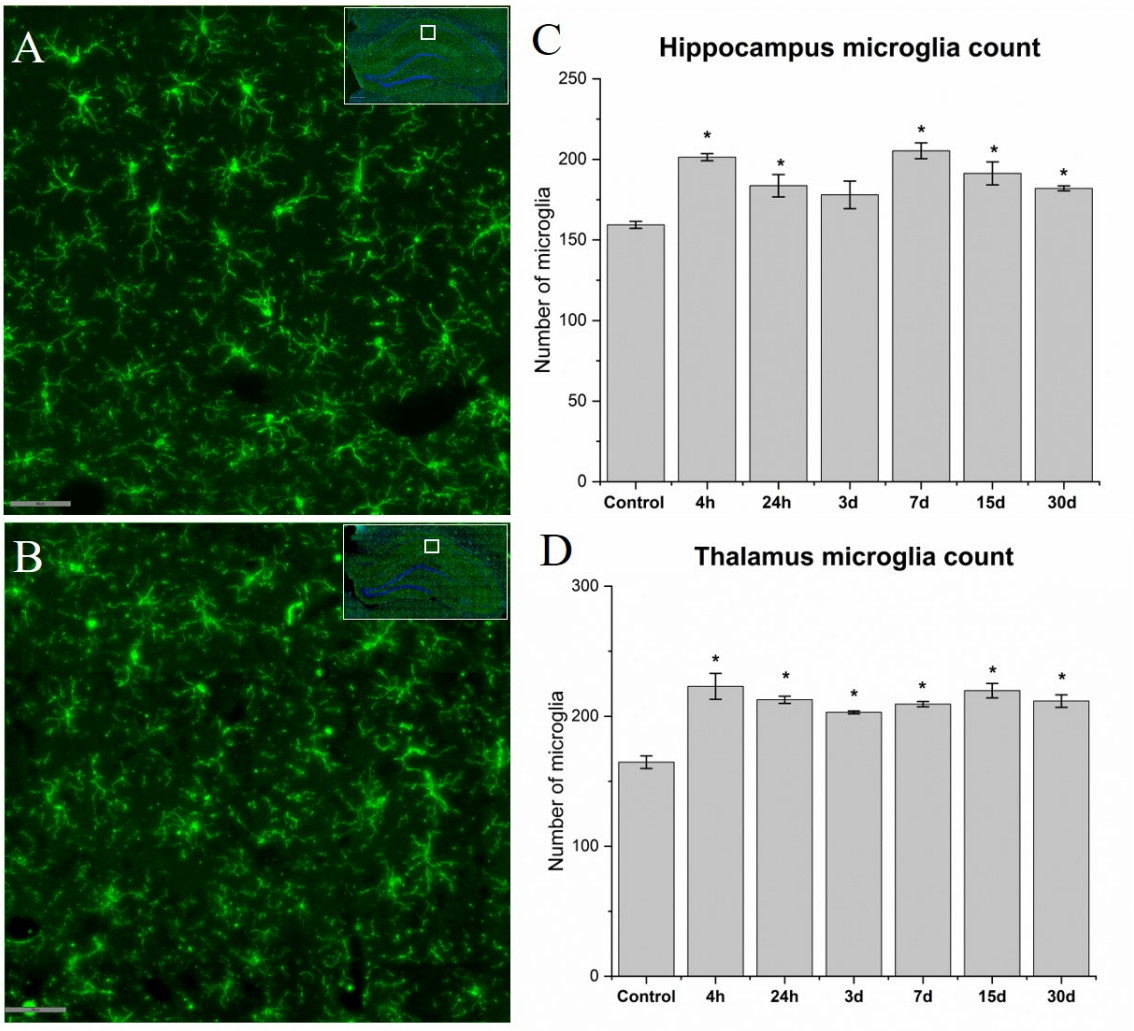

Figure 3.3 Quantitative assessment of microglial number following blast TBI. (A) Number of Iba1-positive microglia in hippocampus from control. (B) Increased number of (Iba1-positive) microglia in hippocampus from animals 4h post-blast showing significant increase in their number. Quantification of number of Iba1-positive microglia in hippocampus (C) and thalamus (D) showing significant increase as early as 4h post-blast. Scale bars = 300μm. *, $p<0.05$ vs. control; Microglial number were quantified from five areas ROIs of 1 mm^2 in each section from five different animals in each group. df=6.

Qualitative evaluation of morphological status of microglia stained with Iba1 showed a statistical decrease in resting microglia in both the hippocampus and thalamus in animals exposed to blast TBI at all time points (Figure 3.5 (A E). Hippocampus and thalamus from sham animals showed the majority of microglia were

in the "resting state" as observed by the extensive arborization of their processes; although a few primed and activated microglia were occasionally observed (Figure 3.5). However, blast injury caused a significant increase in the percentage of microglia within the area of region of interest to activate and transform into the primed and active morphological state indicated by increased soma size and fewer number of processes at all the time points (Figure 3.5 B,C,F,G). There was a statistical increase in the percentage of amoeboid microglia in hippocampus at 4h, 3d, 7d, 30d and in the thalamus at 4h, 24h, 15d and 30d (Figure 3.5 D, H).

3.2.2 Acute activation of Microglia in the Vicinity of Vasculature

At 4h post injury, microglia <100 μm from the vasculature increased in number in both the hippocampus and thalamus. The percent of resting microglia decrease following blast while primed microglia remained unchanged. The two active forms of microglia (active and amoeboid) both increased significantly in both regions (Figure 3.7). At 4h post injury, the number of microglia in the vicinity of the vasculature as compared a vasculature free area was statistically increased in both the hippocampus and thalamus (Figure 3.7). The percentage of resting microglia near the vasculature was significantly decreased while the percentage of primed and active microglia remained unchanged as compared to the non-vascularized region. Virtually all amoeboid microglia were found adjacent to the vasculature and almost none were found in vasculature free area.

Within the blast animal, the number of microglia near the vasculature was significantly elevated compared to an adjacent vasculature free region. The number of resting microglia was significantly decreased near the vasculature and the number of amoeboid microglia was significantly elevated near the vasculature. The number of primed and active microglia did not differ significantly between the two groups (Figure 3.8).

3.3 Discussion

It is interesting to note that at all time points qualitatively examined, microglia at different stages of activation co-existed within hippocampus and thalamus. Our finding on microglia activation following blast align with other laboratories findings that these cells activate immediately and remain active chronically [65, 203]. In the healthy brain microglia are found in a lattice like organization with each cell surveying its own individual section of the brain parenchyma [64]. Following injury, we observed that this even distribution of microglia was disturbed specifically the distribution of activated microglia in the acute time points. We observed higher activation of microglia were present in the vicinity of the vasculature and microglia present away from the blood vessels were primarily in the resting stage. This change in the activity profile of microglia in the vicinity of the vasculature was also accompanied by an increase in microglia number. The increase in of microglia number indicates that the microglia are actively migrating to the vasculature in order to help return the microenvironment. These finding suggest the mechanical opening of the BBB caused by the shockwave is the primary activating stimuli for microglia in the acute phase of injury.

Further, a quantitative increase in microglia number at all-time points was observed in both the hippocampus and thalamus with the largest increase in microglia number at 7 and 10 days, respectively. The activation profile of microglia at the 3 day time point and later were more evenly distributed compared to the acute time points. There was still higher activation in the vicinity of the vasculature but not to the same degree. This activation profile observed in more indicative of the presence of a chronic inflammatory microenvironment. With the higher activation of microglia in the vicinity of the vasculature likely due to the increase BBB permeability induced by IL-1β. This data taken together strongly suggest that a) injury progression in bTBI is a continuous event and b) variable degree of BBB disruption within the same region

could be a stimulating factor for differential activation of microglia. The latter is highly probable due to previous studies from our lab that indicate blood vessels with larger diameter showed great leakage than smaller blood vessels [63]. Such a tenet also holds validity since as shown in Figure 3.6, microglia near larger blood vessels are more highly activated compared to microglia e in the vicinity of small diameter blood vessels. The opening of the BBB allows for the entry of DAMPS from the blood to the brain parenchyma and hence may be a pathological signal for microglia activation since our earlier studies [150] in conjunction with other reports [204] consistently reported BBB disruption is a major component in the pathology of blast TBI. Co-existence of microglia at different stages of activation has also been observed in other neurological conditions including stroke, ischemia, and where BBB disruption is a prominent feature [1]. These This experiment validates my sub hypothesis for aim 1 that microglia activation is a chronic event in bTBI in both the hippocampus and thalamus.

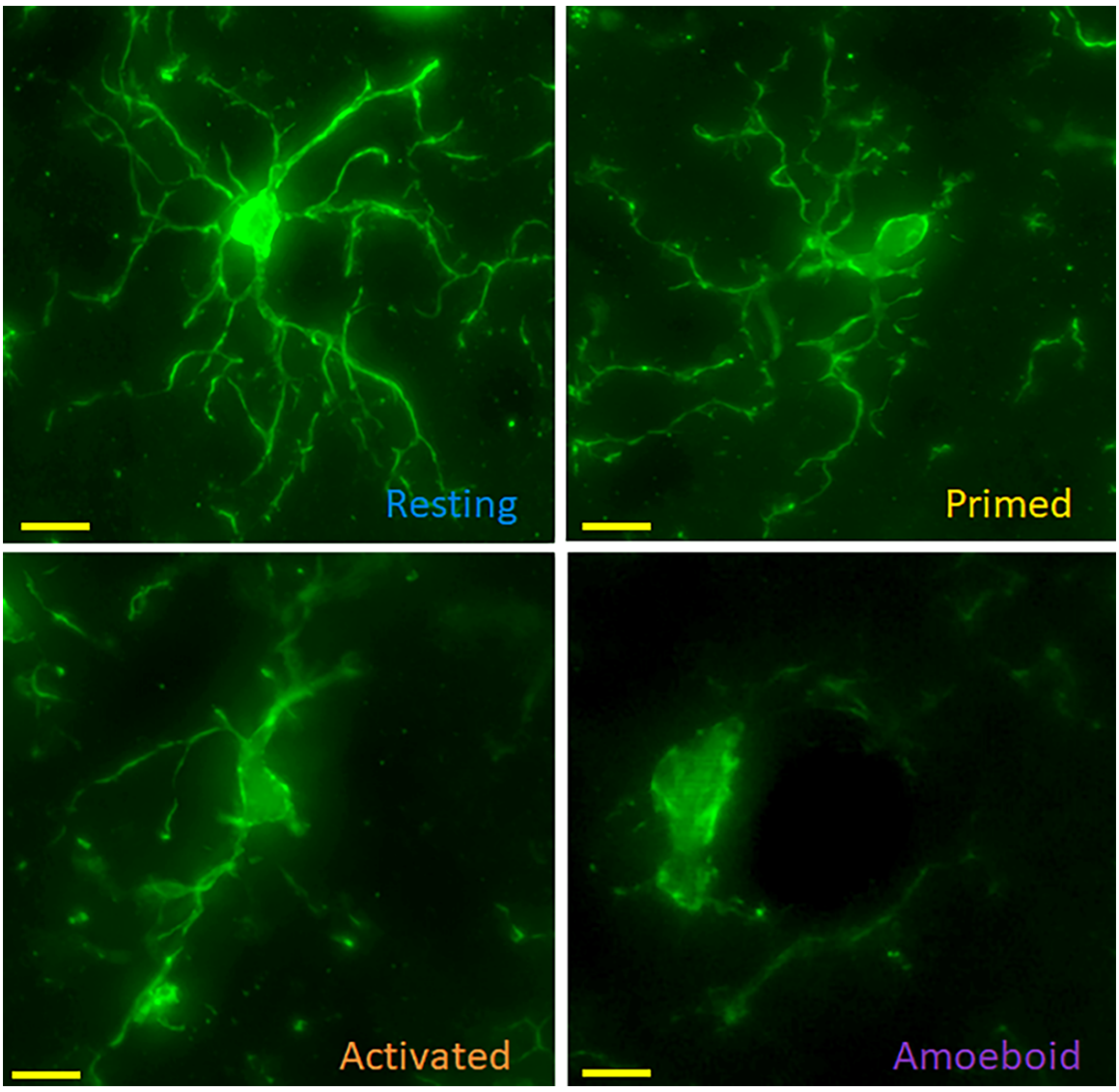

Figure 3.4 Representative immunofluorescent images of microglia stained with Iba-1 4h following blast TBI showing different stages of microglia activation co-existing within hippocampus. Scale bars = 300μm.

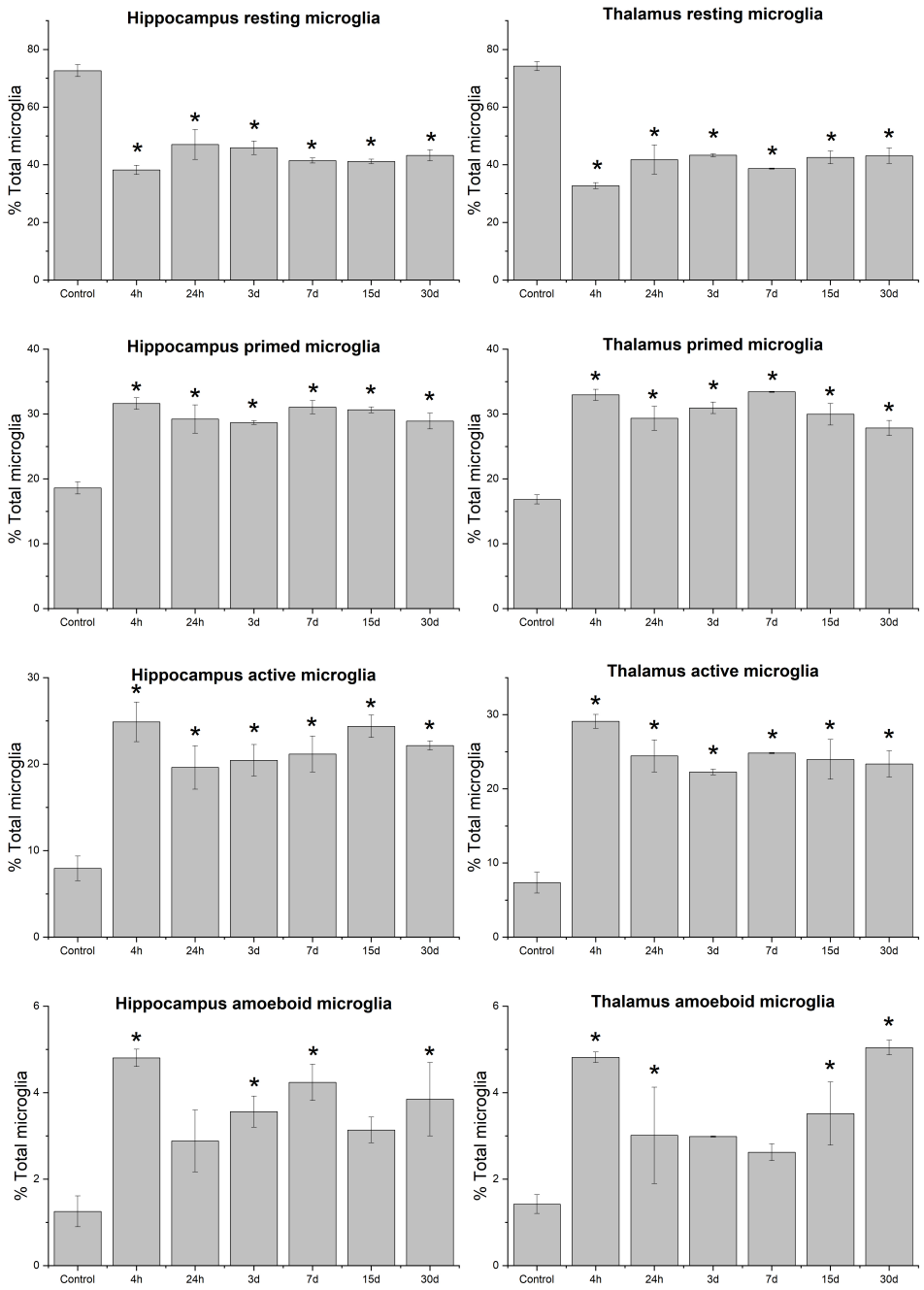

Figure 3.5 Quantitation of fluorescent images of Iba1 stained microglia showing different stages of microglial activation in hippocampus and thalamus. Note the number of resting microglia significantly decreased in both hippocampus and thalamus 4h after bTBI while primed, activated and amoeboid microglial % show a significant increase at different time points after blast which is consistent with robust activation of microglia. *, $p<0.05$ vs. control; Microglial number were quantified from five ROIs of 1 mm^2 in each section from five different animals in each group. df=6.

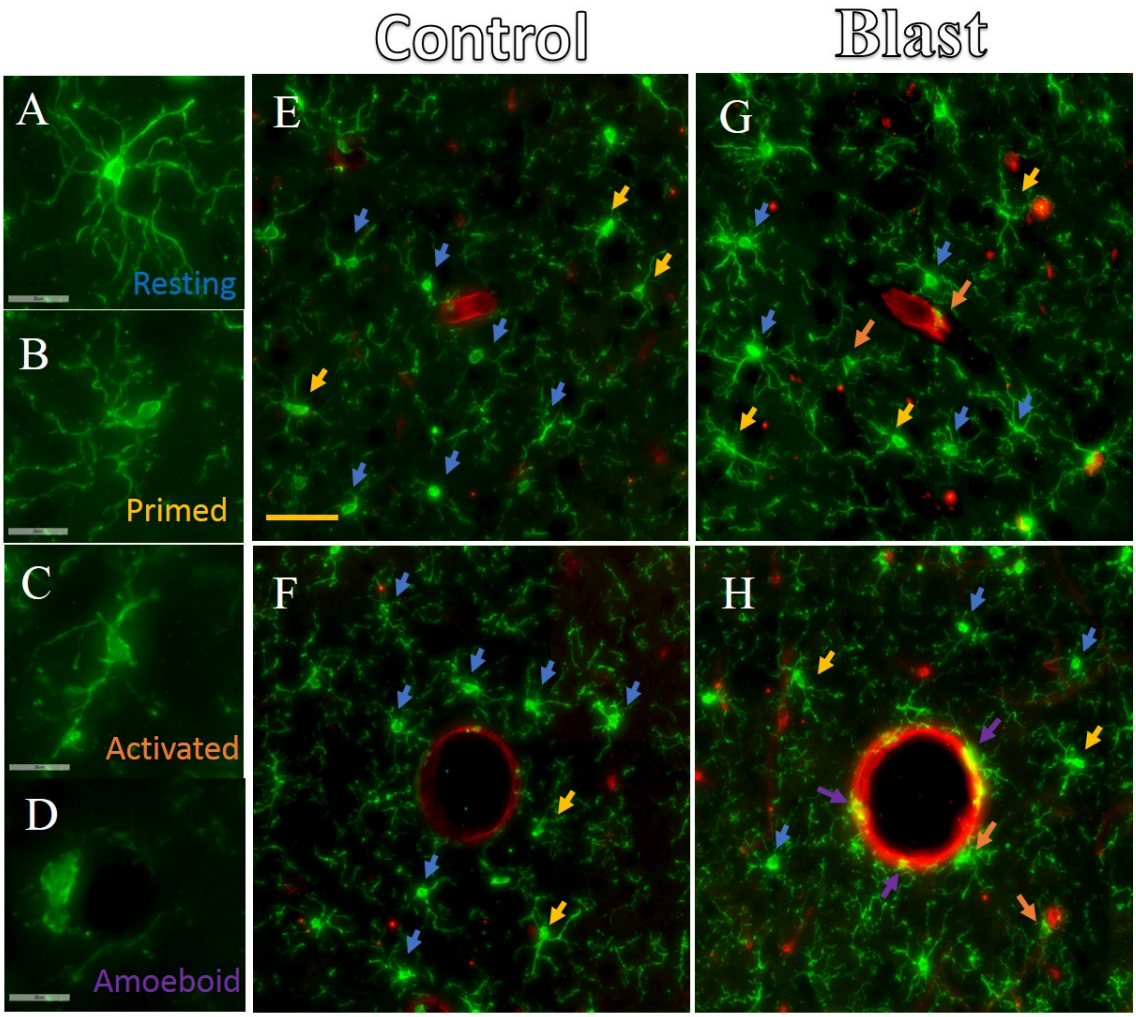

Figure 3.6 Presence of microglia (immune-stained with Iba1) in hippocampus in control and blast-induced animals near blood vessels of small diameter showing extensive arborization of their processes (resting stage). (B) Microglia in the resting state from control animals near larger blood vessel. (D). Ameboid microglia from animals 4h after blast exposure showing higher activation state near blood vessel with larger diameter (pink arrows). Scale bars = 300μm.

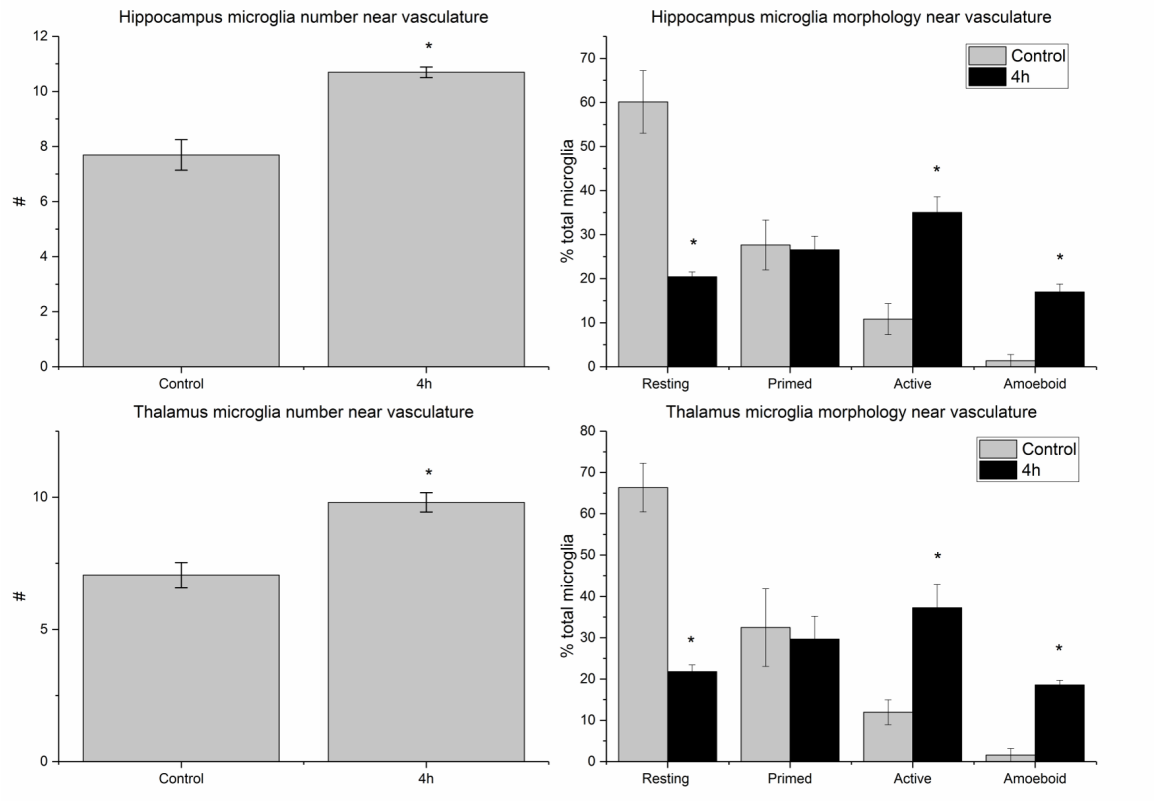

Figure 3.7 Quantitation of fluorescent images of Iba1 stained microglia showing differential activation near blood vessels of smaller and larger diameter in hippocampus and thalamus. Note the number of resting microglia significantly decreased in both hippocampus and thalamus 4h after bTBI near vasculature, while primed, activated and amoeboid microglial percentage show a significant increase which strongly suggests that the extent of vascular rupture dictates the microglia activation stage following blast. *, $p < 0.05$ vs. control; Microglia at different stages were quantified as noted in Methods from five areas ROIs of 1 mm^2 in each section from five different animals in each group. df =6.

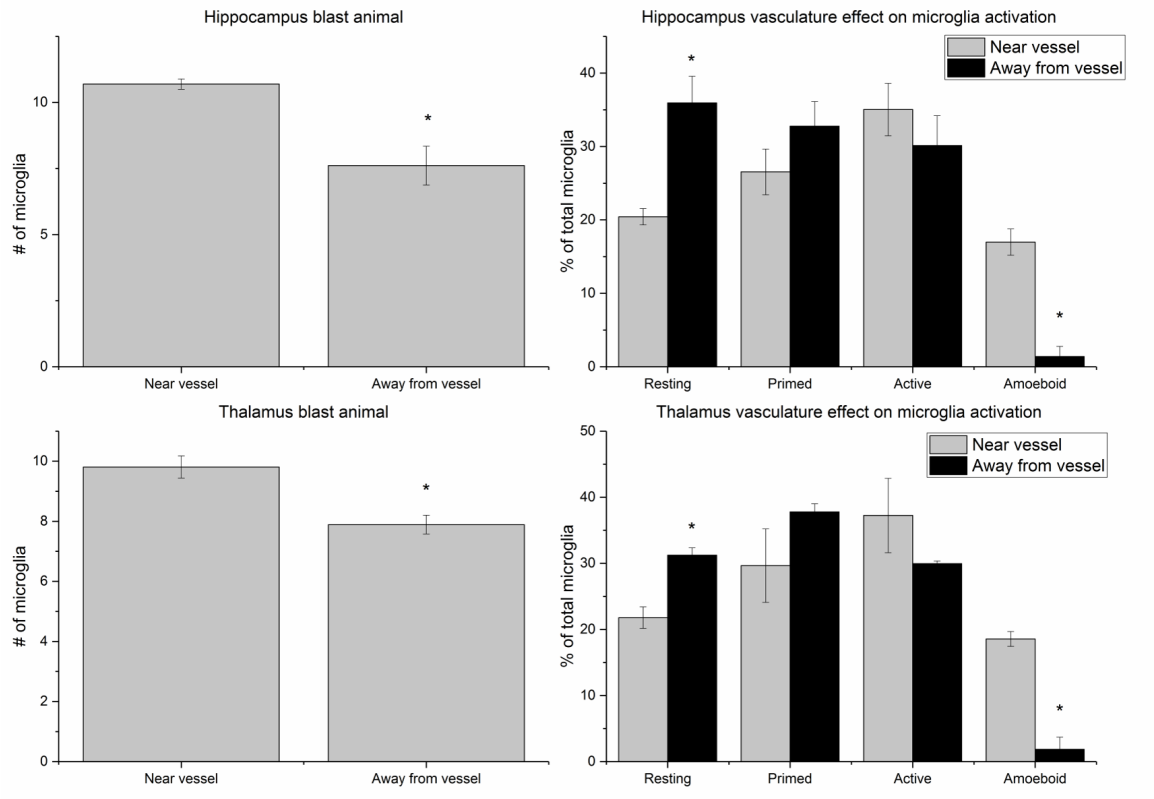

Figure 3.8 Quantitation of fluorescent images of Iba1 stained microglia showing differential activation near blood vessels of smaller and larger diameter in hippocampus and thalamus as compared to adjacent non vascularized tissue. Note the number of resting microglia was significantly decreased, and the number of ameoboid microglia significantly increased after bTBI near vasculature which strongly suggests that the extent of vascular rupture dictates the microglia activation stage following blast. *, $p < 0.05$ vs. control; Microglia at different stages were quantified as noted in Methods from five areas ROIs of 1 mm^2 in each section from 5 different animals in each group df=2.

CHAPTER 4

MAP THE TEMPORAL ACTIVATION OF THE NLRP3 INFLAMMASOME IN TWO VULNERABLE BRAIN REGIONS (HIPPOCAMPUS AND THALAMUS).

4.1 Introduction

In this chapter, we will address the second aim and further investigate the exact mechanism in which body's immune system responds to blast injury. Here we hypothesize that the NLRP3 inflammasome in the biological sensor in microglia that is responsible for their activation and their subsequent production of IL-1β. Identification of the receptor responsible for the activation of microglia is necessary to develop effective treatment for bTBI. Pinpointing the cellular origin of the inflammasome in required to identify vulnerable brain regions to injury as well as time course for potential treatment. Upon completion of this aim, we will be able to identify a potential mechanism for the development of cognitive decline.

4.1.1 Role of Inflammasome in Chronic Inflammation

Microglia the innate immune cells of the central nervous system are able to initiate and sustain inflammatory responses to either infection or injury [205]. To detect the changes to the brain parenchyma following insult, microglia possess membrane bound and cytosolic pattern recognition receptors. These membrane bound receptors include TLR 1-9 and coreceptors including triggering receptor expressed on myeloid cells 2 (TREM2), CD14, CD33, and CD36 [169]. The cytosolic PRR include different inflammasomes receptors such as NLRP3 [206]. These PRR recognize DAMPS release from injured or dying cells, misfolded protein, or protein aggregates produced by neurodegenerative diseases or injury [207, 208, 209].

Astrocytes the most numerous of the cell types found in the brain can also play a role in the innate immune response. Their normal primary function is to

maintain the BBB, and to support neurons by regulating their microenvironment and releasing trophic factors. Astrocytes possess a variety of PRRs including TLR 2-5 and 9, scavenger receptor, and complement receptors [210]. Recent RNA studies have found that astrocytes only express TLR3 in vivo, and microglia are responsible for the remainder of the TLR expression [211, 212]. Astrocytes also express cytosolic PRR in vitro including NLR family CARD domain containing protein 4 (NLRC4) [213], NLRP2 [214], NLRP3 [213, 215, 216].

Upon NLRP3 activation, the NLRP3 protein undergoes conformational changes that allow it to interaction with never in mitosis A-related kinase 7 (NEK7) [217, 218]. ASC is then recruited and undergoes oligomerization to form a spec complex with the NLRP3 protein. Procaspase-1 is then recruited by the inflammasome spec and activated [219, 220]. The active caspase-1 then cleaves pro-IL-1β to produce its active form which is then released from the cell [221]. Caspase-1 is also involved in an inflammatory programmed cell death called pyroptosis. This process causes the cell to lysis and release pro inflammatory molecules [222]. Pyroptosis is initiated by the cleavage of gasdermin D (GSDMD) by caspase-1. The N terminus of GSDMD then forms cell membrane pores [223, 224]. Upon pore formation the inflammasome specs are release and further contribute to neuroinflammation through activity in the extracellular space.

4.1.2 What is NLRP3?

Inflammasomes are complex of proteins the consist of (1) cytosolic pattern recognition receptor (PRR) (2) an adaptor protein called apoptosis associated speck-like protein containing a caspase-recruitment domain (CARD) (ASC), and (3) an effector such as caspase-1 [225]. The most studied of theses protein complex is the nucleotide-binding domain, leucine-rich repeat (NLR) family, pyrin domain containing 3 (NLRP3) inflammasome. The NLRP3 sensor molecule is tripartite protein in the NLR

family and consists of amino terminal PYRIN (PYD) domain, a nucleotide-binding NACHT domain, and carboxy terminal leucine-rich repeat (LRR) domain. The NACHT domain possesses ATPase activity and is believed to require ATP for NLRP3 activation [226]. NLRP3 is known as an integration point for the detection of cellular stress, due to its ability to respond to a wide range of stimuli. It has the ability to respond to stimuli including but not limited to extracellular ATP, viral RNA, lysosomal damage, inhibition of glycolytic or mitochondrial metabolism, or potassium ion efflux [227]. Thus, NLRP3 is able to sense a particular dyshomeostatic cellular state by the loss of its autoinhibition [228].

4.1.3 NLRP3 Activation

The basal expression of the PRR NLRP3, is insufficient to allow for activation in of the inflammasome complex in response to standard activators. The basal levels of ASC and procaspase-1 are sufficient for inflammasome activation and therefore the level of NLRP3 protein in the limiting factor for inflammasome activation. The activation of the NLRP3 inflammasome is a two-step process that requires first a priming step to induce the production of sufficient NLRP3 protein to allow for inflammasome activation [187]. Then a second activating signal is required to induce inflammasome complex formation and activation [229].

Priming The first signal or priming signal initiates the transcription of the NLRP3 protein and other key proinflammatory genes such as pro-IL-1β. The priming step occurs through the activation of membrane bound PRR that activate the pro inflammatory nuclear factor κB (NF κB) [229]. The PRR can include but not limited to toll-like receptor (TLRs) specifically TLR4 in TBI, tumor necrosis factor (TNF) receptor TNFR1 and TNFR2, granulocyte–macrophage colony-stimulating factor (GM-CSF) receptor, nucleotide-binding oligomerization domain-containing protein 2 (NOD2), and the IL-1β receptor ILR1 [187, 230]. The priming step is regulated by

several factors such as microRNAs used to inhibit the translation of NLRP3 [231], input from G protein couples receptors(GPCR) signaling cascade, and activity of cellular metabolic pathways [227].

Post Translational NLRP3 Modification After translation of the NLRP3 protein, it undergoes several post translational modification including several ubiquitylation and phosphorylation [229]. This is done to hold the NLRP protein in an inactive conformation to prevent unwanted activation. Phosphorylation of NLRP3 can occur at three location, in the PYD domain (S3), the linker between the PYD domain and the NACHT domain (S198), as well as one in the LRR domain (Y861 [232, 233, 234]. The phosphorylation of S3 [234] and Y861 [233] both inhibit the NLRP3 activation where phosphorylation at S198 which is mediated by JUN N-terminal kinase-1 (JNK1 or MAPK8) is needed for NLRP3 activation [232]. A fourth location at S295 has been found to be phosphorylated, but its exact function is yet to be determined. It has been found that protein kinase A (PKA) [235] and PKD[236] have been involved in phosphorylating this residue. NLRP3 function is potentiated by PKD and inhibited by PKA.

The phosphorylation state of both S3 and S198 are controlled by TLR activation. The phosphorylation of the S3 and dephosphorylation S198 inhibit the activation of NLRP3 by preventing the homo-oligomerization of the NLRP3 protein and prevents its interaction with ASC [229]. The phosphorylation of Y861 inhibits the activation of the NLRP3 by targeting the NLRP3 protein for autophagic degradation. Thus, lowering the free concentration of NLRP3 below the threshold required for activation [233]. The dephosphorylation of Y861 mediated by PTPN22 which is activated by ATP and monosodium urate (MSU) crystals as well as muramyl dipeptide (MDP) a know priming stimulus [233].

Ubiquitylation another form of post transcriptional modification that controls NLRP3's ability to self-assemble and its rate of degradation [229]. It occurs when an isopeptide linkage is created between ϵ- amino group of a lysine residue and the C-terminus of ubiquitin. Ubiquitin has seven lysine residues (K6, K11, K27, K29, K33, K48, K63) each with the ability for form linkages. Two major types of ubiquitination exist for NLRP3 regulation the K48 and the K68 [237, 238]. TLR activation induces the expression F-box only protein 3 (FBXO3). FBXO3 then degrades ubiquitinase F-box/LRR-repeat protein 2 (FBXL2). The reduction of FBXL2 decreases its K48 linked ubiquitylation which marks NLRP3 for degradation [239]. This allows NLRP3 to reach levels required for activation. BRCA1/BRCA2-Containing Complex Subunit 3 (BRCC3) which is activated by priming signals deubiquitylates NLRP3 in in its LRR domain enabling homo oligomerization [238].

Tripartite motif-containing protein 31 (TRIM31) a type of E3 ubiquitin ligase following priming signal directly interacts with the PYD domain of NLRP3 causing K48 linked ubiquitination targeting the NLRP3 protein for degradation [240]. TRIM31 is found to be upregulated by LPS (TLR) and IL-1β (IL1R) indicating that it is part of a negative regulatory feedback loop [241]. MARCH7 another type of E3 ubiquitin ligase has shown to upregulate upon activation of the dopamine D1 receptor. MARCH7 then ubiquitinates NLRP3 in both its NACHT and LRR domain via K48 linked ubiquitination marking NLRP3 for degradation [242]. Both TRIM31 and MARCH7 are believed to play a role in negative feedback of NLRP3 activation.

Secondary Activation Activation of the NLRP3 occurs only when a primed cell is subjected to a second activating stimulus to an already primed cell. Analyte detection by NLRP3 results in first the recruitment of ASC followed by the activation of caspase-1 [229]. Inhibition at this step of the activation process is preferable as it is specific to NLRP3 and will not influence other biological responses [229]. The exact mechanism

of how NLRP3 is activated is still unknown but five models of NLRP3 activation have been developed. These models are not mutually exclusive and multiple models may contribute to overall NLRP3 activation [229].

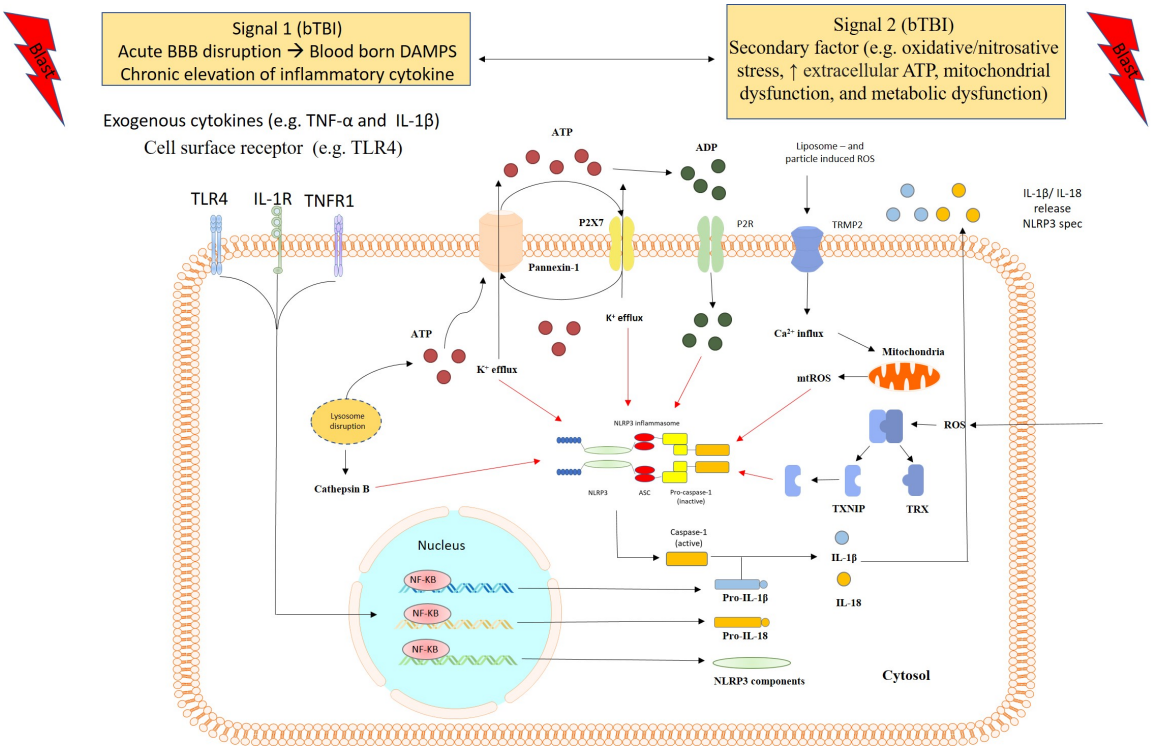

Figure 4.1 NLRP3 mechanism of priming and activation following exposure to blast.

Pore Formation and Ion Distribution The first model developed was the pore formation of ion distribution model. The first know activator of NLRP3, were molecules that permeabilize the cell membrane to the flow of potassium ions. Petrilli et al. have demonstrated the potassium efflux, the only common mechanism in NLRP3 activating ionophores, is sufficient for inflammasome activation in primed cells [189]. High levels of extra cellular ATP found in the vicinity of damages or dying cells induce NLRP3 activate via the P2X purinoceptor 7 (P2X7) channel. Upon ATP binding, the ion channel opens allowing for potassium efflux from the cell balance by calcium influx into the cell [243].

Chloride channels in addition to potassium channels have now also been implicated in the activation of NLRP3. Evidence supporting this assertion stem from non-steroidal anti-inflammatory drugs (NSAIDs) inhibits NLRP3 by blocking chloride movement across the membrane by inhibiting activation volume-regulated anion channel (VRAC) in the plasma membrane [244]. Two separate studies came to a similar conclusion in that chloride intracellular channel proteins (CLICs) 1–4 are required to facilitate the chloride efflux required for NLRP3 activation [245, 246].

Lysosomal Disruption The second model for NLRP3 activation is the lysosomal disruption model. It occurs following phagocytosis of either proteinaceous aggregates or crystalline material that cause disruption to the lysosome [229]. The exact mechanism in which the lysosome undergoes damage is still unknown but is said to mediated by cathepsin release from the lysosome [185]. This model is not mediated by a specific cathepsin in that only cells that lack multiple cathepsins are unable to activate NLRP3 through this methods. Known protein aggregate that trigger NLRP3 activation through this pathway include β-Amyloids [176] and amylin [247, 248]. Crystalline structures that activate NLRP3 are either foreign crystals that are inhaled (silica [185] or asbestos crystals [192]) or produced due to pathological conditions (uric acid [184] and cholesterol crystals [249]). Lysosomal disruption and the subsequent cathepsin release induces an increase in extracellular ATP [250]. This model is believed to then follow the pore formation and ion distribution model and is mediated through the P2X7 channel induced potassium efflux.

Metabolic Dysfunction Recently, metabolic dysfunction has been found to cause the action of the NLRP3 inflammasome. The metabolic dysfunction model is based on activation of NLRP3 due to changes in metabolism caused by injury or infection. Currently 3 triggers exist for the activation of NLRP3 due to metabolism. Activation can occur after displacement of hexokinase-2 from the mitochondria [251], inhibition

of NADH oxidase (complex 1 of the oxidative phosphorylation chain) in concert with depolarization of lysosomes [252], or general inhibition of glycolysis [253], The metabolic dysfunction model is thought to be the only NLRP3 activation model to be potassium efflux independent [229].

Mitochondrial Dysfunction Unlike the other models of NLRP3 activation, the mitochondrial dysfunction model has multiple mechanisms. The first is through the production of mitochondrial ROS (mtROS) which is able to be inhibited by administration of antioxidants [254]. The release of mitochondrial DNA into the cytosol is believed to be a second mechanism of NLRP3 activation but it is still unclear as to whether this event occurs upstream or downstream of caspase 1 activation [196]. A third mechanism proposed is the release of the mitochondrial lipid protein cardiolipin as ligand for the inflammasome [255]. Cardiolipin has been shown to control mitochondrial fission, fusion, and mitophagy [256]. Disruption of mitophagy increases NLRP3 activation in response to potassium dependent activators [257]. These studies suggest that either mitochondrial metabolites or components of the damaged mitochondria are able to activate the NLRP3 inflammasome.

Non Canonical Alternative and Necroptotic The final model for NLRP3 activation is considered non canonical alternative is that in human it is triggered by caspase-4 and caspase-11 in rodents. This activation of this pathway is in response to either the detection of LPS [258, 223], or oxidized phospholipids derived from 1-palmitoyl-2-arachidonoyl-sn-glycero-3-phosphorylcholine (PAPC) which are endogenous DAMPs found in dying cells that signal cellular stress and cellular demise [259]. The detection of these two molecule induce the activation of caspase-4 and 11. Upon activation, caspase-4 and 11 cleave pro gasdermin D. The derived gasdermin D amino terminals oligomerize to form pores in the cell membrane allowing for potassium efflux and pyropoptosis [229]. The potassium efflux then activated NLRP3

and produces IL-1β. In macrophages and dendritic cells, the production of IL-1β by this model can occur in the absence of cell death [260, 261]. It should however be noted that we did not investigate detailed mechanisms of NLRP3 activation by primary and secondary signals since it is beyond the scope of my dissertation.

4.2 Materials and Methods

4.2.1 Animal Preparation

Adult 10-week-old male Sprague-Dawley (Charles River Laboratories) rats weighing 300–350 g were used in this study. The animals were housed with free access to food and water in a 12h dark-light cycle at 22 °C with 40% humidity. All procedures followed the guidelines established in the Guide for the Care and Use of Laboratory Animals and were approved by Rutgers University Institutional Animal Care and Use Committee. Rats were divided into two groups (sham controls and animals exposed to a mild blast of 180 kPa). A total number of 70 rats was used in this study as follows: immunoblotting and ELISA (five controls and 30 blast injured n=5 per time point); immunofluorescence studies (five controls and 30 blast-injured n=5 per time point); For immunofluorescence studies, each brain tissue was processed to obtain several coronal sections (>20) of the hippocampus and thalamus. Each of those sections were used for identification of NLRP3 and caspase-1 proteins in neurons, and microglia by double immunofluorescence analysis.

4.2.2 Primary Blast Wave Exposure of Animals

Using the 9-inch by 9-inch cross section compressed gas driven shock tube at the Center of Injury Biomechanics, Materials, and Medicine (CIBM3) (New Jersey institute of technology, Newark) we determined the effects single mild 180 kPa blast exposure on 10-week old male Sprague Dawley rats [13, 63, 150, 198, 199, 200]. The primary shockwave generated in this shock tube was validated against the

pressure-time profiles measured experimentally in the live-fire explosion experiments [201] and against theoretical pressure-time profiles associated with the detonation of C4 explosive [199, 202].

Before exposing to blast, rats were anesthetized with isofluorane. Sham controls were placed next to the shocktube and only exposed to noise without shockwave exposure. Animals for blast exposure were mounted inside the test section located 2.80 meters from the breach and 3.05 meters from the exit with their head oriented to the direction of the shockwave (Figure 3.1). The rats were strapped securely to the bed with cotton cloth restraints to eliminate head motion. This is done to minimize the contributions of secondary blast injury to total head injury. The animals were exposed to single blast at 180 kPa in a prone position.

Immediately after blast exposure, animals were monitored for any signs of apnea and their neurological status was assessed using modified neurological severity score as reported earlier [199].

4.2.3 Sample Collection

Both sham control and animals exposed to bTBI were sacrificed 4h, 24h, 3 d, 7d, 15d, and 30d after blast exposure. Animals were anesthetized with a mixture of ketamine and xylazine (10 mg/kg) at 10:1 ratio administered via intraperitoneal injection mixture. For immunoblot analysis, rats were transcardially perfused with phosphate buffered saline (PBS, pH 7.0) whereas for immunofluorescence studies, rats were first perfused with PBS followed by 4% paraformaldehyde (PFA).

4.2.4 Immunofluorescence and Microscopy

To evaluate NLRP3 inflammasome activation over time as well as to identify cellular specific activation, we performed double labeled immunofluorescence studies of NLRP3 with NeuN and Iba-1 markers of neurons and microglia, respectively, in the

hippocampus and thalamus. At 4h, 24h, 3d, 7d, 15d, and 30d post-injury after perfusion, the brains were removed from cranial vaults and incubated in 4% PFA for an additional 48h and cryoprotected by immersing in 30% sucrose. Coronal brain tissue sections (20 μm thickness) were prepared using the Leica VT1000S vibratome taken from a single locations along the longitudinal axis of the brain interaural 6.72 mm Bregma -2.28 mm and immunofluorescence was performed. Tissue sections were mounted on glass slides prepared from five individual animals in each group and were washed with 10 mM PBS, fixed in ice-cold methanol (100%) solution for 10 min at -20 °C. The tissue sections were blocked with 10% donkey serum at room temperature for 1h in 1X PBS containing 0.03% Triton X-100. Fixed tissues were incubated overnight at 4 °C with respective primary antibodies to NLRP3 (ab214185 1:100), RECA-1 (NeuN (ab104224 1:150), and Iba1 (Pa5-18039 1:250) in 2% donkey serum. A second 1-hour incubation with Biotin-SP affiniPure Donkey Anti-rabbit IgG to increase the sensitivity of NLRP3 and caspase-1 primary antibody.

Double immunofluorescence was performed using Streptavidin Alexafluor 594 conjugate (S32356) for NLRP3 and Caspase-1, donkey anti mouse Alexafluor 488 (A21202) for NeuN, and donkey-anti goat Alexafluor 488 for Iba-1. The specificity of each antibody staining was validated by excluding each primary antibody (negative controls) and visualized for any nonspecific fluorescence. The primary antibody specificity, however, was not validated independently by blocking the binding to tissue with the corresponding antigen. Slides containing different brain regions were digitized (20x magnification) using Leica Aperio Versa 200 fluorescent microscope and slide scanner. Fluorescence intensities in each region were quantitated using AreaQuant software (Leica Biosystems) and expressed as average fluorescence intensity*unit area.

4.2.5 Cell Counting

Four separate regions of interest in the hippocampus 500 x 500 μm in size were used for cell counting. For hippocampus, the different regions of interest comprised of images from CA1, CA3, and the dentate gyrus for each rat. Five separate regions of interest in the thalamus 500 x 500 μm in size was used for cell counting. The number of microglia (Iba-1) and neurons (NeuN) positively co-labeled with NLRP3 protein within the specified brain regions were counted manually and expressed cells per mm^2.

4.2.6 Western Blotting

Using immunoblot, we examined protein levels of active and the inactive pro form of caspase-1 in the hippocampus and thalamus. After perfusion with 1X PBS, brains were excised from the cranial vaults, sliced into 1 mm coronal sections using adult rat brain slicer matrix. The hippocampus and thalamus were dissected from the 1 mm coronal sections and separately homogenized in ice-cold conditions using RIPA buffer (Sigma) and protease inhibitors. Samples were then centrifuged at 14,000 $\cdot$ g at 4 °C. The protein concentration in the samples was estimated via bicinchoninic acid (BCA) method (Thermo Scientific, Rockford, IL). Subsequently, 10 μg of protein per lane was loaded into 4– 20% SDS-PAGE gradient gels (Bio Rad). Proteins separated according to their molecular size were then transferred onto polyvinylidene difluoride (PVDF) membranes using Transblot turbo transfer system (Bio Rad Laboratories) according to manufacturer instructions. Membranes were blocked with 5% milk dissolved in Tris-Buffered saline containing 0.1% Tween-20 (TBS-T) and incubated overnight at 4 ℃ with caspase-1 (ab1871) at a dilution of 1:50. Bands were visualized using WesternBright ECL (advansta K-12045-D20) on Chemi Doc MP Imaging System (Bio Rad Laboratories). For densitometric quantitation of Western blots, the digitized images were analyzed with BioRad Imagelab version 5.21.

4.2.7 Enzyme Linked Immunosorbent Assay (ELISA)

Levels of pro-inflammatory cytokine IL-1beta in brain tissue was estimated by ELISA. Homogenized hippocampus and thalamus samples were diluted in RIPA buffer and loaded onto ELISA plate (ab100768). All the steps of ELISA procedure (washings, incubation time) were conducted in accordance with manufacturer instructions. Plates were visualized and absorption was measured using SpectraMax i3 (Molecular Devices) microplate reader and analyzed using SoftMax Pro 6.5 software. Output concentration was converted into micrograms per ml of loaded sample.

4.2.8 Data Analysis

Statistical analysis on immunofluorescence, western blotting, and ELISA will expressed as the mean +/- SEM. Statistical analysis of the data was performed using SPSS version 25. Comparison between sham and blast exposed samples was performed by multi-factorial ANOVA. For activation near vessel, an Independent sample t-test was used. Differences will be considered significant at $p < 0.05$. If significance is found, a post hoc Dunnett's test will be conducted for main effect of time following primary blast injury. Boxblot analysis was performed to identify outliers and Shapiro-Wilk and Levene's tests were performed to assess normality of data distribution and homogeneity of variances, respectively.

4.3 Results

4.3.1 Mild Blast TBI Increases Proinflammatory Cytokine IL-1β

Following observation of sustained microglial activation caused by blast TBI, we next examined the levels of IL-1β in hippocampus and thalamus acutely and in chronic conditions. The increase in IL-1β showed a biphasic response in both hippocampus with an acute stage of 4h and 24h and a chronic stage at 15d and 30d. In both hippocampus and thalamus, levels of IL-1β peaked at 4h post injury with an increase

of 210% and 236%, respectively. IL-1β levels in both regions began to increase at 7 days but failed to meet significance in the hippocampus. The chronic stage (7d-30d) peaked at 30 days post injury with a 150% and 232% increase relative to the sham animals (Figure 4.2).

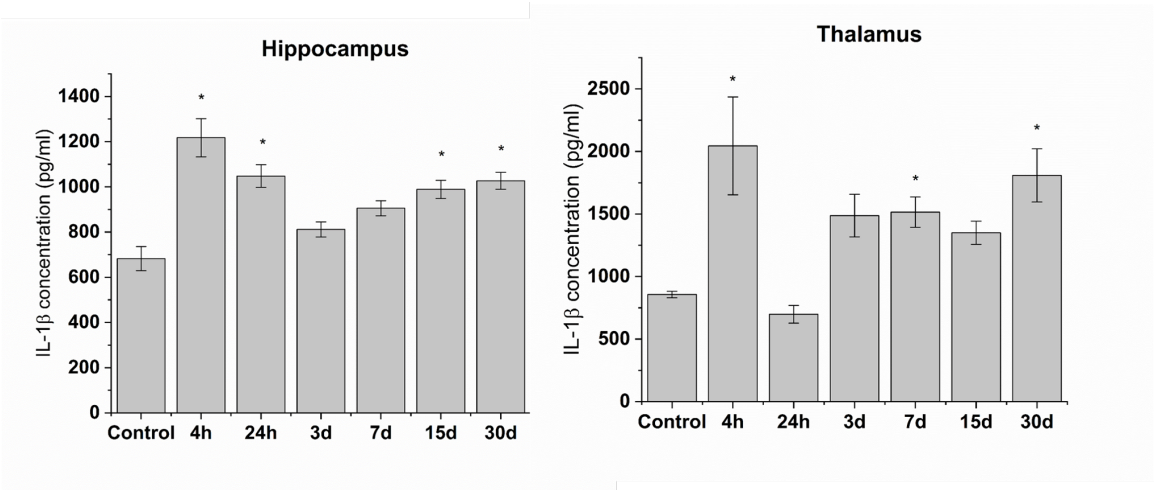

Figure 4.2 Time course of changes in IL-1β levels in hippocampus and thalamus following blast TBI. IL-1β levels were measured by quantitative ELISA in homogenates of hippocampus and thalamus. A biphasic response of change was observed in animals exposed to blast TBI. *, $p<0.05$ vs control (n=5 in each experimental group). df=6.

4.3.2 Blast TBI Increases NLRP3 Inflammasome in Cell-Specific Manner

To determine the source of IL-1β production, we first examined the time course of total NLRP3 inflammasome expression in hippocampus and thalamus (Figure 4.3). Although there was no statistical significance ($\alpha = 0.053$), we observed a strong trend towards an increase of NLRP3 expression at 4h followed by a return to sham levels of NLRP3 expression at 24h and 3d post injury. However, at 7d post injury NLRP3 levels increased in both the hippocampus and thalamus which continued to rise and peaked in both regions at 30d with a 560% increase in the hippocampus (Figure 4.2 D) and 1055% increase in the thalamus (Figure 4.3 E).

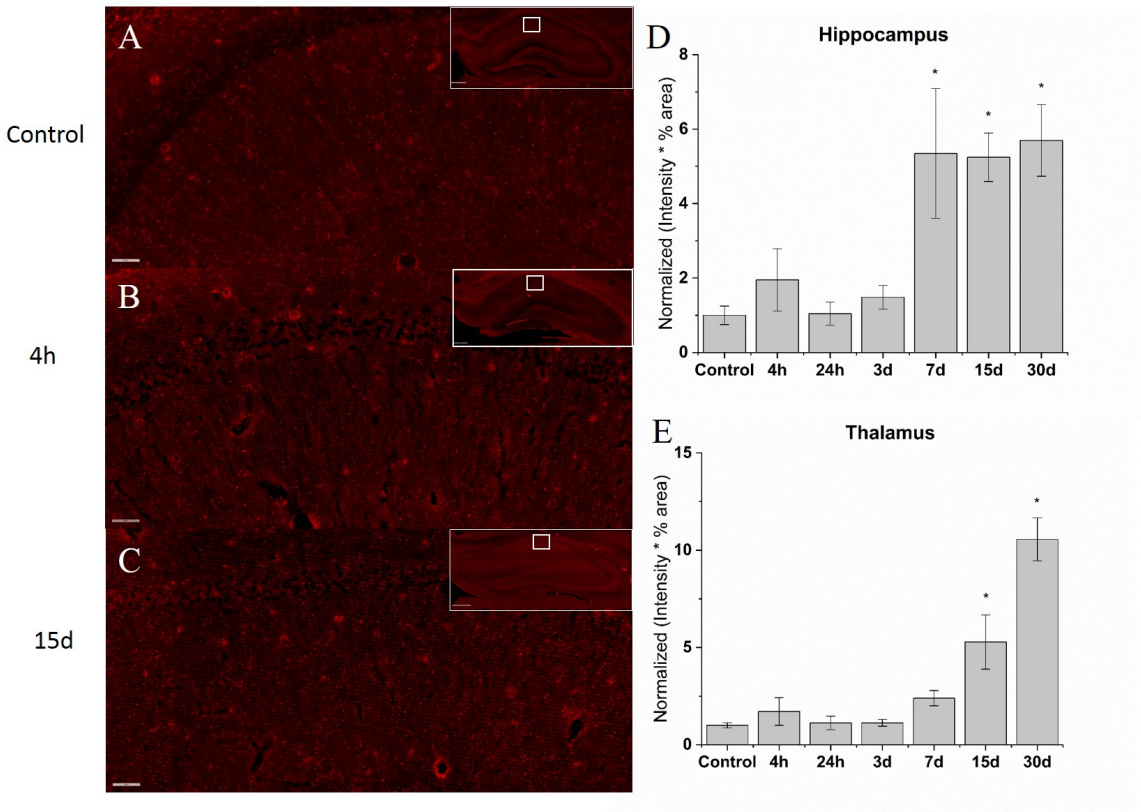

Figure 4.3 NLRP3 expression in the hippocampus and thalamus following blast. A biphasic response in NLRP3 expression was observed following blast*, $p<0.05$ vs control (n=5 in each experimental time point). df=6.

We next investigated the cellular source of the NLRP3 inflammasome in both the hippocampus and thalamus (Figures 4.4 and 4.5). At 3d post-injury, both hippocampus and thalamus showed significantly increased in microglia as indicated by increased number of NLRP3 positive microglia and continued to rise in number until peaking at 30d following injury (Figure 4.4). This chronic production of microglial NLRP3 may be the underlying cellular source of chronic IL-1β production.

Interestingly, examination of NLRP3 expression in neurons showed an early increase in its expression at 4h and 24h after blast injury whereas at later time points, the levels of NLRP3 in neurons returned to that of control (Figure 4.5).

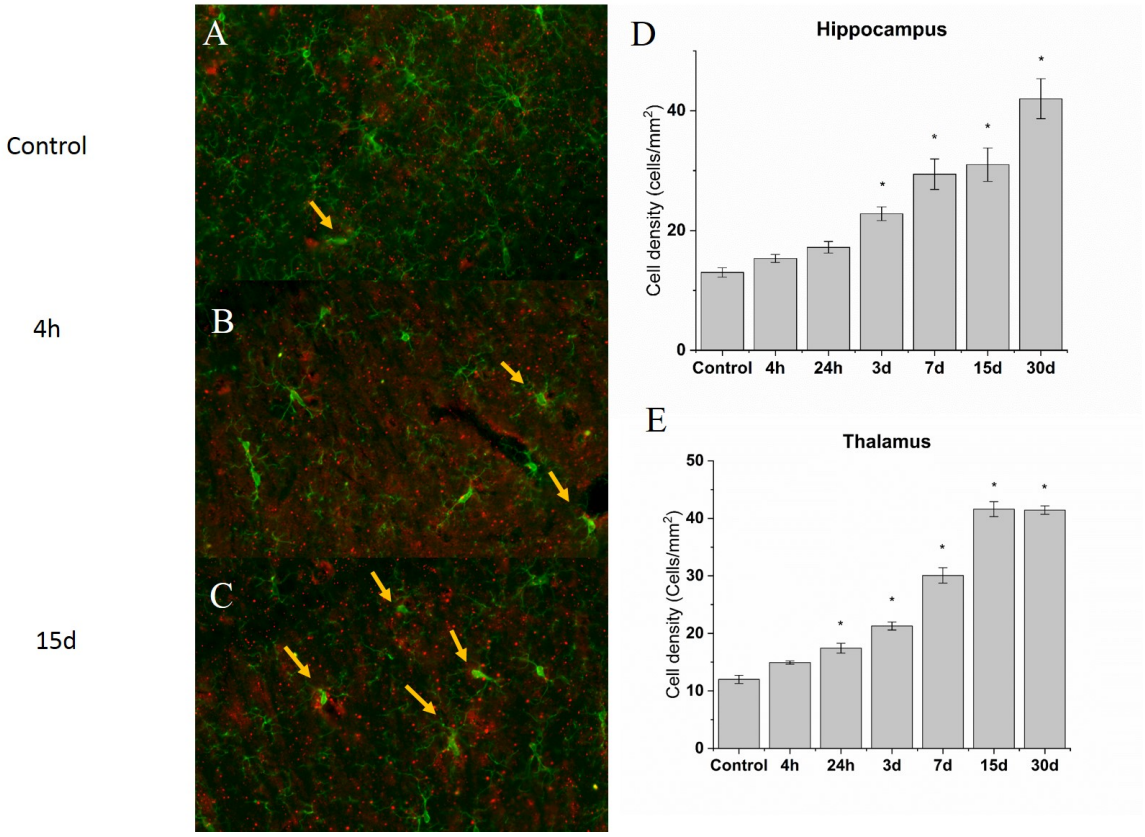

Figure 4.4 NLRP3 protein expression in microglia following blast TBI. (A) Double immunofluorescence image of NLRP3 (red) and Iba1 (green) showing localization of NLRP3 in microglia from hippocampus and thalamus of control and blast exposed animals. (B) & (C) Expression of NLRP3 in microglia from hippocampus in animals 4h and 15d post-blast showing an increase in its content. (D) & (E): Quantification of fluorescence intensity of NLRP3 protein expression in microglia hippocampus and thalamus. *, $p<0.05$ vs. control (n=5 in each experimental time point). df=6.

4.3.3 Blast TBI Increases the Levels of Active Caspase-1

To determine whether increased expression of NLRP3 inflammasome activates downstream events, we investigated the levels of procaspase-1 (signal 1) and levels of active caspase-1 (signal 2). We observed an upward trend for procaspase-1 production culminating in a significant increase of 84% increase relative to control at 7d post injury in the hippocampus. At 15d and 30d post injury procaspase levels fell below control levels but did not reach significance. Procaspase levels at all time points in the thalamus did not differ significantly from control levels. Levels of active caspase-1

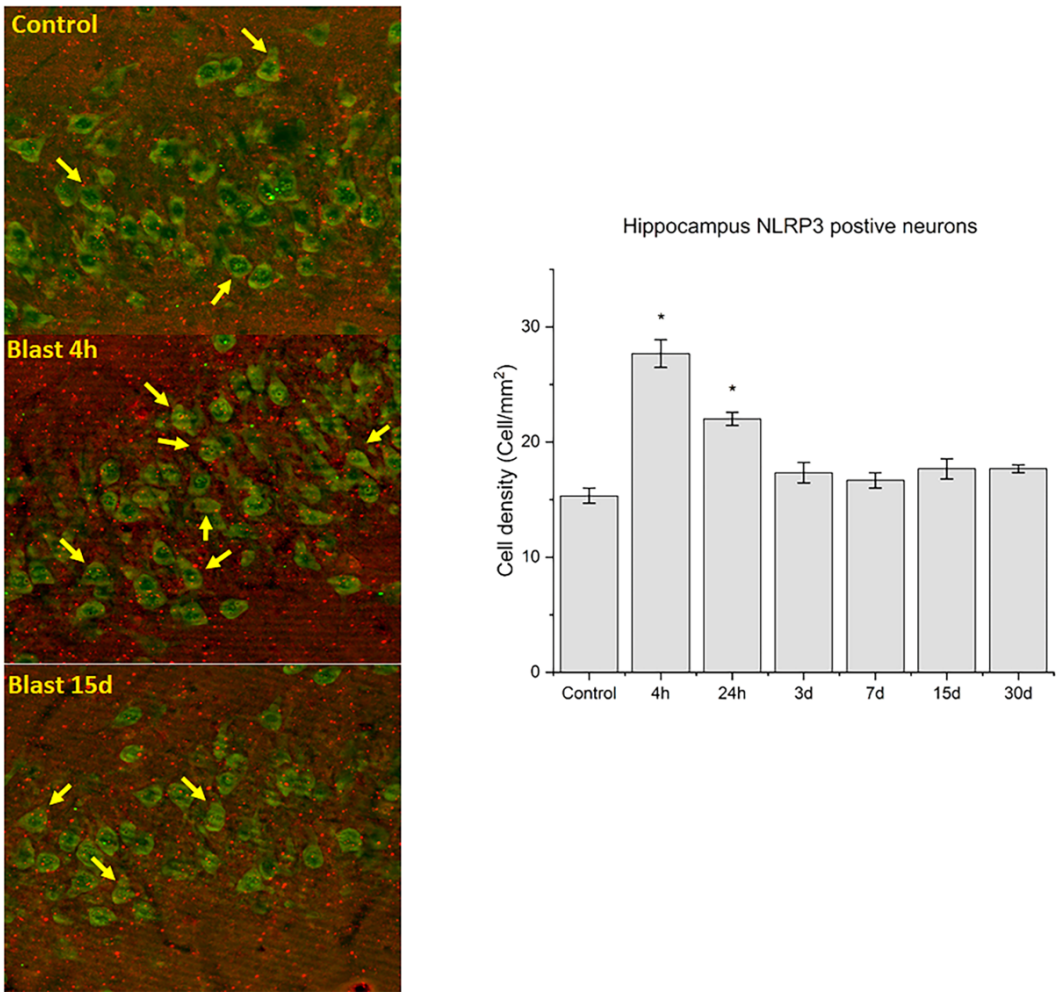

Figure 4.5 NLRP3 protein expression in neurons following blast TBI. Double immunofluorescence image of NLRP3 (red) and NeuN (green) showing localization of NLRP3 in neurons from hippocampus of control and blast exposed animals. Quantification of fluorescence intensity of NLRP3 protein expression in neurons in hippocampus and at different time points display an early increase at 4h and 24h which thereafter restored back to control levels by 3d. *, $p<0.05$ vs. control (n=5 in each experimental group) df=6.

showed a biphasic increase following blast. The acute phase lasted for 24h after injury returning to control levels at 3d in both the hippocampus and thalamus. At 7d post injury active caspase-1 levels became significantly upregulated in the hippocampus but failed to reach significance in the thalamus. In both regions, the levels of active caspase-1 was significantly upregulated at 15d and peaked its expression at 30d post

injury with a 471% increase and 590% in the hippocampus and thalamus, respectively (Figure 4.6).

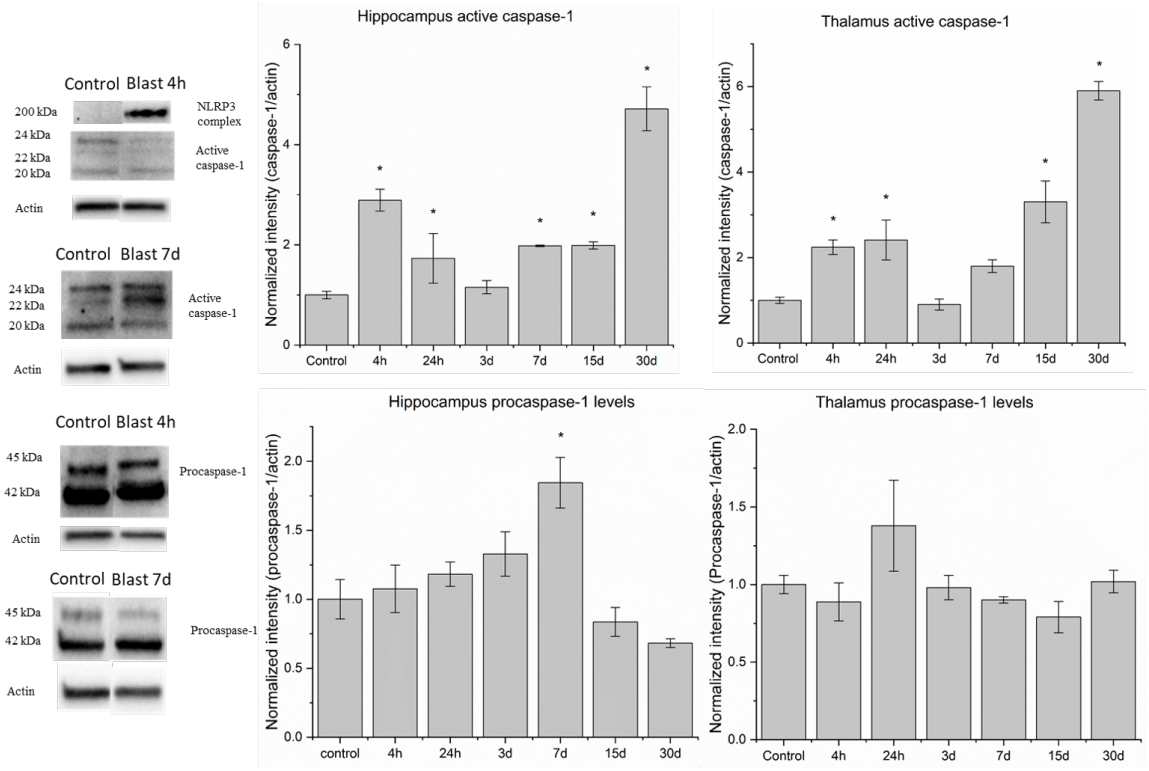

Figure 4.6 Immunoblot of levels of procaspase-1 and active-caspase-1 (cleaved product) in control and blast animals. Procaspase-1 with higher molecular weight band (upper panel) represents aggregated bands of NLRP3 containing other proteins including NLRP, ASC and procaspase-1, see text) show a significant increase at all time points investigated (4h-30d), whereas active caspase-1 (20-24 & 45 kDa) show a biphasic response with an increase at 7 d post-injury followed by completely normalization thereafter to that of control level. *, $p<0.05$ vs. control (n=5 in each experimental group). df=6.

4.4 Discussion

This work aims to investigate the pattern of neuroinflammatory paradigms and its temporal profile in vulnerable brain regions including hippocampus and thalamus following mild bTBI. Blast TBI caused a progressive increase in microglia activation in both hippocampus and thalamus and such microglia activation could further resulted in activation of NLRP3 inflammasome and down-stream pathways ultimately

leading to increased IL-1β production. A schematic illustrating the events that activate microglia and produce proinflammatory cytokine IL-1β by activating NLRP3 inflammasome is presented in Figure 4.1. This data collectively indicates that chronic neuroinflammation and associated production of proinflammatory cytokines via NLRP3 is an important injury mechanisms in bTBI.

Microglia once activated, synthesize and secrete a repertoire of inflammatory factors including proinflammatory cytokines, anti-inflammatory cytokines, chemokines, and cell adhesion molecules by activating variety of cell surface receptors in several neurological conditions including TBI (for review see [262]). Noteworthy, a sustained increase in the levels of IL-1β following blast not only correlated with increased microglia number at similar time points, but also indicate that IL-1β is a major cytokine produced by activated microglia in blast TBI. However, there are a number of pathways by which activated microglia produce IL-1β. These include activation of toll-like receptor 4 (TLR4) which once bound by either DAMPS or PAMPS activated nuclear factor kappa B (NF-kβ) [263] while the other pathway includes the activation of various inflammasomes [264, 265]. Therefore, it is also possible that these pathways could additionally contribute to the production of IL-1β in addition to NLRP3 activation we observed in the present study.

This study employed two vulnerable regions hippocampus and thalamus, for the analysis of various inflammatory events since these regions are involved in the regulation of a variety of cognitive and neurobehavioral tasks including learning and memory, short-term memory, anxiety, and depression like behavior [266, 267, 268, 269] and that chronic neuroinflammation is known to cause neurobehavioral deficits in various neurological conditions including TBI (for reviews see [270, 271]).

Increased expression of NLRP3 inflammasome displayed a biphasic response: 4h post-injury, there was a strong tendency of increase while such increase was completely normalized by 24h and 3d. However, at 7d post-injury onwards a second phase of

increase in NLRP3 was observed which persisted for up to 30d in both hippocampus and thalamus. Such biphasic response highly correlated with the temporal profile of IL-1β levels in both hippocampus and thalamus which strongly suggest that increased IL-1β in bTBI is mediated by activation of NLRP3 inflammasome.

NLRP3 inflammasome is a major mediator of IL-1β production via activation of caspase-1. Studies have reported the activation of NLRP3 inflammasome in blunt models of TBI, whereas very few studies were performed in bTBI [264, 265]. The inflammasome complex consists of three major components: cytosolic pattern recognition receptor (NOD like receptor (NLR)), caspase1, and an adaptor protein (apoptosis-associated speck-like protein containing a CARD (ASC)) that assists with the interaction between the two. Our present study focused on the NLRP3 inflammasome due to its abundant expression in microglia [176, 177, 272, 273]. As illustrated in Figure 4.1, the sequential events that result in the activation of NLRP3 and production of IL-1β is a two-step process that requires two activating stimuli[186]. The first signal usually through TLR receptors prime the cell to transcribe and translate pro-IL-1β and in some cases, NLRP3 expression [187]. The second signal typically ATP, induces the formation of the inflammasome complex which cleaves the pro-IL-1β into its active form and subsequently released from the cell. In the present study, it is possible that blast TBI could be a primary signal causing acute mechanical injury to vasculature that could further amplify to induce secondary mechanisms (signal 2) such as oxidative stress which ultimately contribute to the activation of NLRP3 inflammasome and maintenance of chronic inflammation.

Our observation of early increase in NLRP3 in neurons instead of microglia strongly suggest that neurons are more vulnerable acutely following blast injury. In fact, such early activation of NLRP3 in neurons was also observed in blunt and penetrating injury TBI models [206, 274]. The pattern of NLRP3 activation in bTBI which induces an early effect on neurons, is also plausible since our recent studies

indicate higher vulnerability of neurons to acute oxidative stress as compared to microglia [132]. Therefore, secondary injury mechanisms such as oxidative stress may affect neurons more than in microglia, which may account for the delayed increase in the expression of NLRP3 in microglia ($\geq$3d post-injury) in bTBI.

In the current study, we did not perform studies to assess the changes in ASC component since several reports indicate that ASC is a critical integral component of more than one inflammasome such NLRP1, NLRP3, NLRP4 and AIM2 (see review [275]) and analysis of ASC in the present study will not yield any specific involvement of NLRP3 inflammasome in bTBI.

The present study showed increased levels of activated caspase-1 in parallel with increased expression of NLRP3 indicating that bTBI induces functionally activate NLRP3 inflammasome. Active caspase-1 bands in rodents consist of four separate bands. The first band at 20 kDa is the standard cleavage product formed. Caspase-1 autocleavage occurs at the aspartic acid residue at the 296 and 314 amino acid in the human caspase-1. In rodent caspase-1 there are additional aspartic acid residues at the 300, 304, 308, and 313 amino acids. Due to increased number of possible cleavage sites in rodent caspase-1 gene compared to the human gene, there are two more isoforms that occur and 22 and 24 kDa [276]. A fourth band at $\geq$200 kDa was also observed and can be explained as the "death complex" where two NLRP3 protein dimerize with 2 caspase-1 protein or the "ASC foci" where the entire inflammasome complex (NLRP, ASC, and the caspase-1) are bound together [276]. Sometime in these complex the caspase-1 p20 fragments are not cleaved from its CARD domain leaving a 35 kDa fragment active fragment [277, 278] These semi-active caspase (p35) remain bound the with the CARD domain of either NLRP or ASC protein. Although the preparation of brain homogenates for western blot analysis has multiple steps to dissociate protein complexes (β-mercaptoethanol and heating to 95°C), the complexes remained intact for western blot analysis. Improper preparation of samples would

result in smearing of protein band found in the gel. This was not observed and therefore we believe that the 200 kDa band observed truly are these complexes. The 200 kDa band was observed in all 5 animals at 4 hours, 3 of the 5 animals at 24h, absent in the 3d and 7d animals, and present in all 5 animals and 15 and 30 day. Increased levels of activated caspase-1 in the present study is consistent with increased levels of IL-1β, a final product in the pathway of NLRP3 inflammasome activation.

CHAPTER 5

EXAMINE THE EFFECTS OF NLRP3 INFLAMMASOME INHIBITION ON COGNITIVE OUTCOME

A large motivating factor for our aim 3 was that thus far, no effective treatment strategies have been perfectly identified. Previous promising drugs tested in clinical trials were targeted at the downstream products of chronic inflammation and therefore were unsuccessful. Here in Chapter 5 we investigate the inhibition of the NLRP3 inflammasome following blast with low dose continuous MCC950 treatment. Here we hypothesize that inhibition of NLRP3 activation will cause the subsequent reduction in the production and release of IL-1β. This reduction in IL-1β will mitigate the anxiety like symptoms, memory decline, and motor coordination deficits.

5.1 Introduction

5.1.1 Blast TBI Pathology

Victims of severe blast injury present with clear neurological changes that include diffuse cerebral vasospasm, lowered level of consciousness or confusion, and formation of a pseudoaneurysm a pathology not seen in blunt forms of TBI [279]. Most of the neuropathological consequences of severe blast TBI are observed in other forms of TBI such as blunt or penetrating. Due to the extreme limitation of neuropathological data from blast injured humans, distinct differences between these pathologies may yet arise [62] A distinct pathology unique to bTBI is the increased risk of hearing loss and tinnitus [280].

Determining the pathologies of mild TBI is problematic due to no demonstrable abnormalities appear on standard MRI scans [50] as well as lack of pathological data due to mild bTBI rarely being fatal. These soldier returning with mild bTBI present with compromised executive function, confusion, retrograde amnesia, headache,

mood disturbances, anxiety, altered sleep patterns, and difficulty concentrating [33]. Therefore, the use of animals models is required to uncover the pathology of bTBI.

Animal models of blast are characterized by vasospasm, edema, hyperemia, and diffuse axonal injury [62]. DAI is the most prominent histological change found in animals following blast, and would correspond to the diffuse tractography changes found in blast exposed military personnel [62, 281]. Blast exposed mice show pathological changes associated with DAI such as phosphorylated tauopathy, myelinated axonopathy, chronic neuroinflammation and neurodegeneration. These finding align with reports of chronic traumatic encephalopathy found in returning military personnel. Functional deficits such as ones in social recognition, spatial memory, and motor control were found in the same animals [282] MRSI studies in a porcine model of mild bTBI found the same metabolic injury observed in soldiers at eight months post injury. The metabolic changes were only present in the hippocampus and not found in the Thalamus, basal ganglia, or cortical areas. This change in metabolic activity was mirrored by a significant decrease in neurons in the CA1 of the hippocampus [59]. The similarity between the human and animals models of blast TBI would indicate that the pathologies between the two species are equivalent.

5.1.2 IL-1β Pathology

Interleukin (IL) -1 is an inflammatory cytokine plays and import role in the immune response to injury and infection [283]. It was originally described to be produced by macrophages external to the CNS, but now have been found to be produced in both microglia and neurons [284, 285, 286, 287, 288]. IL-1 exist in an alpha and beta forms, which differ in amino acid composition [289], but function on the same receptors type and produce a similar biological response [290]. IL-1β receptors are distributed throughout the brain but have the highest density in the

hippocampus [291, 292]. Various biochemical and pharmacological have demonstrated the IL-1β affects on monoamine and neuropeptide transmitters release, and turnover [293, 294], endocrine function and induces changes in behavior in rodents [295, 296]. The behavioral changes observed in rodents include impaired spatial memory, sleep disturbances, decreased exploratory activity, anxiety related behaviors, and sleep disturbances [297, 298, 299, 300]. These behavioral changes were observed when IL-1β was administered either peripherally via IP injects or centrally via intracranial injection [301, 287].

These behavioral changes observed are believed to reflect the changes in neurotransmitters and neuromodulators such as, corticotrophin releasing factor (CRF), prostaglandin (PG) E2 [302, 303], and nitric oxide [304] when IL-1β is introduced to the CNS [304]. Most studies investing the effects of IL-1β and the brain have focused on sickness behavior [301, 305, 306]. The neurotransmitter found to be affected include noradrenaline (NA), where IL-1β increases its turnover rate [307]. NA is known to regulate learning and memory, attention and wakefulness-sleep cycle [308], as well as dopamine, GABA, and glutamate.

In vivo production of IL-1β in the CNS is believed to be produced by microglia and infiltrating peripheral immune cells and its effect depends on which cell type the inflammatory cytokine acts on [309]. Both acute and chronic increase in IL-1β levels in vivo have be shown to result in microglia and astrocyte activation [310, 311, 312]. When IL-1β acts on vascular endothelial cells it induces upregulation of chemokine and adhesion molecules which increases BBB permeability [313, 314]. This increase of BBB permeability allows for peripheral neutrophils and monocytes from the circulation to cross the BBB [310, 311, 312, 315, 316, 317, 318] depending on both the response of endothelial and astrocyte [309, 315, 316]. These invading leukocytes while in the brain parenchyma exacerbate the inflammatory response by producing more cytokines, ROS, and proteases. The increase in inflammatory modulators stimulate

lymphocyte recruitment and increase the level of tissue damage [319, 320]. These invading peripheral immune cell have been shown to contribute to the pathogenesis of not only TBI [319, 320] but other disorders such as AD [321, 322], MS [323], and epilepsy [324].

5.1.3 MCC950, a New Target for Neuroinflammation

MCC950 also known as CRID3 or CP-456,773 is a selective small molecule NLRP3 inhibitor derived from the anti diabetic drug class sulfonylurea. MCC950 has been found to bind directly to NACHT domain of NLRP3 preventing its ATPase activity required for activation [325]. It has an IC_{50} 7.5 nM in BMDMs [326, 327, 328] and 8.1 nM in human monocytes [326]. Preclinical studies have shown that MCC950 to have good bioavailability and good CNS penetration making it ideal for treating inflammatory disordered in the CNS [325, 326]. MCC950 has been shown to be specific to only the NLRP3 inflammasome [326, 329]. It is able to block all forms of NLRP3 activation known to date including canonical and non-canonical, and alternative [229, 252, 326, 330].

MCC950 was chosen over other inhibitors for NLRP3 for several reasons. First, it is the most studies of all NLRP3 inhibitors; and therefore, the most information is available. Second, MCC950 has also been used in a variety of animals models with success indicating in its ability to act in many cell systems. Third, the exact mechanism of action is known for MCC950 with is not the case for other NLRP3 inhibitors. This ensures that this inhibitor is specific to the activation of NLRP3 and will not have an effect on other bodily responses. The fourth reason is that it is able to cross the BBB ensuring that IP injecting will have an affect on neuroinflammation. The final reason is that the dosage for MCC950 is know for rats; therefore, we would not have to investigate dosing [229].

MCC950 have be tested in a variety of neuroinflammatory diseases and has been shown to abrogate their symptoms. It has been shown to abrogate the neonatal lethality in a mouse model of cryopyrin associated periodic syndrome (CAPS) [326]. CAPS is an autoinflammatory disease caused by a gain in function mutation in NLRP3. It has also been shown to reduce microglia activation and inhibit inflammasome activation in mouse model of Alzheimers disease [331]. MCC950 has also been tested in animal model of TBI. Following CCI injury administration of MCC950 reduced the neurological deficits at 72 hours post injury [332]. CP-456,773 was tested in phase II clinical trials for rheumatoid arthritis but was not devel¬oped further as it was found to elevate serum liver enzyme levels in the clinic. The cause of this liver toxicity signal is not clear, although the combination of its metaboli¬cally reactive furan moiety and very high clinical dose of 1,200 mg per day, two well-known causes of drug-induced liver injury, might underlie the observed toxicity [333]. In vivo is has also been shown to reduce IL-1β levels in EAE models [326].

5.1.4 Common Neurohavioral Changes: Anxiety Neural Circuit

The anxiety response are due to corticolimbic circuit interpreting the environmental stimuli as a threat [334]. Disturbances to any point in the circuit causes an imbalance in its self-regulatory system. The imbalance in this system causes the misinterpretation sensory inputs as threatening which leads to the inappropriate anxiety response [334]. The neural system found to be involved with rodent are consistent with those found in humans [335, 336, 337, 338, 339, 340, 341, 342, 343]. Therefore, conclusions made while assessing a rodent model can be translated into humans. To produce the observable anxiety response four steps are required, detection, interpretation, evaluation, and response initiation.

In order to assess the level of threat a situation presents, the human or animal must first detect the environmental stimuli through the use of their sensory system

[334]. Next the animal interprets the sensory inputs through the coordination of amygdala, BNST, vHPC, and PFC as potentially dangerous or a threat [334]. The major neural structure that determines if an environmental stimulus is interpreted as threatening is the amygdala. The amygdala nuclei involved in anxiety can be divided into the basolateral amygdala (BLA) and the central amygdala (CEA) [344]. Here the sensory information is given an emotional value [334]. The amygdala first receives excitatory afferents into the (BLA) from the sensory cortices through the thalamus [345]. The BLA is a cortex like structure that consists of 80% glutamatergic spiny projection neurons and 20% consist of GABAergic interneurons [346, 347, 348]. The BLA then processes the sensory information to form association between neutral predictive stimuli with either a negative or positive valence [349]. This association is accomplished via the Hebbian mechanism [350, 351, 352]. Depending on the emotional valence determined either the reward or fear pathways are recruited downstream of the BLA [353]. In the anxiety related pathway the BLA sends activating signals to the central amygdala (composed of medium spiny GABAergic neurons with projections out to brains areas important in anxiety like behaviors [354] and is divided into the lateral (Cel) and medial (Cem) nuclei [355, 356]) and the bed nucleus of the stria terminalis (BNST) [340, 357]. The BLA possesses projections to the Cel, Cem [342], mPFC [341], vHPC [358, 359], and the anterodorsal BNST (adBNST) to control the overall anxiety response. The activation of most of these projections leaving the BLA are anxiolytic except for the projection to the Cel [342].

To initiate the fear and the sustained anxiety response, the BNST must be recruited [360]. The BNST is composes of 12 recognized nuclei with the oval, anterodorsal, and ventral cortices each differentially regulating separate features of the anxiety response [340, 361]. The activation of the adBNST by the BLA, induces the activation of projections of the BNST to the lateral hypothalamus, ventral tegmental area (VTA) and to the parabrachial nucleus. The activation of

the lateral hypothalamus and VTA has been shown to cause anxiogenic behavior such as the ones that regulate subjective preference and risk avoidance in the EPM and OFT. The projections of the BLA to the parabrachial nucleus are responsible for the autonomic anxiety response observed [340]. The adBNST is regulated by the oBNST through local inhibition [340]. Thus the activation of the oBNST is anxiogenic by inhibiting the activation of the adBNST. BLA inputs into the vBNST promote freezing and uncontrollable stress and diminishes social interactions [362]. The ventral BNST (vBNST) send both glutamatergic and GABAergic projections to non dopaminergic cells in the VTA. Activation of the glutamatergic neurons induce the avoidance response found in anxiety [363].

The activation of these structures can be accomplished by direct innervation by the BLA or through glutamatergic inputs from the hippocampus [364, 365], and cortical areas (mPFC, entorhinal cortex and insular cortex [345, 366]). The BLA has also shown to receive monosynaptic input and output for the mPFC and the ventral hippocampus (vHPC) [358, 367, 368]. The Lateral septum (LS) plays a role in stress induced anxiety. It is believe to be activated by the vHPC and is characterized by the expression corticotropin-releasing factor receptor 2 and targets the anterior hypothalamus promoting the stress response [369]. The vHPC has projections to the mPFC, BLA [358], and LS that regulate the anxiety response. Activation of somatostatin positive Cel by the BLA [370] and the paraventricular nucleus of the thalamus [371, 372] have been implicated in fear learning and anxiety responses by inhibition of the Cel.

A second point of regulation exist after the interpretation of the stimuli to prevent unchecked activation of the anxiety response. Here the brain evaluates weather the interpretation of the environmental threat matches the internal and external conditions. To evaluate the threat, the majority of the interpretation occurs in the mPFC. The mPFC receives inputs from midline thalamic nuclei, hippocampus,

and the BLA and possesses efferent projections to the striatum [373]. Disturbances in any of these brain regions will have an effect on the evaluation of the threat.

Other regions implicated to contribute the anxiety state include midbrain serotonergic raphe nucleus [374] the corticotropin-releasing factor system that originates in the paraventricular thalamic nucleus [360, 375] and the noradrenergic locus coeruleus [376]. The exact mechanism as to how these structures effect anxiety is still unknown.

In summary, each brain structure is responsible for specific aspects of anxiety such as the BNST (sustained fear or anxiety), the CEA (conditioned fear), the ventral hippocampus(contextual fear), and the LS (stress-induced anxiety).

5.1.5 Memory Neural Circuit

Normal memory function is controlled by a set of structured in the medial temporal lobe including the hippocampus and adjacent cortical areas entorhinal, perirhinal, and parahippocampal cortex [377]. The cortical areas are involved in object recognition for short intervals while the hippocampus is responsible for long term object recognition [378]. The sensory organs feed (visual, olfactory, and somatosensory stimuli) information to the perirhinal cortex which then feeds the processed information to the hippocampus [379]. The hippocampus does not discriminate the features of the incoming inputs, but compares them to previously stored information determining in the sensory inputs are novel or not [379]. To summarize the hippocampus and perirhinal function differently in object recognition. The perirhinal cortex forms basic information about if the object is novel or familiar. The hippocampus is involved in storing the information about the experience with the object. Without this long term consolidation of information done in the hippocampus, the animal would only be able to retain object memory for very short intervals [380]. Since majority of veterans who experienced one or more blast episodes display neurocognitive and neurobehavioral

deficits, we undertook studies investigating neurobehavioral outcomes in animals exposed to mild blast injury and examined the efficacy of MCC950 to improve neurocognitive outcomes.

5.2 Materials and Methods

5.2.1 Animal Preparation

Adult 10-week-old male Sprague-Dawley (Charles River Laboratories) rats weighing 300–350 g were used in this study. The animals were housed with free access to food and water in a 12h dark-light cycle at 22 °C with 40% humidity. All procedures followed the guidelines established in the Guide for the Care and Use of Laboratory Animals and were approved by Rutgers University Institutional Animal Care and Use Committee. Rats were divided into two groups (sham controls and animals exposed to a mild blast of 180 kPa). A total number of 60 rats were used in this study as follows. Behavior two groups of 30 animals each group consisting of ten control animals ten blast animals and ten blast + MCC950 treated animals.

5.2.2 Primary Blast Wave Exposure of Animals

Using the 9-inch by 9-inch cross section compressed gas driven shock tube at the Center of Injury Biomechanics, Materials, and Medicine (CIBM3) (New Jersey institute of technology, Newark) we determined the effects single mild 180 kPa blast exposure on 10-week old male Sprague Dawley rats [13, 63, 150, 198, 199, 200]. The primary shockwave generated in this shock tube was validated against the pressure-time profiles measured experimentally in the live-fire explosion experiments [201] and against theoretical pressure-time profiles associated with the detonation of C4 explosive [199, 202].

Before exposing to blast, rats were anesthetized with isofluorane. Sham controls were placed next to the shocktube and only exposed to noise without shockwave

exposure. Animals for blast exposure were mounted inside the test section located 2.80 meters from the breach and 3.05 meters from the exit with their head oriented to the direction of the shockwave (Figure 3.1). The rats were strapped securely to the bed with cotton cloth restraints to eliminate head motion. This is done to minimize the contributions of secondary blast injury to total head injury. The animals were exposed to single blast at 180 kPa in a prone position.

Immediately after blast exposure, animals were monitored for any signs of apnea and their neurological status was assessed using modified neurological severity score as reported earlier [199].

5.2.3 MCC950 Treatment

MCC950 was obtained from MedchemExpress LLC (Monmouth Jn, NJ). Just prior to injection, required doses (5 mg/kg) of MCC950 will be dissolved in sterile phosphate buffered saline (PBS). For multiple injections of MCC950, first dose will be given 30 min post-blast and subsequent doses will be given once in every 48h until the day of termination of the experiments.

5.2.4 Behavioral Test Timeline

Animal timeline can be seen in Figure 4.2

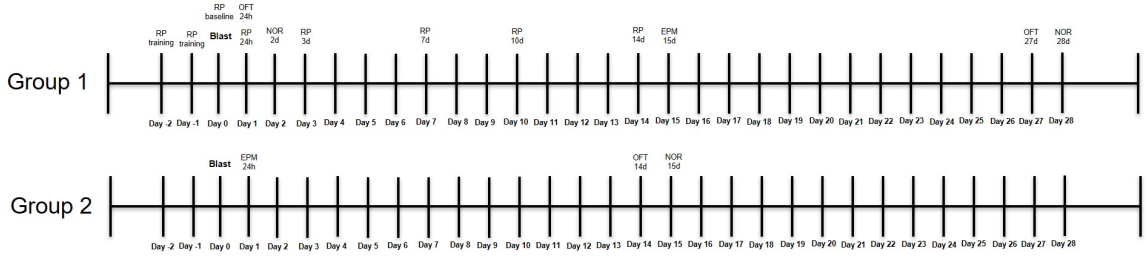

Figure 5.1 Timeline for behavioral tasks in both animal groups.

5.2.5 Rotary Pole Test

Overview In this task, the ability of rats to traverse an elevated wooden pole (elevation 914.4 mm, diameter = 40 mm, length 1500 mm) when the pole is rotating 4.5 RPM in a clockwise direction will be tested as a measure of its vestibular and motor functions.

The WRAIR study was based on the paper by Mattiasson et al. [381]. The testing consists of two pre experiment training days in which the rats learn to traverse the clockwise rotating pole at 4.5 RPM (revolutions per minute). Animals are then tested on the day of blast to form a baseline score. The animals are then tested on day 1, 3, 7, 10, and 14 following blast. The post experiment test consists of the learned behavior (traversing the pole rotating counterclockwise at 4.5 RPM).

Apparatus The rotary pole apparatus consists of four parts (motor unit, wooden pole, black rat enclosure, and two tripod stands) The motor unit was placed on one of the tripod stands and tighten the knob so the motor is held securely. The black rat enclosure consisting of a rat caged covered in black vinyl (to prevent light entering the cage) and a sliding door was affixed to a wooden platform on the second tripod stand. The pole is placed onto the square drive shaft on the motor and the other end mounted at the end of small round wire attached to the wooden platform. Testing apparatus appears in Figure 5.2.

Training To begin training, the pole was turned on to 4.5 RPM in the clockwise direction. The rat was placed on the pole near the black rat enclosure and gently pushed into the box. The door on the box was closed and the animals was allowed to remain for 30 seconds undisturbed. Next, the animal was placed about a foot away from the box and was encouraged to enter the box by prodding and clapping behind the animal. Again, the door was closed, and the animal was left alone for 30 seconds. The animal was then placed half way on the pole and the lab user clapped behind

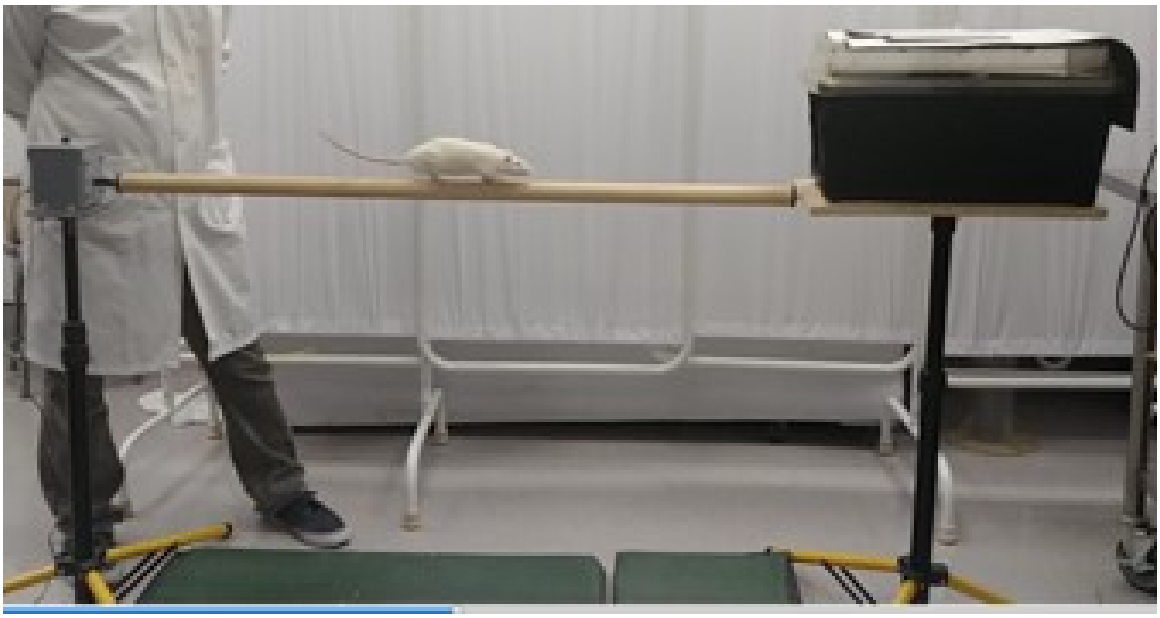

Figure 5.2 Picture of Rotary pole apparatus.

the animal to encourage it to enter the black box. The training was continued in this manner until the rat can traverse the entire length of the pole every time. The objective of the training is for the rats are to be able to make three consecutive timed runs down the entire length of the pole.

Baseline On the day of blast exposure, a baseline test was done which consists of three test runs as a reference to all test runs performed blast post experiment. The baseline test is conducted with the same setup as the two previous training day with the pole set horizontally three feet above the ground and set to rotate clockwise at 4.5RPM. Three consecutive times runs must be completed to get a good baseline score. Between each timed run the door of the box is closed and the animal is left undisturbed for 30 seconds. If the animal falls or reverses during any of the trial the animal will restart from the beginning until three consecutive timed runs are completed. No clapping or prodding is allowed during the baseline and during the post blast tests.

Testing phase The rotary pole was set the same during testing like it was during training and baseline. Three feet in height and set to rotate clockwise at 4.5 RPM. A total of three runs are recorded on each of the 5 testing days. The animals were test on day 1, 3, 7, 10, and 14 post blast exposure.

Timing All test were run using a stopwatch or a three button timer. The timer is started when the rat is released by the experimenter. The rats are timed for reaching the edge of the pole (stop timer one), entering the box (stop timer two), and a 30 second "rest period" accomplished by stopping timer three, 30 seconds after timer two is stopped. Animals are given a total of 120 seconds (2 minutes) to traverse the pole and enter the box.

Scoring While traversing the pole the animal may reverse or fall. The time the animal reverses is recorded, and the animal is allowed to complete the trail until the 2 minutes are completed. If the animal falls while attempted to traverse the pole the location of the fall (beginning, middle, and end third of the pole) will be recorded and used for scoring. A minimum score of 0 will be achieved if the two scored runs the animal falls in the first third of the pole. A score of 0.25 is achieve if both scored runs the animal reversed at any time point. A maximum score of 3.0 can be achieved if two out of the trial are completed in a time approximate the same as the baseline score. The exact scoring algorithm used can be seen here [382].

Statistics To determine whether animals following blast would have motor deficit a mixed design ANOVA was used. To test assumptions for Within-subjects a shipiro-wilks test was run to determine normality of the data distribution. Sphericity was tested using Mauchly's test and when appropriate Huynh-Feldt correction was used. An independent samples Kruskal-wallis test was to determine significance at all time points.

5.2.6 Short-Term Memory Assessment: Novel Object Recognition

Overview To test human amnesia in an animal model, the ability to recognize a previously presented stimuli is the basis for the behavioral tests [377]. For rodents the memory tests include, novel object recognition (NOR) test, delayed nonmatching to sample (DNMS), and the open field test (OFT). These test assess how the animal responds to both a novel and familiar objects [383]. The DNMS test reward the animal upon recognition of the novel object, where the NOR test has no reward and therefor able to assess the animals index of stimulus recognition [377]. Depending on the configuration of the test NOR can be set to measure working memory, attention, anxiety, and preference for novelty [384, 385]. The NOR test has also been used to determine the effectiveness of different pharmacological treatments for TBI [384]

The NOR test is particularly attractive because unlike other behavioral tests that assess memory NOR does not require any external motivation, has relatively little training time, and can be completed in a short period of time [385]. It also has the ability to study short, intermediate, and long term memory by modifying the time between the familiarization and testing phase [386]. This test evaluates object recognition memory in rodent but has shown to be good at making cross species generalization [378, 387].

Apparatus The open field chamber consists of an empty open particle board box with dimensions of (60 x 60 x 60 cm). The box was covered by black waterproof vinyl to prevent urine and other liquids from absorbing into the porous particle board (Figure 5.3)

Procedure This was used to assess short term memory loss specifically object recognition. The task consists of three phases habituation, familiarization, and the test phase. The animals were test 2d, 15d, and 28d following blast exposure. The

Figure 5.3 Picture of open field chamber including the novel and familiar object.

habituation phase occurs one day prior to the familiarization and testing phases. The animals were brought into the testing room 20 minutes prior to testing. In the habituation the animals were allowed to explore the open field chamber in the absence of any objects for 10 minutes. During this phase, the number and time of grooming and rearing episodes were recorded manually during the testing procedure. This habituation phase was used for the open field test data recorded on day 1, 14, and 27. The familiarization and testing phase both occurred on the same day, one day after the habituation phase. During the familiarization phase, a single animal is place in the open field chamber for 10 minutes with two identical new objects place equidistant from opposite corners of the chamber. To prevent coercion to explore the objects, rats were placed in the center of the chamber facing a corner where no objects were located. The number and time of each interaction was recorded manually later using video recordings. Following the familiarization phase the rats were kept in their housing cage for one hour. After the 1 hour retention interval, during the testing phase, the animals are returned to the open field chamber for 5 minutes with two objects. One is identical to the objects used in the familiarization phase, the other

is a new object never seen by the rat. After each trial the open field chamber was cleaned using Clorox antimicrobial wipes and allowed to dry completely.

Scoring For most studies, exploration was defined as the orientation of animal's snout toward the object, sniffing or touching with snout, while running around the object, sitting or climbing on it was not recorded as exploration [378, 379, 388, 389, 390, 391, 392].

Discrimination index

$$DI = \frac{T_N - T_F}{T_N + T_F}$$

T_N = time spent exploring novel object

T_F = time spent exploring familiar object

Scores can range between +1 and -1 where a positive score indicated more time spent with the novel object, a negative score indicated more time spent with the familiar object, and a zero score indicates no preference for either object.

5.2.7 Anxiety assessment: Open Field Test

Overview The habituation phase of the novel object recognition test was evaluated as an open field test to assess the animals anxiety levels following blast.

Anxiety can be defined as an emotional state associated with the perception of potential or ambiguous threats. It is considered a defensive reaction like fear. In human, anxiety is characterized by the feeling of tension, uneasiness, uncertainty, or worries that stem from the anticipation of a negative outcome or a potential threat [393, 394, 395, 396]. Anxiety consists of heightened state of arousal associated with a negative valence [397]. This manifests itself with a heightened vigilance of the environment when no immediate threat is present. Fear while similar to anxiety, differs in that it is transient emotional state that dissipates when the perceived threat

is removed [360]. Anxiety can also manifest itself physiologically with increased heart rate, increased blood pressure, dizziness, and sweating [334].

Anxiety is a normal aspect of the emotional repertoire. It enabled rapid responses to threats due to heightened awareness. Anxiety only becomes pathological when it becomes persistent, disruptive, or disproportionate to the actual danger [334]. The DSM V categorizes pathological anxiety into three categories bases on the precipitating stimuli. Obsessive compulsive and related disorders, trauma and stressors related disorders, anxiety disorders [48]. All three categories the somatic, behavioral, and cognitive effects of anxiety cause substantial economic burden [398, 399, 400]

The anxiety response consists of three steps. First, the animal detects the stimuli using its sensory system. Second, the anxiety neural circuit interprets the meaning of those stimuli to determine the levels of danger. Third, the animals evaluate if the actions taken in response to the stimulus is appropriate or must be changed. Anxiety disorders arise when the highly interconnected circuit that processes the stream of stimuli from the outside world gets disrupted [334].

Anxiety behavioral tests fall into four categories (1) approach avoidance conflict which include elevated plus maze (EPM), zero maze (ZM), open field test (OFT), light dark box, and staircase test. (2) Active avoidance tasks which includes shock probe burying and marble burying. (3) Hyponeophagia which includes novelty suppressed feeding. (4) social test which include ultra-sonic vocalizations and social interactions [401]. Most anxiety assay for rodents are ethologically based. The animals avoidance response or natural aversion is what is used to assess levels of anxiety [402]. The behavioral test are based on the assumption that there is a natural conflict within the rodent which have a drive to both explore or avoid a perceived threatening stimulus [403, 404, 405, 406, 407].

Apparatus The open field chamber consists of an empty open particle board box with dimensions of (60 x 60 x 60 cm). The box was covered by black waterproof vinyl to prevent urine and other liquids from absorbing into the porous particle board. During familiarization and testing phases objects were place is opposite corner (Figure 5.4).

Figure 5.4 Picture of open field chamber used.

Procedure Animals were moved into the testing room 30 minutes prior to testing and allowed to acclimate. Each open field test run lasted 10 minutes where the animal was placed in the center of the open field chamber facing a wall and was left undisturbed. At the end of testing the animal was removed from the chamber and returned back to its housing cage. The number of fecal boli and urine puddles were counted after animals removal. Our lab uses Any-Maze video tracking system attached to an overhead 720p camera to record each trial for later analysis After each trial the open field chamber was cleaned using Clorox antimicrobial wipes and allowed to dry completely. All tests were run during the animals light phase, between the hours of 9 AM and 7 PM.

Monitoring and Analysis of Behavior Using the anymaze software the open field chamber is split into three regions the four corners, walls, and the center region. The system automatically calculated the number of entries and time spent in each of the regions. The number and time of rearing and grooming episodes was assessed at a later date manually using the video recording.

5.2.8 Elevated Plus Maze

Overview EPM is an effective method to assess anxiety, preferred by many investigators since it relies solely on a natural behavior of the rat. It does not involve artificial cues (acoustic or temperature change), stressors (predator odor and foot shock), or any form of motivated or conditioned response (levers and food rewards). It evaluates the rat's behavior via timing the preference to stay in the dark areas (increased anxiety) versus exploration of the novel regions (normal behavior).

Apparatus The elevated plus maze is a plus-shaped apparatus with four arms: two open and two closed arms [408]. Our maze is made with matte black acrylic surface and consists of four arms (two enclosed by 30 cm tall walls and two open arms with no walls). Each arm is 50 cm in length, a 10x10 cm center section separated the the opening to each of the arms, and the entire apparatus elevated 60 cm above the ground. The elevated plus maze was situated in the center of a brightly lit room that had roughly equal illumination in both the open and closed arms (Figure 5.5). Our lab uses Any-Maze video tracking system attached to an overhead 720p camera to automatically detects and record entries and time spent in the open and closed arms and the center region. All test video were saved locally to the computer to allow for future review.

Protocol On a test day, rats will be transferred to a behavioral room and acclimated for 30 min. Rats will then be placed in the center zone of the EPM facing towards

Figure 5.5 Picture of the elevated plus maze apparatus used.

open arm and recordings will be initiated using ANYMAZE software. The rat will be allowed to explore for 5 min, which will be recorded using the video camera controlled by ANYMAZE.

5.2.9 Data analysis

Statistical analysis for all parameters of the OFT, EPM and NOR was expressed as the mean +/- SEM. Statistical analysis of the data was performed using SPSS version 25. Comparison between sham, blast, and blast + MCC950 treated animal at each time point was performed by one way ANOVA. If significance was found, a tukey HSD post hoc test was conducted.

5.3 Results

5.3.1 Blast Induces Acute Motor Deficit Mitigated by NLRP3 Inhibition

The rotary pole test was used to assess motor control, coordination, and integration of motor movement. Following blast injury, blast exposed animals began a decline in motor function compared to the control animals on day one with a decrease of 15.927% but failed to meet statistical significance (p=0.165) (Figure 5.6). The decline in motor function continued until its peaked at 7d with a decrease in rotary pole (RP) score of 37.04%. At 10 and 14 days post blast, animals began to recover towards control level but an approximate 15% decrease in RP score was still observed, although this difference failed to meet significance p=0.08. The blast exposed animals treated with MCC950 did not show motor decline at any of the time points. These MCC950 treated animals had a statistically significant increase in RP score compared to non treated blast exposed animals at both 10d and 14d (Figure 5.6).

5.3.2 Blast Induces Acute, Subacute, and Chronic Anxiety Like Symptoms Mitigated by NLRP3 Inhibition

We next investigated the levels of anxiety like behavior found in rat at 1d, 14d, and 28d following blast. We assessed these behaviors with two separate behavioral tests, (elevated plus maze (1d, and 14d) and the open field test (1d, 14d, and 28d)). Both of theses test are based the rats natural tendency to explore new environments which would be reduced by anxiety like symptoms. In the open field test, a statistically significant increase in corner time was observed in blast exposed animals compared to the control animals at all there time points increasing 36.62%, 52.21%, and 50.0%, respectively. Blast + MCC950 treated animals corner time values fell between the control and blast exposed animals and showed an increase of 17.69%, 39.39%, and 16.26% compared to the control but did not differ significantly at any time point. The Blast + MCC950 animals had a significant decrease compared to the blast animals

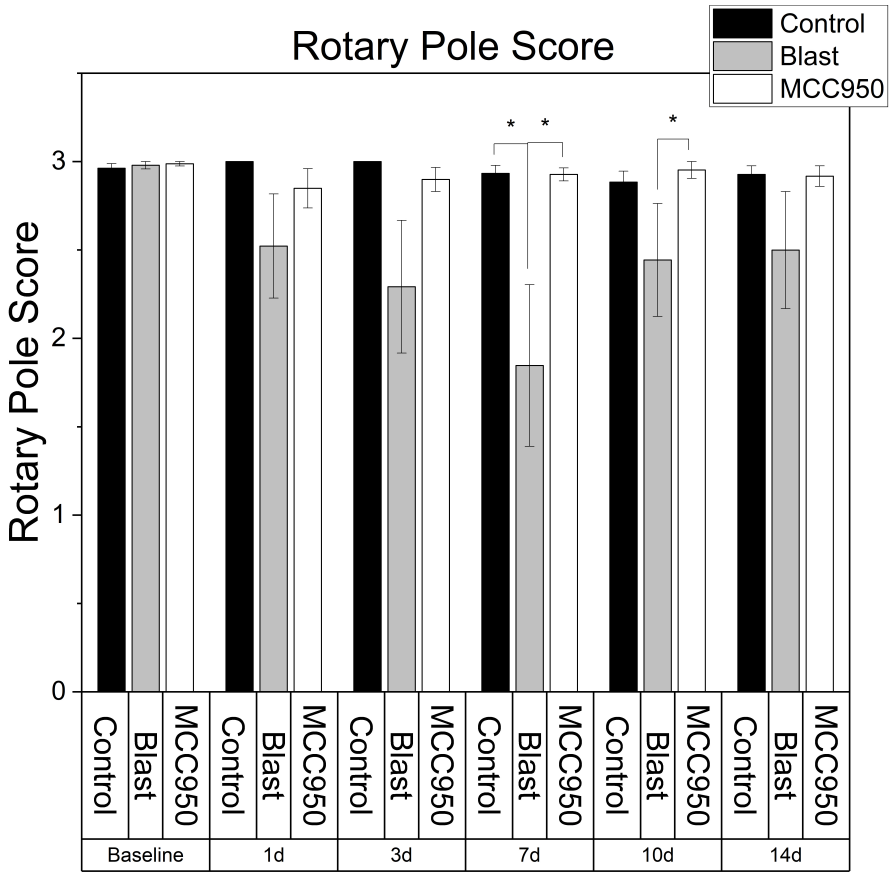

Figure 5.6 Rotary pole results in control blast and blast + MCC950 treated animals over the course of 14 days following blast exposure. *, $p<0.05$ vs control vs Blast + MCC950 (n=10 in each experimental group) df within subject = 10 or 5.406 with Huynh-Feldt correction, df between subjects = 2.

(13.85%) only at the 1 day time point. A significant decrease in center time was observed in blast animals compared to control animals with a decrease of 54.34%, 48.08%, and 51.34%. Although Blast + MCC950 treated animal had a decrease in center time compared to control (35.15%, 20.85%, and 39.35%) it failed to meet significance at any of the time points. At all the time points, blast exposed animals had a significant increase in total distance traveled compared to both control and blast + MCC950 treated animals. This results indicated the motor deficit did not contribute to the differences observed in the other parameter of the open field test. Although no significance was observed a strong trend for a decrease in grooming time

which was mitigated by MCC950 treatment following blast was observed at both 24h and 14d post blast. At 28 days, no changes in grooming time was observed between the three groups. The final parameter asses was rearing time a sign of exploratory behavior. Blast exposed animals showed a decrease in rearing time compared to the control at all three time points (25.32%, 39.68%, 32.01%) but only reach significance at 14 days post injury. Blast + MCC950 treated animals has increased rearing time compared to the blast exposed animals (26.37%, 20.33%, and 34.32%) but failed to meet significance at all three time points. The increase in corner time, and decrease in center time, grooming time, and rearing time observed in blast exposed animals indicate the blast exposure induced acute, subchronic, and chronic anxiety like symptoms following blast exposure. This increase in anxiety like symptoms was partially mitigated by MCC950 treatment.

To confirm the results found in the open field test the elevated plus maze was used. At the acute time point (1d), a general trend for an increase in closed arm time (4.35%) and decrease in open arm time (66.19%) in blast animals was observed but failed to meet significance indicating an increase in anxiety like symptoms. The Blast + MCC950 treated animals did not differ from control in closed arm time and their open arm time value fell between the control and blast exposed animals indicating a partial mitigation of anxiety like symptoms. At the subchronic time point (14d), blast exposed animals a significant increase in closed arm time (20.19%) and significant decrease in open arm time (53.68%) compared to control animals. Blast + MCC950 treated animals has a statistically significant increase of 89.2% in open arm time compared to the blast exposed animals. The blast + MCC950 treated animals closed arm time value fell between the control and the blast animal but did not differ significantly from either (Figure 5.8).

The open field test and elevated plus maze results taken together indicated the blast exposure causes an increase in anxiety like symptoms at acute, subchronic,

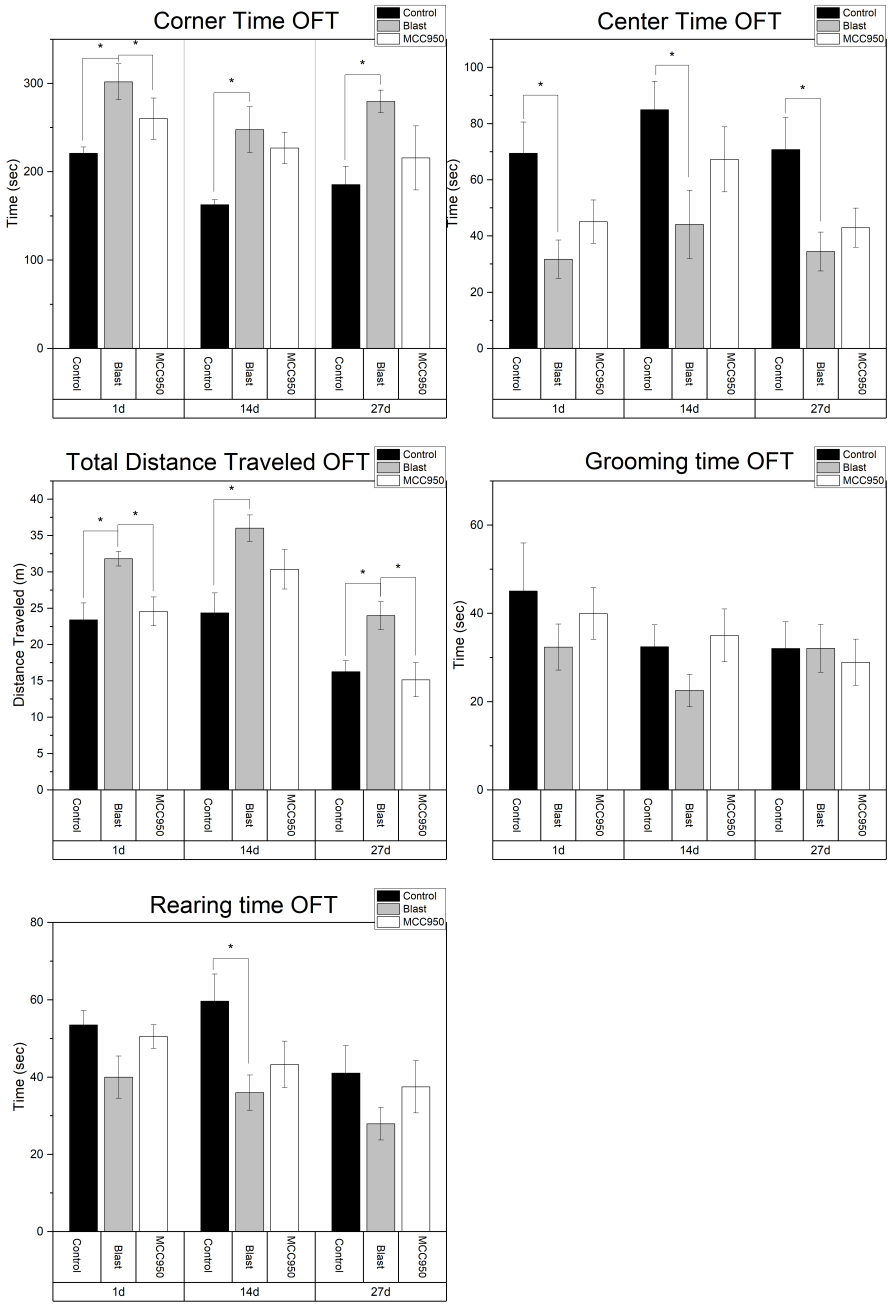

Figure 5.7 Outcomes of the open field test in control, blast, and blast + MCC950 treated animals at 1d, 14d, and 27d following blast exposure. *, $p<0.05$ vs control vs Blast + MCC950 (n = 9 or 10 in each experimental group) df between groups = 2 df within group = 26.

and chronic time points. This increase is partially mitigated by low dose MCC950 treatment.

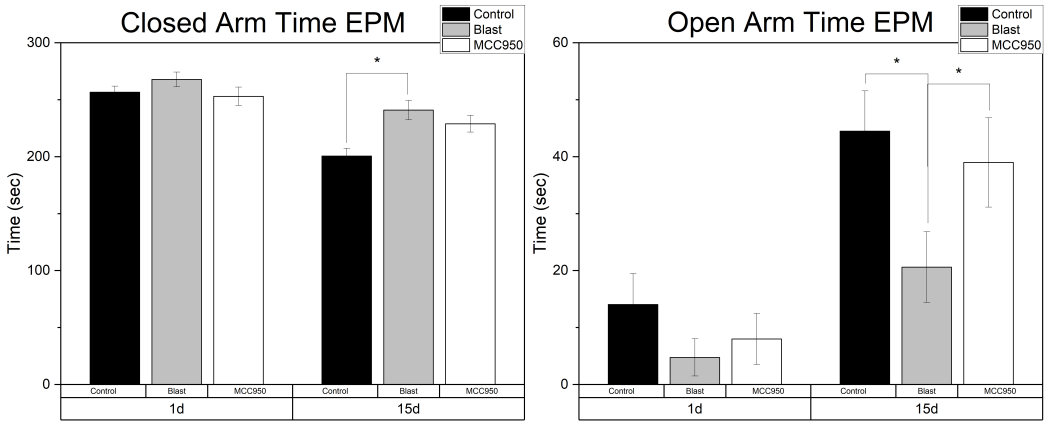

Figure 5.8 Results for the elevated plus maze in control blast and blast + MCC950 treated animals at 1d and 14d following blast exposure. *, $p<0.05$ vs control vs Blast + MCC950 (n = 9 or 10 in each experimental group) df between groups = 2 df within group = 26.

5.3.3 Blast Induces Acute, Subacute, and Chronic Memory Deficits Mitigated by NLRP3 Inhibition

The final behavior task we used was the novel object recognition task. This tasks uses the preference of the rat for a novel object over a familiar objects to determine the effect of blast on short term and recognition memory. At all time points following blast, the control animals had a preference index of of 0.3 indicating the animal's preference to explore the novel object. The blast animals differed significantly from the control animals all time points with a negative preference index ranging form -0.1 to -0.03. This finding suggests that blast induces a persistent deficit in short term memory. Blast + MCC950 treated animals had almost identical discrimination indexes to the control animals at all time points. These finding taken together indicate that the deficit in short term memory induced by blast are mitigated by inhibition of NLRP3 by MCC950.

5.4 Discussion

Following blast the most common symptoms found in soldiers include retrograde amnesia, compromised executive function, headache, confusion, amnesia, difficulty

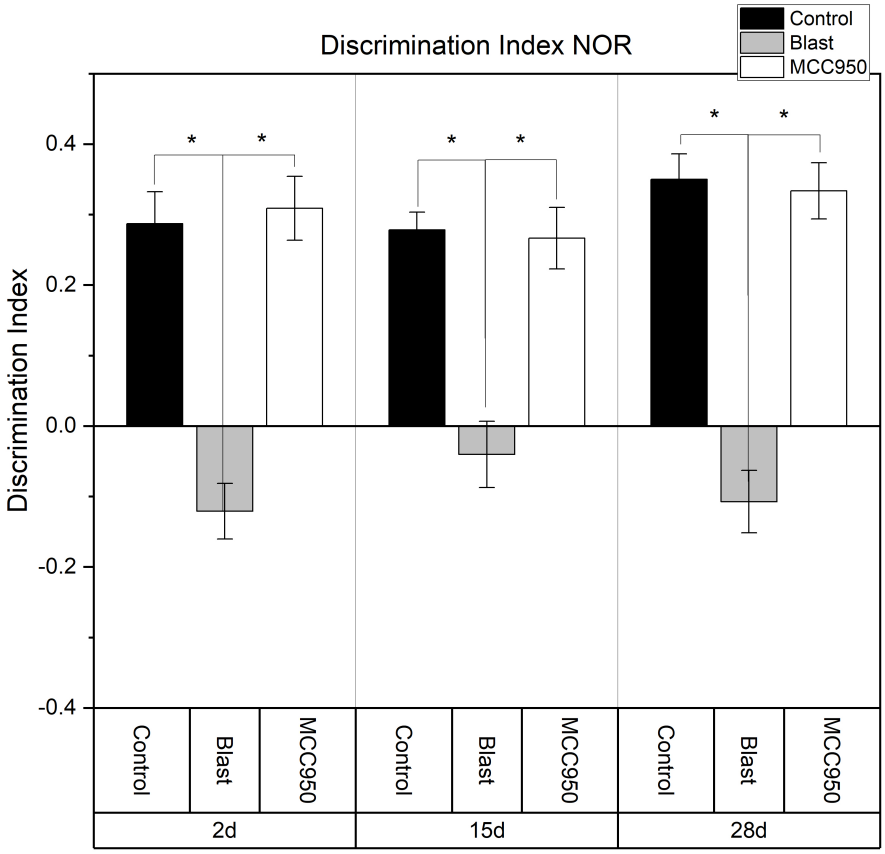

Figure 5.9 The effect of blast on short term memory assessed by the novel object recognition test. The preference for novel objects was significantly reduced at all three time points in blast animals but was not seen in the blast + MCC950 treated animals *, $p<0.05$ vs control vs Blast + MCC950 (n = 9 or 10 in each experimental group) df between groups = 2 df within group = 26.

concentrating, mood disturbance, alterations in sleep patterns, and anxiety [33]. These psychological changes observed in humans are also found to occur following blast in animal models memory [409]. Due to the lack of data for the pathological evidence for mild bTBI the major point of comparison between human and animal models is the cognitive decline observed. Other blast animal models have validated their model to cause behavioral deficits following mild bTBI, but due to the high variability between different blast models we could not assume that our model would produce similar results. Here we show our blast model was able to replicate

these behavioral deficits observed in humans and other animal models thus further validating our blast model accurately represents the pathology of bTBI in humans.

Both the EPM and the OFT were used to assess anxiety like symptoms following blast. The findings can be grouped into changes in exploratory behavior (number of entries and time spent in the center, rearing number, and rearing time), motor function (distance traveled), and grooming behavior. Following blast, it is expected that animals will show an decrease in exploratory behavior possibly a deficit in motor function, as well as a decrease in grooming behavior.

The behavioral test are based on the assumption that there is a natural conflict within the rodent which have a drive to both explore or avoid a perceived threatening stimulus [403, 404, 405, 406, 407]. In a state of anxiety the drive to avoid the perceived threatening stimulus will dominate. This manifests itself in the OFT as a decrease in time spent in the center area (perceived threatening stimulus) and an increase in time spent in the corners and near the edges which amounts to a decrease in exploratory behavior. The decrease in exploratory behavior can also be evaluated investigating the rearing response which can be considered a marker of environmental novelty [410, 411], an exploratory behavior [412, 413], a means of scanning the environment [414, 415], or an orienting response [416]. Following exposure to a stressor a reduction in rearing number of duration occurs indicating an heightened anxiety response [417, 418, 419, 420]. In the EPM the decrease in exploratory behavior manifests itself into decreased time in the open arms (threatening stimulus) and increased time in the open arm. As hypothesized, blast exposed animals showed increase in anxiety like symptoms at all time points. At the 24h time point in the EPM, the blast animals were statistically different from the control animals but at 24h blast exposed animals were significantly different from the control in the OFT. This slight contradiction may be due to the fact that all the animals control and blast exposed animals spent more than 95% of their time in the closed arms of the EPM. Thus, it was not able to

reach significance with a heightened anxiety response shown in the controls. By 15d, the control animals only spent approximately one third of their time in the closed arm and at this time point both the time in open arm and time in closed arm were statistically significantly different.

Body grooming is considered to be a body care behavior [421, 422], a form a scent dissemination for olfactory communication, and sexual attraction [423, 424]. It is believed to occupy up to 40% of the waking hours of the animal in adult rats [422, 425]. Following exposure to a stressor grooming behavior is seen to either decrease or unaffected [426, 427, 428, 429]. Decreases in grooming behavior is believed to be a sign of anxiety and is associated with inflammation, pain, and CRF excretion [430, 431, 432]. Both rearing and grooming have been shown to occur mostly in the enclosed arms of the EPM [419, 433, 434, 435, 436, 437] or in the corners or against the wall of the OFT [412, 434, 438, 439]. Although the time spent grooming was decreased in the blast exposed animal at all time points it failed to reach significance. This may be due to the low injury severity or like in other studies grooming was not affected by exposure to a stressors.

The motor activity was assessed in the OFT to ensure that the times spent in the center and edges of the apparatus were due to animals preference and not due to inability to explore the environment. At all three time points, the blast exposed animals actually travels approximately 30% further distance indicating that their motor activity was not impaired and that the differences in time spent in the center and edges of the OFT apparatus were due anxiety like symptoms.

Motor coordination was assessed via rotary pole test following blast. The blast exposed animals showed a gradual decrease in motor coordination until 7 days with a recovery until day 14. The blast animals did not return to control level by 14 days but did not differ significantly. These results mirror rotary pole results from Arun et al who found a deficit in rotary pole scores at 6 days following 130 kPa blast

and recovered later [382]. Taking the motor activity results and motor coordination results indicate that the animals motor function was not affected while the animal's coordination was. This would indicate possible injury to the cerebellum or other coordinating structures and minor to no injuries to the motor cortex.

Following blast, soldiers memories can be affected. To assess this in our rodent model of blast we conducted the NOR test to assess long term memory. NOR test results from rodents have allowed for accurate cross species generalizations about object recognition memory [378, 387]. The natural tendency for a rodent in an uninjured state is to approach and spend more time exploring novel objects than familiar ones [440]. This assumption is predicated on the novel object already existing in the animals memory [440]. NOR results have been shown to be influenced by both cortical and hippocampal lesions [392, 441]. Specifically the perirhinal cortex which has been shown to play a role in object recognition memory in both rodents and primates [388]. We have shown in aim 2 that there is an increase in IL-1β in the hippocampus following blast. Therefore, as we hypothesized there was a significant loss of object recognition memory in blast exposed animals at acute, subchronic, and chronic time points. The blast exposed animals were unable to differentiate between the novel and familiar object (discrimination index at or below 0) indicating that they were unable to consolidate the memory of the familiar object during the familiarization stage.

IL-1β is shown to elicit diverse behavioral changes similar to those observed following blast These behavioral changes are thought be induced by the changes in neurotransmitters (decrease noradrenaline in the hippocampus and amygdaloid cortex [283, 307], elevated serotonin levels [442], increases dopamine concentration in midbrain and amygdala[283], neuromodulators such as nitric oxide [304], corticotrophin releasing factor (CRF), and prostaglandin (PG) E2 [302, 303]. Also IL-1β induces an increase in serotonin metabolite 5-HIAA in the hippocampus, amygdala

and midbrain [283, 442]. These changes induced by IL-1β classified as sickness behavior [443, 444] are believe to be cause by the stress response induced by the action of IL-1β on serotonergic, catecholaminergic and Cef neurons [445, 446].

In anxiety related behavioral tests, IL-1β administration specifically has been shown to increase motor activity in the OFT [283], increase anxiogenic symptoms in both OFT (number of entries and time spent in the center, rearing number and rearing time) and EPM(decrease time in open arm and increase in time in the closed arm) [283]. The blast animals treated with MCC950 in the OFT test showed an intermediate value between the control and blast exposed animals in all measure. The dosage of MCC950 used in this study was able to significantly reduce corner time between blast and blast + MCC950 treated animals at 24 hours but reduces the corner time by 8.42% and 22.89% at 14d and 27d respectively. MCC950 has the greatest affect on the total distance traveled where MCC950 treated animals had approximate control values and significantly differ from the blast animals at 24h and 27d. At no time point did the values form center time, grooming time, and rearing time did the MCC950 treated animals differ significantly from the blast animals. At all time points their values fell between those of the control and blast animals and did not differ significantly from the control for every variable at every time point.

In the EPM, both peripheral and central administration of IL-1β have been shown to reduce the number of entries and time spent in the open arm of the EPM [297, 299, 307]. At 24 hours following blast none of three groups differed from each other significantly in both time in open and closed arm time. There was a trend for blast animals to have a decrease in open arm time and an increase in closes arm time. Although blast animals had a 66.19% decrease in open arm time it failed to reach significance. The MCC treated animals had a 68.67% increase in open arm time compared to blast exposed animals but failed to reach significance. At 14 days post injury, the anxiogenic symptoms were more pronounce where the blast animals

had a significant increase in closed arm time and a significant decrease in open arm time. The blast + MCC950 animals had significant increase of 89.19% compared to the blast with only 12% decrease compared to the control. Both of my OFT and EPM results align with the behavioral changes observed following IL-1β indicating the IL-1β produce following blast my the NLRP3 inflammasome play a critical role in the anxiogenic symptoms observed.

In line with MCC950 significant effect on motor function on the OFT, MCC950 had also a significant effect on motor coordination. Unlike the blast animals who showed a steady decline in motor coordination until day 7 with a slight recovery by day 14. Blast + MCC950 treated animals did not differ significantly from the control animals at any time point. In fact, they showed no decline in motor coordination at any time point. The blast + MCC950 animals also had significant improvement from the blast animals at both 7 days and 10 days where the control animal only differed from the blast at 7 days.

Peripheral administration via IP injection of IL-1β induce an impairment [306, 447, 448] and increase of anhedonia [449]. With central administration of IL-1β via ICV injection, rodents showed memory impairment [283], and enhance condition fear memory [299]. IL-1β is believe to cause these memory impairments by disrupting long term potentiation in the hippocampus which is the basis for memory formation [450, 451]. The reduction in long term potentiation is believe to be cause by the reduction of noradrenaline in the hippocampus [452]. Unlike the other behavioral tests, we performed to assess anxiety inhibition of NLRP3 via low dose MCC950 was able to completely prevent the memory decline associated with mild bTBI. The blast + MCC950 treated animals displayed almost identical results to the control animals showing no memory impairments.

In conclusion, continuous low dose MCC950 treatment following exposure to mild blast can prevent the memory and motor coordination deficit observed following

blast. This low dose MCC950 was able to mitigate the anxiogenic symptoms following blast but was unable to completely prevent some decline. Due to the lack of biological data following blast, we are am unable to make any definitive conclusions as to the exact mechanism to which these cognitive declines were abrogated.

CHAPTER 6

CONCLUSION

6.1 Summary and Conclusion

In Chapter 3, we investigated the activation profile for microglia following blast. We found that immediately following blast the microglia near the vasculature activated at a higher rate indicating the opening of the BBB is the primary activator of microglia while elevated inflammatory cytokine are responsible for the activation of microglia at the chronic time points. In Chapter 4, we found that early activation of NLRP3 was found in neurons while chronic activation of NLRP3 was found in microglia. This allowed us to conclude that the microenvironment due to injury differs from the acute and chronic time points. In Chapter 5, we examined the effect of inhibition of NLRP3 on cognitive decline. Here we found the inhibition of NLRP3 inflammasome by MCC950 mitigated cognitive deficits associated with bTBI. Below we summarize the mechanism we propose is responsible for the cognitive decline observed (Figure 6.1).

The injury following blast exposure initiated with the primary injury caused by exposure to energy transfer due to the shockwave. This primary injury can be composed of some or all of BBB disruption, localized hypoxia-ischemia, subdural hemorrhage, axonal injury, microvascular injury, and vasospasm. At the cellular level this vascular disruption could result in spillage of blood borne macromolecules and subsequent release of DAMPS including free radicals, activated proteolytic enzymes (matrix metalloproteinases, (MMPs)) to name a few. These DAMPs could be responsible for the breakdown of neuronal membranes which trigger an inflammatory response via the release of excitotoxic amino acids (glutamate), an increase in intracellular calcium, and possibly causing neuronal death [453].

The priming of microglia cells and other cell types that express NLRP3 inflammasome in the acute phases of the bTBI may differ from the priming signal in the subchronic and chronic stages of the progression of bTBI. This conclusion stems from our lab's earlier finding that the BBB open immediately following blast and remains open for at 24h [63], and our observation that microglia cells activate in the vicinity of the vasculature at a higher rate in the acute time points. These two findings lead to the conclusion that these cells are responding to blood components normally absent in the brain parenchyma or through DAMPs induced by the opening of the BBB through the activation of TLR4 or other receptor such as the thrombin receptor [137, 454].

During the chronic time points, the activated microglia appear to be more evenly distributed across the ROI. This would indicate a more diffuse priming signal is responsible. This signal is likely due to the statistical increase of inflammatory cytokines such as the one we observed in IL-1β or other cytokines such as TNF-α, GM-CSF, and NOD [187, 230] following blast in the subchronic and chronic time points. However, our observation of increased IL-1β that is commensurate with the time course of microglia activation suggest more towards the possibility that IL-1β is primary end product of chronic neuroinflammation in bTBI.

Similar to the priming signal, the activation signal for the NLRP3 inflammasome likely changes with the progression of the pathology. Of the five proposed mechanisms for the activation of NLRP3, we believe that only the lysosomal disruption model doesn't play any role, as its standard activators β-Amyloids [176] and amylin [247, 248], silica [185] or asbestos crystals [192], uric acid [184] and cholesterol crystals [249] are absent from the cellular microenvironment at any time point following blast. However, the other four models (pore formation and ion distribution, metabolic dysfunction, mitochondrial dysfunction, and non-canonical) we believe

play an essential role. Their overall contribution in unknown, but all are likely to contribute in a meaningful way.

One speculation is that the pore formation and ion distribution model is likely mediated through the release of ATP. In the acute phased of injury high levels of extra cellular ATP found in the vicinity of damages or dying cells induce NLRP3 activation via the P2X purinoceptor 7 (P2X7) channel. Upon ATP binding, the ion channel opens allowing for potassium efflux from the cell and balance by calcium influx into the cell [243]. This model also likely contributes to the chronic time point as well as cell death has been found to occur in the chronic time points [203]. The mitochondrial dysfunction models likely occurs in both the acute and chronic phase as both early damage and later neuronal dysfunction caused by changes in neurotransmitter level and cytotoxic level of inflammatory cytokines. The metabolic dysfunction model is the only model that has some precedent in human models of mild bTBI. Due to the lack of pathological samples and the lack of imaging data abnormalities, the changes in metabolic function in the ventral hippocampus observed in returning veterans is the only biological parameter that can be shown to be a known activator of NLRP3 in humans [51, 52]. The non-canonical pathway is believed to be active as well, as its activation is in response to oxidized phospholipids derived from PAPC which are endogenous DAMPs found in dying cells which are a signal cellular stress and cellular demise [259]. The requirement for cell death is not required for this model as macrophages and dendritic cells have been shown to produce IL-1β by this model in the absence of cell death [260, 261]. This proposed mechanism of priming and activation can be seen in Figure 4.1.

Following activation of the NLRP3 inflammasome and subsequent production of IL-1β, this cytokine could be released and act on other brain cells including neurons in an paracrine manner and alter cellular homeostasis which causes neuronal dysfunction or death. Supporting this tenet, neurons found in the hippocampus

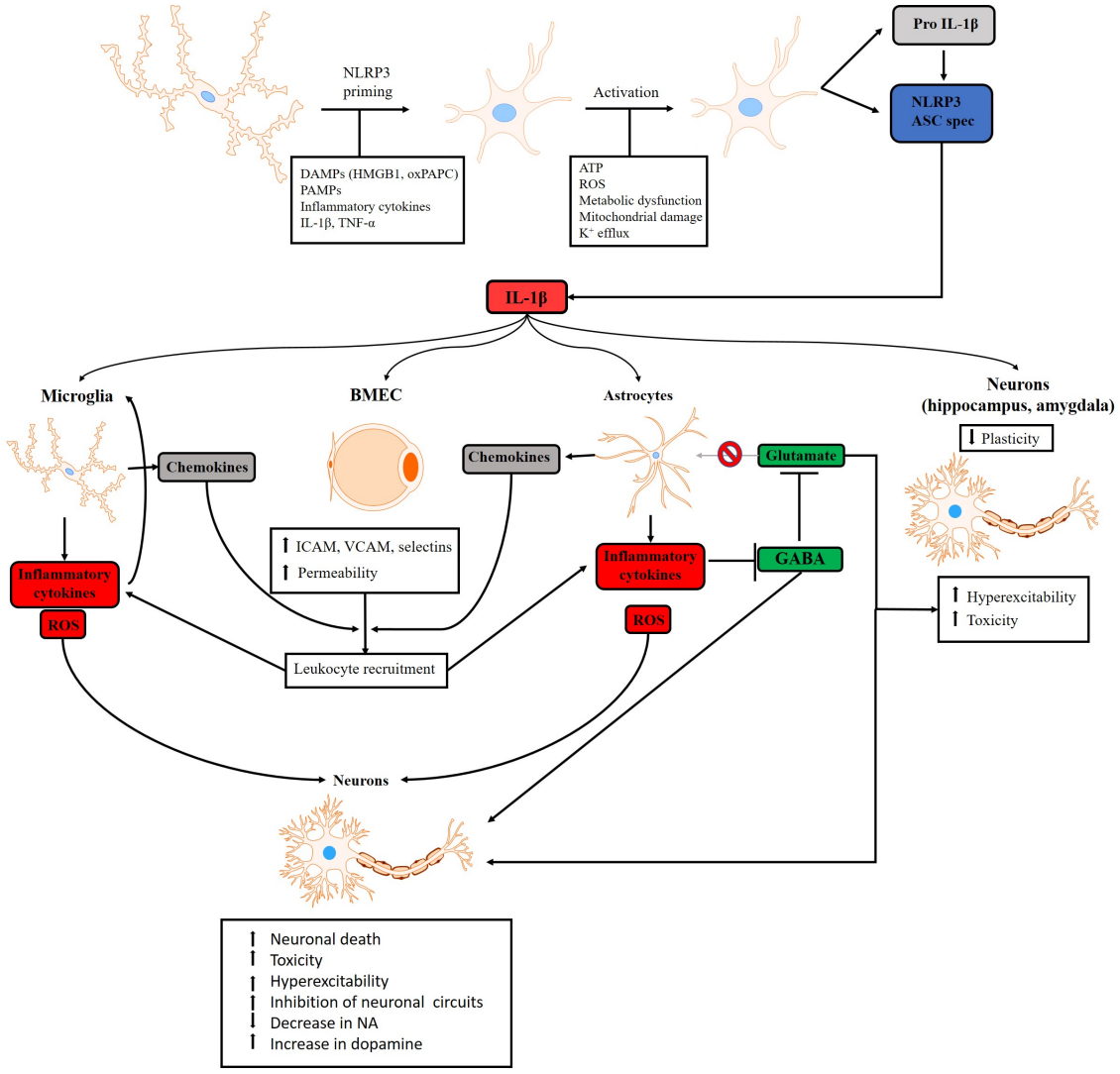

Figure 6.1 Schematic of proposed mechanism from exposure to primary shockwave to cognitive decline.

and amygdala have IL1R and are able to be directly affected by the presence of its ligand. Brain microvascular endothelial cells upon detection of IL-1β could also increase the production of ICAM, VCAM, and selectins which subsequently increase vascular permeability. The detection of IL-1β by microglia and astrocytes can induce further production and release of inflammatory cytokines, chemokines as well as the release of reactive oxygen species. These inflammatory molecules may then recruit peripheral immune cells that are able to further contribute to the aberrant neuroinflammatory environment. These inflammatory molecules specifically

IL-1β then through various mechanism induce in the increase in glutamate, decrease in GABA, increase in dopamine and decrease in noradrenaline. These changes in neurotransmitter in conjunction with cell death and synaptic pruning by microglia are responsible for the cognitive abnormality observed.

The anxiogenic symptom can be cause by a disturbance to any point in the anxiety neural circuit described above. The ventral hippocampus has been implicated in anxiety [455, 456, 457, 458, 459]. Following blast, the release of IL-1β primary from microglia have been shown to induce the release of glutamate into the extracellular environment from membrane depolarization of neurons as well as change in glutamate metabolism by astrocytes. Glutamatergic activation of BLA fibers onto pyramidal neuron of the CA1 of the vHPC have been shown to control the anxiety like behavior in EPM and OFT [358]. Increased activity in this pathway due to glutamate releases enhances the anxiety like response while inhibition reduces the anxiety like response [336, 358]. The release of CRF triggered by IL-1β is proposed as a second potential mechanism for the anxiogenic response observed. However, neutralizing CRF via CRF antagonist alpha-helical CRF, was only able to block anorexic and neuroendocrine effects while not affecting the anxiogenic effects [290]. These changes observed in the hippocampus may play a role in the anxiogenic symptoms that develop following blast but these same biochemical changes occur in other brain regions of anxiety circuit such as the amygdala, mPFC, thalamus, and sensory system. The contribution of these structures to the anxiety like symptoms cannot be determined without further investigation.

The changes in memory observed following blast may be due to the release of noradrenaline (NA) triggered by IL-1β. Central and peripheral administration of IL-1β increases the activity of the sympathetic nervous system and increase NA efflux from the spleen [460]. Changes in NA concentration has an effect on attention, wakefulness-sleep cycles, learning, and memory [308]. The reduction in

long term potentiation is believe to be cause by the reduction of noradrenaline in the hippocampus [452]. To confirm this hypothesis, the levels of NA must be assess. Our results were similar to those found in other types of TBI where IL-1β neutralization following CCI decreases neutrophil infiltration, lesion volume, as well as cognitive deficits found [461, 462].

6.2 Contributions of the Proposed Dissertation Research

Blast induced TBI is a signature injury in soldiers who returned from recent combat operations, in service members in training, as well as law enforcement officers. It is apparent that the majority of these individuals suffer from major neurocognitive and neurobehavioral abnormalities that are similar to PTSD. Additionally, mild blast injuries subtly alter neurocognitive abilities including reaction time to a stimulus, ability for swift responses, as well as short term memory. A clear understanding of the mechanisms responsible for these functional deficits in these individuals is still incomplete. This has largely resulted in failure over 30 clinical trials that reached formal approval by the FDA to treat TBI in general and bTBI inparticular. This dissertation has shown that mild primary blast injury alone (exposure to 180 kPa blast) is able to induce cognitive decline in a rodent model. The cognitive decline consists of a transient motor coordination decline with both a chronic memory and anxiogenic symptoms. This indicates that the cause of motor coordination decline is separate from memory and anxiety like symptoms. We believe that chronic microglia activation via NLRP3 activation and associated release of proinflammatory cytokine IL-1β and its further effect in by autocrine signaling is responsible for the chronic neuroinflammation. The major highlight of my dissertation is the observation that MCC950, which targets NLRP3 inhibition an ideal drug target for bTBI.

6.3 Future work

This work is intended to elucidate the mechanism in which cognitive decline is induced following blast exposure in rodent. This study has shown the microglia NLRP3 is responsible for the chronic inflammation found following blast. Elucidating the pathology of injury is import first step into developing effective treatments. Still further research is needed in order to achieve this goal. Though many possible future directions exist, here we outline the most important studies that are need in order to develop pharmaceutical interventions.

1. Perform biochemical analysis on brain tissue of MCC950 treated rats to determine the reduction of IL-1β production and NLRP3 activation
2. Perform a dosing analysis to determine the ideal dose for treatment
3. Study the timeline for treatment to enable for better treatment of injury in the field
4. Drug design to find an MCC950 analog that will not cause the liver damage found in humans
5. Perform similar experiment in other animal model to ensure that the mechanism for injury in rodent accurately represent the injury found humans
6. Investigation other brain regions such as the prefrontal cortex, amygdala and hypothalamus to determine the exact points in the memory and anxiety circuits that are disrupted after blast

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