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ABSTRACT

REPEATED LOW-LEVEL BLAST INDUCES CHRONIC NEUROINFLAMMATION AND NEUROBEHAVIORAL CHANGES IN RAT MODELS

by

Arun Reddy Ravula

Blast-induced neurotrauma (bTBI) is a signature medical concern for military personnel when they are exposed to explosions in active combat zones. However, soldiers as well as law enforcement personnel are also repeatedly exposed to low-level blasts during training sessions with heavy weaponries as part of combat readiness. Service personnel who sustain brain injuries from repeated low-level blasts (rLLBs) do not display overt pathological symptoms immediately but rather develop cognitive impairments, attention deficits, anxiety, and sleep disturbances over time. An improved rat model of rLLB was developed in this thesis by applying controlled low-level blast pressures (10 psi) repeated five times to model the true mechanism of injuries sustained by service members.

Neither the etiology of rLLB nor the consequences of repeated exposure to this low-level blast are well understood. Thus, this study examined rLLB-induced acute and chronic pathological and behavioral consequences in our rat model. The first aim investigated anxiety, motor, and memory impairments at acute (1-3 days) and chronic (>25 days) time points following rLLB using elevated plus maze (EPM), novel object recognition (NOR), and Rotarod tasks. Rationales for choosing these behavioral tasks were based on the literature; for example, EPM has been widely used in bTBI to assess anxiety-like symptoms, and the NOR test has shown consistent memory impairments at different

BOPs whereas Morris water maze (MWM) has not. Finally, compared to other motor assessments, Rotarod has sensitivity for detecting motor coordination impairments in low-level blast. Results indicated that animals exposed to rLLB significantly displayed acute and chronic anxiety-like symptoms, motor, and short-term memory impairments compared to control (unexposed) and single low-level blast rats.

The second aim explored the molecular mechanisms involved in neurobehavioral changes, including superoxide-producing NADPH oxidase (NOX1), microglial activation, and reactive astrocytosis as likely contributing factors. Of the many pathological mechanisms present following brain injury, chronic neuroinflammation has been observed for up to 17 years post-TBI and has neuroinflammation been observed even months after bTBI. Chronic persistent neuroinflammation can induce neurotoxicity. Microglia are the innate immune cells of the central nervous system and have both beneficial and detrimental effects based on their activation period. Chronic microglial activation causes continued release of free radicals, cytokines (IL-1β, TNF-α), chemokines, and other signaling molecules, resulting in neurobehavioral and pathological changes. Microglial-mediated behavioral deficits can be caused by proinflammatory cytokine IL-1β. Studies have shown that excessive IL-1β levels have an influence on anxiety, motor, and short-term recognition memory impairments in clinical and preclinical research. A molecular mechanism that is involved in IL-1β release is the inflammasome complex, particularly NLRP3. NOX1-mediated oxidative stress and NLRP3-mediated IL-1β release have not been investigated in rLLB. This aim explored the role of the NLRP3 inflammasome complex in regulating caspase-1 activation and subsequent release of the proinflammatory cytokines IL-1β in chronic neuroinflammation. Using immunofluorescence, this study examined NOX1 and

NLRP3 expression, microglial activation, and astrocytosis using specific primary antibodies such as NOX1, NLRP3, Iba-1, and GFAP, respectively in the hippocampus due to its high susceptibility for blast wave compared with any other brain region. Results indicated that there was an increase NOX1 and NLRP3 protein expression, microglial activation, and reactive astrocytosis following rLLB.

The third aim investigated the therapeutic effects of MCC950, a specific NLRP3 inhibitor, on neurobehavioral and neuropathological abnormalities following rLLB. This study mainly examines MCC950's protective role against rLLB-induced chronic microglial activation and inflammation, especially NLRP3-IL-1β-mediated neurobehavioral changes. This study performed single immunofluorescence to assess microglial activation and double immunofluorescence followed by colocalization analysis to evaluate NLRP3+ microglia in the hippocampus and perirhinal cortex following rLLB. Treatment with MCC950 prevented short-term recognition memory impairments and mitigated NLRP3 and cleaved caspase-1 expression and IL-1β release. Furthermore, inhibition of the NLRP3 inflammasome by administration of MCC950 displayed an improvement in behavioral and pathological changes caused by rLLB, validating the original hypothesis. Therefore, targeting microglial activation by inhibiting the NLRP3 inflammasome activation may have a therapeutic potential to counteract rLLB-induced chronic neurobehavioral changes.

REPEATED LOW-LEVEL BLAST INDUCES CHRONIC NEUROINFLAMMATION AND NEUROBEHAVIORAL CHANGES IN RAT MODELS

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Arun Reddy Ravula

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APPROVAL PAGE

REPEATED LOW-LEVEL BLAST INDUCES CHRONIC NEUROINFLAMMATION AND NEUROBEHAVIORAL CHANGES IN RAT MODELS

Arun Reddy Ravula

Dr. Namas Chandra, Dissertation Advisor — Date
Distinguished Professor of Biomedical Engineering, NJIT.

Dr. Farzan Nadim, Committee Member — Date
Professor and Chair of Biological Sciences, NJIT.

Dr. Bryan Pfister, Committee Member — Date
Professor and Chair of Biomedical Engineering, NJIT.

Dr. Bruce Citron, Committee Member — Date
Professor of Pharmacology, Physiology and Neuroscience,
Rutgers University New Jersey Medical School, Newark, New Jersey.

Dr. Tracy Tran, Committee Member — Date
Associate Professor of Biological Sciences,
Rutgers University, Newark, New Jersey.

BIOGRAPHICAL SKETCH

Author: Arun Reddy Ravula

Degree: Doctor of Philosophy

Date: May 2022

Undergraduate and Graduate Education:

- Doctor of Philosophy in Biological Sciences,
  New Jersey Institute of Technology-Rutgers University, Newark, NJ, 2022.

- Master of Science in Biomedical Sciences,
  Rowan University, Stratford, NJ, 2017.

- Master of Pharmacy in Pharmacology,
  Jawaharlal Nehru Technological University Hyderabad, India, 2014.

- Bachelor of Pharmacy in Pharmaceutical Sciences,
  Kakatiya University, India, 2011.

Major: Biological Sciences

Publications:

Ravula, AR., K. Murray, KVR. Rao, B. Pfister, B. Citron, N. Chandra (2021). "MCC950 Attenuates NLRP3 Mediated Neuroinflammation and Memory Impairments Following Repeated Low-Level-Blast in Rat Model" (Manuscript under preparation).

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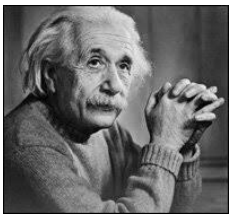
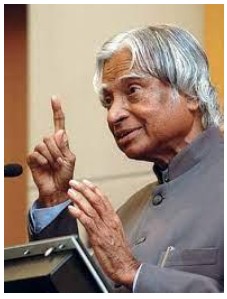
If we knew what it was we were doing, it would not be called research, would it?



-Albert Einstein

All birds find shelter during a rain. But eagle avoids rain by flying above the clouds. Problems are common, but attitude makes the difference.



-APJ Abdul Kalam.

< Dedicated to my Family>

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CHAPTER 1
INTRODUCTION

1.1 Blast-Induced Traumatic Brain Injury

Traumatic brain injury (TBI) is a major cause of hospitalizations [1] and resulted in nearly 61,000 deaths in 2019 (CDC.gov/TBI, 2019). In the civilian population, TBI occurs as a result of motor vehicle crashes, falls, and assault [2]; in soldiers, blast exposures from explosive devices are the most common source of TBI (bTBIs), accounting for 50% of all injuries [3]. Over the past two decades, there has been an increase in bTBI cases in both military and civilian population due to the increased usage of improvised explosive devices in acts of terrorism and warfare domestically and abroad [4-6].

Blast injuries are caused by an impact of pressure from a blast wave generated by explosive devices which travels at supersonic speed outwards from the explosion site. When a blast wave comes in contact with a human, it induces a sudden rise of pressure within the person's biological system and tissues. Air filled organs such as the ears, lungs, stomach, intestines and cerebrospinal fluid filled brain and spinal cord are highly susceptible to these detrimental pressure changes [7]. The damage associated with explosive detonation (blast explosions) can be classified based on its source: 1) primary damage caused by pure shock waves, 2) secondary damage resulting from penetration of fragmentation (shrapnel) and other projectiles into the brain parenchyma, 3) tertiary damage originating from impact with other objects, and 4) miscellaneous damage caused by exposure to heat and toxic gases [8]. In bTBI, the blast or shock wave induced injuries are categorized as mild, moderate, or severe based on the blast over pressure (BOP) (defined as local pressure minus atmospheric ambient pressure, in kilopascals, kPa)

experienced from the blast wave. Based on mortality rate, our lab has standardized the dose-response curve for a single blast exposure in rodents and categorized them into low (≤70 kPa), mild (70-130 kPa), moderate (130-180 kPa) and severe (≥190 kPa) [9]. Mild blast exposure is characterized by 0% mortality with no visible changes in neurological severity score (**Figure 1.1**).

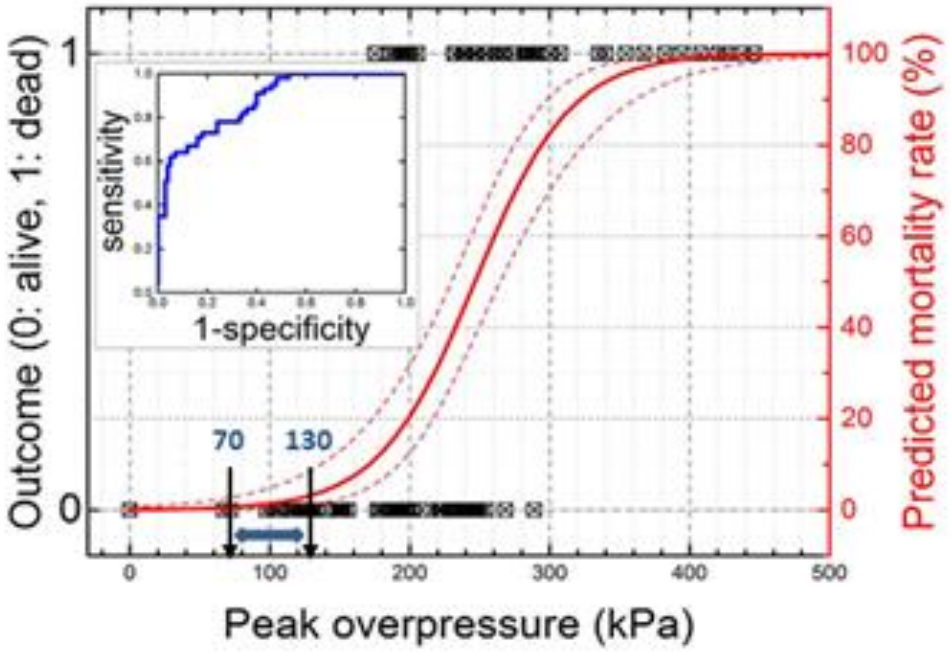

Figure 1.1 Dose response curve for the rats exposed to single blast exposure ranging from 60-450kPa.
Source: [9, 10].

Despite the increase in studies related to bTBI in recent years, there is only a limited understanding of how blast waves interact with the brain to cause injury, which has precluded the establishment of comprehensive diagnostic criteria for bTBI and potential therapeutic strategies.

1.2 Repeated Low-level Blast

Repeated low-level blast (rLLB) exposure is the mildest form of bTBI. Military and law enforcement personnel experience these low-level blasts (about 13-70 kPa) repeatedly throughout their training when routinely using heavy weaponry, including 0.50 caliber rifles and Carl Gustaf recoilless rifles, as well as during breacher training programs [11] (**Figure 1.2**).

Figure 1.2 Images of 0.50 caliber sniper rifle, Carl-Gustav recoilless rifle and breaching exercise.
Source: [11-13].

Despite a tremendous amount of preclinical research done on single mild-moderate blast exposure and its detrimental effects on the brain, there has been limited understanding of low-level blast, especially rLLB and its influence on the brain. rLLB is becoming a pressing issue for military personnel as service members who experience rLLB do not overtly exhibit the acute neurological and pathological symptoms seen in moderate and severe bTBI. However, personnel often complain of chronic symptoms such as fatigue, headache, mild cognitive impairments, and sleeplessness. It is currently unclear whether these symptoms are due to physical exhaustion or neurological effects of rLLB. This delayed presentation of symptoms has misguided service members or soldiers to return to work where they continue to be exposed to low-level blasts multiple times a day throughout their short training. These repeated exposures lead to long-term neurological problems,

including memory decline, anxiety, sleep disturbances, attention deficits, and post-traumatic stress disorder (PTSD) [14, 15].

Clinical assessments of service members exposed to rLLB have been limited and rarely include longitudinal studies [12, 14]. Additionally, true animal models of rLLB have not yet been developed. Earlier studies on repetitive blast have focused on cumulative effects of repeated exposures over longer intervals (i.e., 24 hours between blasts) and within a range of BOPs far higher (i.e., 90-140 kPa) than those experienced by service members [16]. In order to mimic the exposures normally experienced by military personnel, our lab developed a rat model of rLLB in which animals receive five successive low-level (70 kPa) exposures within a 10-minute period to represent the use of carl Gustov recoilless rifles and/or 0.5 caliber rifles but not breachers [16]. Generating such successive blasts within a short period is an engineering challenge within a laboratory setting. Our laboratory has established innovative ways of achieving the relevant loading and these methods of rLLB exposure described used in this thesis work.

1.3 Background on Neuropathology and Behavior in TBI and bTBI

In TBI, an external physical or blast insult directly damages the brain, renders individuals unconscious, impairs cognition, deteriorates motor abilities, and triggers psychological disorders [17]. TBI is characterized by primary and secondary injuries. The primary injury is a result of direct mechanical forces (i.e., impact, blast and penetration) applied to the skull and brain at the time of impact. Primary injury leads to skull fractures, diffuse axonal injuries, and brain hemorrahages, and most of these damages are instantaneous and irreversible. In contrast, secondary injuries are non-mechanical, progressively evolve over

an extended period (minutes to years) after the primary injury and manifest as alterations in brain homeostasis which ultimately lead to neuronal cell death [18].

Unlike primary injury, secondary injuries can be reduced and are reversible. These effects develop from complex biochemical cascades and mechanisms that are activated after the primary injury and include blood-brain barrier disruption, neuroinflammation, oxidative stress, mitochondrial dysfunction, brain edema, cerebral hypoxia, neuronal apoptosis, and neurodegeneration [19]. Although multiple mechanisms are involved in secondary injury following TBI, neuroinflammation plays a vital role in the development of chronic secondary injury following aTBI event.

1.3.1 Blood-brain barrier dysfunction

Among the aforementioned primary and secondary injury mechanisms in bTBI, altered blood -brain barrier integrity has been observed in many cases [20, 21]. The blood-brain barrier (BBB) is a selectively permeable membrane that separates the brain from the circulatory system. The BBB is mainly composed of brain microvascular endothelial cells, astrocytes, basement membrane, pericytes, and neurons, collectively known as a neurovascular unit [22]. Unlike peripheral vascular endothelial cells, brain vascular endothelial cells are tightly connected with tight junctional proteins (TJs) such as occludin, claudin -5 and zonal occludens (ZO1-3). Under normal physiological conditions, the BBB constantly supplies essential nutrients to the brain for proper functionality. However under pathological conditions, BBB integrity is compromised. For instance, BBB integrity was found to be compromised in many neurological disorders such as Alzheimer's, Parkinson's, multiple sclerosis and stroke [23]. Several studies have reported BBB opening

in various TBI models including, controlled cortical impact (CCI) [24, 25], weight drop models, fluid percussion injury(FPI) [26, 27] and blast exposure [28-31].

BBB disruption is regarded as a hallmark of bTBI, since altered BBB integrity has been observed through various methods in many models of mild to moderate blast exposure. In earlier work from our lab, Kuriakose et al reported BBB breakdown in a rat model of bTBI at BOPs of 35, 70, 130, and 180 kPa by observing extravasation of Evans Blue (EB) and sodium fluorescein (NaF) tracers as a measure of BBB permeability. Increased EB and NaF intensity was observed as early as 15 minutes post-injury and persisted for 4 hours in the frontal cortex, striatum, hippocampus, thalamus and cerebellum but returned to the control levels by 24 hours [20]. This study also reported translocation of tight junctional proteins such as occludens and claudin-5 in the brain and blood serum as early as 15 minutes to 4 hours following blast exposure. In another study, Kuriakose et al found a relationship between oxidative stress, NADPH oxidase enzyme 1 (NOX1) and tight junctional proteins in single moderate blast. This study found vascular endothelial NOX1 expression elevated as early as 4hrs along with tight junctional proteins, occludin and claudin 5 displacements, whereas apocynin, NOX inhibitor, treatment preserved tight junctional protein level [32]. Similarly, Stokum et al. also reported a significant increase in EB extravasation at 3 and15min and 3hrs post-injury, which returned to the control level by 24hrs [33].

Conversely, other groups have reported significant BBB opening at later time points such as 3 and 24 hours in the same regions following moderate blast with BBB resealing at 3 days post-injury [30, 31]. This temporal discrepancy in the resealing of the BBB post-injury may reflect the different blast intensities used during these exposures such

that the impact of the blast wave on the BBB integrity positively correlates with BOPs. Indeed, Kabu et al observed an increased BBB opening at 24 hours with increased blast intensity [34]. Besides extravasation tracer BBB breach has also been quantified in bTBI by analyzing tight junctional proteins such as occludin, claudin-5 and ZO-1. Abdul et al demonstrated a significant decrease in the concentrations of tight junctional proteins at 6 and 24 hours following mild to moderate blast exposure [35].

These studies not only demonstrated that BBB is an essential mechanism of injury in bTBI but also showed that the intensity of breakage is a function of both blasts overpressure and elapsed time after exposure. Although these effects were observed in models of single moderate bTBI, the repetitive exposures experienced in rLLB may have a cumulative effect on the mechanical rupture since there is not sufficient time for the BBB to recover.

1.3.2 Oxidative stress

Oxidative stress results from an imbalance between free radical production and endogenous antioxidant enzymes (i.e., superoxide dismutase, catalase, glutathione peroxidase, glutathione reductase, glutathione-S-transferase). Free radicals are highly reactive molecules that have unpaired outer electrons. Under pathological conditions free radical generation surpasses antioxidant enzyme levels. These free radicals aggressively attack lipids, proteins, and DNA, inducing irreversible oxidative modification and resulting in cellular necrosis or apoptosis. Oxidative stress is one of the known mechanisms contributing to secondary injury in TBI pathology [36].

Reactive oxygen species (ROS) and reactive nitrogen species (RNS) are the main sources of oxidative stress in brain injuries. ROS include superoxide ($O^{2\cdot-}$), hydroxyl radical (HO·), hydrogen peroxide (H_2O_2), and hypochlorous acid (HOCl). RNS refers to nitric oxide (NO)-derived compounds such as peroxynitrite (ONOO−) and nitrogen dioxide (NO2). Superoxide is the most common free radical generated immediately following TBI, which is produced when an oxygen molecule gains one electron from other molecules [37]. Increased $O^{2\cdot-}$ reacts with NO to form peroxynitrite, an extremely neurotoxic RNS, which causes damage of lipids, proteins and DNA through oxidation, inactivation of cellular antioxidant enzymes, activation of proinflammatory nuclear factors and eventual tissues damage [38]. Enzymes involved in ROS generation include NADPH oxidase (NOX), inducible nitric oxide synthase (iNOS), endothelial nitric oxide synthase (eNOS), cytochrome P450, cyclooxygenase (COX), lipoxygenase, and xanthine oxidase [23]. Of these, NOX is the only enzyme whose primary function is ROS generation whereas for others ROS is a byproduct. Furthermore, recent work from our lab and others identified NOX as a major contributor to the generation of superoxide in the cell following TBI highlighting its role in mediating secondary injury [9, 32, 35, 39-43].

1.3.2.1 NOX activation leads to oxidate stress in TBI.

NOX is a membrane-bound multi-subunit enzyme complex that is mostly present in phagocytic cells (PHOX/NOX2) and acts as a defense mechanism via generating superoxide ions to kill invading pathogens.The NOX enzyme complex consists of both plasma membrane-bound components: cytochrome b558, composed of p^{22PHOX}, gp^{91PHOX}, cytosolic components, p^{47PHOX}, p^{67PHOX}, and p^{40PHOX} and Rac G-protein. There are seven known NOX complex isoforms that are widely distributed throughout the body: NOX1,

NOX2, NOX3, NOX4, NOX5, dual oxidase DUOX1, DUOX2. The pathological role of the NOX1, 2 and 4 isoforms have been studied extensively in neurodegenerative disorders, stroke, and TBI [44]. Within the CNS, neurons express both NOX1 and NOX2, microglia predominantly express NOX2 and astrocytes express mostly NOX4 as well as small amount of NOX1 and 2 [44].

NOX acts as a key producer of non-mitochondrial ROS in TBI which initiates further damage after primary injury directly caused by shock waves due to its presence within the plasma membrane of neurons (**Figure 1.3**). Several studies have examined NOX expression in TBI pathology. Post-mortem human brain cerebral cortex samples revealed an increase in NOX2 expression in neurons as early as 6 and 24 hours post-injury accompanied by an increase in DNA oxidation [45]. Similarly, elevated expression of NOX4 was observed in the cortex of athletes suffering from chronic traumatic encephalopathy (CTE) [40]. A separate group observed an increase in NOX2 expression as early as 1 hour which persisted for 96 hours post-injury after a controlled cortical impact injury (CCI). This study also demonstrated that the acute (1 hour post-injury) increase in NOX2 was observed in neurons whereas chronic (96 hours) NOX2 expression was observed in microglia [42, 46]. These data were supported by another study that showed elevated NOX2 expression at 7 and 28 days post-blast compared to sham controls [47].

Our lab reported an upregulation in NOX1 expression in rat brain microvessels as early as 1hour post-blast which persisted for 8 days [9, 35]. Subsequent research from our lab revealed an increase in spatial and temporal expression of NOX1 and NOX2 enzymes as early as 4 hours post-blast in neurons and microglia of the cerebrum and cerebellum [41]. Similarly, reports from our lab showed a relationship between NOX1 expression in

brain endothelial cells and BBB dysfunction. This dysfunction resulted due to oxidative stress induced by superoxide generation post-blast exposure. Administration of apocynin (5 mg/kg,i.p, NOX inhibitor) 30 minutes prior to blast exposure reduced BBB dysfunction, oxidative stress and NOX expression [32]. In support of this data, studies from Luke-Wold et al demonstrated an increase in NOX4 expression and superoxide production in the cortex at 24 hours post-blast after bTBI in rodent model [40].

However, research has not been conducted thus far to examine NOX1 mediated oxidative stress in a repetitive blast injury model. The hippocampus has a high vulnerability for oxidative stress compared to any other regions and especially repeated exposure in particular has a profound impact on the hippocampus [48]. Hence, this study investigated rLLB induced NOX1 mediated oxidative stress in hippocampal subregions such as CA1,CA3 and DG.

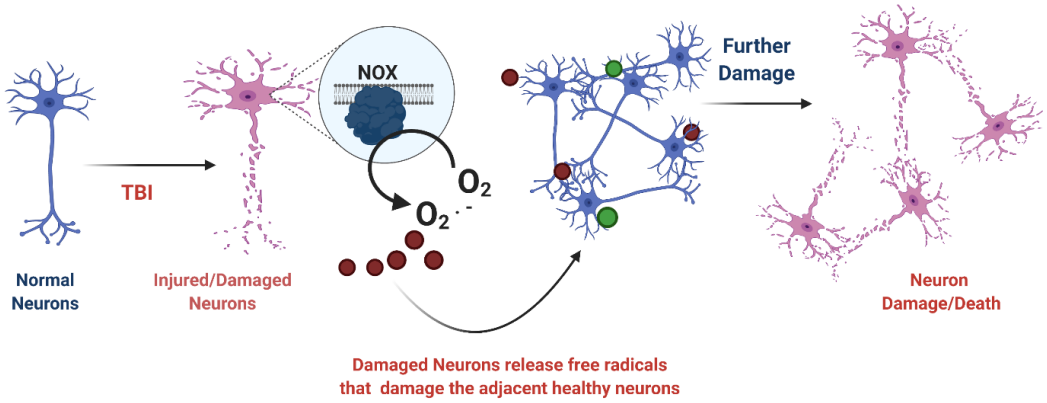

Figure 1.3 NOX involvement in secondary TBI pathology; Oxidative stress.

1.3.3 Neuroinflammation

Neuroinflammation is a known secondary injury mechanism in various forms of TBI, which can persist up to 17 years post TBI [49]. Inflammation in the brain after trauma occurs in two ways: first, by disruption of BBB integrity when microglia become activated locally near the vascular rupture [50] and second, by gradual propagation of glial cells i.e., astrocytes and microglia activation due to neuronal damage [51].

The acute neuroinflammatory response includes secretion of pro-inflammatory cytokines , chemokines and free radicals within minutes following injury. The activation of resident microglia (CNS) and infiltration of macrophages (PNS) are key players in neuroinflammatory response after a TBI event. In the CNS, microglia and astrocytes are the two main types of glial cells involved in innate immune response. Astrocytes are majorly involved in BBB formation and provide protection from neuronal excitotoxicity by clearing extracellular glutamate levels [52]. After injury, astrocytes work with brain derived neurotrophic factors to aid neuronal recovery and protect neurons from injury-induced cell death [53]. However, prolonged/hyperactivation of astrocytes can contribute to neuronal damage [54].

1.3.3.1 Role of microglia in TBI pathology.

Microglial cells are the primary immune cells of the CNS which constantly survey their environment for any changes in homeostasis via their long and branched processes. These processes are motile with an average extension of 1.47 μm/minute and a retraction rate of 0.4-3.8 μm/minute. Like human microglia, rodent resident microglia display four main phenotypes: resting, primed, activated and amoeboid [55, 56]. In the resting stage, microglia possess a rod shaped soma with processes extending out symmetrically in all

directions. Primed microglia possess an oblong cell body with long processes and multiple branches and act as an intermediate stage between resting and activated microglia. Activated microglia have an enlarged soma and fewer, shorter and unramified processes compared to those in the resting and primed stages. Microglia can also release cytokines, chemokines and free radicals during the activation stage [57]. Amoeboid microglia possess an amoeboid cell body and few unramified processes with phagocytic activity.

Boche et al. classified microglial activation into two stages: M1 state (proinflammatory, detrimental) and M2 (anti-inflammatory, defensive/protective) [58]. In the M1 stage, microglia are activated in the presence of pathogens, infection, neurotoxins or tissue damages due to brain injury and produce oxidative metabolites (superoxide, NO), proinflammatory cytokines and chemokines. These factors are responsible for host defense but also cause damage to the surrounding tissues in autocrine (acting on microglia) or paracrine (acting on other neural cells including neurons and astrocytes) manner [59]. In the M2 stage, microglia produce anti-inflammatory cytokines such as IL-4, IL-10,IL-13 and IL-18 which promote matrix remodeling, angiogenesis and tissue repair and tissue regeneration [60]. Moreover, activated microglial cells are highly receptive to pro-inflammatory as well as anti-inflammatory substances such as interleukin (IL-1β, IL-6, Il-18), interferons (INF-γ, δ), tumor necrosis factors (TNF-α), NO and ROS (**Figure 1.4**), which are produced by neighboring microglia, astrocytes, neurons and endothelial cells [19]. Microglial activation is considered to be a double-edged sword since activated microglial cells initially protect the brain after injury by separating healthy and injured tissue inorder to minimize the spread of damage in the brain [19]. However, a chronic microglial activation results in the upregulation of several pro-inflammatory cytokines

including TNF-α, COX-1 and 2, IL-1β, 6 and 12, and INF-δ, and the release of a variety of free radicals which eventually result in neuronal damage [61].

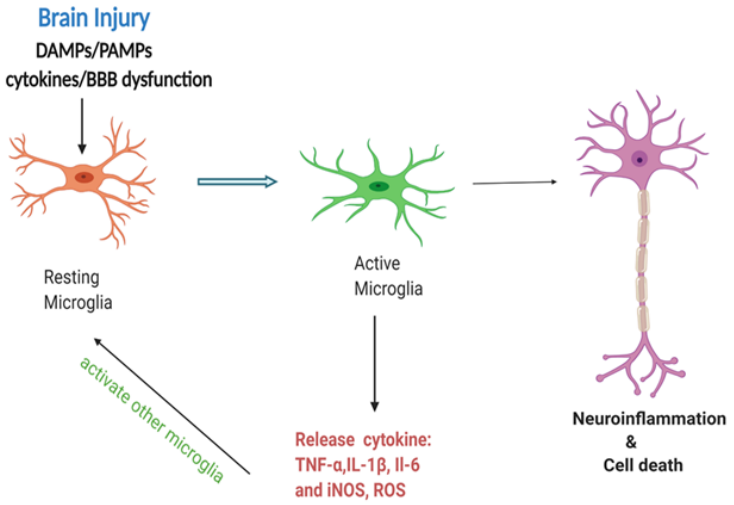

Figure 1.4 Microglia mediated chronic neuroinflammation and cell death.

1.3.3.2 Cytokine release following microglial activation.

Cytokines are small molecular nonstructural proteins with diverse roles, in the CNS including the regulation of key cell functions during development, maintenance homeostasis in mature organisms and coordination of host-defense mechanisms in response to infection or traumatic events. Cytokine levels are low under normal physiological conditions but rapidly increase in response to insult including brain infections, ischemia and TBI. Almost all neuronal cells can produce these mediators and also possess receptors to these mediators [62]. TBI triggers various cells mainly microglia to secrete pro-inflammatory cytokines: TNF-α, IL-1 , IL-18 and IL-6 or anti-inflammatory cytokines: IL-4, Il-10 and IL-13, which inhibits further proinflammatory cytokine secretion.

1.3.3.3 Role of pro-inflammatory cytokine IL-1β in TBI pathology.

IL-1 is the first member of the IL family and it is not a single molecule it exists in IL-1α and IL-1β. IL-1α, β both cytokines exert their action through same IL-1RI. On contrary, there is another cytokine within IL-1 family IL-ra, IL-1receptor antagonist which selectively blocks all IL-1 cytokines actions. All three forms IL-1α, IL-β and IL-ra exist in precursors form such as pro-IL-1α, pro-IL-β and pro-IL-ra. Except for pro-IL-β, remaining pro isoforms are biologically active whereas pro-IL-β need cleavage by caspase-1 to release as active IL-1β [63]. NLRP3 inflammasome is one of the mechanisms involved significantly in activation of caspase-1 and releasing active IL-1β from the cells.

IL-1 cytokines expressed a low-level concentration in the healthy brain, whereas at moderate level, they play an important role in normal physiological process including development, sleep, synaptic plasticity, synaptic pruning and memory consolidation and formation during adulthood [64]. On the other hand at raised levels IL-1 cytokines shows detrimental effects during traumatic event. A rapid upregulation of IL-1β, IL-1Ra, IL-1 and II receptors (IL-1R, IL-1RII) expression in rat stroke model [65]. Similarly, IL-1α expression increased in microglia as early as 4 hours after cerebral ischemia [66]. Exogenous administration of IL-1β exacerbates ischemic damage [67] whereas in IL-1β knock out experiments revealed an improvement [68]. Earlier studies demonstrated an increase in IL-1β and IL-18 levels in brain homogenates after controlled cortical impact focal injury. In this study, they have found early raise in IL-1β at 6hr post-injury which came to control levels by day 7 whereas IL-18 levels gradually increased at day 7 [69]. A study investigated the influence of p38α (MAPK14) protein kinase involvement in controlling IL-1β release from microglia following fluid percussion injury. They found an

early raised in IL-1β (acute, 0-12hrs) levels in p38α knock out (KO) compared to wild type animals whereas, at chronic time (day 7) the IL-1β levels reduced in the same p38α KO animal group [70].

IL-1β receptors are widely distributed all over the brain but are most densly populated in the hippocampus [71]. Earlier studies demonstrated the influence of IL-1β on neurotransmitter release and turnover, endocrine functions and behavioral changes including impaired spatial memory, sleep disturbances, decreased exploratory activity, anxiety through exogenous administration (I.P injection or intracranial injections) of IL-1β [72-78].

1.3.4 NLRP3 inflammasome in chronic neuroinflammation

Microglial cells are the innate immune cells of the CNS which are activated in response to the presence of pathogens associated molecular patterns (PAMPs) or endogenously produced damage-associated molecular patterns (DAMPs) through pathogen recognition receptors (PRRs) such as Toll like cell surface receptors (TLRs), NOD-like receptor proteins (NLRPs) and RIG-I like receptor [19]. Chronic neuroinflammation due to sustained microglial activation is a frequent observation in bTBI [79, 80]. One of the key players contributing to chronic neuroinflammation is the inflammasome complex. The inflammasome is a multi-component cytosolic complex which induces proteolytic cleavage of proinflammatory cytokines such as pro-IL-1β and pro-IL-18. The inflammasome complex consists of three major components: cytosolic pattern recognition receptor (NLR, acts as a sensor), caspase1 (effector protein), and an adaptor protein ASC (apoptosis-associated speck-like protein containing a caspase-recruitment domain).

There are various inflammasome complexes including NLRP1,3,6,12, NLRC4 and AIM2. Of these NLRP3 (pyrin domain 3 containing NLLR family) is the most studied inflammasome complex due to its reception for wide range of stimuli. The NLRP3 molecule contains three major components: an amino-terminal PYRIN (PYD) domain, a nucleotide-binding NACHT domain, and a carboxy-terminal leucine-rich (LRR) domain (autoinhibition). The NACHT domain possesses ATPase enzyme activity, which plays a key role in NLRP3 activation (self-association and function). NLRP3 is a multi-protein ensemble that serves as a molecular sensor to detect microbial antigens, environmental irritants, and internal "danger signals" such as ROS, extracellular ATP, amyloid β, mitochondrial dysfunction and cellular damage [81-84]. In response to pathogens or endogenous signaling (i.e., TBI), NLRP3 becomes activated and assembles the inflammasome complex [85]. Assembly and activation of the NLRP3 inflammasome lead to the production and release of activated/cleaved caspase-1 and proinflammatory cytokines (e.g., IL-1 β and IL-18). Besides IL-1β, cleaved caspase-1 is also involved in an inflammatory programmed cell death known as pyroptosis [86].

1.3.5 NLRP3 activation

In general, activation of NLRP3 is a two-step process that requires two signals; priming and activation [84]. The priming signal serves two major functions: 1) transcriptional upregulation of inflammasome components such as NLRP3, caspase-1, ASC, and pro-IL-1β via NF-kB, and 2) post-translational modifications of NLRP3 such as ubiquitylation, phosphorylation and sumoylation, which autosupresses to maintain its stable inactive form [87-89]. The activation signal is typically initiates the oligomerization and activation of the inflammasome complex that cleaves pro-IL-1β into its active form (IL-1β) and is

subsequently released from the cell (**Figure 1.5**). So far multiple upstream signals that have been involved in the activation of NLRP3 inflammasome complex including PAMPS, DAMPS, ATP, ionic imbalance (K^+ efflux, Ca^{+2} flux, Cl^- efflux), lysosomal disruption, release of cathepsins, mitochondrial DNA or cardiolipin, mitochondrial dysfunction, pore-forming toxins, crystalline substances, ROS, TNF-α and cytokines [90].

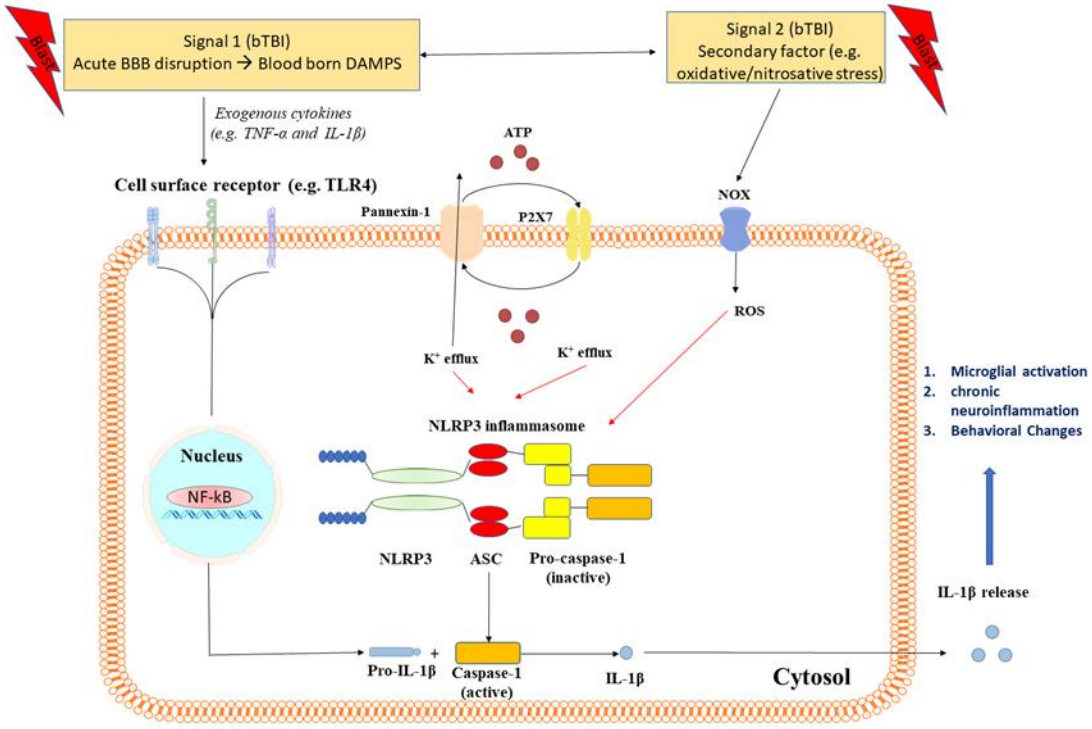

Figure 1.5 A schematic of NLRP3 activation and IL-1β release.

The NLRP3 inflammasome is a major mediator of IL-1β production via activation of caspase-1. An increase in IL-1β levels exacerbates secondary injury by enhancing oxidative stress and inflammation which eventually lead to neurobehavioral and neurological deficits. Earlier studies observed behavioral changes such as anxiety [91], depression, and cognitive decline [92] associated with increased IL-1β and TNF-α levels [93] in the animal model. A prolonged and overactive pro-inflammatory state can lead to

tissue damage via apoptosis, glutamate mediated excitotoxicity, immune activation, and cytotoxicity [94] and has been associated with the neurocognitive and behavioral deficits observed in the development of Alzheimer's disease, Parkinson's disease, amyotrophic lateral sclerosis (ALS), stroke, multiple sclerosis, and prion disease [95].

There is a relationship between NLRP3 inflammasome and acute and chronic brain injuries. A study observed an increased NLRP3 level in CSF in TBI patients. In this study they observed peaked NLRP3 level (>6.63ng/ml) in CSF notice in TBI patients with less than 4-year-old compared with more than 4 years old. They also notice the pattern of NLRP3 levels expression for instance immediately increased NLRP3 level 1 day post-injury, whereas low at day 2 but again increased at 3 and 4 days post-injury [96]. It was previously thought that NLRP3 was exclusively in microglia, but it is now found to be expressed in neurons [88, 97]. The time course for the expression following TBI is different between these two cells. For instance, immediately after the injury, the NLRP3 levels increased in neurons, whereas, over time, the NLRP3 level increased in microglia [98]. Similarly, another group noticed an increase in caspase-1, ASC levels in adult severe TBI patients even after 5 months post-injury [99]. Compared with other TBI models, NLRP3 inflammasome was not studied in the blast TBI field. For instance, there is only one study that reported an increase in NLRP3 expression, caspase-1 expression, IL-1β release along with oxidative stress in moderate blast at 12 and 24 hours [100]. Recently, our lab noticed an increase in microglial activation, NLRP3 protein expression, NLRP3+microglia (colocalization), caspase-1enzyme expression, and IL-1β release as early as 4hrs to 30days in the hippocampus following moderate blast TBI (unpublished data). Hence, blast exposure might influence NLRP3 expression, particularly in microglia.

1.3.6 MCC950

In recent years, several inhibitors of NLRP inflammasomes have been developed, among which MCC950 is the most potent and specific inhibitor of NLRP3 [101]. MCC950 is one of the compounds that has been widely used to treat systemic and neuroinflammatory diseases [102]. MCC950 (CRID3 or CP-456,773) is a sulfonylurea compound that has a specific binding site on the NLRP3 inflammasome complex and inhibits the cleavage of pro-IL-1β to active IL-1β, a process mediated by caspase-1 [103]. MCC950 has been found to bind directly to the NACHT domain of NLRP3 and prevent ATPase activity which is required for inflammasome complex activation [101]. MCC950 has been shown to be specific to only NLRP3 inflammasome [104] and it blocks all forms of NLRP3 activation known to date including canonical and non-canonical inflammasome pathways [104-106].

Compared to other inflammasome inhibitors, MCC950 has several advantages. Firstly, MCC50 has a selectivity for NLRP3 inflammasome and its precise mechanism of action is known. Secondly, can easily cross the BBB ensuring that a systemic route of administration such as intraperitoneal (IP) injection will have a therapeutic effect at minimal dose. Thirdly, MCC950 has been used in a variety of animal models with good compatibility. Fourthly, there have been several research studies on MCC950 in inflammatory and other diseases. Finally, the optimal dosage of MCC950 for rodents is known, so additional experiments are not necessary to investigate dosing [105].

Preclinical studies have shown MCC950 to have good bioavailability and CNS penetration making it ideal for the treatment of neuroinflammatory disorders [101, 104, 107, 108]. Several lines of evidence show that increased levels of proinflammatory cytokines, particularly IL-1β result in several neurocognitive impairments in acute and

chronic neurological disorders including TBI [109]. MCC950 was first developed by Inflazome Inc, and several preclinical studies have shown that it significantly improves neuroinflammation in stroke [110], Parkinson's disease [111], and Alzheimer's disease [112, 113]. It is noteworthy that MCC950 not only inhibits chronic neuroinflammation in various neurological conditions but also improves neurocognitive functions in animal models of diabetes [114], blunt TBI [115], stroke [116], and several other inflammatory conditions where the brain is affected. Also, MCC950 has shown good bioavailability and CNS penetrability [101, 104].

1.4 Neurobehavioral Changes in TBI

According to the Defense and Veterans Brain Injury Center, approximately 380,000 military personnel have been diagnosed with TBI since 2000-2018 [117]. Of these, more than 50% of combat TBI occureddue to blast exposures [118]. Most soldiers diagnosed with bTBI develop cognitive, behavioral and psychological impairments such as PTSD, attention deficits, headaches, anxiety, depression, memory loss, suicidal ideation, tinnitus and vestibular deficiencies [119-122]. Despite significant research on behavioral deficits, the etiology of these deficits following a blast injury is not well understood. This proposal focused on some of these findings with respect to rLLB.

1.4.1 Cognitive deficits

Cognitive deficits have been reported in patients with mild TBI which often resolve within 6 months; however, moderate and severe TBI frequently cause long-term impairments including dementia or other neurodegenerative disorders [123]. Soldiers exposed to bTBI have been diagnosed with retrograde or anterograde amnesia and decreased executive

function [124, 125]. In animal models, bTBI also affects the prefrontal cortex and hippocampal regions involved in cognition [126]. Recognition memory in rodents following bTBI has been mostly assessed using the novel object recognition test while spatial working memory has been studied using Morris water maze (MWM) and Barnes maze.

The novel object recognition test (NOR) measures both short-term and long-term deficits in recognition memory. Animals exposed to single low-level blast (74kPa) do not manifest any recognition memory impairment after 2 weeks [127], whereas animals to higher BOPs exhibited both acute (4-5 days [128], and chronic (2 weeks [79] and 3 months [129]). However, some studies have reported deficits in NOR with low-level blast exposure [130, 131].

Allocentric spatial memory involving the hippocampus and entorhinal cortex has been studied using several different mazes, with the most popular form of assessment being the MWM [132]. In contrast NOR animals tested on MWM only exhibited chronic spatial memory impairment at higher BOPs [133, 134] and acute deficits in lower exposures [135, 136]. Similarly, animals tested on Barners maze test only showed chronic deficits at higher BOPs [137, 138]. This suggests that NOR is likely more sensitive to changes in behavior over a wide range of blast exposures.

1.4.2 Anxiety

Veterans diagnosed with bTBI also report developing anxiety and depression disorders over time. Treatment for military personnel with PTSD and depression has been estimated to cost approximately $6.2 billion in the first two years post-deployment [139]. In a sample population of veterans exposed to bTBI, it was identified that 50% of individuals exposed

to bTBI developed anxiety disorders, 88% developed depression and 60% showed PTSD traits [140].

Anxiety has been evaluated in rodent models of bTBI using tests such as the elevated plus-maze (EPM), open field test, elevated zero maze and light/dark box test. Anxiety measured by the EPM test was identified at chronic time points (i.e .,7 and 15 days post-injury) in most studies, demostrating long-term deficits irrespective of an animal's location in the blast tube [141-144]. Similarly, Awwad et al observed anxiety in EPM at 9 days post bTBI [145]. Another study also observed anxiety using EPM and light/dark box test at acute (1 day [146]) and chronic time points (28 days [147]). Acute deficits in the open field test were observed at 1-7 days post-injury [128, 148] and these changes were also observed at chronic (>25 days) time points [137, 149].

1.4.3 Motor deficits

Veterans diagnosed with bTBI have been known to exhibit balance and vestibular motor coordination issues with symptoms ranging from dizziness, vertigo, postural instability and impaired tandem gait [150-152]. Furthermore, balance deficits are amplified in veterans with a comorbidity of TBI and amnesia or PTSD [153].

Balance and gait impairments in rodent models of bTBI are typically measured using Rotarod, the horrizontal ladder test and beam balance tasks.The Rotarod apparatus is more sensitive in identifying motor deficits in low to mild TBI [154]. Motor learning refers to the rodent's ability to develop a strategy to remain on the rotating rod rather than as a result of improved locomotor skills [155, 156]. While a single low-level blast did not result in any motor deficits in Rotarod test, animals exposed to higher BOPs exhibited acute [157]

and chronic motor defects [149]. Animal exposed to blast did not manifest any acute and chronic motor deficits in the beam balance walk or ladder run test [127, 135, 158].

These behavioral data demonstrated that compared with single low-level blast, deficits are more prominent at higher BOPs and can be observed at both acute and chronic time points. In these studies, behavioral deficits were associated with neurodegeneration and increases in oxidative stress, neuroinflammation, brain edema and several other biochemical factors. This suggests that multiple molecular mechanisms are leading to abnormalities in behavioral outcomes such that no single mechanism is proposed to be soley responsible for eliciting these behavioral deficits post-blast. Furthermore, very few studies were conducted on rLLB and these studies demonstrated that deficits were prominent chronically. However, these studies employed a different approach wherein the repetition of blast occurs every 24 hours which is not a typical time interval experienced by military and law enforcement personnel during training with heavy weaponaries.

1.5 Significance of the Dissertation Research

A pressing question in military medical research is the longitudinal effect of rLLB on the human brain over time. Service members who employ artillery, mortars, heavy weaponry and explosive breaching devices are frequently exposed to these low-level blasts as a part of the training program non-combat training program. This rLLB setting is distinct from the higher-level blasts experienced in a combat setting. For instance, in active combat zones, soldiers experience sporadic high-intensity blast exposures, whereas in non-combat training they experience repeated low-level blast exposures. Standard military training protocols involving blasts restrict the exposure to 28 kPa BOP based on damage to the

unprotected human eardrum rather than damage to the brain [159]. However, there are no guidelines regarding the total number of exposures or blast intensity per training session that a single trainee can experience without detrimental effects on health and neurological performance. There are very few studies reporting long-term consequences such as attention deficits or memory impairments in personnel who have been exposed to rLLB for 5 years [7]. Furthermore these studies were self-reported and lack etiological evidence. Unlike higher intensity blast exposure, the rLLB may not induce any diagnosable injury or symptoms during or immediately after the training program which leads to an important question, **"Does rLLB induce chronic neurological or behavioral deficits ?".**

Currently, there are very few animal models on rLLB that aim to study the cumulative effects of multiple exposures. These models lack in simulating the real-life scenario as these models focus on blasts that are given repeatedly (one blast per day) over consecutive days. For example, one model induces repeated low-level blasts on animals giving 24hrs intervals between blasts and the blast exposure ranges between 100-145kPa [13]. However, in a real scenario, soldiers experience between 1-25 low-level blast exposures in a single day and the number of days to exposure varies [12, 13, 160]. Thus, there is no appropriate animal model that could mimic real-time rLLB scenario. The primary reason that researchers resort to longer time intervals is perhaps due to their inability to quickly induce multiple shocks within a short time which is a design and operational problem of the shock tubes.

Furthermore, there is a lack of understanding of the etiology underlying rLLB and its chronic effects on the brain in the long run. To address this, our lab has developed an animal model in which an animal receives 5 to 7 successive low level (70 kPa) shots within

a 10 minute period under anesthesia. This model is relatively precise because it mimics both number of blasts and frequency of exposures that soldiers experience in a single day. This study examined the effect of rLLB on neurological defects, especially neuroinflammation, known secondary injury mechanisms, at acute and chronic time points after rLLB injury. Data from these experiments provided insights into long-term neurological defects that result from repeated blast exposure. Furthermore, data from these experiments is useful to design an appropriate animal model that accurately represents rLLB that service members experience during real training conditions. In summary, the findings from this study unveiled a mechanism that is contributes to the long-term neurological impairments and neurobehavioral deficits following rLLB injury.

1.6 Hypothesis and Specific Aims

Based on existing literature and previous work and preliminary data, I hypothesized that *"repeated low-level blast injury induces chronic neuroinflammation through activation of NLRP3 inflammasome leading to neurobehavioral changes".* A corollary to this hypothesis is that effective inhibition of NLRP3 activation improves neurocognitive outcomes in rLLB. Based on hypothesis, I formulated three specific aims (**Figure 1.6**).

Specific Aim 1: Characterize acute and chronic neurobehavioral abnormalities following rLLB

I examined neurobehavioral changes exposing rats to rLLB. Animals subjected to various neurobehavioral tasks that are aimed to assess a) anxiety, b) memory loss and c) motor deficits at acute (24 hours) and chronic (>20 days) time points post-injury. I expected to observe chronic behavioral deficits in rLLB animals compared to native animals. Changes

in these behavioral patterns were also investigated in rats exposed to a single blast at 70 kPa to compare the effects of single vs. repeated low-level blast. This aim examined if rLLB induces neurobehavioral changes in our rodent model comparable to clinical and how these effects compare in unexposed and singly exposed animals.

Specific Aim 2: Characterize acute and chronic neuroinflammation following rLLB.

I focused on the pattern of acute and chronic neuroinflammation by microglial activation, as well as the activation of NLRP3 inflammasome and the pro-inflammatory cytokine such as IL-1β production, that may lead to secondary injury after rLLB at acute and chronic time points. I expected to observe chronic neuropathological changes in the rLLB animal group.

Specific Aim 3: Determine whether the NLRP3 inhibitor MCC950 promotes neuroprotection and neurobehavioral improvements following rLLB.

I intraperitoneally administered MCC950, a sulfonylurea compound that inhibits NLRP3 inflammasome complex formation and IL-1β production, to a rLLB group of animals following blast for 4 days. Then, I used the behavioral tests in aim 1 and neuroinflammation assessment in aim 2 to determine whether MCC950 improves the neurobehavioral outcome in the rLLB animals. I anticipated that NLRP3 inhibitor reduce the intensity of neurobehavioral changes observed in aim 2 exhibit that this is a possible pathway and can open up therapeutic opportunities. **Figure 1.6** shows the schematic of the thesis with the three aims and their interactions clearly delineated. This study examined and observed chronic neurobehavioral cahnges and neuroinflammation following rLLB and MCC950 treatment improved behavioral outcomes and prevented chronic microglial activation and neuroinflammation. Thus, these three aims combinedly tested the hypothesis and also established well-designed rLLB animal model to understand rLLb eitiology.

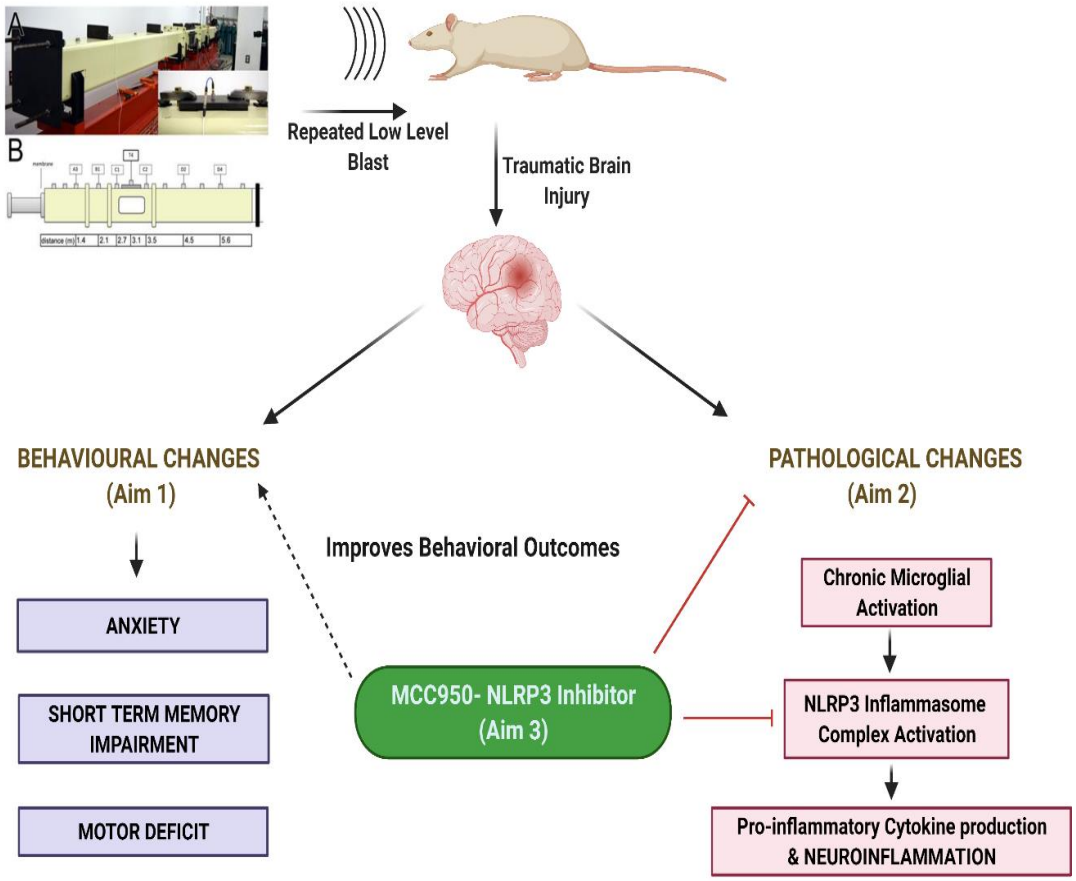

Figure 1.6 A schematic of specific three aims of the proposal.

CHAPTER 2

CHARACTERIZATION OF ACUTE AND CHRONIC NEURO BEHAVIORAL ABNORMALITIES FOLLOWING rLLB

This chapter explores aim 1 in detail, wherein the rationale is established first. Later, some details on the selection of sample size and the methods are described. Results are shown next followed by discussion and the relationships of the outcome of aim 1. In aim 1, I looked at the effect of repeated low-level blast rLLB on chronic neurobehavioral changes in rats. Specifically, I examined the role of rLL in anxiety, memory loss and motor deficits in animals at acute and chronic time points compared to naive and singly exposed animals.

2.1 Rationale for Specific Aim 1

Earlier studies have observed behavioral deficits in NOR at both acute and chronic time in animals exposed to a single moderate blast (around 180kPa) whereas these deficits were not observed in single low-level blast exposed animals [127, 130, 131]. However, chronic recognition deficits were observed when animals exposed to repeated moderate blast exposures with one-day interval (existing repeated model) [142, 147]. Thus, this study examined both acute (1-2 days) and chronic effects (>25 days) of memory using NOR. As per earlier studies, persistent motor deficits in Rotarod were observed in single mild-moderate blast [149, 161]. One study noticed motor deficits that lasted for 14 days but did not persist at 21 and 30 days in a single moderate blast [162]. Similarly, other groups also observed motor deficits at 2 and 24hours and up to 3 days following a single moderate blast

[128, 157], whereas animals exposed to a single low-level blast did not show any motor deficits [127].

Also, a study examined repeated low-level blast (75 kPa) influence, 1 blast/day for 3 days, on motor deficits and they did not find any deficits [163]. In my study, I have observed an acute and chronic motor deficit in rLLB. Elevated plus maze (EPM) is one of the known tests for assessing anxiety in the rodent model. As per earlier studies, a single mild-moderate blast induces acute and chronic anxiety in EPM [133, 137, 145, 164], whereas, a single low-level blast did not induce anxiety in animals [130, 131]. Interestingly, in the existing repeated blast model, animals exhibited anxiety at a chronic time only [142, 147].

2.2 Material and Methods

For this study, I used the control and injured group and conducted behavioral tasks (NOR, EPM, Rotarod) at different time points. Independent samples t-test with a confidence level of 0.05 (two-tailed) was used for statistical analysis. To design appropriately powered study, G*power 3.1.9.2 software was used for power analysis to determine sample size.

For NOR, using n=4 for control group and n=3 for the injured group, no significant difference in discrimination index was found between two groups at both PID 2 ($p=0.068$, effect size=1.734) and PID 25 ($p=0.0693$, effect size). Power analysis revealed a sample size of n=8 needed per group to obtain 80% power to achieve significant difference between two groups, if one exists.

For the Rotarod test, using n=4 for the control group and n=3 for the injured group, no significant difference in latency to fall was found between the two groups at PID 1 ($p=0.071$, effect size=2.121). But at PID 25, a significant difference in latency to fall was

observed (p=0.03, effect size=2.471) between the control and injured groups. Power analysis revealed a sample size of n=7 needed per group to obtain 80% power to detect such effect if one exists.

For EPM, using n=4 for the control group and n=3 for injured group, no significant difference in time spent in the closed arm was found between two groups at PID 1 (p=0.106, effect size=1.733). Power analysis revealed a sample size of n=8 needed per group to obtain 80% power to obtain the significant difference between two groups if one exists.

2.2.1 Animals

Adult male Sprague-Dawley rats (10 weeks old, 300-350 g, Charles River Laboratories) were used in this study. All animals were housed under standard animal room conditions (22^{o}C temperature, 40% humidity, and 12h dark-light cycle) with ad libitum access to food and water. All procedures followed the guidelines established in the Guide for the Care and Use of Laboratory Animals and were approved by the Rutgers University Institutional Animal Care and Use Committee, which monitors the vivarium facility for NJIT. Rats were divided into four different groups: Control groups; repeated blast (n=8) and single blast (n=6), blast injury groups: single blast (70 kPa, n=6, SB), (70 kPa x5, five successive times with a one-minute interval between exposures, n=8, rLLB). Here, control group of animals only get anesthesia (Isoflurane) along with injury group (rLLB/SB) and experienced sound generated by blast whereas injury group received blast exposures.

2.2.2 Rat model of repeated low-level blast

Rats were exposed to incident overpressures of 70 kPa using the 6-meter 23x23 cm cross-section shock tube that has been well validated for field- relevant blast exposures [9, 165-167]. Throughout the entire period of blast exposures, animals received a continuous

supply of 5% isoflurane into the exposure chamber through secured tubing with a hose to directly delivered to the animal's nostrils (**Figure 2.1**). Immediately after the blast exposure, animals from both groups were monitored for any signs of apnea and/or respiratory distress. Any animals that displayed these conditions were eliminated from the remainder of the experiment [168].

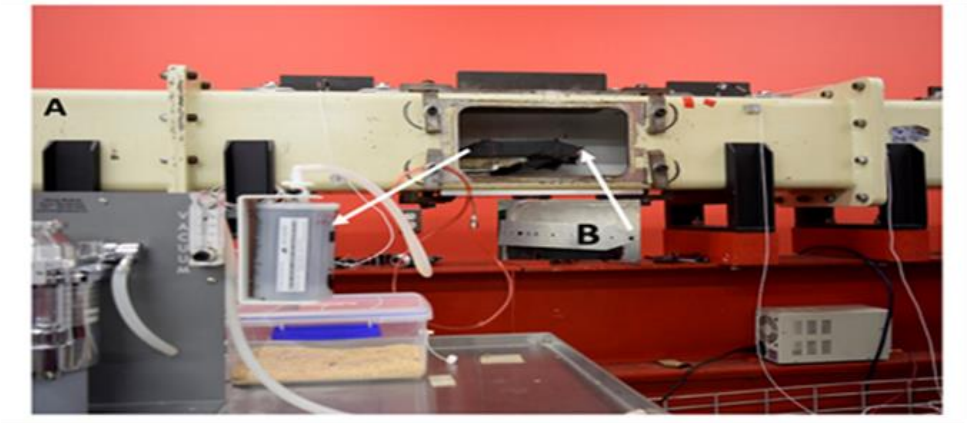

Figure 2.1 A The shock tube at NJIT. For repeated blasts within short interval (<5min), the surfboard (where rats are placed) is directly connected to anesthesia chamber by tubing (blunt arrow) and isoflurane is continuously supplied directly into the shock tube**. B.** The tip of the surfboard where the animal inhales the isoflurane (pointed arrow).

The blast conditions include the use of Ultra-high purity helium (99.99%) as the driver gas. The driver and driven sections of the shock tube were separated by a 1 mm thick Millar membrane which, upon bursting, developed pressure profiles with peak incident overpressures of 70 kPa. Incident pressure profiles were recorded at 1.0 MHz sampling frequency using PCB Piezotronics Model 132A24 (Depew, NY) sensors. Details of the shock tube and the experimental timeline are depicted in **Figure 2.2.**

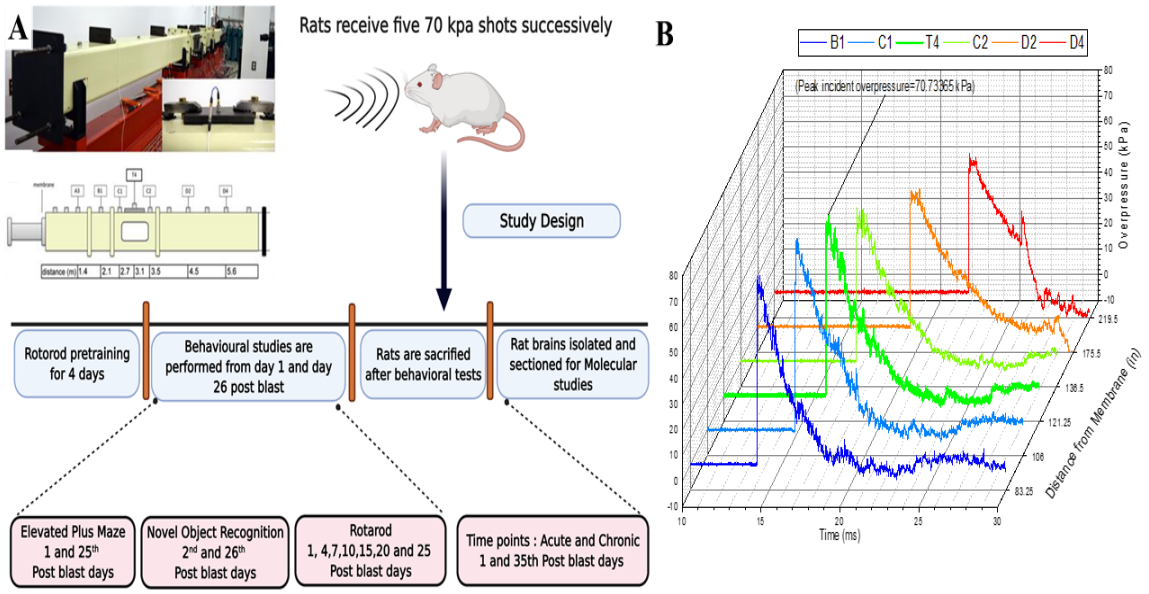

Figure 2.2 A. A schematic representation of the current study. The 9-inches square cross-section shock tube at NJIT facility and timeline of the study. **B.** Shock wave curve for five 70kPa successive blasts.

2.2.3 Neurobehavior studies

Animals subjected to sham, single, and repeated blast conditions were used for behavior tests, including elevated plus maze, rotarod test, and novel objective recognition test at different time points after injury.

2.2.3.1 Elevated plus maze (EPM)

The elevated plus maze (EPM) is a very convenient method to assess anxiety and is preferred by many investigators due to its dependence on the natural behavioral preference of the rat. It does not involve a trigger (acoustic or temperature change) nor a fearful stimulus (predator odor or foot shock) or any form of themotivated or conditioned response (levers and food rewards) for it to occur. The main natural behavior assessed is the rat's preference to stay in the dark areas (due to anxious) and its curiosity to explore novel areas when not experiencing anxiety.

Apparatus The Elevated plus maze is a plus-shaped apparatus with four arms: two open and two closed arms [169]. Our maze is made with matte black acrylic surface and consists of four arms (2 enclosed by 30 cm tall walls and 2 open arms with no walls). Each arm is 50 cm in length, a 10x10 cm center section separated 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 2.3**). 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 central region. All test videos were saved locally to the computer to allow for future review.

Figure 2.3 Picture of elevated plus maze apparatus used.

Protocol This test was used to determine anxiety at different time points, including as 1day and 25 days after blast exposure. On a test day, the rat will be transferred to a behavioral

room and acclimated for 30 min. Rats were placed at the center of the maze facing towards the opened arm and given 5min to explore the maze. Anxiety-like behavior was determined by calculating the time spent in closed arms in the maze. Animal movements in the maze were recorded using a video camera positioned over the maze, and data were analyzed in ANYMaze software.

2.2.3.2 Novel object recognition.

Memory is often accessed through spoken or written language in humans wheras, in animals, cognitive functions must be assessed through different kinds of behavioral experiments. Memory tests are important indexes of the brain functions for rodent's behavior. Many memory tasks require external forces (e.g. electric shocks) or intrinsic forces (e.g. hunger and thirst) to trigger the responses. Under those conditions, rodents are under stress, such as pain, tiredness, malnutrition, or dehydration, which potentially affect the natural neural responses. Novel object recognition is a non-force driving and spontaneous memory test. NOR task is used to evaluate cognition, particularly recognition memory, in rodent models of CNS disorders

In recent times, researchers are showing more interest to examine a relationship between novelty and behavior. The novel object recognition test can be evaluated by the differences in the exploration time of novel and familiar objects. This test is based on the spontaneous tendency of rodents to spend more time exploring a novel object than a familiar one. The choice to explore the novel object reflects the use of learning and recognition memory. Its application is not limited to a field of research and enables various issues can be studied, such as memory and learning and the preference for novelty.

To test human amnesia in an animal model, the ability to recognize a previously presented stimuli is the basis for the behavioral tests. For rodents the memory tests include, novel object recognition (NOR) test, delayed nonmatching to sample (DNMS), and the open field test (OFT). This test assesses how the animal responds to both a novel and familiar object [170]. The DNMS test rewards the animal upon recognition of the novel object, whereas the NOR test has no reward and is, therefor, able to assess the animals' index of stimulus recognition [171]. Depending on the configuration of the test NOR can be set to measure working and recognition memory [172]. The NOR test has also been used to determine the effectiveness of different pharmacological treatments for TBI [173].

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 [172]. It also can study short, intermediate, and long-term memory by modifying the time between the familiarization and testing phase [174, 175].

Apparatus The open field chamber consists of an empty open particle board box with dimensions of (60 x60 x 60 cm). The box was covered by black waterproof vinyl to prevent urine and other liquids from absorbing into the porous particleboard (**Figure 2.4**).

Figure 2.4 Picture of open field chamber including the novel and familiar object.

This test was used to measure short-term memory loss, specifically object recognition in single and repeated blast groups. Rats were assessed for cognitive decline at 2days (2D) and 26 days (26D) post-blast exposure [176].

Procedure Briefly, the NOR test consists of three phases; habituation, familiarization, and testing phase. In the habituation phase, each rat was allowed to acclimatize in the testing chamber for 5 mins 1day prior to the testing phase. I also minimized the learning behavior acquired by the animal for the same objects by changing familiar and novel objects for each time point, which will avoid habituation and potential bias towards only one object by the animals. On testing day, each rat was placed in the testing chamber with two identical objects to familiarize itself with objects for 5min (familiarization phase), then placed back in the housing cage for 1 hour. After an hour interval, each rat was returned to the testing chamber for 3 min, one object replaced with a novel one (Testing phase).

Scoring The total time spent exploring each of the two objects was measured using ANY Maze software. A discrimination index (DI) for the novel object was calculated as the difference between time spent with the novel object (tnovel) and time spent with the

familiar object (tfamiliar) divided by the total time (ttotal) with both objects, which give discrimination ability of the animal between novel and familiar object.

$$DI = [(tnovel - tfamiliar)/(ttotal)].$$

2.2.3.3 Rotarod test.

This test is used for assessing motor coordination, balance and motor learning in rodents. Animals must keep their balance on a rotating rod. It is measured the time (latency) it takes the mouse to fall off the rod rotating at different speeds or under continuous acceleration (e.g., from 4 to 40rpm). The latency to fall from a rotating rod is scored automatically using a rotarod apparatus (Touch screen Rotarod (Panlab, Harvard apparatus, **Figure 2.5**). Time is taken by the animal to fall (latency to fall) was recorded as a measure of motor performance [177]. The speed of the rotarod is mechanically driven and may either be held constant or accelerated. Motor coordination can be tested by comparing the latency to fall on the very first trial between treatment groups.

Before the blast, all groups of animals were trained, 3trials/day for 4days in an accelerated mode from 4-40 RPM over a five-minute session. After the blast, animals undergo rotarod test for 25 days at various intervals, and the latency to fall was calculated as the average of three successive trials.

Figure 2.5 Picture of Rotarod apparatus used to assess the motor deficits.

Procedure: Rota Rod test consists of two phases

1) **Pre-Training Phase:** All animals were trained for 4 days prior to blast exposure.

a) Day 1 (Familiarize the rat with the apparatus)

- *First exposure:* Let the rat stand on the Rotarod, while it is switched off for 30sec. If the rat jumps off, place it back on.

- *First trial:* Keep Rota rod on constant mode and set 7rpm as a constant speed. Now put the rat on a non-rotating drum for 5 secs then start. If rat runs for 3min without falling put rat back in cage if not, then put rat again on a rotating drum until it walks continuously 2min.

- *Second trial:* After 15 min break, now set 12rpm as a constant speed and repeat.

- *Third/fourth trials:* After 15min break, now switch Rota rod to acceleration mode with 4-40rpm in 90sec (2.5secs intervals for 1rpm increment) and repeat as described above.

b) Day 2

Three trials at 4-40rpm in 150sec (4.2sec intervals for 1rpm increment) with 15 minute breaks. Calculate average latency time and rpm for three trials.

c) Day 3: Three trials at 4-40rpm in 260 secs (7.2sec intervals for 1rpm increment) with 15 minute breaks. Calculate average latency time and rpm for three trials.

d) Day 4: Three trials at 4-40rpm in 600 secs (16 sec intervals for 1rpm increment) with 15 minute breaks. Calculate average latency time and rpm for three trials. Now mix and match all animals for control and blast groups before the blast.

2) **Testing Phase:** Three trials at 4-40rpm in 150sec (4.2sec intervals for 1rpm increment) with 15 minute breaks. Calculated mean +/- S.E.M of latency time for all trials for each day.

2.3 Statistical analysis

Statistical analysis on behavioral studies, immunofluorescence, and western blotting was performed using Graph Pad Prism 8.0.1 and with values expressed as mean ± SEM. Based on our preliminary studies, a confidence interval of 80% for power analyses, seeking a $p<0.05$ for a two tailed t-test when comparing groups, I can predict the sample size to avoid false positive results. Factorial ANOVA with Bonferroni correction was used for behavioral studies. Shapiro-Wilk and Levene's tests were performed to assess the normality of data distribution and homogeneity of variances, respectively. Mann-Whitney's test was used when the samples failed the Shapiro-Wilk test.

2.4 Results

2.4.1 rLLB induces acute and chronic anxiety-like symptoms

Analysis of each animal performance in EPM showed that animals exposed to rLLB felt anxious and spent more time in the closed arm ($p<0.05$) immediately after injury at day 1 (acute) and this exious behavior persisted for 25 days (chronic). Conversely, animals

exposed to SB did not display any anxiety-like symptoms as indicated by their normal exploratory behavior and time spent more in open arm area simar sham. Statistical analysis showed a significant difference between rLLB and sham animals at acute and chronic time points ($p<0.05$) post-blast period. This experiment supports the tenet that repeated low-level blast exerts a substantial influence on animals' composive behavior immediately as well as chronically over a period of time after the blast (**Figure 2.6**).

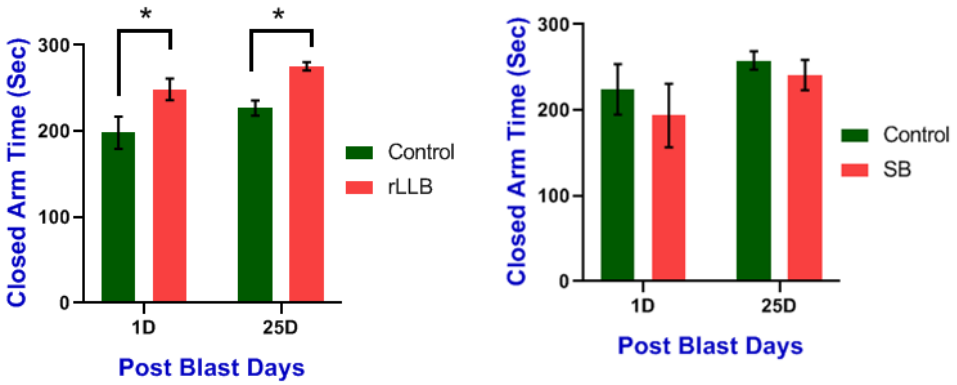

Figure 2.6 Anxiety/depression symptoms in rats exposed to rLLB**. Left panel:** Amount of time spent in the closed arm by animals exposed to rLLB showing a significant increase (* compared with sham, $p^*<0.05$). **Right panel:** Absence anxiety-like symptoms in animals exposed to single blast as indicated by time spent in closed arms similar to that of a sham. Data is mean ± S.E.M of 6-8 animals in each group, and the data analyzed by factorial ANOVA with Bonferroni's correction as post-hoc test.

2.4.2 Motor functions are altered in animals exposed to rLLB

Animals were assessed for motor performance using the rotarod test. Animals exposed to rLLB displayed persistently decreased motor performance initiated at 1-day post-injury and reached significance by 4-7 days ($p<0.05$) and displayed a non-significant recovery; however, such performance significantly deteriorated from 20-day ($p<0.05$) post-injury onwards. Sham control animals as well as animals exposed to single blast, on the other hand, displayed consistently normal endurance (**Figure 2.7**).

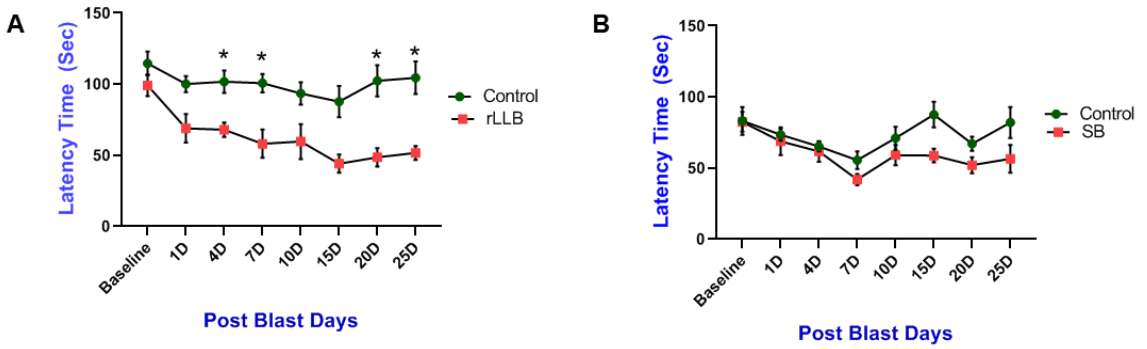

Figure 2.7 Motor incoordination in rats exposed to rLLB. Left panel: Latency to fall by animals exposed to rLLB showing a significant decreased latency time (* compared with sham, p*<0.05). Right panel: Animals exposed to single blast display normal latency time as that of controls showing longer endurance. Data is mean ± S.E.M of 6-8 animals in each group and the data analyzed by factorial ANOVA with Bonferroni's correction as post-hoc test.

2.4.3 Animals exposed to rLLB display short term memory loss

Novel objective recognition tests were performed 2days (2D), and 26days (26D) after bTBI to examine whether rLLB has any influence on short term memory impairments (**Figure 2.8**); persistent memory impairments were observed in animals exposed to rLLB at 2 ($p<0.05$) and 26 days ($p<0.01$) post-injury. The discrimination index showed that rats exposed to rLLB were unable to differentiate a novel object from a familiar object in the testing phase compared with the sham control rats.

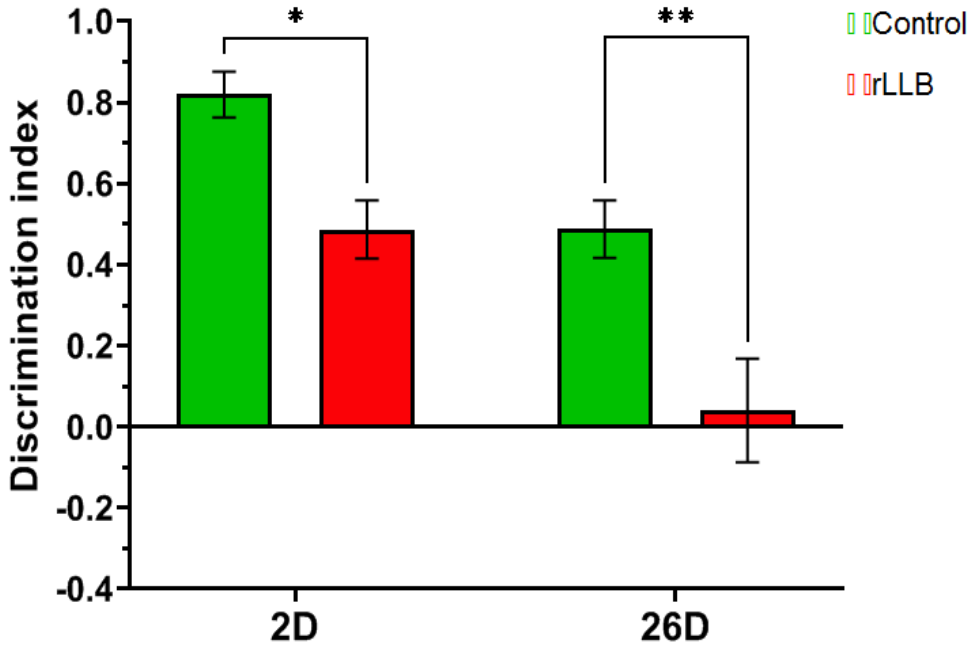

Figure 2.8 Animals exposed to rLLB display short-term memory loss. Discrimination index shows that animals with rLLB have significantly less (p*<0.05, p**<0.01) ability to recognize the novel object. Data is mean ± S.E.M of 8 animals in each group and the data analyzed by factorial ANOVA with Bonferroni's correction as post-hoc test.

2.5 Discussion

These experiments investigated the effects of the rLLB on acute and chronic neurobehavioral and neuropathological changes in our rat model. One of the highlights of this study was establishing a reliable model for rLLB akin to blast exposure paradigms that service members experience in various training facilities by using a BOP of ≤70 kPa at 1-minute intervals [13]. It is also noteworthy that, unlike repeated blasts using higher BOPs as noted in the previous paragraph, the few studies using BOPs around or < 70 kPa with longer intervals (24 hours or more) between blasts did not find any significant behavioral changes [147, 178, 179]. This invokes the possibility that a longer time interval gap between blasts (24 hours or more) could either mask any symptoms or induce compensatory mechanisms to counteract injury pathology. It is of importance here that our

current model adapting 5 successive blast exposures to animals instantaneously (within 5 min) did display significant neurobehavioral as well as neuropathological changes, reinforcing the fact that our model is a true representation of occupational rLLB wherein service members also experience milder symptoms such as headache, disorientation, fatigue as well as subtle memory impairments following training. Our model of rLLB showed a significant alteration in neurobehavioral functions as indicated by increased anxiety/depression-like symptoms, decreased motor performance, and loss of short-term memory loss compared with control and single low-level blast. Thus, rLLB although mild still able to produce injury in animals that leads to substantial alterations in neurobehavioral patterns.

Several studies have documented changes in neurobehavioral and neuropathological sequelae in moderate single blast induces acute and chronic behavioral deficits such as anxiety, memory impairments and conditions [180]. In contrast to single moderate blast, preclinical studies on rLLB are very few and do not represent a true rLLB. An initial study by Ahlers et al. noticed a transient memory impairment in the Morris water maze task when animals were exposed to 36.6 kPa repeatedly for 12 consecutive days with one-day intervals [135]. Likewise, using BOPs higher than 10 psi with longer inter-exposure intervals, studies have shown anxiety/depression, spatial memory loss and motor impairments [137, 181-186]. Unlike studies with higher BOPs, studies using BOPs around 70 kPa with longer intervals did not observe any significant behavioral changes and biochemical changes [142, 147, 163].

As a measure of anxiety like symptoms, this study included data on the increased amount of time animals spent in closed arms while the total distance traveled in the EPM

did not show changes between rLLB and control animals. Although the precise reason is not known, previous reports emphasize that time spent in either open arms or closed arms by the animals is considered true representation of anxiety-like behavior [130, 141, 164]. Further, our recent report also showed anxiety-like symptoms in animals exposed to single moderate blast as indicated by increased closed-arm time spent by the animal [176]. One possibility for the absence of changes in total distance traveled by the animal could be that blast did not affected the normal locomotor activity but rather aggravated the restrictive behavioral response of the animal towards moving into open fields. Such abnormality also often referred to as thigmotaxis, would have prevented the animal from withstanding light and height in the open field.

Although as noted above while blast exposure would not have impaired the normal locomotor activity as noted above data from the Rotarod test showed acute and chronic impairment in motor coordination which may pose a contradictory statement. However, it is possible that while normal locomotor functions are intact in blast injury, motor coordination might have been impaired. Supporting this, several studies reported peripheral auditory damage (hearing loss) and associated vestibular disturbances in both preclinical and in soldiers exposed to blast [187-190]. Additionally, any small acoustic disturbance during the Rotarod task would also have increased startle responses in these animals at risk for early fall from Rotarod [191]. In this study, the observation of significant changes in neurobehavioral patterns in rLLB but not a single low-level blast strongly indicates that milder BOPs when repeated, exert a cumulative detrimental effect over a period.

Overall the current studies have clearly shown that rLLB impacts the normal behavioral functioning of the animal over a period of time which to some extent has also

been observed in the inconsistent behavioral patterns of service members following the repeated use of heavy weaponry [192]. Thus, this aim demonstrated that the repetition of low-level blast causes long-term behavioral deficits as hypothesized. While neurobehavioral changes are prominent, rLLB also appears to induce neuropathological changes. Few molecular mechanisms that activate after blast injury and cause prolonged damage are oxidative stress and neuroinflammation. Oxidative damage and chronic neuroinflammation has been known to cause neurobehavioral changes in several neurological conditions including TBI [193-196]. Therefore it is highly likely that current observation of neurobehavioral changes could be in part mediated by heightened oxidative damage and/or neuroinflammation and astrogliosis. Hence, aim 2 investigates detailed molecular mechanisms that might contribute for secondary injuries and behavioral deficits following rLLB.

CHAPTER 3

CHARACTERIZATION OF ACUTE AND CHRONIC NEUROINFLAMMATION FOLLOWING rLLB

This chapter represents the second aim of the proposal that examined the influence of rLLB on chronic neuropathological changes in rats. Mainly focused on NADPH oxidase 1 mediated oxidative stress and microglial-NLRP3 mediated chronic neuroinflammation.

3.1 Rationale for Specific Aim 2

Oxidative stress is one of the central mechanisms that can contribute to secondary injury. Existing literature has works clearly established that acute and chronic oxidative stress follows bTBI. An increased ROS levels have been reported as early as 4 and 6 hours post-injury and were found to persist at 1, 7, 14, and 28 days following blast exposure [186]. [184], [143] and [197]. These oxidative stress-induced pathological changes were also accompanied by behavioral changes at 1 and 28 days post-injury. However, these studies only examined either ROS levels or 4HNE and 3NT (oxidative by-products) but not NOX mediated oxidative stress. In our lab, we have previously reported an elevation of several different oxidative stress markers including NOX1 and NOX 2 expression, 4-HNE and 3-NT, superoxide production levels as early as 4 hours in combination with BBB disruption which was reversed by apocynin treatment, NOX inhibitor, in a single moderate blast, [9, 32, 41, 198]. Despite these important findings regarding NOX expression in models of single moderate blast, there is lack of comparable understanding of NOX mediated chronic oxidative stress in rLLB.

Other studies have reported a chronic microglial activation and proinflammatory cytokine production following moderate bTBI. For example, Beamer et al. noticed an increase of microglial activation 10 days after a moderate blast [128]. Readnower et al. found microglial activation at days 5 and 10 after blast [199]. Similarly, Cho et al. observed the microglial activation at 1 day and 2 weeks after moderate blast [79]. Hernandez et al. even observed microglial activation 3 weeks after the moderate blast injury [200]. Ning et al. noticed a chronic IL-1β release 1, 4, 8 weeks after a moderate blast injury [201]. Sajja et al. observed microglial activation 3 months after the moderate blast injury [129]. Baalman et al. did not notice any microglial activation for 2 weeks after single low-level blast [127]. Elder et al. did not observe any microglial activation when the animals were exposed to three repeated low-level blasts with 24hrs of interval between blasts [163]. Major studies so far mainly focused on moderate blast induced microglial activation and neuroinflammation, there are very limited studies are reported in rLLB.

Besides tissue injury, one of the reasons for chronic microglial activation is pro-inflammatory cytokines (i.e., IL-1β, IL-18 and TNF-α), free radical generation, and chemokines. Earlier studies reported external administration of IL-1β resulted pathological and behavioral changes such as anxiety, recognition memory and motor deficits. One of the molecular mechanisms involved in releasing chronic IL-1β is the inflammasome complex particularly NLRP3 inflammasome. Earlier studies reported NLRP3 involvement in chronic microglial activation, pathological and behavioral changes in TBI [202-207]. Not many studies examined inflammasome complex (all isoforms) role in blast TBI except 1 study by Ma et al who found an increased NLRP3 expression and IL-1β release at 12 and 24hrs following bTBI [100]. Interestingly, no one yet attempted to understand NLRP3

inflammasome role in rLLB model. In addition to oxidative stress, in this aim this study examined a detailed molecular mechanism; NLRP3 inflammasome, that contributes to chronic neuroinflammation.

Thus, in aim 2 I focused on the effect of rLLB on NOX1-mediated oxidative stress and microglia-NLRP3 mediated chronic pro-inflammatory cytokine IL-1β release and neuroinflammation.

3.2 Material and Methods

For biochemical experiments, my study was based on the effect size calculations on prior studies from our lab and similar techniques; western blotting and immunofluorescence, to examine biochemical changes in; NADPH Oxidase 1 (NOX1), reactive astrocytosis and microglial activation in control and single moderate blast injury group. These studies showed a significant ($p<0.05$) change in NOX1 expression with n=3 per group in western blotting whereas, a significant ($p<0.05$) changes in NOX1 expression, reactive astrocytosis and microglial activation with n=4 per group in immunofluorescence studies.

Based on this, I have chosen n=3 per group to analyze desired protein expression changes in western blotting experiments and n=4 per group to analyze desired protein expression changes in immunofluorescence studies to design an adequately powered study with a power value of 0.8.

3.2.1 Animal Groups

Adult male Sprague-Dawley rats (10 weeks old, 300-350 g, Charles River Laboratories) were used in this study. All animals were housed under standard animal room conditions (22^{o}C temperature, 40% humidity, and 12h dark-light cycle) with ad libitum access to food and water. All procedures followed the guidelines established in the Guide for the Care

and Use of Laboratory Animals and were approved by the Rutgers University Institutional Animal Care and Use Committee, which monitors the vivarium facility for NJIT. Rats were divided into two different groups: Control groups and Repeated blast groups (Day1, Day 35).

3.2.2 Repeated Blast Exposure on Animals

Rats were anesthetized with isoflurane and exposed to incident overpressures of 70 kPa using the 6-meter 23x23 cm cross-section shock tube that has been well validated for field relevant blast exposures [9, 165-167]. In order to maintain continuous anesthesia to animals for the entire period of blast exposures, animals received a continuous supply of 5% isoflurane into the exposure chamber by means of a secured tubing with a hose, wherein the anesthesia is directly delivered to the animal's nostrils (**Figure 2.1**). Immediately after the blast exposure, animals from both groups were monitored for any signs of apnea and/or respiratory distress. Any animals that display this condition were eliminated from the remainder of the experiment [168].

3.2.3 Tissue collection and biochemical studies

Animals were sacrificed 35 days after the blast exposure. For the immunofluorescence studies, rats were anesthetized with a mixture of 10:1 ratio of ketamine and xylazine (10 mg/kg) administered via intraperitoneal injection and subjected to transcardial perfusion first with phosphate-buffered saline (PBS, pH 7.0) followed by 4% paraformaldehyde (PFA) and coronal brain tissue (hippocampus) sections (n=5, 20 µm thickness) were sliced using the Leica VT1000S vibratome. All sections analyzed were between -3.12 mm to -3.60 mm from Bregma and used for immunofluorescence staining as previously standardized and published by our lab [41].

3.2.4 Western blotting

In order to examine NOX-1, NLRP3, Cleaved Caspase 1, proteins levels in the hippocampus, I performed an immunoblot. Briefly, after perfusion with PBS, desired brains region was excised and homogenized in ice-cold condition using sonicator followed by centrifuged at 14,000x g at 4^0C. The protein concentration in the sample was estimated by the bicinchoninic acid method (Thermo Scientific, Rockford, IL). Subsequently, 10-20ug of protein per lane was loaded into 4-15% SDS-PAGE gradient gel (Bio-Rad). Protein separated according to their molecular size were transferred onto polyvinylidene difluoride (PVDF) membranes using a Turbo Protein transfer instrument (Bio-Rad). The membrane was blocked with 5% milk dissolved in Tris-Buffered saline containing 0.1% Tween-20 and incubated overnight at 4^0C with rabbit NOX-1 antibody, rabbit NLRP3 (NBP2-12446, 1:500) and Cleaved caspase-1 followed by secondary antibody. Bands were visualized using Western Pico Chemiluminescence Substrate (Thermo Scientific) on Chemi Doc Imaging (Bio-Rad). For densitometric quantification of western blots, the image was digitized using a Bio-Rad GS800 calibrated densitometer and analyzed with Bio-Rad quantity One software [41].

3.2.5 Immunofluorescence and microscopy

Immunofluorescence was performed using NADPH 1(NOX-1), GFAP, (Iba-1) and NLRP3 to assess oxidative damage, reactive astrocytosis, microglia-NLRP3 inflammasome mediated neuroinflammation respectively. After the blast in the hippocampus brain region. Briefly, fixed tissues were incubated overnight at 4^oC with respective primary antibodies to NOX-1, Iba-1, GFAP, NLRP3 (NBP2-67639, 1:100) (Table 1) in 2% donkey serum. Biotin-SP affinity pure donkey Anti-rabbit IgG was added and incubated for 1 hour to

increase sensitivity to the primary antibody NOX-1. For double immunofluorescence, the secondary antibody, Streptavidin Alexa fluor 594 conjugate (S32356) and donkey anti-goat Alexa Fluor 488 (A11055), were used for NOX-1 and GFAP, respectively. Donkey anti-goat Alexa Fluor 488 (A11055) alone was used in single immunofluorescence staining of Iba-1. The specificity of each antibody staining was validated by excluding each primary antibody (negative controls) and visualizing for any nonspecific fluorescence. 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 Area Quant software (Leica Biosystems) and expressed as average fluorescence intensity*X unit area [208].

Table 3.2 Source, Catalogue Number, and Dilution Factors of Antibodies used in Immunohistochemistry and Western Blot Analyses

Antibody	**Company**	**Catalog No**	**Dilution for IF**	**Dilution for WB**
Gt-Iba-1	Thermo fischer	PA5-18039	1:250	
Rb-NLRP3	NOVUS	NBP1-12446, NBP2-67639	1:100	1:500
Rb-NOX1	Sigma-Aldrich	SAB420097	1:400	1:1000
Ms-GFAP	Thermo Fischer	MA5-12023	1:400	
Rb-Cleaved Caspase-1	Thermo Fischer	PA5-99390		1:500

3.2.6 Cell counting and morphological analysis of microglia.

The total number of microglia were counted in four different regions of the hippocampus (medial and lateral CA1, CA3, and the Dentate gyrus. In each region of the hippocampus, 500^2 µm areas (4-5) were selected for microglial counting. The total number of microglia within the region of interest (ROI) was determined by the presence of Iba-1 positive cells

and expressed as the number of cell/unit areas. Further, different stages of microglia during their activation were identified morphologically. Resting microglia were identified by their small spherical cell body with several highly branched processes radiating in all directions, whereas activated microglia were distinguished from resting microglia by their larger and oblong cells soma with shorter and thicker processes, which would later transform into amoeboid shape reported earlier [209-211].

3.3 Statistical analysis

Statistical analysis on immunofluorescence and western blotting was performed using Graph Pad Prism 8.0.1 and with values expressed as mean ± SEM. Based on our preliminary studies, a confidence interval of 80% for power analyses, seeking a $p<0.05$ for a two-tailed t-test when comparing groups, we can predict the sample size to avoid false-positive results. One-way ANOVA and Student's t-tests was used for immunofluorescence and western blotting analysis. Shapiro-Wilk and Levene's tests was performed to assess the normality of data distribution and homogeneity of variances, respectively. Mann-Whitney's test was used when the samples failed the Shapiro-Wilk test.

3.4 Results

3.4.1 Animals exposed to rLLB display oxidative damage

Immunofluorescence analysis of the protein levels of superoxide-producing enzyme NADPH oxidase (NOX1) showed a strong tendency to increase as early as 1 day following the blast and showed a significant increase at 35 days post-injury ($p<0.05$). Additionally,

immunoblot analysis and subsequent quantitation of the protein bands of NOX1 also showed a similar increase ($p<0.05$) at identical time points (**Figure 3.1**).

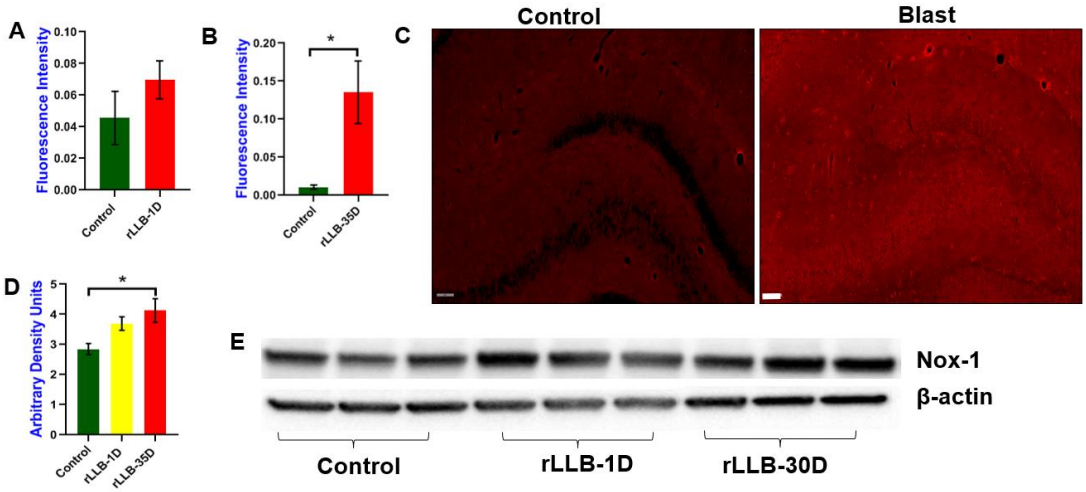

Figure 3.1 Representative images of NOX1 protein expression in control and animals exposed to rLLB. **A-C** control express less NOX1 expression whereas animals exposed to rLLB express significantly more NOX1 at 35 days ($p^*<0.05$, student's t test, n=5). **D-E** Immunoblot analysis also revealed similar pattern as immunofluorescence, there is a significantly increased NOX1 expression at 35 days ($p^*<0.05$, one way ANOVA, n=3). Data is mean ± S.E.M of 3-5 animals in each group, scale bar= 70um.

3.4.2 rLLB induces acute and chronic microglial activation

We found that exposure of animals to rLLB showed changes in microglial number as well as the activation state of microglia. At acute time point (1-day post-injury) the total number of microglia did not show any change, but the number significantly increased at 35 days ($p<0.0001$) whereas, the activated number increased significantly ($p<0.001$) in the acute state and such increase continued even at 35 days post-injury ($p<0.01$) as indicated by the presence of shortened processes and increase in the size of cell soma (**Figure 3.2** and **3.3**).

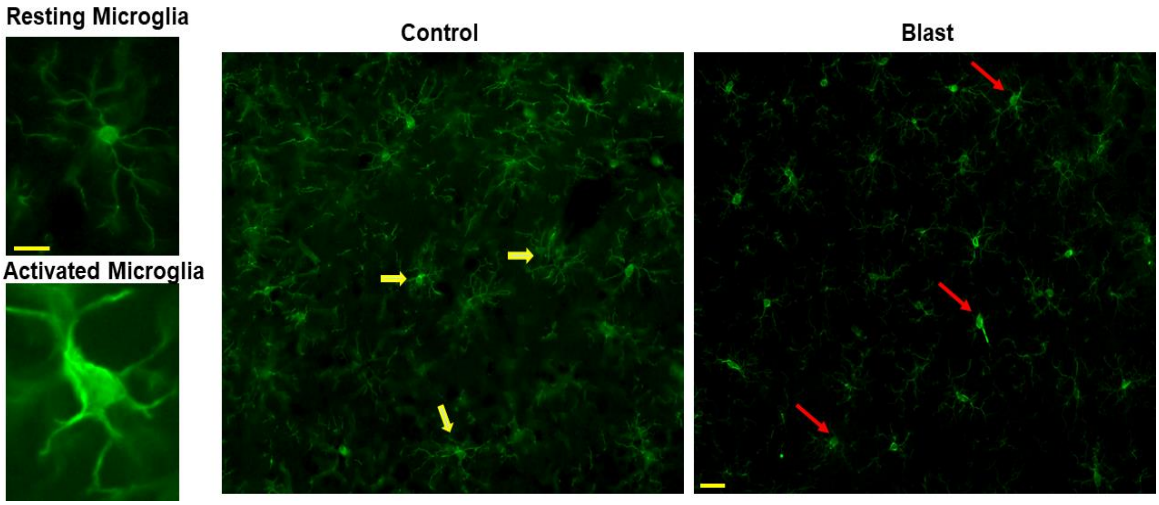

Figure 3.2 Representative images of the total number and activated form of microglia in animals exposed to rLLB. Control animals display a normal microglial morphology containing smaller cell soma and robust processes radiating in all the directions (indicated by yellow arrows), whereas animals exposed to rLLB display shortened processes with enlarged cell soma consistent with the activation. Left panel shows enlarged images of activated microglia in animals exposed to rLLB. Scale bar= 40 µm.

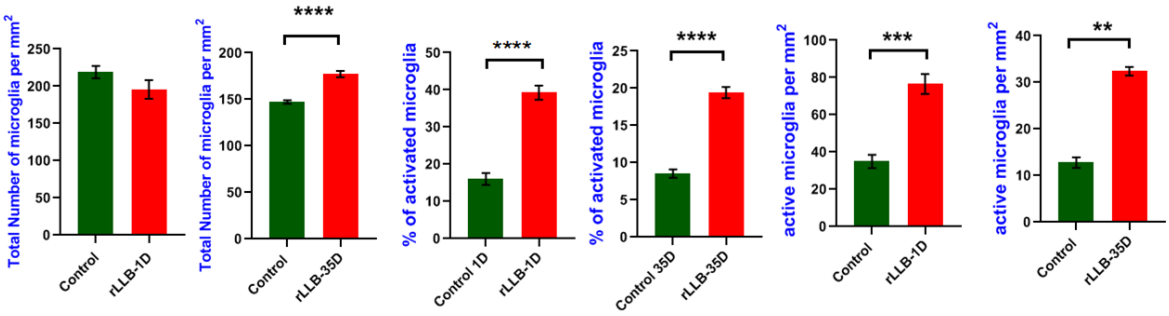

Figure 3.3 Quantitation of total and activated microglial number in control and animals exposed to rLLB. Note increased microglia number in rats exposed to rLLB only at 35 days post-blast but not acutely, suggesting that microglia proliferation is a chronic phenomenon. Unlike the total change in the number of microglial, the number of activated microglia significantly increased in both acute (24h) and chronic stages (35 days post-injury), strongly suggesting that the activation process is instantaneous following injury. Data is mean ± S.E.M of 5 animals in each group and the data analyzed by Student's t test; p****<0.0001, p***<0.0002, Mann Whitney test; p**<0.01.

3.4.3 rLLB increased acute and chronic nlrp3 proteins expression

Immunofluorescence analysis of the protein expression of NLRP3 showed a significant increase at day 1(p**<0.01) and an increasing trend at 35 days (p<0.056) following the blast injury (**Figure 3.4**). Similarly, immunoblot analysis and subsequent quantitation of the protein bands of NLRP3 also showed a significant increase (p<0.05) at 35 days post-injury (**Figure 3.5**).

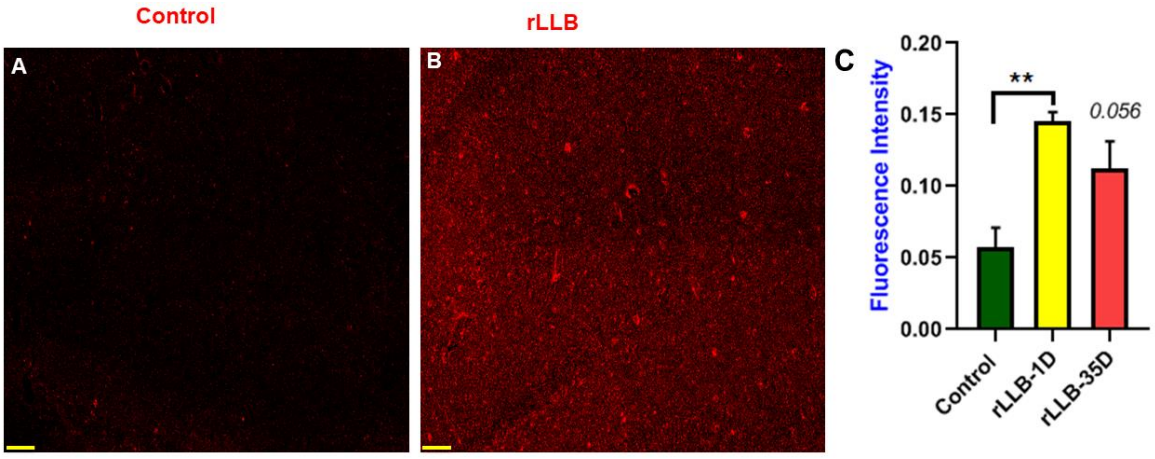

Figure 3.4 Chronic NLRP3 protein expression following repeated low-level blast in hippocampus regions. A-C represents fluorescence images of NLRP3, N=3 per group, one way ANOVA, **p<0.01. Scale bar of 70um.

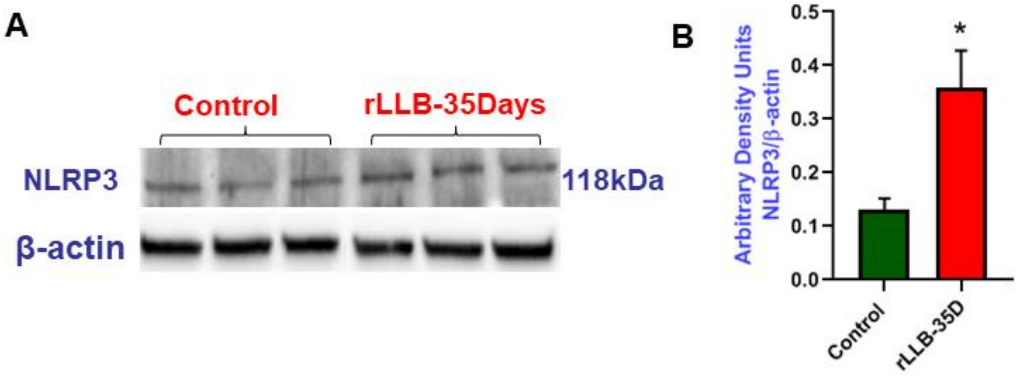

Figure 3.5 Chronic NLRP3 protein expression following repeated low-level blast in hippocampus regions. A, B represents western blot images and band intensity of NLRP3 protein, N=3 per group, Student T-test, *p<0.05.

3.4.4 rLLB induces acute and chronic reactive astrocytosis

To examine the impact of rLLB on astrocyte response, we stained the cells with GFAP (glial fibrillary acid protein), an astrocyte marker. We observed a strong trend of increase in GFAP intensity at 1 day post-injury time point, whereas such change was significant at the chronic time point (35 days post-injury, $p<0.05$)(**Figure 3.6**). These findings suggest that rLLB induces astrocytosis immediately after the injury, and these effects persist chronically.

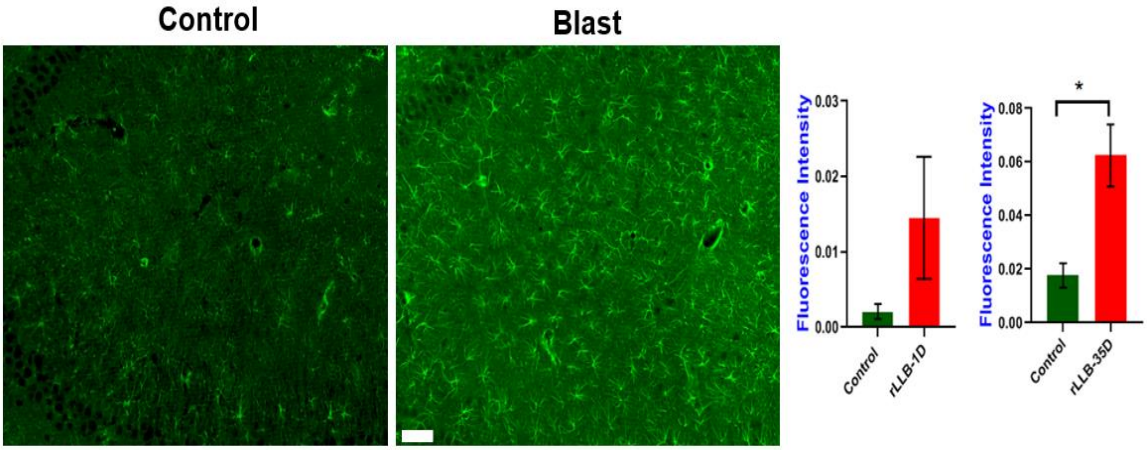

Figure 3.6 Representative images of the GFAP immunofluorescence in animals exposed to rLLB. **Left** Control animals display a normal GFAP immunoreactivity, whereas animals exposed to rLLB display a robust increase of GFAP immunoreactivity at 35 days post-injury, suggesting that chronic blast induces reactive astrocytosis. **Right** Quantitation GFAP immunofluorescence. Data is mean ± S.E.M of 5 animals in each group and the data analyzed by Student's t test; $p^*<0.05$. Scale bar= 70 μm.

3.5 Discussion

This study aimed to investigate the effects of acute rLLB exposure on chronic behavioral and biochemical changes in response to the activation of secondary injury mechanisms namely neuroinflammation and oxidative stress. Earlier studies suggested that mild to moderate blast exposure can trigger secondary injury mechanisms such as oxidative stress and neuroinflammation via glial activation and BBB disruption [31, 35, 212]. Since previous aim explored the role of rLLB in these neurobehavioral deficits, I examined the influence of rLLB related neuropathological events in the hippocampus due to its involvement not only in memory consolidation but also in the regulation of motor coordination and control of anxiety-related behavioral functions [213-215]. Also studies observed the hippocampus has higher propensity for early metabolic compromise [216], oxidative damage [41, 217] following different BOPs (low-moderate) compared to any other brain regions including frontal cortex, striatum, thalamus, cerebellum and basal ganglia [218-220].

Oxidative stress is one of the known secondary injury mechanisms that evolves after blast exposure due to increased ROS production by NOX1 and 2 in neurons, microglia, and astrocytes [47]. In this study, I examined NOX1 expression at 24 hours and 35 days post-blast in the rat hippocampus and observed an increase in NOX1 at both acute and chronic time points. Earlier studies have reported a relationship between BBB disruption and ROS generation via endothelial NOX1 following single moderate blast [20, 31, 221]. We previously reported increased BBB disruption in the presence of increased endothelial NOX1 expression which was reversed by administration of the NOX inhibitor apocynin [21]. Besides endothelial cells, we also observed a significant increase in NOX1

expression in neurons, microglia, and astrocytes following single moderate blast [41]. Additionally, in the present study, I observed chronic short-term memory deficits in a hippocampal associated NOR task accompanied by NOX1 elevation in the hippocampus at 35 days post-injury. Similarly, other rodent studies also observed behavioral deficits such as cognitive impairments, anxiety, and motor deficits in conjunction with an elevation in NOX1 expression [222-224].

Microglial mediated chronic neuroinflammation is known secondary injury mechanisms following TBI. Microglial activation results in the production of pro-inflammatory cytokines such as TNF-α, IL-1β, IL-6, ROS and iNOS that can cause chronic neuroinflammation and neuronal death [225]. Activated microglia express various receptors for glutamate, ATP, growth factors and cytokines to receive signals from other microglia as well as neighboring neurons and astrocytes [226]. Recently, we also reported microglial activation both at acute (4 hours) and chronic (30 days) time points after single moderate blast [227]. Similarly, several studies also observed acute [79] and chronic microglial activation following single moderate blast [31]. Also, in this study I observed acute and chronic neuroinflammation along with behavioral deficits such as anxiety, motor deficits and short-term memory impairments [176]. Similarly, other studies also observed a relationship between neuroinflammation and behavioral deficits after single mild to moderate blast [79, 126, 228].

However, similar experiments have not been conducted in rodent models of rLLB. Iba-1 staining revealed a significant increase in the total number (including both resting and active forms) of microglia, percentage of activated microglia, and number of activated microglia in the hippocampus at a chronic time point. These findings suggest that rLLB

might induce sustained microglial activation, resulting in chronic neuroinflammation. Oxidative stress and inflammation are linked such that if one mechanism is initiated, it will trigger the other as well [229]. I observed a significant increase in NOX1 and microglial activation at both acute and chronic time points, showing that both are activated immediately after injury but remain dysregulated for an extended period. NOX not only generates superoxide and ROS but also induces microglial activation and neuroinflammation [42, 230, 231].

Chronic neuroinflammation due to sustained microglial activation is a frequent observation in bTBI [79, 80]. One of the key players contributing to chronic inflammation is the inflammasome. Of the various inflammasome complexes, NLRP3 has been the most due to its reception for a wide range of stimuli including ROS, ATP, amyloid β, mitochondrial dysfunction, and cellular damage [81-84]. In response to pathogens or endogenous signaling (i.e., TBI), NLRP3 becomes activated and assembles the inflammasome complex [85], leading to the production and release of active caspase-1 and proinflammatory cytokines (e.g., IL-1β and IL-18). IL-1β then activates neighboring glial cells to cause chronic glial activation and neuroinflammation and interacts with neurons to disturb neuronal function. IL-1β is majorly produced by NLRP3-mediated caspase-1 activation.

Here, I demonstrated an increase in NLRP3 expression in the hippocampus at both 24 hours and 35 days post-blast in a rat model of rLLB. There is an established relationship between NLRP3 expression and detrimental behavioral and pathological changes at acute and chronic time points in other rat models of TBI. For instance, Zou et al. observed a significant increase in ROS production and cytokine release along with increased NLRP3

expression following weight drop TBI [232]. Similarly, Zheng et al. observed increased NLRP3 expression, microglial activation, neuronal loss, and spatial memory impairments after injury in a CCI model [233]. In another CCI study, Chao et al. noted behavioral changes in MWM and beam balance along with increased NLRP3 protein expression [234]. Thus, our findings support earlier works on the relationship between NLRP3 expression and behavioral changes.

Besides microglia, astrocytes are also involved in neuroinflammation after brain injury. Astrocyte cells are long, star-like projections that make up approximately 90% of the human brain with the ratio of astrocytes per neuron depending on the brain's size and complexity. Astrocytes play an important role in the formation of the BBB and also protect against neuronal excitotoxicity by monitoring extracellular glutamate levels [52]. Astrocytes quickly activate within 24 hours after injury and sustained this activation for longer period [235]. Immediately after the injury, astrocytes play a protective role by forming a glial scar within the brain, which isolates damaged areas and prevents the spread of inflammatory cells to surrounding cells. However, prolonged activation of astrocytes results in neuronal damage [54]. In this study, I examined reactive astrocytosis/astrogliosis using the GFAP antibody, a known astrocyte marker, and observed chronic activation of astrocytes in the hippocampus even after 35 days. Earlier studies reported astrocytes activation as early as 24 to [236] 72 hours [126] and lasting for 6-8 months after blast exposures [237]. Therefore, our results suggest there is a sustained activation of astrocytes following rLLB.

In summary, rLLB caused an increase in NOX1 enzyme expression, reactive astrocytosis and microglial activation at acute and chronic time points. Additionally, rLLB caused an increase in NLRP3 protein expression in the hippocampus at acute and chronic times. Our findings support data from existing literature suggesting that rLLB induces persistent chronic oxidative stress, NLRP3 protein expression, and glial activation, which may lead to behavioral deficits following rLLB. To understand the relationship between NLRP3 expression and behavioral changes in rLLB, this study further examined the role of selective NLRP3 inhibitors in mitigating chronic inflammation and improving behavioral deficits.

CHAPTER 4

ADMINISTRATION OF SPECIFIC NLRP3 INHIBITOR PROMOTES NEUROPROTECTION AND NEUROBEHAVIORAL IMPROVEMENTS FOLLOWING rLLB

4.1 Rationale for Specific Aim 3

Earlier studies reported microglial activation and cytokine release especially release of IL-1β, Il-18 and TNF-α following bTBI. One of the mechanisms involved in cytokines release in activated microglial cells are inflammasome complexes. NLRP3 is the most studied inflammasome complex and plays a major role in microglia in IL-1 β release. Chen et al. observed an increase in NLRP3 protein expression as early as 6hours and continued until 7 days and an increase in IL-1β in a weight drop model of TBI and this effect was mitigated with MCC950 [238]. Also, the same group again observed an increased expression of NLRP3 inflammasome complex after neonatal hypoxic-ischemic brain injury accompanied by behavioral changes for motor (Rotarod) and memory deficits (MWM) [239]. Irrera et al. noticed a faster recovery in object recognition memory and IL-1β release in mouse model of TBI due to a reduction in NLRP3 inflammasome complex expression [240].

Lin et al. observed an increase in NLRP3 activation, IL-1β release and behavioral changes in a CCI model [234]. Zheng et al. observed an increase in NLRP3 activation and microglial activation in hippocampus and behavioral changes in CCI model [233]. Yi et al. observed an increase in NLRP3 expression and IL-1β release as early as 1 day and continuing for 7 days post-injury in the weight drop model [241]. Xin et al. observed an increase in NLRP3 inflammation as well as chronic neuroinflammation and long-term

neurological changes following TBI in a CCI model and these effects were minimized by treatment with MCC950 [115]. Thus, although effect of NLRP3 and its inhibitors have been studied in various models of TBI and other neurodegenerative disorders there have been only a few studies in blast TBI and none in rLLB.

4.2 Material and Methods

4.2.1 Animal groups

Adult male Sprague-Dawley rats (10 weeks old, 300-350 g, Charles River Laboratories) were used in this study. All animals were housed under standard animal room conditions (22^{o}C temperature, 40% humidity, and 12h dark-light cycle) with ad libitum access to food and water. 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, which monitors the vivarium facility for NJIT.

Rats were divided into three groups: Control groups+PBS, rLLB+PBS, rLLB+MCC950, n=3-8 rats/group/task (based on Aim 1 and 2 data). MCC950 was obtained from Medchem Express LLC (Monmouth Jn,NJ). The drug is dissolvable in PBS, as per earlier studies and was administered 10mg/kg intraperitoneally to the rats. The first dose was 30 min post-blast and the remaining dose were given once in every 48h until the last day of experiments. MCC950 has beeen found to bind directly to NACHT domain of NLRP3 and prevents ATPase activity which fails NLRP3 inflammasome complex activation [101].

4.2.2 Repeated blast exposure on animals

Rats were anesthetized with isoflurane and exposed to incident overpressures of 70 kPa using the 6-meter 23x23 cm cross-section shock tube that has been well validated for field relevant blast exposures [9, 165-167]. In order to maintain continuous anesthesia to animals for the entire period of blast exposures, animals received a continuous supply of 5% isoflurane into the exposure chamber by means of a secured tubing with a hose, wherein the anesthesia is directly delivered to the animal's nostrils (**Figure 2.1**). Immediately after the blast exposure, animals from both groups were monitored for any signs of apnea and/or respiratory distress. Any animal that displays this condition was eliminated from the remainder of the experiments [168].

4.2.3 Novel object recognition

This test was used to measure short-term memory loss, specifically object recognition in single and repeated blast groups. Rats were assessed for cognitive decline at 2days (2D) and 26 days (26D) post-blast exposure [176]. Briefly, the NOR test consists of three phases; habituation, familiarization, and testing phase. In the habituation phase, each rat was allowed to acclimatize in the testing chamber for 5 mins 1day prior to the testing phase. I also minimized the learning behavior acquired by the animal for the same objects by changing familiar and novel objects for each time point, which will avoid habituation and potential bias towards only one object by the animals. On testing day, each rat was placed in the testing chamber with two identical objects to familiarize itself with objects for 5min (familiarization phase), then placed back in the housing cage for 1 hour. After an hour interval, each rat was returned to the testing chamber for 3 min, one object replaced with a novel one (Testing phase).

The total time spent exploring each of the two objects was measured using ANY Maze software. A discrimination index (DI) for the novel object was calculated as the difference between time spent with the novel object (tnovel) and time spent with the familiar object (tfamiliar) divided by the total time (ttotal) with both objects, which give discrimination ability of the animal between novel and familiar object.

$$DI= [(tnovel-tfamiliar)/(ttotal)]$$

4.2.4 Tissue collection and biochemical studies

Animals were sacrificed 30 days after the blast exposure. For the immunofluorescence studies, rats were anesthetized with a mixture of 10:1 ratio of ketamine and xylazine (10 mg/kg) administered via intraperitoneal injection and subjected to transcardial perfusion first with phosphate-buffered saline (PBS, pH 7.0) followed by 4% paraformaldehyde (PFA) and coronal brain tissue (hippocampus, perirhinal) sections (n=5, 20 µm thickness) were sliced using the Leica VT1000S vibratome. All sections analyzed were between -3.12 mm to -3.60 mm from Bregma and used for immunofluorescence staining as previously standardized and published by us [41].

4.2.5 Western blotting

In order to examine NLRP3, Caspase-1 (and active) proteins levels in the hippocampus, I performed an immunoblot. Briefly, after perfusion with PBS, desired brains region was excised and homogenized in ice-cold condition using sonicator followed by centrifuged at 14,000xg at 4^0C. The protein concentration in the sample was estimated by the bicinchoninic acid method (Thermo Scientific, Rockford, IL). Subsequently, 10-20ug of protein per lane was loaded into 4-15% SDS-PAGE gradient gel (Bio-Rad). Protein separated according to their molecular size were transferred onto polyvinylidene difluoride

(PVDF) membranes using a Turbo Protein transfer instrument (Bio-Rad). The membrane was blocked with 5% milk dissolved in Tris-Buffered saline containing 0.1% Tween-20 and incubated overnight at 4^0C with rabbit NLRP3 (NBP1-12446, 1:500) and Caspase-1 (cleaved caspase-1, p20) antibodies followed by secondary antibody. The concentration of the primary antibodies is listed in table 2. Bands were visualized using Western Pico Chemiluminescence Substrate (Thermo Scientific) on Chemi Doc Imaging (Bio-Rad). For densitometric quantification of western blots, the image was digitized using a Bio-Rad GS800 calibrated densitometer and analyzed with Bio-Rad quantity One software [41].

4.2.6 NLRP3+ microglia cell counting

Double Immunofluorescence was performed using NLRP3 and Iba-1 antibodies to assess NLRP3-microglia-mediated neuroinflammation in the hippocampus; CA1, CA3 and DG and peripheral (PrH) cortex. All sections analyzed were between -3.12 mm to -3.60 mm from Bregma. Briefly, fixed tissues were incubated overnight at 4^oC with respective primary antibodies to Iba-1 (goat polyclonal, PA5-18039,1:250), rabbit NLRP3 antibody (Novus-NBP1-67639, 1:100) in 2% donkey serum. For double immunofluorescence, the secondary antibody, donkey anti-rabbit Alexa Fluor 594 and donkey anti-goat Alexa Fluor 488 (A11055), were used for NLRP3 and Iba-1, respectively. The specificity of each antibody staining was validated by excluding each primary antibody (negative controls) and visualizing for any nonspecific fluorescence. 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 Area Quant software (Leica Biosystems) and expressed as average fluorescence intensity*X unit area [208].

NLRP3+ microglia were identified using an automated batch processing macro in cellSens. Colocalization analysis was first performed to generate a separate channel displaying colocalized pixels which were positive for both Iba1 and Nlrp3. The channel containing only Iba1/NLRP3 colocalized pixels was then overlaid on the Iba1 channel to create an image that highlighted Nlrp3+ microglia for consistent counting. Microglia containing colocalized pixels in the soma were manually counted in ImageJ. Regions of interest (ROIs) were outlined using the polygon tool to define CA1, CA3, DG, and Prh in each image. Counts were normalized to each ROI area and reported as Nlrp3+ microglia per mm^2 for each region. Normalized counts within each region in an image were averaged by sample, and sample averages were used to analyze counts by region per group.

Table 4.2 Source, Catalogue Number, and Dilution Factors of Antibodies used in Immunohistochemistry and Western Blot Analyses

Antibody	**Company**	**Catalog No**	**Dilution for IF**	**Dilution for WB**
Gt-Iba-1	Thermo Fischer	PA5-18039	1:250	
Rb-NLRP3	NOVUS	NBP1-12446 NBP2-67639	1:100	1:500
Rb-Caspase-1	Thermo Fischer	PA5-99390		1:500

4.2.7 Microglia morphological analysis or skeleton analysis

Microglia manifest different morphology based on brain homeostasis; mainly resting and activated form. Resting microglia were identified by their small spherical cell body with several highly branched processes radiating in all directions, whereas activated microglia were distinguished from resting microglia by their larger and oblong cells soma with

shorter and thicker processes, which would later transform into amoeboid shape reported earlier [209-211]. In this study, mean process length per cell and number of processes per cell were measured to determine microglial activation after rLLB exposure.

Images for cell counting and microglial morphology were acquired with a Fluoview 3000 laser scanning confocal microscope (Olympus, Waltham, MA). Regions of interest were selected on a 1.25x stitched map of the entire slide showing only the nuclear staining. Multichannel Z stacks were collected by multi-area time lapse under a 20x objective with consistent light and detection parameters using 405, 488, 561, and 640 diode lasers. All sections analyzed were between -3.12 mm to -3.60 mm from Bregma.

Automated analysis for cell counting and intensity measurements was conducted using the cellSens Dimension Count & Measure module (version 3.2, Olympus). An adaptive thresholding algorithm was applied to the whole frame for each channel to detect objects compared to the background, and noise was removed using an object filter by area. Class measurements for images of the same region in both hemispheres were averaged by sample. Sample averages were then used to analyze cell counts per region for each group. All counts were normalized by area and reported as number of microglia per mm2.

Morphological changes in microglia were quantified automatically by batch processing all images in ImageJ. Maximum Z projections were generated from multichannel Z stacks for each image and then separated into images containing individual channels. Only images containing Iba1 staining (488 nm) were processed for morphology. All steps were carried out iteratively for each image using a macro. First, the contrast was enhanced by to improve the Iba1 signal against the background. Automated thresholding was performed using the built-in Triangle algorithm to create a binary image [242]. Objects

were then skeletonized using the ImageJ Skeleton plugin, and any objects that were too small to be analyzed were removed using the Particle Remover plugin. Skeletons and branches were measured using the Analyze Skeleton plugin to the record number of branches and mean branch length per cell in each image [243]. This procedure has been previously shown to identify reactive microglial morphology [244-246]. Total process length was calculated per cell by multiplying the number of branches by the mean branch length. Overall mean process length for images of the same region in both hemispheres were averaged by sample. Sample averages were then used to analyze morphology by region for each group.

4.2.8 Enzyme-linked immunosorbent assay (ELISA)

Pro-inflammatory cytokine IL-1β levels in the brain tissue were estimated by ELISA. The homogenized hippocampus sample was 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 Spectra Max i3 (Molecular Devices) microplate reader and analyzed using SoftMax Pro 6.5 software.

4.3 Statistical Analysis

Statistical analysis on immunofluorescence and western blotting was performed using Graph Pad Prism 8.0.1 and with values expressed as mean ± SEM. Factorial ANOVA with a Tukey HSD post hoc test was used for behavioral studies whereas, one-way ANOVA with a Tukey HSD post hoc test was used for immunofluorescence and western blotting analysis. Shapiro-Wilk and Levene's tests were performed to assess the normality of data distribution and homogeneity of variances, respectively.

4.4 Results

4.4.1 MCC950 mitigated rllb induced acute and chronic short term memory deficits

The behavioral task that was examined to check MCC950 effects on behavioral outcomes was NOR test**.** This task uses the preference of the rat to novel objects over familiar objects to determine the effect of rLLB on short term recognition memory. rLLB group of rats preferences for novel objects was significantly decreased at 1D ($p^{*}<0.05$) and 30D ($p^{***}<0.001$) compared to control group of rats. MCC950 treated rats showed preference to novel objects almost similar to control ($p^{*}<0.05$) at 1D whereas a trend at 30D ($p<0.09$) compared to the injured group (**Figure 4.1**).

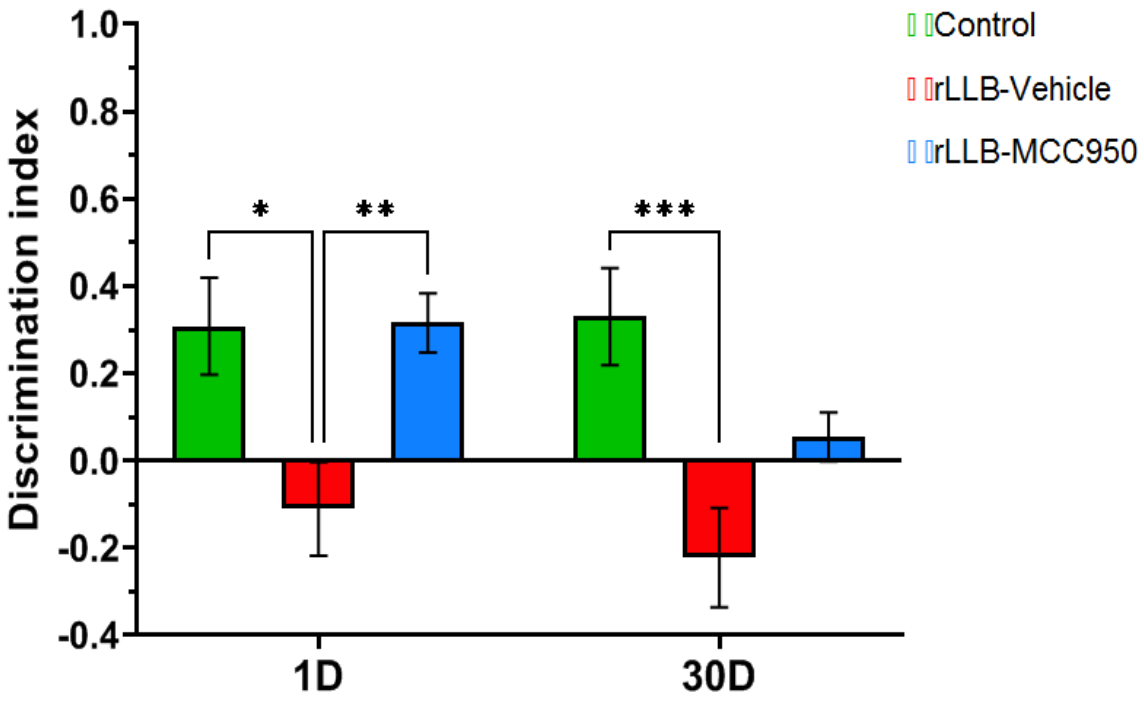

Figure 4.1 MCC950 treatment improved rLLB induced short term memory deficits. Discrimination index shows that animals with rLLB have significantly less (p*<0.05, p***<0.001) ability to recognize the novel object whereas, MCC950 administration improved (p*<0.05). Data is mean ± S.E.M of 8 animals in each group and the data analyzed by factorial ANOVA with a tukey HSD as post-hoc test p**<0.01, p***<0.001.

4.4.2 MCC950 decreased rLLB induced chronic microglial activation

Our earlier works revealed an acute and chronic microglial activation [168] following rLLB. This study examined MCC950 influence on chronic microglial activation. This study mainly evaluated microglial activation based on the number of processes per cell and mean process length in hippocampus; CA1,CA3,DG and perirhinal cortex (PrH) as both are involved in object recognition memory. rLLB showed significant decreases in processess (p*<0.05, p**<0.01, p***<0.001) as well as mean process length (p*<0.05, p**<0.01, p***<0.001) per microglia cell due to microglial activation in hippocampus and perirhinal cortex compared to control whereas MCC950 treatment significantly prevented microglial activation in both brain regions (**Figure 4.2-4.4**).

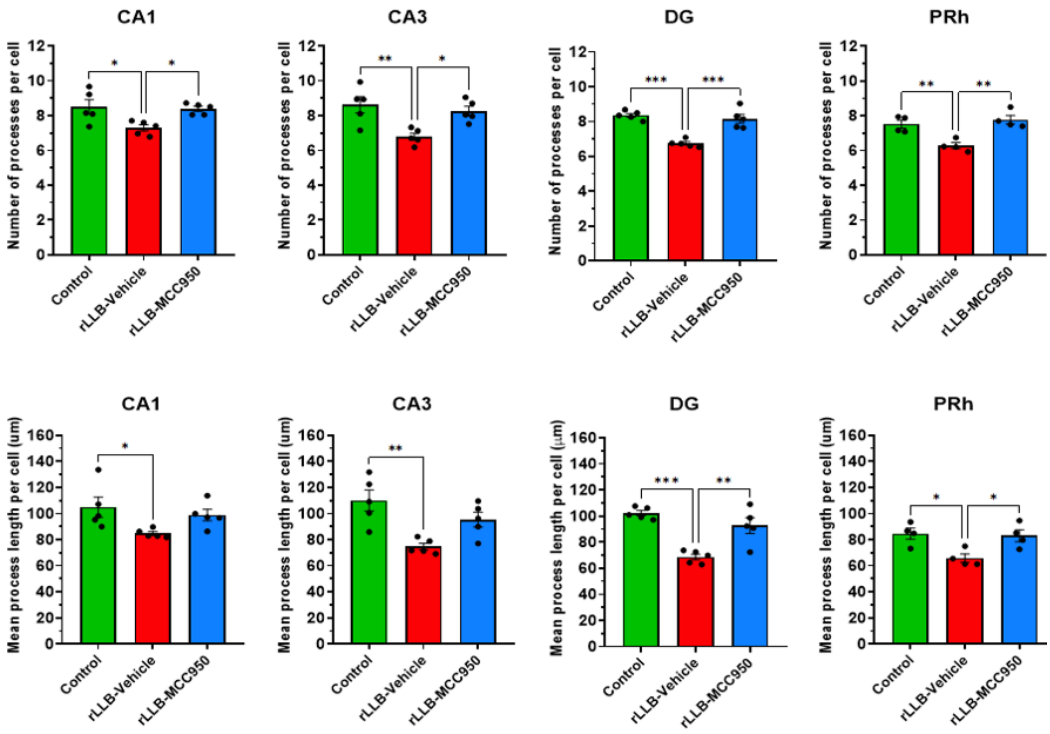

Figure 4.2 MCC950 suppressed rLLB Induced chronic microglial activation. Graphs represents mean process length and number of processes of microglial cell in hippocampus and perirhinal cortex at day 30 in control, treatment (rLLB+MCC950) and rLLB groups (n=4-5), one-way ANOVA, p*<0.05, p**<0.01, p***<0.001 Mean ± SEM.

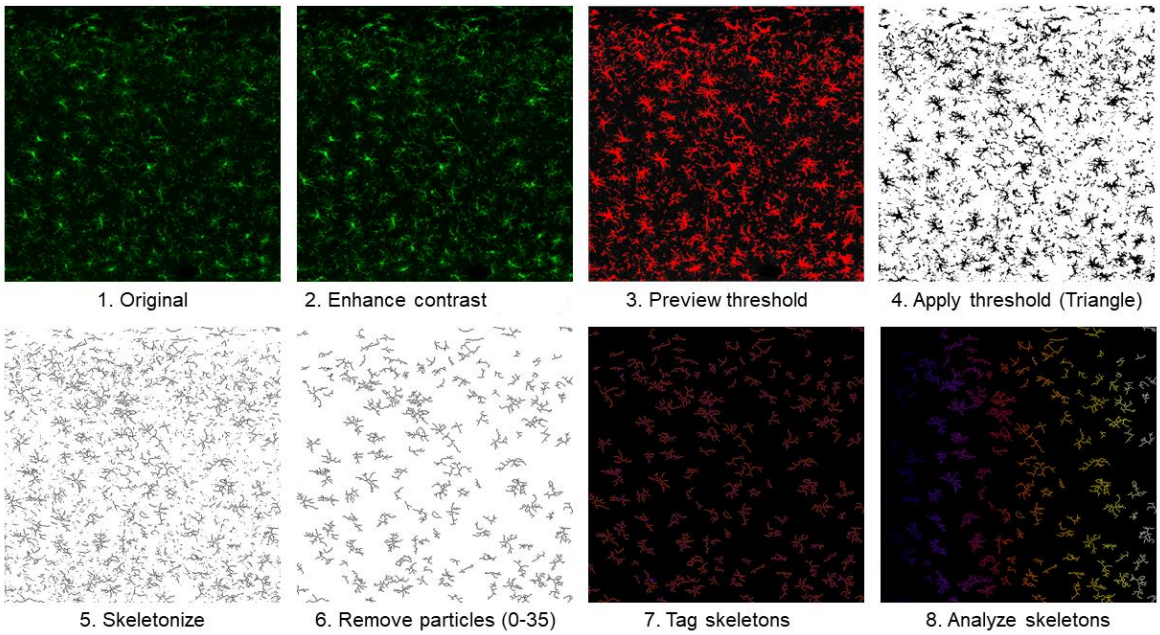

Figure 4.3 Step by step process of microglia skeleton analysis performed using ImageJ.

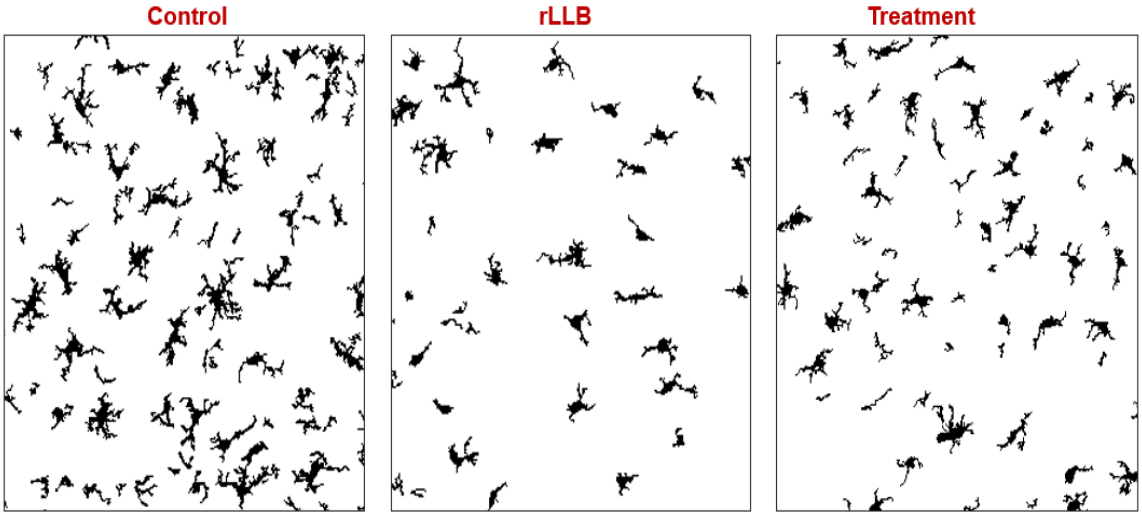

Figure 4.4 Representative images of microglia morphology (number of processes and processes length) in control, rLLB and MCC950 treated groups. Microglia cells in control group has a greater number of processes per cell in all direction and mean process length per cell whereas, injured group has a smaller number of processes per cell and mean process length per cell.

4.4.3 MCC950 decreased rLLB induced chronic microglial activation and nlrp3 protein expression.

Similar to our earlier work, there is a significant increase in microglial activation following rLLB at chronic time points**.** This study also examined NLRP3 expression in microglia as our hypothesis is mainly based on microglia-NLRP3 mediated pro-inflammatory cytokine IL-1β release and behavioral deficits. So double immunofluorescence was performed on; Iba-1+NLRP3 to examine NLRP3 positive microglial cells in hippocampus and perirhinal cortex at chronic time point. There is a significant increase ($p^{*}<0.05$, $p^{**}<0.01$, $p^{***}<0.001$) in total microglial cells (like our earlier work) in hippocampus and a trend in perirhinal cortex whereas, MCC950 treatment decrease total microglial number. Furthermore, I observed a significant increase in NLRP3+ microglial cells ($p^{*}<0.05$,

p**<0.01, p***<0.001) in injured group compared with control. MCC950 treatment significantly decreases NLRP3 expression in microglia (p***<0.001) compared to the injured group in hippocampus and perirhinal cortex (**Figure 4.5-4.9**)

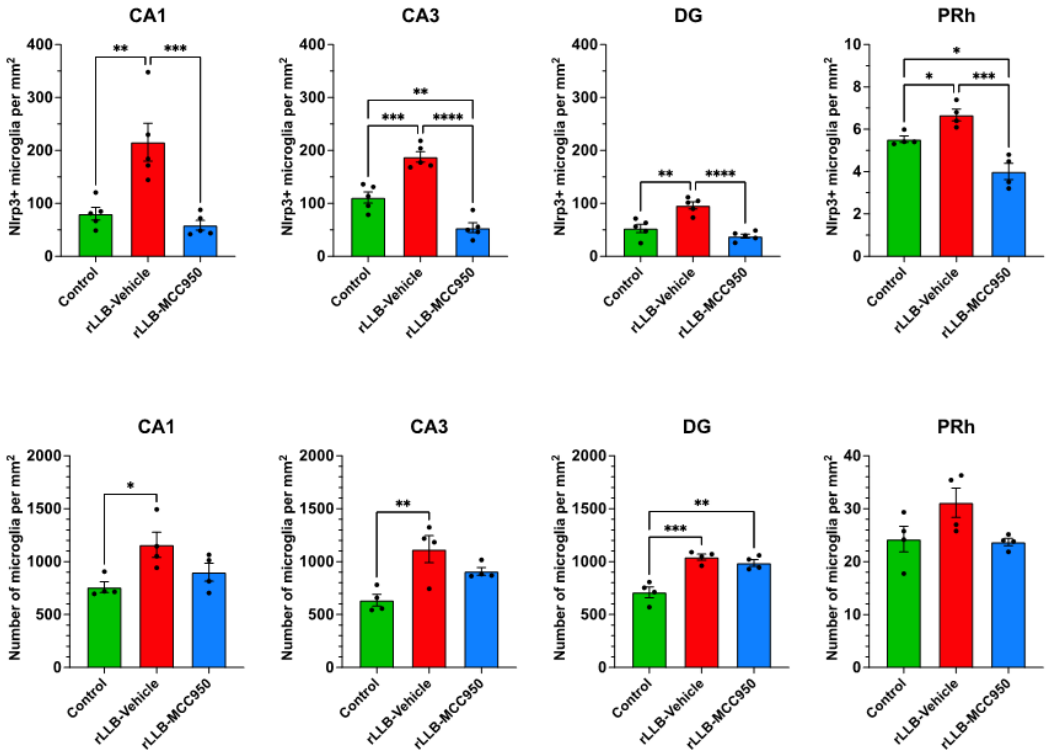

Figure 4.5 MCC950 suppressed rLLB Induced chronic microglial activation and NLRP3 protein Expression. A) Graph represents total microglial number, B) graphs represents NLRP3+microglial cells in hippocampus and perirhinal cortex at day 30 in control, treatment (rLLB+MCC950) and rLLB groups (n=4-5), on-way ANOVA, p*<0.05, p**<0.01, p***<0.001 Mean ± SEM.

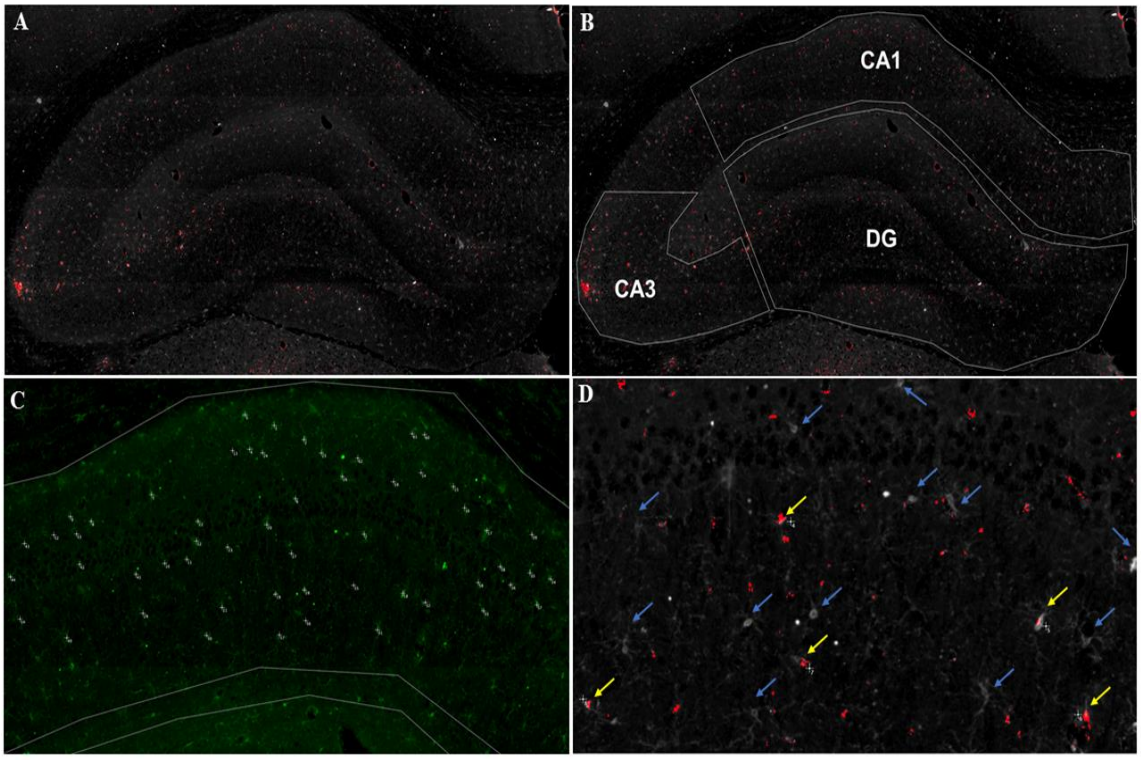

Figure 4.6 NLRP3+ Microglia counting procedure**.** An image represents whole hippocampus region, B images CA1, CA3 and DG region of hippocampus selected for counting NLRP3+ microglia (C, D). C image represents counting total microglia within the region. D represents NLRP3 positive microglia (yellow arrow) and NLRP3 negative microglia (blue arrow).

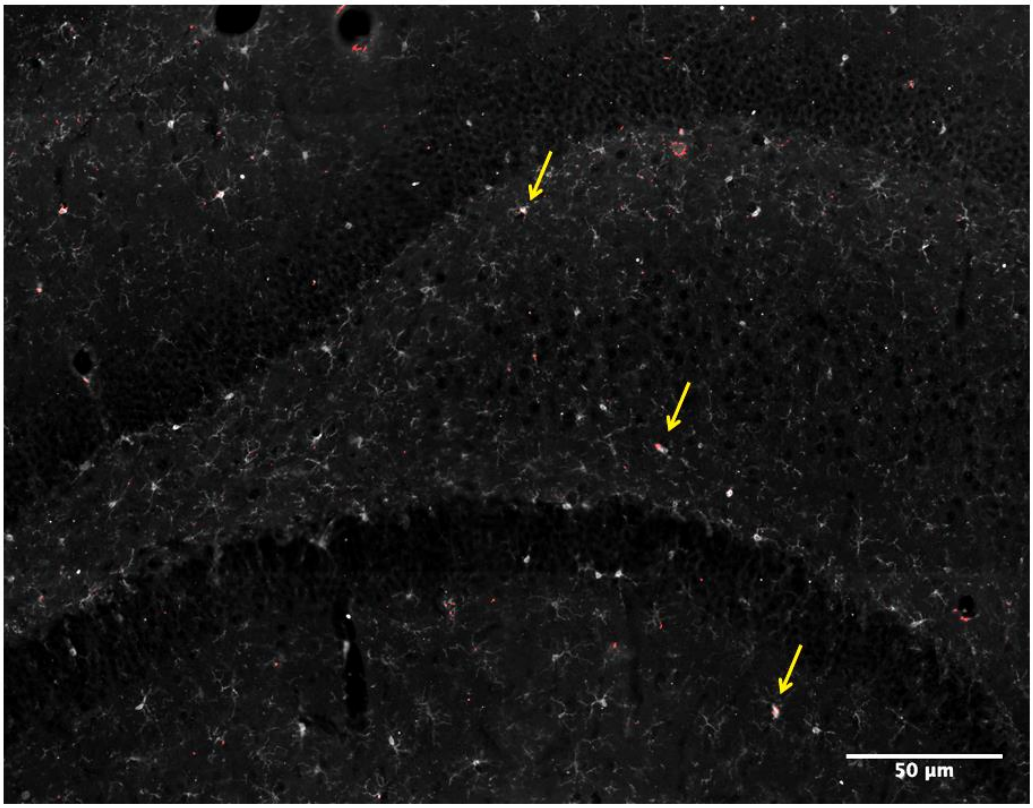

Figure 4.7 Representation of NLRP3+Microglia (colocalized cells) in hippocampus (DG) in Control.

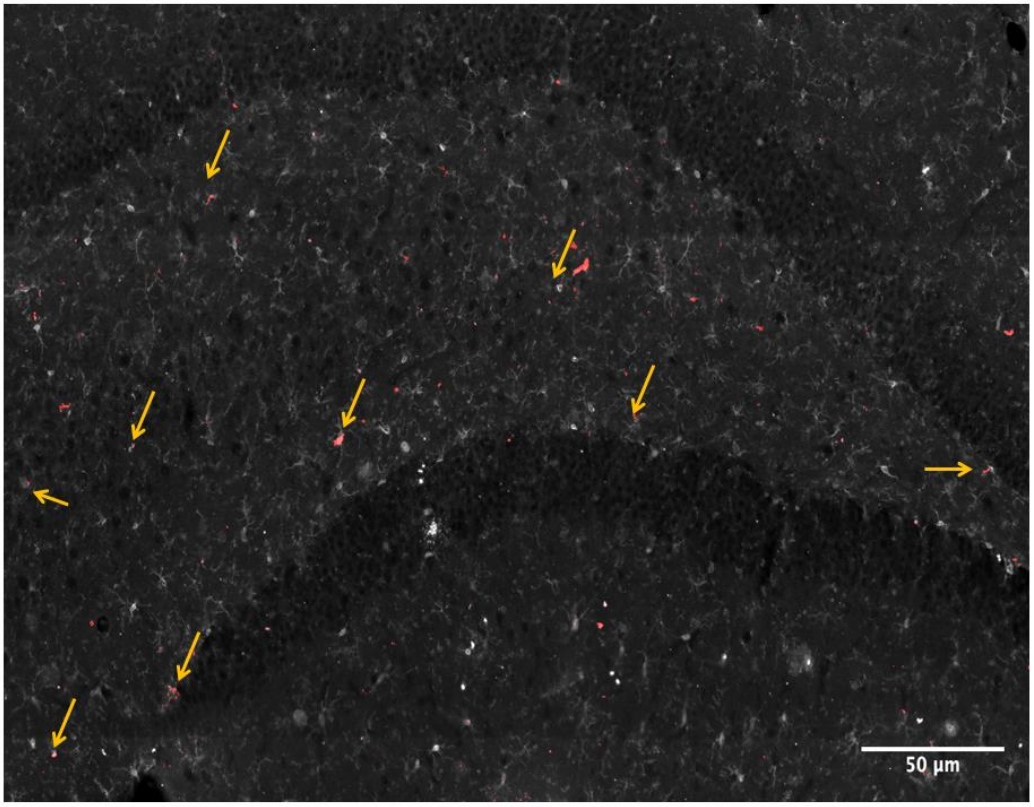

Figure 4.8 Representation of NLRP3+Microglia (colocalized cells) in hippocampus (DG) in rLLB**.** More expression of NLRP3+microglial cell in rLLB group compared with control.

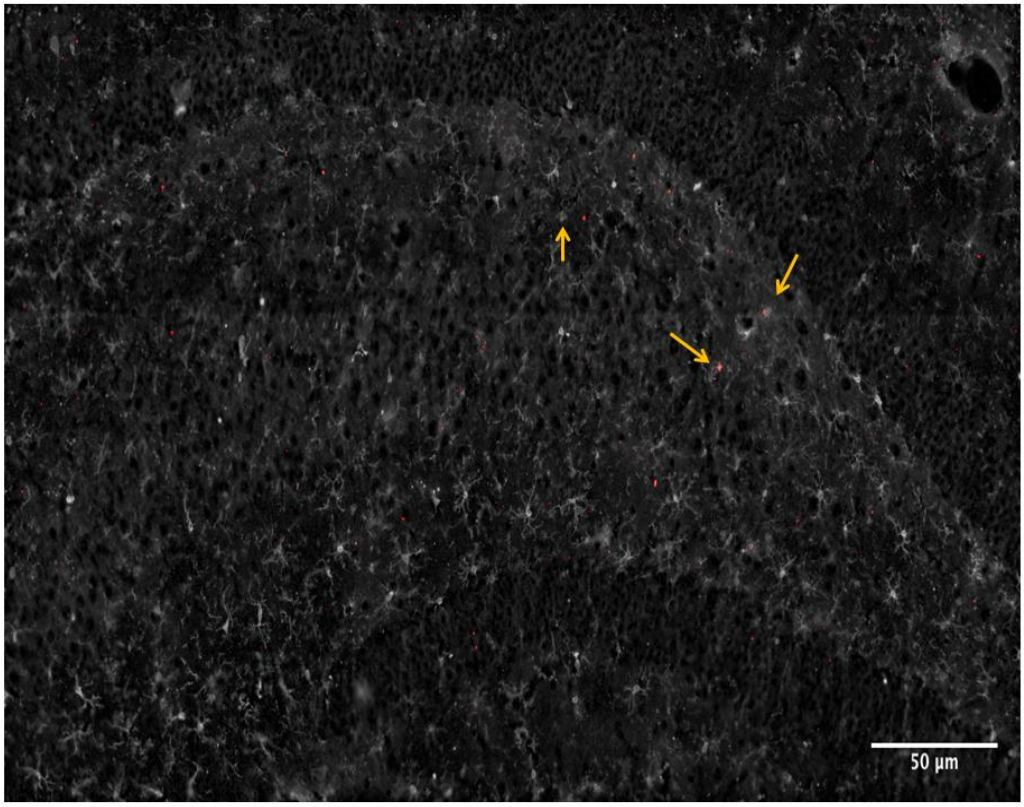

Figure 4.9 Representation of NLRP3+Microglia (colocalized cells) in hippocampus (DG) in MCC950 treated group**.** Colocalized NLRP3+microglial cell was less in treatment group compared to injured (rLLB) group**.**

4.4.4 MCC950 decreased rLLB induces acute and chronic nlrp3 protein expression

This study mainly examined total NLRP3 inflammasome protein expression in hippocampus region. There is a significant increase in NLRP3 proteins expression as early as day 1($p^{**}<0.01$) which persisted until 30 days ($p^{***}<0.001$) after rLLB compared to the control group. On other hand, MCC950 treatment significantly decreased NLRP3 expression at both time points ($p^{*}<0.05$) compared to the injured group (**Figure 4.10**).

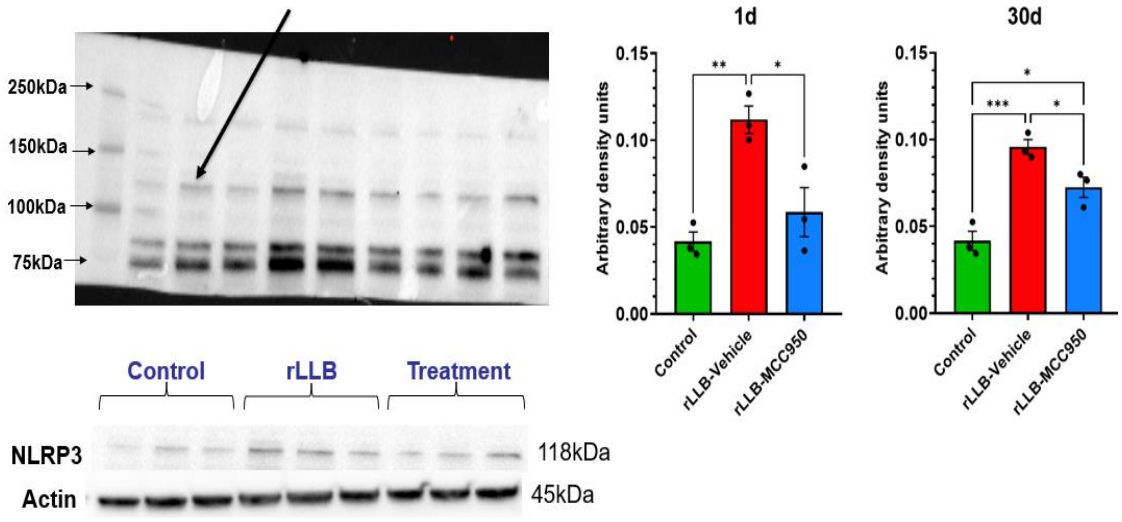

Figure 4.10 MCC950 decreased rLLB Induced acute and chronic NLRP3 inflammasome protein expression. Graphs represents NLRP3 expression at day 1(1D) and day 30 (30D) in control, treatment (rLLB+MCC950) and rLLB groups (n=3), one-way ANOVA, p*<0.05, p**<0.01, p***<0.001, Mean ± SEM.

4.4.5 MCC950 decreased rLLB induced acute and chronic active caspase-1 enzymes expression

As this study noticed an increase in NLRP3 protein expression and chronic pro-inflammatory cytokine IL-1β release, this study further examined total cleaved/active caspase-1, p20 enzyme in hippocampus region. There is a significant increase as early as day 1(p***<0.001) which almost persisted until 30 days (p<0.06) after rLLB compared to control group. On other hand, MCC950 treatment decreased cleaved/active caspase 1, p20 enzyme expression at both time points compared to the injured group (**Figure 4.11**).

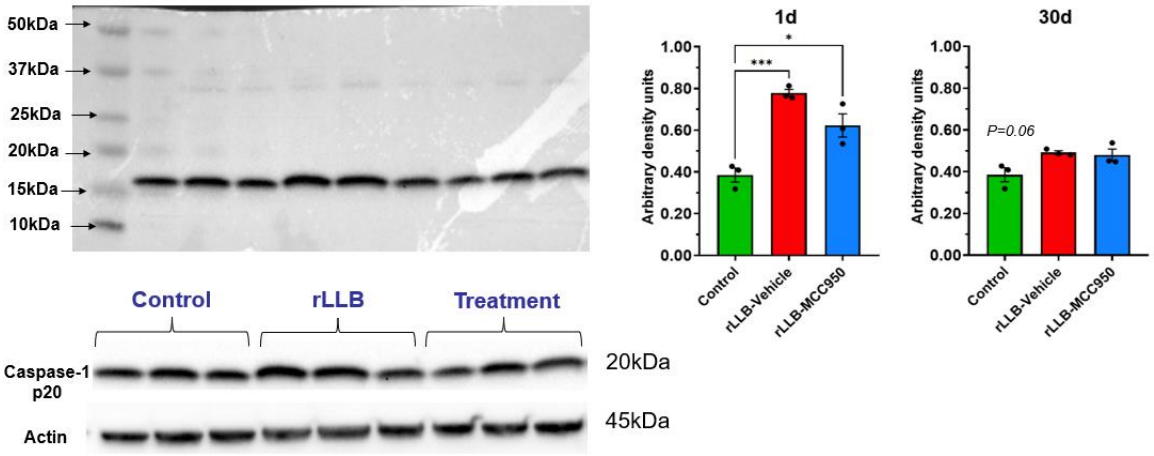

Figure 4.11 MCC950 decreased rLLB Induced acute and chronic active caspase-1 enzyme expression. Graphs represents active caspase-1 expression at day 1(1D) and day 30 (30D) in control, treatment (rLLB+MCC950) and rLLB groups (n=3), one-way ANOVA, p***<0.001, Mean ± SEM.

4.4.6 MCC950 decreased rLLB induces chronic pro-inflammatory cytokine il-1β release

ELISA results indicate that rLLB induced an increase in IL-1β release as early as 1D (p***<0.001) and persisted even after 30D (p***<0.001) of post-injury compared to control group (**Figure 4.12**). On the other side, MCC950 treatment showed a significant decrease in IL-1β release at 1D and 30D compared to the injured group (p**<0.01).

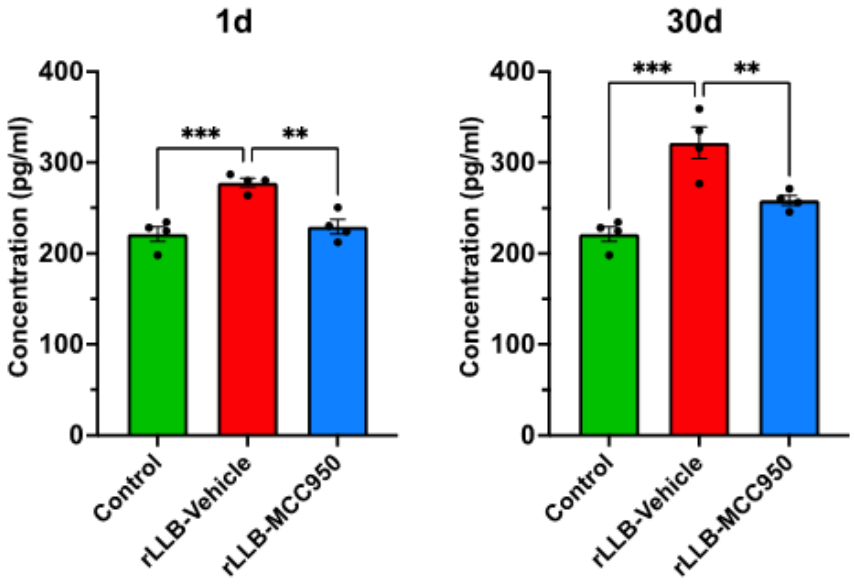

Figure 4.12. MCC950 suppressed rLLB induced chronic pro-inflammatory cytokine IL-1β release. Left and Right graphs represent concentration (pg/ml) of IL-1β at day 1(1D) and day 30 (30D) respectively in vehicle, treatment (rLLB+MCC950) and rLLB groups (n=4), one-way ANOVA, p**<0.01, p***<0.001, Mean ± SEM.

4.5 Discussion

Recent studies demonstrated the involvement of the NLRP3 inflammasome in chronic neuroinflammatory response following a TBI event [247]. The current study explored the role of the NLRP3 inflammasome in rLLB and provided evidence that administration of a selective NLRP3 inhibitor, MCC950, significantly ameliorated rLLB-induced chronic neuroinflammation and long-term memory impairments in our rat model.

In this study, I found that MCC950 treatment improved short-term object recognition memory as assessed by discrimination for the novel object in the NOR task. Reason for choosing NOR test was based on previous literature where wide range of BOPs can show impact on short-term recognition memory compared to spatial memory [180] and

hippocampus has high susceptibility wide range of BOPs and this vulnerability increased with repetition [48]. As I examined pathological changes in hippocampus and perirhinal cortex where both regions involved in novel object recognition test. I observed significant improvements at 1 day post-blast with a similar trend at 30 days. Neurocognitive outcomes have improved following treatment with several drugs targeting the NLRP3 inflammasome in other rodent models of TBI. MCC950 itself has been shown to improve neurological severity score at 1 and 3 days after TBI in rat and mouse injury models [248]. A study by Zheng et al. examined the effect of dexmedetomidine (Dex), an α2-selective adrenergic receptor agonist, on NLRP3 inflammasome expression after TBI and found that Dex administration improved spatial learning and memory. This effect was further enhanced when Dex was co-administered with the NLRP3 inhibitor BAY-11-7082 [233]. Additionally, Lin et al. found a neuroprotective role of omega-3 fatty acid against NLRP3-mediated chronic neuropathological changes which improved memory following TBI [234]. Thus, the significant improvement in object recognition memory after MCC950 administration as reported in this chapter suggests that the NLRP3 inflammasome is an appropriate target for improvement of cognitive outcomes following rLLB as well as other forms of TBI.

When examining the effects of treatment on proteins and cytokines involved in NLRP3-mediated neuroinflammation, I found that MCC950 decreased NLRP3 and cleaved caspase-1 expression and reduced IL-1β release in the rat hippocampus and perirhinal cortex at 1 and 30 days post-blast. Previously, Ismael et al. reported that MCC950 administration mitigated TBI-induced NLRP3 and caspase-1 expression and IL-1β release at 1 and 3 days post-injury and simultaneously decreased brain water content

and neurological severity score in mice [116]. In rats, Zhao et al. found that MCC950 treatment decreased apoptotic cell death, brain water content, NLRP3 expression, caspase-1 enzyme levels, and IL-1β release at 1 and 3 days after TBI [248]. Dex administration decreased NLRP3 and caspase-1 expression, reduced IL-1β release, and increased neuronal viability in a rat model of CCI; these effects were also enhanced by co-administration of BAY-11-7082 [233]. Several other studies have also demonstrated NLRP3 inhibition-mediated neuroprotection following TBI [206, 232, 239, 240, 249-251].

Activation of the NLRP3 inflammasome and subsquent caspase-1 cleavage and IL-1β release are key components in a neuroinflammatory mechanism that is specific to microglia compared to other cell types in the CNS [115]. Consequently, I evaluated microglial activation based on process length and number of processes per cell and assessed the number of microglia expressing NLRP3 in the soma after injury and/or treatment. I observed a significant reduction in microglial process length and number of processes after rLLB, and this effect was reversed by treatment with MCC950. In addition to microglial activation, I also observed significant increase in NLRP3+microglial number following rLLB at chronic time points whereas, MCC50 treatment decreased NLRP3 expression in microglia.Xu et al. examined MCC950 treatment after TBI and observed suppressed microglial activation and decreased pro-inflammatory cytokine release, BBB permeability, and brain water content at 3 days post-injury. Furthermore, this group also evaluated microglia specific NLRP3 expression and its role in chronic neuroinflammation and long-term neurological changes by inducing microglial depletion via PLX3397. They found that the protective role of MCC950 was abolished in microglial-depleted animals. Treatment

with Dex also resulted in suppression of microglial activation which was enhanced when co-administered with the NLRP3 inhibitor BAY-11-7082 [233]. _

Although there are a small number of studies referenced above using MCC950 as a treatment in rodent models of TBI, these studies only examined the therapeutic efficacy of MCC950 at acute time points after TBI. In aim 3, the effects of MCC950 were also assessed at chronic time points (30 days post-blast) in our rat model of rLLB. I found that MCC950 treatment ameliorated short-term memory deficits and suppressed NLRP3 expression and microglial activation at both acute and chronic time points. Hence, rLLB-induced chronic behavioral deficits could be mediated through the NLRP3 inflammasome pathway.

CHAPTER 5

GENERAL DISCUSSION

Low-level blast is the mildest form of bTBI. Military and law enforcement personnel attend training sessions periodically throughout their service as part of combat readiness in which they are exposed to these low-level blasts repeatedly [11, 13]. Unlike single moderate-severe bTBIs, TBIs caused by rLLB exposure do not show any diagnosable symptoms immediately after a training session; however, studies on soldiers participating in these trainings reported chronic neuropsychological, neuromotor, and neurocognitive problems [252-254]. However, these reports are anecdotal and have not been validated with preclinical research, especially using animal models. The major gap in understanding the impact of these repetitive low-level exposures on the CNS over time is due to the lack of an accurate animal model. In this study, we have developed a rat model of rLLB in order to examine acute and long-term neurobehavioral and neuropathological changes in this form of TBI.

In Chapter 2, I investigated the influence of rLLB on neurobehavioral changes at 1 and 30 days post-blast. I found both acute and chronic anxiety, short-term recognition memory impairments, and motor deficits. In Chapter 3, I investigated several possible factors contributing to the behavioral changes observed following rLLB exposure, including NOX1-mediated oxidative stress, microglial activation, NLRP3 inflammasome expression, and astrocytosis, in the hippocampus. I noticed a significant increase of all these factors at both acute and chronic time points following rLLB. In Chapter 4, I examined NLRP3 inflammasome-mediated IL-1β release and its influence on chronic microglial activation, neuroinflammation, and neurobehavioral changes.

For this, I administered MCC950, a selective NLRP3 inflammasome inhibitor, and analyzed its therapeutic efficacy in terms of suppression of microglial activation, NLRP3 protein expression and IL-1β release as well as improvement in behavioral outcomes. Here, I found that MCC950 treatment mitigated chronic neuroinflammation mediated through NLRP3 and IL-1β release and improved short-term recognition memory.

The physical mechanism to explain how a blast wave causes damage to the brain is still controversial. However, like other forms of TBI, bTBI causes a primary injury (exposure to blast wave) and secondary injury (develops over time). Primary injury occurs mainly due to the transmission of energy in the form of a blast wave to the brain, which might induce the shearing of brain tissues as in diffuse axonal injury (DFI). Known blast-induced primary injuries include BBB disruption, hemorrhage, and microvascular injury; of these, BBB disruption has been well documented at different BOPs in bTBI [20, 21, 30, 31, 255]. Possible secondary injury mechanisms that result in rLLB-induced chronic microglial activation might occur due to (1) disruption of BBB and vascular endothelium, which might induce compromised permeability and spillage of bloodborne macromolecules respectively and (2) induction of acute and chronic free radical generation and oxidative stress [256]. One known enzyme that documented in single moderate bTBI is NOX1 and 2 [208]. I observed an increase in NOX1 expression which persisted for 30 days post-injury. At day 1, I found that the total number of microglia in the control and rLLB group was not significantly different, but the number of activated microglia was greater in rLLB rats compared to control. This discrepancy might be accounted for by several possible primary and secondary injury mechanisms that could induce microglial activation, such as (1) physical displacement of the surrounding extracellular fluid, (2)

rLLB-induced BBB disruption, or (3) NOX-mediated oxidative stress and free radical generation. I also observed an increase in the total number of microglia as well as the number of activated microglia, which might be caused by NOX and IL-1β-mediated microglial proliferation [257]. Microglial activation and proliferation not only cause chronic release of inflammatory mediators such as cytokines (e.g., IL-1β, TNF-α), chemokines, and ROS but also disrupt the integrity of the BBB [258]. Furthermore, proinflammatory cytokine IL-1β induces BBB disruption via downregulation of sonic hedgehog production in astrocytes, which plays a crucial role in BBB formation, and upregulation of hypoxia-inducible factor 1 (HIF1) and its target vascular endothelial growth factor A (VEGFA) in astrocytes [259, 260]. Hence, chronic microglial activation could result in biphasic opening of the BBB after injury.

Chronic neuroinflammation due to sustained microglial activation is a frequent observation in bTBI [79, 80]. One of the key players for contributing to chronic neuroinflammation is the NLRP3 inflammasome complex, which predominently expressed in microglia [115]. Activation of the NLRP3 inflammasome complex requires (1) a priming signal consisting of Nf-kB-mediated upregulation of NLRP3 components and pro-IL-1β and (2) an activation signal and results in caspase-1-mediated clevage of pro-IL-1β to IL-1β. In the present study, I did not examine the mechanism behind the priming step, but I speculate that rLLB might cause BBB disruption or neuronal damage which might result in the release of DAMPs such as heat shock proteins (HSPs), high mobility group box protein 1 (HMGB1), S100β, DNA, etc. These components have receptors on microglia (e.g., TLR, RAGE receptors) which can induce Nf-kB activation. Increased Nf-kB activation has been reported along with inflammation and ROS generation as early as 2

hours to 3 days following blast [261]. Activation and oligomerization of NLRP3, which are the focus of this study, are triggered by a wide range of signals including ATP, ionic imbalance (K^+ efflux, Ca^{+2} flux, Cl^- efflux), mitochondrial DNA, ROS, and proinflammatory cytokines IL-1β and TNF-α [90]. I observed acute and chronic ROS generation via NOX1 expression, so I assume that initial NLRP3 activation in microglia might be due to ROS. In contrast, chronic NLRP3 activation resulting in microglial activation and proliferation is likely mediated by IL-1β, as I observed a significant increase in levels of this cytokine at days 1 and 30.

NLRP3 inflammasome activation results in cleavage of pro-IL-1β to IL-1β. Increased IL-1β release can activate neighboring microglia and astrocytes, which causes an amplification of glial activation and induces chronic neuroinflammation. Studies are well documented that external adminstration of IL-1β can cause altered neurotransmitter release and behavioral changes including impaired spatial memory, anxiety [262], motor deficits [263], decreased exploratory activity, and sleep disurbances [72-78]. IL-1β receptors are widely distributed all over the brain but are most densly populated in the hippocampus [71]. A study has shown that IL-1β can induce structural changes (plasticity) in the hippocampus, especially CA1, by reducing dendritic spines which can cause memory impairments [264]. In this study, I observed acute and chronic IL-1β levels, NLRP3 and cleaved caspase-1 expression in hippocampus which accompanied by acute and chronic short-term memory impairments (NOR) following rLLB. These behavioral deficits improved after treatment with MCC950, a selective NLRP3 inhibitor, with a corresponding decrease in NLRP3 and cleaved caspase-1 expression and IL-1β levels. Similarly, in an lipopolysaccharide (LPS) injury model, researchers observed an improvement in short-

term recognition memory (NOR) and anxiety-like symptoms (elevated zero maze, EZM) in hippocampal lentivirus-mediated IL-1β knock-down mice [265]. The increased anxiety-like symptoms that I observed in EPM also might be due to the involvment of IL-1β. Studies have shown that IL-1β can interact with the endocannabinoid system, particularly type-1 cannabinoid receptors (CB1R) [266]. CB1Rs are crucial for regulating excitatory and inhibitory synaptic transmission in different brain regions such that increased IL-1β can downregulate GABAergic synaptic transmission via interactions with CB1Rs [266, 267]. Similarly, persistent motor defcits following rLLB might be due to elevated IL-1β. Though this study did not examine IL-1β levels in any brain regions other than the hippocampus, I speculate that similar increases might occur in the frontal cortex, motor cortex, and cerebellum, resulting in altered motor coordination after rLLB. Elevated levels of IL-1β due to microglial activation have been previously shown to cause motor deficits following LPS injection, whereas blocking IL-1β reversed these deficits [263]. Furthermore, increased IL-1β also alters neurotransmitter concentrations within the brain, especially levels of the monoamines norepinephrine, serotonin, and dopamine [268]. Of these, altered norepinephrine levels lead to attention, learning, and memory deficits [269]. Thus, an increase in IL-1β might induce acute and chronic pathological and behavioral changes through diverse pathways following rLLB.

As previously mentioned, an increase in IL-1β can also have a detrimental impact on neurons, which could result in IL-1β-mediated behavioral deficits following rLLB. My preliminary data have shown a significant decrease in neuronal cell counts in the CA1, CA3, DG, and PRh regions following rLLB at 30 days post-injury which was significantly prevented by treatement with MCC950 after rLLB. Thus, I speculate that this neuronal loss

is mediated by IL-1β because the treatment prevented neuronal loss, implying that MCC950 supressed NLRP3-mediated IL-1β release and subsequent IL-1β-mediated neurotoxicity. This neuronal cell loss can occur through several different mechanisms, including oxidative stress, apoptosis, necrosis, pyroptosis, and glutamate-mediated excitotoxicity. The neuronal cell loss that I observed in rLLB could be the result of any of these pathways. First, the blast wave itself causes NOX-mediated free radical generation and lipid, protein, and DNA oxidation, which can cause neuronal cell death [270-276]. Second, increased IL-1β release can cause additional glial activation and proliferation [277] followed by excessive release of ROS and proinflammatory cytokine which results in further neurotoxicity. Third, IL-1β can induce caspase-3 activation, cytochrome C release, downregulation of Bcl-2, activation of p53 (tumor supressor gene), upregulation of p38 (mitogen-activated protein kinase), thereby triggering neuronal apoptosis [278-280]. Fourth, IL-1β can cause glutamate-mediated neuronal excitotoxicity. Proinflammatory cytokines IL-1β and TNF-α released by glial cells can increase enzyme activity of glutaminase 1, an amidohydrolase which converts glutamine to glutamate within neurons. Excess extracellular glutamate interacts with NMDA receptors to induce excitotoxicity. A study observed glutaminase 1 activity, glutamate production, and NMDA-mediated excitotoxicity in the presence of elevated IL-1β which was blocked by administration of NMDA receptor antagonist MK-801 [281]. Increased extracelluar glutamate can also serve as a signal for NLRP3 inflammasome activation which causes further release of IL-1β. In addition to neuronal cells, microglia also release excess glutamate when activated or in the presence of oxidative stress or lipid peroxidation [282]. In the presence of DAMPs released from necrotic cells, microglial glutaminase levels are

elevated via TLR-MyD88-mediated Nf-kB activation, resulting in excess glutamate release from microglia [283]. Fifth, neuronal loss could occur via pyroptosis, as IL-1β interacts with neurons and serves as a signal for inflammasome complex activation. Besides producing IL-1β, the inflammasome complex can also induce pyroptosis via pore formation through caspase-1-mediated gasdermin D activation. Neuronal cells predominantly express the NLRP1 isoform compared to NLRP3, so chronic IL-1β release via microglial NLRP3 can activate NLRP1 in neurons, leading to neuronal cell death via pyroptosis [284-286].

In summary, the current animal model using low-level blast exposures successively within a short span of about 5-10 minutes closely mimics a true exposure, allowing for investigation of rLLB-mediated chronic neurological changes that may potentially occur in service members. Accordingly, this model is also a useful preclinical model to formulate therapeutic interventions which are greatly needed to prevent cognitive and behavioral deficits in those who are required to participate in training sessions where they will inevitably be exposed to rLLB **(Figure 5.1)**.

As a future study, the following are suggested:

1. This study is limited to anxiety, motor, and memory impairments. There is a need to examine PTSD, depression, sleep disturbances, attention deficits, hearing loss, and fear conditioning following rLLB. These are the other behavioral symptoms manifested by soldiers or law enforcement personnel after the training.

2. Blood-brain barrier dysfunction (BBB) is a secondary injury mechanism that is widely observed after single moderate blast. However, there is a possibility of that BBB is involved in the chronic neuroinflammation following rLLB.

3. This study investigated one possible secondary injury mechanism, neuroinflammation after rLLB. Neuronal loss or synaptic protein changes may also

play an important role in manifesting behavioral abnormalities and need to be studied.

4. In addition to neuronal loss and the influence of altered synaptic transmission on behavior, there is a need to examine electrophysiological changes following rLLB.

5. This study mainly focused on designing an appropriate animal model for rLLB and investigated rLLB influence on neurological changes at 30 days (chronic time points). However, there is a need to examine long-term (3, 6, 9, 12 months) effects of rLLB.

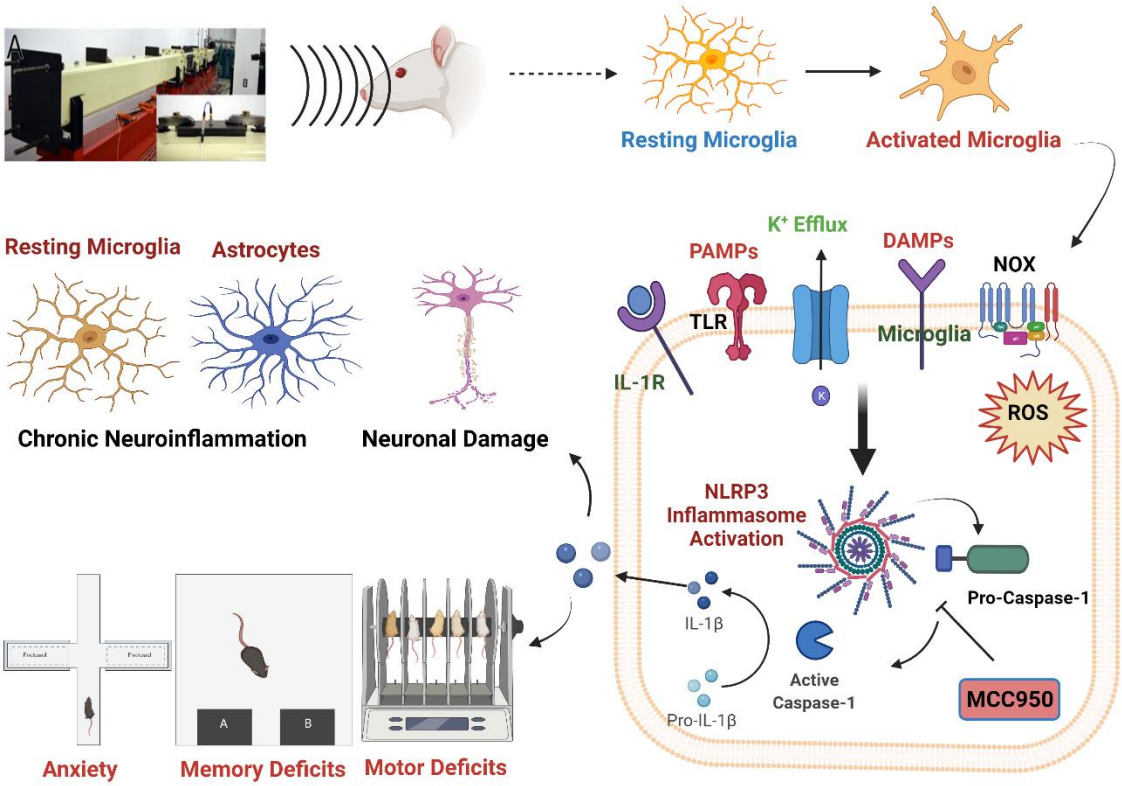

Figure 5.1 Schematic of dissertation work on rLLB induced NLRP3 mediated neurological changes. rLLB induced chronic microglia activation through NLRP3 mediated pro-inflammatory cytokine IL-1β release which leads to pathological and behavioral changes. MCC950 treatment mitigated pathological changes and ameliorated short-term memory impairments.

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