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14. ABSTRACT Mild traumatic brain injury (mTBI), such as mild "blast" injuries due to improvised exploding devices, result in long term impairment of cognition and behavior. Our hypothesis is that there are inflammatory outcomes, via the IL-1α/β and TNFα signaling pathways, to mTBI over time that cause the neuropathogenesis responsible for the clinical outcomes. We developed and characterized an adaptation of the rat moderate brain lateral fluid percussion brain injury model (mLFP injury) and a mild blast brain injury model (mBBI) developed by us with similar resulting righting reflex response times (RRRT). Both showed increased IL-1β and TNFα levels, macrophage/ microglial and astrocytic activation, blood brain barrier disruption, neuronal losses and behavioral impairment. mBBI showed increased phosphorylated Tau protein (p-Tau) levels, a neuroencephalopathy marker. For mLFP injury, we showed beneficial outcomes after IL-1 receptor blockade with FDA-approved Kineret and of TNFα receptor with FDA-approved Etanercept, singly or in combination, decreased neuropathology and improved outcomes. We determined an optimal time course of treatment. For mBBI, we showed that there were increases in IL-1β and TNFα levels, macrophage/ microglial and astrocytic activation, and phosphorylated Tau (p-Tau) levels, the latter indicative of neuroencephalopathy, in the injured cortex, hippocampus, thalamus and amygdala. Whereas there was an apparent correlation between the RRRT values and the p-Tau levels, general inflammatory responses were more threshold-triggered. For mBBI we showed a beneficial outcome after blockade of IL-1 receptor (IL-1R) with Kineret. These results suggest potential therapies for mild blast injuries.

15. SUBJECT TERMS

Blast head injury; mild head injury; anti-inflammatory therapy; cytokine receptors; IL-1; TNFα; encephalopathy

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INTRODUCTION

Mild traumatic brain injuries (**mTBI**) are common among our military personnel (Hogue et al., 2008; Kamnash et al., 2012), of these mild blast brain injuries (**mBBI**) are responsible for over 80% of casualties in the last decade (Levin et al., 2010). Mild "blast" injuries due to improvised exploding devices have long term cognitive and behavioral deficits due to what has been labeled as mild traumatic brain injury (mTBI). Mild traumatic brain injury, particularly mild "blast type" injuries due to improvised explosive devices, result in long term impairment of cognition and behavior (Cantu, 2007; Dept. VA and DOD, 2010; Okie, 2005; Omalu, 2006). Rat models of mTBI have shown an important role for hippocampus, cortex, thalamus and amygdala as evidenced by the resultant neuropathophysiology (Elder et al., 2012; Perez-Polo et. al., 2013; Perez-Polo et al., in preparation).

Our goal has been to develop and characterize mTBI models that can serve as useful tools to study the role of inflammation in mTBI and provide platforms for the development and assessment of intervention strategies that will lead to clinical therapies relevant to the military theatre. The two models developed and characterized are: a "mild" adaptation of an established TBI model, the fluid percussion rat TBI model (**mLFP injury**; Dixon et al., 1987) and the Vandenberg blast model (mBBI; developed by us, see previous reports). In both instances, we defined mTBI to occur when rats displayed righting reflex response times (**TTTRs**) that were more than 4 minutes and less than 10 minutes in duration, as compared to sham-treated rats displaying a range of 1-4 minutes. For the injured rats, with mLFP injury or mBBI, there was a significant impairment in their ability to balance on a beam or transverse a mesh.

We showed that for mild lateral fluid percussion (mLFP) injury, there are increased levels of Interleukin 1 (**IL-1**) and Tumor Necrosis Factor alpha (**TNFα**) that are likely to increase inflammation by activating astrocytes, microglia and macrophages that have been shown to be responsible in part for the observed neuronal and myelin losses in cortex, hippocampus, thalamus and amygdala reported by us (Perez-Polo et al., 2013). In the mLFP-injured rats, we observed a significant impairment of the blood brain barrier (**BBB**). For the mBBB we saw similar incrases in inflammatory cytokines, activation of microglia and macrophages, neuronal losses and BBB impairment. Interestingly, mBBI stimulated increases in the levels of phosphorylated Tau protein (**p-Tau**) that has been established to be a biomarker for neuroencephalopathy. These increases were significant across all brain structures sampled: parietal cortex, hippocampus, thalamus and amygdala. We also saw cognitive impairments in both mTBI rat models as reflected in working memory assessments.

We hypothesized that blocking the IL-1 receptor with Kineret agonist or TNF-α receptor with its cognate antibody Etanercept, both FDA-approved drugs, would improve outcomes. Thus, as a corollary to our hypothesis that mTBI stimulates inflammatory cytokine activity, we tested the two drugs in our mTBI models using a delivery protocol and a time frame consistent with battlefield conditions. In both instances, the interleukin receptor blockade improved outcomes.

Body of the Report

SPECIFIC AIMS

Specific Aim #3: **To develop new and innovative treatment strategies for mTBI**
Specific Aim #3.3: To study the role of IL-1 and TNF receptor activation in neurological deficits after TBI
Specific Aim #3.3.1: To serially measure brain cytokine levels after MTBI
Specific Aim #3.3.2: To study the role of IL-1 receptor activation in neuronal cell death and in the inflammatory response after MTBI
Specific aim #3.3.3: To study the role of TNF receptor activation in neurological deficits after TBI

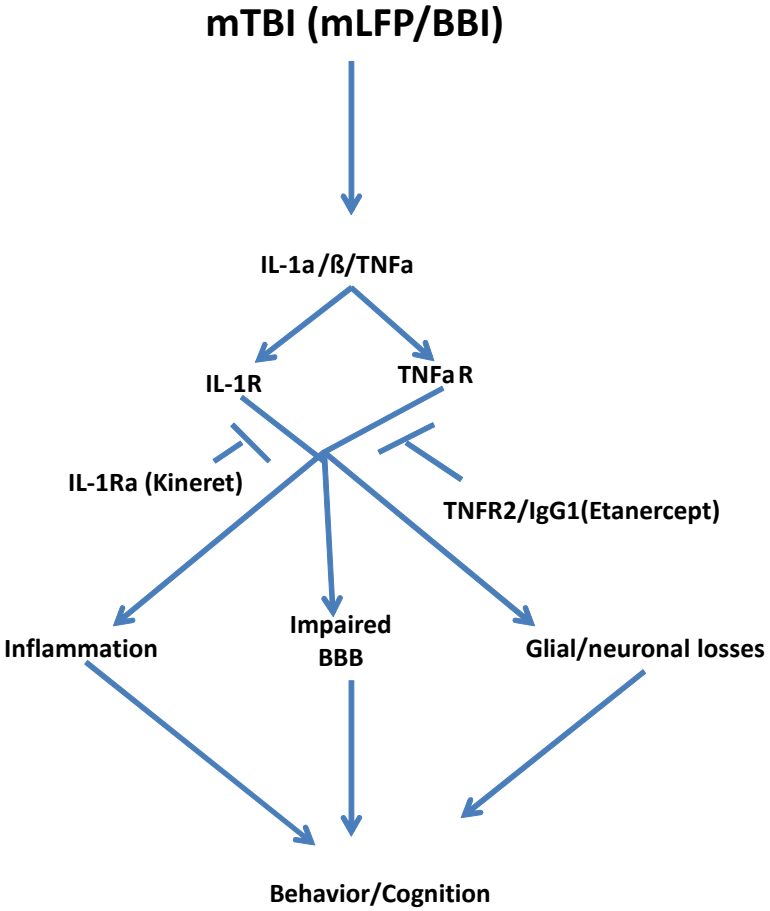
Progress to Date (August 1, 2008 to August 31, 2014)

Specific Aim #3: To develop new and innovative treatment strategies for mTBI

Hypotheses (Figure 1).

• mTBI resulting from a mild lateral fluid percussion brain injury (mLFP injury), or a mild blast injury (**mBBI**), trigger increases in inflammatory cytokines resulting in significant neuropathology as measured by neuronal axonal and cell losses as well as decreases in measurable myelin; astrocytic, macrophage and microglial activation, impairment of the blood brain barrier and behavioral and cognitive impairment.
• Prompt post-injury blockade of the receptors for the injury-induced inflammatory cytokines with Kineret and/or Etanercept w

Figure 1. Specific Aim 3.3 Schematic



mTBI Rodent Models.

Rats are anesthetized with 4.0% isoflurane in an anesthetic chamber, intubated with a pediatric endotracheal tube using a custom modified pediatric laryngoscope. The rats are mechanically ventilated with 2.0% isoflurane in Medical O2:Medical air (30:70) using a volume ventilator. The rats are be prepared for mLFP injury as described in Perez-Polo et al., 2013 and for blast injury rats are anesthetized with isoflurane and their heads shaved, plugging the external auditory canals with foam plugs and covering their heads with a silicone pad. The silicone pad transmits the blast pressure wave but protects the rats from flash burn injury. The rats are then positioned on a foam pad under the Vandenberg blast injury device to be concussed 2 cm from device, (**Figure 2**). Once the rat is positioned, the isoflurane is discontinued, and when the animal exhibits a withdrawal reflex in response to a paw pinch, the rat is subjected to blast injury or sham injury.

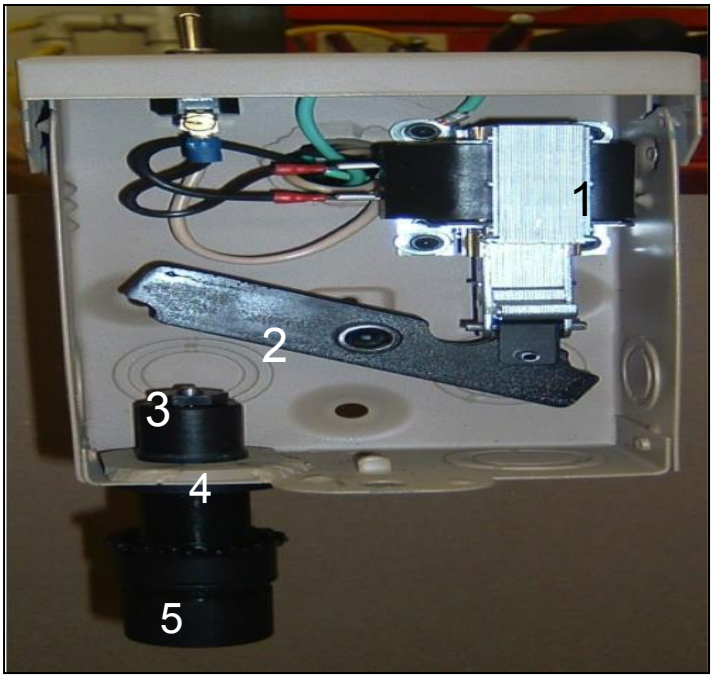


Figure 2. The Vandenberg brain blast injury apparatus uses an electric solenoid (#1) to fire blank cartridges. Force from energized solenoid is transmitted via hammer (#2) to firing pin (#3) is housed in standing breech (#4), igniting blank cartridge in the firing chamber (#5).

Additional animals that are identically prepared but not injured serve as a sham-treated control group. Within 15 seconds following injury, the animals are removed from under the injury device and placed on a table for neurological assessment. A battery of tests is applied (Dixon et al., 1987), to characterize certain acute neurological consequences of mechanical brain injury, including tests analogous to some motor components of the Glasgow Coma Scale. This battery is derived in part from neurological scales previously developed us to evaluate the behavioral effects of brain injury on rats. After a brief postoperative recovery to ensure the general health of the rats (absence of pulmonary edema, cerebral hemorrhaging, etc.), the animals are allowed to recover. The assessment of the paw withdrawal, consists of gradual application of pressure on the hind paw until paw withdrawal occurs. Postural somatosensorymotor functions are assessed by recording the duration of suppression of the righting response. The righting response reflex is defined as the animal's ability to right itself three times consecutively after being placed on its back. The time interval until the animal can right itself is defined as the righting reflex response time (RRRT) ans is a close analogue of return to consciousness in patients suffering TBI where it is used as an important measure of seriousness of injury. After recovery of the righting response, spontaneous locomotion is evaluated by regularly placing the rat in a marked 25 × 25-cm area until the rat walks spontaneously out of the area.

We relied on righting reflex response times (RRRTs) to either mLFP or mBBI-treated rats where RRRTs of 4 - 10 minutes defined a mild traumatic brain injury and RRRTs of 1-3 minutes defined sham-treated animals for both the mLFP and BBI models of mTBI (**Figure 3**).

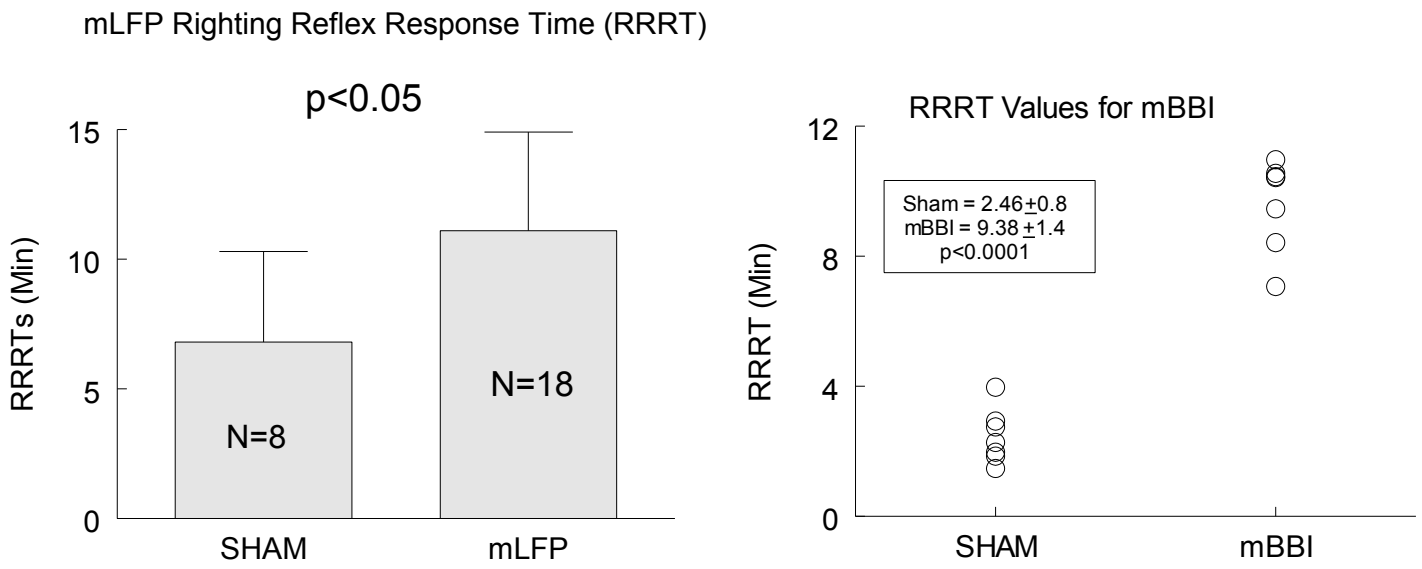
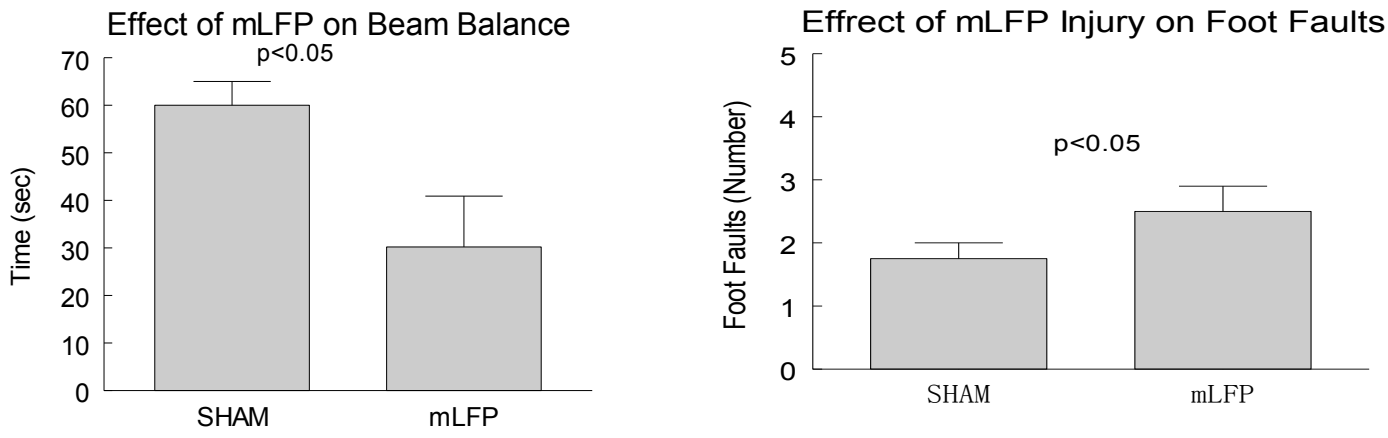


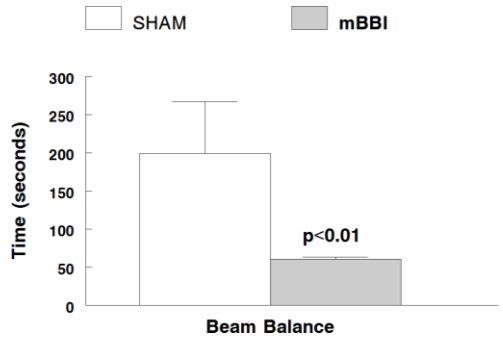
Figure 3. Rightng reflex response times (RRRTs) for mLFP injured and mBBIed rats.

Two other measures used to assess sensorimotor function were the balance beam assay where a a rat is placed on the center of the beam beam 91.5cm L x 1.7cm W and elevated 30cm off the surface below and secured to a platform on either end and released (start time) and allowed to walk to either end. The animal is returned to the center of the platform if he walks onto one of the end platforms. This is continued for1 minute or until the rat falls off (stop time). The foot fault assessment is similar. Here a rat is placed on a wire mesh 69.5 cm W x 45 cm L with 3 cm gaps that is stretched out over a wooded frame. The number of times out of 10 that the forelimb or hindlimb falls through the gaps is counted and is recorder as % foot faults. We showed significant impairment of the amount of time that injured rats could balance on a beam

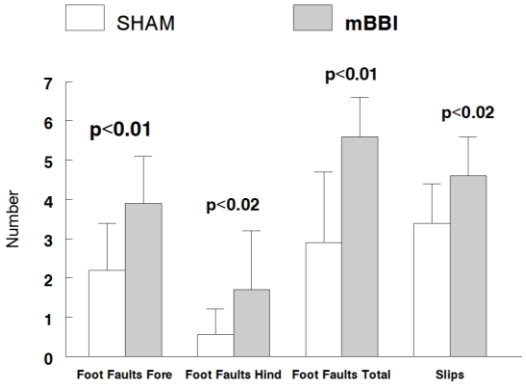
Figure 4. Effects of mLFP injury and mBBI on Beam Balance and Foot Faults (N=6)



Effect of mBBI on Beam Balance



Effect of mBBI on Foot Faults



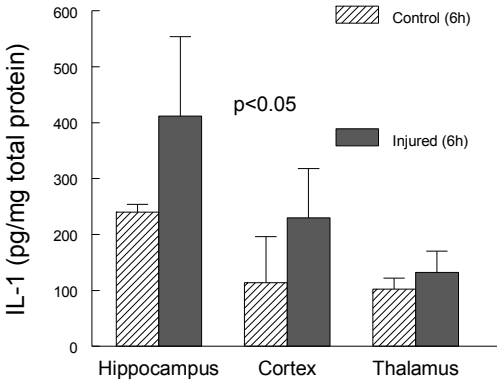
and their ability to transverse a mesh in terms of the number of foot faults committed (**Figure 4**). The three measures: RRRTs, beam balance and foot fault performance defined our classification of mTBI for both mLFP injury and mBBI. The foot fault impairment applied equally well to front or hind paws.

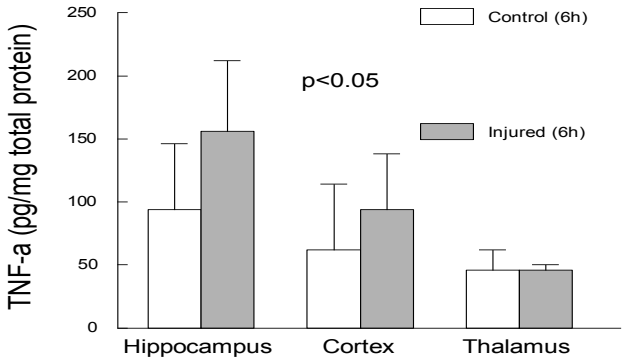
Specific Aim #3.3.1: **To serially measure brain cytokine levels after mTBI**

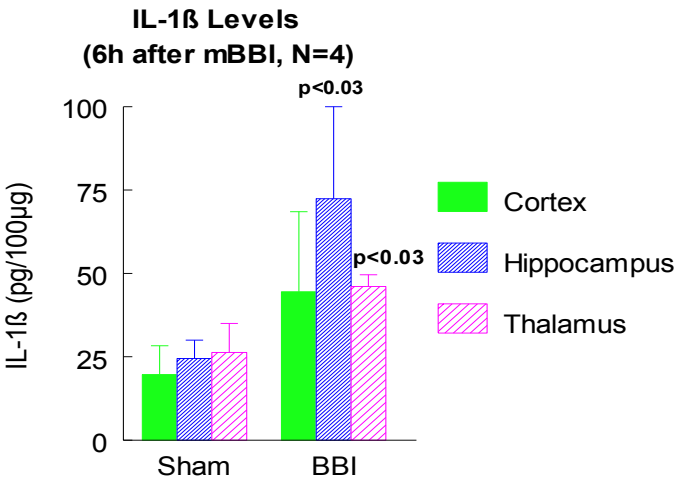
Cytokine Immunoassay: We measured cytokines using the Bio-Plex assay kit from Bio Rad, a capture sandwich immunoassay that is very sensitive detecting cytokines in the pg/ml range.
The Bio- Plex cytokine assay is a multiplex bead based assay designed to detect cytokines using color-coded bead sets conjugated with different specific reactants specific for the different cytokines: IL-1α, IL-1β, TNF-α, IL-2, IL-6, IL-4, IL-10. GM-CSF and IFN-γ.

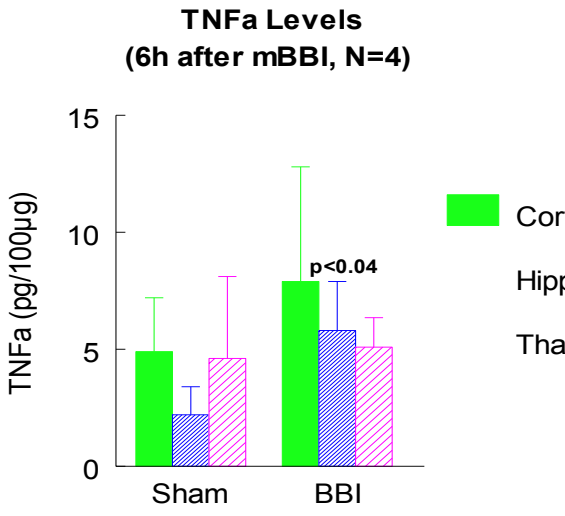
We showed in the mLFP injury model that the key brain inflammatory cytokine Interleukin 1 (**IL-1α/β**) and Tumor Necrosis Factor alpha (**TNFα**) protein levels increase as early as 3 and 6 hours across several brain structures (cortex, hippocampus, and thalamus; **Figure 5**) with a return to basal levels by 18 days post-injury, except at the injury site where there was a small but detectable IL-1β presence. This was also true for the amygdala after mLFP injury although the small size of the amygdala made accurate determinations of the cytokine increases in mBBI difficult. We therefore restricted our comparisons between the two models to the parietal cortex, hippocampus and thalamus at 6 hours for both models and 18 days post injury for mLFP and 30 days for mBBI by which time there was a return to basal levels for both mLFP injury and BBI. The increases showed different time courses and brain region specificity in terms of amount of increases (Perez-Polo, et al., 2013). In all instances for mBBI, the hippocampus was the brain structure most consistently affected, albeit there were also significant increases in thalamus for IL-1β but not TNF-α. There was a trend to higher IL-1β and TNF-α levels after mBBI in cortex but these were not significant due to the high inter-animal variability. We believe that the differences in time course and brain region diversity of magnitude of response would suggest that different aspects of mTBI and blast mTBI in the varied environments associated with combat situations are likely to dictate the necessity for the development of different therapeutic modalities based on several preclinical model studies

Figure 5 Effect of mLFFP Injury and mBBI on IL-1β & TNFα (N=3)









Specific Aim #3.3.2 and #3.3.3 : **To study the role of IL-1 and TNF receptor activation in the inflammatory response, neuronal cell death, and neurological deficits after mTBI**

Given the prompt and robust nature of the increases in the inflammatory cytokines, we were not surprised to see significant increases in the neuroinflammatory biomarkers GFAP and Iba 1 in the same time frames

as early as 6 hours after injury for both mLFP injury and mBBI (**Figure 6 and 7**; Perez-Polo et al., 2013). While the increased display of inflammatory biomarkers did not crease over time, they did persist for 18 days for mLFP injury and 30 days for mBBI but did show significant increases over earlier 6 hour time measurements of both GFAP and Iba1 reflecting changes in astrocytic activation and microglia/macrophage activation respectively.

Figure 6. mLFP Injury Cortical Effect (N=3)

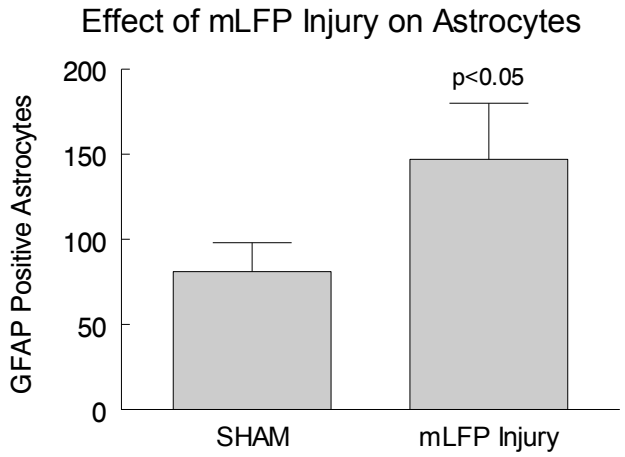
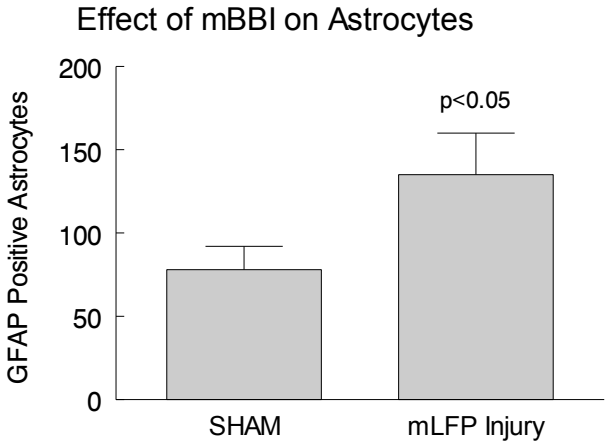
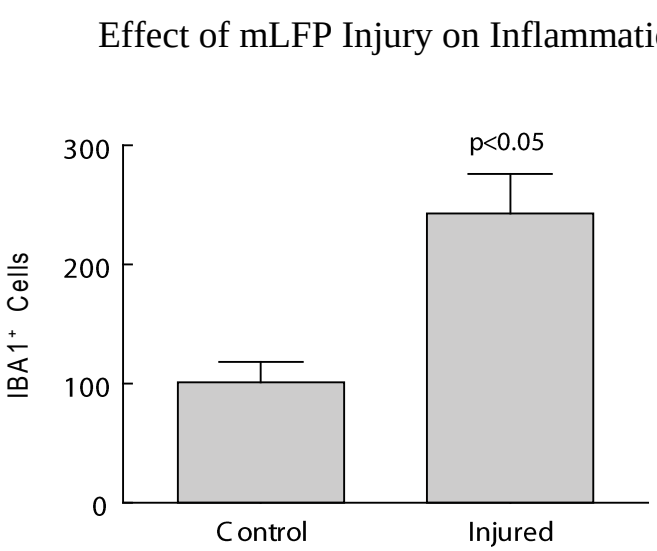
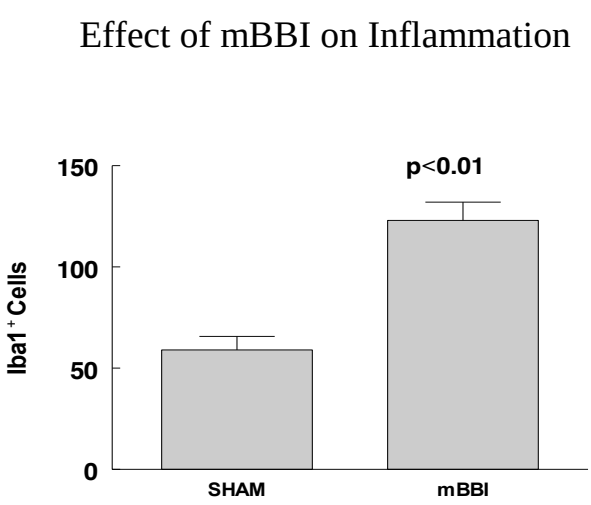


Figure 7. mBBI Cortical Effect (N=3)

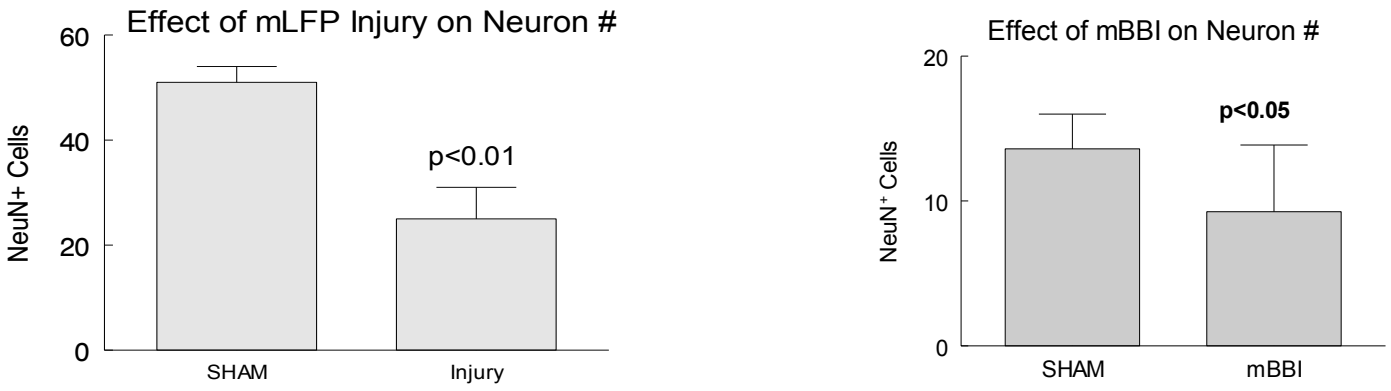






In order to determine whether there were significant neuronal losses in rats exposed to mLFP injury after 18 days, cells were stained for NeuN, a marker for neuronal nuclei. As seen in **Figure 8**, there were significant neuronal losses in hippocampus after both mLFP injury and mBBI that persisted for up to 18 days and 30 days respectively. Although the maintenance of neuronal loss due to injury would suggest that neuronal loss principally is an acute phenomena, this does not preclude a significant delayed ongoing neuronal loss that could become apparent at times beyond the 18 and 30 day time course here pursued.

Figure 8. Effect of mLFP Injury and mBBI on Hippocampal Neuronal Cell Number ($NeuN^+$ Cells)
(SHAM N=10; mLFP N=6) **(SHAM N=10; mBBI N=10)**



Brain trauma often results in impairments of the blood brain barrier. An indicator of blood brain barrier function is endothelial barrier antigen (EBA). When EBA, recognized by SMI-71, was measured 18 days aftermLFP injury or mBBI there was an acute decrease in SMI71 levels as early as 6 hours consistent with impaired blood brain barrier functionality (**Figure 9**).

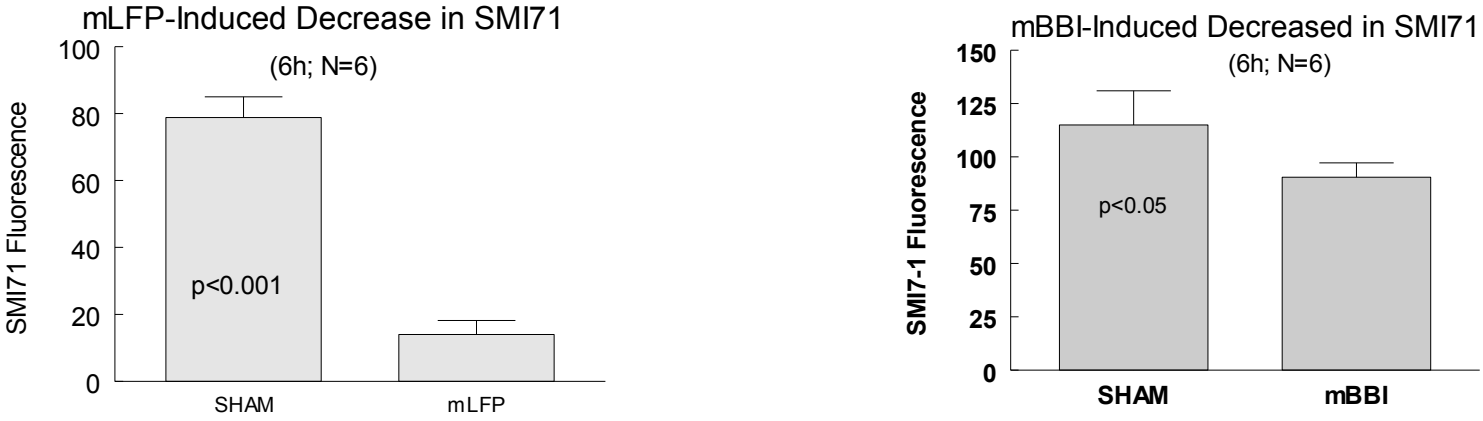


Figure 9. Effect of mLFP Injury and mBBI on Cortical Blood Brain Barrier

Effect of mLFP injury and mBBI on Cognitive Function

Having shown an acute development of several components of neuropathology subsequent to mLFP injury or mBBI, we turned our attention to an assessment of cognitive function. While there are many assays of cognitive function that are applicable to rodent models; we found, after a large number of trials using different protocols, that the most reliable, after a series of rigorous statistical analyses, was the measurement of working memory using a Morris Water Maze.

The Morris Water Maze is a 6' diameter tank, filled to 2 cm above the invisible platform that is 4 inches diameter. The water temperature is held at 22-24 degrees. The platform is stationary throughout the experiment. The tank is divided into four quadrants and stationary cues are marked on the wall in each quadrant. Before the first trial, the rat is placed on the platform for 30 seconds. The animals' starting point is randomly chosen each day based on these quadrants, one trial is started from each quadrant. The SMART computer system is used to track and monitor the animal during the trials. After placing the animal in the water facing the wall of the tank, the handler leaves the room. The animal is allowed two minutes to find and climb the platform and escape the water (latency to platform); he must remain on the platform for 30 seconds. If the animal does not find the platform, the handler places him on it for 30 seconds before removing him from the maze. Working memory tests rely on the improved performance of a rat at the end of the second of four trials run on the fifth day post injury. The animals are given a four-minute rest period in a warming chamber between each trial. Rats are then allowed to recover Evaluation of the effects of mLFP and mBBI on working memory, provided similar results consistent with the neuropathology results (**Figure 10;** Perez-Polo et al., 2013).

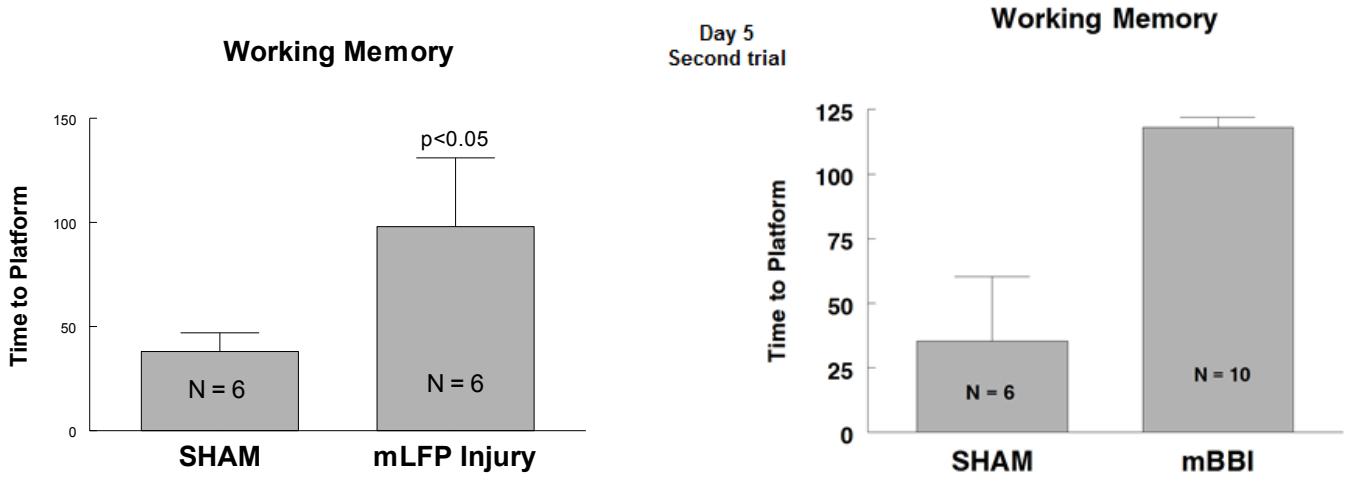


Figure 10. Effect of mLFP Injury and mBBI on Working Memory

Effect of mBBI on neuroencephalopathy (p-Tau protein)

It is clear from the reported cognitive and behavioral outcomes to mTBI due to blasts that there is likely to be involvement of the parietal cortex, hippocampus, thalamus and amygdala; our chosen immunohistological target tissues. One clinical outcome of these neuronal and glial losses could be the

long term encephalopathy frequently documented years after a TBI incident or incidents (Cantu, 2007; Omalu et al., 2006). Both for injured athletes and blast TBI rodent models, there are documented increases in the levels of phosphorylated Tau protein (p-Tau) taupathy associated with significant neuroencephalopathy (McKee et al., 2009).

In addition to the similar neuropathology observed in both the mLFP injury and mBBI results, mBBI showed a significant increase in phosphorylated Tau protein (**p-Tau)**, associated with significant neuroencephalopathy as early as 6 hours and persisting up to 30 days that appeared to show some correlation with increased RRRT in the injured rats (**Figure 11)** even as an overall biomarker for activated macrophages and microglia, Iba 1, did not show such a correlative response. This would be consistent with the concept of a broader spectrum of downstream damage to the brain and not a simple increase in p-Tau as a sole damaging agent.

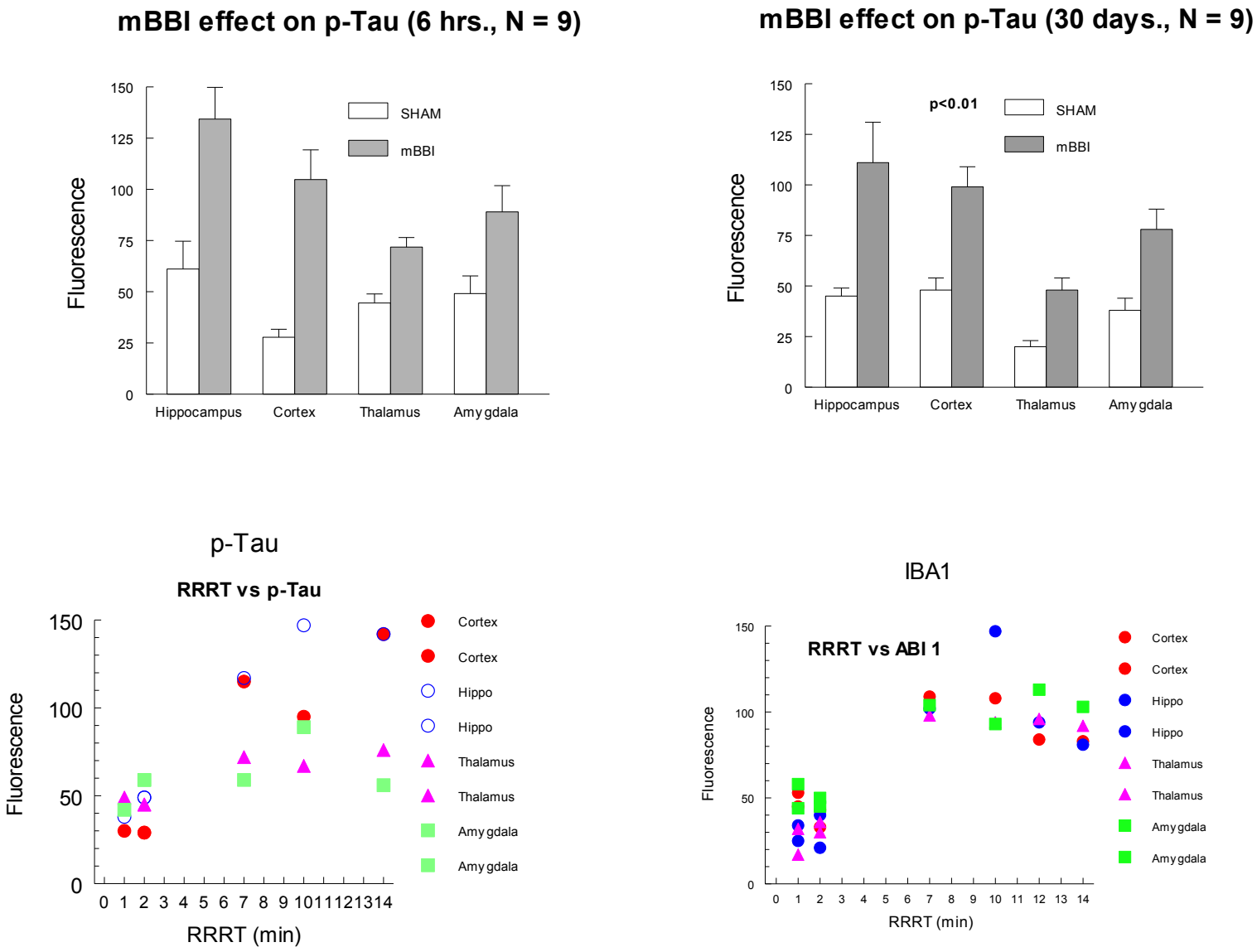


Figure 11. Effect of mLFP Injury and mBBI on p-au expression

Inflammatory cytokine receptor blockade after mLFP injury and mBBI (Kineret and Etanercept)

Given our initial demonstration of increased IL-1α/β and TNF-α after either mLFP injury or mBBI, we hypothesized that blocking the IL-1 receptor with Kineret agonist or the TNF-α receptor with its cognate antibody Etanercept would improve outcomes by ameliorating inflammation. Our results showed that 18 days after the treatments described in **Figure 12**, there were significant ameliorations of the neuropathology observed in cortex, hippocampus, thalamus and amygdala and the behavioral deficits resulting from mLFP injury (**Figure 13**). Combined treatments with Kineret and Etanercept did not significantly improve the beneficial effects observed after individual treatments. Also, there were no significant improvements at 18 days compared to those observed at 6 hours, leading to the conclusion that single treatment within the first 6 hours after injury (**Figure 13**) was as effective as the combined more extensive treatment protocol for 11 days described in **Figure 12**. Therefore, an early Kineret alone treatment 1 and 6 hours after injury was as beneficial as the chronic treatment with both therapeutic agents.

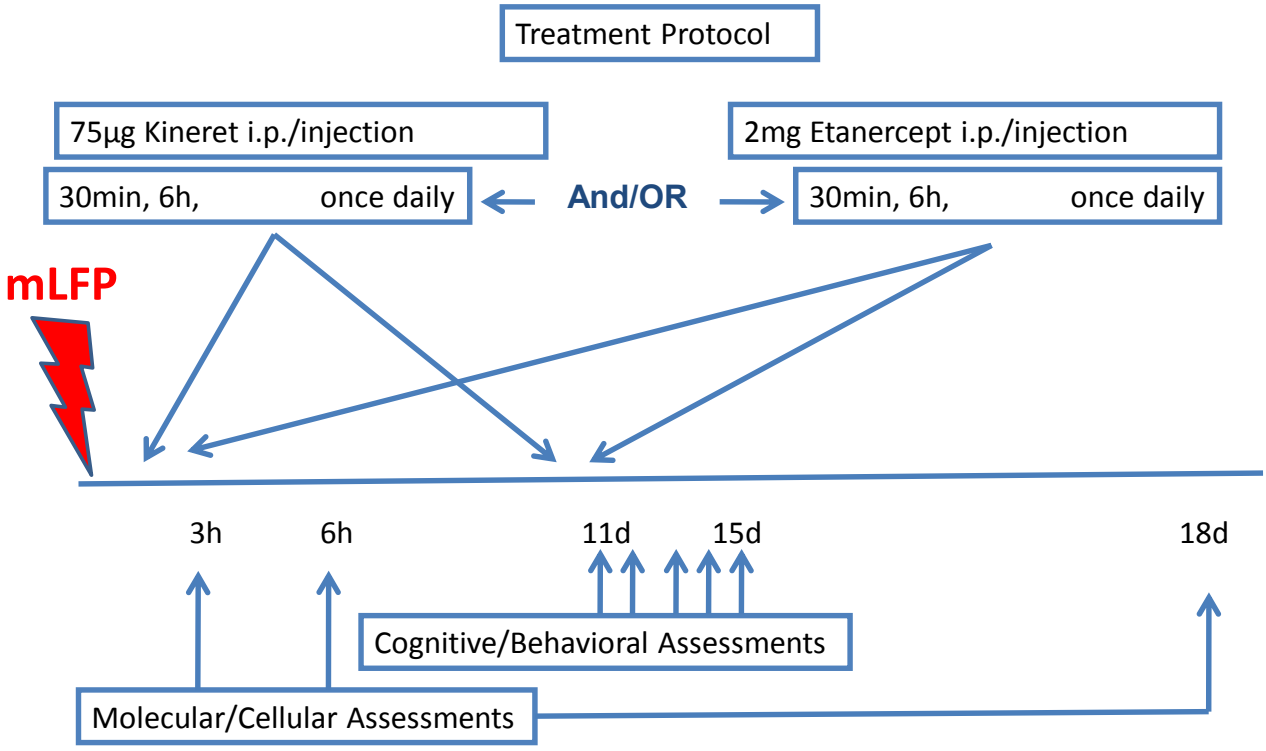
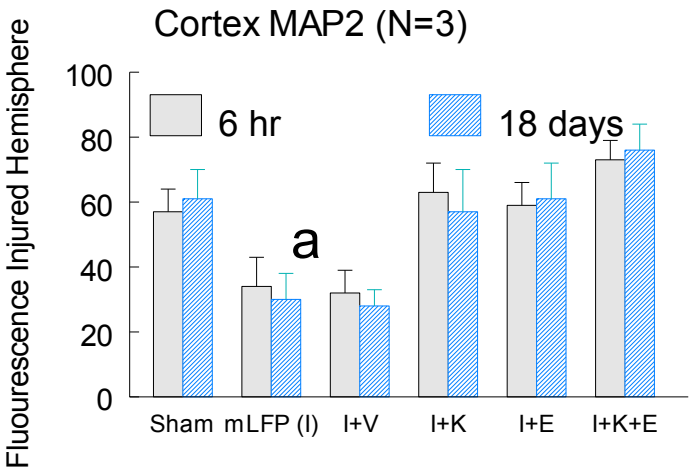
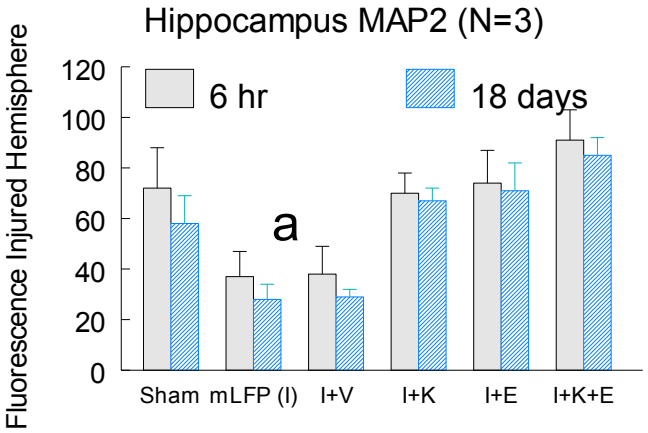
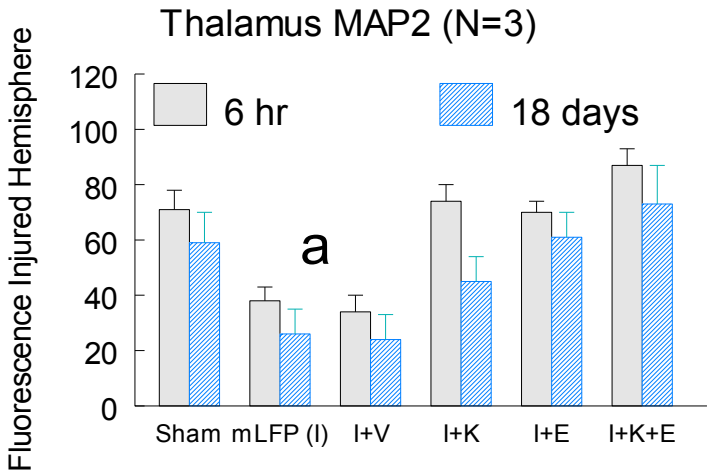


Figure 12. Treatment Protocol with Kineret and Etanercept blockade of IL-1 α/β and TNFα receptors respectively. Groups: naïve, sham, vehicle- and drug-treated.







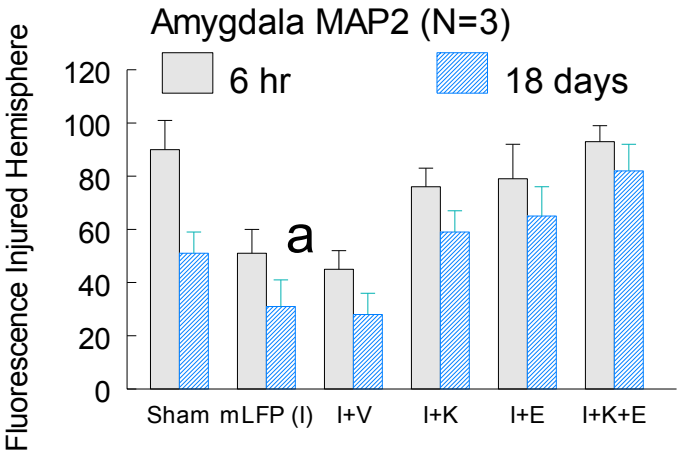
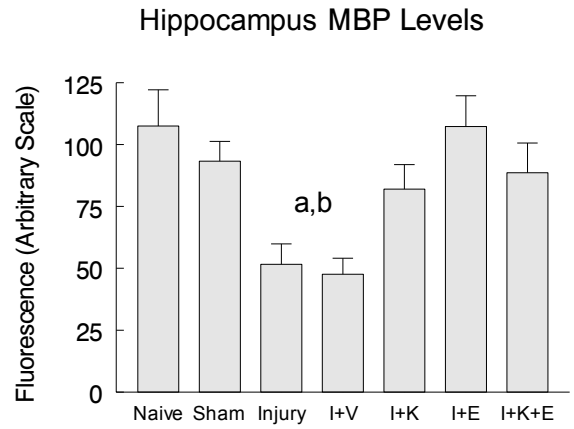
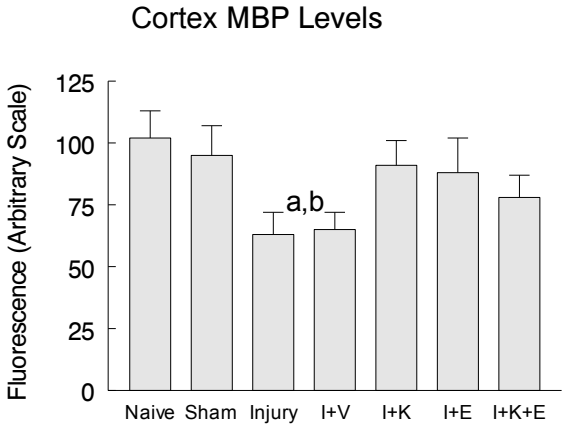
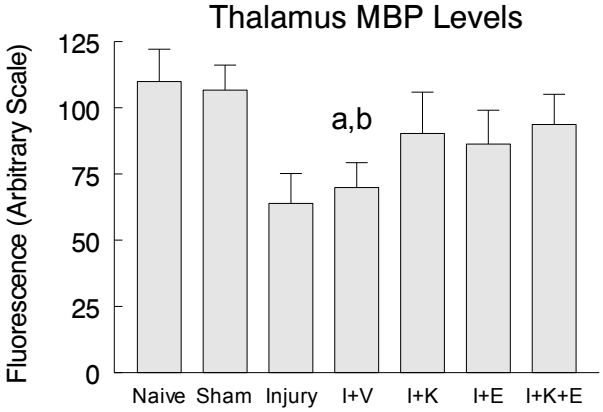


Figure 13a. Quantitation of immunofluorescence 18 days after mLFP injury. (a) = p<0.01 naïve or sham vs Injury or injury + vehicle (I+V); p<0.05 Injury or I+V vs I+Kineret (I+K) or injury + Etanercept (I+E) or both (I+K+E); all other comparisons are not significant.







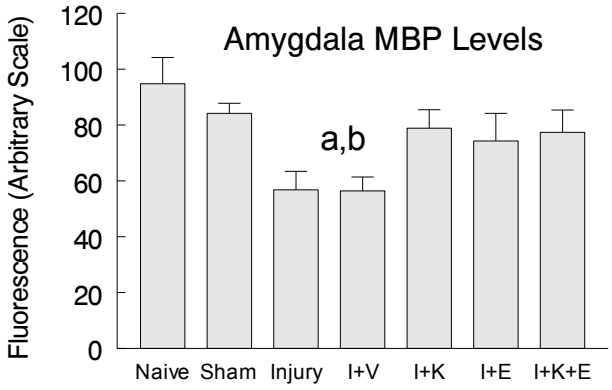
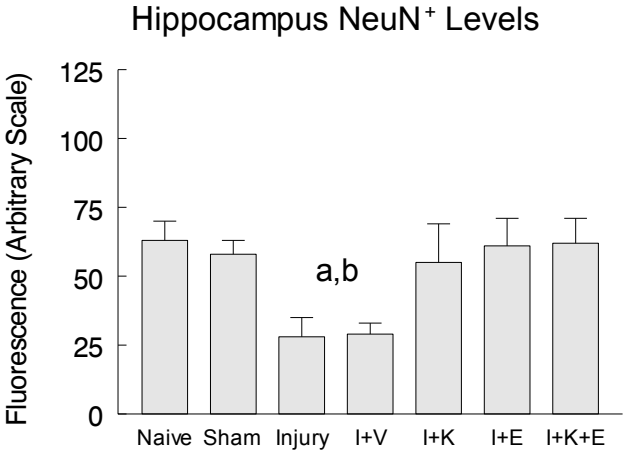


Figure 13b. Quantitation of immunofluorescence 18 days after mLFP injury. (a) = p<0.01 naïve or sham vs Injury or injury + vehicle (I+V); (b) = p<0.05 Injury or I+V vs I+Kineret (I+K) or injury + Etanercept (I+E) or both (I+K+E); all other comparisons are not significant. N = 9.



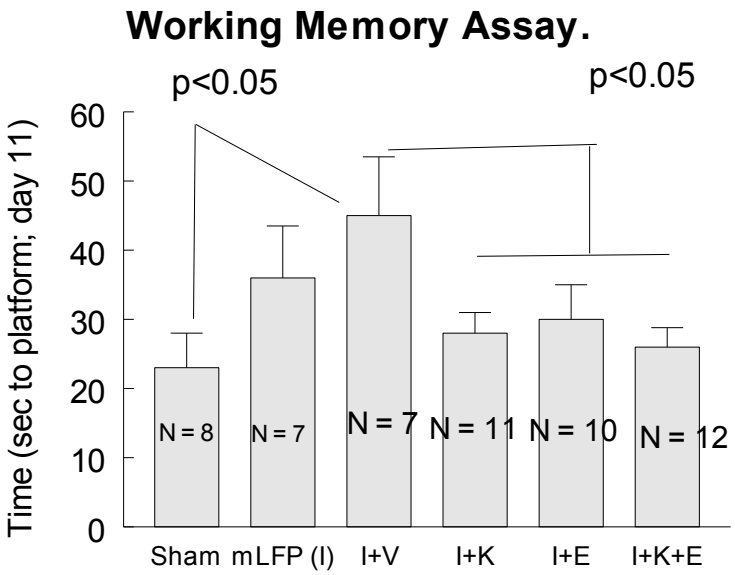


Figure 13c. Effect of treatments on working memory as assessed Quantitation of immunofluorescence 18 days after mLFP injury. (a) = p<0.01 naïve or sham vs Injury or injury + vehicle (I+V); (b) = p<0.05 Injury or I+V vs I+Kineret (I+K) or injury + Etanercept (I+E) or both (I+K+E)

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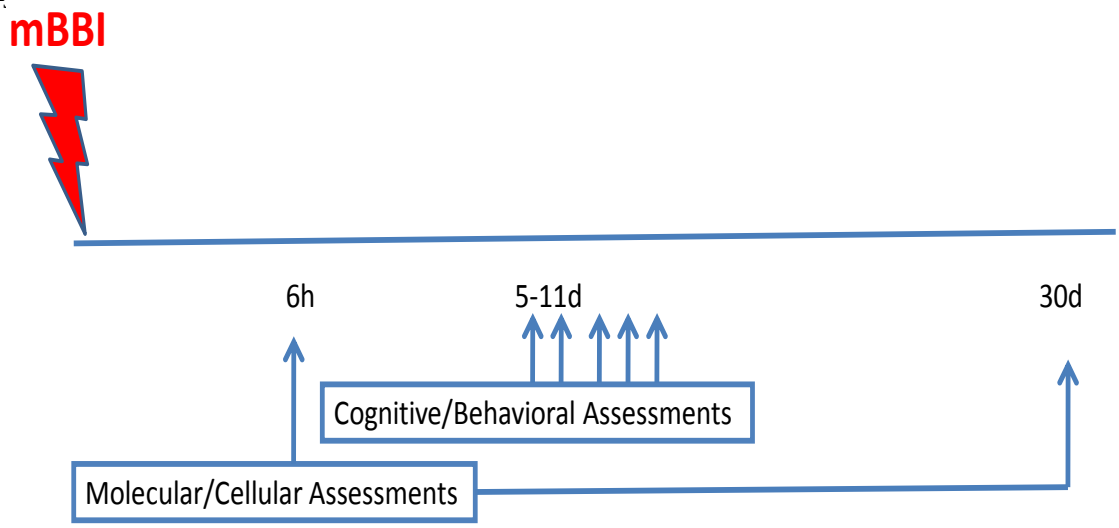

Figure 14. mBBI Protocol Groups: naïve, sham, and injured.

When rats were treated with Kineret alone acutely following mBBI using the same regime applied to mLFP injured rats, we observed a significant but not robust amelioration of p-Tau level increases at 30 days post injury (**Figure 15**). This can be interpreted as suggesting that while inflammatory triggered events contribute to cytokine mediated increases in p-Tau, there are other mechanisms at work.

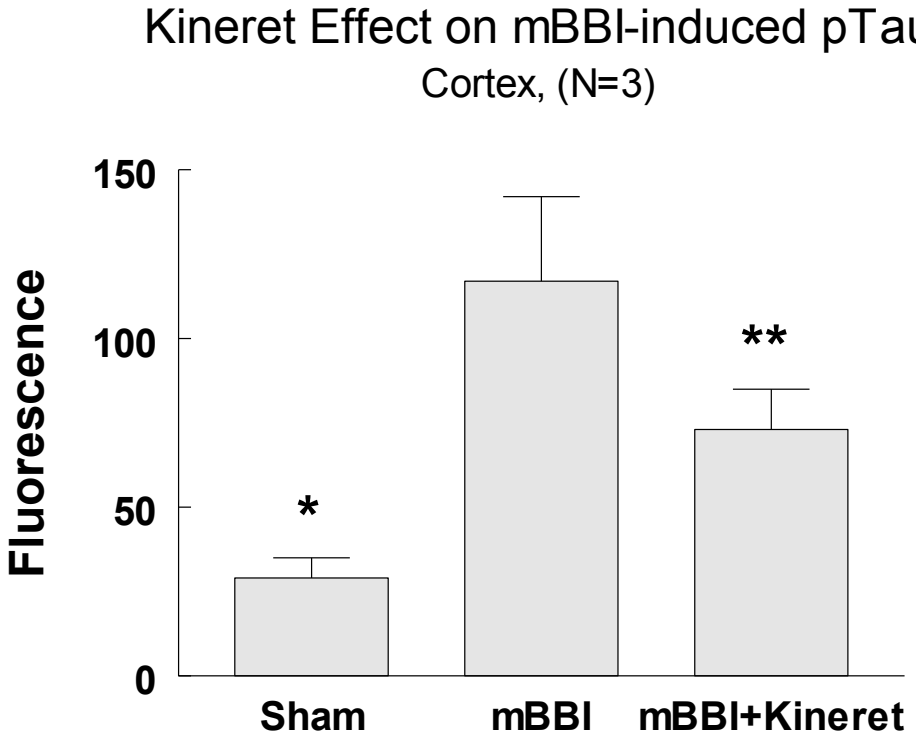

Figure 15. Effect of Kineret on mBBI-Induced pTau (30 days; * is p<0.01 compared to mBBI and p<0.05 compared to mBBI+Kineret; ** mBBI is p<0.05 compared to mBBI+Kineret.

Taken together, these results would confirm the value of using the RRT time as a measure of severity of insult and likely resultant neuropathology and psychomotor/behavioral/cognitive impairment to mTBI and more specifically based on our data to date mBBI. They also present substantive evidence in favor of the hypothesis that mBBI is a high risk factor for the development of encephalopathy over time given the increased presence of p-Tau as early as six hours after injury that persisted for up to 30 days. Lastly, our data to date on mBBI would suggest that aside from simple inflammatory responses, there may be other mechanisms responsible for the extent of encephalopathy in common with those present in age-related neurodegenerative disease. While there is a lack of direct clinical demonstrations of encephalopathy in mBBI perhaps eventual autopsy studies of VA patients with a clinical history of mBBI may determine the clinical importance of the observations we made in a rat model of mBBI.

Conclusion

Blocking the cytokine IL-1 and TNFα receptors with FDA-approved drugs can ameliorate mTBI outcomes in the rat.

Mild brain blast injury in the rat results in significant increases in established neuroencephalopathy and inflammation markers

Preclinical studies of these two drug treatments are necessary given their potential for therapeutic use

Reportable Outcomes

Perez-polo et al, 2013 see Appendix

Key Research Accomplishments

- We demonstrated that mTBI using two models of injury, mLFP and mBBI, stimulate the inflammatory cytokines IL-1 and TNFα
- We characterized inflammatory responses in mLFP impacting neuronal and glial losses and blood brain barrier function
- We showed that single treatments blocking the cytokine IL-1 receptor within 6 hours of the injury ameliorate the mLFP-induced neuropathology and behavioral deficits as well as having a beneficial effect on mBBI induced increases in p-Tau
- We demonstrated that measuring RRRT values provides good criteria for defining mTBI
- We determined the effect of mBBI on markers for neuroencephalopathy and inflammation

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Appendices n/a