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14. ABSTRACT Mild traumatic brain injury (mTBI), particularly mild "blast" injuries due to improvised exploding devices, result in long term impairment of cognition and behavior. Our hypothesis is that there are inflammatory consequences to mTBI that persist over time and in part cause the resultant pathogenesis and clinical outcomes. We used an adaptation (1 atm pressure) of the moderate to severe brain lateral fluid percussion (LFP) brain injury rat model. Our "mild" LFP (mLFP) injury resulted in acute increases in IL-1 α / β and TNF α levels, macrophage/ microglial and astrocytic activation, and blood brain barrier (BBB) disruption; which may contribute to brain pathology. These results suggest therapeutic opportunities to treat mTBI via blockade of the IL-1 α / β and TNF α receptors. We further hypothesize that blockade of these receptors, IL-1 α and IL-1 β , both bind to the IL-1R receptor, will block inflammatory cytokine signaling after mTBI and improve outcomes by ameliorating inflammation. Our goal is to develop, implement and assess interventions with two FDA-approved drugs (Kineret or Interleukin-1 Receptor Antagonist, IL-1Ra and Etanercept or antibody to the Tumor Necrosis Factor Receptor alpha, TNF α) singly or in combination in a rat model of mTBI (mild lateral fluid percussion or mLFP) that will ameliorate the mTBI-induced inflammation and therefore the resultant neuropathology and neurological deficits. We also proposed to test our hypothesis on a blast model of mTBI on two models. An earlier model relied on an injury consisting of a primary blast exposure followed by blunt impact injury, the Vanderberg model. The other more recently implemented model is the Advanced Blast Simulator. During this last year, we assessed the beneficial effects of individual vs combined treatment with Kineret and Etanercept; we also determined an optimal time course of treatment.					
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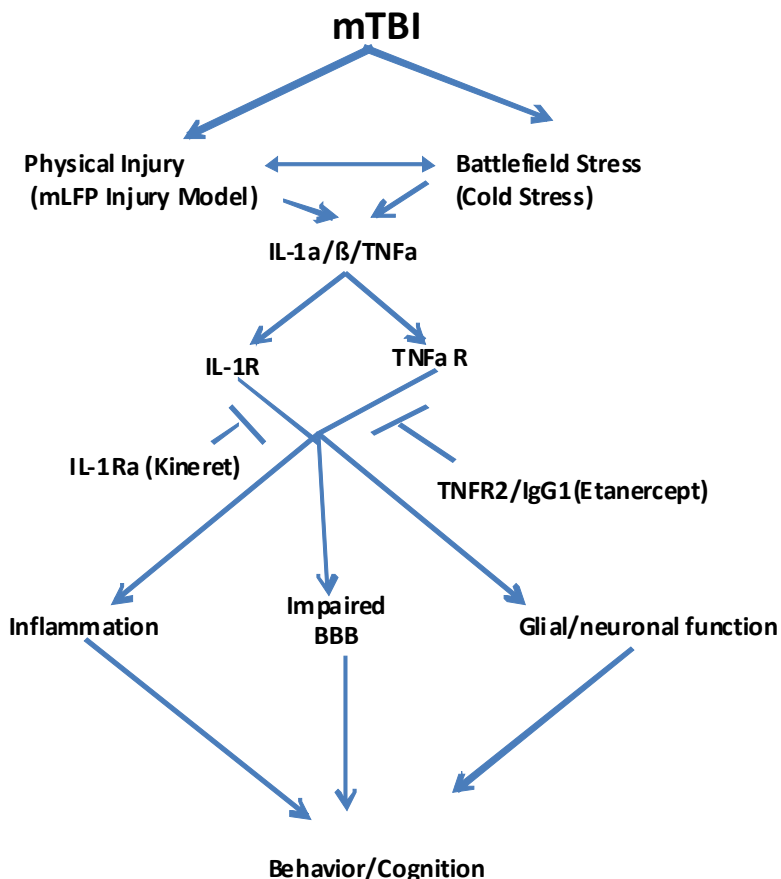
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INTRODUCTION

Mild traumatic brain injury (mTBI), particularly mild “blast type” injuries due to improvised exploding devices, result in long term impairment of cognition and behavior. Our central hypothesis is that there are inflammatory consequences to mTBI that persist over time and in part are responsible for resultant pathogenesis and clinical outcomes. Because of the diffuse nature of the injuries classified as mTBI, no single model of mTBI is likely to match 100% the spectrum of mechanisms and outcomes that have been documented for clinical mTBI in a military context. We used an adaptation (1 atm pressure) of a well characterized moderate to severe brain lateral fluid percussion (LFP) brain injury rat model. This is one model that presents many of the clinical issues associated with mTBI (see also report by Dr. Pramod Dash). Our “mild” LFP (mLFP) injury resulted in acute increases in IL-1 α/β and TNF α levels, macrophage/microglial and astrocytic activation, and blood brain barrier (BBB) disruption; all of which may contribute to brain pathology. These results suggest therapeutic opportunities to treat known cognitive and behavioral dysfunction associated with mTBI via blockade of the IL-1 α/β and TNF α receptors. We hypothesize that blockade of these receptors, IL-1R and TNFR2, will block inflammatory cytokine signaling after mTBI and improve outcomes by ameliorating inflammation. Our approach is to implement and assess interventions with the FDA-approved drugs Kineret an Interleukin-1 Receptor Antagonist (IL-1Ra) and Etanercept an antibody to the Tumor Necrosis Factor Receptor 2 (TNFR2) singly or in combination in mLFP injury expecting to ameliorate mLFP injury-induced inflammation and resultant neuropathology and neurological deficits (**Figure 1**). We also proposed to test our hypothesis on a blast model of mTBI. At present there are two models available to us (See also report by Dr. Douglas DeWitt). The two blast models rely on the use of two different instruments. An earlier model relied on an injury consisting of a primary blast exposure followed by blunt impact injury, the Van Den Berg model, named after A.C. Van Den Berg. The other more recently implemented model is the Advanced Blast Simulator (ABS) described in a previous report and in Dr. DeWitt’s report to you. The basic approach being for Dr. DeWitt to perform vascular characterization while we perform characterization focused on inflammation. During this last year, we

assessed the beneficial effects of individual vs. combined treatment with Kineret and Etanercept; we also determined an optimal time course of treatment.

Figure 1. Specific Aim 3.3 Schematic



Body of the Report

SPECIFIC AIMS

Specific Aim #3: To develop new and innovative treatment strategies for MTBI and provide the preclinical and phase 1-2 testing of treatments found to improve outcome.

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

As presented in previous reports and described in two manuscripts under review at the Journal of Neurotrauma, we have shown in an experimental rodent model of brain injury adapted from the moderate to severe lateral fluid percussion (LFP) brain injury model (mLFP) that the key brain inflammatory cytokine (Interleukin 1 (IL-1) and Tumor Necrosis Factor alpha (TNF α) protein levels increase as early as 3 and 6 hours across several brain structures and there is a return to basal levels by 18 days post-injury, except at the injury site where there is an IL-1 β presence. There was also a significant increase in activation of astrocytes and microglia which were shown to be the source for the increased levels of IL-1 and TNF α . We also demonstrated the mLFP injury resulted in impairment of the blood brain barrier (BBB) as evidenced by an increased presence of blood borne proteins such as albumin and IgG as well as an increase in the BBB dysfunctional marker SMI-71 by 6 hours post injury and that this impairment persists up to 18 days (Perez-Polo et al., submitted to J. Neurotrauma; DeWitt et al., submitted to J. Neurotrauma).

Progress to Date (August 1, 2011 to July 31, 2012)

There were mLFP injury-induced gross alterations in the neuronal cell pattern displayed by mLFP injured hippocampi that were not present in the sham-treated rats. The hippocampal structures were clearly distorted 18 days after injury as a result of the insult. This was evidenced in all injured brains.

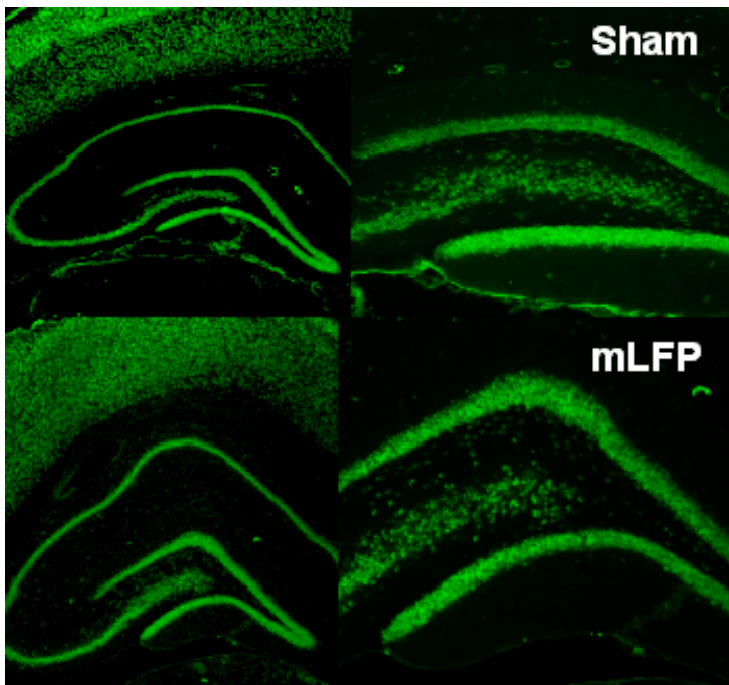


Figure 2. Effect of mLFP injury on hippocampal structure.

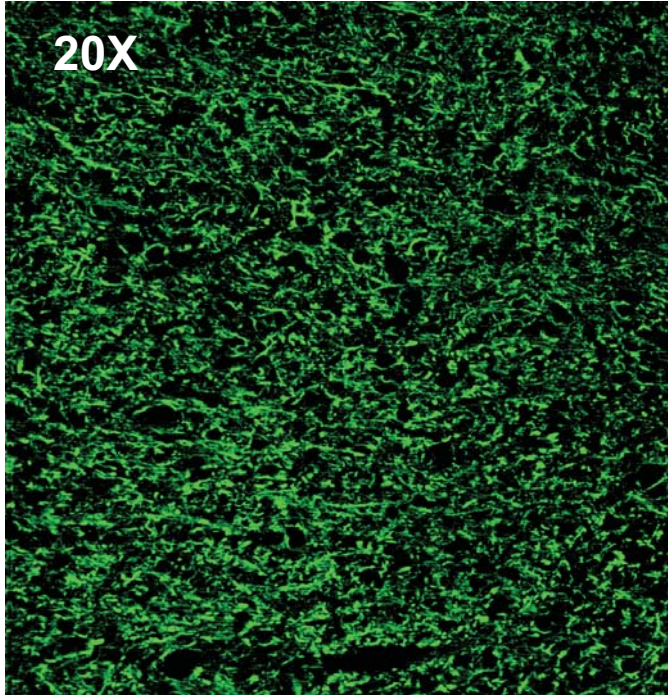
A more molecular/cellular representation of functional loss relied on assessments of glial and neuronal damage to structures vital to brain function, such as neuronal cell loss and amounts of axonal and myelin structures as represented by the levels of Neuronal Nuclear protein (NeuN), microtubule associated protein 2 (MAP2) and myelin basic protein (MBP) after mLFP injury as detected by immunohistochemistry analysis. NeuN is a soluble nuclear protein that binds DNA. Antibodies to NeuN cross-react immunohistochemically with nervous tissue in mouse, rat, and human neurons. MAP2 is involved in microtubule assembly and serves to stabilize microtubules. MAP2 is prevalent in neuronal dendrites and is critical to synaptic function. MBP is a major constituent of the myelin sheath of oligodendrocytes and Schwann cells in the nervous system. Deficits in both these measures are likely outcomes of cytokine-mediated inflammation and are likely to contribute to working memory

deficits and behavioral impairments, consistent with reports of the clinical impairments documented for many military personnel that have experienced mTBI in the battlefield.

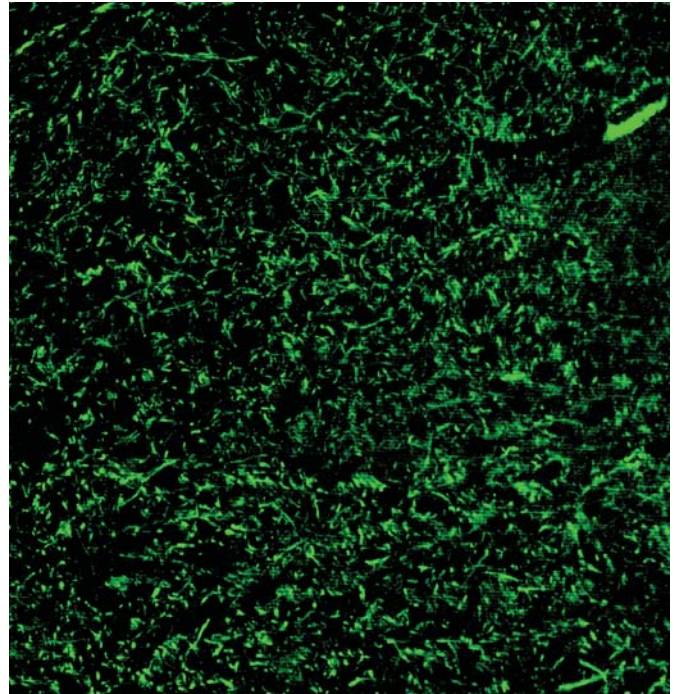
Although the functions of the thalamus and the amygdala are complex and inter-related, thus difficult to isolate the function of the two individual brain structures, it is well known that the thalamus plays a major role in regulating emotional function and the ability to interact socially with others and the amygdala is responsive to stress and regulates stress response mechanisms in brain and interactions that have been shown to be mediated in part by the neuroendocrine, sympathetic and sensory systems. Thus, we included both in our assessments of MAP2 and MBP alongside hippocampus and cortex in many of our experiments.

Figure 3. Representative thalamic MBP Immunofluorescence 18 days post-mLFP Injury (20X). $p < 0.01$ sham vs injury. N = 3.

(A) Sham



Injury



(B)

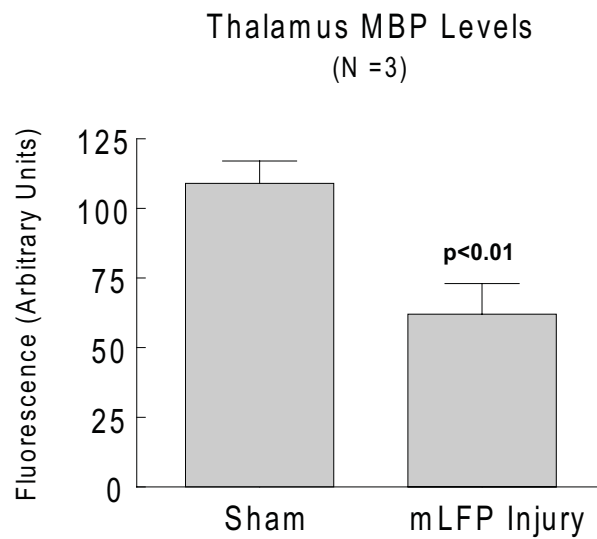
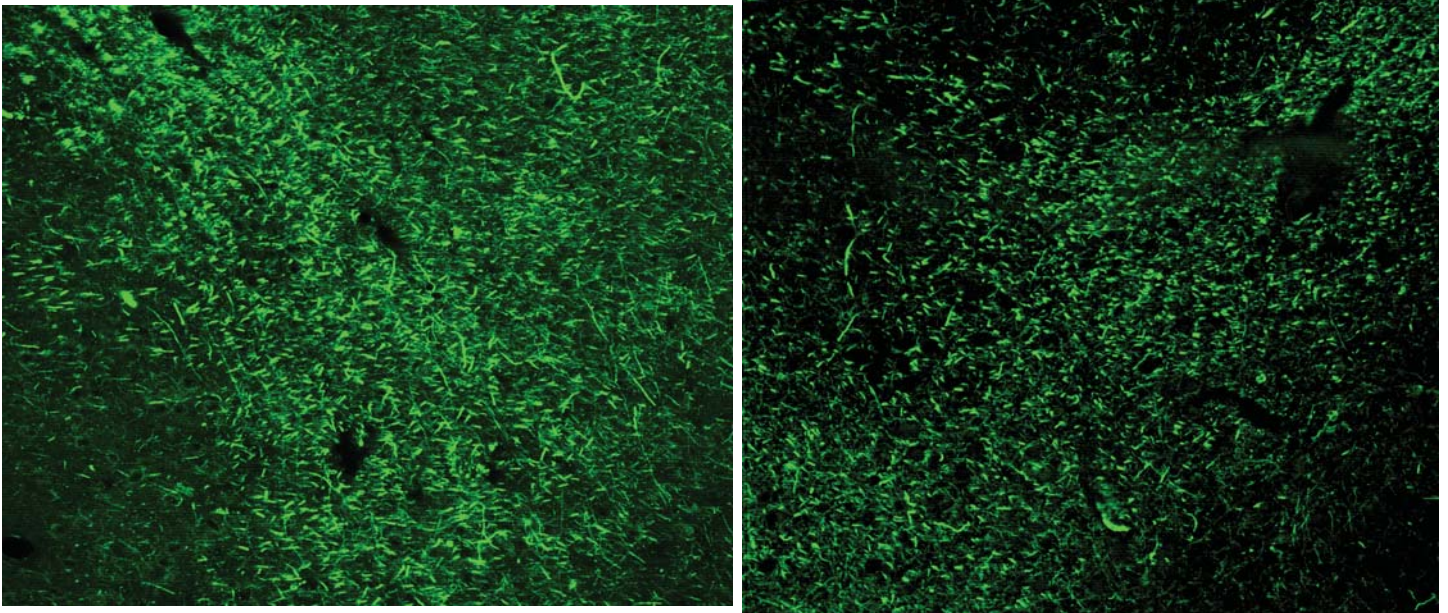


Figure 4. Representative amygdala MBP Immunofluorescence 18 days post-mLFP Injury (20X). $p < 0.01$ sham vs injury. N = 3.

(A) Sham

Injury



Amygdala MBP Levels
(N = 3)

(B)

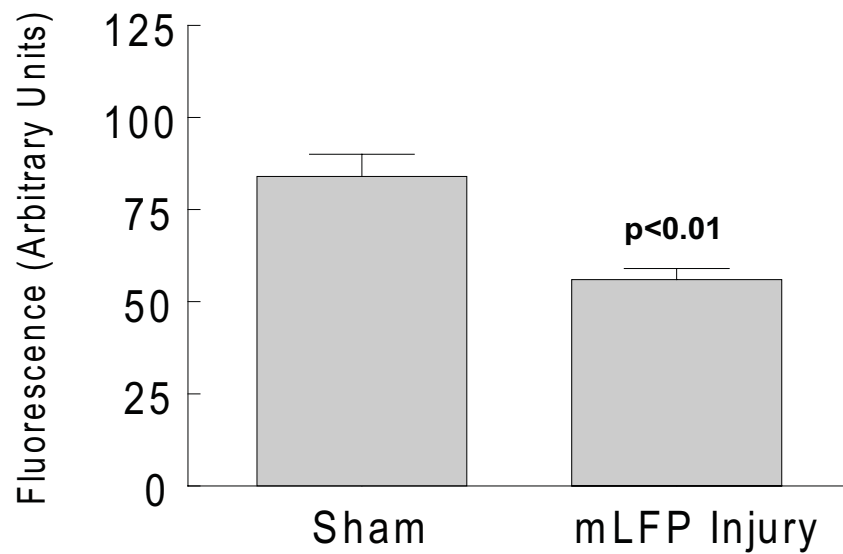


Figure 5. Quantitation of Thalamus MAP2 Immunofluorescence. ($p < 0.01$ Sham vs mLFP Injury; N = 3)

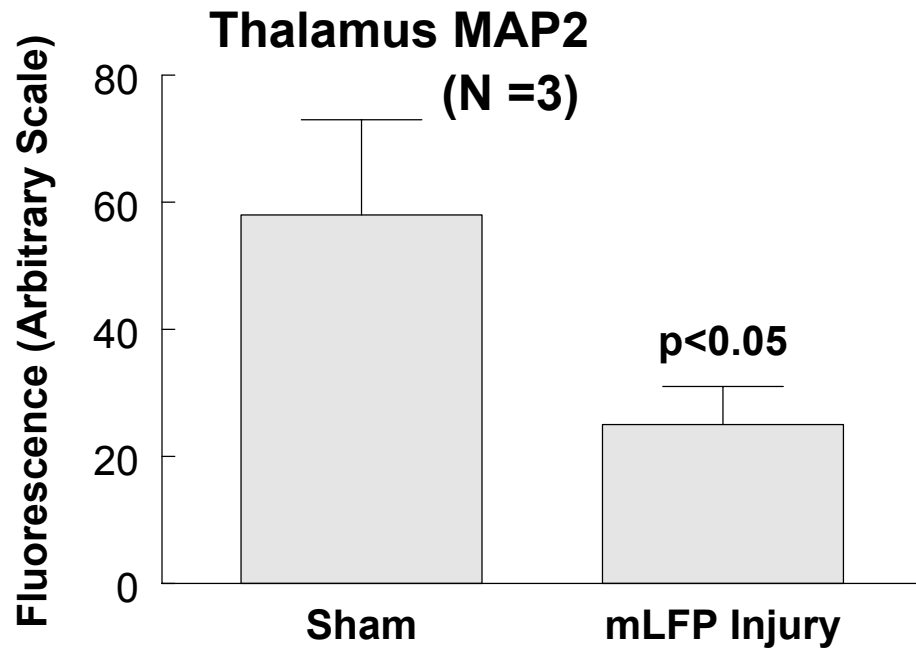
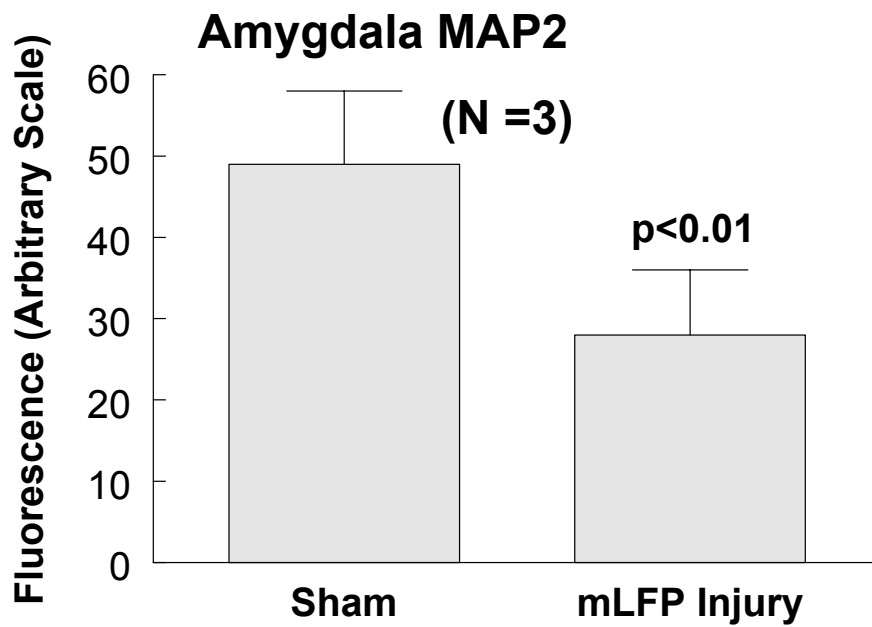


Figure 6. Quantitation of Amygdala MAP2 Immunofluorescence. ($p < 0.01$ Sham vs mLFP Injury; N = 3)



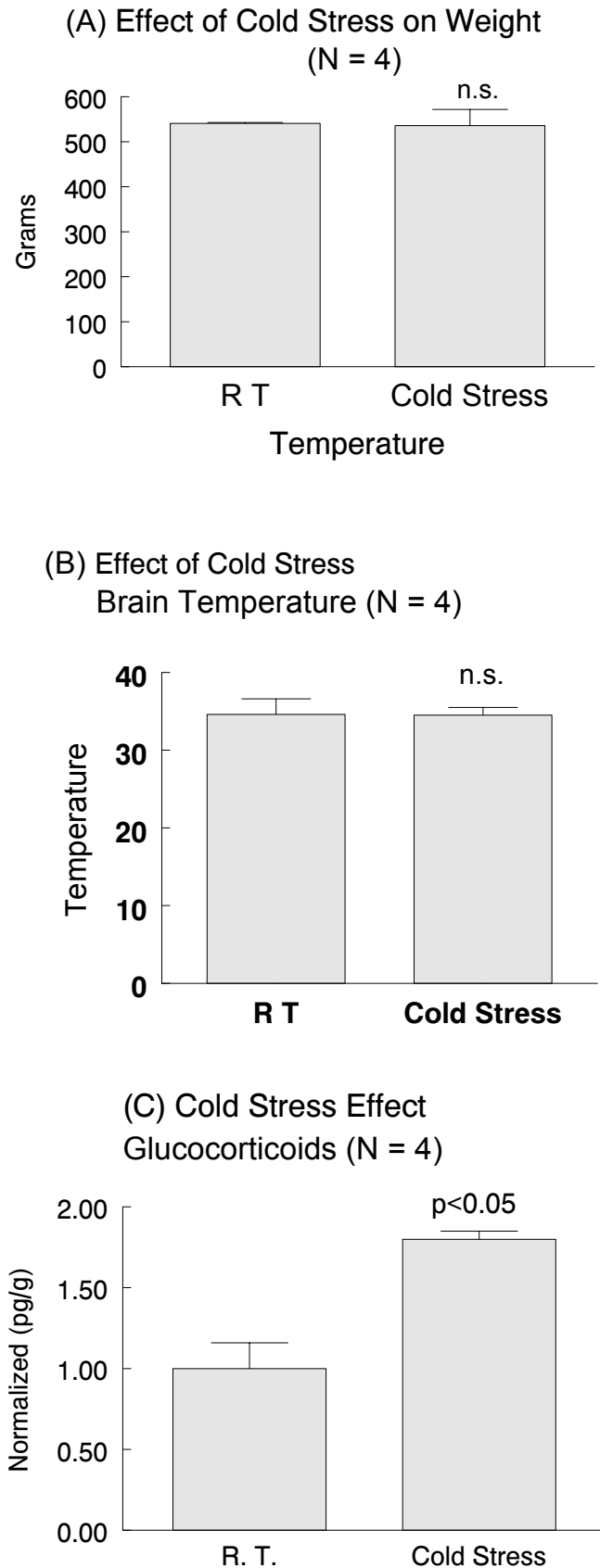


Figure 7. Stress Effects. One hour cold stress (5°C) effect on weight, brain temperature (n.s.=not significant) and corticosteroid fecal content ($p<0.05$), N =4.

Mild blast injuries experienced in a battlefield take place against a background of physical, emotional and mental stress. One could argue that there is a PTSD component to all battlefield mTBI experiences. In response to our behavioral findings and the known literature on the roles of the thalamus and amygdala in stress responses, we began preliminary development of a protocol to our rodent model of mLFP injury to better fit the battlefield mTBI context by incorporating stress into the mLFP injury model based on exposure of rats to cold stress. While measures such as righting reflex times, footfall errors, and beam balance ability are gold standard measures in moderate and severe TBI, these did not appear to be adequate to fully comply with our goals. For example, critical analyses of the clinical data suggested that working memory assessments are more likely to be more appropriate compared to standard Morris water maze tests for long-term memory acquisition. We found two reproducible and sensitive measures that reflect stress-derived anxiety in assays based on determination of fecal pellet number and glucocorticoid fecal levels in response to stress. “Cold stress” is a stressor, which we have used in the past (Foreman et al., 1992). Exposure of rats to 1 hour cold stress (5°C) blocks neurotrophin action in hippocampus and induces microglial and astrocytic activation in hippocampus and hypothalamus where there is increased IL-1 β -immunoreactivity (Foreman et al., 1992). “Cold stress” has also been shown to have significant effects on thalamus and amygdala. We found that 1 hour cold stress (5°C) had no confounding effect on brain temperatures while stimulating significant increases in number of fecal pellets and glucocorticoid concentrations in fecal pellets (**Figure 7**). This would suggest that incorporating cold stress into mLFP injury would be practical and valuable to a preclinical study, albeit this is beyond the scope of the present study.

Our major focus during this past year has been to block the IL-1 α/β and TNF α signaling pathways with FDA-approved Kineret and Etanercept and reduce the neuropathology resulting from this injury-triggered inflammatory cascade so as to improve outcomes using treatment modalities that best mimic the military injury scenario. We used i.p. delivery, more suitable to the battlefield environment. Individual treatments were given at 30min, 6hr and then daily for 11 days (**Figure 8**).

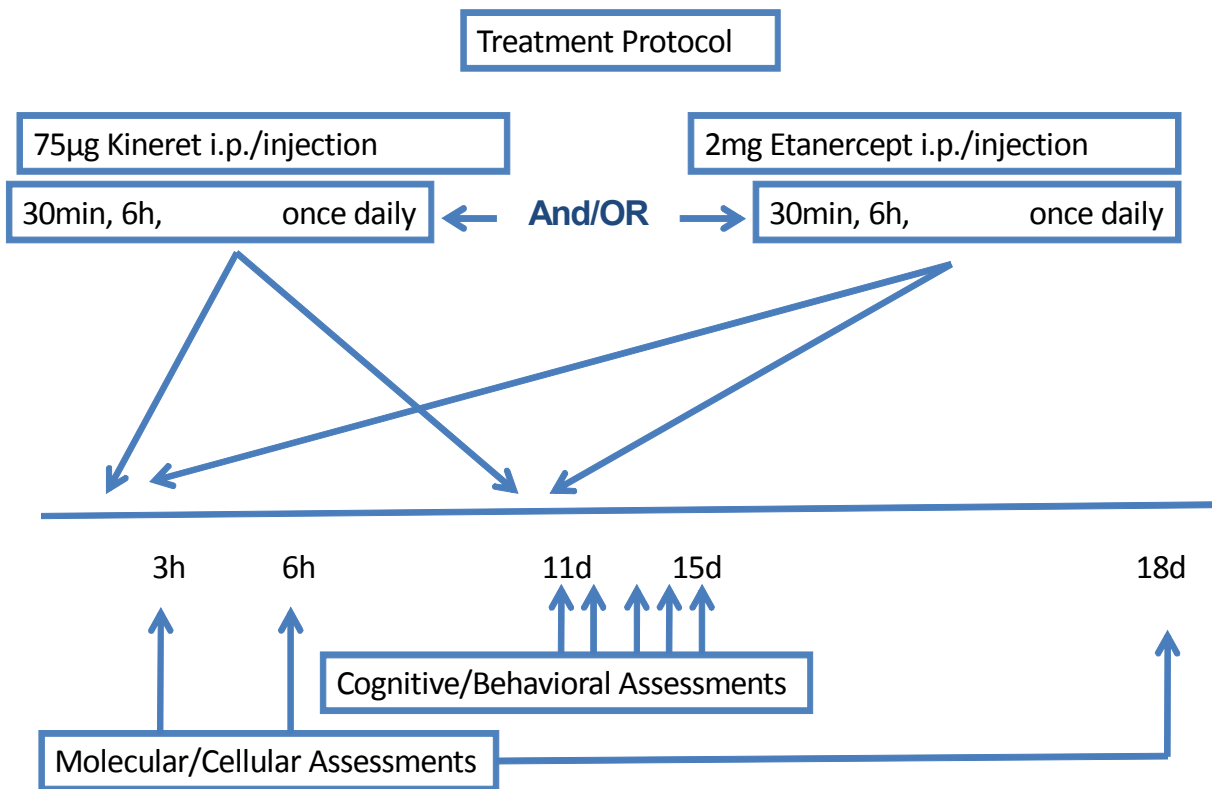
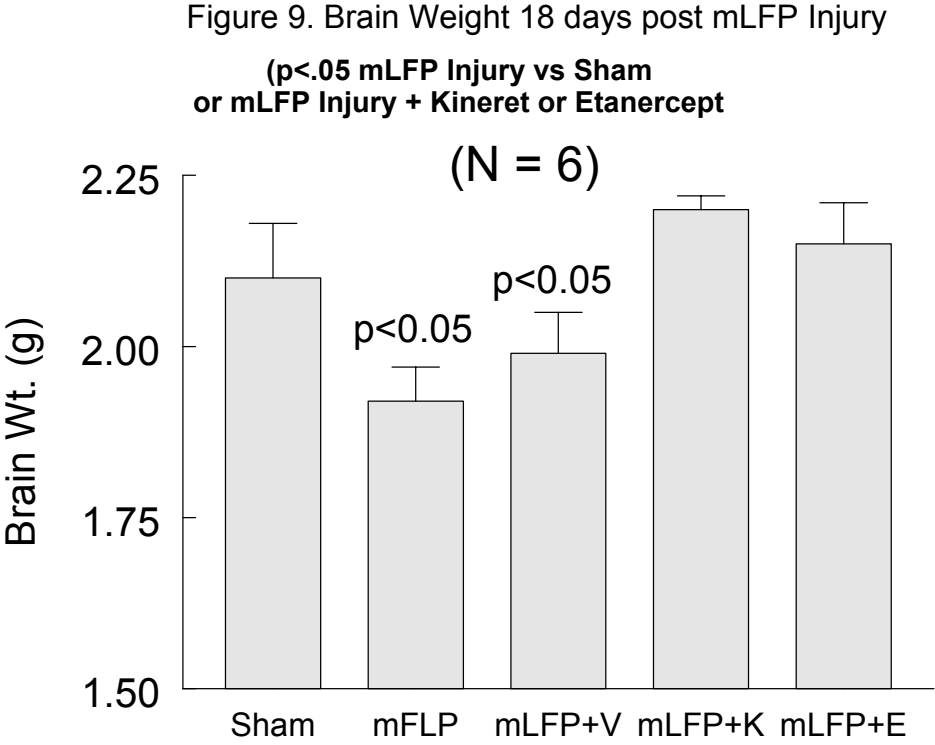


Figure 8. Treatment Protocol with Kineret and Etanercept blockade of IL-1 α/β and TNF α receptors respectively. Groups: naïve, sham, vehicle- and drug-treated.

When rats were subjected to mLFP injury and treated with Kineret or Etanercept individually, there was a significant amelioration in the mLFP injury-induced decrease in brain weight. There was no significant difference between the outcomes from the two individual treatments after 18 days (**Figure 9**).



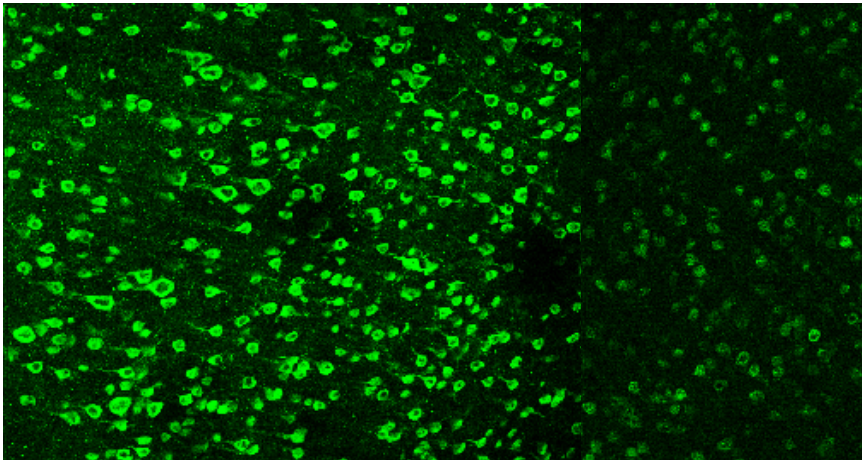
One interesting observation, not unique to our project as other members of the consortium have made similar observations in their evaluations of neuronal losses after injury (Grill, personal communication), is that mTBI in the rat elicits neuronal morphological changes best viewed with NeuN immunohistochemistry (**Figure 10**). We observed two distinct patterns of staining for NeuN after mLFP injury. For example, when injured rats were treated with vehicle vs. combined Kineret and Etanercept, there were groups of cells that displayed the classical bright stain pattern of neurons that is well documented in the literature, as well as a more “faded” less distinct pattern of NeuN staining that we and others in the consortium are seeing in several different brain structures after TBI irrespective of the particular model being studied. The latter is likely to be a pathological response that is characteristic of TBI itself. This “fade out” of NeuN stain was most obvious in the thalamus and cortex. Our hypothesis for this phenomenon is that early on after injury, the inflammatory disruption of the blood brain barrier activates astrocytes and microglia, triggering synaptic function dysfunction due to apoptotic cell death processes that result in chromatin clumping and a pronounced DNA presence in cytoplasm prior to the onset of cell death. Given the DNA binding properties of NeuN, it is not surprising to find a more diffuse and hence “faded” NeuN presence in these cells. The second observation was a shift from a preponderance of “bright” NeuN+ cells with a minor component of “faded” cells at 6 hours to the opposite, a preponderance of “faded” NeuN+ cells with a minor component of “bright” cells at 18 days after injury. This is consistent from an early appearance of an event that is eventually lethal, “fading”; to the eventual loss of cells to an apoptotic like neuronal cell death, an absence of “bright” cells.

Figure 10. Representative images of “bright” NeuN+ cells and “fade d” NeuN+ cells 18 days after mLFP injury (Parietal Cortex)

mLFP + Kineret

mLFP + Vehicle

20X



We assessed neuronal survival by counting NeuN+ cells, a well accepted protocol for neurons that lends itself well to cell counting due to its more confined nuclear presence. We counted cell profiles of (nuclei, cell soma, etc.) were counted in specific anatomic regions, such as hippocampus CA1, dentate gyrus, parietal cortex, thalamus, and amygdala on histological sections that were consistently 3 um in thickness, from at least three sections per animal, for at least three different animals, averaged and calculated as profile counts within that specific region/per animal/per side and considered as one data point per side (ipsilateral or injured vs. contralateral). Thus, the final number is a mean number of profiles counted within a three dimensional region (area of anatomical region x thickness of section). Since each region compared is similar, and the thickness of the sections were not statistically significant between different animals or brain hemispheres, we report our counts as number of cells with the understanding that the counts are stereological estimates based on profile counts. All counts were repeatedly done blind by three different individuals to eliminate bias on images obtained at 20X. Thus, we averaged for each individual brain the average of counts as determined by the three individuals counting each slice and then determined the mean for the slice +/- SD. While there were differences in resulting counts for any slice for the three different individuals counting, the relative differences between samples were consistent and there was a 10% or less variation for any sample count across individual counts from the three individuals performing counts. For myelin and MAP2 images we measured image intensity by Meta Morph offline Meta Imaging Series 6.1 where the intensity values from color images are weighted equally (intensity + [R+G+B]/3).

The individual Kineret and Etanercept treatments also significantly ameliorated mLFP-induced cell, axon and myelin hippocampal losses as early as 6 hours after injury (**Figure 11-12**). These beneficial changes persisted up to 18 days after the mLFP injury (**Figure 10-14**). However, there was no further enhancement of benefits at 18 days when compared to benefits observed at 6 hours post mLFP injury. Interestingly, we saw no significant differences in this outcome measure when comparing the two treatments although there was a trend showing an advantage to the Kineret or Etanercept treatment for different biomarkers in different brain structures. We observed a similar pattern at 18 days post-mLFP injury (**Figures 13-14**).

Figure 11. Effect of Kineret and Etanercept Individual Treatments on Hippocampal Neuronal Survival measured as number of NeuN⁺ cells. ($p < 0.01$ mLFP Injury (I) vs Sham, mLFP+Kineret (I+K) or Etanercept (I+E); but not mLFP injury+Vehicle (I+V)(N = 3)

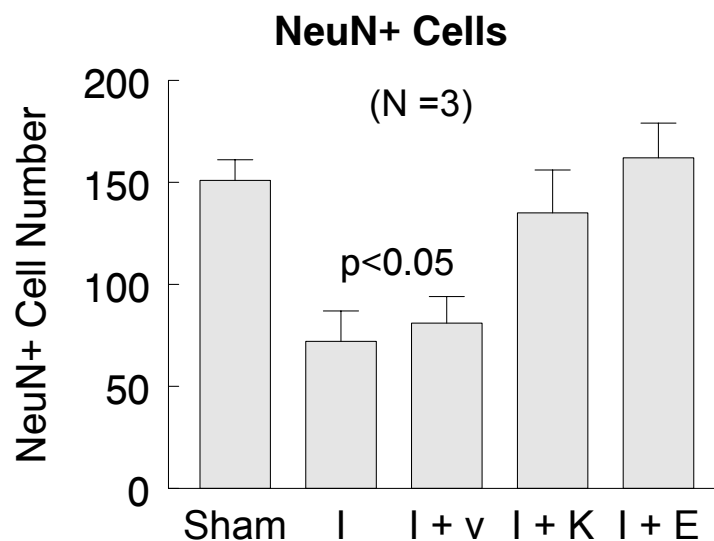
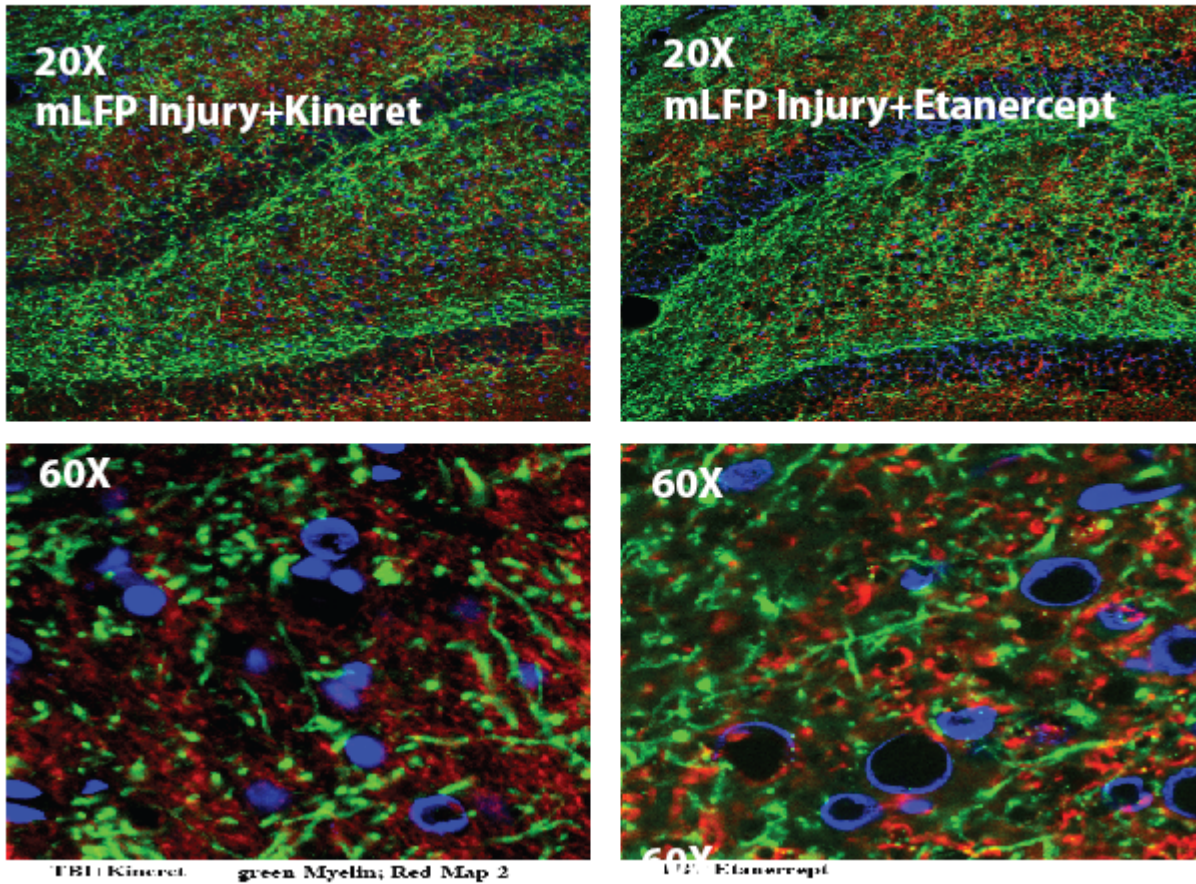


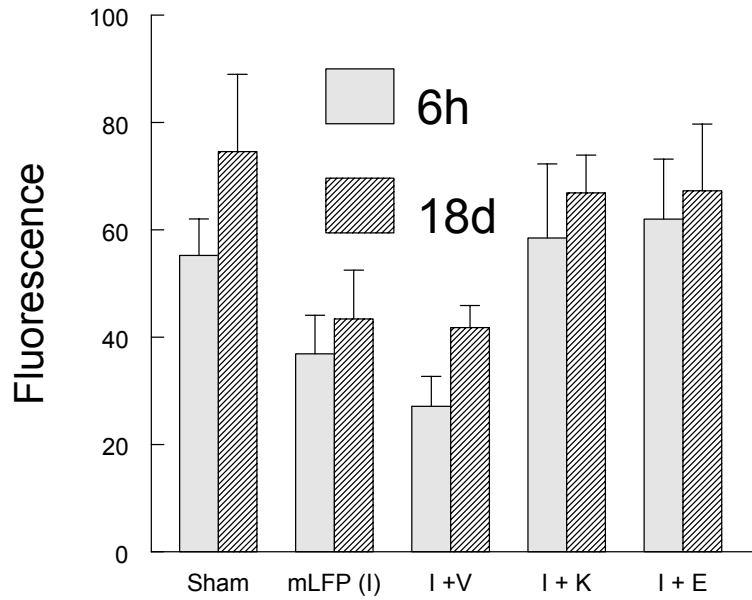
Figure 12. Effect of Kineret and Etanercept Treatment on Hippocampal neurons. (A) Representative Immunohistochemistry. MAP2 = Red; MBP + Green; DAPI = Blue (20X; 60X). (B) Quantitation at 6h and 18 days post-injury. Injured values are $p < 0.05$ significantly lower than shams or treated hippocampi.

(A)

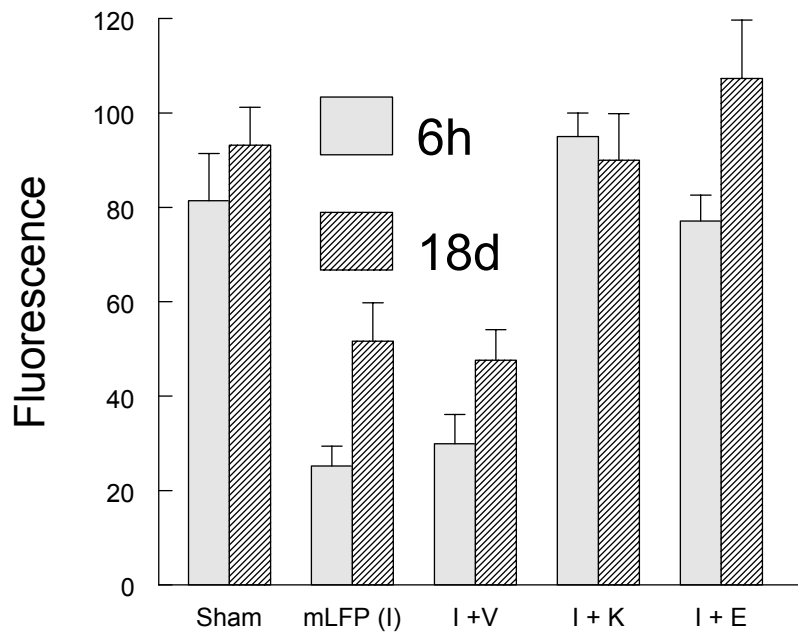


B)

MAP2 (6h;18d)
(N = 3)



MBP (6h; 18 days)
(N = 3)



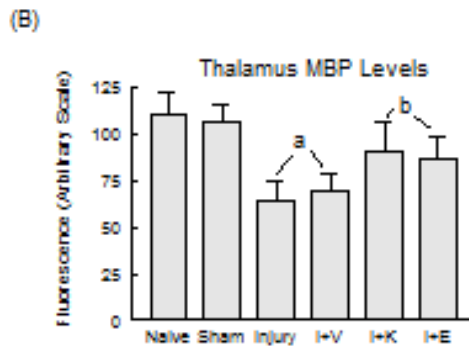
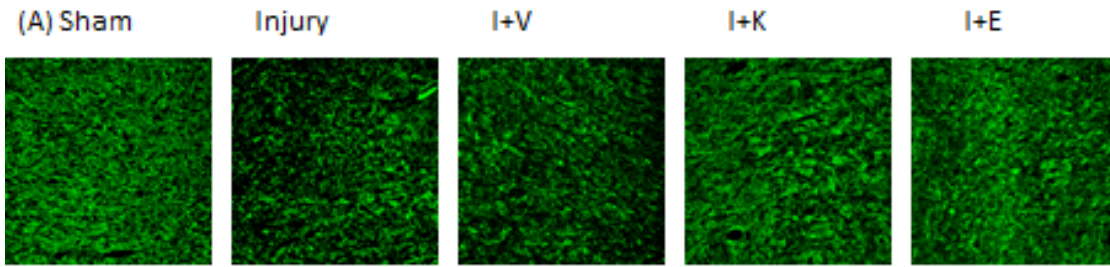


Figure 13. (A) Representative immunofluorescence from Thalamus sections for MBP (GREEN) levels 18 days post-mLFP Injury with Vehicle, Kineret and Etanercept Treatment (20X). (B) Quantitation of immunofluorescence. a = $p < 0.01$ naïve or sham vs Injury or injury + vehicle (I+V); b = $p < 0.05$ injury + Kineret or injury (I+K) + Etanercept (I+E); all other comparisons are not significant. N=3.

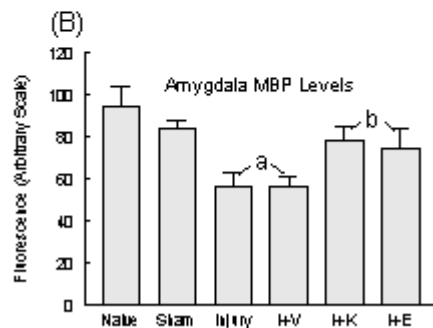
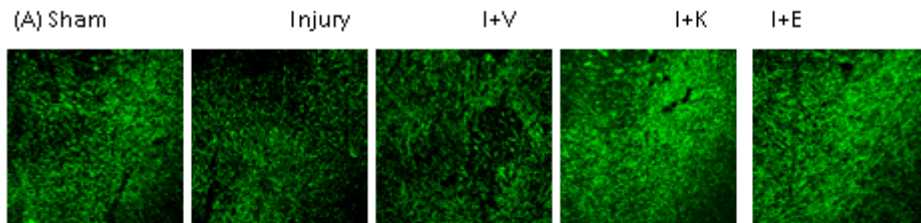


Figure 14. (A) Representative immunofluorescence from Amygdala sections for MBP (GREEN) levels 18 days post-mLFP Injury with Vehicle, Kineret and Etanercept Treatment (20X). (B) Quantitation of immunofluorescence. a = $p < 0.01$ naïve or sham vs Injury or injury + vehicle (I+V); b = $p < 0.05$ injury + Kineret or injury (I+K) + Etanercept (I+E); all other comparisons are not significant. N= 3.

Combined Treatments. Since February we have treated mLFP-injured rats **simultaneously** with Kineret and Etanercept using the same research design we found useful in the individual treatments (See **Figures 15-18**). We had some concerns about using a combination treatment, given the likely immunosuppressive effects of these drugs consistent with their anti-inflammatory role. However, given the relatively short duration of treatment, eleven days, we felt that a clinical regimen of similar duration can easily be managed. Initially, we focused on cell markers for neuronal death and axonal integrity (NeuN and MAP2) and myelin (MBP) as indices of pathology.

We performed mLFP injury and combined treatments of both Kineret and Etanercept to determine if there were synergistic effects given the different time responses in IL-1 and TNF α induction after mLFP injury. A thorough analysis showed that there was no benefit from the combined treatment compared to either of the two individual treatments as assessed by neuronal cell number, axon or myelin density in hippocampus, thalamus, or amygdala or behavior (**Figure 15-18**). Thus, individual treatment with either drug for up to 6 hours after mLFP injury is as beneficial as combined treatment for up to 6 hours or 18 days.

Figure 15. Thalamus MAP2 Intensity after 18 days

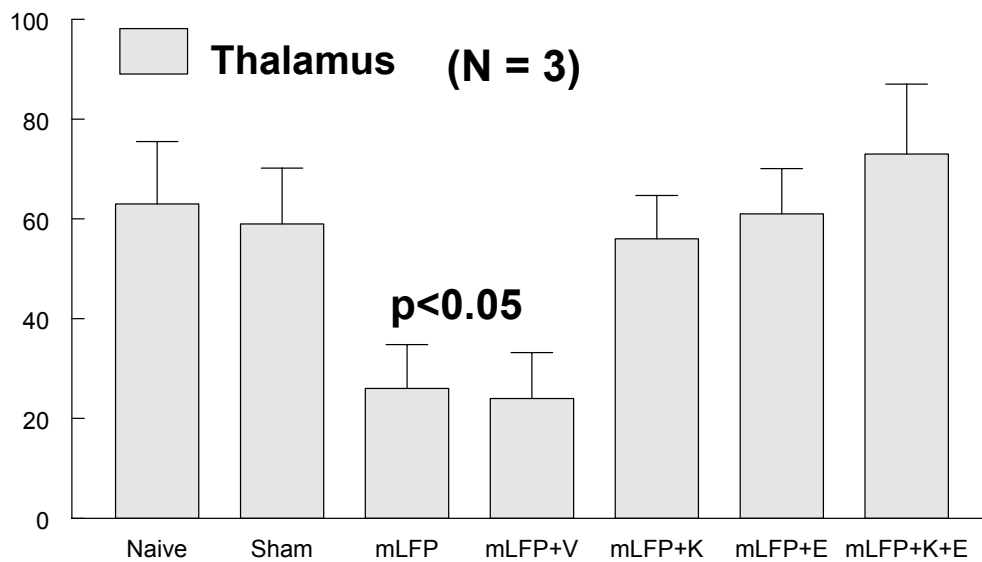
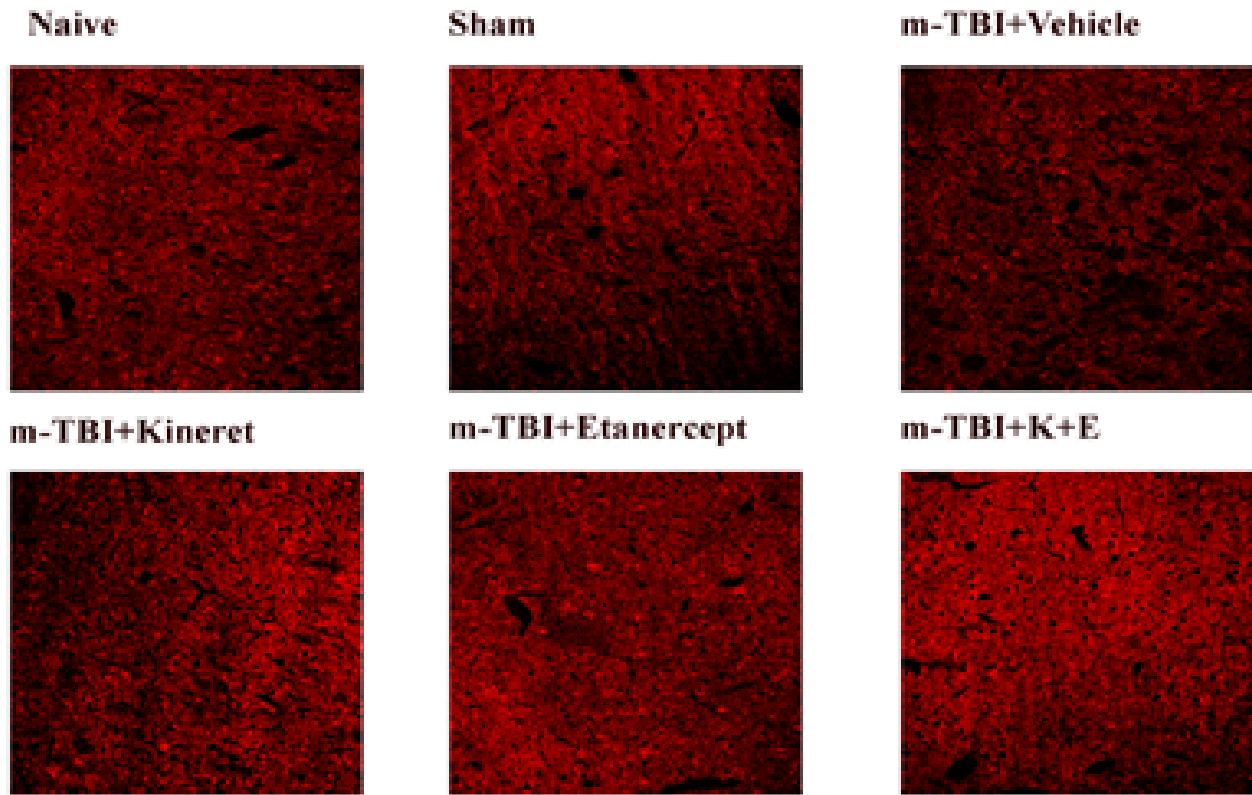


Figure 16. (A) Representative Ipsilateral Hippocampi MAP2 Intensity 18 days after m-LFP Injury and Treatment Groups; (B) Quantitation based on immunofluorescence ($p < 0.05$ m-LFP Injury vs all except m-LFP Injury+Vehicle)(N=3).

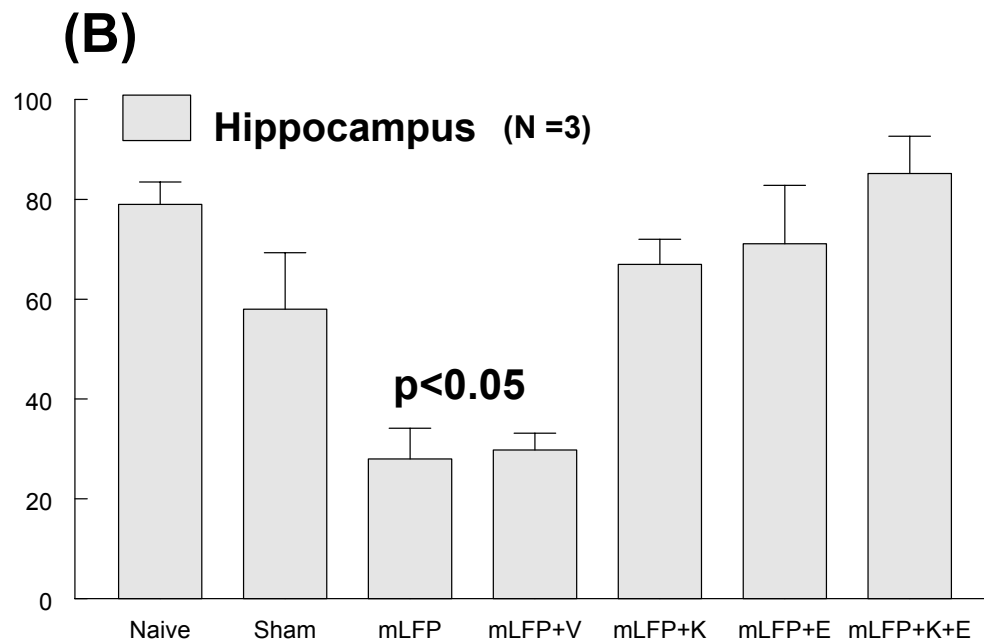
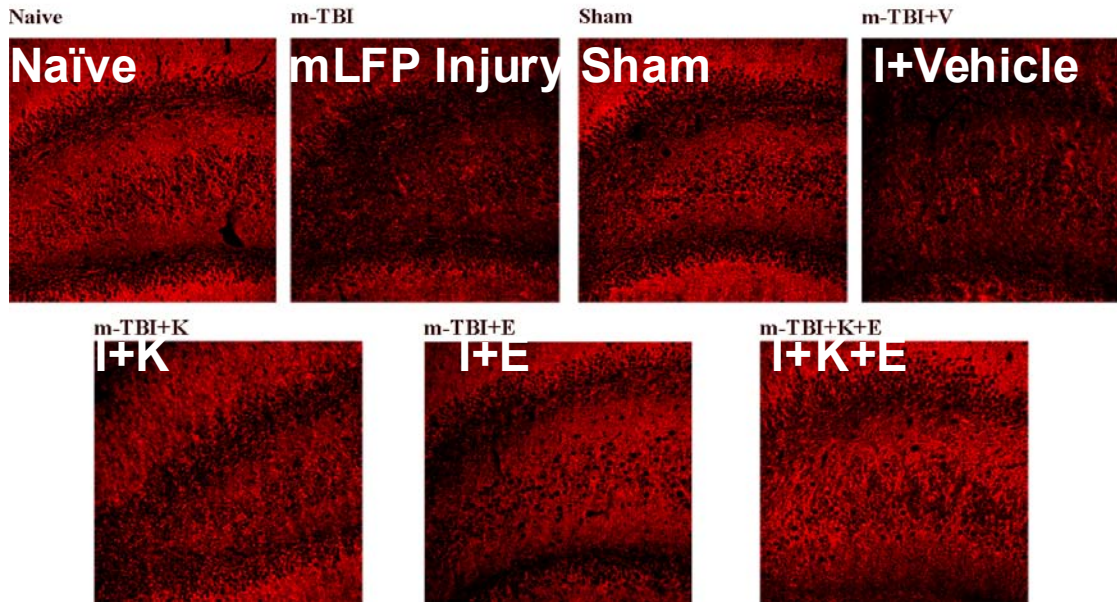


Figure 17. Effect of Kineret+Etanercept treatment on hippocampal NeuN+ cell number 6h and 18 days after mLFP injury (N =3)

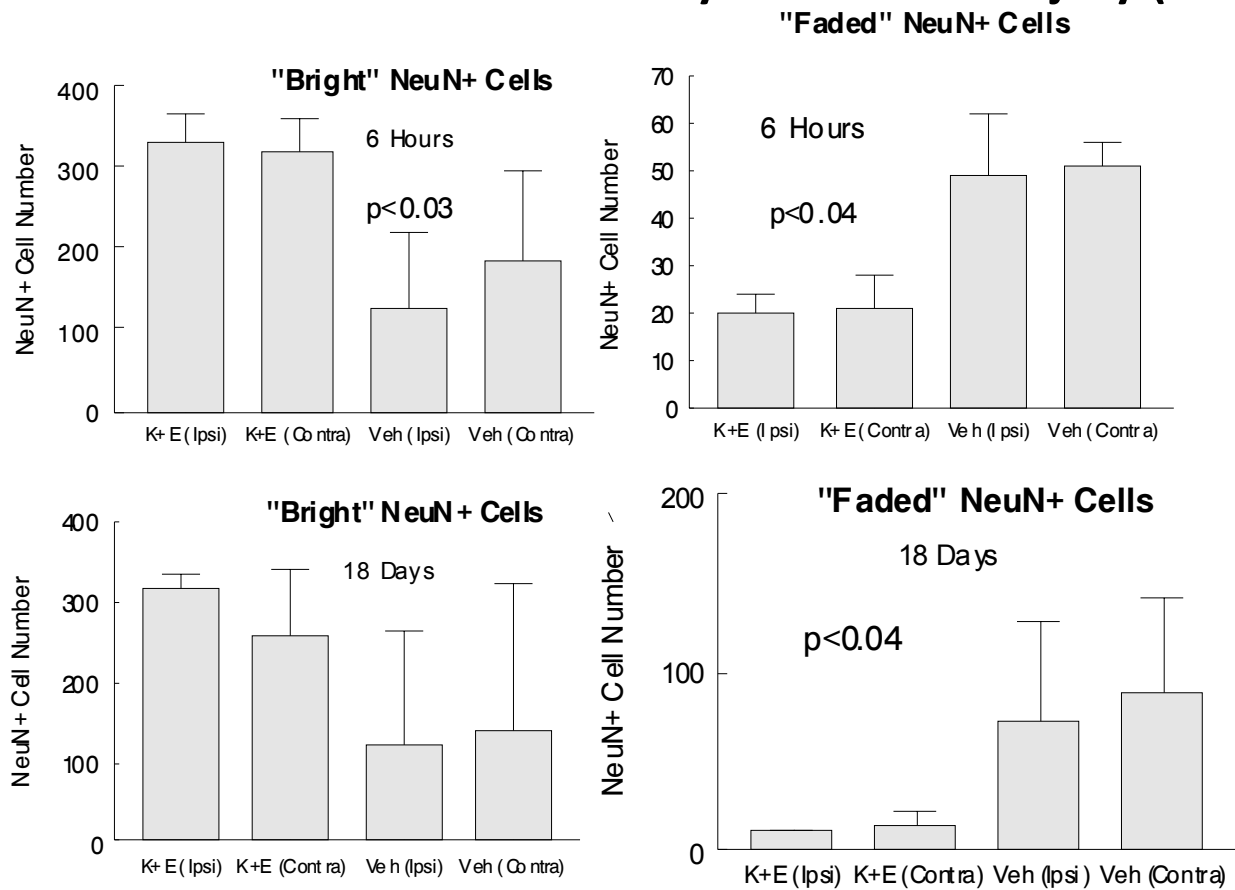
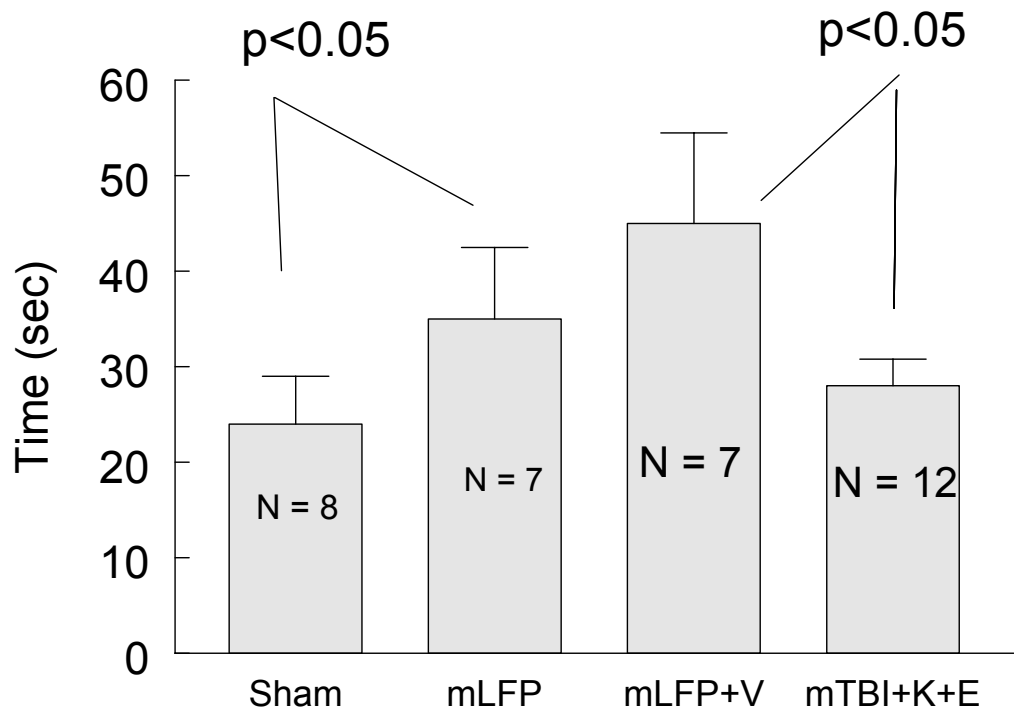


Figure 18. Working Memory Assay.



Blast models of mTBI.

For detailed progress on the Van Den Berg and Advanced Blast Simulator Blast (ABSB) models, please see DeWitt's Summary Report. We have begun using the Van Den Berg model beginning on August 1. We will be using it to test the hypothesis that blast injured animals treated at one hour; two and three days with Kineret will exhibit beneficial outcomes. This will provide a test of our intervention strategy on two mTBI-relevant models, mLFP and a blast model. Due to scheduling difficulties and state of characterization of the two blast models and in order to assure progress, in consultation with the DeWitt group we will perform our blast model assessments of Kineret treatment relying on the Van Den Berg blast model. We will be doing about 240 rats to be sacrificed at 6 hours and 18 days and assessed for pathological outcomes at these two time points and behavioral and cognitive improvements at 5 days to allow for a useful comparison to the mild fluid percussion model being tested by Dr. Pramod Dash (see his annual report). Animal groups will consist of naïve, shams, injured and injured treated with vehicle or Kineret.

In a pilot study that Dr. DeWitt finished of ABSB blast levels of up to 22 psi (low to moderate blast injury depending on source) there was significant impairment of dilator responses to reduced intravascular pressure in cerebral arteries harvested from the blasted rats. They are now measuring arterial blood pressure and cerebral perfusion. We are presently performing comparison tests of ABSB and Van Der Berg blast injuries.

KEY RESEARCH ACCOMPLISHMENTS: Bulleted list of key research accomplishments emanating from this research.

We have shown in the past that after mLFP injury:

- The key brain inflammatory cytokine (Interleukin 1 (**IL-1**) and Tumor Necrosis Factor alpha (**TNF α**) protein levels increase as early as 3 and 6 hours across several brain structures and there is a return to basal levels by 18 days post-injury;
- There is a significant increase in activation of astrocytes and microglia and the latter were found to be the source of the increased levels of IL-1 and TNF α ;
- The blood brain barrier integrity is impaired by 6 hours post injury and this impairment persists up to 18 days.

During this past year we determined that:

- There are mLFP-induced decreases in wet brain weight and significant neuronal cell and axonal, and myelin losses in the hippocampus, thalamus and amygdala, structures associated with cognitive function and behavioral responses.
- There is persistent inflammation that may contribute to the development of working memory deficits consistent with reports of the clinical impairments documented for many military personnel that have experienced mTBI in the battlefield.
- We found that 1 hour cold stress (5°C) had no confounding effect on brain temperatures while stimulating significant increases in number of fecal pellets and glucocorticoid concentrations in fecal pellets.
- We tested two interventions that block the injury-induced increases in IL-1 α/β and TNF α and reduce the neuropathology resulting from the injury-triggered inflammatory cascade and thus improve outcomes. Since a corollary of our overall goal is to apply the FDA-approved treatments using modalities that best mimic as best possible the military injury scenario, we used an i.p. delivery system that is more suitable to the battlefield environment. Thus, individual treatments were given at 30min, 6hr and then daily for 11 days.
- The individual treatments with Kineret and Etanercept both ameliorated the mLFP-induced neuropathology (brain weight loss; astrocytic and microglial activation; cell, axon and myelin losses; blood brain barrier impairment) as early as 6 hours after injury and these changes persisted up to 18 days. However, there was no further enhancement of benefits at 18 days. Interestingly, we saw no significant differences in this outcome

measure when comparing the two treatments although there was an overall trend showing an advantage to the Kineret treatment.

- We also performed mLFP injury and **combined** treatments of both Kineret and Etanercept to determine if there were synergistic effects given the different time responses in IL-1 and TNF α induction after mLFP injury. A thorough analysis showed that:

a. There was no benefit from the combined treatment compared to either of the individual treatments based on changes in neuropathological effects of mLFP as assessed by neuronal cell number, axon density, myelin density, astrocytic activation, and blood brain barrier integrity in parietal cortex, hippocampus, thalamus, and amygdala.

b. There was no significant amelioration of pathology or increased benefit of cognitive/behavioral outcome measures after 11 days of treatment as compared to the significant beneficial effects of a 6 hr treatment as measured at 6hr and 18 days (pathology) or 11-15days cognitive/behavioral measures.

REPORTABLE OUTCOMES: Provide a list of reportable outcomes that have resulted from this research to include: manuscripts, abstracts, presentations; licenses applied for and/or issued; degrees obtained that are supported by this award; development of cell lines, tissue or serum repositories; informatics such as databases and animal models, etc.; funding applied for based on work supported by this award; employment or research opportunities applied for and/or received based on experience/training supported by this award

We have presented abstracts at the Society for Neurotrauma Meeting and Society for Neuroscience Meeting in 2011.

We have also submitted two manuscripts; now being reviewed in revised form in Journal of Neurotrauma.

A rodent model of mild traumatic brain injury by DeWitt et al.

Experimental Models of Mild Traumatic Brain Injury by Perez-Polo et al.

CONCLUSION: Summarize the results to include the importance and/or implications of the completed research and when necessary, recommend changes on future work to better address the problem. A "so what section" which evaluates the knowledge as a scientific or medical product shall also be included in the conclusion of the report.

Our work to date suggests that i.p. injection of two FDA-approved anti-inflammatory agents targeting inflammatory cytokine receptors applied after mLFP injury can ameliorate the injury-induced neuropathology and behavioral/cognitive deficits characteristic of the injury.

We also have observed that a shorter time course of treatment or treatment with one agent alone is as useful as the longer time course treatment or the treatment with both drugs.

REFERENCES: List all references pertinent to the report using a standard journal format (i.e. format used in Science, Military Medicine, etc.).

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APPENDICES: Attach all appendices that contain information that supplements, clarifies or supports the text. Examples include original copies of journal articles, reprints of manuscripts and abstracts, a curriculum vitae, study questionnaires, and surveys, etc.

SUPPORTING DATA: All figures and/or tables shall include legends and be clearly marked with figure/table numbers.