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14. ABSTRACT

Results from studies conducted during this funding period have provided evidence that a double dose of EC enzyme degradation inhibitors are effective at reducing NSS scores. Further analysis of these tissue samples will examine how the double dose of EC enzyme degradation inhibitors affected the MPO activity and mRNA expression of IL1β, IL-6, TNF-α, and MCP-1. In addition, samples from perfusion fixed brains with one dose of the EC enzyme degradation inhibitors will undergo immunostaining to evaluate the injury lesion size and detection of neuronal injury and gliosis, using specific antibodies (CD11b and ED-1) to detect activated microglia during the next funding period.

15. SUBJECT TERMS

Lateral fluid percussion, traumatic brain injury, blood brain barrier, neuroinflammation, neurological dysfunction, endocannabinoids

Table of Contents

INTRODUCTION:

Long term effects of traumatic brain injury (TBI), including neuroendocrine dysregulation and neurobehavioral recovery may be ameliorated by intervention aimed at reducing short term neuroinflammation, oxidative stress, and altered neuroendocrine and behavioral functions. Our working hypothesis is that elevating levels of the endocannabinoids (EC), 2-arachidonoyl glycerol (2-AG) and N-arachidonoyl ethanolamine (AEA) should ameliorate neuroinflammatory changes following TBI. During this funding period, selective pharmacologic inhibitors have been used to decrease the degradation of 2-AG and/or AEA. Studies during this funding period are currently underway in an effort to prolong the duration of drug treatment to establish the optimal length of intervention. This is accomplished by administrating pharmacotherapy 30 min post-TBI, as well as an additional dose of the selective EC enzyme degradation inhibitors 24 hrs post TBI. Ongoing studies will continue to examine the extent to which this intervention has modulated cytokine release, neutrophil influx, blood brain barrier permeability, and neurological and neurobehavioral severity impairments following TBI.

BODY:

Progress Report 3rd Annual Funding Period:

Studies to date have been directed towards the completion of Milestones 1, 2, and 3 as defined in the Statement of Work. The goal of milestone 1 is to describe the impact of EC degradation inhibition on neutrophil influx, pro-inflammatory cytokine expression, oxidative injury, edema, and blood barrier permeability. Additionally, histological assessment of the protective effects of EC following brain injury will be demonstrated. The goal of milestone 2 is to examine the effectiveness of decreasing EC degradation in maintaining neuroendocrine integrity following TBI. The goal of milestone 3 is to examine the efficacy of elevated EC levels to provide neuroprotection and improve neurobehavioral outcome as reflected in motor and cognitive function. Current efforts are focused primarily on Tasks 1 and 3.

Task 1: Determine the effectiveness of specific inhibitors of endocannabinoid degradation in reducing neutrophil influx, pro-inflammatory cytokine expression, oxidative injury, edema, and blood barrier permeability.

a. Inflammation & oxidative stress (2h, 4h, 24h, 72h post-TBI) . Brain tissue (area of injury, prenumbral region, contralateral region, frontal cortex) content of cytokines and chemokines, oxidative stress (lipid peroxidation and catalase activity). Inflammatory cell infiltration examined by immunohistochemistry.
b. Brain edema (4h, 24h, 72h post-TBI). Wet/dry ratio determined.
c. Blood brain barrier permeability analyzed by dye tracer extravasation (24h & 72h post-TBI).
d. Cell injury by histological analysis (7d & 30d post TBI).
e. Endo-Cannabinoid Levels measure in extracted brain tissue lipids

Progress:

Efforts under Task 1 have been primarily focused towards Task 1a (Inflammation and oxidative stress), and Task 1d (Cell injury by histological analysis). For Task 1a samples were analyzed using two separate IL-6 ELISA kits to measure IL-6 at 24 and 48 hrs post-TBI. In addition, samples collected 24 and 72 hrs post-TBI treated with only a single dose of EC enzyme degradation inhibitors 30 min post-TBI were analyzed using RT-PCR mRNA expression of IL-1β, IL-6, TNF-α and MCP-1 in sham, vehicle and drug treated groups.

As of mid-February, we began using a new lateral fluid percussion system (LFPI) which required optimization, We began by establishing a level of injury we felt was consistent with our previously reported Neurological Severity Score (NSS) and Neurobehavioral Score (NBS) findings using a force that produced a similar level of injury compared to our previous studies. In addition we examined the IL-6 expression and MPO activity in the ipsilateral cortex at 72 hrs post-TBI.

Studies were completed to establish the optimal length of pharmacologic intervention by prolonging the duration of drug treatment for optimal neuroprotective effects. This is accomplished by administering a second dose of the selective endocannabinoid enzyme degradation inhibitors (JZL184; 16 mg/kg and URB597 0.3 mg/kg, IP); initial dose 30 min post-TBI followed by a second dose 24 hrs later. We hypothesize that this second dose of the EC breakdown inhibitors should produce a significant reduction in inflammatory and oxidative tissue damage (Task 1). We have completed one set of animals through the 72 hrs time point and plan to repeat with another set of animals to increase sample size prior to running analysis of tissue for inflammation and oxidative stress measures. In the next funding period, we plan to complete a set of animals each for brain edema, blood brain barrier and immunohistochemistry as set out in the tasks 1a-d. It is important to note that a different set of animals is required for doing analysis for each of these tasks mentioned above.

Additional progress has been made towards Task 1d: Cell Injury by histological analysis from sham (un-injured) and post-TBI animals. Brains have been perfused and sliced to evaluate the injury lesion size through a series of coronal sections stained with cresyl violet. For detection of neuronal injury and gliosis, sections were stained using specific antibodies (CD11b and ED-1) to detect activated microglia. Preliminary results to date show increased microglia activation on the ipsilateral side in TBI animals when compared to sham. It is important to note that a different set of animals is required for doing analysis for each of the above mentioned tasks.

Summary of Findings:
Task 1a

Effects of Inhibiting Endocannabinoid Degradation in Reducing pro-Inflammatory Cytokine Expression Following Traumatic Brain Injury

TBI initiates a neuroinflammatory cascade characterized by an increased production of proinflammatory cytokines and chemokines, such as interleukin IL-6. In the case of TBI, this complex neuroinflammatory cascade can promote neuroinflammation and potentially lead to neurodegeneration. We have previously demonstrated that treatments to the endocannabinoid system 2-arachidonoyl glycerol (2-AG) and N-arachidonoyl-ethanolamine (AEA) ameliorate the neuroinflammatory response at 24hrs post-TBI.

Tissues samples were analyzed for IL-6 protein cytokine expression 24 and 72hrs post-TBI using an Invitrogen IL-6 Rat Elisa Kit. Current data demonstrates no significant differences between Sham and TBI/Vehicle treated animals (Fig. 1a-b). Furthermore, drug interventions (JZL184 16mg/kg, IP, 30min's post-TBI) and (URB597 0.3mg/kg, IP, 30 min's post-TBI) did not effectively decrease IL-6 activity as anticipated. In fact, addition of a naive (no surgery) group identified a small yet significant rise in IL-6 at 72 h post TBI in the uninjured animals. It is possible that the sensitivity of the current analytical approach may preclude us from detecting differences in tissue cytokine levels.

Fig:1a

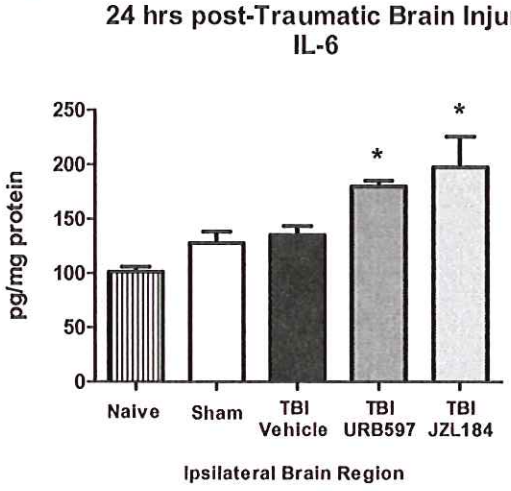

Fig:1b

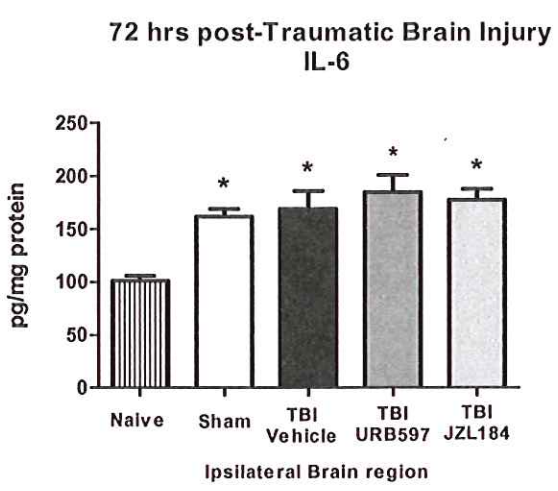

Fig 1a-b: IL-6 Activity (pg/mg protein) Measured in the Ipsilateral Brain Region Excised at 24hr and 72hrs post-TBI. Inhibition of 2-AG and AEA degradation by the use of the selective inhibitors (JZL184; 16 mg/kg and URB597 0.3 mg/kg, respectively, IP) administered 30 min post-TBI does not appear to reduced the proinflammatory cytokine IL-6 when compared to vehicle treated animals. Measured by IL-6 Rat ELISA (n=8-10/group).

In an effort to confirm these findings, we re-ran our samples using a Rat IL-6 ELISA kit from R& D Systems. Additional tissues samples were analyzed for IL-6 protein cytokine expression 24 and 72hrs post-TBI using an IL-6 Rat Elisa Kit from R & D Systems. As seen before using an Invitrogen IL-6 kit, this data demonstrates no significant differences between Sham and TBI/Vehicle treated animals (Fig. 2a-b). Furthermore, drug interventions with JZL184 (16mg/kg, IP) and URB597 (0.3mg/kg, IP) 30 min's post-TBI did not effectively decrease IL-6 expression as anticipated. The lack of significant differences in tissue expression of IL-6 is puzzling, as it does not correlate with our data from the neurological and neurobehavioral studies. Efforts are being directed towards optimizing a new method to evaluate these cytokines for improved accuracy and sensitivity which may lead to more conclusive results.

Fig 2a.

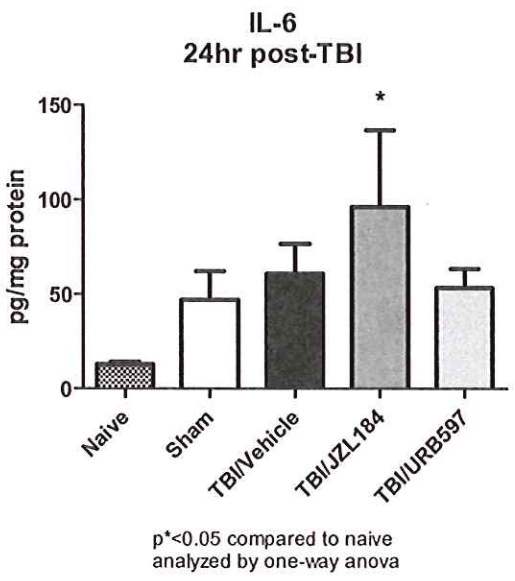

Fig 1b

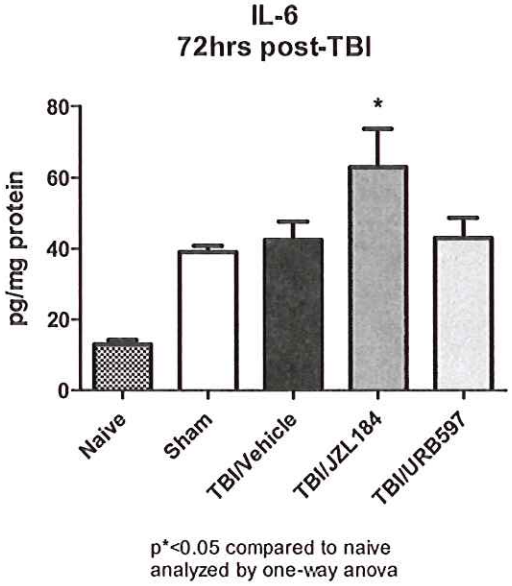

Fig 2a-b: IL-6 Activity (pg/mg protein) Measured in the Ipsilateral Brain Region Excised at 24hr and 72hrs post-TBI. Inhibition of 2-AG and AEA degradation by the use of the selective inhibitors (JZL184; 16 mg/kg and URB597 0.3 mg/kg, respectively, IP) administered 30 min post-TBI does not appear to reduce the proinflammatory cytokine IL-6 when compared to vehicle treated animals. Measured by IL-6 Rat ELISA R&D Systems Kit (n=8-10/group).

Pro-Inflammatory Cytokine Expression and Neutrophil Influx Following Traumatic Brain Injury using the new Lateral Fluid Percussion Injury Device

During this funding year we began using a new LFPI system, initially we had to establish a level of injury we felt was consistent with our previously reported Neurological Severity Score (NSS) and Neurobehavioral Score (NBS) findings. By testing three different levels of injury ranging from 1.0-2.5 atm's we determined that LFPI at 2 atm (~30 PSI) produced a similar level of injury compared to our previous studies. These tissue samples were then analyzed for IL-6 protein cytokine expression, as well as MPO activity 72hrs following TBI. Results to date indicate a noticeable difference between Naïve, Sham and TBI treated animals analyzed for IL-6 expression and MPO activity using the new LFPI device (Fig. 3a-b).

3a. **IL-6 72hrs post-TBI using New LFPI Device**

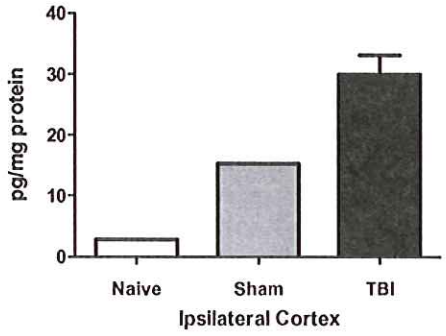

Fig 3a. IL-6 Expression (pg/mg protein) Measured in the Ipsilateral Cortex Excised 72hrs post-TBI.
Measured by R&D Systems Rat IL-6 ELISA
Sham; TBI/Vehicle; (n=6/grp)

3b. **Myeloperoxidase Activity 72hrs post-TBI using New LFPI Device**

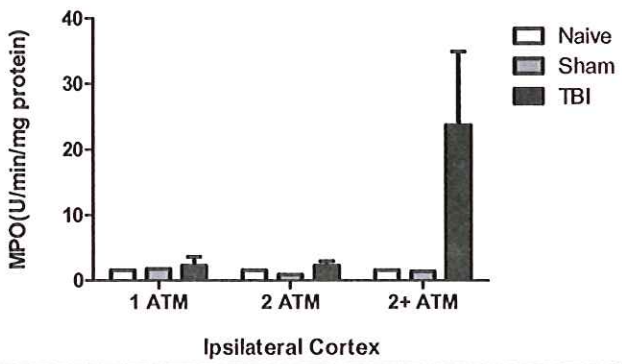

Figure 3b. MPO Activity (U/min/mg protein) in the Ipsilateral Cortex Excised 72 hrs post-TBI.
Sham; TBI/Vehicle; (n=6/grp)

Pro-Inflammatory Cytokine mRNA Expression Following Traumatic Brain Injury

TBI initiates a neuroinflammatory cascade characterized by an increased production of pro-inflammatory cytokines and chemokines, such as IL1β, IL-6, TNF-α, and MCP-1. In the case of TBI, this complex neuroinflammatory cascade can promote neuroinflammation and potentially lead to neurodegeneration. We measured mRNA using RT-PCR to assess the levels of pro-inflammatory cytokines and chemokines at 24 and 72hrs post-TBI (Fig. 4 and 5, respectively). Here we see a trend toward an increased IL-6 and TNF-α at 24 hrs post-TBI when compared to sham and TBI treated with JZL184 (16mg/kg, IP) and URB597 (0.3mg/kg, IP) 30 min's post-TBI The results are in contrast to the peptide expression measured by ELISA.

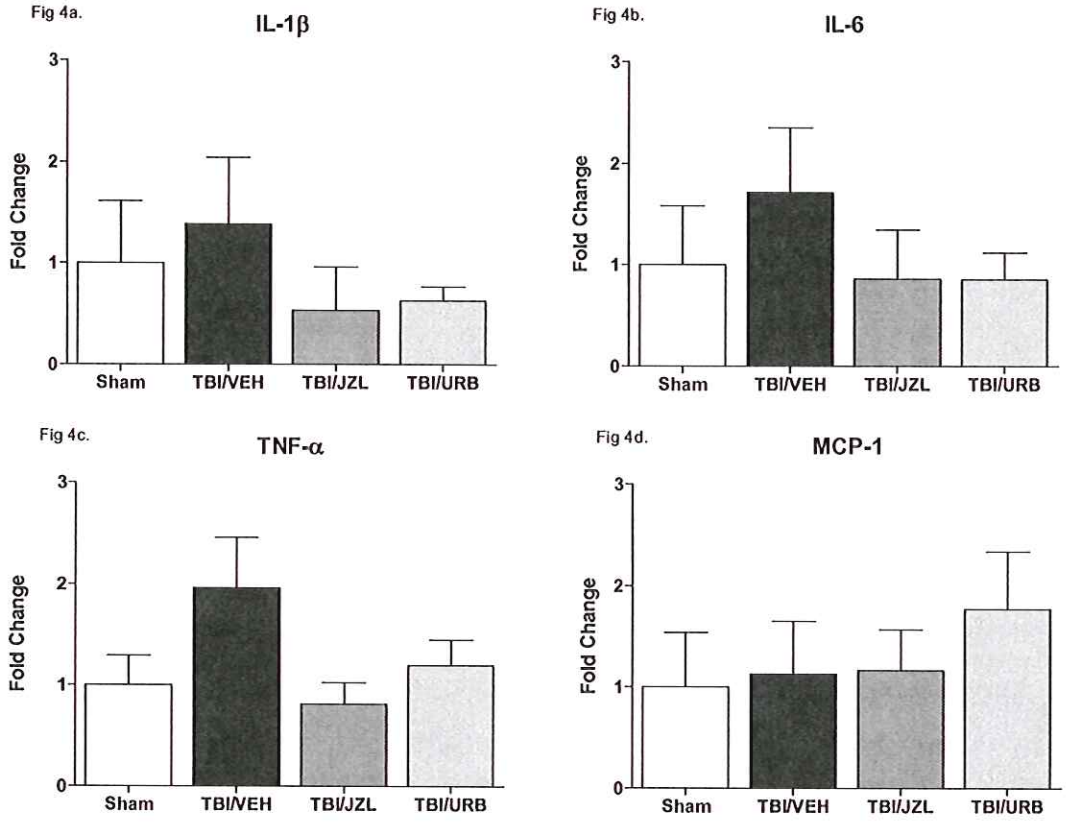

Fig 4a-d: mRNA expression at 24hrs post-TBI. Inhibition of 2-AG and AEA degradation by the use of the selective inhibitors (JZL184; 16 mg/kg and URB597 0.3 mg/kg, respectively, IP) administered 30 min post-TBI does not significantly reduce the pro-inflammatory cytokines IL-1β, IL-6, TNF-α, or MCP-1 when compared to vehicle treated animals. (n=6-9/group).

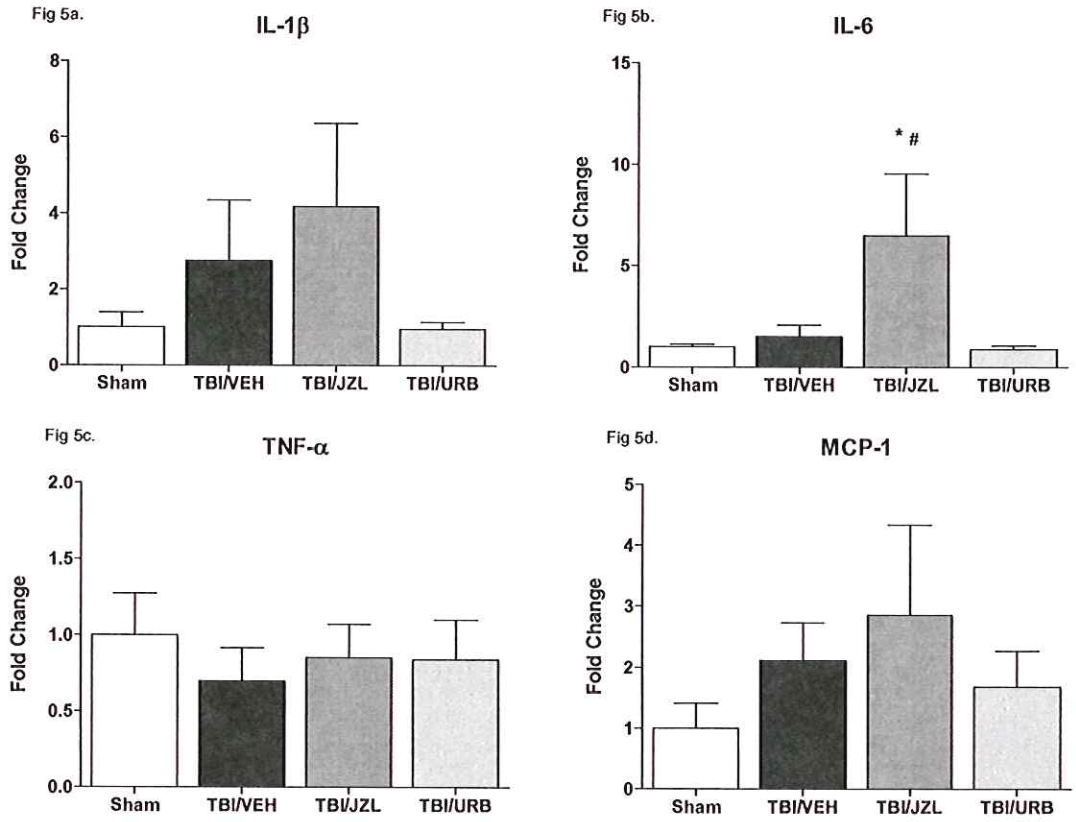

Fig 5a-d: mRNA expression at 72hrs post-TBI. Inhibition of 2-AG and AEA degradation by the use of the selective inhibitors (JZL184; 16 mg/kg and URB597 0.3 mg/kg, respectively, IP) administered 30 min post-TBI does not significantly reduce the proinflammatory cytokines IL-1β, TNF-α, or MCP-1 when compared to vehicle treated animals. While IL-6 was significantly increased with administration of JZL 184 30 min post-TBI when compared to both SHAM and TBI/VEH. (n=6-9/group).

Task 2: Determine the effectiveness of the selective increase in endogenous 2-AG and AEA levels in preventing neuroendocrine dysfunction following TBI.

a. Basal unstimulated neuroendocrine function
b. Autonomic and neuroendocrine response to cardiovascular challenge
c. Autonomic and neuroendocrine response to water deprivation test

Progress:

No progress to report.

Task 3: Determine the capacity of increased EC levels to protect neurobehavioral and cognitive function following TBI.

a. Severity of TBI determined by the righting reflex.
b. Sensory reflex examined by the forelimb and hindlimb reflex.
c. Somatomotor function examined by a beam balance task and beam-walking task.
d. Cognitive function tested by the radial-arm maze.

Progress:

In February we received our new lateral fluid percussion injury (LFPI) device. This device has become the standard icon in the neurotrauma research community worldwide, and has been shown to create more controlled, reproducible and accurate fluid percussion pressures/injuries. It is anticipated that this new equipment will greatly improve the accuracy of our TBI model; it has required a significant period of fine tuning, which included calibration and the use of several sets of practice animals to match our established protocols and results. This equipment is now set up in our laboratory and fully operational.

Our previously described fluid percussion injury was produced by dropping the weight of a steel cylinder (350g) through a hollow guide from a height of 42cm. The steel cylinder hitting the plunger of a 10cc syringe produces a pressure wave of sterile saline delivered to the dura (~1.8atm for a duration of 25ms). Using this TBI model the injury produced marked neurobehavioral dysfunction as indicated by increased NSS and NBS scores out to 72 hrs.

Upon arrival we began the set-up and calibration of the new LFPI device. Due to the construction delay in our animal recovery rooms and lack of animal availability the process of optimizing the new LFPI device took more time than anticipated. By May, several sets of practice animals were run with the new LFPI system in an effort to establish a level of injury that is consistent with our previous findings. Initially we used previous published papers to determine a starting range of force (atm's) to deliver to the dura matter following craniotomy. We tested three different levels of force ranging from 1-2.5 atm's. We determined the LFPI at 2 atm (~30 PSI) produced a similar level of injury compared to our previous studies based on NSS and NBS assessments out to 72 hr post-TBI (Figure 5a-f). Brains from these animals have been collected and are in queue for future tissue analysis of MPO activity and IL-6 expression. These preliminary observations will be further confirmed by additional sets of practice animals with and without drug treatment.

Fig 6a

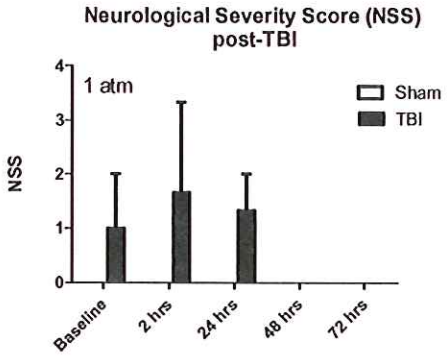

Fig 6b

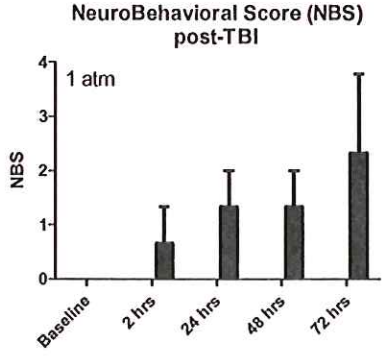

Fig 6c

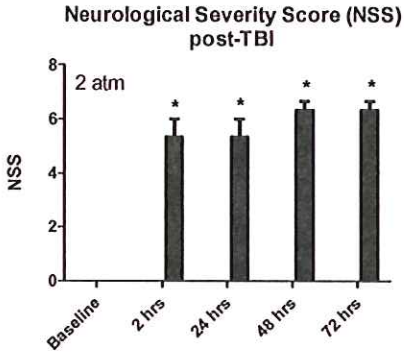

Fig 6d

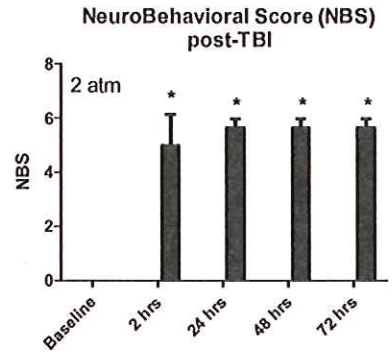

Fig 6e

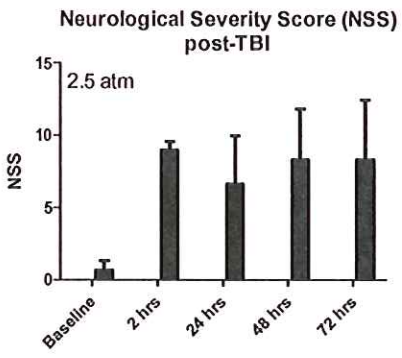

Fig 6f

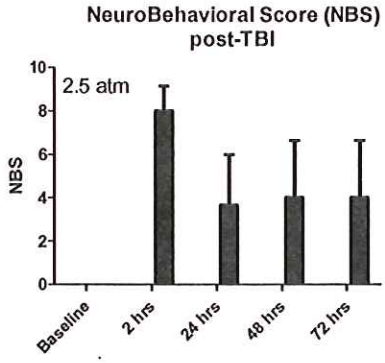

Fig 6a-f: Neurological Severity Score (NSS) & Neurobehavioral Score (NBS) post- TBI

NSS scores (5a, c and e) represent the degree of neurological dysfunction following traumatic brain injury at the following time points: 2hr, 24hr, 48hr and 72 hrs.
NSS scoring (0=normal; 25=severe); Sham (n=3); TBI vehicle (n=3) P* < 0.05 compared to un-injured sham; Analyzed by Two-way ANOVA
NBS scores (5b, d and f) represent the degree of neurobehavioral dysfunction following traumatic brain injury at the following time points: 2hr, 24hr, 48hr and 72 hrs.
NSS scoring (0=normal; 12=severe); Sham (n=3); TBI vehicle (n=3)
P* < 0.05 compared to un-injured shams; Analyzed by Two-way ANOVA

Summary of Findings:

Task 3a

Ongoing efforts have been directed towards the completion of Tasks 3a-d using a double dose of EC enzyme degradation inhibitors. These initial studies have focused on assessing the effects of a double dose of EC enzyme degradation inhibitors on neurological and neurobehavioral outcomes following TBI. Neurological (NSS) and Neurobehavioral (NBS) function is examined by a combination of somatomotor and cognitive assessments. Following TBI, each animal is re-assessed at 2, 24, 48, and 72hrs time points to determine the degree of neurological and neurobehavioral dysfunction. The selective EC enzyme degradation inhibitors (JZL184; 16 mg/kg and URB597 0.3 mg/kg, IP) are administered 30 minutes post-TBI followed by a second dose given 24 hrs later. Previous results have demonstrated that a single dose of JZL184 administered 30 minutes following injury is superior to URB597 in improving tissue markers of inflammation (MPO), brain structural damage (BBB), neurological (NSS) and neurobehavioral (NBS) outcomes following TBI. Our initial studies demonstrate that both EC enzyme degradation inhibitors, given at 30 min and 24 hrs post-TBI, are effective at reducing the NSS score at 48 hrs when compare to vehicle treated TBI animals. While no significant improvement was seen in the NBS scores.

Fig 7a

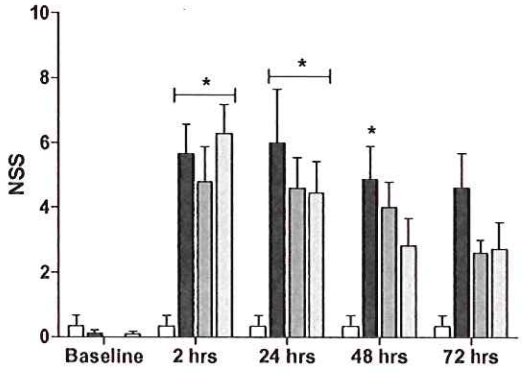

Fig 7b

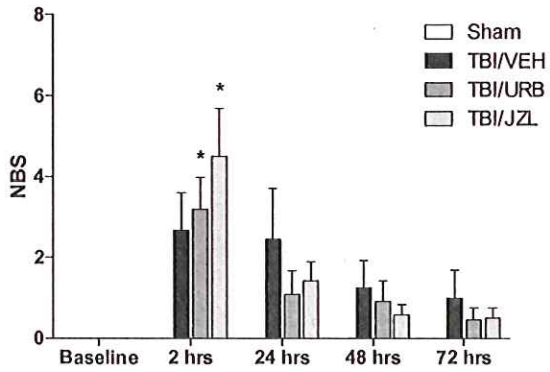

Fig 7a-b Neurological Severity Score (NSS) & Neurobehavioral Score (NBS) post- TBI Double Dose. Inhibition of 2-AG and AEA degradation by the use of the selective inhibitors (JZL184; 16 mg/kg and URB597 0.3 mg/kg, respectively, IP) administered 30 min and 24 hrs post-TBI reduced NSS at 48 hrs. NBS was not significantly different between TBI groups at 24, 48 and 72 hrs (n=6-12/group).

KEY RESEARCH ACCOMPLISHMENTS:

- EC enzyme degradation inhibitors, given at 30 min and 24 hrs post-TBI, are effective at reducing the NSS score at 48 hrs when compare to vehicle treated TBI animals.

REPORTABLE OUTCOMES:

Publications:

Book chapter submitted.
Katz PS, Molina PE. Cannabinoids. ***Neuroinflammation and Neurodegeneration.*** *Peterson and Torborek (eds.), Springer Science + Business Media, New York, NY.*

Manuscript in preparation.
Katz PS, Sulzer J, Impastato R, Teng X, Rogers E, Molina PE. Decreased endocannabinoid degradation attenuates neurobehavioral dysfunction and protects blood brain barrier integrity following traumatic brain injury in rodents.

Presentations:

Poster presentation at Experimental Biolog, April 2013.
Inhibition of endocannabinoid degradation reduces neurological damage and blood brain barrier disruption following traumatic brain injury. Katz PS, Impastato R, Rogers E, Molina P.

CONCLUSION:

The results from our ongoing studies during this funding period have provided evidence that a double dose of EC enzyme degradation inhibitors are effective at reducing NSS scores. Further analysis of these tissue samples will examine how the double dose of EC enzyme degradation inhibitors affected the MPO activity and mRNA expression of IL1β, IL-6, TNF-α, and MCP-1. In addition, samples from perfusion fixed brains with one dose of the EC enzyme degradation inhibitors will undergo immunostaining to evaluate the injury lesion size and detection of neuronal injury and gliosis, using specific antibodies (CD11b and ED-1) to detect activated microglia.

REFERENCES: N/A

APPENDICES: N/A