AWARD NUMBER: W81XWH-11-2-0011

TITLE: Endocannabinoids as a target for the treatment of traumatic brain injury

PRINCIPAL INVESTIGATOR: Patricia E. Molina, MD, PhD

CONTRACTING ORGANIZATION: Louisiana State University Health Sciences Center
New Orleans, LA 70112

REPORT DATE: January 2016

TYPE OF REPORT: Final Report

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;
Distribution Unlimited

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE	2. REPORT TYPE	3. DATES COVERED
January 2016	FINAL - Revised	4Oct2010 - 3Oct2015

4. TITLE AND SUBTITLE

ENDOCANNABINOIDS AS A TARGET FOR THE TREATMENT OF TRAUMATIC BRAIN INJURY

5a. CONTRACT NUMBER
W91ZSQ0191N640

5b. GRANT NUMBER
W81XWH-11-2-0011

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)
PATRICIA E. MOLINA, MD, PhD

E-Mail: pmolin@lsuhsc.edu

5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

Louisiana State University Health Sciences Center
New Orleans, LA 70112

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)

U.S. ARMY MEDICAL RESEARCH AND MATERIEL COMMAND
FORT DETRICK, MARYLAND 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT

Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT
Traumatic brain injury (TBI) is characterized by neuroinflammation and oxidative stress as well as impaired neurocognitive function Our studies have revealed protective effects of endocannabinoid (EC) degradation inhibitors administered systemically 30 min post-TBI. We show marked suppression of astrocyte and microglia activation, improved neurobehavioral function, attenuation of blood brain barrier leak, and decreased glutamatergic excitability. These findings indicate that EC degradation inhibition post-TBI is neuroprotective.

15. SUBJECT TERMS
Traumatic brain injury, endocannabinoids, neuroinflammation, neurobehavioral, blood brain barrier

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			USAMRMC
Unclassified	Unclassified	Unclassified	Unclassified	81	19b. TELEPHONE NUMBER *(include area code)*

Standard Form 298 (Rev. 8-98)
Prescribed by ANSI Std. Z39.18

Table of Contents **Page**

INTRODUCTION

Traumatic brain injury (TBI) resulting from explosive or blast attacks has become increasingly frequent in current warfare (Helmic, 2015). Kevlar body armor and helmets effectively shield soldiers from bullets and shrapnel and have reduced the frequency of penetrating head injuries. However, the number of closed brain injuries has increased significantly. TBI is not limited to the military population, and has become a well-recognized medical problem in contact sports such as football and boxing (McKee, 2009). The majority of TBI victims are young, otherwise healthy adults. TBI, with its associated morbidity, is a major area of unmet medical need that lacks effective therapies. Current investigations on the pathobiology of military blast-induced TBI have revealed that despite similar secondary injury cascades to those seen with other types of injury, the underlying pathophysiology involves systemic, local, and cerebral responses. The neurological manifestations of TBI range from headaches, sleep disturbances, and sensory hypersensitivity to cognitive and behavioral deficits and neuroendocrine dysfunction (Okie, 2005; Elder, 2009). Current available treatment and management is palliative, due to incomplete understanding of the processes involved (Cernak, 2009) with no effective therapies identified to improve outcome from TBI. The studies conducted during the funding period provided an integrative approach to dissecting the mechanisms involved in the neurobehavioral sequelae of TBI, and specifically focused on establishing the effectiveness of endocannabinoid (EC) system modulation as therapeutic approach in the management of this patient population. The premise of the studies conducted was that the long-term effects of TBI, including neurobehavioral dysfunction and neurodegeneration, may be ameliorated by interventions aimed at reducing short-term neuroinflammation, oxidative stress, and altered neuroendocrine and behavioral functions. The working hypothesis that guided the studies conducted was that maintaining levels of the EC, 2-arachidonoyl glycerol (2-AG) and N-arachidonoyl ethanolamine (AEA) should reduce neuroinflammatory changes following TBI. This hypothesis was tested in a rodent model of controlled TBI via fluid percussion in rats and administered EC degradation inhibitors 30 minutes and 24 hours post injury. The outcome measures included were behavioral and cellular outcomes throughout the acute recovery period. Overall the goal of these studies was to determine if this pharmacological regimen would reduce TBI-induced neuroinflammation, blood brain barrier (BBB) permeability, and neurobehavioral dysfunction and if the protective effects were sustained. The most important findings are summarized as follows.

KEYWORDS

Traumatic brain injury, endocannabinoid, MAGL, FAAH, 2-AG, AEA, inflammation, behavior, JZL184, URB597

ACCOMPLISHMENTS

BODY:

Studies were directed towards the completion of Milestones 1, 2, and 3 as defined in the Statement of Work. The goal of Milestone 1 was to describe the impact of EC degradation inhibition on neutrophil influx, pro-inflammatory cytokine expression, oxidative injury, edema, and blood barrier permeability. Additionally, Milestone 1 aimed to demonstrate histological assessment of the protective effects of endocannabinoid degradation inhibition following brain injury. The goal of Milestone 2 was to examine the effectiveness of decreasing EC degradation in maintaining neuroendocrine integrity following TBI. The goal of Milestone 3 was to examine the efficacy of elevated EC levels to provide neuroprotection and to improve neurobehavioral outcome as reflected in motor and cognitive function.

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 (2 h, 4 h, 24 h, 72 h 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 (4 h, 24 h, 72 h post TBI). Wet/dry ratio determined.
- **c.** Blood brain barrier permeability analyzed by dye tracer extravasation (24 h & 72 h post TBI).
- **d.** Cell injury by histological analysis (7 d & 30 d post TBI).
- **e.** Endocannabinoid Levels measure in extracted brain tissue lipids

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

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 hind limb reflex.
- **c.** Somatomotor function examined by a beam balance task and beam-walking task.
- **d.** Cognitive function tested by the radial-arm maze.

KEY RESEARCH ACCOMPLISHMENTS:

TASK 1a

Inhibition of EC degradation following TBI decreases neutrophil activation. This study was completed as a component of Task 1, to determine the effect of EC degradation inhibition on markers of inflammation following TBI. TBI was induced in a subset of animals by lateral fluid

percussion (1.8 atm; ~ 25 ms), while sham surgery animals did not receive any injury. Thirty minutes post-TBI, rats were randomized into experimental groups (n=6-8 per group) and received intraperitoneal injections of vehicle (alcohol, emulphor, and saline; 1:1:18) or a selective inhibitor of 2-AG (JZL184, 16 mg/kg) or AEA (URB597, 0.3 mg/kg) degradation. Overall, TBI resulted in an increase in brain myeloperoxidase (MPO) activity in the ipsilateral region of injury as compared to the sham animals post injury (Figure 3a-c). Treatment with the selective EC inhibitor JZL184 resulted in markedly decreased MPO activity in the ipsilateral brain region when compared to the TBI/vehicle-treated animals at 24 h and 48 h post TBI. However, this protective effect was not evident at the 72 h time point. In contrast, MPO activity measured in brains of animals treated with the selective EC inhibitor URB597 did not show effective suppression in neutrophil influx as our data indicates no differences between that of the TBI/vehicle-treated animals. Our findings imply higher efficacy of JZL184 in attenuating the inflammatory response early on, but also indicate a relatively short-lived anti-inflammatory effect, as by 72 h MPO values are elevated. It is possible that additional doses of the EC degradation inhibitors may prove beneficial at further reducing MPO activity, however most of the outcome measures we examined showed significant response with the single dose of EC degradation inhibitors.

TASK 1a

Effects of inhibiting EC degradation in reducing pro-inflammatory cytokine expression following TBI. This experiment was completed as a component of Task 1 to identify the effect of EC degradation inhibition on reducing inflammation post-TBI. TBI was induced in a subset of animals by lateral fluid percussion (1.8 atm; ~ 25ms), while sham surgery animals did not receive any injury. Thirty minutes post-TBI, injured rats were randomized into experimental groups (n=6-8 per group) and received intraperitoneal injections of vehicle (alcohol, emulphor, and saline; 1:1:18) or a selective inhibitor of 2-AG (JZL184, 16 mg/kg) or AEA (URB597, 0.3 mg/kg) degradation. TBI initiates a neuroinflammatory cascade characterized by an increased production of proinflammatory cytokines and chemokines, such as interleukin IL-1, IL-6, tumor necrosis factor (TNFα) and MCP1. In the case of TBI, this complex neuroinflammatory cascade can promote neuroinflammation and potentially lead to neurodegeneration. We have previously demonstrated that preventing degradation of 2-arachidonoyl glycerol (2-AG) and N-arachidonoyl-ethanolamine (AEA) ameliorate the neuroinflammatory response at 24 h post-TBI. We analyzed our 24 h and 72 h tissue samples post TBI for IL-6 activity using an Invitrogen IL-6 Rat Elisa Kit. Our results showed no significant difference between sham and TBI/vehicle-treated animals. Furthermore, drug interventions (JZL184 16mg/kg, IP, 30 min post TBI) and (URB597 0.3mg/kg, IP, 30 min post TBI) did not effectively decrease IL-6 expression as anticipated. Therefore, to provide a more sensitive measure of the pro-inflammatory response following TBI, we measured cytokine mRNA expression by RT-PCR analysis. Data for 72 h post TBI indicates that MCP1 is the only upregulated cytokine in TBI animals (Figure 4).

TASK 1b

Brain edema. Nothing to report. Findings from the blood brain barrier studies (mentioned in Task 1c) confirm brain edema, so this sub-aim was not pursued further.

TASK 1c

Inhibition of EC reduces BBB disruption following TBI. To determine if these biochemical changes resulted in structural protection, we next examined the impact of inhibition of EC degradation on blood brain barrier (BBB) integrity. This study was completed as a component of Task 1 to identify the effects of EC degradation inhibition on improving BBB disruption following TBI. The experimental groups were divided as described above with animals randomly split into four groups (sham, TBI-VEH, TBI-JZL, TBI-URB; n=6-8 per group), with drug-treated animals receiving either JZL184 (16 mg/kg IP) or URB597 (0.3 mg/kg IP). We examined the effectiveness of inhibiting endocannabinoid enzymatic degradation on blood brain barrier integrity at the 24 h and 72 h time points using Evan's Blue dye tracer extravasation. TBI alone results in a significant increase in blood brain barrier leak (~two-fold) as compared to the sham animals (Figure 5). Results from this study suggest that inhibition of 2-AG and AEA degradation using (JZL184; 16 mg/kg and URB597 0.3 mg/kg) administered IP, 30 min post TBI improves BBB integrity in the targeted ipsilateral brain region of injury when compared to the TBI/vehicle group. Specifically, animals treated with the selective inhibitor JZL184 sacrificed 24 h post TBI experienced significant ($p<0.05) improvements in BBB integrity when compared to the vehicle-treated animals (below). The compromise in BBB structure does not appear resolved at 72 h post injury as seen in the TBI/vehicle animals without the use of EC drug interventions. While not statistically significant, both JZL184 and URB597 are effective at minimizing BBB leak in the ipsilateral region 72 h after injury. In subsequent studies, we showed that BBB permeability has been restored at day 7 post TBI, and there are no differences between TBI and sham animals, with JZL and URB-treated animals remaining similar to sham values.

TASK 1d

EC degradation inhibition attenuates astrocyte and microglial activation 24 h and up to 7 d following TBI. This study was also completed as a component of Task 1 to determine the effect of EC degradation inhibition on markers of inflammation following TBI. TBI was induced in a subset of animals by lateral fluid percussion (1.8 atm; ~ 25ms), while sham surgery animals did not receive any injury. Thirty minutes post-TBI, injured rats were randomized into experimental groups (n=6-8 per group) and received intraperitoneal injections of vehicle (alcohol, emulphor, and saline; 1:1:18) or a selective inhibitor of 2-AG (JZL184, 16 mg/kg) or AEA (URB597, 0.3 mg/kg) degradation. In addition to oxidative stress, activation of resident support cells (microglia and astrocytes) post injury can play a role in sustained neuroinflammation that increase secondary injury. In the second quarter of this funding year, perfusion fixed brains were sliced, mounted and stained with GFAP, a marker of astrocyte activation, and ED1, a marker of microglial activation. Analysis was completed on perfusion fixed brains 24 h post-TBI. (Figure 2 A-N).

TASK 1e

Determination of EC levels. Despite the fact that our studies had shown significant neuroprotection with the use of EC degradation inhibitor administration during the post-TBI period, we had not obtained measurements of the actual levels of the EC in tissues. In collaboration with Dr. Cecilia Hillard, we performed measurements in tissues obtained 24 h post-TBI (Figure 8). Because we had seen more robust protection using the JZL compound, we limited the analysis to tissues obtained from JZL-treated animals and did not include URB treatment in this set of studies. Frozen tissue was used for extraction and the amounts of AEA, 2-AG and 3 additional

lipid analogs (palmitoylethanolamide (PEA); N-oleoylethanolamine (OEA) and 2-oleoylglycerol (2-OG)) were quantified using isotope dilution, LC/MS/MS using an Agilent Technologies 6460 Triple Quad LC/MS with a 1290 Infinity liquid chromatography unit and were analyzed using Agilent MassHunter Qualitative Analysis B.04.00 software. Unfortunately, the results from these showed that JZL treatment did not change EC levels in ipsilateral cortex 24 h after injury. Although JZL appeared to have a long-lasting therapeutic effect on TBI animals, ipsilateral cortex measurements of 2-Arachidonoylglycerol (2-AG), 2-Oleoylglycerol (2OG), Oleoylethanolamine (OEA), N-arachidonoylethanolamine (AEA), and Palmitoylethanolamide (PEA) were not significantly different between experimental groups. It remains to be seen whether a different time point (perhaps closer to the time of TBI) would yield different results.

TASK 2a

Effectiveness of the selective increase in endogenous 2-AG and AEA levels in preventing neuroendocrine dysfunction following TBI. These studies were completed as a component of Task 2 to identify the role of the EC system in alleviating neuroendocrine dysfunction following TBI. The experimental groups were divided as described above with animals randomly split into four groups (sham, TBI-VEH, TBI-JZL, TBI-URB; n=6-8 per group), with drug-treated animals receiving either JZL184 (16 mg/kg IP) or URB597 (0.3 mg/kg IP). Collection of blood samples past the initial 24-h time point was a challenge in some animals, as the indwelling catheters have a high rate of failure at that time. Blood pressure and heart rate measurements were recorded immediately prior to, during, and after TBI in 30-s intervals. Our results indicate a significant decrease in heart rate immediately following TBI when compared to the sham (Figure 6). No significant alteration in mean arterial blood pressure was observed during the immediate and early time post-TBI. However, it is important to note that animals were lightly anesthetized with 3% isoflurane during the time of injury, and that may explain the lack of significant modulation of blood pressure response. This lack of significant TBI effect on hemodynamics did not warrant additional investigations with the use of the EC degradation antagonists.

TASK 2b

Characterization of Neuroendocrine and Cardiovascular Alteration Following TBI. Studies were conducted to characterize the model for determining the effectiveness of the selective increase in endogenous 2-AG and AEA levels in preventing neuroendocrine dysfunction following cardiovascular system challenge post TBI. We examined the neuroendocrine and cardiovascular system response in sham vs. TBI animals 24 h post-TBI when challenged with hydralazine HCl (10mg/kg, administered IV). The goal was to reveal the extent of subclinical neuroendocrine dysregulation following TBI. Animals were subjected to a pharmacological challenge to induce hypotension. Mean arterial blood pressure (MABP) was recorded from TBI and sham animals only. TBI animals present with lower baseline MABP as compared to un-injured sham animals (Figure 6a) p= NS. After 15 min of stable blood pressure recordings, hydralazine hydrochloride (10 mg/kg) was slowly injected intravenously through the jugular catheter. Mean arterial blood pressure decreased substantially (~50% of baseline) within 10 min of hydralazine HCl injection, and remained suppressed during the monitoring period of 90 min. Initial results suggested that there appeared to be a trend for more persistent hypotension in the TBI animals vs. sham animals. This study was repeated to include a more frequent time point for data collection, to ensure that

any early differences in response were not missed by the initial set of studies. The results showed that the hydralazine-induced decrease in blood pressure was similar in sham and TBI animals (Figure 7). While preliminary findings suggest that hydralazine appropriately lowers MABP, it does not appear to unmask neuroendocrine dysregulation of the cardiovascular system. Sodium nitroprusside has a rapid onset of action. We proceeded to challenge the animals with sodium nitroprusside following the same protocol described above for hydralazine. Post-TBI animals challenged with sodium nitroprusside (10mg/kg) intravenously through the jugular catheter appeared to demonstrate dramatic reductions in MABP seen at 30-s post injection, coupled with slower and incomplete recovery to baseline compared to time-matched sham controls (Figure 7). These findings suggest that integrity of cardiovascular regulation may be compromised following TBI, and that the use of sodium nitroprusside, as compared to hydralazine hydrochloride, may be of more use in unmasking cardiovascular dysregulation. Additional studies did not show this to be a reproducible finding, therefore no additional studies in inhibitor-treated TBI animals were performed.

TASK 2c

Water deprivation test. Nothing to report. Because of the lack of statistical significance in the cardiovascular challenge experiment (Task 2b) we decided not to further pursue this route of investigation, instead focusing cost and energy on other elements of this project.

TASK 3a

Righting reflex. The severity of injury was determined by the amount of time it takes the animal to right itself following the injury. This measure reflects a loss of consciousness following the injury and was used as a point of reference for neurological and neurobehavioral assessments post-TBI. All animals were first placed in a 3% isoflurane (pre-charged) anesthesia induction chamber. Once anesthetized, each animal was then placed into the stereotaxic frame and fitted with the cranial female luer loc connected to the lateral fluid percussion (LFP) system. Although the animals were not actively receiving isoflurane at the time of injury, they were still anesthetized. Upon the return of a hind-limb toe pinch response, the traumatic brain injury (1.8 atm; ~ 25 ms) was delivered to the dura. Following the injury, the animal was removed from the stereotaxic frame and placed on its right side to recover. The time it took for the animal to regain complete consciousness was recorded. Sham control animals were connected to the LFP system in the exact same manner, but not subjected to the TBI. Our results to date indicate that TBI produces a significant delay in righting reflex time when compared to the uninjured sham animals ($p^*<0.0001$) (Figure 11). Because the inhibitors were administered following TBI, this outcome measure is not expected to be affected by the drugs.

TASKS 3b, 3c

Inhibition of EC degradation reduces sensorimotor dysfunction following TBI. To determine if the protection conferred by inhibition of EC degradation on the biochemical and structural outcome measures detailed above were associated with improved sensorimotor function, sensory reflex and somatomotor function were measured by a modified neurological severity score (NSS). These experiments were completed as a component of Task 3 to determine the protective effects of the EC system of behavior. The EC degradation inhibitor JZL184 was utilized to specifically

inhibit MAGL and therefore increase 2-AG concentrations, and the EC degradation inhibitor URB597 was utilized to specifically inhibit FAAH and therefore to increase AEA concentrations. Animals were randomly divided into four groups (sham surgery, TBI/VEH, TBI/JZL, TBI/URB; n=6-8 per group) and 30 min post-TBI animals either received one injection (16 mg/kg i.p.) of JZL184, one injection of URB597 (0.3 mg/kg i.p.), or one injection of vehicle (alcohol, emulphor, and saline; 1:1:18), followed by behavioral testing. The modified NSS consisted of an 18-point scale comprised of motor and sensory tests, beam balance tests, and reflex tests. Animals received scores based on their ability to carry out the tests. The ΔNSS is representative of sensory and somatomotor outcomes and is well characterized in the lateral fluid percussion model of TBI. There was a significant increase of ΔNSS seen in vehicle-treated animals as compared to sham animals, and this remained elevated (but no longer statistically different from pre-TBI or sham values) until 14-days post TBI (Figure 1). Administration of JZL and URB attenuated the increase in NSS and NBS beginning at 24 h following TBI. These findings suggest that the benefit of the selected intervention is sustained and extends beyond the biochemical changes described above and produces functional protection as well.

TASK 3d

Cognitive impairment with radial arm maze. To determine if the protection conferred by inhibition of EC degradation on the biochemical and structural outcome measures detailed above were associated with improved cognitive function, animals were subjected to the Y-maze task for assessing cognitive function 3 days post TBI. These experiments were completed as a component of Task 3 to determine the protective effects of the EC system of behavior. The EC degradation inhibitor JZL184 was utilized to specifically inhibit MAGL and therefore increase 2-AG concentrations, and the EC degradation inhibitor URB597 was utilized to specifically inhibit FAAH and therefore increase AEA concentrations. Animals were randomly divided into three groups (sham surgery, TBI/VEH, TBI/JZL; n=6-8 per group) and 30 min post TBI animals either received one injection (16 mg/kg IP) of JZL184, one injection of URB597 (0.3 mg/kg i.p.), or one injection of vehicle (alcohol, emulphor, and saline; 1:1:18) followed 3 days later by cognitive behavioral testing. Testing was accomplished using the Y-maze task, which tests an animal's ability to recall spatial information in deciding which arm of the Y-maze to enter upon each trip to the middle intersection of the device. A normal rat will recall the arm that he recently explored and will choose to explore the new arm (usually picking the new arm correctly with 65% accuracy). A cognitively impaired rat will not recall the arm that was just explored and will therefore pick an arm at random (resulting in a 50% success rate). In the current study, TBI/VEH animals were not significantly different than cognitively impaired rats, failing to correctly pick the unexplored arm more than 50% of the time. JZL184-administered animals, however, showed significant improvement on this task, picking the correct arm upwards of 70% of the time (Figure 12). These findings suggest that the benefit of the selected intervention is sustained and extends beyond the biochemical changes described above and produces functional protection as well**.**

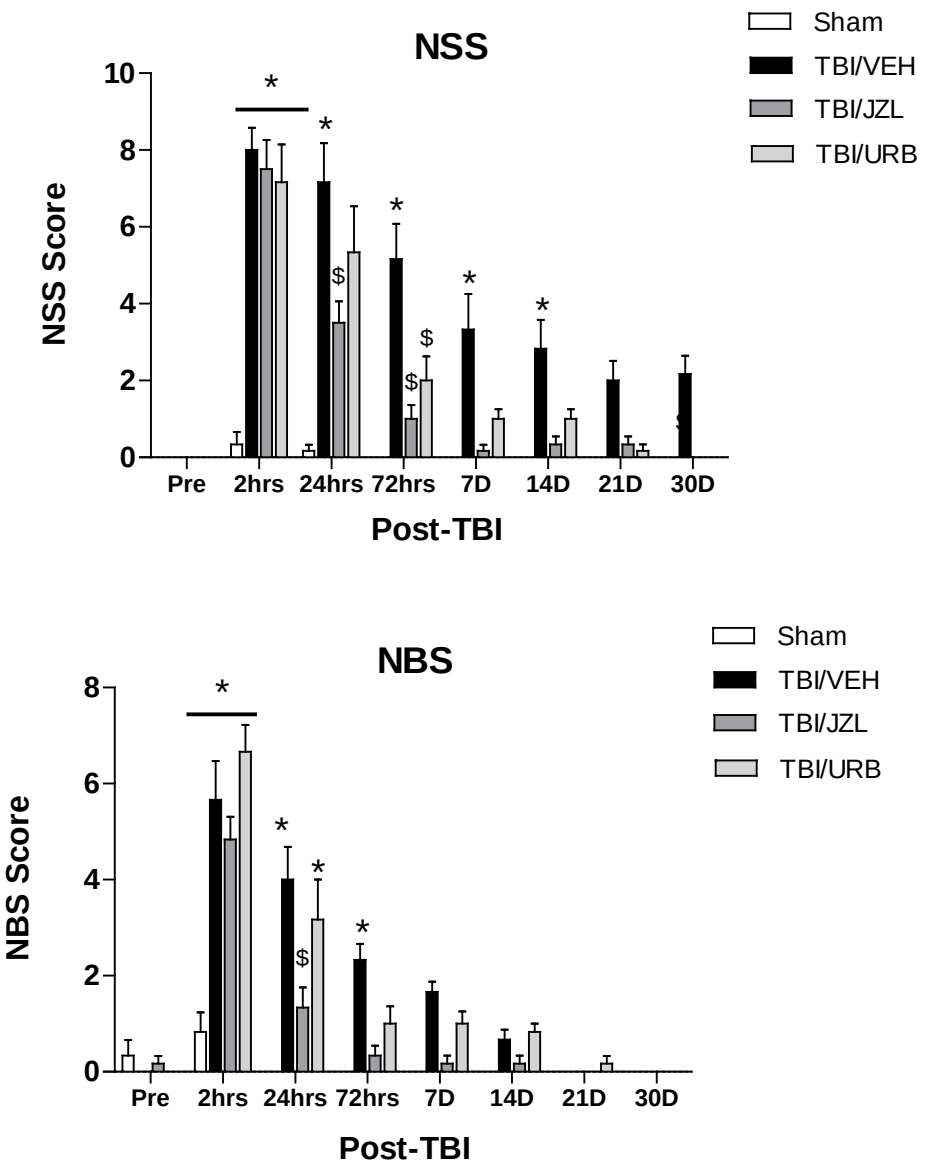
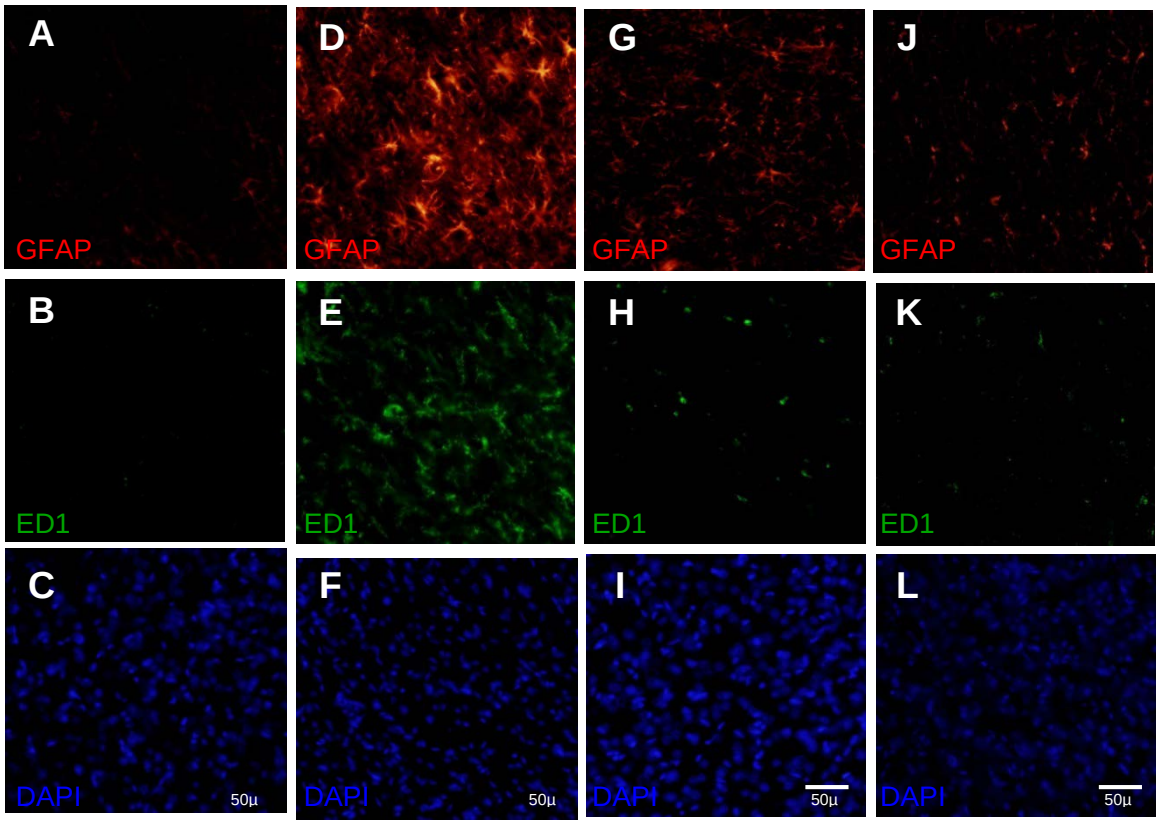


Figure 1. NSS and NBS scores 30 days post TBI. Inhibition of 2-AG degradation by the use of the JZL184, administered 30 min post TBI, reduced NSS starting at 24 h post TBI and restored NSS scores to sham levels through 14 d post TBI. In addition, inhibition of AEA degradation the use of URB597, administered 30 min post TBI, reduced NSS starting at 72 h post TBI and restored NSS scores to sham levels through 14 d post TBI. 2B.) JZL 184 treatment reduced NBS starting at 24 h and restored NBS scores to sham levels through 72 h post-TBI. URB597 treatment was able to restore NBS scores to Sham levels at 72 h post-TBI (Top). Total Neurological Severity Score (NSS) and neurobehavioral score (NBS) prior to TBI and at selected time points post TBI. (Bottom) NSS; examines Gross Motor Function – Flexion of limbs, Beam walking, Sensory – placement of palms, and reflexes pinnal, corneal, startle, righting (max score 25). NBS; examines lateral pulsion resistance, ability to stand on an inclined plane, exploratory behavior, novel object exploration (max score 12).



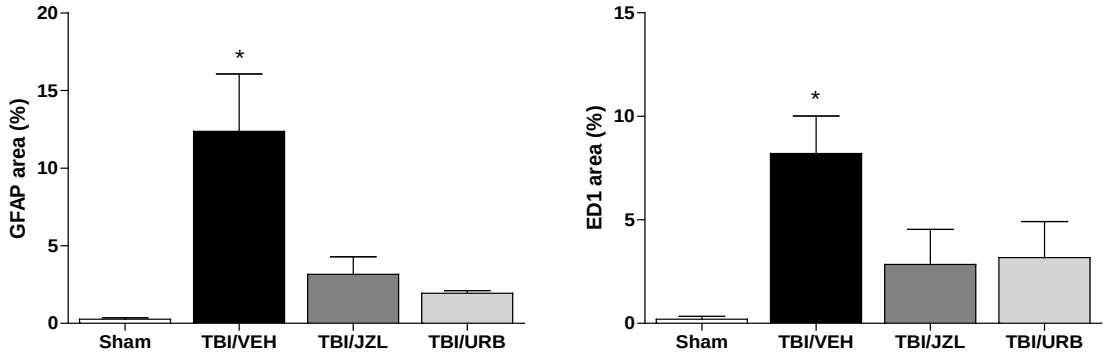


Figure 2. Immunohistochemistry of perfusion fixed brains 24 h post TBI. Inhibition of 2-AG and AEA degradation by the use of the selective inhibitors (JZL184; 16 mg/kg and URB597 0.3 mg/kg, respectively, IP) administered 30 min post-TBI, significantly reduced astrocyte (GFAP) and microglial (ED1) activation at 24 h post-TBI. (n=4-8/group).

Fig: 1a

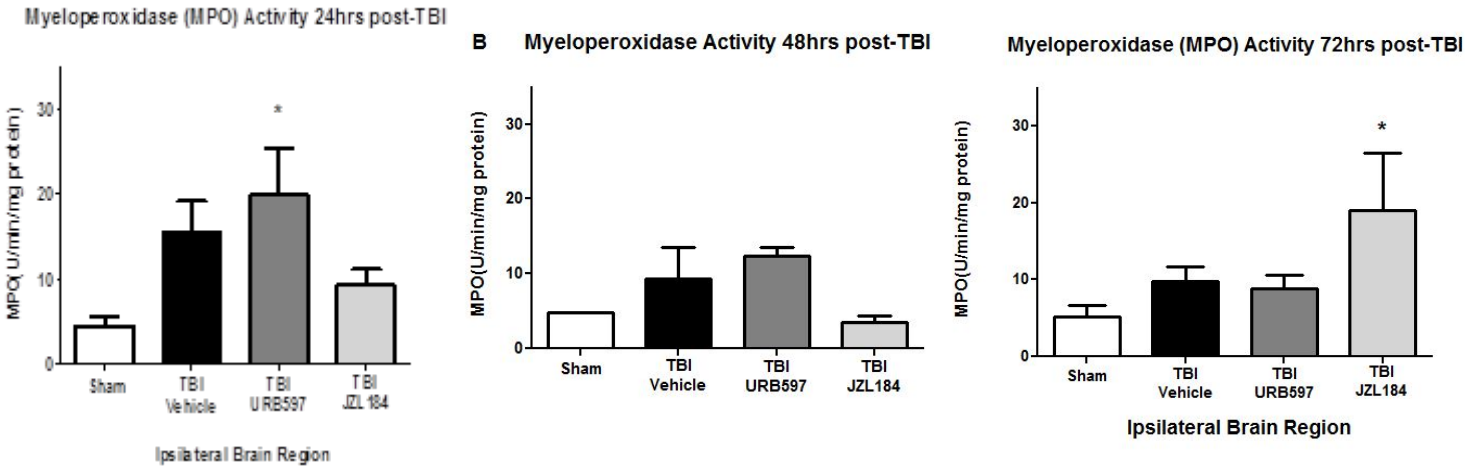


Figure 3. Myeloperoxidase (MPO) activity (U/min/mg protein) in the ipsilateral brain region excised at 24 h, 48 h and 72 h post TBI. Inhibition of EC. degradation proves to be effective at decreasing MPO activity 24 h and 48 h following TBI when treated with the selective inhibitor JZL184 (16mg/kg, IP, 30minutes post-TBI) Sham; TBI/Vehicle (alcohol, emulphor, saline 1:1:18); TBI/JZL 184 (16mg/kg, IP); and TBI/URB 597 0.3mg/kg, IP) (n=6/group); P*<0.0001 compared to sham; Analyzed by One-way ANOVA.

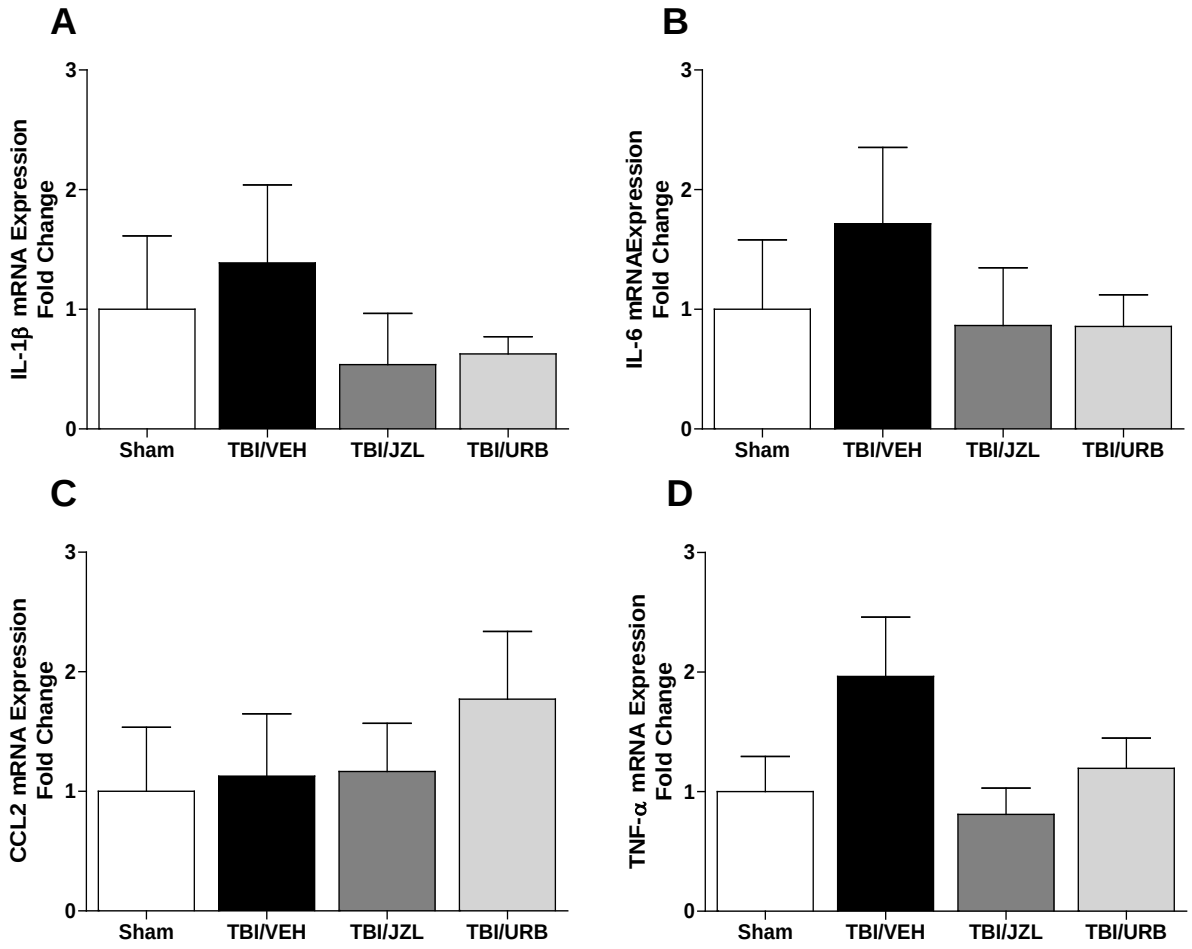


Figure 4. Effects of inhibiting endocannabinoid degradation in reducing pro-inflammatory cytokine expression 72 h following traumatic braininjury. Brain tissues were analyzed for TNFα, MCP1, IL-6 and IL-1 by RT-PCR 72 h post injury in Sham; TBI/vehicle- (alcohol, emulphor, saline 1:1:18); TBI/JZL 184 (16mg/kg, IP); and TBI/URB 597 0.3mg/kg, IP) treated animals. (n=6 per group); P* < 0.0001; Analyzed by One-way ANOVA.

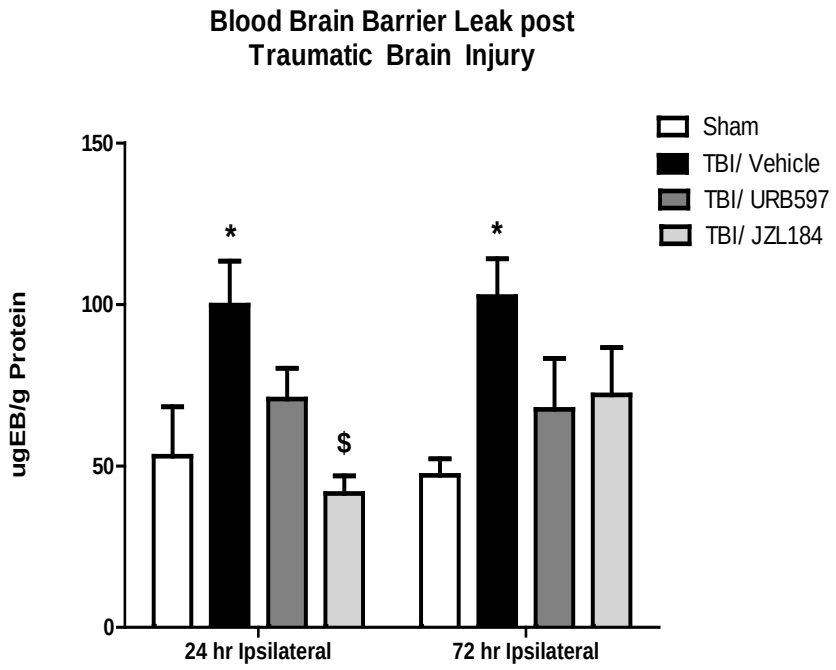


Figure 5. Inhibition of EC degradation reduces blood brain barrier breakdown 24 h and 72 h post-TBI. Traumatic brain injury significantly disrupts the integrity of the blood brain barrier when compared to the sham uninjured animals. Inhibition of 2-AG and AEA degradation with the use of the selective inhibitors (JZL184; 16 mg/kg and URB597 0.3 mg/kg, respectively, IP) administered 30 min post-TBI helps to reduced blood brain barrier dysfunction when compared to vehicle treated animals. 24 h post-TBI animals treated with JZL184 is significantly different than that of the TBI/vehicle-treated animals. P*<0.05 compared to sham; P$<0.05 compared to TBI/vehicle; Analyzed by Two-way ANOVA (n=5-19 per group).

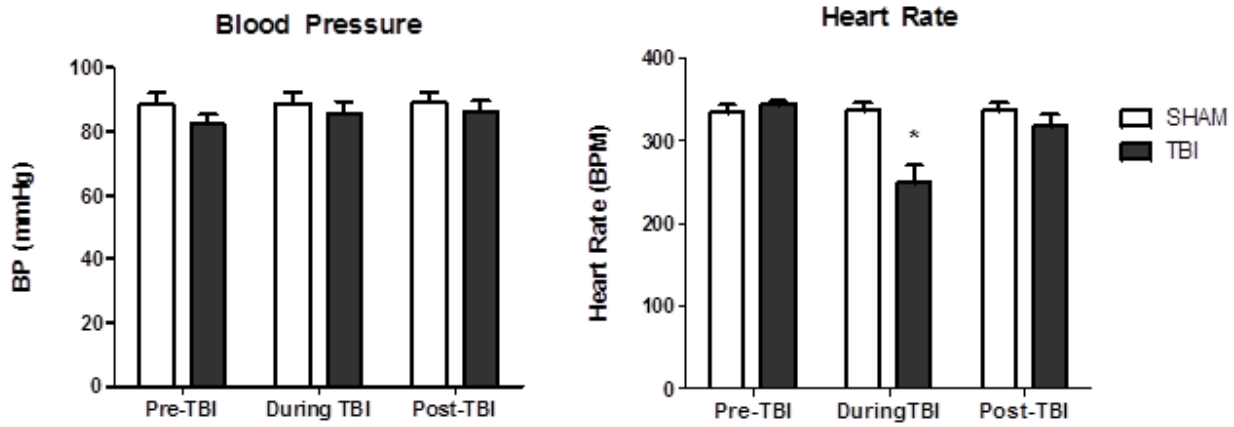


Figure 6. Blood pressure (mmHg) and heart rate (BPM) response to TBI. All animals were anesthetized using 3% isoflurane and were continuously monitored via the arterial catheter connected to a pressure transducer for changes in blood pressure and heart rate using the LabPro System. Blood pressure and heart rate were recorded and analyzed for a period of 30 s at each of the following time point intervals (pre-TBI, during TBI, post-TBI). Results to date indicate significant heart rate changes during the time of the traumatic brain injury. (n=18/Sham; n=50/TBI). P * <0.01 sham vs. TBI; Analyzed by Two-way ANOVA.

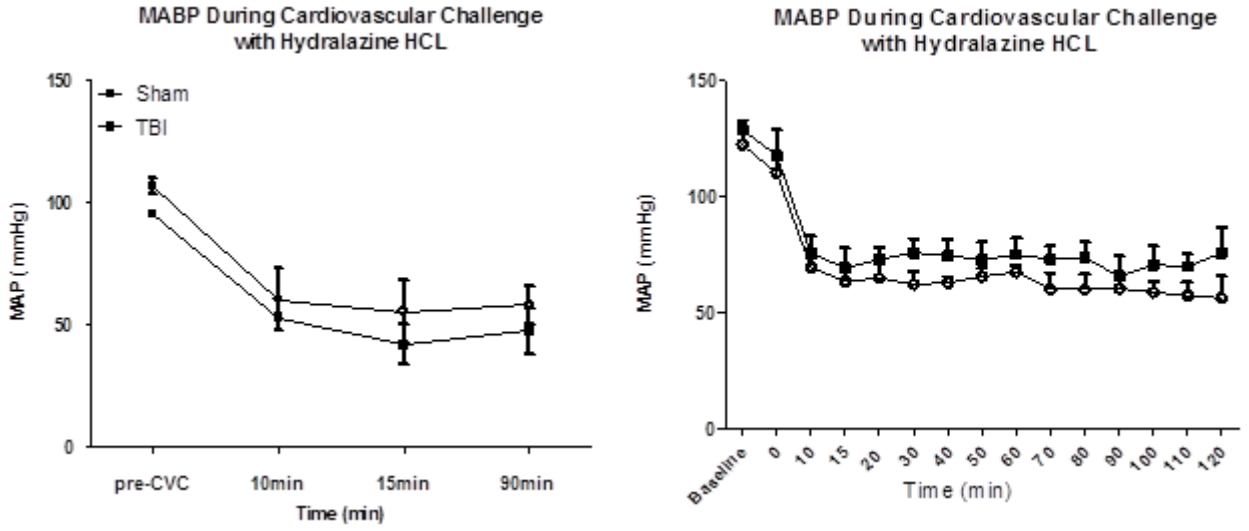


Figure 7. MABP in response to cardiovascular challenge with hydralazine HCL (10mg/kg). Animals subject to TBI appear to have a lower baseline mean arterial pressure (MABP) 24 h post-TBI compared to sham. The graph on the right depicts measurements obtained at greater frequency than those on the left. Because there was no apparent TBI effect, studies with inhibitors were not performed.

During the no cost extension of the grant, we completed studies establishing the optimal length of pharmacologic intervention. We hypothesized that prolonging the duration of drug treatment would achieve optimal neuroprotective effects. This is accomplished by administering a second dose of the selective EC enzyme degradation inhibitors (JZL184; 16 mg/kg and URB597 0.3 mg/kg, IP); the initial dose was administered 30 min post-TBI followed by a second dose 24 h later. NSS and NBS were determined at 2, 24, 48, and 72 h time points. Our results demonstrate that both EC enzyme degradation inhibitors, given at 30 min and 24 h post-TBI, are effective at reducing the NSS score at 48 h when compare to vehicle treated TBI animals, with no significant advantage over that seen with a single EC inhibitor injection. These studies warrant further development.

Future directions

The results obtained from the studies performed during the funded period have provided exciting, reproducible, and significant proof of the effectiveness of EC degradation inhibition in ameliorating the sequelae from TBI. We performed additional measures using tissues obtained from the funded studies and have obtained remarkable results showing that JZL184 treatment attenuated expression of markers of synaptic hyperexcitability including pGluR1^{S845}/GluR1 and pERK/ERK ratios and mEPSC frequency as well as amplitude in cortical neurons 10 days post-TBI. TBI/VEH treated animals expressed greater ratio of phosphorylated ERK 1 and 2 to total ERK (pERK)/ERK (approximately 2-fold) and greater ratio of phosphorylated AMPA glutamate receptor subunit GluR1 to total GluR1 (pGluR1^{S845})/GluR1 (approximately double) compared to sham animals.

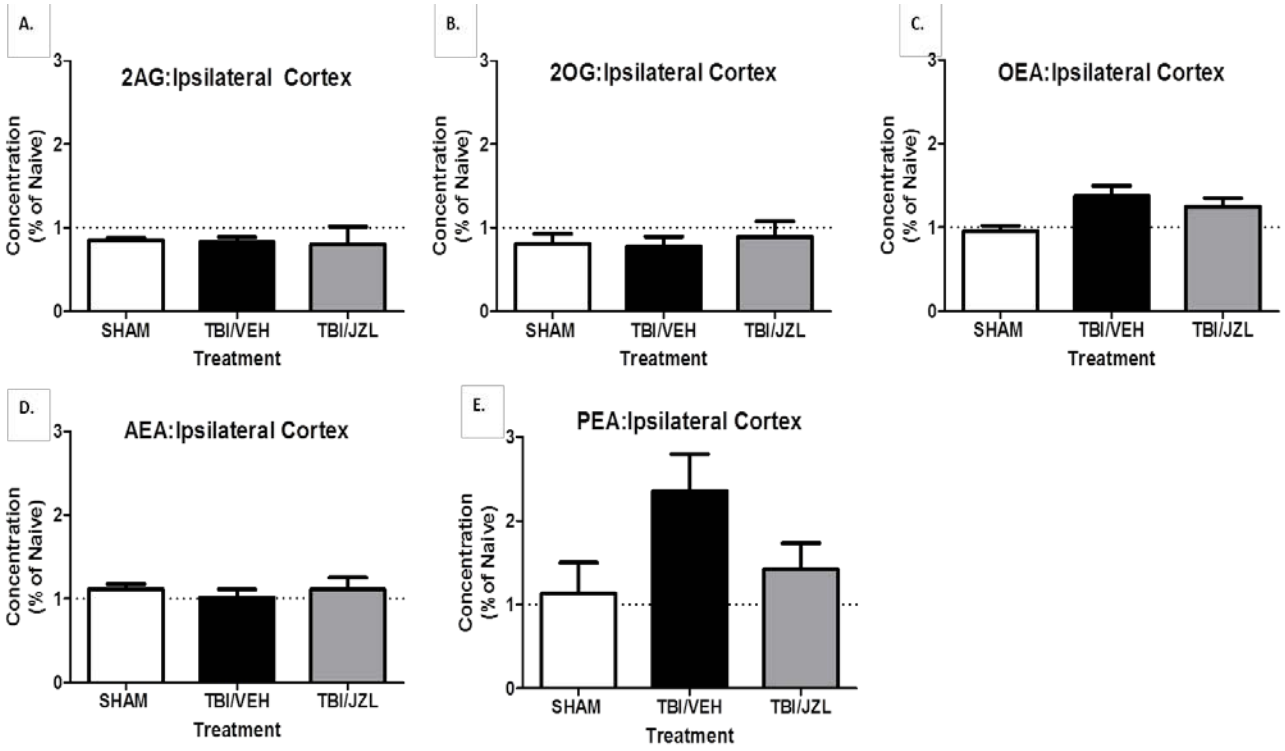


Figure 8. Ipsilateral concentrations of various endogenous EC 24 h post-TBI in the presence and absence of EC enzyme degradation inhibitors. Values are concentrations relative to those of naïve (sham) animals.

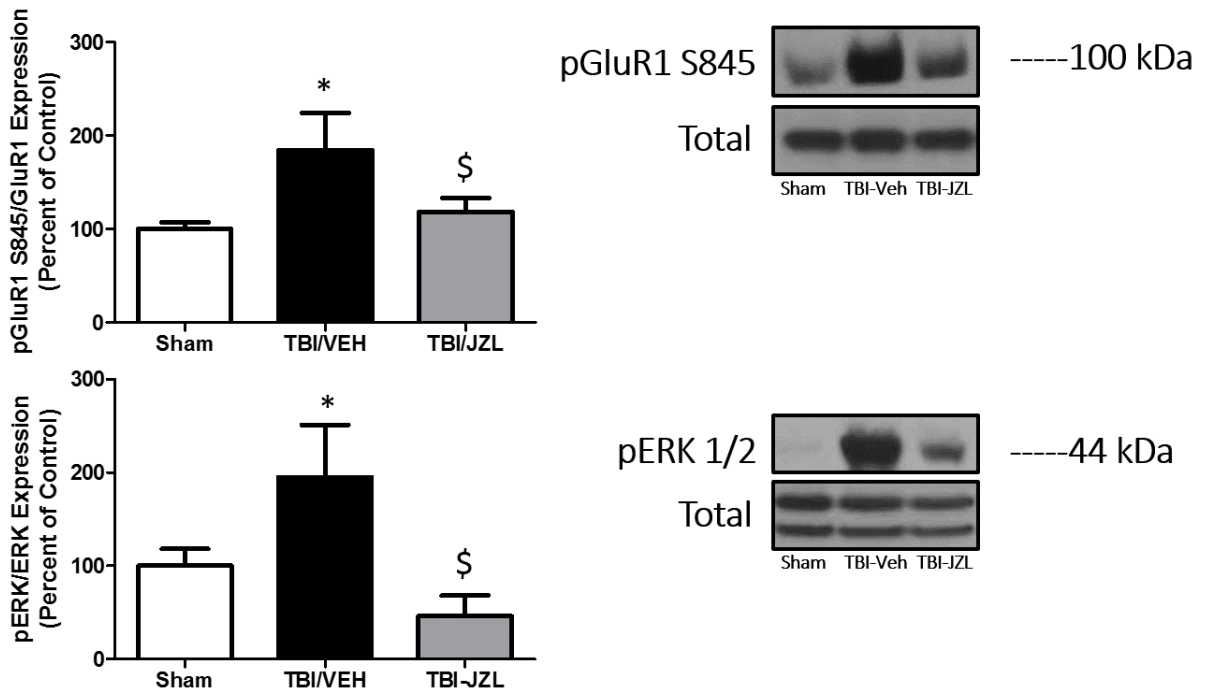


Figure 9. Ipsilateral cortex phosphoprotein changes in response to TBI and JZL treatment. JZL treatment significantly attenuated ($pGluR1^{S845}$)/GluR1 and (pERK)/ERK, reducing the ratios to approximately sham (uninjured) levels. *P<0.05 compared to sham levels, $P<0.05 compared to TBI/VEH levels. Analyzed via one-way ANOVA.

These findings suggest that the attenuation in neuroinflammation seen with EC degradation inhibitors translates to further neuroprotection and likely attenuates excitotoxicity. Though this is an early stage hypothesis, the results from these studies are quite promising and encouraging of an area that needs further investigation. We believe our results showing significant white matter inflammation as well as synaptic changes indicating a hyper-glutamatergic state following TBI and improvement in these outcome measures with EC degradation inhibitors indicate the elucidation of a critical window for therapeutic intervention. Furthermore, our results suggest that a therapeutic intervention can prevent the transition from the primary injury to the secondary injury, improving long-term outcomes. We believe these findings show that the EC system plays a critical role in blocking the transition to secondary injury by modulating synaptic activity and exerting neuroprotective and anti-inflammatory effects.

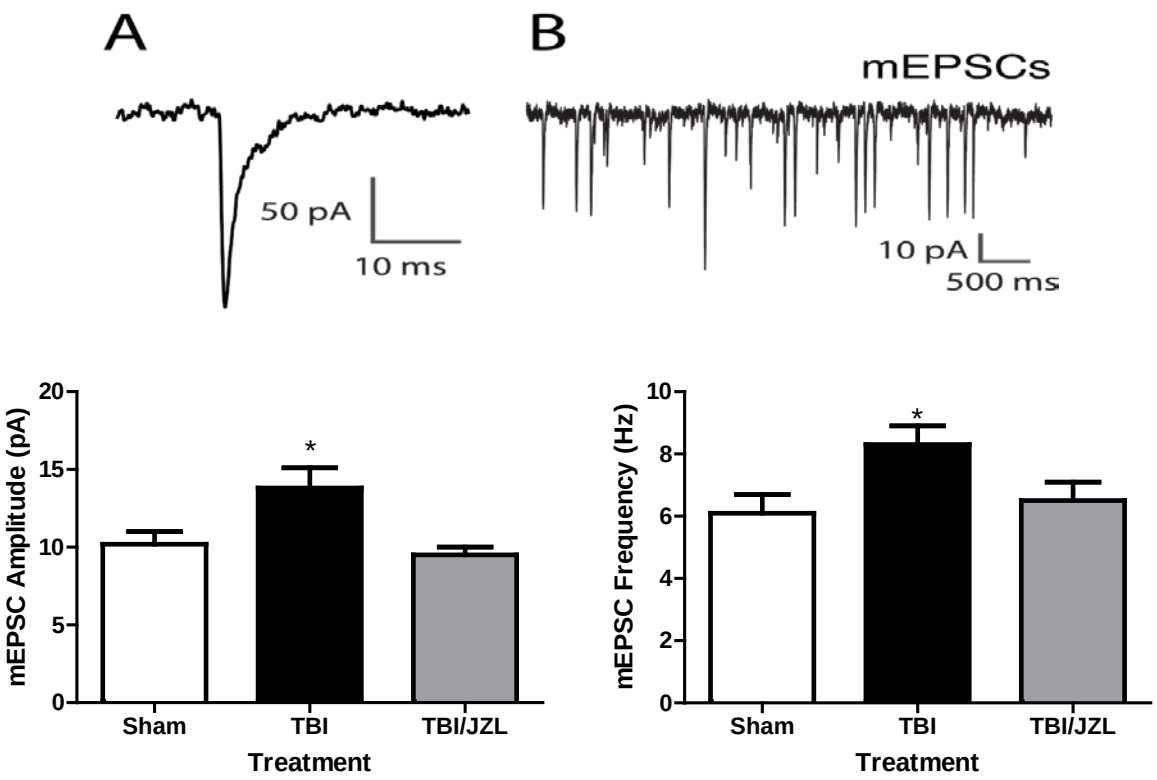


Figure 10. Mini excitatory post-synaptic currents (mEPSCs) recorded from rat cortex (site of injury) via brain slice electrophysiology 10 days post injury. A downward deflection is a depolarizing current. (A) Representative mEPSC and (B) representative trace recording. JZL treatment significantly attenuated mEPSC frequency (C) and amplitude (D) compared to TBI-vehicle animals. *P<0.05 analyzed via one-way ANOVA.

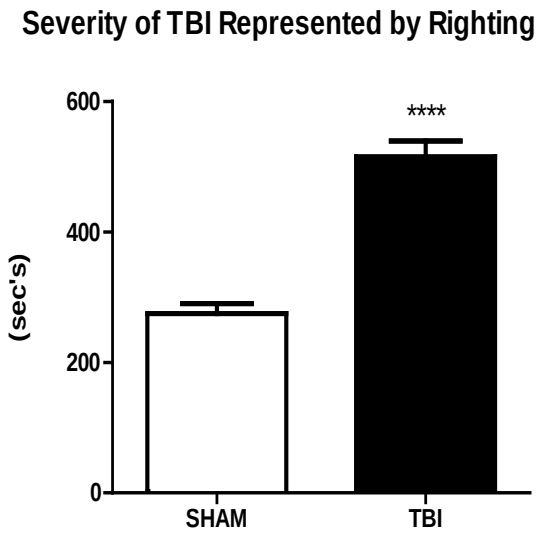
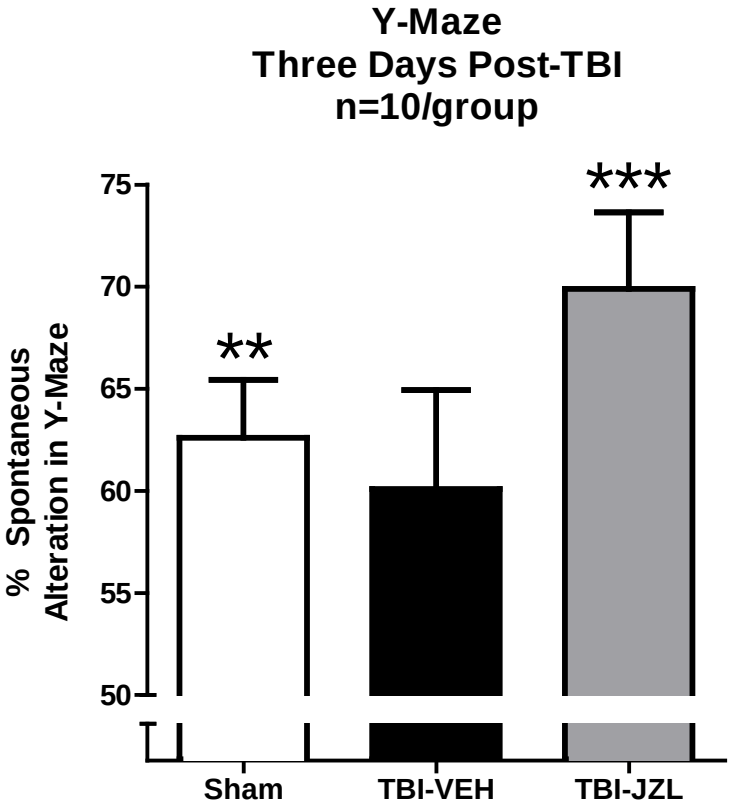


Figure 11: Severity of TBI Represented by Righting Reflex time immediately post TBI. Animals subject to traumatic brain injury have a significantly increased duration of loss of consciousness determined by the time it takes the animal to right itself when compared to the sham uninjured animals. P * value =0.0001 sham vs. TBI analyzed by a 2-tailed unpaired T-test.



**p<0.01, ***p<0.001 compared to 50% alternation (chance)

Figure 12: Severity of cognitive impairment based on Y-maze task Animals subjected to TBI exhibit cognitive impairment based on poor performance on the Y-maze task. JZL-184 treatment significantly improved cognitive performance. Analyzed via one-sample T-test compared to 50% (cognitively impaired rats).

REPORTABLE OUTCOMES:

Opportunities for Training and Professional Development:

This project has allowed training opportunities in laboratory techniques, animal experimentation, tissue preparation and processing, and data analysis for the numerous students who have had the opportunity to work on it. In addition, students were able to present the data listed here at intramural and extramural conferences, which allowed for students to increase their professional development through presentation preparation, public speaking, and networking.

Dissemination of results:

Publications:

Journal Articles:

1. Katz PS, Sulzer JK, Impastato RA, Teng SX, Rogers EK, Molina PE. Endocannabinoid degradation inhibition improves neurobehavioral function, blood-brain barrier integrity, and

neuroinflammation following mild traumatic brain injury. J Neurotrauma. 2015;32(5):297-306. Epub 2014/08/29. doi: 10.1089/neu.2014.3508. PMID: 25166905; PMCID: PMC4348366.

2. Mayeux JP, Katz PS, Edwards S, Middleton J, Molina PE. Inhibition of Endocannabinoid Degradation Improves Outcomes from Mild Traumatic Brain Injury: A Mechanistic Role for Synaptic Hyperexcitability. J Neurotrauma 2016; Submitted.

Book Chapter

Cannabinoids. In: Neuroinflammation and Neurodegeneration. Katz PS, Edwards S, Molina PE. Peterson and Torborek (eds.), Springer Science + Business Media, New York, NY, 2014.

Presentations:

1. Oral presentation. Inhibition of endocannabinoid degradation improves outcome following traumatic brain injury (2011). Sulzer JK and Molina PE. 34th Annual Conference on Shock. Norfolk, VA. June 11-14.
2. Poster presentation. Inhibition of endocannabinoid degradation reduces neurological damage and blood brain barrier disruption following traumatic brain injury (2013). Katz PS, Impastato R, Rogers E, Molina PE. Experimental Biology. Boston, MA. April 21.
3. Oral presentation. Inhibition of Endocannabinoid Degradation Improves Cellular and Behavioral Outcomes from Mild Traumatic Brain Injury (2016). Mayeux JP, Katz P, Edwards S, Middleton J, Molina P. Experimental Biology. San Diego, CA. April 8.
4. Poster presentation. Inhibition of Endocannabinoid Degradation Improves Cellular and Behavioral Outcomes from Mild Traumatic Brain Injury (2016). Mayeux JP, Katz P, Edwards S, Middleton J, Molina P. Experimental Biology. San Diego, CA. April 8.

Plans for next reporting period:

Nothing to report.

IMPACT

Principle Discipline:

- Demonstrated that inhibition of EC degradation can limit inflammation and oxidative stress following TBI
- Demonstrated that the benefits of inhibition of EC degradation can limit not only the structural damage and dysfunction following TBI but can confer functional protection as well as assessed by a panel of sensorimotor function tests. Endocannabinoid degradation inhibition effectively improves neurological and neurobehavioral outcomes following TBI
- Demonstrated that inhibition of EC degradation can significantly maintain blood brain barrier integrity following TBI
- 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
- EC enzyme degradation inhibitors attenuate glutamatergic hyperexcitability and modulate synaptic plasticity

Other Disciplines:

- A single dose 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

Technology Transfer:

- Nothing to report

Society:

- Promising therapeutic target/window (administering JZL184 via injection up to 30m post-TBI) allows for improved recovery from common injury usually affecting otherwise healthy individuals

CONCLUSION:

The overall results from studies conducted provide evidence that EC degradation inhibition following TBI can have lasting positive effects up to 30 days post-injury. We have shown that TBI causes inflammation and oxidative stress marked by increased pro-inflammatory cytokine expression, and that JZL and/or URB administration can attenuate this effect. In addition, EC degradation inhibition is effective in reducing microglial and astrocyte activation (the key cells involved in the local inflammatory response in the brain). In addition, we have shown that TBI reduces blood brain barrier function and JZL may be effective in protecting BBB integrity following injury. In addition to these cellular and molecular results, we also demonstrated that TBI causes significantly worse neurobehavioral outcome in the days following injury compared to sham animals. Interestingly, JZL administration after TBI not only attenuated cellular dysfunction (as described above) but also was effective in attenuating behavioral dysfunction.

Together, these results indicate that a single administration of endocannabinoid degradation inhibitor, such as JZL or URB, 30 min post TBI can have strong and long-lasting protective effects from mild TBI. Future studies need to examine whether an additional dose of JZL after TBI further improves outcome from mild TBI.

"So what." The knowledge gained from this study can potentially be further developed to yield a breakthrough therapeutic which, when administered in the early time course post-TBI, can reduce duration of recovery and improve cellular and behavioral outcomes.

CHANGES/PROBLEMS

Nothing to report.

PRODUCTS

As detailed in part 3: Accomplishments – Reportable Outcomes, the project resulted in two publications and four presentations.

PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

Name:	Patricia Molina, MD, PhD
Project Role:	*Principal Investigator*
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	*60 months*
Contribution to Project:	*Dr. Molina has overseen the work completed on the project.*
Funding Support:	

Name:	Jesse Sulzer
Project Role:	*Graduate Student*
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	*36 months*
Contribution to Project:	*Mr. Sulzer has performed work in animal behavior, surgery, and tissue analysis.*
Funding Support:	*NIAAA Institutional T32 Training Grant*

Name:	Renata Impastato
Project Role:	*Laboratory Technician*
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	*36 months*
Contribution to Project:	*Ms. Impastato has performed work in animal behavior, surgery, and tissue analysis.*
Funding Support:	*NIAAA Institutional T32 Training Grant*

Name:	Sophie Teng
Project Role:	*Graduate Student*
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	*36 months*
Contribution to Project:	*Ms. Teng has performed work in animal behavior, surgery, and tissue analysis.*
Funding Support:	*NIAAA Institutional T32 Training Grant*

Name:	Emily Rogers

Project Role:	*Rotating Student*
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	*12 months*
Contribution to Project:	*Ms. Rogers has performed work in animal behavior, surgery, and tissue analysis.*
Funding Support:	*NIAAA Institutional T32 Training Grant*

Name:	Jacques Mayeux
Project Role:	*Graduate Student*
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	*36 months*
Contribution to Project:	*Mr. Mayeux has performed work in animal behavior, surgery, and tissue analysis.*
Funding Support:	*NIAAA Institutional T32 Training Grant*

Name:	Paige Katz
Project Role:	*Post-Doctoral Fellow*
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	*24 months*
Contribution to Project:	*Dr. Katz has performed work in animal behavior, surgery, and tissue analysis.*
Funding Support:	*NIAAA Institutional T32 Training Grant*

Name:	Kylie Mills
Project Role:	*Laboratory Technician*
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	*6 months*
Contribution to Project:	*Ms. Mills has performed work in animal behavior, surgery, and tissue analysis.*
Funding Support:	*NIAAA Institutional T32 Training Grant*

Name:	Marta Montes
Project Role:	*Rotating Graduate Student*

Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	*6 months*
Contribution to Project:	*Ms. Montes has performed work in animal behavior, surgery, and tissue analysis.*
Funding Support:	*NIAAA Institutional T32 Training Grant*

SPECIAL REPORTING REQUIREMENTS

Quad Chart - See page 27

Endocannabinoids as a target for the treatment of traumatic brain injury
DM103009/Final Report
W81XWH-11-2-0011

PI: Patricia E. Molina, MD, PhD **Org:** LSU Health Sciences Center- New Orleans **Award Amount:** $777,197

Study Aim(s)

1: Determine the effectiveness of the selective increase in endogenous 2-AG and AEA levels in ameliorating brain structural damage following TBI.

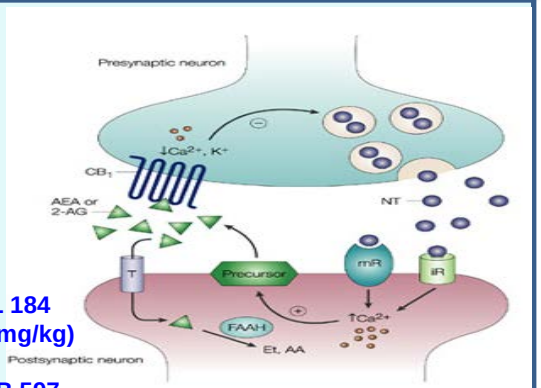
2: Determine the effectiveness of the selective increase in endogenous 2-AG and AEA levels in preventing neuroendocrine dysfunction following TBI

3: Determine the capacity of increased EC levels to preserve neurobehavioral neurobehavioral function following TBI

Approach

TBI is produced by lateral fluid percussion (~1.4J, ~30 ms) resulting in apnea (15±5 sec) and delay in righting reflex (716+118 sec). Specific inhibitors of endocannabinoid degradation (JZL184; 16mg/kg, or URB597; 0.3mg/kg) are administered IP, 30 minutes post-TBI. Neurobehavioral assessments (somatomotor & cognitive), blood brain barrier permeability, and neuroinflammation are examined over time. Resting and stimulated autonomic and neuroendocrine responses to cardiovascular challenge and water deprivation test are measured post TBI.

- **Hypothesis:** Pharmacotherapy aimed at inhibiting endocannabinoid (EC) degradation will improve TBI outcomes by reducing the structural (inflammation & oxidative injury) and functional (neuroendocrine & neurobehavioral) damage post-TBI



JZL 184 (16mg/kg)

URB 597 (0.3mg/kg)

Accomplishments: A single dose of an EC degradation inhibitor, either JZL 184 or URB597, reduced inflammation and oxidative stress, blood brain barrier damage, cell injury, and behavioral impairments including cognitive dysfunction following mild TBI.

Timeline and Cost

Activities FY	10	11	12	13	14
Milestone 1: Establish the impact of EC degradation inhibition on neutrophil influx, pro-inflammatory cytokine expression, oxidative injury, edema, blood brain barrier permeability, histological analysis and brain EC levels					
Milestone 2: Establish the impact of EC degradation inhibition on neuroendocrine and cardiovascular responses under basal and unstimulated conditions					
Milestone 3: Demonstrate the impact of EC degradation inhibition on neurobehavioral function following TBI					
Estimated Budget ($K)		**$193,280**	**Funding Modification**		

Updated: (April 18, 2016)

Goals/Milestones

FY11 Goal – Effectiveness of EC degradation inhibitors in reducing brain structural damage.

- ✓ Inflammation & oxidative stress
- ✓ Brain Edema
- ✓ Blood Brain Barrier Integrity
- ✓ Cell Injury
- ✓ EC Levels in Brain tissue

FY12 Goals – Effectiveness of EC degradation inhibitors in preventing neuroendocrine dysfunction following TBI.

- ✓ Basal unstimulated neuroendocrine function
- ✓ Autonomic & neuroendocrine response to cardiovascular challenge
- ❑ Autonomic & neuroendocrine response to water deprivation test

FY13 Goal – Effectiveness of EC degradation inhibitors in preserving neurobehavioral function following TBI.

- ✓ Severity of TBI – Righting Reflex
- ✓ Sensory Reflex – Forelimb and hind limb reflex
- ✓ Somatomotor Function – Beam walking & beam balancing
- ✓ Cognitive Function – Radial arm maze

Comments/Challenges/Issues/Concerns

None to report

Budget Expenditure to Date

Projected Expenditure: $0

Actual Expenditure: $777,408

REFERENCES:

1. Cernak I, Noble-Haeusslein LJ. Traumatic brain injury: an overview of pathobiology with emphasis on military populations. J Cereb Blood Flow Metab.2010 Feb;30(2):255-66. doi: 10.1038/jcbfm.2009.203. Epub 2009 Oct 7. Review. Erratum in: J Cereb Blood Flow Metab. 2010 Jun;30(6):1262. PubMed PMID: 19809467; PubMed Central PMCID: PMC2855235.
2. Elder GA, Cristian A. Blast-related mild traumatic brain injury: mechanisms of injury and impact on clinical care. Mt Sinai J Med. 2009 Apr; 76(2):111-8. Review. PubMed PMID: 19306373.
3. Helmick KM, Spells CA, Malik SZ, Davies CA, Marion DW, Hinds SR. Traumatic brain injury in the US military: epidemiology and key clinical and research programs. Brain Imaging Behav. 2015 Sep;9(3):358-66. doi:10.1007/s11682-015-9399-z. PubMed PMID: 25972118.
4. McKee AC, Cantu RC, Nowinski CJ, Hedley-Whyte ET, Gavett BE, Budson AE, Santini VE, Lee HS, Kubilus CA, Stern RA. Chronic traumatic encephalopathy in athletes: progressive tauopathy after repetitive head injury. J Neuropathol Exp Neurol. 2009 Jul;68(7):709-35. doi: 10.1097/NEN.0b013e3181a9d503. Review. PubMed PMID: 19535999; PubMed Central PMCID: PMC2945234.
5. Okie S. Traumatic brain injury in the war zone. N Engl J Med. 2005;352(20):2043-2047. Epub 2005/05/20. doi: 10.1056/NEJMp058102. PMID: 15901856.

APPENDICES

1. Katz PS, Sulzer JK, Impastato RA, Teng SX, Rogers EK, Molina PE. Endocannabinoid degradation inhibition improves neurobehavioral function, blood-brain barrier integrity, and neuroinflammation following mild traumatic brain injury. J Neurotrauma. 2015;32(5):297-306. PMCID: PMC4348366.
2. Mayeux JP, Katz PS, Edwards S, Middleton J, Molina PE. Inhibition of Endocannabinoid Degradation Improves Outcomes from Mild Traumatic Brain Injury: A Mechanistic Role for Synaptic Hyperexcitability. J Neurotrauma 2016; Submitted.

JOURNAL OF NEUROTRAUMA 32:297–306 (March 1, 2015)

DOI: 10.1089/neu.2014.3508

Endocannabinoid Degradation Inhibition Improves Neurobehavioral Function, Blood–Brain Barrier Integrity, and Neuroinflammation following Mild Traumatic Brain Injury

Paige S. Katz, Jesse K. Sulzer, Renata A. Impastato, Sophie X. Teng, Emily K. Rogers, and Patricia E. Molina

Abstract

Traumatic brain injury (TBI) is an increasingly frequent and poorly understood condition lacking effective therapeutic strategies. Inflammation and oxidative stress (OS) are critical components of injury, and targeted interventions to reduce their contribution to injury should improve neurobehavioral recovery and outcomes. Recent evidence reveals potential protective, yet short-lived, effects of the endocannabinoids (ECs), 2-arachidonoyl glycerol (2-AG) and *N*-arachidonoyl-ethanolamine (AEA), on neuroinflammatory and OS processes after TBI. The aim of this study was to determine whether EC degradation inhibition after TBI would improve neurobehavioral recovery by reducing inflammatory and oxidative damage. Adult male Sprague-Dawley rats underwent a 5-mm left lateral craniotomy, and TBI was induced by lateral fluid percussion. TBI produced apnea (17 ± 5 sec) and a delayed righting reflex (479 ± 21 sec). Thirty minutes post-TBI, rats were randomized to receive intraperitoneal injections of vehicle (alcohol, emulphor, and saline; 1:1:18) or a selective inhibitor of 2-AG (JZL184, 16 mg/kg) or AEA (URB597, 0.3 mg/kg) degradation. At 24 h post-TBI, animals showed significant neurological and -behavioral impairment as well as disruption of blood–brain barrier (BBB) integrity. Improved neurological and -behavioral function was observed in JZL184-treated animals. BBB integrity was protected in both JZL184- and URB597-treated animals. No significant differences in ipsilateral cortex messenger RNA expression of interleukin (IL)-1β, IL-6, chemokine (C-C motif) ligand 2, tumor necrosis factor alpha, cyclooxygenase 2 (COX2), or nicotinamide adenine dinucleotide phosphate oxidase (NOX2) and protein expression of COX2 or NOX2 were observed across experimental groups. Astrocyte and microglia activation was significantly increased post-TBI, and treatment with JZL184 or URB597 blocked activation of both cell types. These findings suggest that EC degradation inhibition post-TBI exerts neuroprotective effects. Whether repeated dosing would achieve greater protection remains to be examined.

Key words: 2-AG; AEA; endocannabinoids; neuroinflammation; TBI

Introduction

Traumatic brain injury (TBI) is an increasingly frequent occurrence in the military population, resulting from explosive or blast attacks.[1,2] Reports indicate that the number of closed brain injuries has increased with TBI, accounting for 66% of all army war-zone evacuations.[3] TBI is not limited to the military population and has become a well-recognized medical problem in contact sports, such as football and boxing.[4] The majority of TBI victims are young, otherwise healthy adults—in fact, TBI is now recognized as a leading cause of death in young adults (< 45 years of age).[5] The early-period post-TBI is characterized by neuroinflammation and oxidative stress (OS), then followed by neurological and -behavioral changes that include, but are not limited to, increased incidence of anxiety and depression, stress sensitivity, anhedonia, impulse control deficits, sleep disturbances, and pain sensitivity.[6–10]

The initial mechanical insult induces increased and sustained inflammation, which is characterized by acute up-regulation of proinflammatory cytokines (interleukin [IL]-1α, IL-6, and tumor necrosis factor [TNF]-α), activation of astrocytes and microglia, and disruption of blood–brain barrier (BBB).[11] The acute response to TBI is followed by a long-term injury, which involves neuronal damage, cytotoxicity, and cognitive impairment.[12,13] Thus, timely

Department of Physiology, Alcohol and Drug Abuse Center of Excellence, Louisiana State University Health Sciences Center, New Orleans, Louisiana.

modulation of neuroinflammation early on becomes critical in preventing prolonged neuroinflammation that can be damaging when in excess, while not interfering with the reparative contribution of endogenous neuromodulators, and activated astrocytes and glia.[14,15] Current available treatment and management of TBI is palliative, owing to incomplete understanding of its pathophysiology, with no effective therapies identified to improve outcomes after injury.[16] Long-term effects of TBI, including neurobehavioral dysfunction, may be ameliorated by interventions aimed at reducing short-term neuroinflammation, OS, and excessive astrocyte and microglial activation.[10,14,15,17]

Over the past decade, the neuroprotective effects of the endocannabinoid (EC) system have received increased attention.[18–21] The EC system primarily consists of two G-protein-coupled transmembrane receptors, cannabinoid receptor 1 (CB1) and cannabinoid receptor 2 (CB2), and several lipid-derived endogenous ligands. The CB1 receptor is expressed throughout the brain, and CB2 receptor distribution is predominantly in cells and tissues of the immune system.[22] Two principal ECs have been identified, 2-arachidonoyl glycerol (2-AG) and *N*-arachidonoyl-ethanolamine (AEA), of which 2-AG is the most bioactive and abundant EC in the brain.[18,23] In contrast to preformed neurotransmitters, which are stored in secretory vesicles, ECs are synthesized ''on demand'' in response to specific stimuli. Similar to neurotransmitters, degradation of these ECs is rapid. Once released, ECs act locally and their effects are quickly terminated by cellular uptake and enzymatic degradation by fatty acid amide hydrolase (FAAH), monoacylglycerol lipase (MAGL), and cyclooxygenase 2 (COX2).[24] Degradation is predominantly mediated by FAAH for AEA and MAGL for 2-AG; however, COX2 has also been shown to metabolize both AEA and 2-AG.[25–27] Several investigators have reported neuroprotective effects of cannabinoid receptor agonists (CRAs).[18,19,21,28–31] CRAs have been demonstrated to decrease glutamatergic toxicity, OS, and inflammation as well as improve motor function recovery, reduce BBB breakdown, and attenuate cerebral edema after head injury in rodents.[28,30] However, use of CRAs has resulted in conflicting outcomes in clinical trials in severe head injury.[32] In addition, therapeutic cannabinoid agonist administration may produce psychotropic effects through CB1 receptor activation and this has limited their widespread use.

An alternative approach to achieving cannabinoid-mediated neuroprotection is that of modulating EC degradation. ECs are synthesized in response to specific stimuli, including traumatic injury or inflammatory challenges, and their (2-AG and AEA) rapid degradation is mediated primarily by two enzymes, FAAH and MAGL.[20,33–35] Thus, we hypothesized that decreased EC degradation would improve outcomes from TBI, without producing overt neuropsychological dysfunction, thereby presenting a unique pharmacological intervention for TBI.[21] The aim of this study was to test the prediction that inhibition of enzymatic degradation of EC after TBI provides neuroprotection and, in turn, improves neurobehavioral outcomes as reflected in motor and cognitive function.

Methods

Animals

All animal procedures were approved by the institutional animal care and use committee of the Louisiana State University Health Sciences Center (LSUHSC; New Orleans, LA) and were in accord with the National Institute of Health (NIH) guidelines. Adult male Sprague-Dawley rats (Charles River, Raleigh, NC) weighing 250–275 g at the time of arrival were housed in the Division of Animal Care at LSUHSC and were exposed to a 12-h light/dark cycle and fed a standard rat diet (Purina Rat Chow; Ralston Purina, St. Louis, MO) *ad libitum* for 1 week before surgical procedures.

Surgical procedures

Animals were anesthetized (intramuscular injection of ketamine 90 mg/kg and xylazine 9 mg/kg) and positioned in a stereotaxic apparatus (model 900; Kopf Instruments, Tujunga, CA), and craniotomy (5.0 mm) was performed (2.0 mm posterior to bregma, 3.0 mm lateral from mid-line, over the left sensory motor cortex). Extreme care was taken to ensure that the dura matter was not penetrated. A female Luer Loc connector was positioned directly over the craniotomy and secured in place with cyanoacrylate glue. Once the glue was dry, dental cement (Lang Dental Manufacturing, Wheeling, IL) was applied around the female Luer Loc connector and surrounding exposed skull approximately 2 mm in thickness. The female Luer Loc was filled with sterile normal saline and capped. A subset of animals was also surgically implanted with a carotid catheter for BBB measurements. Briefly, using aseptic surgical procedures, catheters (PE50; BD Diagnostic Systems, Sparks, MD) were inserted into the left carotid artery, then advanced approximately 3 cm in length. Catheters were flushed with sterile saline, then sealed, and subcutaneously routed to the nape of the neck, where they were exteriorized through a small incision and secured with tape. After surgery, animals were placed in individual cages, allowed to recover from anesthesia, and given food and water *ad libitum* for 3 days before randomization to either sham or TBI groups.

Traumatic brain injury model

After recovery from surgery, animals were either subjected to TBI by lateral fluid percussion (LFP) or given no injury [shams]). The LFP model is the most extensively used and well-characterized model of nonpenetrating and nonischemic TBI and provides consistent, reproducible injury.[36–39] Animals were anesthetized with isoflurane (4% induction, 3% maintenance) and positioned into a stereotaxic frame; the cranial female Luer Loc was connected to an LFP system by pressure tubing. LFP (pressure wave of $\sim$2 atm and 25-ms duration) was delivered to the dura. Animals were immediately monitored for signs of apnea after TBI, removed from the stereotaxic frame, and placed on their right side for observation of respiratory rate. Righting reflex was recorded as the time it took for the animal to regain complete consciousness and standing on all four limbs. Time-matched sham controls were anesthetized and connected to the LFP system, but not subjected to LFP. All animals were placed back into their individual home cage and continuously monitored for 2 h postinjury with free access to food and water.

Endocannabinoid modulation

Selective inhibitors of MAGL and FAAH, the enzymes responsible for 2-AG and AEA hydrolysis (JZL184 [16 mg/kg] and URB597 [0.3 mg/kg]) were dissolved in alcohol, emulphor, and saline (1:1:18) as vehicle. Either JZL184 (TBI/JZL) or URB597 (TBI/URB) was injected intraperitoneally at 30 min post-TBI. Time-matched controls received equal volumes (10 μL/kg body weight) of vehicle (TBI/VEH). Animals were studied during the acute postinjury period (2–24 h).

Neurological and neurobehavioral assessments

As previously described, neurological (neurological severity scores; NSS) and neurobehavioral (neurobehavioral scores; NBS) function was assessed at baseline (1 h before TBI) and at 2 and 24 h post-TBI, as previously described.[40] All animals were exposed to all tasks, trained, and evaluated before TBI using the testing parameters that were adapted from previously published methods of

assessing cognition and behavior.[39,41] To reduce any impact on behavior assessments potentially caused by the process of relocation, they were transported to the test room 1 h in advance of the start of testing. NSS scores ranged from 0 to 25 and NBS scores ranged from 0 to12 and were based on the animal's ability to carry out each task. A score of 0 represented normal or pass, whereas higher scores correlated to the animal's severity of injury and NSS or NBS impairment. NSS evaluates motor function, sensory, reflexes, beam walking, and beam balancing. Pinna, corneal, startle, and righting reflexes were assessed, where one is no reflex and 0 indicates the reflex is intact. Beam walking assessed motor coordination, animals were placed on beams of decreasing width (10, 8, 5, and 2.5 cm) and allowed 60 min to traverse each beam. In addition, beam balance was assessed where animals were placed on a 1.5-cm-wide beam and given 60 sec to balance. Failure to walk all beams and/or balance for 60 sec resulted in increased NSS total. NBS tests sensorimotor, proprioception, exploratory behavior, and novel object exploration. Proprioception was assessed by pushing each animal laterally (lateral pulsion) on each side of its body. Each side was assessed and failure to resist lateral pulsion on one or both sides increased the NBS total. Exploratory behavior was assessed immediately after the animal's cage top was removed. Uninjured animals actively explore the top of the cage and surroundings.

Blood–brain barrier permeability

Integrity of the BBB was examined by dye tracer extravasation, as previously described.[42,43] Animals received a 1-mL injection of a Ringer's lactate solution containing 2% Evans Blue (EB; Sigma-Aldrich, St. Louis, MO) into the carotid catheter. After 10–15 min, animals were deeply anesthetized with isoflurane and then transcardially perfused for 15 min with normal saline to remove dye from the vasculature. The brain was removed and flash frozen in liquid nitrogen, and brain regions (ipsi- and contralateral) were isolated, weighed, and stored at −80°C before homogenization and extraction. Tissue was homogenized in formamide, and EB was extracted from brain tissue by incubating in formamide (Sigma-Aldrich) at 37°C overnight. Samples were then centrifuged at 4000*g* at 4°C for 10 min. The concentration of EB was measured in the supernatant with a spectrophotometer at 620 nm. A linear standard curve of EB in formamide was used to calculate brain-tissue EB concentration (μg/mL EB) and was then normalized to tissue weight in grams. Data are expressed as μg/mL EB/g of brain tissue.

Tissue collection

After decapitation, the brain was removed from the skull, rapidly sprayed by Richard-Allan Scientific Cytocool II (Thermo Scientific, Waltham, MA), and dipped into liquid nitrogen for approximately 6–10 sec. Using a prefrozen standard adult rodent brain slicer matrix (Zivic Instruments, Pittsburgh, PA), the brain was positioned and cut using three razor blades pressed into slice channels at 4, 8, and 13 mm from the tip of the frontal cortex to excise out a 4-mm width of prefrontal cortex and a 5-mm width of the injured area. A fourth razor blade was pressed at mid-line to separate the ipsilateral (injured) from the contralateral (uninjured) region, and a 5-mm width of uninjured cortex was collected. Brain tissues were collected at 24 h post-TBI and stored at −80°C for further analyses.

Real-time quantitative polymerase chain reaction analysis

Messenger RNA (mRNA) expression of IL-1β, IL-6, chemokine (C-C motif) ligand 2 (CCL2), TNF-α, COX2, and gp91^{phox} (nicotinamide adenine dinucleotide phosphate [NADPH] oxidase; NOX2) was measured 24 h post-TBI at the site of injury. Total RNA was extracted from brain tissue using an RNeasy Plus Universal Mini Kit (Qiagen, Valencia, CA), according to the manufacturer's instructions. Total RNA was reverse transcribed using the TaqMan Reverse Transcription Reagent kit (Life Technologies Corporation, Carlsbad, CA). Primer sequences (Integrated DNA Technologies, Coralville, IA) for IL-1β, IL-6, TNF-α, and CCL2 used in this study are listed in Table 1. COX2 and NOX2 primers were purchased from SA Biosciences (Valencia, CA), and these sequences remain proprietary. Primer concentrations used were 500 nM. The RT^2 SYBR Green FAST Mastermix (Qiagen) was used for real-time polymerase chain reaction (PCR). All reactions were performed on a CFX96 system (Bio-Rad Laboratories, Hercules, CA). Quantitative reverse-transcriptase PCR data were analyzed using the $\Delta\Delta C_T$ method. Target genes were compared with RPS13 and normalized to control values. RPS13 was chosen as the endogenous control to normalize gene expression because it was stably expressed based on a meta-analysis of 13,629 gene array samples.[44]

Table 1. qRT-PCR Primer Sequences for IL-1β, IL-6, CCL2, TNF-α, and RSP13 (Housekeeping Gene)

Target	*Forward primer*	*Reverse primer*
IL-1β	agcagctttcgacagtgaggagaa	tctccacagccacaatgagtgaca
IL-6	aagccagagtcattcagagc	gtccttagccactccttctg
CCL2	tgctgtctcagccagatgcagtta	tacagcttctttgggacacctgct
TNF-α	ccaacaaggaggagaagttcccaa	gagaagatgatctgagtgtgaggg
RSP13	gacgtgaaggaacaaattt acaagttggcc	Gaatcacacctatctgggaa ggagtca

qRT-PCR, quantitative reverse-transcriptase polymerase chain reaction; IL, interleukin; CCL2, chemokine (C-C motif) ligand 2; TNF-α, tumor necrosis factor alpha; RSP13, ribosomal protein 13.

Western blot analysis

Fresh frozen brain tissue from the ipsilateral cortex was powdered and then homogenized in RIPA buffer containing 50 mM of Tris HCL (pH 8), 150 mM of NaCL, 1% NP-40, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate (SDS) with Halt protease, and phosphatase inhibitor cocktail (Pierce Thermo Scientific, Rockford, IL). Protein concentrations from brain tissue lysates were determined using a bicinchoninic acid assay (Bio-Rad). Equal amounts of protein (60 ug) from each sample were separated on 4–20% gradient SDS/polyacrylamide gel electrophoresis gels and then transferred to polyvinylidene fluoride membranes (Millipore, Billerica, MA). Membranes were then blocked, incubated with anti-COX2 (1:200; Abcam, Cambridge, MA), anti-NOX2 (1:1000; Abcam), and anti-β-actin (1:1000; Cell Signaling Technology, Beverly, MA) primary antibodies (Abs) overnight at 4°C, and then incubated with secondary Abs conjugated with horseradish peroxidase (1:2500; Cell Signaling technology) for 1 h at room temperature. Bands were visualized using Chemiluminescence Reagent Plus (PerkinElmer Life Science, Boston, MA), and densitometry was used to quantify protein expression using Carestream molecular imaging software (Carestream Health, Inc., Rochester, NY). β-actin was used as a loading control on all membranes.

Immunohistochemistry

Perfusion-fixed brains were sliced at 35-μm thickness, and sections were mounted on glass slides before staining. Sections were permeabilized with 0.3% Triton-X 100 in phosphate-buffered saline (PBS) and blocked in 1% bovine serum albumin and 2% normal donkey serum for 1 h at room temperature. Sections were incubated with two primary Abs: rabbit anti-glial fibrillary acid protein (GFAP; 1:200; Abcam) and mouse anit-ED1 (1:200;

Table 2. Body Temperature Measured in Pre-TBI and 2 and 24 h Post-TBI

	Body temperature (°C)		
Treatment	*Pre-TBI*	*2 h*	*24 h*
SHAM	37.64 ± 0.16	37.51 ± 0.13	37.49 ± 0.13
TBI/VEH	37.41 ± 0.11	37.48 ± 0.16	37.21 ± 0.23
TBI/JZL	37.40 ± 0.11	37.28 ± 0.3	36.94 ± 0.38
TBI/URB	37.23 ± 0.22	37.51 ± 0.17	37.76 ± 0.61

Data are presented as total mean score ± SEM (SHAM, $n = 18$; TBI/VEH, $n = 20$; TBI/JZL, $n = 18$; TBI/URB, $n = 19$).
TBI, traumatic brain injury.

Abcam) for 24 h at 4°C. Sections were then washed 3 × 5 min with PBS and incubated in a mixture of secondary Abs: Alexa Fluor 555 donkey anti-rabbit (1:200; by Life Technologies, Carlsbad, CA) and Alexa Fluor 488 donkey anti-mouse (1:200; by Life Technologies) for 2 h at room temperature. Slides were washed 3 × 5 min with PBS, dried, and cover-slipped using antifade mounting media with 4′,6-diamidino-2-phenylindole (ProLong Gold; Life Technologies). Sections were observed under a Nikon Eclipse TE2000-U (Nikon, Tokyo, Japan), and images were captured using NIS Elements (Version 3.22.11; Nikon). Images were then quantified using ImageJ software (NIH, Bethesda, MD) at 40× magnification. Values are expressed as percent area of positive staining. At least two pictures were taken of three sections, for a total of six to nine pictures per animal, and 3 animals were analyzed per group.

Statistical analysis

All data are expressed as mean ± standard error of the mean (SEM) with the number of animals per group indicated in the figure legends. Statistical analysis of differences in outcome measures was determined by one-way analysis of variance (ANOVA) and two-way ANOVA with repeated measures using GraphPad Prism 5.0 statistical software (Graphpad Software Inc., La Jolla, CA). Pair-wise multiple comparisons were determined using Tukey's test for one-way ANOVA and Bonferroni's test for two-way ANOVA. Specific tests used for analysis are stated in the table (Tables 1 and 2) and figure legends. Statistical significance was set at $p < 0.05$.

Results

Impact of traumatic brain injury on apnea, righting reflex, and respiratory rate

TBI produced significant apnea (17 ± 5 sec; $p < 0.05$) and a delayed righting reflex (479 ± 21 sec; $p < 0.05$). Respiratory rate was significantly reduced immediately following TBI (61 ± 2 breaths/min; $p < 0.05$), when compared to shams (73 ± 2 breaths/min).

Impact of inhibition of endocannabinoid degradation on neurological severity and neurobehavioral scores after traumatic brain injury

Animals' performance on NSS (Fig. 1A) and NBS (Fig. 1B) was tested pre-TBI and 2 and 24 h post-TBI. There was a significant main effect of treatment (two-way ANOVA: $F_{(3,71)} = 6.68$; $p = 0.0005$) and time (two-away ANOVA: $F_{(2,142)} = 60.33$; $p < 0.0001$) and a significant interaction (two-way ANOVA: $F_{(6,142)} = 8.62$; $p < 0.0001$), as indicated by a marked increase in the NSS at 2 h post-TBI (TBI/VEH: 5.6 ± 0.8, $p < 0.01$; TBI/JZL: 5.8 ± 0.9, $p < 0.01$; TBI/URB: 6.1 ± 0.6, $p < 0.01$), when compared to shams. NSS scores remained significantly elevated in TBI/VEH (6.7 ± 1.2; $p < 0.01$) and TBI/URB (5.1 ± 0.9; $p < 0.01$) animals at 24 h post-TBI, when compared to shams. Animals treated with JZL184 had reduced NSS at 24 h (3.3 ± 1.1). The NBS was also increased in TBI animals 2 h post-TBI (TBI/VEH, 3.5 ± 0.6; TBI/JZL, 3.3 ± 0.8; TBI/URB, 3.4 ± 0.6), when compared to shams, and revealed a significant main effect of treatment (two-way ANOVA: $F_{(3,71)} = 7.70$; $p = 0.0002$) and time (two-way ANOVA: $F_{(2,142)} = 36.54$; $p < 0.0001$) and a significant interaction (two-way ANOVA: $F_{(6,142)} = 4.69$; $p = 0.0002$).

Protection of blood–brain barrier integrity with inhibition of endocannabinoid degradation

TBI (TBI/VEH) significantly disrupted BBB integrity, as reflected by the increased leak (103 ± 14; $p < 0.05$), when compared to shams (31 ± 5; one-way ANOVA: $F_{(3,36)} = 6.98$; $p = 0.0008$) at 24 h post-TBI (Fig. 2). Treatment with either JZL184 or URB597, 30 min post-TBI, was effective at minimizing BBB dysfunction

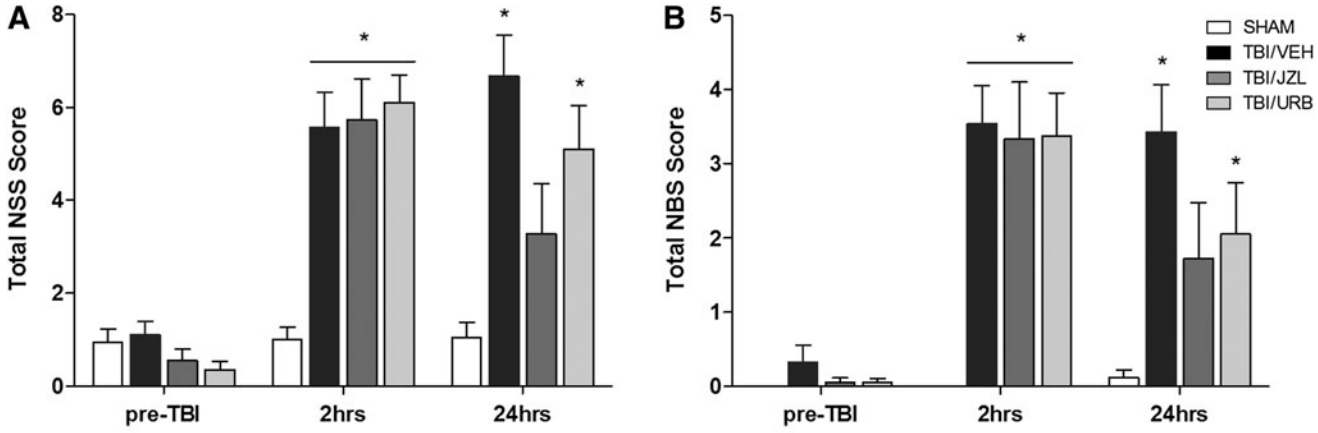


FIG. 1. Neurological severity scores (NSS) (**A**) and neurobehavioral scores (NBS) (**B**) assessed pre-TBI and 2 and 24 h post-TBI. Endocannabinoid degradation inhibitors, JZL184 and URB597, were given intraperitoneally 30 min post-TBI. Data are presented as total mean score ± SEM (SHAM, $n = 18$; TBI/VEH, $n = 20$; TBI/JZL, $n = 18$; TBI/URB, $n = 19$) and were analyzed using two-way ANOVA with repeated measures. *$p < 0.05$ versus time-matched shams. TBI, traumatic brain injury.

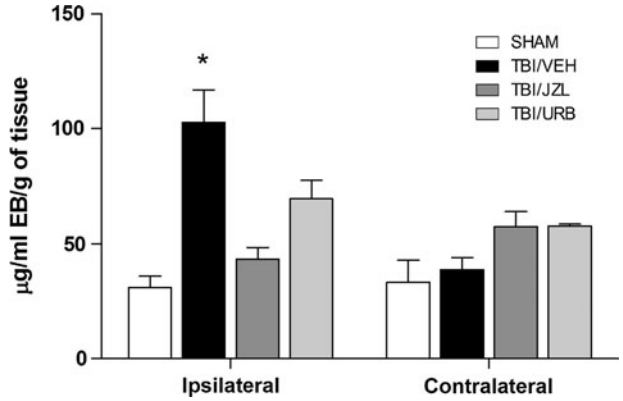


FIG. 2. Blood–brain barrier integrity was assessed in ipsilateral (injured) and contralateral (uninjured) brain regions at 24 h post-TBI. Endocannabinoid degradation inhibitors, JZL184 and URB597, were given intraperitoneally 30 min post-TBI. Data are presented as mean ± SEM (SHAM, $n=9$; TBI/VEH, $n=18$; TBI/JZL, $n=7$; TBI/URB, $n=6$) and were analyzed using a one-way ANOVA. *$p<0.05$ versus time-matched shams. EB, Evans Blue; TBI, traumatic brain injury.

(TBI/JZL: 43 ± 5, not significant [NS]; TBI/URB: 69 ± 8, NS) at 24 h (Fig. 2). BBB integrity remained intact on the contralateral side in the sham and TBI/VEH groups, and treatment with either JZL184 or URB597 did not affect BBB integrity on the contralateral brain region (Fig. 2).

Effects of inhibiting endocannabinoid degradation on cytokine messenger RNA expression

mRNA expression of IL-1β, IL-6, CCL2, and TNF-α was measured at 24 h post-TBI (Fig. 3A–D). No statistically significant differences were detected at 24 h post-TBI in mRNA expression of any of the measured cytokines (one-way ANOVA: IL-1β, $F_{(3,22)}=0.51$, NS; IL-6, $F_{(3,22)}=0.62$, NS; CCL2, $F_{(3,22)}=0.39$, NS; TNF-α, $F_{(3,22)}=1.80$, NS).

Effects of inhibiting endocannabinoid degradation on oxidative-stress–related protein and messenger RNA expression

Protein and mRNA expression of COX2 and NOX2 were measured at 24 h post-TBI (Fig. 4A–D). No significant differences in protein (one-way ANOVA: COX2, $F_{(3,12)}=0.79$, NS; NOX2, $F_{(3,12)}=0.33$, NS) and mRNA (one-way ANOVA: COX2, $F_{(3,24)}=2.56$, NS; NOX2, $F_{(3,24)}=1.56$, NS) expression of COX2 and NOX2 were detected in any of the experimental groups.

Inhibiting endocannabinoid degradation increased astrocyte activation

Brain slices were double-stained with GFAP and ED1 to determine protein expression levels 24 h post-TBI (Fig. 5A–N). Astrocyte-specific GFAP immunoreactivity post-TBI was significantly increased (12.4 ± 3.7; $p<0.05$) above sham levels (0.3 ± 0.1;

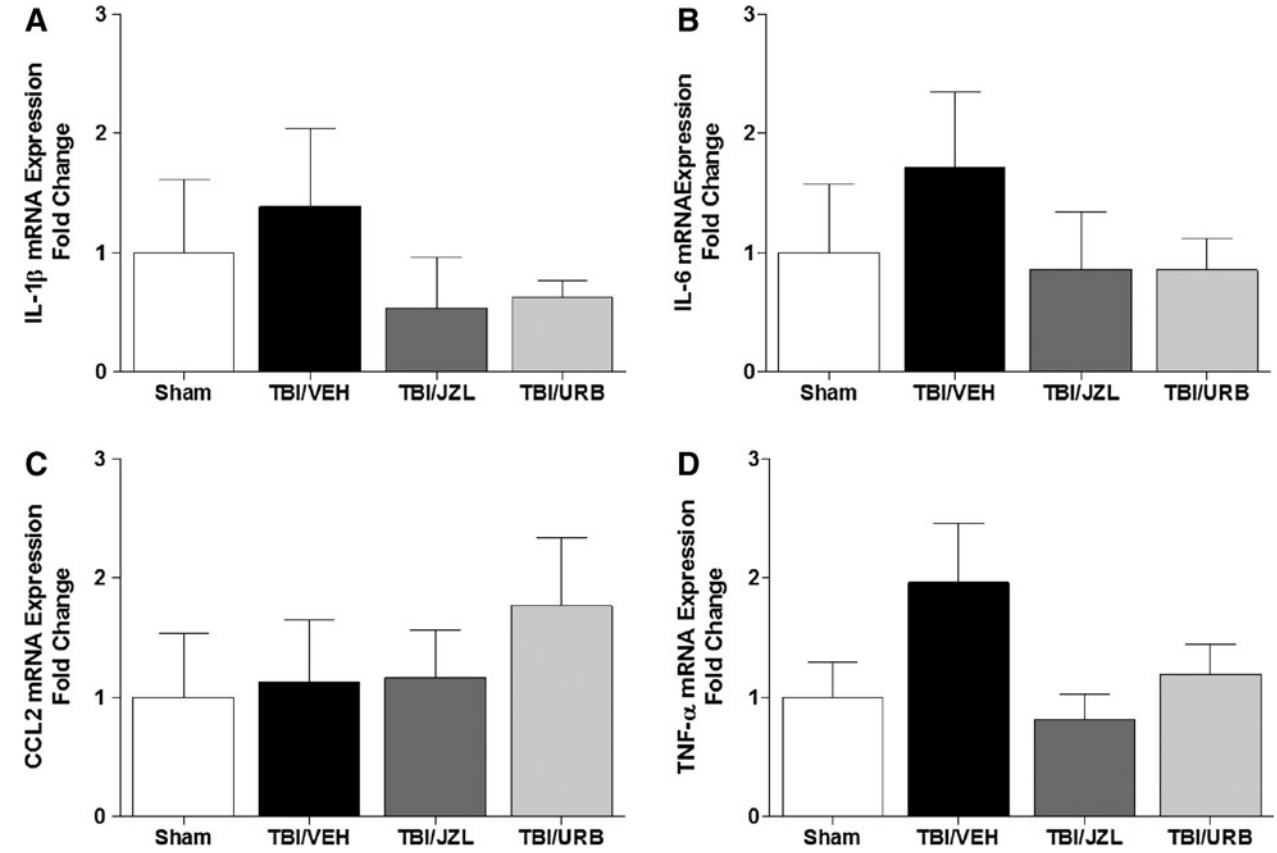


FIG. 3. Brain tissue from the site of injury was analyzed for mRNA expression of interleukin (IL)-6 (**A**), IL-1β (**B**), CCL2 (**C**), and TNF-α (**D**) by quantitative reverse-transcriptase polymerase chain reaction 24 h post-TBI. Endocannabinoid degradation inhibitors, JZL184 and URB597, were given intraperitoneally 30 min post-TBI. Data are presented as mean ± SEM (SHAM, $n=6$; TBI/VEH, $n=9$; TBI/JZL, $n=5$; TBI/URB, $n=6$) and were analyzed by one-way ANOVA. IL, interleukin; mRNA, messenger RNA; CCL2, chemokine (C-C motif) ligand 2; TNF-α, tumor necrosis factor alpha; TBI, traumatic brain injury.

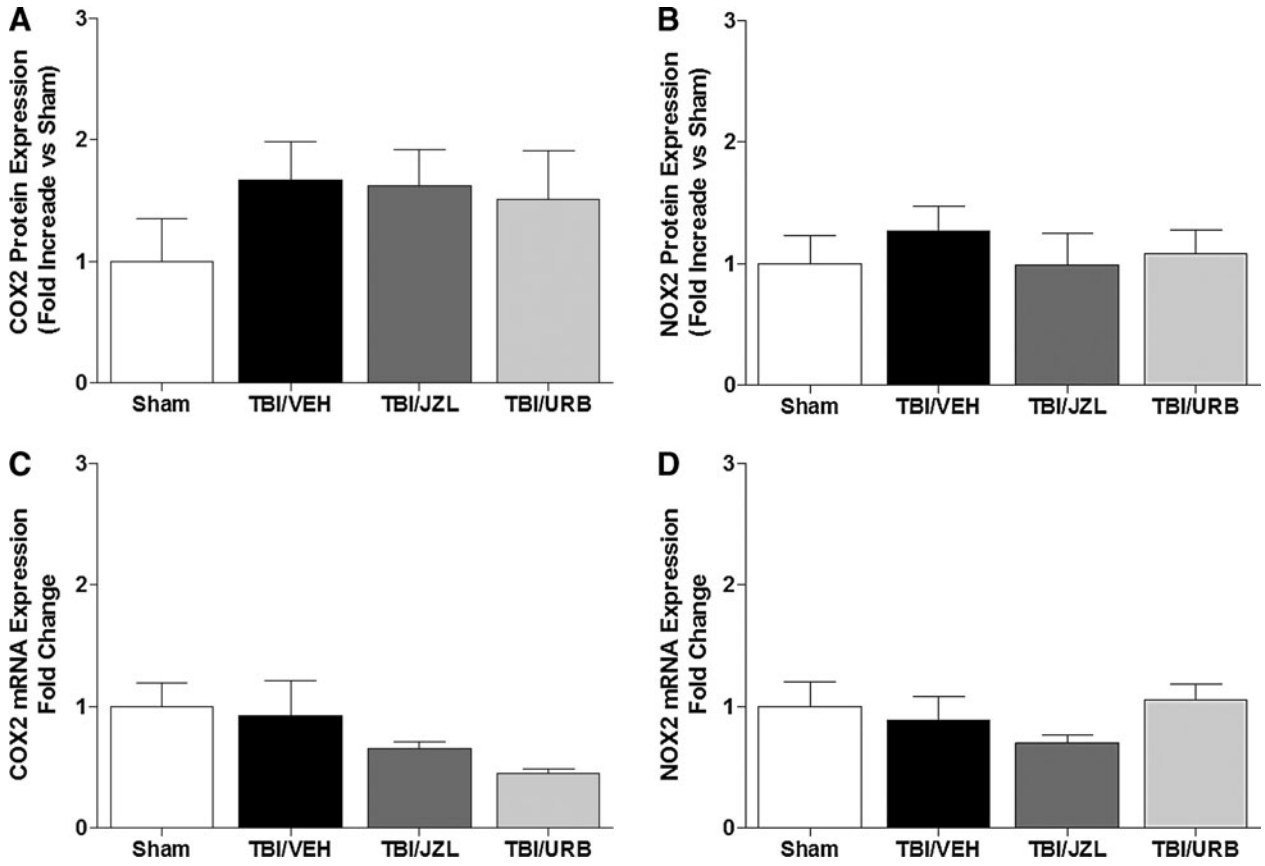


FIG. 4. Brain tissue from the site of injury was analyzed for protein and mRNA expression of COX2 (**A** and **C**) and NOX2 (**B** and **D**) by quantitative reverse-transcriptase polymerase chain reaction 24 h post-TBI. Endocannabinoid degradation inhibitors, JZL184 and URB597, were given intraperitoneally 30 min post-TBI. Data are presented as mean ± SEM (protein, $n = 4$/group; mRNA, SHAM, $n = 6$; TBI/VEH, $n = 9$; TBI/JZL, $n = 5$; TBI/URB, $n = 6$) and were analyzed by one-way ANOVA. COX2, cyclooxygenase 2; NOX2, nicotinamide adenine dinucleotide phosphate oxidase; mRNA, messenger RNA; TBI, traumatic brain injury.

one-way ANOVA: $F_{(3,8)} = 7.82$; $p = 0.009$), indicating increased astrocyte activation, and treatment with either JZL184 (3.2 ± 1.1) or URB597 (1.9 ± 0.2) inhibited the increased astrocyte activation observed in brains from TBI/VEH animals (Fig. 5M). In addition to activation, TBI alone induced visible astrocyte hypertrophy, as compared to sham, TBI/JZL, or TBI/URB animals (Fig. 5A,D,G,J). Expression of ED1, a marker of microglial activation, was significantly increased in TBI/VEH animals (8.2 ± 1.8; $p < 0.05$), when compared to shams (0.2 ± 1.4; one-way ANOVA: $F_{(3,8)} = 4.87$; $p = 0.03$), and this activation was abolished in both TBI/JZL (2.8 ± 1.7) and TBI/URB (3.2 ± 1.7) animals (Fig. 5B,E,H,K,N).

Discussion

The present study examined the effects of EC degradation inhibition on TBI outcomes. Previous studies have demonstrated potential neuroprotection with exogenous administration of ECs.[21,45] Therefore, we hypothesized that inhibiting the degradation of endogenously released ECs, 2-AG, or AEA with JZL184 or URB597, respectively, would extend EC signaling, provide neuroprotection, and improve outcomes after TBI. Our results demonstrate that inhibition of MAGL with JZL184 (30 min post-TBI) improved neurological and -behavioral function at 24 h, whereas inhibition of FAAH with UBR597 did not improve neurological and -behavioral function at 24 h. In addition, TBI-induced BBB dysfunction was reduced with inhibition of both MAGL and FAAH. mRNA expression of proinflammation cytokines IL-1β, IL-6, CCL2, and TNF-α were unchanged across groups at 24 h post-TBI. In addition, oxidative-related protein and mRNA expression of COX2 and NOX2 were unchanged. Finally, increased expression of GFAP, a marker of astrocyte activation, was observed in TBI/VEH that was reversed with both JZL184 and URB597 treatment. These results suggest that the selective EC degradation inhibitor, JZL184, is extremely effective at improving neurological and -behavioral impairment as well as protecting BBB integrity post-TBI, which is associated with increased astrocyte activation.

The neuroprotective effects of cannabinoid agonists and synthetic cannabinoids have been reported by several investigators.[28–30] Mauler and colleagues demonstrated, with BAY 38-7271, a CRA, neuroprotection in a rat model of TBI.[29] In addition, cannabinoid receptor 2 agonist, 0-1966, significantly reduced brain edema and improved locomotor performance in mice when given after cortical contusion impact injury. Moreover, treatment using a synthetic cannabinoid, HU-211, improved motor function recovery, which was accompanied by reduced BBB dysfunction and edema, in a model of closed head injury in rats.[28] Interestingly, these experimental findings were confirmed in one clinical trial using HU-211 for treatment after severe closed head injury, but these improvements were not evident in another clinical trial using HU-211 for treatment after TBI.[32,46] Although some experimental studies have shown neuroprotection, exogenous EC administration can impair learning and executive

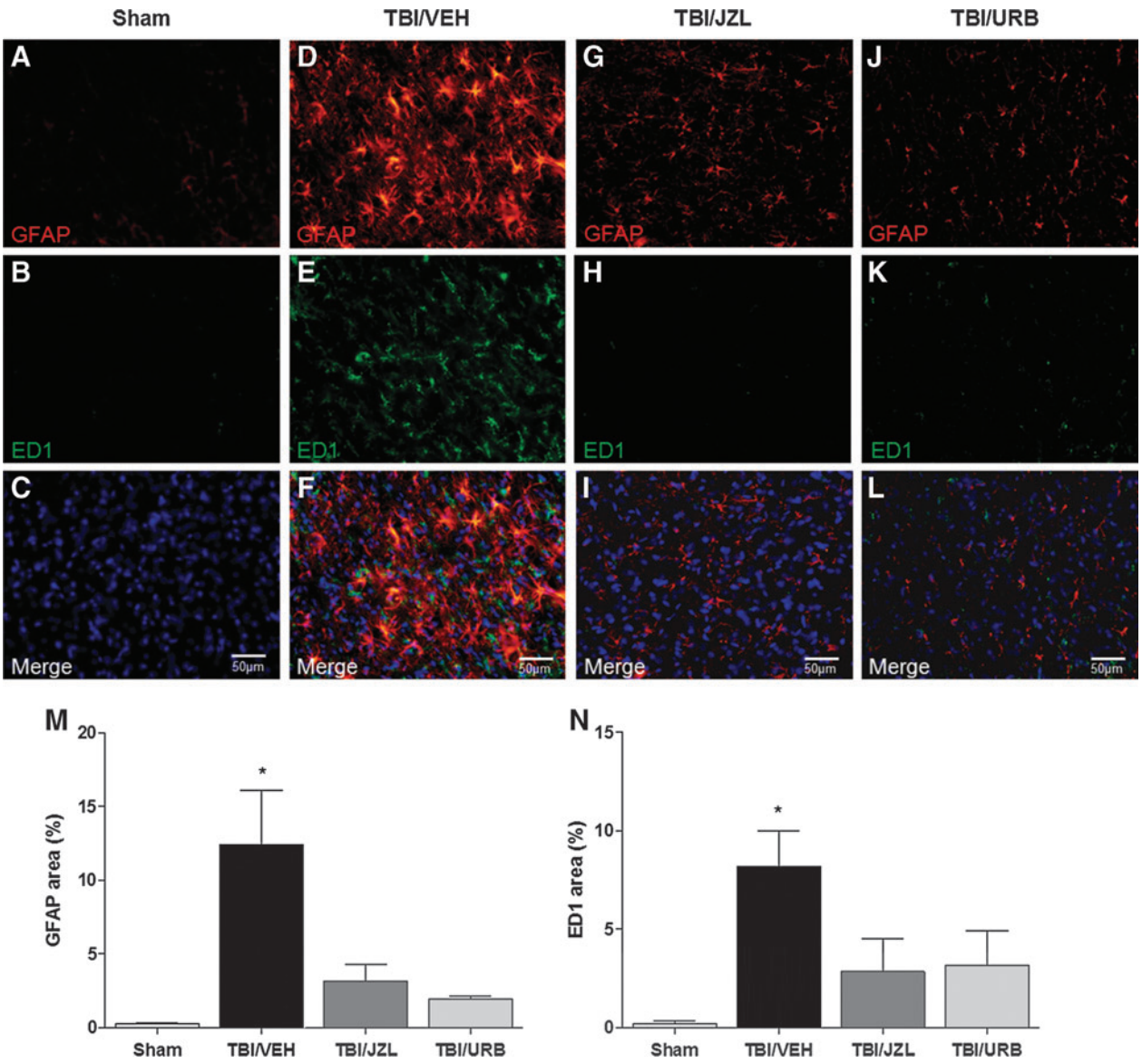


FIG. 5. Perfusion-fixed brains at 24 h post-TBI were double-stained with GFAP and ED1 and counterstained with 4′,6-diamidino-2-phenylindole for sham (**A–C**), TBI/VEH (**D–F**), TBI/JZL (**G–I**), and TBI/URB (**J–L**). Endocannabinoid degradation inhibitors, JZL184 and URB597, were given intraperitoneally 30 min post-TBI. All images were captured at 40× magnification. Data are summarized as positive staining area percent for GFAP (**M**) and ED1 (**N**). Data are presented as mean ± SEM ($n = 3$/group) and were analyzed by one-way ANOVA. *$p < 0.05$ versus time-matched shams. GFAP, glial fibrillary acidic protein; TBI, traumatic brain injury. Color image is available online at www.liebertpub.com/neu

function as a result of their psychoactive effects and the CB1-dependent behavioral alterations (hypomotility, analgesia, and catalepsy).[47] Alternatively, use of selective EC degradation inhibitors could avoid these behavioral alterations while increasing the effective time of endogenously released ECs in response to neuronal injury and promoting an intrinsic neuroprotective response through an activated EC system.[24] In these studies, the model of TBI induced by LFP produced immediate apnea and a delayed righting reflex. In addition, TBI significantly impaired somatomotor and cognitive function (NSS) and neurobehavior (NBS) as early as 2 h post-TBI and this impairment persisted to 24 h. These deficits were reversed by post-TBI treatment with JZL184 and partially improved with URB597. Notably, JZL184 was most effective at improving somatomotor and cognitive function as well as improving neurobehavior.

TBI-induced brain damage results from direct and indirect events. The direct (immediate or primary) injury results from mechanical disruption of brain tissue. This is followed by an acute inflammatory response, breakdown of the BBB, edema formation, and swelling. Our findings show that inhibiting EC degradation with either JZL184 or URB597 was effective at protecting the BBB integrity by attenuating EB extravasation in the area of injury (ipsilateral) after TBI at 24 h. These findings are consistent with previous studies using exogenous administration of 2-AG after closed head injury and ischemia.[21,45,48]

After the BBB breakdown, infiltration of peripheral blood cells with activation of immunocompetent cells leads to intrathecal release of numerous immune mediators, such as cytokines and chemokines. The neuroinflammatory cascade characterized by activation of astrocytes and microglia, increased production of

immune mediators, along with excitotoxic and oxidative responses, has been proposed as the principal underlying mechanisms of cell injury and has been confirmed by postmortem pathology in experimental and clinical studies.[17,49] Although the early inflammatory response plays an important role in recovery from injury, its sustained duration contributes both to the acute pathological processes after TBI, including cerebral edema, and the longer-term neuronal damage and cognitive impairment.[9,50] Inhibition of proinflammatory responses has been identified as a potential mechanism responsible for the improved outcome.[21] Thus, timely modulation of neuroinflammation becomes critical in not interfering with the reparative contribution of activated glia. The current results were unable to detect any significant increase in mRNA expression of IL-6, IL-1β, CCL2, or TNF-α in TBI animals, when compared to time-matched shams at 24 h post-TBI. We believe this is because of the time point measured given that our previous studies have demonstrated increased IL-6 and CCL2 at 6 h post-TBI, which are not significantly different from sham values at 24 h.[40] Earlier time points may reveal an increase in cytokine mRNA expression, which is no longer detectable at 24 h, and is therefore a limitation of these studies. Though these results are in contrast with more-robust and sustained neuroinflammatory changes after TBI, it is possible that differences in the severity of the injury, or time frame when we performed our measures, can explain these differences. In addition to the proinflammatory cytokines, we examined two key OS-related factors, COX2 and NOX2, at the level of protein and mRNA expression.

Cyclooxygenase and NADPH oxidases are two primary generators of ROS, and increased OS has been proposed to be a mediator of TBI-associated tissue injury.[51–53] Classically, COX2 is an inducible enzyme involved in the formation of prostaglandins from arachidonic acid, which are potent mediators of cytotoxic inflammation during pathological conditions and is the predominant COX isoform found in inflammatory cells and the brain.[54] Previous studies have demonstrated an increased expression of brain COX2 post-TBI, and administration of COX2 inhibitors has been shown to improve neurological reflexes and memory, as well as reduce inflammation after lateral cortical concussion in rats.[55,56] In this study, we did not observe any changes in COX2 expression at 24 h post-TBI. Similar to the lack of significant increase in inflammatory cytokine expression at this time point, it is possible that we did not observe significant up-regulation in COX2 expression owing to a more modest injury severity in our studies, compared to that used in other studies.[55] NOX2 is one isoform of the catalytic subunit of NADPH oxidase enzyme, and NOX2 deficiency in mice has been shown to be protective in ischemic stroke.[57] The role NOX2 plays in TBI has not been fully explored, but a recent study using gp91phox$^{-/-}$ mice showed reduced ROS production and contusion area, when compared to wild-type mice.[58] Although we did not observe increases in either NOX2 protein or mRNA expression at 24 h post-TBI, we believe this may be owing to species difference and/or injury severity.

Resident glial cells, including astrocytes and microglia in the brain, have been shown to play a role in the inflammatory process after injury.[59] Reactive astrogliosis involves hypertrophy and proliferation of astrocytes, which alters morphology, and expression of a structural protein, GFAP.[60] Following injury, astrocytes are activated, which induces increased GFAP expression along with cell projection hypertrophy.[61] In addition to activation of astrocytes, microglia can be activated and migrate to the area of injury. Activation of microglia involves altered cell morphology, whereby microglia transform from a ramified shape to an amoeboid shape and concomitantly increase expression of ED1, a specific marker on lysosomal membranes. The increased expression of ED1 in microglia serves as a marker of activation. Our results showed increased expression of both GFAP and ED1 in the ipsilateral cortex of TBI/VEH animals, indicating activated astrocytes and microglia, respectively. Treatment with either JZL184 or URB597 was effective at reversing astrocyte activation, which may contribute to maintenance of the BBB and improved neurological and -behavioral recovery in this study. Cannabinoid receptors are present on both astrocytes and microglia, which may explain the lack of astrocyte and microglia activation in both treatment groups.[62,63] The role astrocyte and microglia activation play in BBB dysfunction is not fully understood, but some evidence indicates inflammatory mediators release by activated astrocytes modulate cell migration through the BBB.[64]

Taken together, the results from the present study suggest that inhibition of EC degradation, primarily by JZL184, is effective in attenuating BBB dysfunction and neurological and -behavioral impairments, which are associated with increased astrocyte and microglia activation post-TBI. These findings provide additional support for the potential therapeutic benefit of EC modulation with the use of selective inhibitors of EC degradation after TBI. Whether the protective effects are sustained or improved with additional drug dosing after the initial 24-h post-TBI remains to be examined.

Acknowledgments

The authors thank Drs. Nicole LeCapitaine and Robert Siggins for their scientific guidance, Jane Schexnayder for her technical assistance, as well as Rebecca Gonzales for her editorial support. This work was supported by the Department of Defense (DOD-W81XWH-11-2-0011) and the National Institute on Alcohol Abuse and Alcoholism (NIAAA-007577 and NIAAA-19587).

Author Disclosure Statement

No competing financial interests exist.

References

1. Warden, D. (2006). Military TBI during the Iraq and Afghanistan wars. J. Head Trauma Rehabil. 21, 398–402.
2. Terrio, H., Brenner, L.A., Ivins, B.J., Cho, J.M., Helmick, K., Schwab, K., Scally, K., Bretthauer, R., and Warden, D. (2009). Traumatic brain injury screening: preliminary findings in a US Army Brigade Combat Team. J. Head Trauma Rehabil. 24, 14–23.
3. Mayorga, M.A. (1997). The pathology of primary blast overpressure injury. Toxicology 121, 17–28.
4. McKee, A.C., Cantu, R.C., Nowinski, C.J., Hedley-Whyte, E., Gavett, B.E., Budson, A.E., Santini, V.E., Lee, H.S., Kubilus, C.A., and Stern, R.A. (2009). PMC2945234; chronic traumatic encephalopathy in athletes: progressive tauopathy after repetitive head injury. J. Neuropathol. Exp. Neurol. 68, 709–735.
5. Faul, M., Xu, L., Wald, M.M., and Coronado, V.G. (2010). Traumatic brain injury in the United States: emergency department visits, hospitalizations and deaths, 2002–2006. Centers for Disease Control and Prevention, National Center for Injury Prevention and Control: Atlanta, GA.
6. Elder, G.A., and Cristian, A. (2009). Blast-related mild traumatic brain injury: mechanisms of injury and impact on clinical care. Mt. Sinai J. Med. 76, 111–118.
7. Okie, S. (2005). Traumatic brain injury in the war zone. N. Engl. J. Med. 352, 2043–2047.
8. Riggio, S. (2011). Traumatic brain injury and its neurobehavioral sequelae. Neurol. Clin. 29, 35–47, vii.
9. Lenzlinger, P.M., Morganti-Kossmann, M., Laurer, H.L., and McIntosh, T.K. (2001). The duality of the inflammatory response to traumatic brain injury. Mol. Neurobiol. 24, 169–181.

10. Werner, C., and Engelhard, K. (2007). Pathophysiology of traumatic brain injury. Br. J. Anaesth. 99, 4–9.
11. Schmidt, O.I., Heyde, C.E., Ertel, W., and Stahel, P.F. (2005). Closed head injury—an inflammatory disease? Brain Res. Brain Res. Rev. 48, 388–399.
12. Chen, Y., Constantini, S., Trembovler, V., Weinstock, M., and Shohami, E. (1996). An experimental model of closed head injury in mice: pathophysiology, histopathology, and cognitive deficits. J. Neurotrauma 13, 557–568.
13. Lloyd, E., Somera-Molina, K., Van Eldik, L.J., Watterson, D.M., and Wainwright, M.S. (2008). PMC2483713; suppression of acute proinflammatory cytokine and chemokine upregulation by post-injury administration of a novel small molecule improves long-term neurologic outcome in a mouse model of traumatic brain injury. J. Neuroinflammation 5, 28.
14. Jain, K.K. (2008). Neuroprotection in traumatic brain injury. Drug Discov. Today 13, 1082–1089.
15. Loane, D.J., and Faden, A.I. (2010). Neuroprotection for traumatic brain injury: translational challenges and emerging therapeutic strategies. Trends Pharmacol. Sci. 31, 596–604.
16. Cernak, I., and Noble-Haeusslein, L.J. (2010). Traumatic brain injury: an overview of pathobiology with emphasis on military populations. J. Cereb. Blood Flow Metab. 30, 255–266.
17. Pitkänen, A., Longhi, L., Marklund, N., Morales, D.M., and McIntosh, T.K. (2005). Neurodegeneration and neuroprotective strategies after traumatic brain injury. Drug Discov. Today Dis. Mech. 2, 409–418.
18. Di Marzo, V., Bifulco, M., and De Petrocellis, L. (2004). The endocannabinoid system and its therapeutic exploitation. Nat. Rev. Drug Discov. 3, 771–784.
19. Mackie, K., and Stella, N. (2006). Cannabinoid receptors and endocannabinoids: evidence for new players. AAPS J. 8, E298–E306.
20. Mechoulam, R., Panikashvili, D., and Shohami, E. (2002). Cannabinoids and brain injury: therapeutic implications. Trends Mol. Med. 8, 58–61.
21. Panikashvili, D., Shein, N.A., Mechoulam, R., Trembovler, V., Kohen, R., Alexandrovich, A., and Shohami, E. (2006). The endocannabinoid 2-AG protects the blood-brain barrier after closed head injury and inhibits mRNA expression of proinflammatory cytokines. Neurobiol. Dis. 22, 257–264.
22. Wilson, R.I., and Nicoll, R.A. (2002). Endocannabinoid signaling in the brain. Science 296, 678–682.
23. Savinainen, J.R., Jarvinen, T., Laine, K., and Laitinen, J.T. (2001). Despite substantial degradation, 2-arachidonoylglycerol is a potent full efficacy agonist mediating CB(1) receptor-dependent G-protein activation in rat cerebellar membranes. Br. J. Pharmacol. 134, 664–672.
24. Hwang, J., Adamson, C., Butler, D., Janero, D.R., Makriyannis, A., and Bahr, B.A. (2010). Enhancement of endocannabinoid signaling by fatty acid amide hydrolase inhibition: a neuroprotective therapeutic modality. Life Sci. 86, 615–623.
25. Yu, M., Ives, D., and Ramesha, C.S. (1997). Synthesis of prostaglandin E2 ethanolamide from anandamide by cyclooxygenase-2. J. Biol. Chem. 272, 21181–21186.
26. Kozak, K.R., Rowlinson, S.W., and Marnett, L.J. (2000). Oxygenation of the endocannabinoid, 2-arachidonylglycerol, to glyceryl prostaglandins by cyclooxygenase-2. J. Biol. Chem. 275, 33744–33749.
27. Kozak, K.R., Prusakiewicz, J.J., and Marnett, L.J. (2004). Oxidative metabolism of endocannabinoids by COX-2. Curr. Pharm. Des. 10, 659–667.
28. Nadler, V., Biegon, A., Beit-Yannai, E., Adamchik, J., and Shohami, E. (1995). 45Ca accumulation in rat brain after closed head injury; attenuation by the novel neuroprotective agent HU-211. Brain Res. 685, 1–11.
29. Mauler, F., Horvath, E., De Vry, J., Jager, R., Schwarz, T., Sandmann, S., Weinz, C., Heinig, R., and Bottcher, M. (2003). BAY 38-7271: a novel highly selective and highly potent cannabinoid receptor agonist for the treatment of traumatic brain injury. CNS Drug Rev. 9, 343–358.
30. Biegon, A. (2004). Cannabinoids as neuroprotective agents in traumatic brain injury. Curr. Pharm. Des. 10, 2177–2183.
31. Tchantchou, F., and Zhang, Y. (2013). Selective inhibition of alpha/beta-hydrolase domain 6 attenuates neurodegeneration, alleviates blood brain barrier breakdown and improves functional recovery in a mouse model of traumatic brain injury. J. Neurotrauma 30, 565–579.
32. Knoller, N., Levi, L., Shoshan, I., Reichenthal, E., Razon, N., Rappaport, Z.H., and Biegon, A. (2002). Dexanabinol (HU-211) in the treatment of severe closed head injury: a randomized, placebo-controlled, phase II clinical trial. Crit. Care Med. 30, 548–554.
33. Shohami, E., Cohen-Yeshurun, A., Magid, L., Algali, M., and Mechoulam, R. (2011). Endocannabinoids and traumatic brain injury. Br. J. Pharmacol. 163, 1402–1410.
34. Correa, F., Mestre, L., Molina-Holgado, E., Arevalo-Martin, A., Docagne, F., Romero, E., Molina-Holgado, F., Borrell, J., and Guaza, C. (2005). The role of cannabinoid system on immune modulation: therapeutic implications on CNS inflammation. Mini Rev. Med. Chem. 5, 671–675.
35. Downer, E.J. (2011). Cannabinoids and innate immunity: taking a toll on neuroinflammation. ScientificWorldJournal 11, 855–865.
36. Dixon, C.E., Lyeth, B.G., Povlishock, J.T., Findling, R.L., Hamm, R.J., Marmarou, A., Young, H.F., and Hayes, R.L. (1987). A fluid percussion model of experimental brain injury in the rat. J. Neurosurg. 67, 110–119.
37. McIntosh, T.K., Vink, R., Noble, L., Yamakami, I., Fernyak, S., Soares, H., and Faden, A.L. (1989). Traumatic brain injury in the rat: characterization of a lateral fluid-percussion model. Neuroscience 28, 233–244.
38. Thompson, H.J., Lifshitz, J., Marklund, N., Grady, M.S., Graham, D.I., Hovda, D.A., and McIntosh, T.K. (2005). Lateral fluid percussion brain injury: a 15-year review and evaluation. J. Neurotrauma 22, 42–75.
39. Ling, G.S.F., Lee, E.Y., and Kalehua, A.N. (2004). Traumatic brain injury in the rat using the fluid-percussion model. Curr. Protoc. Neurosci. Chapter 9, Unit 9.2.
40. Teng, S.X., and Molina, P.E. (2014). Acute alcohol intoxication prolongs neuroinflammation without exacerbating neurobehavioral dysfunction following mild traumatic brain injury. J. Neurotrauma 31, 378–386.
41. Boyko, M., Ohayon, S., Goldsmith, T., Novack, L., Novack, V., Perry, Z.H., Gruenbaum, B.F., Gruenbaum, S.E., Steiner, O., Shapira, Y., Teichberg, V.I., and Zlotnik, A. (2011). Morphological and neurobehavioral parallels in the rat model of stroke. Behav. Brain Res. 223, 17–23.
42. Euser, A.G., Bullinger, L., and Cipolla, M.J. (2008). Magnesium sulphate treatment decreases blood-brain barrier permeability during acute hypertension in pregnant rats. Exp. Physiol. 93, 254–261.
43. Xu, Q., Qaum, T., and Adamis, A.P. (2001). Sensitive blood-retinal barrier breakdown quantitation using Evans blue. Invest. Ophthalmol. Vis. Sci. 42, 789–794.
44. de Jonge, H.J., Fehrmann, R.S., de Bont, E.S., Hofstra, R.M., Gerbens, F., Kamps, W.A., de Vries, E.G., van der Zee, A.G., te Meerman, G.J., and ter Elst, A. (2007). Evidence based selection of housekeeping genes. PLoS One 2, e898.
45. Panikashvili, D., Simeonidou, C., Ben-Shabat, S., Hanus, L., Breuer, A., Mechoulam, R., and Shohami, E. (2001). An endogenous cannabinoid (2-AG) is neuroprotective after brain injury. Nature 413, 527–531.
46. Maas, A.I., Murray, G., Henney, H., Kassem, N., Legrand, V., Mangelus, M., Muizelaar, J., Stocchetti, N., and Knoller, N. (2006). Efficacy and safety of dexanabinol in severe traumatic brain injury: results of a phase III randomised, placebo-controlled, clinical trial. Lancet Neurol. 5, 38–45.
47. Cravatt, B.F., Demarest, K., Patricelli, M.P., Bracey, M.H., Giang, D.K., Martin, B.R., and Lichtman, A.H. (2001). Supersensitivity to anandamide and enhanced endogenous cannabinoid signaling in mice lacking fatty acid amide hydrolase. Proc. Natl. Acad. Sci. U. S. A. 98, 9371–9376.
48. Nagayama, T., Sinor, A.D., Simon, R.P., Chen, J., Graham, S.H., Jin, K., and Greenberg, D.A. (1999). Cannabinoids and neuroprotection in global and focal cerebral ischemia and in neuronal cultures. J. Neurosci. 19, 2987–2995.
49. Povlishock, J.T. (1992). Traumatically induced axonal injury: pathogenesis and pathobiological implications. Brain Pathol. 2, 1–12.
50. Lloyd, E., Somera-Molina, K., Van Eldik, L.J., Watterson, D.M., and Wainwright, M.S. (2008). Suppression of acute proinflammatory cytokine and chemokine upregulation by post-injury administration of a novel small molecule improves long-term neurologic outcome in a mouse model of traumatic brain injury. J. Neuroinflammation 5, 28.
51. Lewen, A., Matz, P., and Chan, P.H. (2000). Free radical pathways in CNS injury. J. Neurotrauma 17, 871–890.

52. Tyurin, V.A., Tyurina, Y.Y., Borisenko, G.G., Sokolova, T.V., Ritov, V.B., Quinn, P.J., Rose, M., Kochanek, P., Graham, S.H., and Kagan, V.E. (2000). Oxidative stress following traumatic brain injury in rats: quantitation of biomarkers and detection of free radical intermediates. J. Neurochem. 75, 2178–2189.
53. Pratico, D., Reiss, P., Tang, L.X., Sung, S., Rokach, J., and McIntosh, T.K. (2002). Local and systemic increase in lipid peroxidation after moderate experimental traumatic brain injury. J. Neurochem. 80, 894–898.
54. Dubois, R.N., Abramson, S.B., Crofford, L., Gupta, R.A., Simon, L.S., Van De Putte, L.B., and Lipsky, P.E. (1998). Cyclooxygenase in biology and disease. FASEB J. 12, 1063–1073.
55. Strauss, K.I., Barbe, M.F., Marshall, R.M., Raghupathi, R., Mehta, S., and Narayan, R.K. (2000). Prolonged cyclooxygenase-2 induction in neurons and glia following traumatic brain injury in the rat. J. Neurotrauma 17, 695–711.
56. Gopez, J.J., Yue, H., Vasudevan, R., Malik, A.S., Fogelsanger, L.N., Lewis, S., Panikashvili, D., Shohami, E., Jansen, S.A., Narayan, R.K., and Strauss, K.I. (2005). Cyclooxygenase-2-specific inhibitor improves functional outcomes, provides neuroprotection, and reduces inflammation in a rat model of traumatic brain injury. Neurosurgery 56, 590–604.
57. Wang, Z., Wei, X., Liu, K., Zhang, X., Yang, F., Zhang, H., He, Y., Zhu, T., Li, F., Shi, W., Zhang, Y., Xu, H., Liu, J., and Yi, F. (2013). NOX2 deficiency ameliorates cerebral injury through reduction of complexin II-mediated glutamate excitotoxicity in experimental stroke. Free Radic. Biol. Med. 65, 942–951.
58. Dohi, K., Ohtaki, H., Nakamachi, T., Yofu, S., Satoh, K., Miyamoto, K., Song, D., Tsunawaki, S., Shioda, S., and Aruga, T. (2010). Gp91phox (NOX2) in classically activated microglia exacerbates traumatic brain injury. J. Neuroinflammation 7, 41.
59. Ridet, J.L., Malhotra, S.K., Privat, A., and Gage, F.H. (1997). Reactive astrocytes: cellular and molecular cues to biological function. Trends Neurosci. 20, 570–577.
60. Pekny, M., and Nilsson, M. (2005). Astrocyte activation and reactive gliosis. Glia 50, 427–434.
61. Eng, L.F., Ghirnikar, R.S., and Lee, Y.L. (2000). Glial fibrillary acidic protein: GFAP-thirty-one years (1969–2000). Neurochem. Res. 25, 1439–1451.
62. Stella, N. (2009). Endocannabinoid signaling in microglial cells. Neuropharmacology 56, Suppl 1, 244–253.
63. Stella, N. (2010). Cannabinoid and cannabinoid-like receptors in microglia, astrocytes, and astrocytomas. Glia 58, 1017–1030.
64. Weiss, J.M., Downie, S.A., Lyman, W.D., and Berman, J.W. (1998). Astrocyte-derived monocyte-chemoattractant protein-1 directs the transmigration of leukocytes across a model of the human blood-brain barrier. J. Immunol. 161, 6896–6903.

Address correspondence to:
Patricia E. Molina, MD, PhD
Department of Physiology
Alcohol and Drug Abuse Center of Excellence
Louisiana State University Health Sciences Center
1901 Perdido Street, Room 7205
New Orleans, LA 70112-1393

E-mail: pmolin@lsuhsc.edu

Journal of Neurotrauma

Journal of Neurotrauma: http://mc.manuscriptcentral.com/neurotrauma

Inhibition of Endocannabinoid Degradation Improves Outcomes from Mild Traumatic Brain Injury: A Mechanistic Role for Synaptic Hyperexcitability

Journal:	*Journal of Neurotrauma*
Manuscript ID	NEU-2016-4452.R1
Manuscript Type:	Regular Manuscript
Date Submitted by the Author:	01-Apr-2016
Complete List of Authors:	Mayeux, Jacques; Louisiana State University Health Sciences Center, Physiology Katz, Paige; Louisiana Health Sciences Center, Department of Physiology Edwards, Scott; Louisiana State University Health Sciences Center, Physiology Middleton, Jason; Louisiana State University Health Sciences Center, Cell Biology and Anatomy Molina, Patricia; Louisiana State University Health Sciences Center, Physiology
Keywords:	TRAUMATIC BRAIN INJURY, NEUROEXCITATION, INFLAMMATION, Behavior, ELECTROPHYSIOLOGY

Inhibition of Endocannabinoid Degradation Improves Outcomes from Mild Traumatic Brain Injury: A Mechanistic Role for Synaptic Hyperexcitability

Jacques Mayeux, MS[1], Paige Katz, PhD[2], Scott Edwards, PhD[3], Jason W. Middleton, PhD[4], and Patricia Molina, MD, PhD[5].

[1]Graduate Research Assistant and PhD Candidate, Department of Physiology and Alcohol and Drug Abuse Center of Excellence, Louisiana State University Health Sciences Center, New Orleans, LA 70112

Louisiana State University Health Sciences Center

Alcohol and Drug Abuse Center of Excellence

Department of Physiology

1901 Perdido Street Rm 7212

New Orleans, LA 70112

[2]Postdoctoral Fellow, Department of Physiology and Alcohol and Drug Abuse Center of Excellence, Louisiana State University Health Sciences Center, New Orleans, LA 70112

Louisiana State University Health Sciences Center

Alcohol and Drug Abuse Center of Excellence

Department of Physiology

1901 Perdido Street Rm 7212

New Orleans, LA 70112

[3]Assistant Professor, Department of Physiology and Alcohol and Drug Abuse Center of Excellence, Louisiana State University Health Sciences Center, New Orleans, LA 70112

Louisiana State University Health Sciences Center

Alcohol and Drug Abuse Center of Excellence

Department of Physiology

1901 Perdido Street Rm 7212

New Orleans, LA 70112

[4]Assistant Professor, Department of Cell Biology and Anatomy and Alcohol and Drug Abuse Center of Excellence, Louisiana State University Health Sciences Center, New Orleans, LA 70112

Louisiana State University Health Sciences Center

Alcohol and Drug Abuse Center of Excellence

Department of Cell Biology and Anatomy

1901 Perdido Street Rm 6103

New Orleans, LA 70112

[5] **CORRESPONDING AUTHOR;** Professor and Chair, Department of Physiology and Alcohol and Drug Abuse Center of Excellence, Louisiana State University Health Sciences Center, New Orleans, LA 70112

Louisiana State University Health Sciences Center

Alcohol and Drug Abuse Center of Excellence

Department of Physiology

1901 Perdido Street Rm 7212

New Orleans, LA 70112

Corresponding Author:

Patricia E. Molina, MD, PhD

Running/Table of Contents title: Inhibition of EC Degradation Improves mTBI Outcomes

Key Words: 2-AG, MAGL, TBI, neuroinflammation, endocannabinoids

Abstract

Traumatic brain injury (TBI) is an increasingly prevalent condition affecting soldiers, athletes, and motor vehicle accident victims. Unfortunately, it currently lacks effective therapeutic interventions. TBI is defined as a primary mechanical insult followed by a secondary cascade involving inflammation, apoptosis, release of reactive oxygen species, and excitotoxicity, all of which can cause synaptic changes, altered neuronal signaling, and ultimately, behavioral changes. Previously we showed that preventing degradation of the endocannabinoid (EC) 2-acylglycerol (2-AG) with JZL184 following mild TBI attenuated neuroinflammation and improved recovery of neurobehavioral function during the early 24 h post-TBI period. The aim of this study was to extend the timeline of observations to two weeks post-injury and to investigate JZL184's impact on synaptic transmission, which we view as potential mechanism for TBI-induced cellular and behavioral pathology. Adult male rats were subjected to mild TBI (mTBI) followed by a single intraperitoneal injection of JZL184 or vehicle thirty minutes post-injury. JZL184 administered-TBI animals showed improved neurobehavioral recovery compared to vehicle-injected TBI animals beginning 24 hours post-injury and persisting for two weeks. JZL184-treated animals had significantly diminished gray and white matter astrocyte activation when compared to vehicle-treated animals at day 7 post-TBI. JZL184 administration significantly attenuated the increased pGluR1^{S845}/GluR1 and pERK 1/2 / ERK and the increases in miniature excitatory postsynaptic potential (mEPSC) frequency and amplitude observed in layer 5 pyramidal neurons at 10 days post-TBI. These results suggest a neuroprotective role for ECs in ameliorating the TBI-

induced neurobehavioral, neuroinflammatory and glutamate dyshomeostasis from mTBI. Further studies elucidating the cellular mechanisms involved are warranted.

Introduction

Traumatic brain injury (TBI) commonly affects otherwise healthy individuals such as athletes, victims of motor vehicle accidents, and soldiers, and currently lacks effective therapeutic intervention.[1] Mild TBI (commonly called a concussion) is an often underreported injury due to the transient nature of clinical manifestations such as loss of consciousness.[1] Recent evidence is mounting, however, that even after these transient effects dissipate, less obvious symptoms at the cellular and molecular level including inflammation, glutamate homeostasis disruptions and excitotoxicity, blood brain barrier disruption, and synaptic changes may persist.[2] If left untreated, these cellular and molecular changes may increase the risk for a number of short term (pain, headaches, anxiety, depression)[3-9] and long term (cognitive problems, neurodegenerative disease)[10] neurobehavioral sequelae. It is for this reason that studying the mechanisms driving cellular and behavioral pathology during the acute recovery period following mild TBI and researching potential therapeutic targets is of great importance.

There are two phases of cellular pathology following TBI. There is initially a primary mechanical insult, commonly caused by acceleration-deceleration or rotational forces.[11] These forces can stretch and strain neurons and microvessels resulting in diffuse axonal injury (DAI) and membrane permeability changes, triggering cellular apoptosis and glial cell activation.[11] The initial mechanical injury can also destroy or disrupt normal glial cell function which may impair blood brain barrier integrity.[11] Acute inflammation is a necessary and beneficial step following the initial mechanical insult. However, TBI is often characterized by a secondary “sustained” inflammatory response that actually exacerbates tissue damage in a feed forward mechanism.[1, 12-14] Activated neuroimmune

cells (microglia and astrocytes) and infiltrating peripheral immune cells release large amounts of proinflammatory cytokines triggering neuronal apoptosis, which in turn promotes increased glial proinflammatory cytokine release.[1, 12-14] The end result of this inflammatory sequelae is membrane permeability changes on both glial cells and neurons, potentially triggering massive release of glutamate and synaptic changes that could lead to neuronal damage or death by excitotoxicity.[1, 12-14] Pathologic changes in glutamate system homeostasis may be caused by post-TBI inflammation[15] and can be measured by examining protein level changes following injury (such as glutamate receptor subunit phosphorylation) and by measuring changes in spontaneous excitatory synaptic transmission. In all, the common neurobehavioral pathologies associated with TBI outlined above may be ameliorated if interventions can be identified that attenuate the development of this secondary sustained inflammatory and excitotoxic cascade.

There is mounting evidence, including work from our own lab[16], which indicates a protective role of the endocannabinoid (EC) system in reducing inflammation and neuronal death following injury[17]. ECs such as 2-arachidonoyl glycerol (2-AG) (the most bioactive and abundant EC in the brain) have been shown to have anti-inflammatory properties.[18-20] In addition, 2-AG has also been shown to be effective in decreasing glutamate toxicity.[17, 19] Much like neurotransmitters, though, once ECs are released in response to injury they are rapidly degraded. The primary enzyme that breaks down 2-AG is monoacylglycerol lipase (MAGL).[21-22] Previously, we showed significant improvement of behavior and neuroinflammation post-TBI in MAGL inhibitor JZL184-treated rats. Our results showed that treatment with one dose of JZL184 30 minutes post-TBI attenuated inflammation, blood brain barrier permeability, and neurobehavioral

and neurological severity scores for up to 24 hours post-injury. Our studies showed that of the two EC degradation inhibitors, URB597 and JZL184, animals treated with JZL184 showed more consistent improvement in all outcome measures examined. Because of these data, we sought in the current study to focus exclusively on MAGL inhibition and to extend the timeline for the studies to examine whether JZL184 treatment improved glutamate homeostasis disruptions and synaptic hyperexcitability, which we believe to be involved mechanistically in pathologic outcomes from mild TBI. We hypothesized that treatment with the EC degradation inhibitor JZL184 would attenuate behavioral, cellular, and synaptic dysfunction during the acute recovery period up to 14 days following mild TBI.

Methods

Animals

Two cohorts of adult male rats (Sprague-Dawleys for the first set of studies and Wistar rats for the second study) weighing between 250–275 g were purchased from Charles River Laboratories (Wilmington, Mass.) and pair-housed in a temperature and humidity controlled animal housing room with a 12 hour (h) light/dark cycle. We used Sprague Dawley rats for neurobehavioral analysis, immunohistochemical analysis, and phosphoprotein expression analysis to stay consistent with (and extend the findings of) our previous study.[16] To further explore the mechanisms involved in neuroprotection, we used Wistar rats exclusively for the electrophysiology studies. Wistar rats tend to show pronounced cortical changes following injury[23] and in parallel studies conducted by our group they are being used to examine alcohol drinking behaviors following TBI because of their more ease in acquiring alcohol self-administration behavior. Our studies have shown similar neurobehavioral and neuroinflammatory changes following identical protocols of TBI in both strains of rats.[16, 24, 25] Both sets of animals were exposed to identical experimental procedures. Animals had *ad libitum* access to water and standard rat chow, and were allowed one week to habituate to housing conditions prior to any experimental procedures. All animal procedures were approved by the Institutional Animal Care and Use Committee of the Louisiana State University Health Sciences Center (LSUHSC; New Orleans, LA) and were in accord with the National Institute of Health (NIH) guidelines.

Craniotomy

Surgeries were completed as previously detailed.[24] Briefly, animals were anesthetized with a mixture of ketamine (90 mg/kg) and xylazine (9 mg/kg) prior to craniotomy (-2mm bregma and 3mm lateral to bregma; over the left sensorimotor cortex). A female Luer Loc connector was superglued over the craniotomy site and secured with dental cement (Lang Dental Manufacturing, Wheeling, IL). The female Luer Loc was filled with sterile normal saline and capped. After surgery, animals were allowed to recover for three days in single housed cages with *ad libitum* access to food and water before randomization to either sham (craniotomy-only) or TBI groups.

Traumatic Brain Injury via Lateral Fluid Percussion

TBI was produced by lateral fluid percussion (LFP) as previously described.[24] Briefly, rats were anesthetized via inhalant isoflurane (4% induction, 3% maintenance) and placed in a stereotaxic frame (model 900; Kopf Instruments, Tujunga, CA). The Luer Loc that was previously attached to the rat skull was connected to the LFP apparatus via pressure tubing. Dropping the mallet on the LFP apparatus from a predetermined angle (approximately 18^{o}) produced a consistent saline pressure wave that impacted the dura (2 atm pressure for 25 ms) and resulted in injury. Sham control animals were subject to the same procedure and connected to the LFP apparatus but did not receive an injury. All animals were placed back into their individual home cage and continuously monitored for 2 h post-TBI with free access to food and water.

Endocannabinoid Degradation Inhibition

JZL184 is a selective inhibitor of MAGL, the enzyme responsible for 2-AG hydrolysis. MAGL inhibition following JZL184 administration is rapid (maximal inhibition achieved

within 0.5 h post-treatment) and potent (> 80% inhibition of 2-AG hydrolysis activity resulting in a 7-9 fold increase in brain 2-AG levels). The half-life of JZL184 is approximately 7h[62]. JZL184 (16 mg/kg) was injected intraperitoneally 30 minutes following TBI (TBI/JZL) in both cohorts of rats. Time-matched TBI and sham animals received equal volumes of vehicle (0.64 mL of vehicle per kg body weight; TBI/VEH or SHAM/VEH; vehicle injections contained alcohol, emulphor, and saline (1:1:18)). Animals were studied during the 7-14 days post-TBI recovery period.

Assessment of Neurobehavioral Function

Neurological (neurological severity scores; NSS) and neurobehavioral (neurobehavioral scores; NBS) function were assessed at baseline (1 h before TBI) and at 2 h, 24 h, 72 h, 7 d, 14 d, 21 d, and 30 d post-TBI, as previously described (Teng et al., 2014). All animals were exposed to all tasks, trained, and evaluated before TBI using the testing parameters that were adapted from previously published methods of assessing cognition and behavior. Animals were allowed 30 minutes to habituate to the testing room prior to assessment. NSS scores range from 0 to 25 (least to most impaired) and NBS scores range from 0 to12 (least to most impaired) and were based on the animal's performance on each task.

NSS evaluates motor function, sensory, reflexes, beam walking, and beam balancing. Pinna, corneal, startle, and righting reflexes were assessed, where a score of 1 indicates impaired reflex and a score of 0 indicates the reflex is intact. Animals were placed on beams of decreasing width (10, 8, 5, and 2.5 cm) to test motor coordination (allowed 60 seconds to traverse each beam). In addition, beam balance was assessed

where animals were placed on a 1.5-cm-wide beam and given 60 sec to balance. Failure to walk all beams and/or balance for 60 sec resulted in increased NSS total.

NBS tests sensorimotor, proprioception, exploratory behavior, and novel object recognition. Proprioception was assessed by pushing each animal laterally (lateral pulsion) on each side of its body. Each side was assessed and failure to resist lateral pulsion on one or both sides increased the NBS total. Exploratory behavior was assessed immediately after the animal's cage top was removed - uninjured animals actively explored the top of the cage and surroundings (lower NBS score) while injured animals tended to avoid exploratory behavior (increased NBS score).

Tissue collection

After behavioral tests were completed, animals were euthanized by decapitation under light isoflurane anesthesia. Brains were rapidly dissected and snap frozen in isopentane. The site of injury was isolated using a prefrozen standard adult rodent brain slicer matrix (Zivic Instruments, Pittsburgh, PA) and divided at mid-line to separate the ipsilateral (injured) from the contralateral (uninjured) region. Brain tissues were then stored at −80°C for further analyses.

Immunohistochemistry and immunofluorescence

Immunohistochemistry was performed as previously described.[24-25] Briefly, a subset of animals were perfusion-fixed and brains isolated and sectioned in 40 micron slices at -20^{o} C prior to permeabilization with 0.3% Triton-X 100 in PBS for 30 minutes. Sections were then blocked at room temperature with blocking buffer (bovine serum albumin, normal donkey serum, Triton-X 100, PBS) for one hour.

Sections were incubated with rabbit anti-glial fibrillary acid protein (GFAP; 1:200; Abcam) for 24 h at 4°C in a humidification chamber. The next day slides were washed for 15 minutes (3 x 5 min in fresh PBS) prior to secondary antibody incubation with Alexa Fluor 555 donkey anti-rabbit (1:200; by Life Technologies, Carlsbad, CA) for 2 h at room temperature in the dark. The slides were dried and coverslipped using mounting media with DAPI (ProLong Gold, Life Technologies Carlsbad, CA). Images were captured at 20x or 4x magnification using a three millisecond exposure time on a Nikon Eclipse TE2000-U (Nikon, Tokyo, Japan). The imaging software was NIS Elements (Version 3.22.11, Nikon, Tokyo, Japan). IHC was quantified using ImageJ software at 40x magnification. Images were then quantified using ImageJ software (NIH, Bethesda, MD) at 40× magnification. Values are expressed as percent area of positive staining. At least two pictures were taken of three sections, for a total of six to nine pictures per animal, and 5 animals were analyzed per group.

Western Blot

To determine whether TBI induced sustained disruption of excitatory signaling and whether JZL184 could attenuate these changes, we examined changes in glutamate receptor subunit GluR1 and levels of extracellular signal-related kinase (ERK), a marker of neuronal activity. Individual ERK and GluR1 phosphorylation levels in the brain regional homogenates were determined as previously described.[26] Regional tissue samples were obtained using 12–16 gauge punches from frozen coronal brain slices (0.5 mm thick) obtained by the use of a cryostat. Tissue samples were homogenized by sonication in lysis buffer (320 mM sucrose, 5 mM HEPES, 1 mM EGTA, 1 mM EDTA and 1% SDS, with protease inhibitor cocktail and phosphatase inhibitor cocktails II and

III diluted 1:100; Sigma, St Louis, MO, USA), heated at 100 °C for 5 min and stored at −80 °C until the determination of protein concentration by a detergent-compatible Lowry method (Bio-Rad, Hercules, CA, USA). Protein samples (15 µg) were subjected to SDS-polyacrylamide gel electrophoresis on 4-15% gradient acrylamide gels by using a Tris/Glycine/SDS buffer system (Bio-Rad), followed by electrophoretic transfer to polyvinylidene difluoride membranes (GE Healthcare, Piscataway, NJ, USA). Membranes were blocked overnight in 5% non-fat milk at 4 °C and were then incubated in primary antibody recognizing the dual phosphorylated form of ERK (1:2500, 5% non-fat milk; Cell Signaling, Danvers, MA, USA) and the protein kinase A (PKA)-phosphorylated AMPA glutamate receptor subunit pGluR1S845 (1:1000; 5% non-fat milk; Cell Signaling, Danvers, MA, USA). Membranes were washed and labeled with species-specific peroxidase-conjugated secondary antibody (1:10 K; Bio-Rad) for 1 h at room temperature. Following chemiluminescence detection (SuperSignal West Pico; Thermo Scientific, Rockford, IL, USA), blots were stripped for 20 min at room temperature (Restore; Thermo Scientific) and were reprobed for total protein levels of ERK (1:5000; Cell Signaling) and GluR1 (1:2500; Cell Signaling). Immunoreactivity was quantified by densitometry (ImageJ 1.45S; NIH) under linear exposure conditions. Densitized values were expressed as a percentage of the mean of control values for each gel to normalize data across blots. Individual phosphoprotein levels were normalized to individual total protein levels to generate ERK phosphorylation (pERK)/ERK and GluR1 phosphorylation (pGluR1)/GluR1 ratio values for statistical comparison.

Electrophysiology

To further validate whether TBI caused sustained changes in glutamate homeostasis disruptions and whether JZL184 could attenuate these changes we used electrophysiology to examine spontaneous excitatory activity in the form of mini excitatory post-synaptic currents (mEPSCs). Animals underwent the same experimental procedures described above and were sacrificed by decapitation under deep isoflurane anesthesia 10 days post-TBI. Brains were removed and sliced in an ice-cold artificial cerebrospinal fluid (ACSF) containing (in mM): 125 Choline Chloride, 2.5 KCl, 1.25 NaH_2PO_4, 2 $MgSO_4$, 25 $NaHCO_3$, 7 dextrose, 0.5 $CaCl_2$, 7 $MgCl_2$ saturated with 95% O_2–5% CO_2. Coronal slices containing somatomotor cortex underlying the site of injury were then transferred to a holding chamber in a water heat bath where the holding/recording solution was kept at 36˚C. The holding/recording solution consisted of (in mM): 125 NaCl. 25 $NaHCO_3$, 1.25 NaH_2PO_4, 2.5 KCl, 10 dextrose, 1.25 $CaCl_2$, 2 $MgCl_2$, 1.3 Na-ascorbate and 3 Na-pyruvate. After 30 mins, the holding chamber was removed from the heat bath and allowed to rest at room temperature for ~ 45 minutes before slices were used for electrophysiological recording experiments. Whole-cell recordings were performed using borosilicate glass micropipettes (3–7 MΩ) filled with internal solution containing (in mM): 130 K-gluconate, 10 HEPES, 10 Na_2-phosphocreatine, 4 $MgCl_2$, 4 Na_2-ATP, 0.4 Na-GTP, 3 ascorbic acid, 0.2 EGTA (pH 7.25, 290–295 mOsm). Recordings were obtained from pyramidal neurons because they are the primary excitatory neurons in the cortex and, because of to their location, may be vulnerable to damage and altered signaling by TBI. Postsynaptic current was measured while clamping the membrane voltage at -70 mV; mEPSCs were detected in current traces and both frequency and amplitude were calculated to characterize

glutamatergic synaptic transmission in cortical brain slices. Changes in the amplitude, kinetics or frequency of mEPSCs were analyzed to garner information about both presynaptic and postsynaptic alterations (Isaacson & Walmsley, 1995) that may occur as a result of TBI.

Statistical analysis

All data are expressed as mean ± standard error of the mean (SEM). Statistical differences were determined by either one-way analysis of variance (ANOVA) or two-way ANOVA with repeated measures using GraphPad Prism 5.0 statistical software (Graphpad Software Inc., La Jolla, CA). Tukey's test for one-way ANOVA and Bonferroni's test for two-way ANOVA were utilized for pair-wise multiple comparisons. Statistical significance was set at $p<0.05$.

RESULTS

JZL Treatment Attenuated TBI-Induced Impairments in Neurobehavioral Scores (NBS) and Neurological Severity Scores (NSS)

NBS and NSS for both TBI/VEH and TBI/JZL animals were greater than sham animals 2 hours post-injury (indicating greater impairment, Fig. 1). However, 24 hours post-TBI the TBI/JZL treatment significantly attenuated NBS and NSS dysfunction (50% score reduction) compared to TBI/VEH animals. By 72h following TBI the TBI/JZL treated animals still exhibited reduced NBS/NSS compared to TBI/VEH animals. One week post-TBI, JZL treatment still had a significant protective effect in the form of lower NBS/NSS scores compared to TBI/VEH animals. Two weeks post-TBI, NSS scores were still significantly reduced in TBI/JZL treated animals compared to TBI/VEH animals, while NBS scores were attenuated for all animals by this time point.

JZL Treatment Attenuated Astrocyte Activation

A hopeful therapeutic window following mild TBI is immediately after the primary mechanical insult prior to the start of the secondary injury (development of sustained neuroinflammation). While acute inflammation is important and necessary in tissue recovery, sustained inflammation may actually potentiate pathology following TBI. Here we wanted to examine astrocyte activation (which often accompanies inflammation and

thus serves as an indirect marker of neuroinflammation) following TBI with and without the JZL administration during a critical therapeutic window (30 min post-TBI). Astrocyte activation (as measured by GFAP immunoreactivity) was significantly increased in the ipsilateral cortex (gray matter) one week post-TBI, and was attenuated by JZL treatment (Fig. 2). The contralateral cortex showed no significant astrocyte activation in any experimental condition. Interestingly, TBI induced white matter astrocyte activation in both the ipsilateral and contralateral corpus callosum and this astrocyte activation was also attenuated by JZL treatment. Representative images show the diffusivity of astrocyte activation spreading from the cortex to the corpus callosum in TBI/VEH animals (Fig. 2, M) and the reduction of this astrocyte activation in TBI/JZL treated animals (Fig. 2, N).

JZL Treatment Attenuated Phosphoprotein Markers of Increased Glutamate Receptor Activity

The nature of TBI lends itself to glutamate homeostasis disruptions, whether caused by stretch/strain of cortical neurons resulting in diffuse axonal injury and neuronal membrane permeability changes, or whether caused by inflammation-mediated excitotoxicity. To identify if these glutamate homeostasis disruptions resulted in any stable phosphoprotein expression changes at glutamate synapses, we examined two protein markers associated with glutamate activity. pGluR1 S845 is an AMPA glutamate receptor subunit that, when phosphorylated, regulates opening probability of the AMPA receptor in the post-synaptic neuron. In addition we examined ERK phosphorylation which is a protein correlate of post-synaptic neuronal activity.

At the site of injury, TBI/VEH-treated animals expressed higher levels of PKA-mediated GluR1 phosphorylation (twofold change, P<0.05) as well as greater phosphorylation of ERK (twofold change, P<0.05) compared to sham animals (Fig. 3). JZL treatment significantly attenuated the heightened GluR1S845 and ERK phosphorylation. No changes in total GluR1 or ERK were observed. *P<0.05 compared to Sham, $P<0.05 compared to TBI/VEH analyzed via one-way ANOVA with Tukey post-hoc analysis.

JZL Treatment Attenuated Increases in Mini Excitatory Post Synaptic Current and Amplitude at Site of Injury

We have shown that TBI results in phosphorylation of postsynaptic proteins associated with glutamatergic synaptic transmission at the site of injury. To investigate whether these protein changes result in measurable functional changes 10 days post-injury we recorded mini excitatory post-synaptic currents (mEPSCs) in layer 5 cortical pyramidal neurons at the site of injury (Fig. 4A,B). Alterations in spontaneous mEPSCs may reflect changes in presynaptic transmitter release or postsynaptic strength.[27] We found that pyramidal neurons in the cortex of TBI animals had increased mEPSC amplitude (13.9 ± 1.3 pA) and frequency (8.3 ± 0.6 Hz) compared to sham neurons (amplitude: 10.2 ± 0.8, frequency: 6.1 ± 0.6; p = 0.025; Fig. 4C, D). Treatment with JZL resulted in mEPSC amplitudes and frequencies that are similar to sham levels (amplitude: 9.5 ± 0.5 pA, p = 0.513 vs. sham, p = 0.005 vs. TBI; frequency: 6.5 ± 0.6 pA, p = 0.639 vs. sham, p = 0.04 vs. TBI). Increased excitatory synaptic inputs into a cortical pyramidal neurons would act to drive its action potential output, which may then further increase synchronous neural network activity.[28] Our findings suggest that both pre- and postsynaptic alterations have occurred as a result of TBI.[27,29]

DISCUSSION

In the current study we examined whether single-dose EC degradation inhibition post-TBI improved long term (up to two weeks) synaptic dysfunction, our proposed mechanism for TBI-induced cellular and behavioral pathology. Numerous studies implicate that exogenous EC administration may be neuroprotective[17] and previously we showed that preventing EC degradation was effective in improving short term (up to 24h) outcomes from mild TBI.[16] For these reasons we hypothesized in the current study that treatment with the EC degradation inhibitor JZL184 would attenuate behavioral, cellular, and synaptic dysfunction during the acute recovery period up to 14 days following mild traumatic brain injury. Our results show that JZL184 treatment improved NSS and NBS scores up to 14 days post-TBI, attenuated astrocyte reactivity at both the cortex (gray matter) and corpus callosum (white matter) 10 days post-TBI, and attenuated cortical (site of injury) expression of markers of synaptic hyperexcitability including pGluR1S845 and pERK and mEPSC frequency and amplitude in cortical neurons 10 days post-TBI. Overall, these results suggest that inhibiting the degradation of the endocannabinoid (EC) 2-AG with JZL184 is a promising therapeutic strategy that attenuates behavioral and cellular outcomes from mild TBI for up to 14 days post-injury.

The rationale for this study came from the wealth of literature indicating an important role for ECs in synaptic plasticity[30-33] and neuroprotection.[34-39] It is well known that TBI causes a host of cellular changes including neuroinflammation, excitotoxicity, and oxidative stress, all of which can potentially cause neuronal damage and death.[2, 12-14] As TBI transitions from the primary injury (mechanical insult) to the secondary injury (sustained neuroinflammation), the risk increases for long-term neurodegenerative

disease, including hallmark signs of neuropathology such as beta-amyloid plaque formation, tau phosphorylation, and white matter degeneration.[11] In fact, results from the current study show significant white matter astrocyte activation as well as synaptic changes indicating a hyper-excitable state following a single mild brain injury. Therefore the most critical window for therapeutic intervention is the acute period immediately following the primary mechanical insult. If a therapeutic can block the transition from the primary injury to the secondary injury, long-term prognosis might improve. The EC system may play a critical role in blocking the transition to secondary injury because of its role in modulating synaptic activity and exerting neuroprotective and anti-inflammatory effects.

Synaptic plasticity occurs with changes in synaptic transmitter release and/or postsynaptic receptor binding and transmembrane current mediation. Synaptic plasticity is widely believed to be one of the most important neurochemical changes involved in learning[40-43], memory[44-45] and behavioral modification.[46] The EC system plays a large role in retrograde signaling at glutamatergic synapses throughout the brain where it inhibits the release of neurotransmitter (net suppression of excitability).[47-48] The primary EC involved in short term depression of synaptic transmission is 2-AG, and this effect has been documented in numerous brain regions including hippocampus, amygdala, cerebellum, basal ganglia, brain stem, hypothalamus, and most relevant to the present study, cerebral cortex.[17, 49-50] Pharmacological manipulation of the EC system to increase 2-AG, thereby preventing excitotoxicity and oxidative stress, is a promising strategy for treatment. The EC system also has a well-known anti-inflammatory and neuroprotective effect.[51] In fact, Panikashvili and colleagues demonstrated a

neuroprotective role for the EC system in a mouse model of closed-head TBI; they demonstrated that direct 2-AG administration reduces inflammatory cytokine expression, edema formation, and blood brain barrier permeability. While 2-AG is innately elevated in the brain following TBI, it is rapidly metabolized due to its nature as an unstable fatty acid. We hypothesized in the current study that blocking the metabolism of 2-AG to allow for strengthened EC signaling during the critical window of transition from primary to secondary injury after mild TBI would have long lasting therapeutic benefit.

Importantly in the present study, we showed that even a single mild TBI can result in sustained neuropathology including white matter astrocyte activation and a hyper-excitable state, both of which were predictably associated with neurobehavioral dysfunction. It is well known that repetitive mild TBIs or a single severe TBI can increase the risk for developing a neurodegenerative disease now officially classified as chronic traumatic encephalopathy (CTE). Growing evidence supports the development and persistence of neuroinflammation in CTE development and interestingly, neuroinflammation has recently been implicated in the development of other neurodegenerative diseases such as Alzheimer's.[52-55] White matter inflammation is of particular interest in brain injury due to the unique nature of how TBIs occur (stretch/strain, rotational forces, coup-contra-coup injuries) and the common occurrence of DAI (diffuse axonal injuries).[56-57] In the present study we show dramatic corpus callosum astrocyte activation up to 10 days following a single mild TBI. The corpus callosum is a highly vulnerable region to trauma which contains the axons of large neurons stretching between the two hemispheres of the brain.[56-57] Interestingly,

damaged axons in the corpus callosum remain damaged long after the initial injury according to analysis of post-mortem human brains, and at least one study implicates axonal damage in the white matter tract as an important source of beta-amyloid plaques in Alzheimer's disease.[58-61] In the present study it was therefore particularly interesting that JZL184 administration following mild TBI not only attenuated cortical astrocyte activation but white matter tract astrocyte activation as well.

Overall the present study showed that single mild TBI was enough to result in some of the hallmark signs of neurodegeneration including hyper-excitable signaling, white matter tract astrocyte activation, and behavioral dysfunction. We proposed that increasing the efficacy of EC signaling by inhibiting MAGL-induced EC breakdown could improve behavioral outcomes by attenuating inflammation and synaptic plasticity changes. We showed that a single dose of JZL184 30 minutes post mild TBI improved neurobehavioral outcomes, synaptic plasticity, and astrocyte activation up to two weeks after injury. These data indicate a protective role for the EC system following mild brain injury and future work should be done exploring this translational potential of these findings.

Acknowledgements

This work was supported by NIH training (AA007577, JM, PK) and research grants AA020839 (SE), and DOD-W81XWH-11-2-0011 (PM). The authors would like to thank Dr. Nicholas Gilpin for scientific discussions during the preparation of this manuscript and Kylie Mills for assistance with animal behavior and surgery experiments. Paige Katz was a postdoctoral fellow at LSUHSC during the completion of these studies but has since completed her fellowship.

Author Disclosure Statement.

No competing financial interests exist.

References

1. Corrigan JD, Selassie AW, Orman JA. (2010). The epidemiology of traumatic brain injury. J Head Trauma Rehabil 25(2):72-80.
2. Al Nimer F, Lindblom R, Ström M, Guerreiro-Cacais AO, Parsa R, Aeinehband S, Mathiesen T, Lidman O, Piehl F. (2013). Strain influences on inflammatory pathway activation, cell infiltration and complement cascade after traumatic brain injury in the rat. Brain Behav Immun. 27(1):109-22.
3. Garden N, Sullivan KA. (2010). An examination of the base rates of post-concussion symptoms: the influence of demographics and depression. Appl Neuropsychol. 17(1):1-7.
4. Garden N, Sullivan KA, Lange RT. (2010). The relationship between personality characteristics and postconcussion symptoms in a nonclinical sample. Neuropsychology. 24(2):168-75.
5. Konrad C, Geburek AJ, Rist F, Blumenroth H, Fischer B, Husstedt I, Arolt V, Schiffbauer H, Lohmann H. (2011). Long-term cognitive and emotional consequences of mild traumatic brain injury. Psychol Med. 41(6):1197-211.
6. Levin HS, Brown SA, Song JX, McCauley SR, Boake C, Contant CF, Goodman H, Kotrla KJ. (2001). Depression and posttraumatic stress disorder at three months after mild to moderate traumatic brain injury. J Clin Exp Neuropsychol. 23(6):754-69.
7. Moore EL, Terryberry-Spohr L, Hope DA. (2006). Mild traumatic brain injury and anxiety sequelae: a review of the literature. Brain Inj. 20(2):117-32. Review.

8. Rao V, Bertrand M, Rosenberg P, Makley M, Schretlen DJ, Brandt J, Mielke MM. (2010). Predictors of new-onset depression after mild traumatic brain injury. J Neuropsychiatry Clin Neurosci. 22(1):100-4.
9. Vanderploeg RD, Curtiss G, Luis CA, Salazar AM. (2007). Long-term morbidities following self-reported mild traumatic brain injury. J Clin Exp Neuropsychol. 29(6):585-98.
10. Washington PM, Villapol S, Burns MP. (2015). Polypathology and dementia after brain trauma: Does brain injury trigger distinct neurodegenerative diseases, or should they be classified together as traumatic encephalopathy? Exp Neurol. 275 Pt 3:381-8.
11. Johnson VE, Stewart W, Smith DH. (2013). Axonal pathology in traumatic brain injury. Exp Neurol. 246:35-43.
12. Kumar A, Loane DJ. (2012). Neuroinflammation after traumatic brain injury: opportunities for therapeutic intervention. Brain Behav Immun. 26(8):1191-201.
13. Loan DJ, Faden AI. (2010). Neuroprotection for traumatic brain injury: translational challenges and emerging therapeutic strategies. Trends Pharmacol Sci. 31(12):596-604.
14. McIntosh TK, Smith DH, Meaney DF, Kotapka MJ, Gennarelli TA, Graham DI. (1996). Neuropathological sequelae of traumatic brain injury: relationship to neurochemical and biomechanical mechanisms. Lab Invest. 74(2):315-42.
15. Prow NA, Irani DN. (2008). The inflammatory cytokine, interleukin-1 beta, mediates loss of astroglial glutamate transport and drives excitotoxic motor neuron injury in the spinal cord during acute viral encephalomyelitis. J Neurochem. 105(4):1276-86.

16. Katz PS, Sulzer JK, Impastato RA, Teng SX, Rogers EK, Molina PE. (2015). Endocannabinoid degradation inhibition improves neurobehavioral function, blood-brain barrier integrity, and neuroinflammation following mild traumatic brain injury. J Neurotrauma. 32(5):297-306.
17. Xu JY, Chen C. (2015). Endocannabinoids in synaptic plasticity and neuroprotection. Neuroscientist. 21(2):152-68.
18. Chen X, Zhang J, Chen C. (2011). Endocannabinoid 2-arachidonoylglycerol protects neurons against β-amyloid insults. Neuroscience.178:159-68.
19. Panikashvili D, Simeonidou C, Ben-Shabat S, Hanus L, Breuer A, Mechoulam R, Shohami E. (2001). An endogenous cannabinoid (2-AG) is neuroprotective after brain injury. Nature. 413(6855):527-31.
20. Zhang J, Chen C. (2008). Endocannabinoid 2-arachidonoylglycerol protects neurons by limiting COX-2 elevation. J Biol Chem. 283(33):22601-11.
21. Cravatt BF, Giang DK, Mayfield SP, Boger DL, Lerner RA, Gilula NB. (1996). Molecular characterization of an enzyme that degrades neuromodulatory fatty-acid amides. Nature. 384(6604):83-7.
22. Dinh TP, Carpenter D, Leslie FM, Freund TF, Katona I, Sensi SL, Kathuria S, Piomelli D. (2002). Brain monoglyceride lipase participating in endocannabinoid inactivation. Proc Natl Acad Sci U S A. 99(16):10819-24.
23. Fuzik J, Gellért L, Oláh G, Herédi J, Kocsis K, Knapp L, Nagy D, Kincses ZT, Kis Z, Farkas T, Toldi J. (2013). Fundamental interstrain differences in cortical activity between Wistar and Sprague-Dawley rats during global ischemia. Neuroscience. 228:371-81.

24. Teng SX, Molina PE. (2014). Acute alcohol intoxication prolongs neuroinflammation without exacerbating neurobehavioral dysfunction following mild traumatic brain injury. J Neurotrauma. 31(4):378-86.

25. Mayeux JP, Teng SX, Katz PS, Gilpin NW, Molina PE. (2015). Traumatic brain injury induces neuroinflammation and neuronal degeneration that is associated with escalated alcohol self-administration in rats. Behav Brain Res. 279:22-30.

26. Edwards S, Baynes BB, Carmichael CY, Zamora-Martinez ER, Barrus M, Koob GF, Gilpin NW. (2013). Traumatic stress reactivity promotes excessive alcohol drinking and alters the balance of prefrontal cortex-amygdala activity. Transl Psychiatry. 3:e296.

27. Zhang J, Yang Y, Li H, Cao J, Xu L. (2005). Amplitude/frequency of spontaneous mEPSC correlates to the degree of long-term depression in the CA1 region of the hippocampal slice. Brain Res. 1050(1-2):110-7.

28. Kimura A, Pavlides C. (2000). Long-term potentiation/depotentiation are accompanied by complex changes in spontaneous unit activity in the hippocampus. J Neurophysiol. 84(4):1894-906.

29. Oliet SH, Malenka RC, Nicoll RA. (1996). Bidirectional control of quantal size by synaptic activity in the hippocampus. Science. 271(5253):1294-7.

30. Alger BE. (2002). Retrograde signaling in the regulation of synaptic transmission: focus on endocannabinoids. Prog Neurobiol. 68(4):247-86. Review.

31. Kano M, Ohno-Shosaku T, Hashimotodani Y, Uchigashima M, Watanabe M. (2009). Endocannabinoid-mediated control of synaptic transmission. Physiol Rev. 89(1):309-80.

32. Wilson RI, Nicoll RA. (2002). Endocannabinoid signaling in the brain. Science. 296(5568):678-82.

33. Xu JY, Chen R, Zhang J, Chen C. (2010). Endocannabinoids differentially modulate synaptic plasticity in rat hippocampal CA1 pyramidal neurons. PLoS One. 5(4):e10306.

34. Bisogno T, Di Marzo V. (2010). Cannabinoid receptors and endocannabinoids: role in neuroinflammatory and neurodegenerative disorders. CNS Neurol Disord Drug Targets. 9(5):564-73. Review.

35. Du H, Chen X, Zhang J, Chen C. (2011). Inhibition of COX-2 expression by endocannabinoid 2-arachidonoylglycerol is mediated via PPAR-γ. Br J Pharmacol. 163(7):1533-49.

36. Eljaschewitsch E, Witting A, Mawrin C, Lee T, Schmidt PM, Wolf S, Hoertnagl H, Raine CS, Schneider-Stock R, Nitsch R, Ullrich O. (2006). The endocannabinoid anandamide protects neurons during CNS inflammation by induction of MKP-1 in microglial cells. Neuron. 49(1):67-79.

37. Sarne Y, Mechoulam R. (2005). Cannabinoids: between neuroprotection and neurotoxicity. Curr Drug Targets CNS Neurol Disord. 4(6):677-84. Review.

38. Stella N. (2009). Endocannabinoid signaling in microglial cells. Neuropharmacology. 56 Suppl 1:244-53.

39. van der Stelt M, Di Marzo V. (2005). Cannabinoid receptors and their role in neuroprotection. Neuromolecular Med. 7(1-2):37-50. Review.

40. Bliss TV, Collingridge GL. (1993). A synaptic model of memory: long-term potentiation in the hippocampus. Nature. 361(6407):31-9.

41. Barnes CA. (1995). Involvement of LTP in memory: are we "searching under the street light"? Neuron. 15(4):751-4. Review.

42. Jeffery KJ. (1997). LTP and spatial learning--where to next? Hippocampus. 7(1):95-110. Review.

43. Shors TJ, Matzel LD. (1997). Long-term potentiation: what's learning got to do with it? Behav Brain Sci. 20(4):597-614; discussion 614-55.

44. Abraham WC, Mason-Parker SE, Logan B. (1996). Low-frequency stimulation does not readily cause long-term depression or depotentiation in the dentate gyrus of awake rats. Brain Res. 722(1-2):217-21.

45. Abraham WC, Bear MF. (1996). Metaplasticity: the plasticity of synaptic plasticity. Trends Neurosci. 19(4):126-30. Review.

46. Hawkins RD, Kandel ER. (1984). Is there a cell-biological alphabet for simple forms of learning? Psychol Rev. 91(3):375-91.

47. Diana MA, Marty A. (2004). Endocannabinoid-mediated short-term synaptic plasticity: depolarization-induced suppression of inhibition (DSI) and depolarization-induced suppression of excitation (DSE). Br J Pharmacol. 142(1):9-19.

48. Wilson RI, Nicoll RA. (2001). Endogenous cannabinoids mediate retrograde signalling at hippocampal synapses. Nature. 410(6828):588-92.

49. Kreitzer AC, Regehr WG. (2001). Cerebellar depolarization-induced suppression of inhibition is mediated by endogenous cannabinoids. J Neurosci. 21(20):RC174.

50. Ohno-Shosaku T, Tsubokawa H, Mizushima I, Yoneda N, Zimmer A, Kano M. (2002). Presynaptic cannabinoid sensitivity is a major determinant of depolarization-

induced retrograde suppression at hippocampal synapses. J Neurosci. 22(10):3864-72.

51. Panikashvili D, Shein NA, Mechoulam R, Trembovler V, Kohen R, Alexandrovich A, Shohami E. (2006). The endocannabinoid 2-AG protects the blood-brain barrier after closed head injury and inhibits mRNA expression of proinflammatory cytokines. Neurobiol Dis. 22(2):257-64.
52. Akiyama H, Barger S, Barnum S, Bradt B, Bauer J, Cole GM, Cooper NR, Eikelenboom P, Emmerling M, Fiebich BL, Finch CE, Frautschy S, Griffin WS, Hampel H, Hull M, Landreth G, Lue L, Mrak R, Mackenzie IR, McGeer PL, O'Banion MK, Pachter J, Pasinetti G, Plata-Salaman C, Rogers J, Rydel R, Shen Y, Streit W, Strohmeyer R, Tooyoma I, Van Muiswinkel FL, Veerhuis R, Walker D, Webster S, Wegrzyniak B, Wenk G, Wyss-Coray T. (2000). Inflammation and Alzheimer's disease. Neurobiol Aging. 21(3):383-421. Review.
53. Yoshiyama Y, Higuchi M, Zhang B, Huang SM, Iwata N, Saido TC, Maeda J, Suhara T, Trojanowski JQ, Lee VM. (2007). Synapse loss and microglial activation precede tangles in a P301S tauopathy mouse model. Neuron. 53(3):337-51.
54. Eikelenboom P, van Exel E, Hoozemans JJ, Veerhuis R, Rozemuller AJ, van Gool WA. (2010). Neuroinflammation - an early event in both the history and pathogenesis of Alzheimer's disease. Neurodegener Dis. 7(1-3):38-41.
55. Brettschneider J, Libon DJ, Toledo JB, Xie SX, McCluskey L, Elman L, Geser F, Lee VM, Grossman M, Trojanowski JQ. (2012). Microglial activation and TDP-43 pathology correlate with executive dysfunction in amyotrophic lateral sclerosis. Acta Neuropathol. 123(3):395-407.

56. Adams JH, Graham DI, Murray LS, Scott G. (1982). Diffuse axonal injury due to nonmissile head injury in humans: an analysis of 45 cases. Ann Neurol. 12(6):557-63.

57. Geddes JF, Vowles GH, Beer TW, Ellison DW. (1997). The diagnosis of diffuse axonal injury: implications for forensic practice. Neuropathol Appl Neurobiol. 23(4):339-47.

58. Smith DH, Chen XH, Nonaka M, Trojanowski JQ, Lee VM, Saatman KE, Leoni MJ, Xu BN, Wolf JA, Meaney DF. (1999). Accumulation of amyloid beta and tau and the formation of neurofilament inclusions following diffuse brain injury in the pig. J Neuropathol Exp Neurol. 58(9):982-92.

59. Stone JR, Okonkwo DO, Singleton RH, Mutlu LK, Helm GA, Povlishock JT. (2002). Caspase-3-mediated cleavage of amyloid precursor protein and formation of amyloid Beta peptide in traumatic axonal injury. J Neurotrauma. 19(5):601-14.

60. Johnson VE, Stewart W, Smith DH. (2010). Traumatic brain injury and amyloid-β pathology: a link to Alzheimer's disease? Nat Rev Neurosci. 11(5):361-70.

61. Tran HT, LaFerla FM, Holtzman DM, Brody DL. (2011). Controlled cortical impact traumatic brain injury in 3xTg-AD mice causes acute intra-axonal amyloid-β accumulation and independently accelerates the development of tau abnormalities. J Neurosci. 31(26):9513-25.

62. Long JZ, Li W, Booker L, Burston JJ, Kinsey SG, Schlosburg JE, Pavón FJ, Serrano AM, Selley DE, Parsons LH, Lichtman AH, Cravatt BF. (2009). Selective blockade of 2-arachidonoylglycerol hydrolysis produces cannabinoid behavioral effects. Nat Chem Biol. 2009 Jan;5(1):37-44.

Figure legends

Figure 1. Neurobehavioral Scores (NBS) and Neurological Severity Scores (NSS) during the first two weeks post-TBI. Higher scores indicate greater motor impairment**.** (A) NBS scores are attenuated with JZL treatment 7 days following TBI, and (B) NSS scores are attenuated with JZL treatment 14 days following TBI. *P<0.05 compared to Sham, $P<0.05 compared to TBI/VEH.

Figure 2.Gray matter (cortex) and white matter (corpus callosum) astrocyte activation following TBI. Ipsilateral (A-C) and contralateral (D-F) cortex (gray matter) astrocyte activation. Ipsilateral (G-I) and contralateral (J-L) corpus callosum (white matter) astrocyte activation. Representative images (A-L) are counterstained with DAPI. Representative images (M-N) show JZL treatment reduces gray and white matter astrocyte activation following TBI. Quantification of IHC represented as area fraction % (O-R) where *P<0.05 compared to Sham, $P<0.05 compared to TBI/VEH. Terms: i.c. – ipsilateral cortex. i.c.c. – ipsilateral corpus callosum. Scale bars (white) are equal to 100μm.

Figure 3. Phosphoprotein expression changes in the ipsilateral cortex (site of injury). (A) Expression of pGluR1 S845 is attenuated in JZL-treated animals compared to TBI/VEH animals, and (B) post-TBI ERK 1/2 phosphorylation is attenuated with JZL treatment. *P<0.05 compared to Sham, $P<0.05 compared to TBI/VEH.

Figure 4. Mini excitatory post-synaptic currents (mEPSCs) recorded from rat cortex (site of injury) via brain slice electrophysiology 10 days post-injury. A

downward deflection is a depolarizing current. (A) Representative mEPSC and (B) representative trace recording. JZL treatment significantly attenuated mEPSC frequency (C) and amplitude (D) compared to TBI-vehicle animals. *P<0.05 compared to Sham; $p<0.05 compared to TBI/VEH.

Figure 1

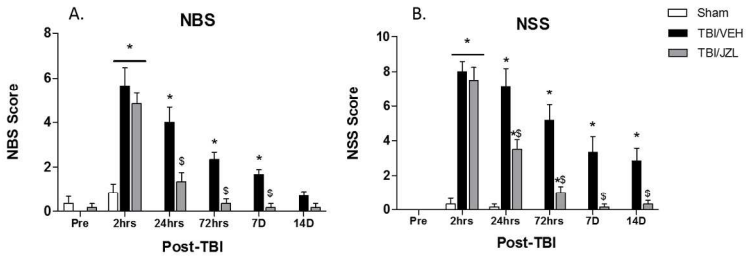


Figure 1. Neurobehavioral Scores (NBS) and Neurological Severity Scores (NSS) during the first two weeks post-TBI. Higher scores indicate greater motor impairment. (A) NBS scores are attenuated with JZL treatment 7 days following TBI, and (B) NSS scores are attenuated with JZL treatment 14 days following TBI. *P<0.05 compared to Sham, $P<0.05 compared to TBI/VEH.

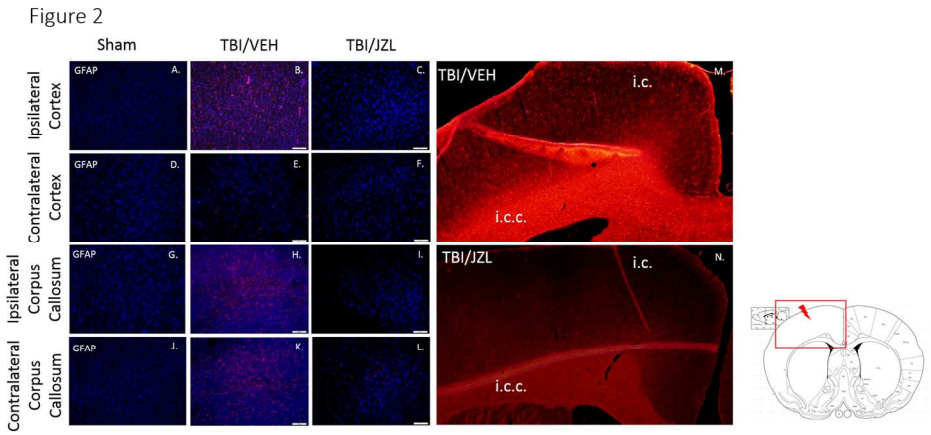


Figure 2.Gray matter (cortex) and white matter (corpus callosum) astrocyte activation following TBI. Ipsilateral (A-C) and contralateral (D-F) cortex (gray matter) astrocyte activation. Ipsilateral (G-I) and contralateral (J-L) corpus callosum (white matter) astrocyte activation. Representative images (A-L) are counterstained with DAPI. Representative images (M-N) show JZL treatment reduces gray and white matter astrocyte activation following TBI. Quantification of IHC represented as area fraction % (O-R) where *P<0.05 compared to Sham, $P<0.05 compared to TBI/VEH. Terms: i.c. – ipsilateral cortex. i.c.c. – ipsilateral corpus callosum. Scale bars (white) are equal to 100μm.

Figure 2

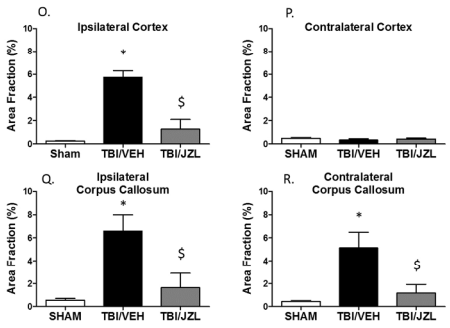


Figure 2.Gray matter (cortex) and white matter (corpus callosum) astrocyte activation following TBI. Ipsilateral (A-C) and contralateral (D-F) cortex (gray matter) astrocyte activation. Ipsilateral (G-I) and contralateral (J-L) corpus callosum (white matter) astrocyte activation. Representative images (A-L) are counterstained with DAPI. Representative images (M-N) show JZL treatment reduces gray and white matter astrocyte activation following TBI. Quantification of IHC represented as area fraction % (O-R) where *P<0.05 compared to Sham, $P<0.05 compared to TBI/VEH. Terms: i.c. – ipsilateral cortex. i.c.c. – ipsilateral corpus callosum. Scale bars (white) are equal to 100μm.

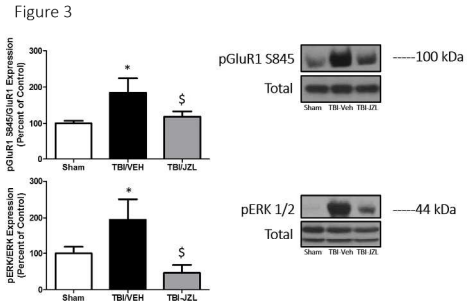


Figure 3. Phosphoprotein expression changes in the ipsilateral cortex (site of injury). (A) Expression of pGluR1 S845 is attenuated in JZL-treated animals compared to TBI/VEH animals, and (B) post-TBI ERK 1/2 phosphorylation is attenuated with JZL treatment. *P<0.05 compared to Sham, $P<0.05 compared to TBI/VEH.

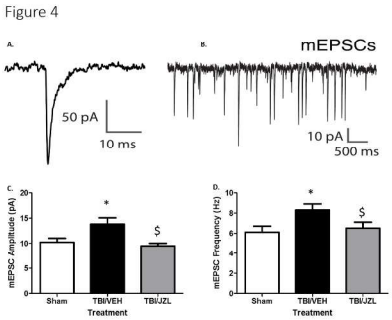


Figure 4. Mini excitatory post-synaptic currents (mEPSCs) recorded from rat cortex (site of injury) via brain slice electrophysiology 10 days post-injury. A downward deflection is a depolarizing current. (A) Representative mEPSC and (B) representative trace recording. JZL treatment significantly attenuated mEPSC frequency (C) and amplitude (D) compared to TBI-vehicle animals. *P<0.05 compared to Sham; $p<0.05 compared to TBI/VEH.