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PRINCIPAL INVESTIGATOR: J. Regino Perez-Polo, Ph.D.

CONTRACTING ORGANIZATION: University of Texas Medical Branch

Galveston, TX 77555-1072

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J. Regino Perez-Polo, Ph.D.
Email: regino.perez-polo@utmb.edu

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14. ABSTRACT Brain injury, particularly mild "blast type" injuries due to improvised exploding devices are difficult to detect clinically, although there are long term cognitive and behavioral deficits. Key inflammatory cytokines are unregulated after traumatic insults that play a role in the development of long-term deficits. Early events are increased IL-1 and TNFalpha cytokine levels, that contribute to cell death and inflammation, and microglial and astrocytic activation, mediators dysfunction via persistent inflammation. Our central hypothesis is that blocking inflammatory cytokine signaling after mild traumatic brain injury (MTBI) will improve outcomes by ameliorating inflammation and the resultant neuronal dysfunction. Our goal is to develop, implement and assess interventions to ameliorate the long term neurological deficits caused by MTBI by ameliorating injury-induced brain inflammation. We used lateral fluid percussion model of MTBI in rat. The results are that we have an appropriate injury that provides detectable damage as early as 6h after injury. We showed by immunohistochemistry that TBI induced significant astrocytic and microglial activation and increased IL-1α and TNFα in brain 6 hours and 18 days. We saw corresponding behavioral deficits based on beam balance and footfall assays. Thus, we have biomarkers to assess therapeutic strategies designed to minimize outcomes associated with MTBI.

15. SUBJECT TERMS
Blast head injury; mild head injury; anti-inflammatory therapy; cytokine receptors; IL-1; TNFα

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Introduction

Brain injury, particularly mild “blast type” injuries due to improvised exploding devices are difficult to detect clinically, but there may be long term consequences resulting in cognitive and behavioral deficits. Key inflammatory cytokines are unregulated after traumatic insults, and may play a role in the development of long-term deficits. There is an increase in brain cytokines and chemokines after CNS trauma that triggers cellular infiltration, neuronal losses, and persistent inflammation. Early events are increased IL-1 and TNFalpha cytokine levels known to contribute to cell death and inflammation, and microglial and astrocytic activation, mediators of behavioral dysfunction. Our central hypothesis is that **blocking inflammatory cytokine signaling after mild traumatic brain injury (MTBI) will improve outcomes by ameliorating inflammation and therefore neuronal dysfunction.** Our goal is to develop, characterize and assess interventions that will ameliorate the long term neurological deficits following MTBI by ameliorating injury-induced acute inflammation in the brain. Our overall work plan is to develop and implement an evaluation and validating scheme for assessment of two treatment modalities for mild head injury in rodents. Assessments are based on increased expression of proteins known to play significant roles in inflammation in specific cell types identified by using well established cell phenotype biomarkers and behavioral outcomes relevant to the clinical outcomes of interest. Thus, we will measure brain cytokine, cell death and inflammation levels after blocking receptors for key inflammatory cytokines (Kineret for IL-1 receptor and Etanercept for TNF α,β receptors) after MTBI. These interventions are FDA-approved, have few adverse effects, and could rapidly be moved into clinical testing if successful in the laboratory.

Body of the Report

Over the initial part of the project year, we hired and trained our scientific staff and established the first of the two rat models to be used. We adapted our UTMB Institutional Animal Care and Use Committee (IACUC) protocols to those required by the U. S. Army successfully so as to be in full compliance with both UTMB and DOD requirements. The discrepancies between the two were minor and consisted in the reliance on different data bases and the extent of detail on the administration of intervention modalities.

We recruited to the project Margaret A. Parsley to carry out the Mild Traumatic Brain Injuries (MTBIs) under the supervision of Doug DeWitt (Blast TBI and fluid percussion models) and Pramod Dash (Contusion) and Kathia Johnson who is experienced in behavioral assays in working with rodent trauma models. We also recruited Harriett Rea who is also experienced in both animal handling and the cell, molecular and biochemical assays to be carried out in the project and will carry out all immunoassays, Western blot analyses and brain dissections and together with another recruit, Geda Unabia, who is experienced in various aspects of imaging and will carry out tissue slicing and staining. As planned J. Regino Perez-Polo designs dosages and treatments and together with Claire Hulsebosch is responsible for all reports, publications, and interpretation of results with Perez-Polo focusing on the IL-1 interventions and Hulsebosch focusing on the TNF interventions (Perez-Polo and Hulsebosch have been collaborators since 1984).

We also established weekly meetings of investigators (excluding the time when the Ike storm precluded such activities) for planning and evaluation purposes with some of these meetings to include Dr. Grill, Director of the Imaging Core, to allow standardization of protocols and interpretation of results across projects. We also held a discussion with the Neuroprotection and the Model of Injury Working Groups to assess common end points to all projects for the three different models to be assessed. Finally, in October and early November, once the UTMB campus became accessible to researchers, we assessed damage caused by Ike and completed all post-Ike recovery efforts affecting the individual laboratories involved. Given that we were not able to order animals until much later we carried out the initial experiments in Houston in the laboratory of Dr. Dash, one of our Co-Investigators.

In these first experiments, we carried out contusion injury, varying the degree of injury (depth) and the time of sacrifice. The results suggest that while we have an appropriate degree of injury that provides detectable damage as early as 24h after injury, which is just above the threshold for no observable damage (**Figure 1**), there is a large amount of interanimal variability. We used 350-400g Sprague Dawley rats with the following Trauma Brain Injury parameters. Rats were anesthetized with the introduction of 5% isoflurane and maintained at 2.5% for the purpose of the experiment. All rats received a unilateral contusion injury. Injury parameters:

velocity= 4.5m/s; Depth= 2.0 or 2.7 mm; Dwell= 150; Angle= 10 degree. Rats were allowed to recover for 24 and 72 hour survival periods. Injuries were evaluated by TTC staining where animals were euthanized with 150mg/kg of Nembutal I.P. and perfused transcardially with 0.9% saline 200ml. The brains were removed and immediately frozen at -20 degree centigrade for 20 min. and then sectioned at 2mm using a rotary hand microtome. All brain tissue was stained with TTC, in order to distinguish viable verses dead tissues after injury. Brains sections were incubated in 2% TTC in Dulbecco's Phosphate buffered saline for 8 min. in the dark at 37degree centigrade. All sections were then fixed in buffered 4% paraformaldehyde pH 7.4 and refrigerated.

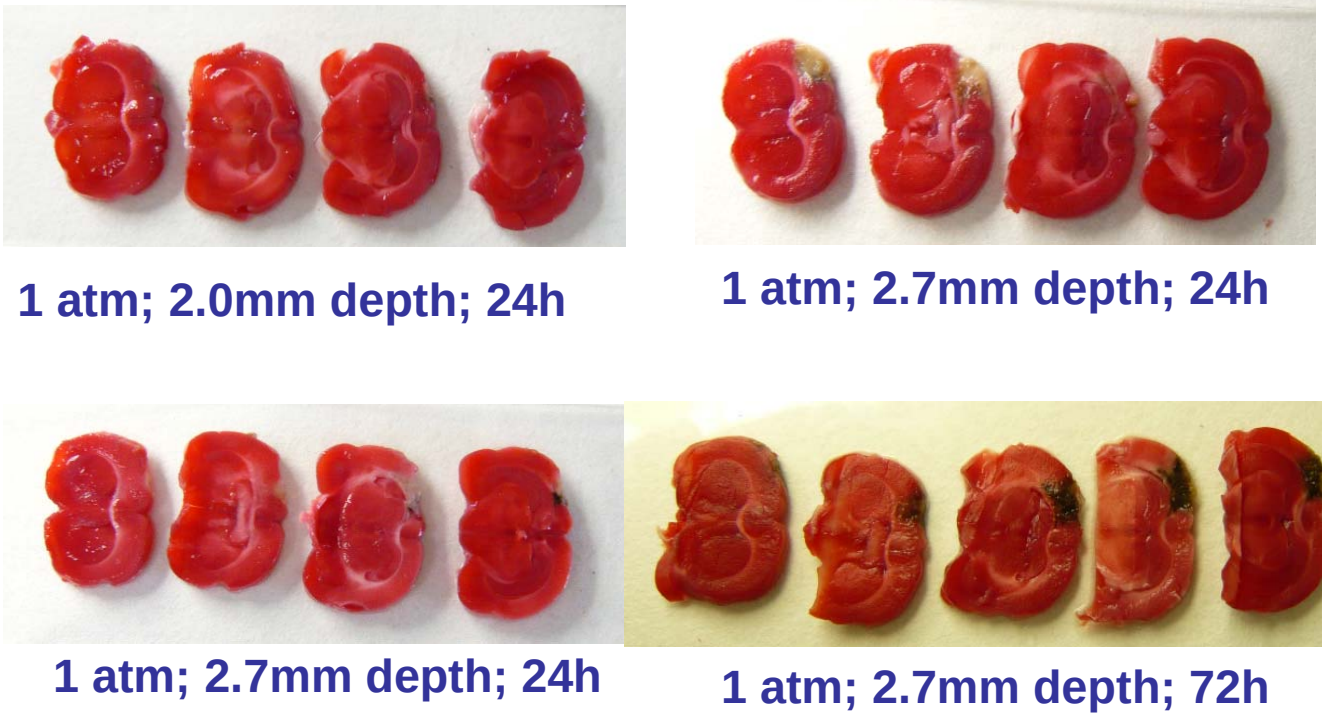


Figure 1. Histochemical analysis of extent of injury in brains exposed to contusion TBI.

As can be seen, the 2.0 mm depth did not yield any detectable injury by TTC at 24h while the 2.7mm depth yielded more significant injury at 24h. There was also an increase in damaged tissue at 72h compared to 24h. However, there was significant variability in the amount of damage at 72h (data not shown). We have found that to be typical for most mild injuries and to be indicative of edema development.

In order to minimize variability without large increases in animal numbers that would delay project progress, we adopted as our first rat model to be assessed for mild brain injury the lateral fluid percussion model (LFPM).

We chose this model as an initial attempt because and, most important, in addition to being well established, one of our Co-PIs, Dr. DeWitt, is an expert in the field, one can use the contralateral side of the cortex as a control for the injury taking place in the ipsilateral cortex. This provides a more sensitive model, a necessary prerequisite at this stage in our study, as it minimizes inter-animal variations.

We performed lateral fluid percussion injury at a level to insure significant signaling of the two key molecules involved in the TBI-induced inflammatory response that are our targets for intervention: IL-1 and TNF-α. Since IL-1 is the earliest inflammatory signal and TNF-α is a later one with more chronic consequences, we chose an intermediate sampling time of six hours after injury for sacrifice based on our experience with CNS trauma in rodent models (Gill et al., 2008a:b; Hu et al., 2005; Rafati et al., 2007), varying the degree of injury and the time of sacrifice. The results suggest that we have an appropriate degree of injury that provides detectable damage as early as 6 hours after injury that is just above the threshold for no observable damage.

We applied the lateral fluid percussion injury model (Dewitt et al., 1997; Dixon et al., 1987). We carried out lateral fluid percussion injury at 1, 2 and 2.4 atmospheres, all levels that insure significant signaling of two key

molecules involved in the TBI-induced inflammatory response that are our targets for intervention: IL-1 and TNF-α. Male Sprague-Dawley rats weighing 350 to 400 g were anesthetized with isoflurane in an anesthetic chamber, intubated, and mechanically ventilated with 1.5-2.0% isoflurane in O2:room air (70:30) using a volume ventilator (EDCO Scientific, Chapel Hill, NC). Rats were prepared for mild lateral fluid-percussion injury as previously described (Dewitt et al., 1997; Dixon et al., 1987). Briefly, rats were placed in a stereotaxic frame and the scalp was sagittally incised. A 4.0 mm diameter hole was trephined into the skull 2.0 mm to the right of the sagittal suture and midway between lambda and bregma. A modified Luerlok syringe hub was placed over the exposed dura, bonded in place with cyanoacrylic adhesive and covered with dental acrylic. Isoflurane was discontinued; the rats were connected to the trauma device and subjected to mild (1.0 atm), moderate (2.0 atm) or severe (2.4atm) fluid-percussion TBI, immediately after the loss of a withdrawal reflex to paw pinch. After TBI or sham injury, rats were disconnected from the fluid percussion device and righting reflex was assessed every 60 seconds until a normal righting reflex was observed. Rats were then placed on 2% isoflurane, wound sites were infused with bupivicaine and sutured with prolene. Isoflurane was discontinued and the rats were extubated and allowed to recover in a warm, humidified incubator.

Rats were allowed to recover for at least 6 hour survival periods, and often longer periods depending on the experiment, prior to sacrifice with 150mg/kg of Nembutal I.P. and perfused transcardially with 4% paraformaldehyde unless earmarked for behavioral assays in which sacrifice was carried out no later than 24 hours after the behavioral assay. The brains were removed, post fixed overnight in 4% paraformaldyde, blocked into saggital sections and transferred to 30% sucrose until penetration was complete. The tissue was then embedded in OCT and frozen at -20 degree centigrade. The tissue was mounted and then sectioned at 50 µm using a sliding microtome. All brain tissue was stained with antibodies to GFAP, a marker for activated astrocytes, IL-1 (α and β), and TNF-α, as well as other cellular phenotypes and inflammatory markers.

After the brains were removed and immediately frozen at -20 degree centigrade for 20 min. and then sectioned at 2mm using a rotary hand microtome, all brain tissue was stained with antibodies to GFAP, a marker for activated astrocytes, IL-1 (α and β), and TNF-α, TUJ1, RECA, GFAP, OX42 using the fluorescence method. The antibodies used were 1:625 Anti-IL-1 alpha (rabbit polyclonal, Abbiotec); 1:200 Anti-IL1 alpha (mouse monoclonal, Sta. Cruz); 1:50,000 Anti-TUJ1 (mouse monoclonal, Covance); 1:200 Anti-RECA-1 (mouse monoclonal, Serotec); 1:1,000 Anti-Iba-1 (rabbit polyclonal, Wako); 1:1,000 Anti-GFAP (mouse monoclonal, Chemicon); 1:200 Anti-GFAP (rabbit polyclonal, Sigma); 1:300 Anti-OX42 (mouse monoclonal, Serotec). We cut **50**um thick sections with sliding microtome and collected sections in a glass petri dish with cold 0.05M TBS. We washed sections with 0.05M TBS---3X, 10 min. each RT (shaking) and blocked sections with 0.05M TBS/Triton + 5% NGS + 0.3% BSA---1hour RT (shaking). We incubated sections with antibodies in Tris/Triton + 0.3% BSA+ 1% NGS overnight at RT (shaking) and the following day we washed sections with 0.05M TBS ---3X, 10 min. each at RT (shaking). We incubated sections with 1:1,000 Alexa Fluor 568 (red) or 488 (green) goat Anti-rabbit IgG or goat anti-mouse IgG for 2 hours RT (shaking). We mounted sections on slide, dried for a few minutes and added regular mounting medium and let slides sit at RT for at least 20 min. then put in refrigerator for at least 24hr. before storage in freezer until viewing.

We focused on two time points after the injurious event as initially valuable in the context of what is known about the clinical picture. Injury to the brain is known to have an early component in which prompt cell death promoting signaling mechanisms are triggered that are followed by activation of signaling pathways responsible for more pervasive delayed chronic inflammation and more persistent plastic changes. Thus, we rely on 6h and 18days after mild head injury as useful time points for our biomarker based assessments, an added advantage of the 18 day time point is that it takes place afer all proposed behavioral outcome tests are performed.

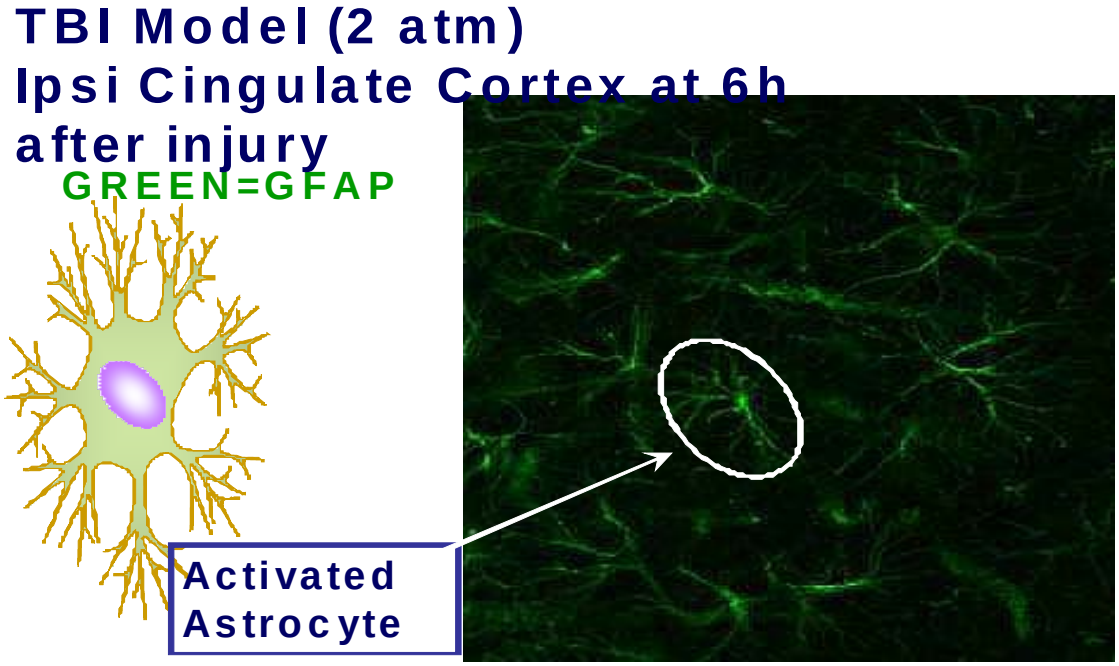
Our initial assessments confirmed that the mild TBI induced significant astrocytic activation in a number of brain regions as shown using the marker for activated astrocytes GFAP. Here we show a representative image from cingulated cortex ipsilateral to the injury site. **Figure 2** shows the classical star-like configuration of astrocytes responding to injury that in part defines the inflammatory cascade induced by TBI, a well contributor to later impairment and pathology. We observed similar levels of activation in thalamus, hippocampus and other cortical structures.

We also used the biomarker for astrocytes, nestin and vimentin, and as can also be seen in **Figure 2.** The TBI induced significant increases in nestin and vimentin labeling of activated astrocytes, providing a good

biomarker for our intervention evaluations. Here we show again a representative image from cingulated cortex ipsilateral to the injury site. **Figure** 2 shows the classical star-like configuration of astrocytes responding to injury that in part defines the inflammatory cascade induced by TBI, a well known contributor to later impairment and pathology. We observed similar levels of activation in thalamus, hippocampus and other cortical structures.

Figure 2. Demonstration of (A) astrocytic inflammatory activation 6 hours after TBI as shown by GFAP ; (B) nestin and (C, next page) vimentin staining (400X).

(A)



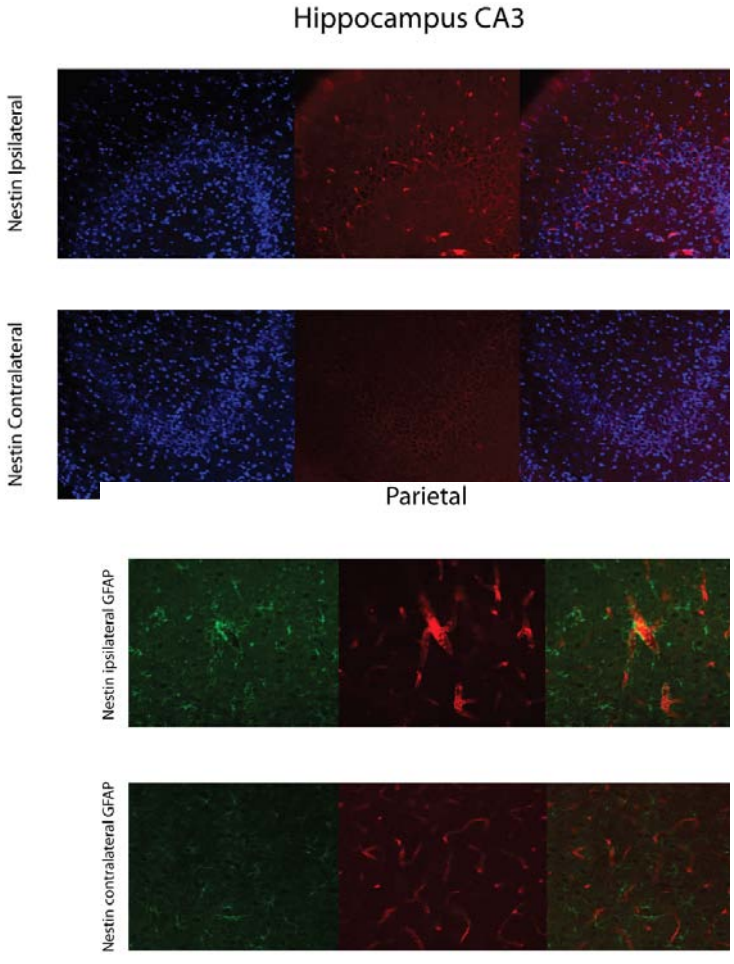
(B)

Demonstration of increased Nestin associated with activated Astrocytes at 6h after 1atm TBI

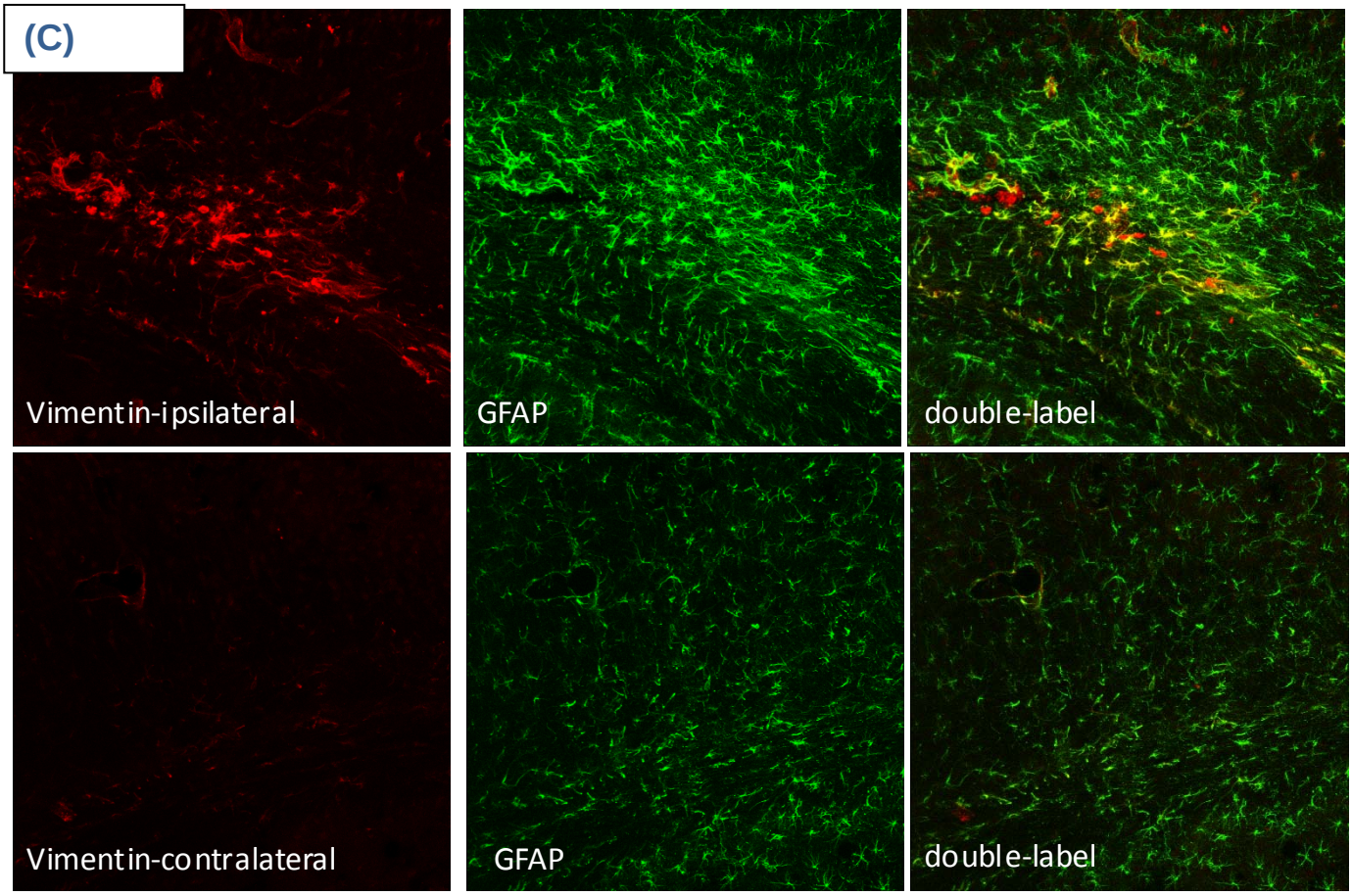
RED is Nestin

Blue is nuclear marker

Green is GFAP



Vimentin, like GFAP, appears upregulated in astrocytes as early as 6 hours post-FPI



While we were able to observe significant damage to the ipsilateral injury site over time, we were not able to detect apoptotic-specific cell death based on an immunoassay for endonuclease directed cleavage of DNA, consistent with findings suggesting that while apoptosis may play a role in more intense injuries likely to involve ischemia, MTBI is likely to involve longer term persistent inflammatory responses.

We then carried out staining in the same brain areas with antibodies to IL-1 (α and β) and TNF-α as well as with GFAP using different filters to be able to show colocalization of the labeled cytokines in situ. In **Figures 3-5 (next page)**, we show representative images from the same brain area where we showed astrocytic activation in the cingulate cortex.

While the presence of glial TNF-α is very significant, it is equally interesting and novel that it is the IL-1α and not the IL-1β that is being synthesized by the activated astrocytes. This is consistent with other reports by us showing that IL-1α has a significant presence after ischemic insults in the neonatal brain using a mild model of hypoxia ischemia (Fabian et al., 2007; Hu et al., 2005; Qiu et al., 2004). These results were consistent in all brain regions that were looked at. We now need to quantitate these results with tissue immunoassays as well as do a more thorough screen of other cyokines and chemokines over time in a milder TBI model that better mimics the clinical situation. Thus, our initial results confirm that we can assess localized inflammatory responses by measuring astrocytic glial activation and the appearance of glial IL-1 (α and β) in selected regions of the brain.

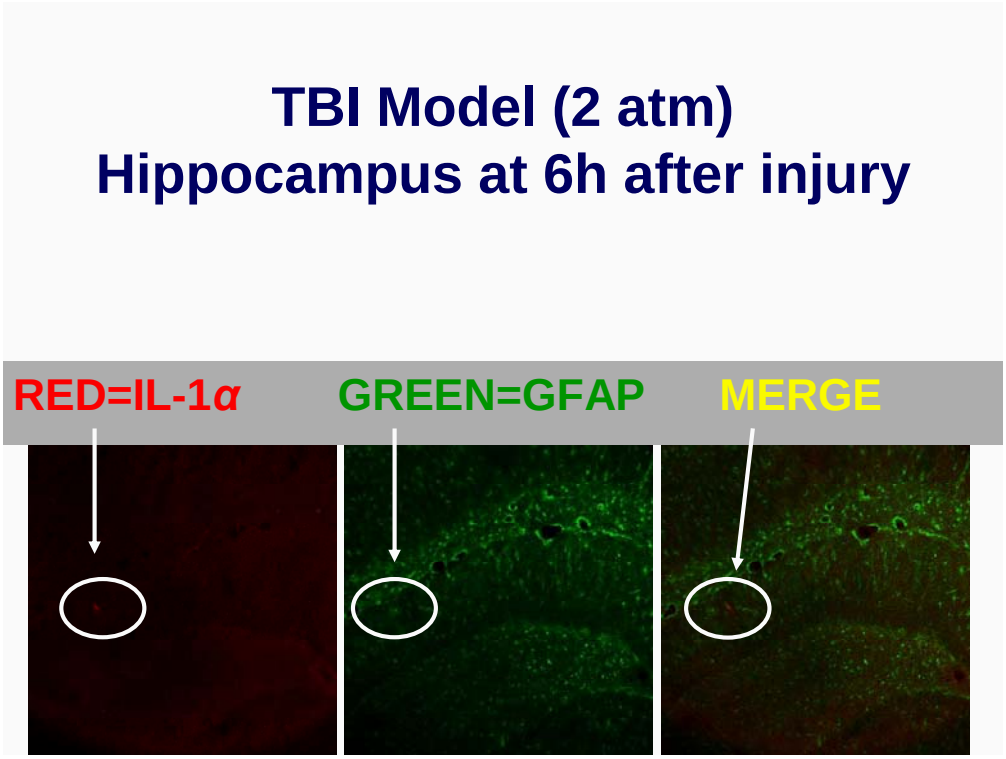


Figure 3. Demonstration of activated astrocyte production of IL-1α 6 hours after TBI.

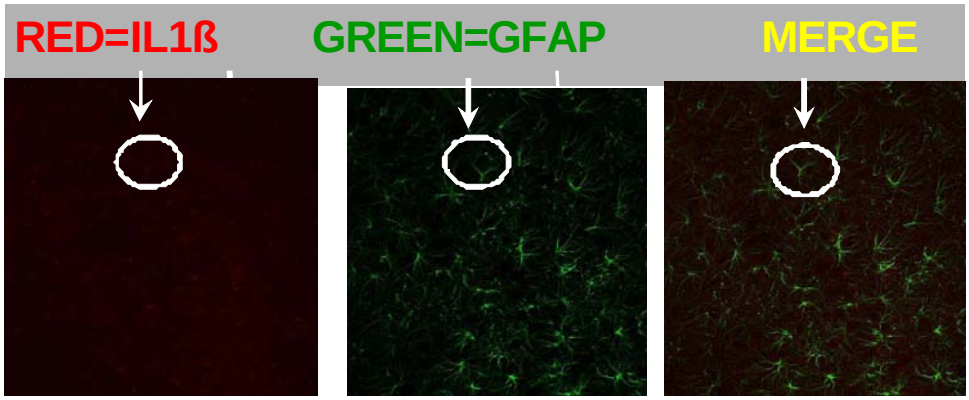


Figure 4. Demonstration of activated astrocyte production of IL-1β 6 hours after TBI.

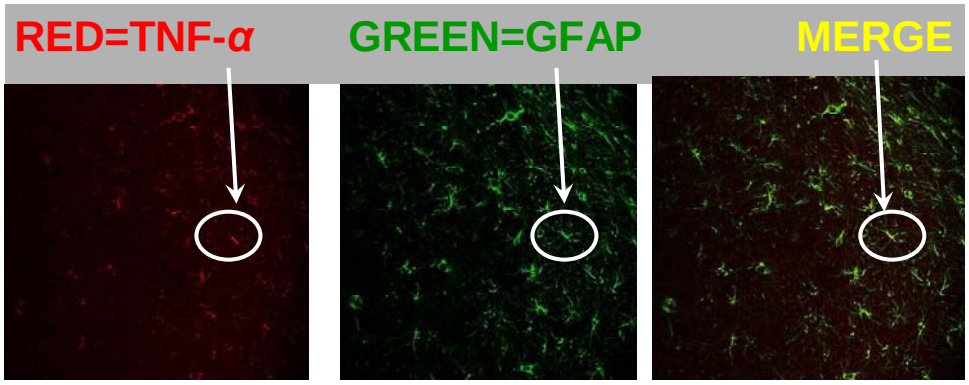
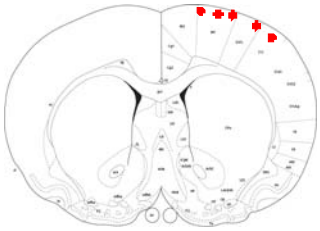
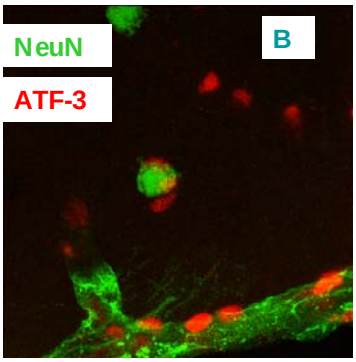


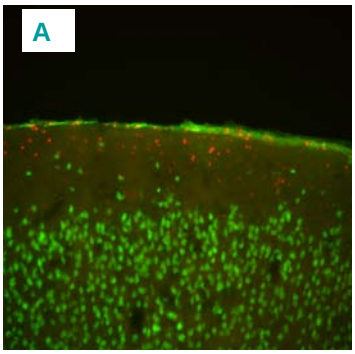
Figure 5. Demonstration of activated astrocyte production of TNFα 6 hours after TBI.

Another biomarker of inflammation after TBI being used by several of the individual projects in the consortium and carried out uniformly by the Core is ATF-3 (**Figure 6**). To date ATF-3 labeling does NOT appear to be neuronal or oligodendroglial in the cortex since there is no colabeling there (**Figure 6A and 6B**) of NeuN or RIP (not shown). There is ATF-3 labeling in neurons of the hippocampus and ventricles. There is no ATF-3 labeling in the thalamic regions. ATF-3 expression is detected in and around hypothalamus where it co-localizes with NeuN. It is remains to be determined with certainty the full range of population of cells expressing ATF-3 after TBI but it is now a reliable marker of mild injury along with nestin and the inflammatory cytokines IL-1 and TNF-α .

Figure 6







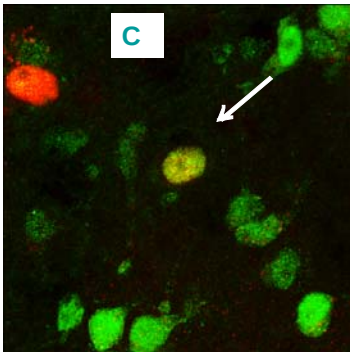
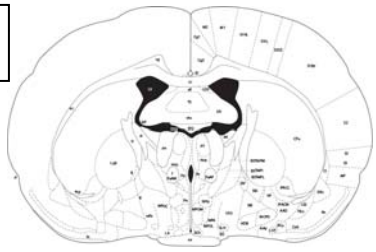
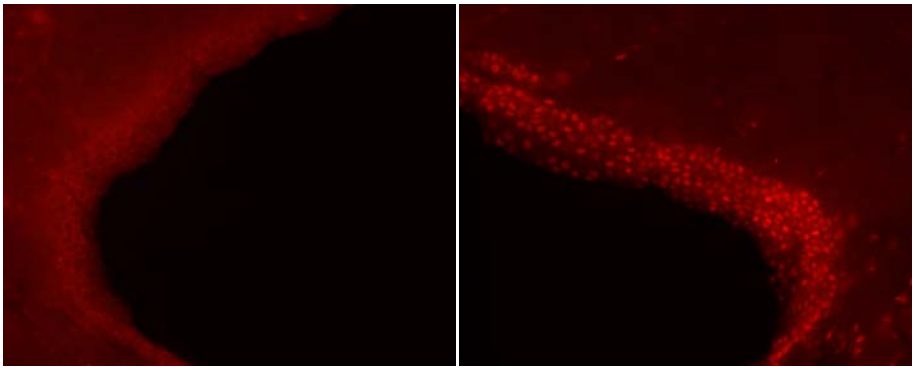


Figure 6. (A - C) ATF-3 (RED) is expressed in some, but not all, forebrain cortex neurons (GREEN) 6 hours after mild lateral fluid percussion injury; **(D)** ATF expression is also increased significantly in ventricles 6 hours after mild lateral fluid percussion injury.

Figure 6 D.





contralateral ipsilateral

An important marker of plasticity is the BDNF neurotrophin. Neurotrophins have been known to play a role in various aspects of immune function at the borders of the neuroimmune axis as well as, more relevant to this study, neuronal plasticity associated with behavior (Dietz et al., 2009; Linker et al., 2009). Thus, we are both excited and intrigued by our finding of BDNF labeling of microglia in the ipsilateral cortex of animals experiencing TBI after 6 hours (**Figure 7**) given our previous demonstration of activation of astrocytes after TBI.

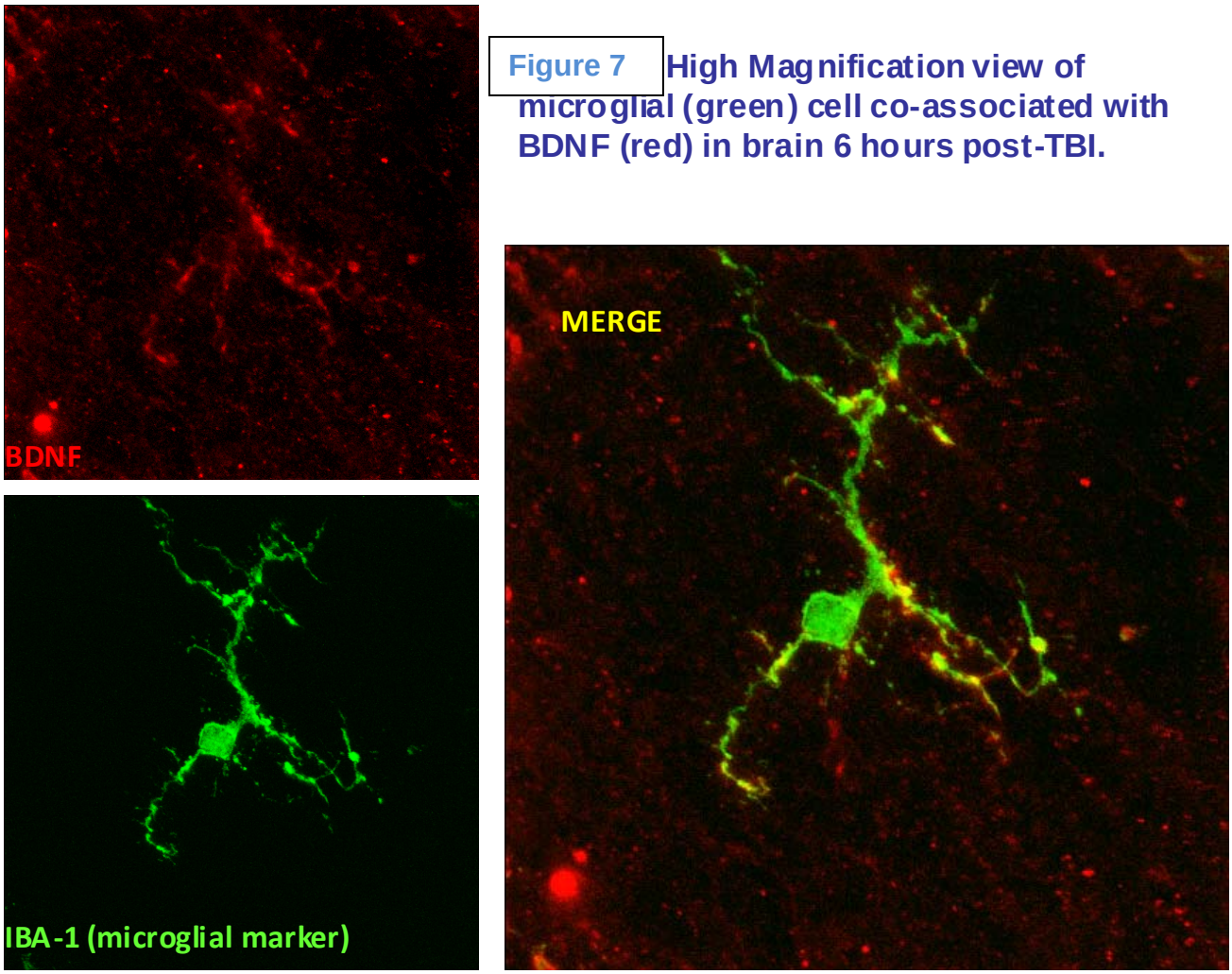


Figure 7 **High Magnification view of microglial (green) cell co-associated with BDNF (red) in brain 6 hours post-TBI.**

Given the clinical indication of a chronic outcome to MTBI, we pursued the hypothesis that inflammatory markers would be present at a late post injury time: 18 days. To our surprise we observed an increased cortical presence of GFAP and IL-1α, both indicative of ongoing astrocytic activation and stimulation of the IL-1 inflammatory pathway, a key element of our therapeutic strategy (**Figure 8 and 9**). A similar assessment for TNFα is underway.

A key element of validation was to perform the same pathological measures at our laboratories at UTMB as well as in the Core facilty in Houston supervised by Dr. Raymond Grill who would compare these outcomes to those he is performing for other components of the consortium. This cross validation process has allowed for comparisons to be made across the diferrent projects and thus provide a level of reliability and reproducibility necessary for the extension of these studies into the clinical setting. Our 18 day data is novel and provides a good biomarker assessment to correlate with behavioral assessments performed, all of which can be concluded by day 18 post-injury.

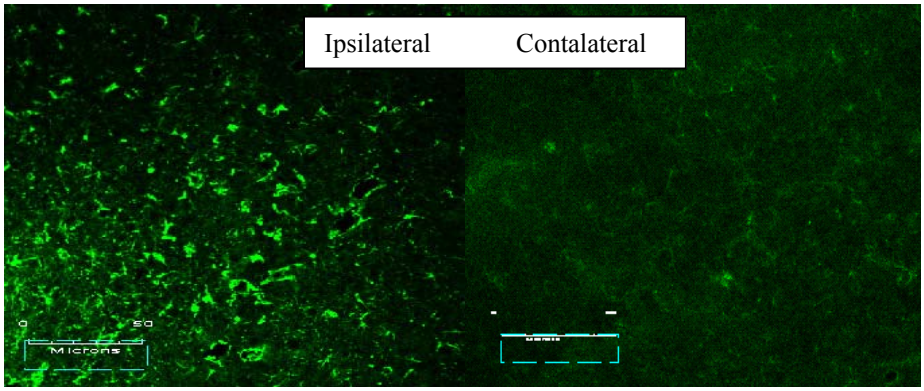


Figure 8. GFAP expression (GREEN) in insular cortex 18 days after mild lateral fluid percusssion injury. (200X).

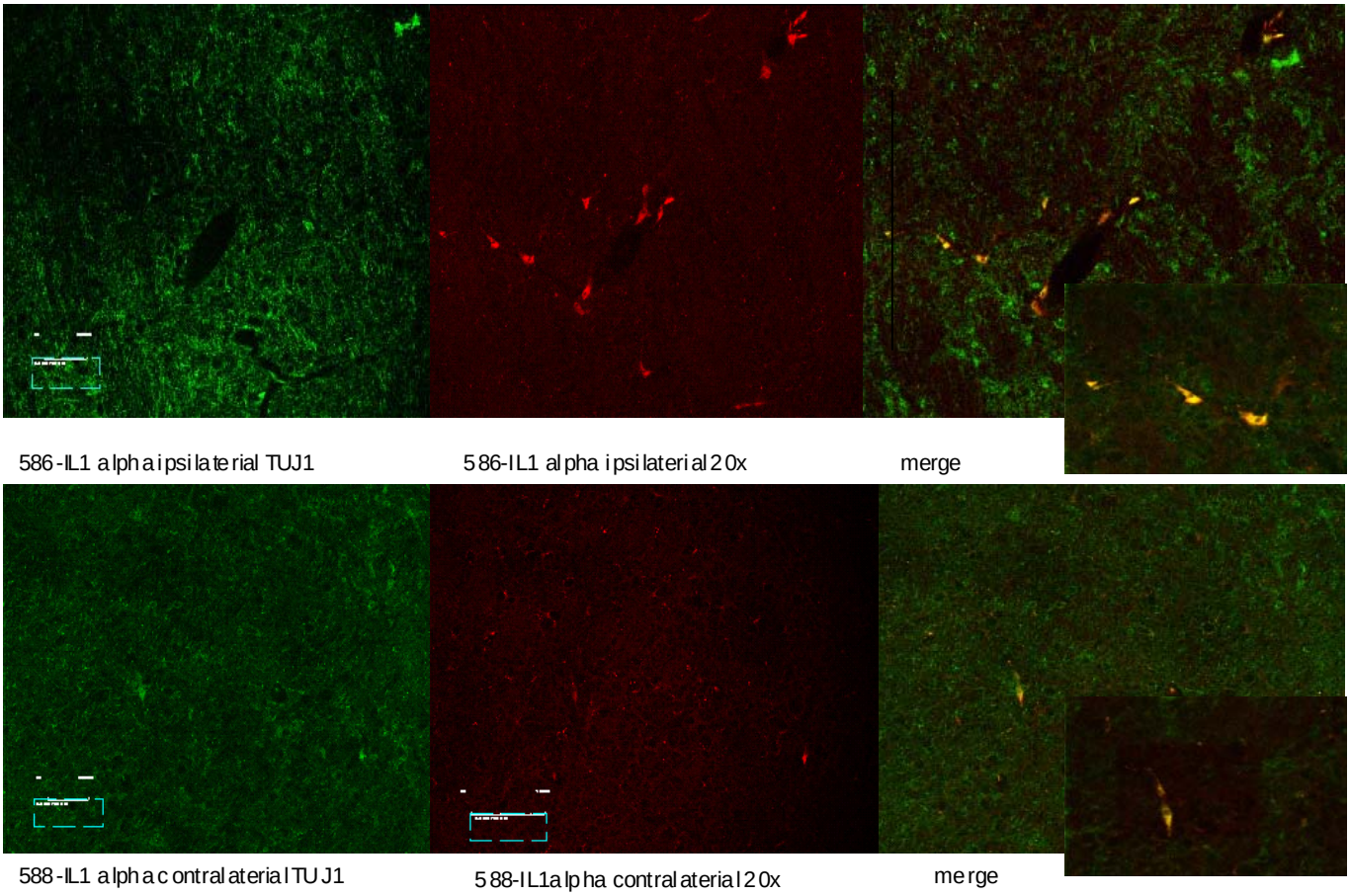


Figure 9. Demonstration of increased IL-1α protein (RED) levels in injured ipsilateral cortical neurons (GREEN) 18 days after 1.0 atmospheres mild lateral fluid percussion injury. (200X)

We also began to evaluate a number of behavioral outcomes using the same bimodal strategy we have applied throughout. Thus, we assessed early recovery processes such as righting reflex time, beam balance and foot faults, all known reliable assays that provide an accepted and fairly global index of behavioral recovery based on coordinated locomotor function. For example we have used the Beam Balance Assay. The balance beam apparatus consists of a beam 91.5cm L x 1.7cm W and elevated 30cm off the surface below and secured to a platform on either end. The rat is placed on the center of the beam and released (start time) and is allowed to walk to either end. The animal is returned to the center of the platform if he walks onto one of the end platforms. This is continued for1 minute or until the rat falls off (stop time).

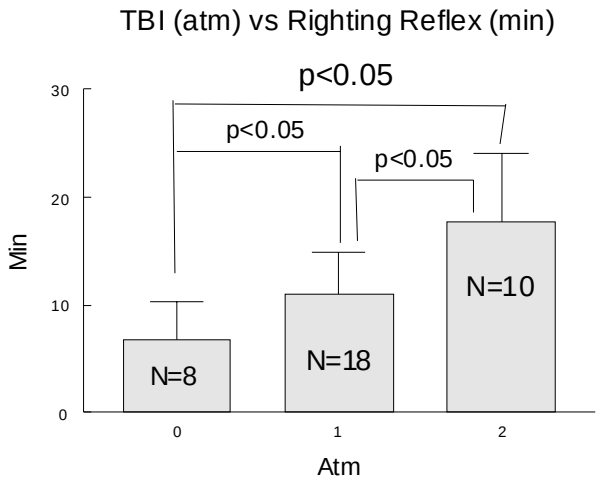


Figure 10. Effect of TBI on Righting Reflex

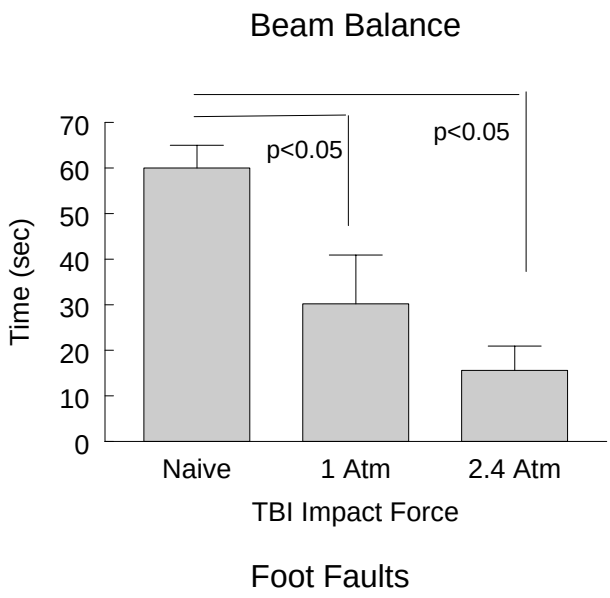


Figure 11. TBI Effect on Beam Balance Performance

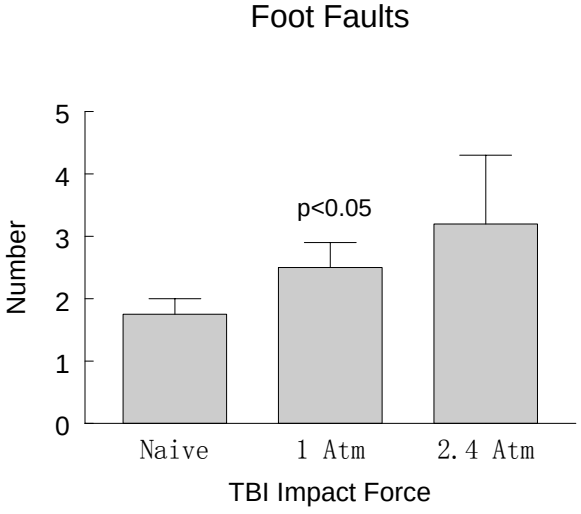


Figure 12. TBI Effect on Foot Fault Assay

In addition, we are using the Foot Fault Assay where a wire mesh 69.5 cm W x 45 cm L with 3 cm gaps is stretched out over a wooded frame. The number of times out of 10 that the forelimb or hindlimb falls through the gaps is counted and is recorder as % foot faults.

We now have our first set of behavioral outcomes after TBI (**Figures 10-12**). In each case, we have shown likely dose response behavior with respect to the severity of the injury. Although we need to test more animals to achieve sufficient power to have meaningful statistical results, it is clear that we now have behavioral measures suitable to the task. These results appear to be consistent in brain regions that were looked at. We now need to both confirm and quantitate these results.

We are presently testing more sensitive cognitive assays that will be particularly useful in assessing long term outcomes; the Barnes and Morris water maze. Not surprisingly, the more complex tasks provide more variability. Rather than simply increasing the number of animals tested to increase power and yield significance, we are taking the approach of fine tuning our cognitive assays in an effort to detect the kind of minimal perturbations that over time may prove a better fit to the clinical counterparts of our rat studies.

The Barnes Maze apparatus consists of an elevated circular table with 20 circular holes along the edge. Under each hole is a slot for a box in which the rat can escape. The rat is first placed in the slot, which will be in the same location for that individual rat for the duration of the experiment, for 30 seconds. Then the surfaces are cleaned and the rat is placed in the middle of the table and covered for 15 seconds. The rat is uncovered (start time) and allowed 4 minutes to find the drop box and put all 4 limbs inside (stop time). He is then allowed to stay in the drop box for 30 seconds. If the rat does not find and enter the drop box after 4 minutes he is then placed in it for 30 seconds. There are 2 trials for each rat with a rest period of 4 minutes between trial 1 and 2. All the surfaces are cleaned in between trials.

In the Morris Water Maxe the animals are assessed on days 11 through 15 post-injury. Four trials are run each day. The water maze is a 6' diameter tank, filled to 2 cm above the invisible platform that is 4 inches diameter. The water temperature is held at 22-24 degrees. The platform is stationary through out the experiment. The tank is divided into four quadrants and stationary cues are marked on the wall in each quadrant. Before the first trial, the rat is placed on the platform for 30 seconds. The animals' starting point is randomly chosen each day based on these quadrants, one trial is started from each quadrant. The SMART computer system is used to track and monitor the animal during the trials. After placing the animal in the water facing the wall of the tank, the handler leaves the room. The animal is allowed two minutes to find and climb the platform and escape the water (latency to platform); he must remain on the platform for 30 seconds. If the animal does not find the platform, the handler places him on it for 30 seconds before removing him from the maze. The animals are given a four-minute rest period in a warming chamber between each trial.

On a parallel track we have made great progress in our development of a true "blast" injury model, which of necessity will most likely provide both more meaningful assessments although of a more complex nature. This part of the project has been under the direct supervision of our Co-investigator Dr. Doug Dewitt. Over the next year, full validation of biomarkers for the 6h and 18day post injury will be quantified via quantitated confocal immunohistochemistry. In addition, we are likely to add an intervening time point of 24h chosen as the time known from the literature on brain trauma to be a time point in which all cellular losses occur and the loss of cognate cell death biomarkers for cell death with both apoptotic and necrotic features.

We also plan to have fully developed all of our behavioral assays. More significantly for the project, we will begin therapeutic trials as described in our proposal.

Key Research Accomplishments

• Development and implementation of appropriate acute (6 hours) and chronic (18 days) biomarkers for assessments of planned therapeutic interventions

• Demonstration of persistent glial activation and inflammation via IL-1 and TNFα at 18 days

• Demonstration of perturbation of BDNF levels after mild TBI

• Development and implementation of appropriate behavioral assays

• Demonstration of behavioral impairments after mild TBI

Reportable Outcomes

We have submitted five abstracts:

Hulsebosch CE, Johnson KM, Dewitt DS, Dash PK, Grill R, Parsley MA, Unabia G, Rea H, Perez-Polo JR. Role of IL-1 and TNF receptor activation in neurological deficits after TBI National Neurotrauma Society, 2010 Johnson KM, Dewitt DS, Dash PK, Grill R, Parsley MA, Unabia G, Rea H, Perez-Polo JR, Hulsebosch CE, Measures of Neurological Deficits after Mild Traumatic Brain Injury. Society for Neuroscience, 2009.

Hulsebosch CE, Johnson KM, Dewitt DS, Dash PK, Grill R, Parsley MA, Unabia G, Rea H, Perez-Polo JR. Role of IL-1 and TNF receptor activation in neurological deficits after TBI. Society for Neuroscience, 2009.

Perez-Polo, J, DeWitt, DS, Rea, HC, Dash PK, Grill RJ, Parsley MA, Unabia G, Hulsebosch CE. Anti-inflammatory treatment of traumatic brain injury (TBI). Society for Neuroscience, 2009.

Perez-Polo, JR; DeWitt, DS, Rea, HC, Dash PK, Grill RJ, Parsley MA, Unabia G, Hulsebosch CE.; Role of IL-1 and TNF receptor activation in neurological deficits at TBI. Military healtlh Research Forum, 2009.

Conclusion

In conclusion, we developed and implemented biomarker and behavioral assessments of mild traumatic brain injury. We have shown a persistent inflammatory response up to 18 days after injury and the involvement of the neurotrophin BDNF known for its regulatory and plasticity properties in both the nervous and immune systems.

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Appendices

None