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| 13. SUPPLEMENTARY NOTES | | | | | |
| 14. ABSTRACT The purpose of this research project is to explore anti-epileptogenic strategies in an animal model of post-traumatic epilepsy (PTE) using lateral fluid percussion injury (LFPI). Our focus is on attenuating damaging effects of hyperexcitability in the brain induced by inflammation resulting from glial cell immune responses to trauma. We are exploring two drugs, MN166 and SLC022, that are known to suppress post-traumatic glial activation and thus inflammation to evaluate their effectiveness in preventing epileptogenesis in the LFPI model of PTE. In this first project year we have developed a high-speed video/EEG recording and analysis system for rapid quantification of chronically recorded epileptiform activity in multiple (24-32) subjects. With this system we have become expert in identifying epileptiform versus normal video/EEG activity in the rodent and have discovered an important source of artifact currently being interpreted in other published reports as seizure activity. We have developed a pilocarpine model of temporal lobe epilepsy to explore the effectiveness of glial cell (neuroimmune) attenuation in preventing or limiting epileptogenesis (development of epilepsy) in this rapidly developing model. We are making changes in our LFPI model to produce earlier developing signs of epilepsy, increasing the probability of succeeding in our long-term study of epileptogenesis following traumatic brain injury. Finally, we discovered and published results concerning development of post-traumatic anxiety in our brain injured animals that we could effectively prevent with peri-injury administration of glial attenuating drug, MN-166, the same drug to be used in our studies concerning prevention of epileptogenesis following traumatic brain injury. | | | | | |
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INTRODUCTION:

Post-traumatic epilepsy (PTE) is a common result of traumatic closed head injury. The development of epilepsy (epileptogenesis) can take many months to several years before the appearance of behavioral seizures. Compared to other forms of epilepsy, PTE is particularly resistant to antiseizure medication once it has developed and there are currently no therapeutic interventions to prevent or attenuate epileptogenesis. The purpose of this research project is to explore anti-epileptogenic strategies in an animal model of PTE using lateral fluid percussion injury (LFPI). Our focus is on attenuating damaging effects of hyperexcitability in the brain induced by inflammation resulting from glial cell immune responses to trauma. We are exploring two drugs, MN166 and SLC022, that are known to suppress post-traumatic glial activation and thus inflammation to evaluate their effectiveness in preventing epileptogenesis in the LFPI model of PTE. If successful, our results could have accelerated impact on translation to preventing PTE in war fighters since one of these drugs (MN166) has already been approved by the FDA and is in clinical trials for human neuropathic pain studies.

BODY:

There were two objectives for year one of this project. The first was to construct a custom video/EEG acquisition/analysis system. The second was to record sensory evoked potentials and spontaneous EEG from acutely anesthetized animals receiving LPS applied directly to the cortical surface to evaluate the effectiveness of MN166 in reducing microglial TLR-4-mediated hyperexcitability.

Progress on objective 1: The custom video/EEG acquisition and analysis system is complete and fully functional (**Fig. 1**). This consists of two racks with 16 recording chambers each. Each recording chamber was custom made and consists of a 12" diameter and 24" high plastic cylinder equipped with a 7 channel electrode harness that is attached on one end to chronic screw electrodes on each rat and on the other end to a slip-ring swivel connector, permitting recording with unimpeded movement. Each recording chamber is also equipped

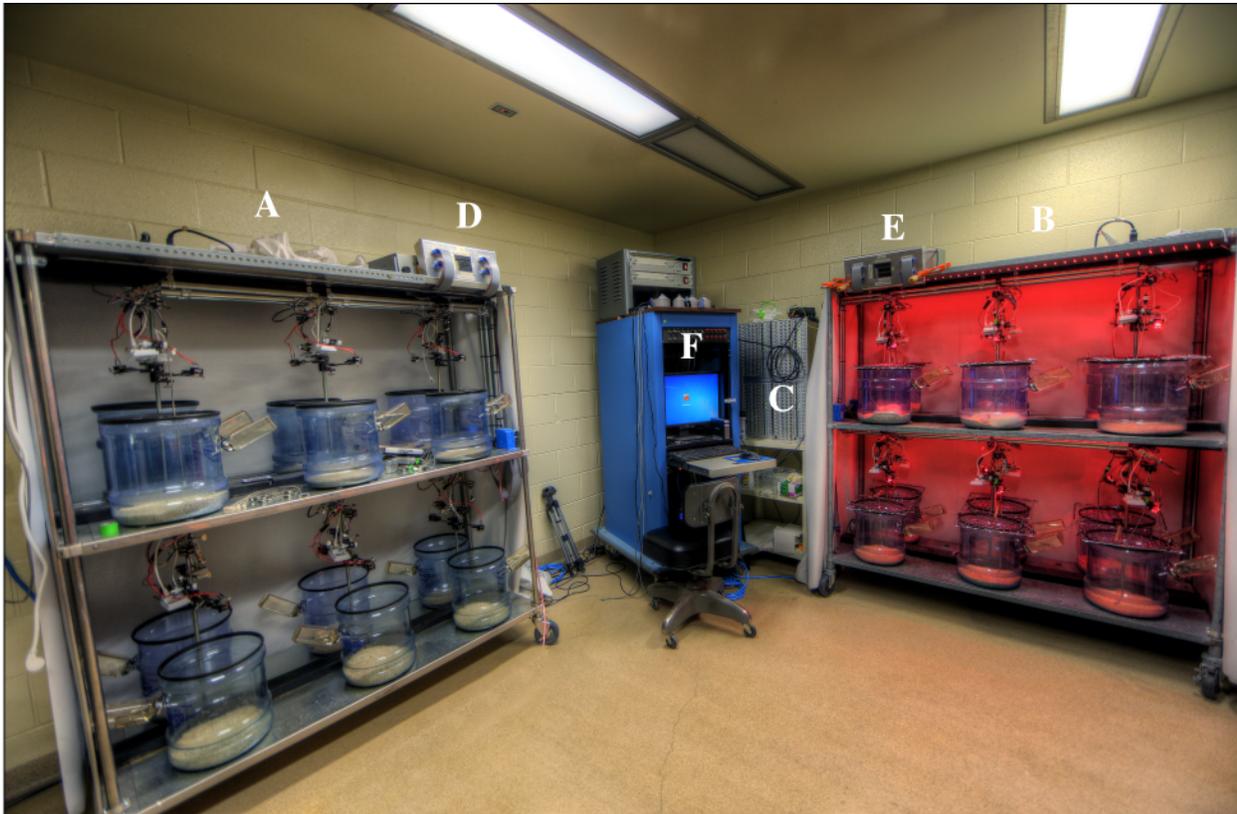


Fig. 1. Wide angle view of chronic recording rigs used for video/EEG of 24 (max 32) rats simultaneously. A&B) Dual level platforms holding 6 chambers per level. Each chamber consists of a containment vessel, swivel electrode harness and video camera (shown in higher resolution in next figure). All cameras are IP surveillance cameras multiplexed through high speed internet switches on the top of each rig. C) 64 channel EEG amplifier used for our first recordings. D&E) These have now been replaced by 2 custom made 64 channel EEG amps of much smaller dimension. F) Rack cabinet with power supplies and DAQ computer.

with a dedicated surveillance camera (Axis M1011) for recording video. We chose cameras that are designed for internet protocol (IP) recording because they can easily be multiplexed through a wired or wireless router and use compression (H264) to reduce bandwidth (**Fig. 2**), which is critically important for chronic video/EEG recording and analysis. Each chamber is also equipped with DC (light emitting diode) lighting for day (white) and night (red) video recording without disturbing sleep cycles. Two compact 64-channel EEG amplifier systems (designed by the P.I.) were also constructed (one for each rack) to buffer signals before digitization and computer storage. The digital acquisition software was written by the P.I. in Visual Basic and provides a flexible means of logging EEG and simultaneous video for each rat in date/time registered folders. The need to log video along with EEG posed a particular challenge due to the bandwidth of video and the need to precisely

time-lock the signal to each rat's EEG. This problem was solved in part through using IP cameras as noted above. The final solution to the problem was to use computers capable very large RAM storage so 30 minute trials of temporally contiguous EEG could be sampled without interrupt from all rats while spooling video to disk and finally writing the EEG at the end of each trial while the cameras are paused. This data collection hardware/software was finished early and has been fully functional for several months, permitting us to get a head start on chronic video/EEG recording.

Our analysis hardware and software for the video/EEG data has also been completed and is fully functional. This turned out to be the most challenging part of the project since there is presently no commercially available software that permits extremely rapid inspection of these enormous data sets recorded 24/7 from large numbers of animals. The hardware finally chosen consists of PCs designed for gaming, providing very fast numerical and video processing at moderate cost. Video data is displayed on two high-resolution monitors mounted in tandem, permitting visual inspection of 30 min of EEG in a single page. All data analysis software was written by the P.I. in the MatLab environment and, to our knowledge, exceeds anything commercially or privately available for exploring these large data sets. From the P.I.'s previous experience of the pitfalls of automated analysis of epileptiform EEG data, the design principal of the present software was to permit initial rapid visual inspection of all data, and to use automated analysis only for subsequent quantification of suspected epileptiform events. As noted above, EEG data for a given rat is rapidly presented in 30 min blocks. The operator can rapidly zoom in on suspected epileptiform events and precisely mark their latency with a mouse click for event logging and subsequent quantification. Zooming also defines a time window within which clicking on a trace plays the video clip associated with that window for verification of

seizures. Thus, unlike existing review software, our program permits quasi-random access to the data accompanied by user defined video review. This software has now been in extensive use and meets our design goal of reviewing a full one-day data set in 5-10 min. Since we finished the data collection and analysis system ahead of schedule, we have had several months to begin looking at spontaneous recordings from normal and brain damaged rats (noted below). This has prompted two additions to the software for quantifying results. The first was designed for automated epileptic spike detection. Typical spike detection programs commercially available are based on attempting to use universal spike descriptors (i.e. amplitude, rise-time, etc.) to separate spikes from noise. These approaches, while easier to implement, suffer from numerous false positives and noise. The approach we took instead was to take advantage of our ability to rapidly visually identify sub-sets of spikes for each rat individually, and from these make a rat specific average spike template that is sequentially matched to the actual data using a covariance measure that is thresholded to separate signal from noise. This approach is quite accurate, and with our fast processors, can count spikes over many days of data in under an hour. We have also added a feature to the software that employs a touch screen to permit rapid but manual

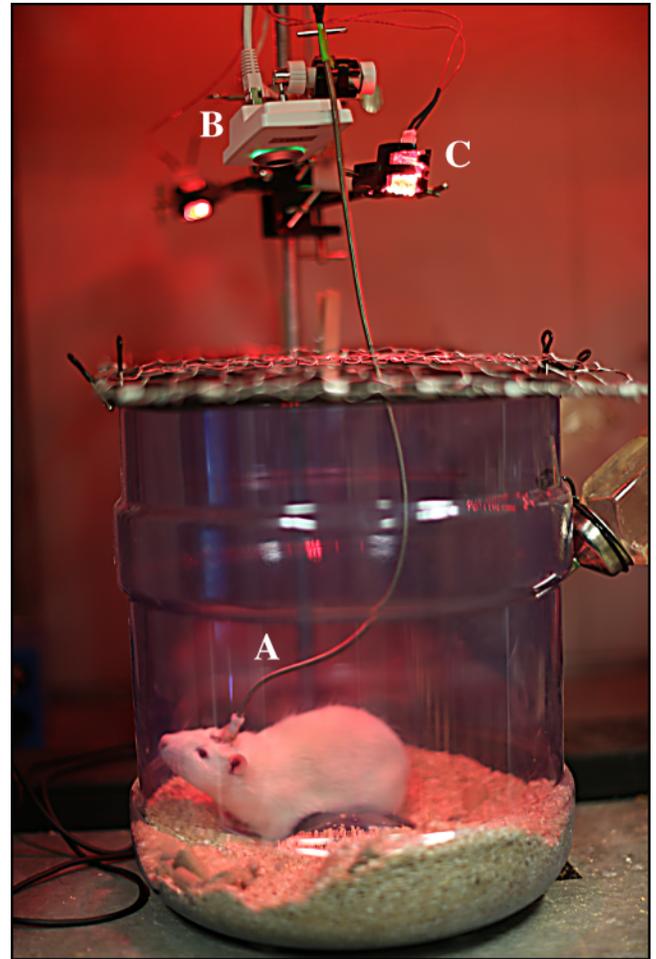


Fig. 2 Close up view of single recording chamber. A) EEG channel plug for rat head mount attached to flexible electrode harness and slip-ring commutator. B) Surveillance camera (1 per rat) providing highly compressed H264 images to limit band-width demands. C) High intensity red LEDs for night recording.

identification and quantification of more prolonged epileptiform events such as seizures and seizure like artifacts (noted below) for subsequent video verification. The speed of this quantification is achieved by using foot pedals to signal the event type and a wand on the touch screen to mark event time and duration. A brief video demonstration of this analysis software was provided to our Science Officer, Dr. Jordan D. Irvin, and is downloadable at <http://dl.dropbox.com/u/11873936/SoftwareDemo1.wmv>. We will also be presenting this work at The 2012 Military Health System Research Symposium held 13-16 August 2012 in Fort Lauderdale, Florida. The abstract for this presentation is included in APPENDICES. We feel our video/EEG data collection/analysis system should serve not just our own research but is sufficiently unique, fast, and inexpensive to be useful for emergency and post-emergency monitoring of soldiers suffering traumatic brain injury in the battlefield.

Progress on objective 2: The second objective of this first year project was to determine the efficacy of attenuating glial cell activation (using MN-166 and SLC022) in decreasing acute hyperexcitability of the brain induced by lipopolysaccharide (LPS) applied directly to the cerebral cortex of anesthetized rats. Upon initial investigation we realized that anesthesia was having an unacceptable and variable influence on cortical excitability induced by LPS. Thus, while we could suppress excitability through glial attenuation, these results were confounded by additional suppressive anesthesia effects. Particularly troublesome was the fact that the effect of various anesthesia regimes we tried (ketamine/xylazine, xylazine alone, isoflurane, urethane) had highly variable effects in both increasing or decreasing the response to LPS independent of glial modulating treatment. With permission of our Scientific Officer, we decided to abandon this study in order to devote our time instead to accelerating work on unanesthetized animals. This turned out to be a good decision for several reasons:

1) We got a head start on examining chronic video/EEG recording from rats with and without lateral fluid percussion injury (LFPI). We were able to examine these initial recordings with unprecedented accuracy since our software relies on visual as opposed to automatic review. It immediately became apparent to us that both our control and LFPI rats displayed a repertoire of EEG patterns associated with chewing, grooming etc., which

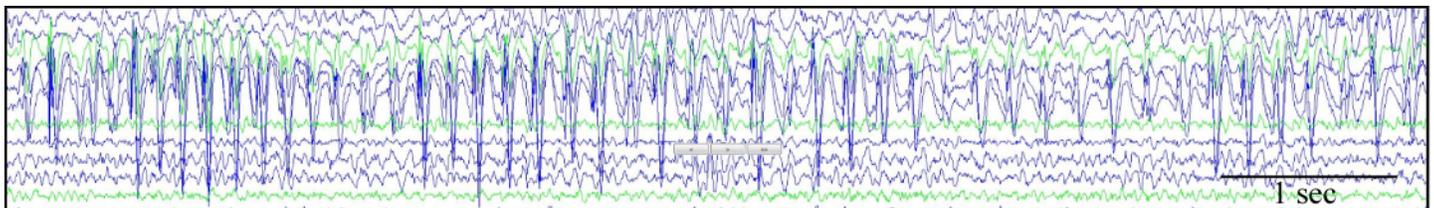


Fig. 3. Typical theta activity recorded from rat sensorimotor cortex during “bruxing” and “eye boggling”.

are normal artifacts that we are now expert in recognizing. However, an unexpected finding was that both the LFPI and the control rats displayed pronounced runs of theta activity (Fig. 3). This drew our attention because the theta activity we recorded was similar in frequency, amplitude, and duration to Epileptiform Electrographic Events (EEE) previously associated exclusively with LFPI (D'Ambrosio et al., 2009; D'Ambrosio and Miller, 2010). By examining restrained animals with high resolution video and simultaneous EEG, it became apparent that the theta was not epileptiform but was instead due to “bruxing”, also referred to as “vacuous chewing” (Rosales et al., 2002; Zeredo et al., 2009), and “eye boggling”, both activities that rats perform normally to dull the front incisors as well as when they are under stress. Please see video clip at <http://dl.dropbox.com/u/11873936/BruxingBoggling.wmv> displaying such behavior in relation to the EEG shown in Figure 3. While this discovery sounds trivial, it is actually at the center of a very recent controversy concerning what can be safely considered to be post-traumatic epileptiform activity in LFPI rats (see (D'Ambrosio and Miller, 2010) and (Dudek and Bertram, 2010) for point/counter-point).

2) In reviewing our initial chronic recordings we realized that we needed more experience discerning normal from epileptiform activity. To this end we received approval to conduct a pilot study using a pilocarpine model of temporal lobe epilepsy. This model involves injecting animals with lithium followed by pilocarpine (a muscarinic receptor agonist) which induces acute status epilepticus for several hours (Curia et al., 2008). Status is followed by a “silent period” of several weeks where epileptiform spikes may be recorded, and then the

appearance of regular temporal lobe seizures. We began an initial study in 8 rats using this model and have just started to see seizures at the 4-week time-point following status. Thus, this brief study has served its purpose of familiarizing us with chronic video/EEG recording and analysis of spikes and seizures. However, having succeeded with this model, it provides us with an ideal opportunity to test the effectiveness of glial attenuation in preventing epileptogenesis presumed to occur during the one month silent period before chronic seizures occur. This study is underway and should provide us with valuable insights concerning prevention of epileptogenesis in this more rapidly developing model before proceeding with LFPI animals and a much prolonged (many months) silent period.

3) Finally, our early start on chronic recording led to an unexpected serendipitous finding concerning post-traumatic anxiety. In piloting LFPI rats, we noticed that brain damaged animals displayed behaviors suggesting increased anxiety when placed in the recording chamber. We pursued this by performing an experiment using a controlled stressor (foot shock) and measuring freezing behavior (the rats natural defensive behavior to danger). Indeed, our LFPI animals showed a reliable over-reaction to stress when compared to controls, suggesting an animal model of post-traumatic anxiety. Most important, we found that glial attenuation with peri-injury administration of MN-166 completely prevented development of post-traumatic anxiety. While not directly related to post-traumatic epilepsy, we believe the enhanced post-traumatic anxiety is reflective of increased excitability of limbic structures due to injury-induced neuroinflammation. In this way, our serendipitous discovery holds promise for our epilepsy studies. This work is now in press (Rodgers et al., 2012) and the manuscript is included in APPENDICES.

Recommended changes or future work to better address the research topic: As noted above, it has been essential for us to get an early start on actual chronic recording of unanesthetized rats to get a better understanding of normal and epileptiform electrographic patterns. We are presently conducting a study with the pilocarpine model to learn if glial attenuation might be effective in preventing rapidly developing epileptogenesis. In our pilot studies of LFPI so far, we have been disappointed that the percussion pressures and parietal location we were initially using has not reliably produced epileptiform spiking within 1-2 months (expected for animals that should go on to develop seizures). We feel it is essential to continue piloting this model with increased impact pressures and with moving our impact location to motor cortex, where recent reports indicate much higher success rates for eventual seizures (Curia et al., 2011). We will therefore be requesting a modification of the original Statement of Work to reflect this additional pilot work for the first several months of the second year.

KEY RESEARCH ACCOMPLISHMENTS:

- Completed and tested chronic video/EEG recording hardware and software.
- Developed high speed, random access, video/EEG review and analysis software.
- Achieved expertise in identifying normal and epileptiform EEG patterns in chronic recording.
- Discovered key discrepancy in current literature concerning “epileptiform” theta activity.
- Began pilocarpine model of temporal lobe epilepsy to test prevention of epileptogenesis.
- Discovered and published effect of glial attenuation on post-traumatic anxiety.

REPORTABLE OUTCOMES:

- Rodgers, K.M., Bercum, F.M., McCallum, D.L., Rudy, J.W., Frey, L.C., Johnson, K.W., Watkins, L.R. and Barth, D.S. Acute neuroimmune modulation attenuates the development of anxiety-like freezing behavior in an animal model of traumatic brain injury. *J. Neurotrauma*, 2012, (*in press*).
- Barth, D.S. A Very High Speed System for Video/EEG Monitoring and Quantification of Post-traumatic Epileptogenesis. To be presented at the 2012 Military Health System Research Symposium to be held 13-16 August 2012 in Fort Lauderdale, Florida.
- Completed an Invention Disclosure Form so that our Technology Transfer Office can investigate whether the video/EEG review and analysis software is patentable or at least can be protected with a copyright.
- Alex Benison received his Ph.D. this year and Krista Rodgers will be receiving her Ph.D. this Fall. Both will be continuing on as a post-doctoral fellows on this project. The last year of their doctoral work was supported by this award.
- Submitted pre-proposal in response to a USAMRMC Broad Agency Announcement for a new project entitled: “The Prevention and Treatment of Post-traumatic Anxiety Through Neuroimmune Modulation”, based on the serendipitous discovery made in the present project.

CONCLUSION:

Achievements: In the first year of this project, we constructed and tested all hardware for chronic video/EEG recording of 24 animals (expandable to 32). Software for logging video/EEG in a time-locked manner has been completed and is in use. Software for the extremely rapid quasi-random review and analysis of video/EEG data has been completed and is in use. We have been using this system to examine normal and brain damaged animals for the past several months. From this work we have discovered that what has been interpreted by others as pathological theta activity is also prominent in normal animals during “vacuous chewing” and we have entered into a very recent controversy in the field about what constitutes a valid post-traumatic epileptic seizure. We have developed a pilocarpine model of temporal lobe epilepsy and have begun recording epileptic spikes and seizures in this model. We will be conducting a study to determine if epileptogenesis (what goes on during the 1 month silent period before regular seizures evolve) can be attenuated or prevented with attenuation of glial activation using MN-166. Finally, we serendipitously discovered that LFPI produces post-traumatic anxiety that can be prevented with administration of MN-166 peri-injury.

Recommendations: We recommend that the second year of this project include completion of the pilocarpine study. It should also include several months more pilot testing of an LFPI injury site that is more rostral, over motor cortex, and uses higher impact pressures. We feel this work is essential to improve our chances of success when we then commit to long-term monitoring of treated and control LFPI animals for the remainder of the second and third project years.

So what: 1) Our data collection and analysis hardware/software comprises a unique and inexpensive approach to chronic monitoring of post-traumatic brain activity that is ideal, not just for the present research, but for emergency battlefield-related medical monitoring. For this reason, our results will be presented at this years MHSRS Symposium. Virtually nothing is known about epileptogenesis following traumatic brain injury in humans due in large part to the fact that video/EEG monitoring is rarely performed post-injury in the absence of a behavioral seizure. Our hardware/software system should be pursued as a tool for making this not only feasible but routine. 2) Our discovery that normal bruxing behavior in rats produces EEG activity closely resembling what has been identified as post-traumatic “pathological theta” turns out to be quite timely and important because there are currently attempts to use theta as a unique sign of early epileptogenesis and to develop drugs that might suppress this activity instead of waiting for development of actual seizures. This issue needs rapid resolution so the field of anti-epileptogenesis drugs does not head in the wrong direction. We are currently collaborating with epilepsy researcher, Dr. Edward Dudek at the University of Utah, on this effort. 3) Our work with the pilocarpine model, while not directly related to post-traumatic epilepsy, could represent a major advance in the field if we are able to block or attenuate epileptogenesis in this more rapidly developing model. 4) Our plan to pilot the best (earliest spiking) model of LFPI should assure us the highest probability of success in our long-term study of post-injury epileptogenesis and its possible attenuation with glial modulation. 5) Our unexpected finding that LFPI produces an animal model of post-traumatic anxiety, and that this development can be prevented by early attenuation of post-injury brain inflammation, may have extremely important implications for post-traumatic stress disorder (PTSD) experienced by many of our war fighters after head injury. It suggests that a strong component of PTSD may in fact be directly produced by the injury and not just the psychological setting within which it occurs. It also opens the way to potential future intervention. We are currently applying to both the DoD and NIMH for funding to separately pursue this discovery.

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APPENDICES:

Attached are copies of our recent manuscript concerning post-traumatic anxiety and an abstract submitted to MHSRS reporting our video/EEG recording analysis system.

Journal of Neurotrauma

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

Acute neuroimmune modulation attenuates the development of anxiety-like freezing behavior in an animal model of traumatic brain injury.

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| Journal: | <i>Journal of Neurotrauma</i> |
| Manuscript ID: | NEU-2011-2273.R1 |
| Manuscript Type: | Regular Manuscript |
| Date Submitted by the Author: | 24-Feb-2012 |
| Complete List of Authors: | Rodgers, Krista; University of Colorado, Psychology and Neuroscience Bercum, Florencia; University of Colorado, Psychology and Neuroscience McCallum, Danielle; University of Colorado, Psychology and Neuroscience Rudy, Jerry; University of Colorado, Psychology and Neuroscience Frey, Lauren; University of Colorado Denver, Neurology Johnson, Kirk; MediciNova, Inc., Watkins, Linda; University of Colorado, Psychology and Neuroscience Barth, Daniel; University of Colorado, Psychology and Neuroscience |
| Keywords: | INFLAMMATION, TRAUMATIC BRAIN INJURY, ANIMAL STUDIES |
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Manuscripts

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3 **Acute neuroimmune modulation attenuates the development of anxiety-like**
4 **freezing behavior in an animal model of traumatic brain injury.**
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11 **Krista M. Rodgers, M.A.¹, Florencia M. Bercum, B.A.¹, Danielle L. McCallum, B.A. ¹,**
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35 Running title: Neuroinflammation and post-traumatic anxiety.
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37 Table of contents title: Neuroimmune modulation of anxiety behavior in a rat model of TBI.
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Abstract

Chronic anxiety is a common and debilitating result of traumatic brain injury in humans. While little is known about the neural mechanisms of this disorder, inflammation resulting from activation of the brain's immune response to insult has been implicated in both human post-traumatic anxiety and in recently developed animal models. In this study, we used a lateral fluid percussion injury (LFPI) model of traumatic brain injury in the rat and examined freezing behavior as a measure of post-traumatic anxiety. We found that LFPI produced anxiety-like freezing behavior accompanied by increased reactive gliosis (reflecting neuroimmune inflammatory responses) in key brain structures associated with anxiety: the amygdala, insula and hippocampus. Acute peri-injury administration of Ibudilast (MN166), a glial cell activation inhibitor, suppressed both reactive gliosis and freezing behavior, and continued neuroprotective effects were evidenced several months post-injury. These results support the conclusion that inflammation produced by neuroimmune responses to traumatic brain injury play a role in post-traumatic anxiety, and that acute suppression of injury-induced glial cell activation may have eventual promise for prevention of post-traumatic anxiety in humans.

Key Words

TBI, LFPI, PTSD, neuroinflammation

Introduction

The long-term consequences of traumatic brain injury (TBI) include heightened risk of neuropsychiatric disorders, of which anxiety disorders are the most prevalent (Rao and Lyketsos, 2000; Moore et al., 2006; Vaishnavi et al., 2009). Studies of the etiology of anxiety disorders implicate exaggerated responses of the amygdala and insula (Rauch et al., 1997; Simmons et al., 2006; Stein et al., 2007; Shin and Liberzon, 2010; Carlson et al., 2011), impaired inhibition of medial prefrontal cortex and anterior cingulate (Davidson, 2002; Shin et al., 2006; Milad et al., 2009; Shin and Liberzon, 2010) and decreased hippocampal volume (Bremner et al., 1995; Sapolsky, 2000; Shin et al., 2006). Yet, whether and how TBI induces neurochemical, structural, and functional abnormality in these structures is poorly understood.

There is increasing evidence that excessive inflammatory actions of the neuroimmune system may contribute to the development of anxiety disorders following TBI (Spivak et al., 1997; Gasque et al., 2000; Tucker et al., 2004; Shiozaki et al., 2005; von Känel et al., 2007; Hoge et al., 2009). Microglial cells are the first line of defense and primary immune effector cells in the CNS and respond immediately to even small pathological changes from damaged cells, producing proinflammatory cytokines and toxic molecules that compromise neuronal survival (Gehrmann, 1996; Gonzalez-Scarano and Baltuch, 1999; Aloisi, 2001; Town et al., 2005). This rapid microglial response often precedes the more delayed, yet prolonged activation of astrocytes and is thought to be involved with the onset and maintenance of astrogliosis (Graeber and Kreutzberg, 1988; McCann et al., 1996; Hanisch, 2002; Iravani et al., 2005; Herber et al., 2006; Zhang et al., 2010). It has been well established that microglia and astrocytes are activated during the innate immune response to brain injury, leading to the expression of high

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3 levels of proinflammatory cytokines, most notably interleukin-1 beta (IL-1 β), interleukin-6 (IL-
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6 6) and tumor necrosis factor alpha (TNF- α). While glial activation is typically neuroprotective
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8 (Aloisi, 2001; Farina et al., 2007), the chronic inflammatory responses and exaggerated
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10 proinflammatory cytokine levels observed following injury initiate neurotoxic processes
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12 resulting in secondary tissue damage (Gasque et al., 2000; Simi et al., 2007; Hailer, 2008;
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14 Lehnardt, 2010), neuronal death (Sternberg, 1997; Brown and Bal-Price, 2003; Schmidt et al.,
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16 2005; Beattie et al., 2010), secondary injury cascades (Bains and Shaw, 1997; Cernak et al.,
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18 2001b, a; Ansari et al., 2008a, b) and neuronal hyperexcitability (Hailer, 2008; Riazi et al., 2008;
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20 Rodgers et al., 2009; Beattie et al., 2010), all of which may contribute to the dysfunction of brain
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22 regions associated with anxiety.
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27 Recently developed animal models of post-traumatic anxiety (O'Connor et al., 2003;
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29 Vink et al., 2003; Fromm et al., 2004; Sönmez et al., 2007; Wagner et al., 2007; Jones et al.,
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31 2008; Baratz et al., 2010; Liu et al., 2010) permit examination of the possible contributions of
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33 brain inflammation. Tests of post-traumatic anxiety in these models have typically included
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35 standard measurements of exploratory preference in mildly stressful environments, such as an
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37 open-field or elevated-plus testing apparatus. However, it has been frequently noted that
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39 measures of exploratory preference may be confounded by a marked overall decrease in
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41 exploration in brain-injured animals (O'Connor et al., 2003; Vink et al., 2003; Fromm et al.,
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43 2004). Decreased exploration cannot be attributed to TBI-induced motor deficits since numerous
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45 studies report only transient (~ 1 week) deficits following trauma (Yan et al., 1992; Taupin et al.,
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47 1993; Dixon et al., 1996; Fassbender et al., 2000; Goss et al., 2003; Cutler et al., 2005; Cutler et
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49 al., 2006b; Cutler et al., 2006a; Kline et al., 2007; Wagner et al., 2007; Bouilleret et al., 2009;
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51 Frey et al., 2009; Baratz et al., 2010; Liu et al., 2010). Rather, TBI-induced decreases in
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3 exploration have been attributed to the indirect effects of freezing (a primary component of the
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5 rodent's natural defensive behavior repertoire; Blanchard and Blanchard, 1988), suggesting an
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7 abnormally heightened response to stress in brain-injured rats (O'Connor et al., 2003; Vink et al.,
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9 2003; Fromm et al., 2004).

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12 Based on these results, we tested the hypothesis that trauma-induced innate immune
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14 responses contribute to the development of anxiety-like behaviors in rats by directly examining
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16 freezing responses to a minor (novel environment) and major (foot-shock) stressor following
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18 Lateral Fluid Percussion Injury (LFPI; a clinically relevant animal model of human closed head
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20 injury). We also tested the effectiveness of a glial cell activation inhibitor, Ibudilast (MN166), in
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22 attenuating post-injury freezing behavior and reducing reactive gliosis in brain regions associated
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24 with hyperexcitability in anxiety disorders.
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Materials and Methods

Sixty adult viral-free male Sprague-Dawley rats (275-325g; Harlan Laboratories, Madison, WI) were housed in pairs in temperature (23 ± 3 °C) and light (12:12 light:dark) controlled rooms with *ad libitum* access to food and water. All procedures were performed in accordance with University of Colorado Institutional Animal Care and Use Committee guidelines for the humane use of laboratory rats in biological research. Rats were randomly assigned to 1 of 10 groups (n = 6/group). Six groups (surgically naïve, sham operated, sham operated+vehicle, sham operated+MN166, LFPI+vehicle and LFPI+MN166) were shocked immediately after behavioral testing at 1 month post-surgery (sham operation or LFPI in the experimental rats). Surgically naïve rats received no injections or surgery, whereas sham operated rats received surgery but were not injected, the final 4 groups received sham or LFPI surgery and either vehicle injections or MN166 treatment. Another 4 groups (sham operated+vehicle, sham operated+MN166, LFPI+vehicle and LFPI+MN166) were run separately in a sucrose preference test to assess anhedonia (the inability to experience pleasure, a core symptom of human depression) without exposure to stressors (anxiety tests and foot shock).

Lateral Fluid Percussion Injury. LFPI rats were anesthetized with halothane (4% induction, 2.0-2.5% maintenance) and mounted in a stereotaxic frame. The lateral fluid percussion injury used in this study has been described previously (McIntosh et al., 1989; Thompson et al., 2005; Frey et al., 2009) utilizing a PV820 Pneumatic PicoPump (World Precision Instruments, Inc., Sarasota, FL) to deliver standardized pressure pulses of air to a standing column of fluid. A 3.0 mm diameter craniotomy was centered at 3 mm caudal to bregma and 4.0 mm lateral of the sagittal suture, with the exposed dura remaining intact. A

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3 female Luer-Loc hub (inside diameter of 3.5 mm) was secured over the craniotomy with
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5 cyanoacrylate adhesive. Following hub implantation, the animal was removed from the
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7 stereotaxic frame and connected to the LFPI apparatus. The LFPI apparatus delivered a moderate
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9 impact force (2.0 atmospheres; 10 ms). The injury cap was then removed, scalp sutured and the
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11 rats returned to their home cages for recovery. Sham operated rats underwent identical surgical
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13 preparation, but did not receive the brain injury.
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17 *Ibutilast (MN166) administration.* MN166 (MediciNova, San Diego, CA) is a relatively
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19 non-selective phosphodiesterase inhibitor with anti-inflammatory actions via glial cell
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21 attenuation, which has been found to reduce glia-induced neuronal death through the suppression
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23 of nitric oxide, reactive oxygen species, and proinflammatory mediators (Mizuno et al., 2004;
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25 Rolan et al., 2009). Treated rats received a 5-day dosing regimen of once-daily MN166
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27 injections (10 mg/kg, 1 ml/kg subcutaneously in corn oil) 24 hr prior to LFPI, the day of surgery
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29 and LFPI, and 3 days following LFPI. Weight was recorded prior to each dosing and treatment
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31 administered at the same time each day to maintain constant levels across a 24 hr period. Dose
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33 selection was based on prior animal pharmacology results (Ellis AL, SFN, 2008) showing
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35 MN166 to be safe and well tolerated, yielding plasma concentration-time profiles commensurate
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37 with high dose regimens in clinical development. MN166 administered via this regimen yields
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39 plasma and CNS concentrations that are linked to molecular target actions including, most
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41 potently, macrophage migration inhibitory factor (MIF) inhibition (Cho et al., 2010) and,
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43 secondarily, PDE's -4 and -10 inhibition (Gibson et al., 2006). The relevance of MIF inhibition
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45 in disorders of neuroimmune function such as neuropathic pain has recently been well
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47 demonstrated (Wang et al., 2011). Such dosing regimens have clearly been linked to glial
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49 attenuation in other animal models (Ledeboer et al., 2007), and the anti-inflammatory actions of
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3 MN166 have recently been shown to suppress cerebral aneurysms in a dose-dependent manner
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8 *Tests of motor, vestibular and locomotive performance.* Baseline testing of motor,
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10 vestibular and locomotive performance in all groups was conducted immediately prior to surgery
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12 and again, following a 1-week recovery period. These tests included ipsilateral and contralateral
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14 assessment of forelimb and hindlimb use to assess motor function, locomotion, limb use and
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16 limb preference (Bland et al., 2000; Bland et al., 2001), toe spread to assess gross motor response
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18 (Nitz et al., 1986), placing to assess visual and vestibular function (Schallert et al., 2000;
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20 Woodlee et al., 2005), catalepsy rod test to assess postural support and mobility (Sanberg et al.,
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22 1988), bracing to assess postural stability and catalepsy (Schallert et al., 1979; Morrissey et al.,
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24 1989) and air righting to assess dynamic vestibular function (Pellis et al., 1991b; Pellis et al.,
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26 1991a). Scoring ranged from 0 (severely impaired) to 5 (normal strength and function). The
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28 individual test scores were summed and a composite neuromotor score (0–45) was then
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30 generated for each animal. In addition to the composite neuromotor score, limb-use asymmetry
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32 was assessed during spontaneous exploration in the cylinder task, a common measure of motor
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34 forelimb function following central nervous system injury in rats (Schallert et al., 2000;
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36 Schallert, 2006) and post-injury locomotor activity was assessed through distance traveled on a
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38 running wheel, both tasks were scored for 5 minutes under red light (~90 lux).
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46 *Behavioral measures.* A novel environment was used to assess freezing behavior in
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48 response to a minor stressor (Dellu et al., 1996). The environment consisted of a standard rat
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50 cage with one vertically and one horizontally striped wall. No aversive stimuli were introduced
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52 in this context and no conditioning occurred. Rats were tested (5 minutes) and the percent of
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3 freezing behavior was assessed. Freezing was defined as the absence of movement except for
4 heart beat/respiration, and was recorded in 10 sec intervals.
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8 Freezing behavior in the novel environment was measured before and after
9 administration of a foot shock in a separate shock apparatus. The shock apparatus consisted of
10 two chambers placed inside sound-attenuating chests. The floor of each chamber consisted of 18
11 stainless steel rods (4 mm diameter), spaced 1.5 cm center-to-center and wired to a shock
12 generator and scrambler (Colbourn Instruments, Allentown, PA). An automated program
13 delivered a 2-sec/1.5 mA electric shock. Rats were transported in black buckets and shocked
14 immediately upon entry to chambers. Following shock, rats were returned to their home cages.
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24 A sucrose preference test was also performed in separate groups of rats that did not
25 receive foot-shock or testing in the novel environment. This task is commonly used to measure
26 anhedonia in rodent models of depression (Monleon et al., 1995; Willner, 1997). The sucrose
27 preference task was included because anxiety and depression share high rates of co-morbidity in
28 humans (Moore et al., 2006) and was assessed as a possible confound to freezing behavior, due
29 to possible co-occurrence of depression-like behavior. Rats were first habituated to sucrose
30 solution, and were tested during the dark phase of the light/dark cycle to avoid the food and
31 water deprivation necessary when testing during the light phase. Day 1 and day 2 consisted of
32 habituation, day 3 and day 4 were baseline (averaged) and day 5 was the first test day. The rats
33 were presented with two pre-weighted bottles containing 2% sucrose solution or tap water for a
34 period of 4 hours. Thirty minutes into the task the bottles were swapped to force preference and
35 counter for placement effects. Total sucrose intake and sucrose preference (sucrose
36 intake/(sucrose intake + water intake * 100) were measured.
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Timeline for behavioral testing: Following a 2-week recovery period from sham operation or LFPI in experimental animals, all groups except those to be evaluated for sucrose preference were tested in the novel context. Testing was performed at 2 weeks, 1, 2 and 3 months post-surgery. Shock was delivered after behavioral testing was completed at the 1 month time-point. Tests for sucrose preference were performed at 2 weeks, 1 month and 3 months post-surgery with no intervening foot-shock.

Immunohistochemistry: Immunoreactivity for OX-42 (targets CD11b/c, a marker of microglial activation) and glial fibrillary acidic protein (GFAP; a marker of astrocyte activation) was measured using an avidin-biotin-horseradish peroxidase (ABC) reaction (Loram et al., 2009). Brain sections (12 μ m) were cut on a cryostat and mounted onto poly-L-lysine-coated slides and stored at -80 °C. Sections were post-fixed with 4% PFA for 15 min at room temperature, then treated with 0.03% H₂O₂ for 30 min at room temperature. The sections were incubated at 4 °C overnight in either mouse anti-rat OX-42 (1:100; BD Biosciences Pharmingen, San Jose, CA) or mouse anti-pig GFAP (1:100; MP Biomedicals, Aurora, OH). The next day, sections were incubated at room temperature for 2 h with biotinylated goat anti-mouse IgG antibody (1:200; Jackson ImmunoResearch, West Grove, PA). Sections were washed and incubated for 2 h at room temperature in ABC (1:400 Vector Laboratories, Burlingame, CA) and reacted with 3', 3'-diaminobenzidine (DAB; Sigma-Aldrich, St. Louis, MO). Glucose oxidase and β -D-glucose were used to generate hydrogen peroxide. Nickelous ammonium sulfate was added to the DAB solution to optimize the reaction product. Sections were air-dried over night and then dehydrated with graded alcohols, cleared in HistoClear and coverslipped with Permount (Fisher Scientific, Fairlawn, NJ). Densitometric analysis was performed using Scion Image software.

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Image Analysis: Slides were viewed with an Olympus BX-61 microscope, using Olympus Microsuite software (Olympus America, Melville, NY) with bright-field illumination at 10X magnification. Images were opened in ImageJ, converted into gray scale and rescaled from inches to pixels. Background areas were chosen in the white matter or in cell-poor areas close to the region of interest (ROI). The number of pixels and the average pixel values above the set background were then computed and multiplied, giving an integrated densitometric measure (integrated gray level). Four measurements were made for each ROI; the measurements were then averaged to obtain a single integrated density value per rat, per region. All measurements were taken while blind to treatment group.

Statistical Analyses: Results are expressed as mean \pm SEM. Analyses for all behavioral variables used analysis of variance (ANOVA) with repeated measures (time after injury), and treatment as the independent variable. The integrated density from the histology was only conducted at one time point and utilized one-way ANOVAs to compare regions between groups. Data were analyzed using SPSS® Statistics software and, in all cases, statistical significance was set at $p < 0.05$.

Results

Neuromotor composite scores of the brain-injured groups (LFPI+MN166, LFPI+vehicle) did not significantly differ from controls ($F(3,20) = 0.803$, $p = 0.508$). Rats in all groups consistently received normal scores on forelimb and hindlimb use, toe spread, placing, catalepsy rod, bracing, and air righting tests, indicating no impairments in motor, vestibular or locomotive functioning due to TBI. There were also no significant between group differences in limb-use asymmetry observed for contralateral ($F(5,29) = 0.544$, $p = 0.741$) and ipsilateral ($F(5,29) = 0.428$, $p = 0.826$) forelimb use during vertical exploratory behavior in the cylinder task,

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3 indicating no limb-use bias due to injury (Fig. 1A). No significant between group differences
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5 were found in locomotor performance evidenced by distance traveled during the running wheel
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7 activity ($F(5,29) = 0.069$, $p = 0.996$), revealing no post-injury impairments in locomotion (Fig.
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9 1B). Nor were there significant between group differences in the sucrose preference task ($F(3,21)$
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11 $= 0.338$, $p = 0.798$), indicating no impairments in hedonic states post-injury.
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15 Despite normal motor, vestibular and locomotive function, LFPI produced large increases
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17 in freezing behavior when rats were placed in a novel context (Fig. 2; $F(5,30) = 9.539$, $p <$
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19 0.0001). Exposed only to this minor stressor (i.e. at 2 week and 1 month post-injury
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21 measurements conducted prior to shock), LFPI rats injected with either MN166 or vehicle (Fig.
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23 2; white and black bars, respectively) froze approximately twice as long as naïve or sham
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25 operated rats (Fig. 2; light and dark grey bars, respectively; $p < 0.01$). At 2 and 3-month
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27 measurement times, following the additional major stressor of shock (Fig. 2; arrows), freezing in
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29 both naïve and sham operated rats remained constant at approximately 10%. Freezing in LFPI
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31 rats treated with MN166 remained consistently higher than these controls ($p < 0.001$), but, while
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33 appearing higher compared to earlier post-injury measurements in the same animals, this
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35 increased freezing compared to naïve and sham operated rats before (1 month) and following (2
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37 month) shock did not reach significance ($p=0.316$). By contrast, LFPI+vehicle rats nearly
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39 doubled their freezing time to approximately 50% (Fig. 2; black bars) compared to pre-shock
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41 values ($p < 0.001$), freezing approximately twice as long as LFPI+MN166 rats ($p < 0.001$) and 5
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43 times as long as naïve and sham operated controls ($p < 0.001$) at the 2 and 3 month post-injury
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45 measurement times.
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52 The behavioral effects of injections alone, independent of LFPI, are reflected in sham
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54 surgery groups with injections of either MN166 or vehicle (Fig. 2; narrow and broad diagonal
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3 lines, respectively). Sham operated rats tended to freeze more than un-injected naïve and sham
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5 operated controls, reaching significance for both groups at the 2 and 3-month measurement
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7 points ($p < 0.01$) and suggesting that injections alone are aversive and can contribute to
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9 subsequent freezing. However, even at pre-shock measurement points, LFPI animals that
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11 received the same injections of MN166 or vehicle froze significantly more than injected controls
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13 ($p < 0.01$), indicating substantial enhancement of freezing produced by LFPI. This effect became
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15 more apparent following shock, where LFPI+vehicle rats froze twice as long as the injected
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17 controls ($p < 0.001$). By contrast, LFPI+MN166 rats were not distinguishable from either
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19 injected control group following shock, suggesting that their elevated freezing compared to naïve
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21 and sham operated animals was the result of injections alone and that MN166 eliminated the
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23 exaggerated freezing response to shock characterizing LFPI+vehicle rats.
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29 OX-42 and GFAP immunoreactivity (reflecting microglia and astrocytic activation) was
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31 assessed in the insula, amygdala and hippocampus in brain-injured rats for comparison to sham
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33 operated and surgically naïve rats. Representative images (40X), showing GFAP
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35 immunoreactivity in several of these regions, are shown in Figure 3, revealing normal astrocyte
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37 morphology in surgically naïve and sham operated rats. LFPI+vehicle rats showed clear signs of
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39 reactive astrocytes (Fig. 3; bottom row). LFPI rats treated with MN166 (Fig. 3; third row) were
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41 difficult to differentiate from sham operated or surgically naïve control groups.
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46 Densitometry of GFAP labeling in all areas examined confirmed that activation of
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48 astrocytes was significantly greater in LFPI compared to all other groups in insula (Fig. 4A; left
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50 bars; $F(3,19) = 13.17$, $p < 0.0001$), amygdala (Fig. 4B; left bars; $F(3,18) = 7.54$, $p < 0.002$) and
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52 hippocampus (Fig. 4C; left bars; $F(3,15) = 8.47$, $p < 0.002$). In contrast, no differences in GFAP
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54 labeling were observed between surgically naïve, sham operated and LFPI+MN166 groups in
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3 any of the regions examined. While MN166 treated LFPI rats were not distinguishable from
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5 surgically naïve or sham operated controls, post-hoc analyses revealed that LFPI+vehicle rats
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7 had significantly greater astrocyte activation in all 3 brain regions as compared to controls (Fig.
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9 4A-C): insula ($p < 0.002$ vs. surgically naïve, sham operated and LFPI+MN166), amygdala ($p <$
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11 0.02 vs. surgically naïve, sham operated and LFPI+MN166) and hippocampus ($p < 0.03$ vs.
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13 surgically naïve, sham operated and LFPI+MN166).
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17 Analysis of GFAP immunoreactivity in sub-regions of the insula (Fig. 4A; right bars),
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19 amygdala (Fig. 4B; right bars), and hippocampus (Fig. 4C; right bars), also revealed no
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21 differences between surgically naïve, sham operated and LFPI+MN166 groups. As in the
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23 regional analysis, LFPI+vehicle rats showed increased astrocyte activation over controls in most
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25 sub-regions examined. In the insula, LFPI+vehicle rats showed significantly increased GFAP
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27 labeling in agranular ($F(3,19) = 16.778$, $p < 0.0001$), dysgranular ($F(3,19) = 6.042$, $p < 0.005$)
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29 and granular ($F(3,19) = 5.277$, $p < 0.008$) regions, as compared to control groups. In the
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31 amygdala, GFAP labeling in LFPI+vehicle rats was significantly increased in the BLA ($F(3,18)$
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33 $= 4.050$, $p < 0.023$) and CE ($F(3,18) = 5.012$, $p < 0.011$) nuclei, as compared to controls.
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35 LFPI+vehicle rats also showed increased GFAP expression in the hippocampus, but this was
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37 only significant in CA3 ($F(3,18) = 3.810$, $p < 0.03$) and approached significance in CA1 ($F(3,17)$
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39 $= 3.234$, $p = 0.055$).
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46 LFPI+vehicle rats also showed significantly increased microglia activation compared to
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48 control groups, as measured by OX-42 labeling, but this was restricted to the insula (Fig. 4D;
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50 $F(3,19) = 5.59$, $p < 0.007$). Analysis of sub-regions of the insula also revealed increases in
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52 microglial activation for LFPI+vehicle rats, and post-hoc comparisons showed that LFPI alone
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54 significantly increased OX-42 labeling in agranular ($F(3,19) = 11.186$, $p < 0.0001$), granular
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3 (F(3,18) = 3.740, p < 0.03), and approaching significance (F(3,19) = 2.742, p < 0.072) in
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5 dysgranular areas. No differences in OX-42 labeling were observed between surgically naïve,
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7 sham operated and LFPI+MN166 groups in any insular regions examined. No significant
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9 between group differences were found in OX-42 expression for the amygdala or hippocampus.
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Discussion

These data suggest a link between injury-induced brain inflammation and post-traumatic anxiety. Rats with LFPI display freezing responses to the minor stress of a novel environment that is 2-3 times normal and which, unlike controls, is nearly doubled by the delivery of a major foot-shock stressor. LFPI also results in marked reactive gliosis in brain regions associated with anxiety. The possibility that post-traumatic brain inflammation and gliosis may contribute to anxiety-like behavior observed here, is supported by the effects of glial-cell activation inhibitor MN166. MN166 reduces reactive gliosis and TBI-induced freezing behavior, rendering these animals histologically and behaviorally indistinguishable from naïve and sham operated controls. To our knowledge, this is the first study to report pharmacological immunosuppression resulting in the reduction of anxiety-like behaviors following TBI.

A possible mechanism for neuroimmune induced post-traumatic anxiety.

Our finding of prolonged reactive gliosis in brain structures including, but likely not confined to, the hippocampus, amygdala and insular cortex, suggests that these structures may contribute to the persistent enhanced freezing of our brain-injured animals in reaction to a novel environment. All three structures have been implicated in rodent research investigating the pathogenesis of anxiety (Davis, 1992; Davis et al., 1994; Davidson, 2002; Vyas et al., 2004; Paulus and Stein, 2006; Rauch et al., 2006; Canteras et al., 2010) and fear behavior in the rat (Sullivan, 2004; Rosen and Donley, 2006; Milad et al., 2009; Liu et al., 2010).

The mechanisms by which immune responses may contribute to dysfunction of these structures remain to be determined. It is well established that LFPI in the rat results in activation of microglia and astrocytes as part of the innate immune response to insult. A number of studies

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3 indicate that LFPI-induced reactive gliosis follows a distinct time-course, beginning with
4 predominant microglia activation that peaks within a week (Hill et al., 1996; Nonaka et al., 1999;
5 Grady et al., 2003; Gueorguieva et al., 2008; Clausen et al., 2009; Yu et al., 2010) but continues
6 for several weeks and overlaps later with persistent astrocytic activation (D'Ambrosio et al.,
7 2004; Yu et al., 2010). Microglia are resident macrophages and first responders to pathogens and
8 neuronal insults in the CNS. They react rapidly, leading to activation of astrocytes and prolonged
9 disruption of neuronal function (Iravani et al., 2005; Herber et al., 2006; Zhang et al., 2009;
10 Zhang et al., 2010). Several lesion paradigms have also shown rapid microglial response
11 followed by delayed astrocyte reaction (Gehrmann et al., 1991; Dusart and Schwab, 1994; Frank
12 and Wolburg, 1996; McCann et al., 1996; Liberatore et al., 1999).

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27 Our results support this well-documented temporal relationship suggesting that microglial
28 activation precedes astrocytic activation and plays a role in the onset and maintenance of
29 astrogliosis (Graeber and Kreutzberg, 1988; McCann et al., 1996; Hanisch, 2002; Iravani et al.,
30 2005; Herber et al., 2006; Zhang et al., 2010). This time-course is consistent with behavioral
31 freezing responses in the present study, appearing rapidly within 2 weeks but persisting unabated
32 for the 3-month post-injury measurement period. It is also consistent with our
33 immunohistochemistry results, indicating injury-induced astrocytic activation in all 3 regions of
34 interest, insula, amygdala and hippocampus at 3 months post-injury, but less activation of
35 microglia, only significant in the insula. The lower levels of microglia expression are likely due
36 to assessment at 3 months post-injury.

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51 Trauma-related reactive gliosis is well known to result in the release of high levels of
52 pro-inflammatory cytokines, specifically tumor necrosis factor- α (TNF- α) (Taupin et al., 1993;
53 Fan et al., 1996; Lloyd et al., 2008), interleukin-1 beta (IL-1 β) (Taupin et al., 1993; Fan et al.,
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3 1995; Fassbender et al., 2000; Yan et al., 2002; Lloyd et al., 2008), and interleukin-6 (IL-6;
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5 (Taupin et al., 1993; Yan et al., 2002; Lloyd et al., 2008), which are central mediators of
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7 neuroinflammation following head injury (Fan et al., 1995; Rothwell and Hopkins, 1995;
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9 Rothwell and Strijbos, 1995; Fan et al., 1996; Simi et al., 2007). Release of these pro-
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11 inflammatory cytokines, particularly IL-1 β and TNF- α , pathologically increases neuronal
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13 excitability in all brain regions where it has been measured (Riazi et al., 2008; Schafers and
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15 Sorkin, 2008; Rodgers et al., 2009; Beattie et al., 2010; Maroso et al., 2010). While neuronal
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17 excitability and proinflammatory cytokine levels were not measured in the present study,
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19 neuroinflammation has been implicated in neuronal excitability of amygdala and insular cortex
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21 and anxiety-like behavior by others using c-Fos labeling (Abrous et al., 1999; Ikeda et al., 2003;
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23 Kung et al., 2010). These same regions have also consistently been reported to be hyperexcitable
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25 in human imaging data across a variety of anxiety disorders (Rauch et al., 1997; Shin et al.,
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27 2006; Simmons et al., 2006; Stein et al., 2007; Shin and Liberzon, 2010; Carlson et al., 2011).

33 *Attenuation of post-traumatic anxiety with MN166.*

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Meta-analysis of the impact of pharmacological treatments on behavioral, cognitive, and motor outcomes after traumatic brain injury in rodent models (Wheaton et al., 2011) indicates that of 16 treatment strategies evaluated to date, improved cognition and motor function have been reported, but almost no treatments have improved behaviors related to psychiatric dysfunction in general and anxiety in specific. Exceptions to this are recent promising reports of treatments such as magnesium sulphate to limit excitotoxic damage (Vink et al., 2003; Fromm et al., 2004; O'Connor, 2003, 533-41) and resevatrol to limit excitotoxicity, ischemia, hypoxia (Sönmez et al., 2007), both increasing open field exploration (resulting from decreased freezing) and therefore presumably decreasing post-injury anxiety.

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Glial targeted immunosuppression has also been found to be neuroprotective following TBI in rodents, resulting in increased structural preservation and improved functional outcomes (Hailer, 2008); including recent reports that MN166 significantly attenuated brain edema formation, cerebral atrophy and apoptosis in neuronal cells following ischemic brain injury in rats, increasing neuronal survival rates (Lee et al., 2011). MN166 may reduce neuronal damage in regions involved in anxiety, mitigating the role of glial activation, neurotoxicity and hyperexcitability in the subsequent development of anxiety-like behaviors. While not focused on post-traumatic anxiety, MN166 has been found to reduce intracellular calcium accumulation (Yanase et al., 1996), apoptosis, functional damage and passive avoidance behaviors following a transient ischemia model in rats (Yoshioka et al., 2002). Increasing evidence supports neuroinflammation, chronic inflammatory responses, proinflammatory cytokines, neuronal hyperexcitability, and secondary injury cascades in the pathophysiology of post-traumatic anxiety. The mechanisms of the effect of MN166 on TBI-induced anxiety-like behavior are not fully known. However, the results of this study provide evidence of a neuroprotective role for MN166 in attenuating and perhaps preventing development of post-traumatic anxiety.

Further establishing a relationship between TBI, neuroimmune responses, neurocircuitry and anxiety disorders, is important to further understand the sequelae of TBI and to the development of effective treatment strategies. The development of anxiety disorders following TBI is a complex and multifaceted problem, and finding treatments that work will require multifaceted approaches. The injury itself initiates many complex biological events including glial activation, breakdown of the blood brain barrier, excitotoxicity and chronic neuroinflammation. While primary injury often cannot be prevented, it may be possible to reduce secondary injury, leading to better functional and behavioral recovery following TBI. The

1
2
3 present results, using peri-injury treatment with MN166 to prevent post-traumatic freezing
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5 behavior, not only suggest a role for neuroimmune inflammation in anxiety physiology, but
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8 similarly successful results with post-injury treatment could introduce a promising and clinically
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10 realistic translational possibility for prevention of post-traumatic anxiety in humans.
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6 **Author Disclosure Statement**
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8 Krista M. Rodgers: No competing financial interests exist.
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Figure Captions

Figure 1. Cylinder task and running wheel activity at 1 week post-injury. **(A)** LFPI rats mean number of spontaneous forelimb placements (ipsilateral and contralateral) during exploratory activity in the cylinder test did not differ from controls at 1 week post-injury. A reduction was seen in contralateral limb-use in injured rats, but this reduction did not reach significance ($p=0.741$). **(B)** LFPI rats mean change in distance traveled in the running wheel activity did not significantly differ from controls at 1 week post-injury. Data represent mean \pm SEM.

Figure 2. Freezing behavior in a novel context. Both surgically naïve and sham operated rats froze approximately 5-10% at post-surgical measurement points before (2 weeks and 1 month) after (2 and 3 month) foot-shock (arrow). In contrast, LFPI rats froze significantly longer (~20%) than these controls before shock. After shock, untreated LFPI rats (LFPI-vehicle) nearly doubled in time freezing (~50%) whereas treated LFPI rats (LFPI+MN166) showed only a slight increase (~25%) that did not reach significance ($p=0.316$). The effect of injections alone (Sham+Mn166 and Sham+vehicle) were to increase freezing behavior compared to un-injected naïve and sham operated rats, particularly at the 2 and 3 month post-shock measurement points where freezing in these rats could not be distinguished from LFPI rats treated with MN166. Data represent mean \pm SEM.

Figure 3. Representative images depicting GFAP immunoreactivity (reflecting astrocytic activation) assessed in the hippocampus, amygdala and insula at 3 months post-injury. LFPI rats injected with vehicle showed clear signs of reactive astrocytes (bottom row), while naïve and sham operated rats appeared to have normal astrocyte morphology. LFPI rats treated with

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3 MN166 (third row) were difficult to differentiate from surgically naïve and sham operated
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10 **Figure 4.** Regional and sub-regional analyses of microglial and astroglial activation in
11 hippocampus, amygdala and insula at 3 months post-injury. (A-C) LFPI vehicle injections
12 induced a significant increase in GFAP labeling in all three regions, compared to surgically
13 naïve, sham operated and LFPI+MN166 treated rats. (D) In the insula, OX-42 activation was
14 greater in LFPI rats compared to surgically naïve, sham operated and LFPI+MN166 treated rats.
15 There were no significant differences found between surgically naïve, sham operated and
16 LFPI+MN166 treated rats in either analysis. Data represent mean± SEM integrated densities of
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Figure Captions

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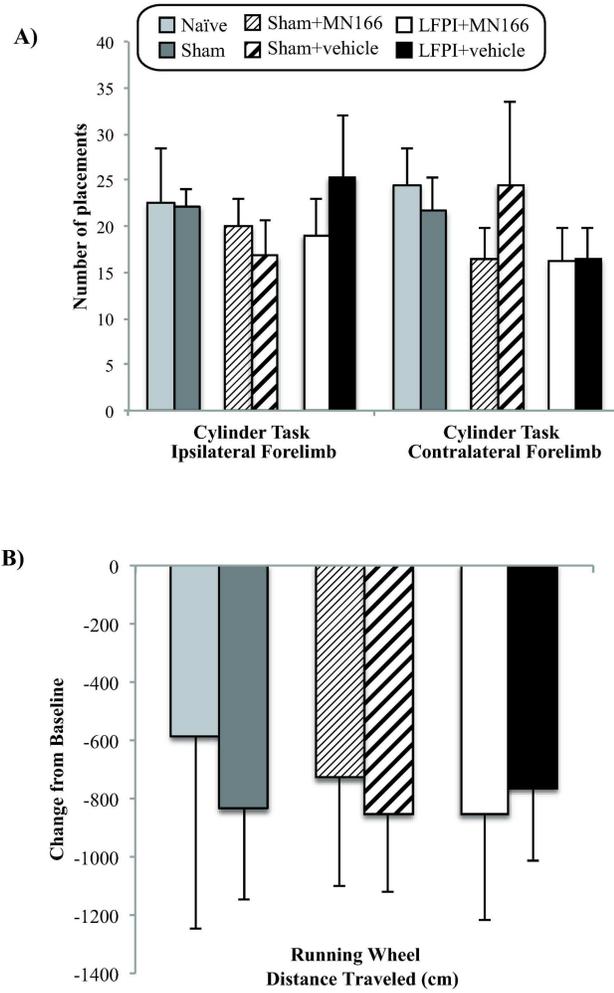
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Figure 4. Regional and sub-regional analyses of microglial and astroglial activation in hippocampus, amygdala and insula at 3 months post-injury. **(A-C)** LFPI vehicle injections induced a significant increase in GFAP

1 labeling in all three regions, compared to surgically naïve, sham operated and LFPI+MN166 treated rats. (D) In
2 the insula, OX-42 activation was greater in LFPI rats compared to surgically naïve, sham operated and
3 LFPI+MN166 treated rats. There were no significant differences found between surgically naïve, sham operated
4 and LFPI+MN166 treated rats. There were no significant differences found between surgically naïve, sham operated
5 and LFPI+MN166 treated rats in either analysis. Data represent mean± SEM integrated densities of
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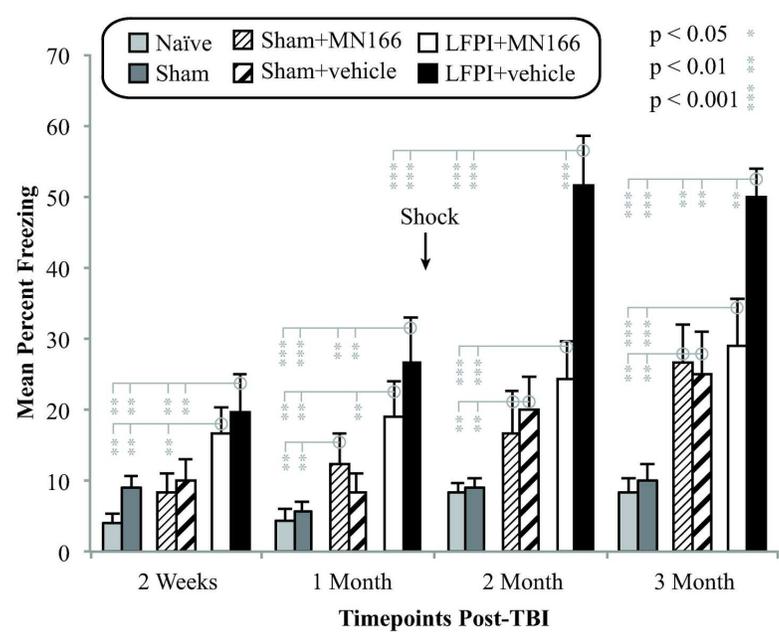


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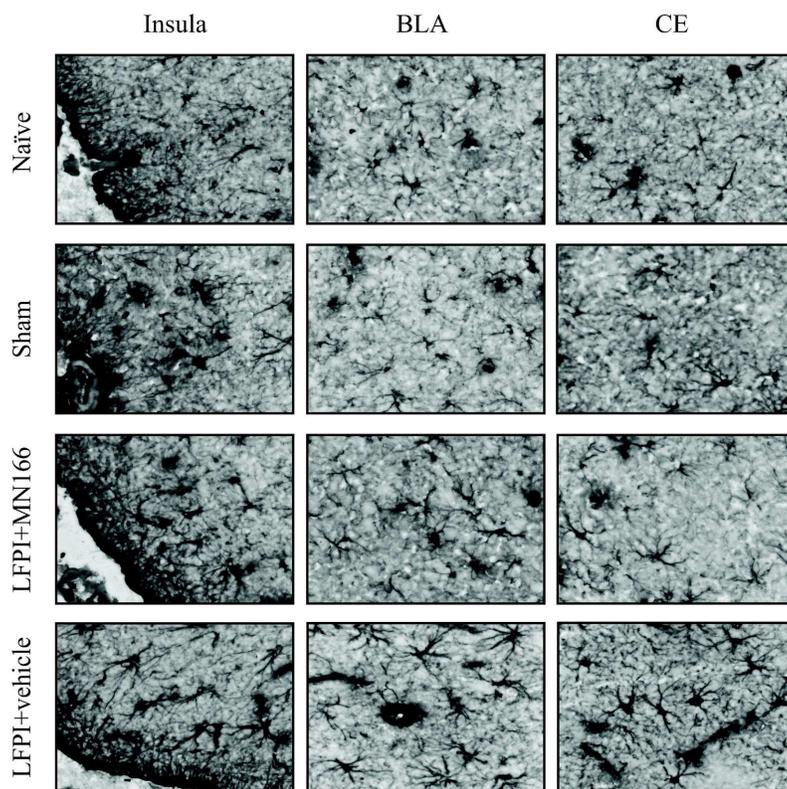
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Fig.2



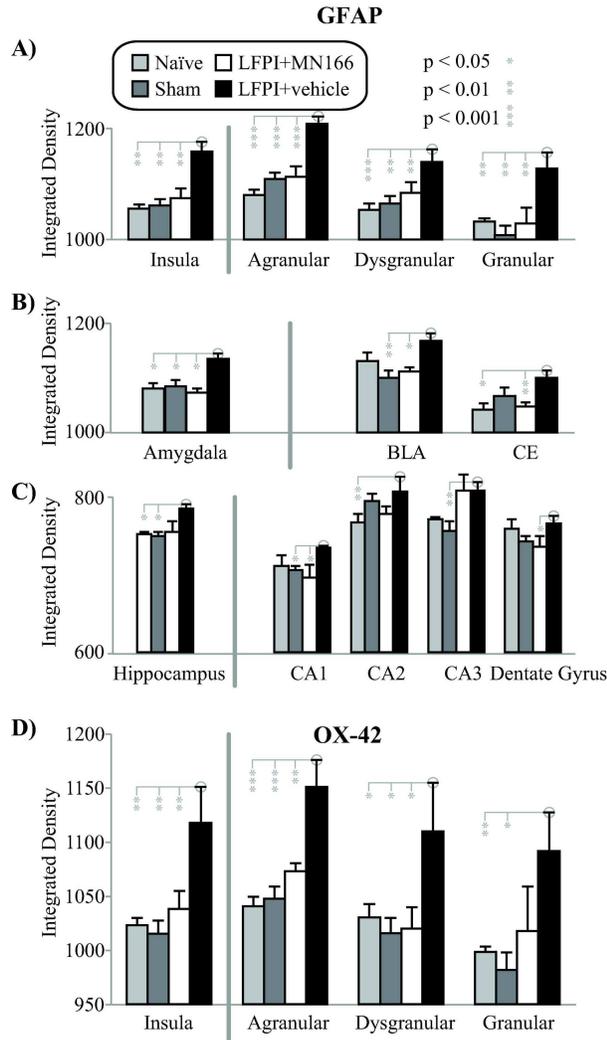
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Fig.3



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Fig.4



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Title of Study: **A Very High Speed System for Video/EEG Monitoring and Quantification of Post-traumatic Epileptogenesis**

This abstract is being submitted for (check one):

- Oral presentation or poster display
- Oral presentation only
- Poster display only

This presentation represents:

- Quantitative research Qualitative research
- Research utilization Combined methods
- Clinical innovation

Consideration for Young Investigators' Forum (check

one): YES NO

Research Topic: Traumatic brain injury / Healthcare informatics and medical systems

If selected, the presenter will be: Daniel Barth

A Very High Speed System for Video/EEG Monitoring and Quantification of Post-traumatic Epileptogenesis

Daniel S. Barth, Ph.D.

PURPOSE/AIMS: The overall goal of this project is to examine the role of brain inflammation in the development of post-traumatic epilepsy (PTE), and to prevent PTE with newly developed drugs that modulate the brain's immune system following injury. We use a lateral fluid percussion injury (LFPI) in the rat, a widely accepted animal model of closed head traumatic brain injury (TBI) experienced by war fighters in the battlefield. Since our major goal is to prevent the development of PTE (epileptogenesis), our first challenge was to devise methods by which we could unambiguously identify electrical (EEG) and behavioral (video) biomarkers of the epileptic brain prior to appearance of the first seizure. This is a daunting task given the vast quantities of long-term video/EEG that must be recorded and analyzed from a large number of animals.

DESIGN: To this end, during the first project year, we developed a unique system for recording, visually reviewing, and quantifying video/EEG from up to 32 animals in parallel.

POPULATION/SAMPLE STUDIED: Sprague Dawley rats with and without LFPI to the parietal and motor cortex are presently under investigation.

METHOD(S): We designed and constructed a unique recording system, based on compact amplifiers and miniature surveillance cameras, that is inexpensive, durable, and performs at a low bandwidth, permitting storage and review of large amounts of data recorded continuously for weeks.

DATA ANALYSIS: The most innovative component of the system is our specially designed software that permits very fast and interactive visual examination and event identification of hours of video/EEG data in minutes. The driving principle behind this software is that the human visual system is far more skilled and reliable than automated systems for identifying epileptiform events in the EEG and validating these events with video-recorded behavior. The core of our software is a graphical user interface that makes this possible for minimally trained users.

FINDINGS: Our system is now allowing us to precisely identify potential EEG biomarkers during epileptogenesis, when intervention may be possible. The system is also allowing us to statistically quantify both abnormal and normal EEG activity from long-term recording in animal models of TBI.

CONCLUSIONS/RECOMMENDATIONS: It is now possible to record and visually analyze long-term video/EEG data following brain trauma at a minimum cost and with sufficient speed and accuracy to make statistical analysis of normal and pathological EEG biomarkers possible for the first time.

IMPLICATIONS: We have begun analyzing epileptogenesis in LFPI and in a pilocarpine model. We have identified archetypical patterns in the normal EEG that could be easily mistaken for epileptiform. This knowledge will serve as critical foundation for studying the effects of neuro-immune modulating compounds following trauma in preventing epileptogenesis during the second project year. We also anticipate an unplanned application to military medicine to detect potential post-traumatic neurological disturbance and facilitate return to duty decisions.

FROM/TO TIME PERIOD OF STUDY: 07/01/11 to 06/30/12

FUNDING: CDMRP #PR100040