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Dear Members of the New Jersey Commission on Brain Injury Research Board:

Attached please find the Final Progress Report for our postdoctoral fellowship grant from the New Jersey Commission on Brain Injury Research, entitled "Investigating the role of MHC class I proteins in traumatic brain injury".

During the course of this fellowship, Dr. Tyler made significant progress in understanding how MHCI immune proteins regulate NMDA receptors in the brain, and may influence neuronal damage and death following Traumatic Brain Injury (TBI). This work identifies new a potential therapeutic target for improving outcomes after TBI.

The results of this work have been presented at several scientific conferences and are reported in a manuscript that will be submitted for publication in the next few months.

A signed original and two hard copies of this Report have been mailed to the New Jersey Commission on Brain Injury Research office. Please feel free to contact me if you have any questions.

Sincerely,

Lisa M. Boulanger
Assistant Professor
Department of Molecular Biology and Princeton Neuroscience Institute

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2) Institution: Princeton University
3) Grant title: Investigating the role of MHC class I proteins in traumatic brain injury
4) Grant number: 10.001.BIR3
5) Grant period covered by the report: 11/1/10-10/31/13
6) Date of submission of the report: 03/09/14

**Fellowship Grant Final Progress Report**

Traumatic Brain Injury (TBI) dramatically increases release of the neurotransmitter glutamate, resulting in prolonged neuroexcitation surrounding the injury site. This neuroexcitation can cause excitotoxic cell death, which contributes to pathophysiology and exacerbates functional deficits following brain injury. TBI-induced excitotoxicity is thought to be mediated, in part, by the *N*-methyl-*D*-aspartate-type glutamate receptor (NMDAR). Under normal conditions, NMDARs mediate synaptic communication, but overstimulation of NMDARs can trigger excitotoxic neuronal death. Given the role of NMDARs in neuronal death after TBI, it is not surprising that current therapies to limit post-TBI excitotoxicity include several NMDAR inhibitors. However, these drugs generally have severe side effects, because they inhibit both the pathological and essential functions of NMDARs. Our objective in this proposal was to better understand *endogenous* regulation of NMDAR function, with the ultimate goal of identifying more selective, better-tolerated approaches to prevent TBI-induced excitotoxicity.

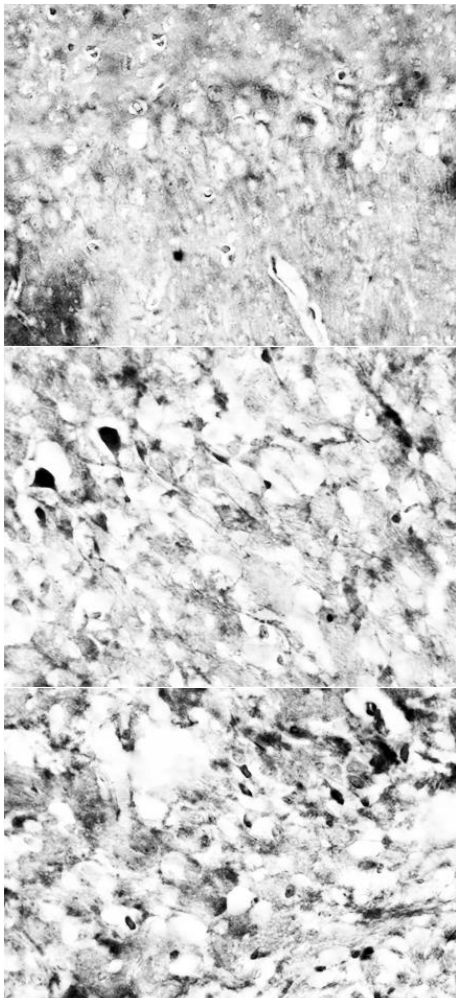
Our central hypothesis was that changes in the levels of proteins of the major histocompatibility class I (MHCI) regulate NMDAR-mediated excitotoxicity following TBI, and affect the extent of injury and the potential for functional recovery. If this is the case, then MHCI might be a novel target for treatments to limit brain damage following TBI. Our hypothesis was based on our recent finding that NMDARs are inhibited by MHCI in the healthy brain (Fourgeaud et al., 2010). MHCI is upregulated following peripheral nerve injury in animal models, but the effect of TBI on central nervous system MHCI expression and the potential role of MHCI regulation of the NMDA receptor during TBI-induced excitotoxicity is unknown. Over the funding period, we addressed these fundamental questions, and collected data that we will use as the basis for a future application for an NIH R01.

At the start of the project, we traveled to the University of Pennsylvania/Childrens' Hospital of Philadelphia to receive training in the lateral fluid percussion injury (FPI) model of traumatic brain injury in mice from collaborator Dr. Akiva Cohen. Dr. Cohen has several years' experience with mouse FPI and has published several studies using this model. During our visit, we observed the technique, received hands-on experience with all steps in inducing the model, and received detailed FPI protocols. In addition,

members of Dr. Cohen's lab advised us on how to construct the FPI equipment and how to perform and analyze these experiments in our lab at Princeton. Following this meeting, we purchased equipment for performing TBI in our own lab, including a Stoelting Lab Standard Mouse/Neonatal Rat Adaptor stereotaxic frame for craniotomy surgeries, the same model used by the Cohen lab, as well as VetEquip Tabletop Laboratory Animal Anesthesia System for craniotomy and FPI procedures. This anaesthesia system is similar to one in use in the Cohen Lab, and allows several craniotomy surgeries and FPIs to be performed in one day, facilitating induction of FPI in a cohort of animals, by enabling quick, reproducible, and highly regulated inhalation anesthesia in multiple animals simultaneously.

A central Aim of this study was to determine if MHCI levels are increased in the central nervous system (CNS) following TBI. To answer this question, we examined MHCI expression in the adult mouse hippocampus following induction of the FPI model of TBI, using immunohistochemical protein labeling techniques and confocal microscopy. We identified appropriate antibodies, optimized protein detection methods, and characterized the baseline levels and distribution of MHCI protein in the uninjured CNS. With these tools in hand, we performed immunostaining experiments in which MHCI expression is compared among animals exposed to FPI (3 days post FPI) as well as sham-treated animals (in which a surgical window was opened, but FPI was not induced). We detected strong, specific MHCI labeling of neurons throughout the brain in uninjured adult mice. Furthermore, we saw that FPI causes a

**Figure 1. FPI increases MHCI immunostaining in adult mouse brain.** Brightfield images of DAB labeling with an anti-MHCI antibody in area CA1 of hippocampus. Top, untreated control; middle, sham; bottom, FPI.



dramatic upregulation of MHCI protein in many regions of the brain, even at sites distal to the injury, including dentate gyrus (not shown) and area CA1 of hippocampus (Figure 1). These results have been replicated using multiple uninjured and FPI-treated adult animals. These studies allow us to conclude that TBI triggers a dramatic and widespread increase in MHCI expression in the adult brain.

This result is exciting for several reasons. First, it shows, for the first time, that an NMDAR inhibitor (MHCI) is upregulated in the wake of TBI. Second, because MHCIs are immune proteins, our findings suggest that changes in immune signaling (e.g., due to infections related to the brain injury, unrelated systemic illness, or anti-inflammatory medications) may influence recovery from TBI in previously-unexpected ways. Third, it raises the possibility that currently-approved immunomodulatory drugs could help accelerate or augment this natural protection, and improve outcomes after TBI.

Relevant to the goals of Aim 2, we performed electrophysiological and biochemical experiments characterizing how MHCI affects NMDAR function. Our initial results indicated that lower levels of MHCI lead to an increase in NMDAR-mediated responses (recently reported in a poster at the Society for Neuroscience meeting, as well as a publication in the Proceedings of the National Academy of Sciences (Fourgeaud *et al.*, 2010)). We have now extended these findings in two key ways that provide mechanistic insights into how MHCI modifies NMDAR function in the normal and injured brain.

First, we identified cellular mechanisms by which MHCI limits NMDAR function. Many proteins regulate NMDAR function by influencing the synthesis of NMDARs, their subunit composition, or their trafficking to and from the synapse. We found that surprisingly, MHCI does not affect any of these parameters, despite its striking effects on NMDAR function. Using sophisticated Non-Stationary Noise Analysis of whole-cell patch clamp electrophysiological recordings, we determined that MHCI negatively regulates the current flowing through individual NMDAR ion channels (Figure 2). Thus MHCI is an endogenous inhibitor of NMDAR single-channel currents.

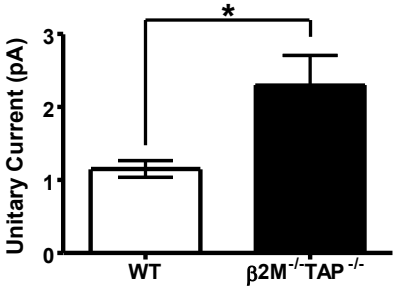



**Figure 2. Increased NMDAR single-channel current in MHCI-deficient mouse hippocampus.** Single-channel properties calculated via non-stationary noise analysis (see text).

Second, our biochemical studies show that MHCI-dependent changes in NMDAR function are associated with altered phosphorylation of specific tyrosines in particular NMDAR subunits. These changes in NMDAR phosphorylation are of particular interest since tyrosine phosphorylation of the NMDAR can modify single-channel currents. These results, which are reported in a paper in preparation, identify MHCI as one of only a handful of known endogenous inhibitors of NMDAR single-channel properties, and a promising target to suppress excitotoxicity in patients with TBI. These findings lay the groundwork for understanding how increased MHCI levels following TBI, identified above, could affect NMDAR function and subsequent excitotoxicity. Ultimately, these findings could help guide the development of MHCI-targeting drugs that reduce post-TBI pathology, while leaving essential NMDAR functions intact.

Our initial hypothesis was that endogenous MHCI limits NMDAR-mediated excitotoxicity following TBI. If this is the case, then the sequelae of TBI—in particular, those mediated by NMDAR over-activation—should be more severe in MHCI-deficient animals. Our preliminary findings suggest, remarkably, that MHCI-deficient animals instead appear to be *relatively protected* from the neuropathological effects of TBI. Thus, rather than being neuroprotective, endogenous MHCI may inhibit survival and/or promote excitotoxicity. Of note, our result parallels recent findings that MHCI exacerbates brain injury in rodent stroke models (Adelson et al., 2012).

This result, while unexpected, was a resounding confirmation of a key element of our hypothesis: it demonstrates that MHCI modifies excitotoxocity in the wake of TBI. Although it is unexpected, the fact that we find MHCI exacerbates TBI-induced damage is perhaps even more exciting outcome than the one we initially predicted, because it suggests that endogenous MHCI is a brake on TBI recovery. Thus the increases in MHCI expression we see following TBI (Figure 1) may actively impair the brain's intrinsic capacity for neuronal survival and recovery. If this is the case, then it may be possible to relieve MHCI's brake on survival, and improve outcomes after TBI.

There were several technical challenges associated this project. As anticipated, both FPI and sham treatment increased nonspecific background immunostaining, as evidenced by an increase in the

intensity of no-primary staining over untreated controls. To minimize this potential confound, we switched to a blocking protocol that is optimized to reduce background, and used avidin-biotin amplification to increase the signal-to-noise ratio. Using this protocol, we found that MHCI levels increase following TBI. Staining was also increased, to a lesser extent, following sham treatment (Figure 1), indicating that opening a surgical window in itself represents a form of brain injury that is sufficient to slightly upregulate MHCI in neurons. In the future, it may be of interest to explore changes in MHCI expression in less severe models of TBI (including micro-FPI and closed-skull models) and to examine MHCI expression earlier than three days post-injury, to further dissociate FPI-induced from post-surgical changes in MHCI expression.

Consistent with the possibility that FPI injury is relatively severe, the histological appearance of the tissue shows evidence of degeneration, even distal from the injury site. In fact, damage is so severe in most cases that immunostaining in the immediate area of the injury site (e.g., superficial layers of cortex) is not possible. To ensure that we could quantify MHCI levels in intact as well as damaged tissue, we used a combination of immunohistochemistry (staining intact brain sections) and western blotting (staining in brain homogenates—samples that can be obtained even from severely damaged brain). For future studies, we have begun to construct a Micro-FPI device, which produces similar results to the larger, standard FPI device, but with finer control over the severity of the injury (Kabadi et al., 2010).

In summary, the studies performed during this Fellowship demonstrate, for the first time, that experimental TBI models significantly increase MHCI expression in the adult brain, and that MHCI inhibits single-channel currents mediated by synaptic NMDARs. They also indicate that increased MHCI following TBI may unexpectedly *exacerbate* the neurotoxic effects of TBI. This result is exciting because it suggests that the brain has an intrinsic capacity for survival and recovery that is actively inhibited in the wake of TBI. If this is the case, then it may be possible to relieve MHCI's brake on the survival pathway to limit cell death and enhance repair and functional recovery following TBI.

**Publications:**

**Tyler, C.M.,** Boulanger, L.M. (2012) Complement-mediated microglial clearance of developing retinal ganglion cell axons. *Neuron.* 74(4):597-599.

Fourgeaud, L., Davenport, C.M., **Tyler, C.M.,** Cheng, T.T., Spencer M.B., Boulanger, L.M. (2010) MHC class I modulates NMDA receptor function and AMPA receptor trafficking. *PNAS.* 107(51): 22278-22283.

**Manuscripts in Preparation**

**Tyler, C.M.,** Aguilar, J.I., Boulanger, L.M. MHCI is an endogenous regulator of hippocampal NMDA receptor single-channel currents.

**Tyler, C.M.,** Frietze, K. K., Melson, J.W., Pappy, A.L., Perlman, D. H., Boulanger, L.M. NMDAR phosphorylation is inhibited by MHCI immune proteins.

**Abstracts**

**C.M. Tyler**, M. Chacon, J.I. Aguilar, D.H. Perlman, L. Fourgeaud, L.M. Boulanger (2013) MHC class I is a voltage-dependent regulator of NMDA receptor-mediated single-channel currents. *Society for Neuroscience Abstr.*

L.M. Boulanger, L. Fourgeaud, **C.M. Tyler**, J.I. Aguilar, M.A. Chacon (2013) Control of synaptic transmission and synaptic plasticity by MHCI immune proteins. *International Society for Neurochemistry Abstr.*

**Tyler, C.M.,** Fourgeaud, L., Aguilar, J.I., Chacon, M., Boulanger, L.M. (2011) MHC class I regulates NMDA receptor function in the hippocampus. *Society for Neuroscience Abstr.*