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# THE DEVELOPMENT OF LASTING IMPAIRMENTS: A MILD PEDIATRIC BRAIN INJURY ALTERS GENE EXPRESSION, DENDRITIC MORPHOLOGY, AND SYNAPTIC CONNECTIVITY IN THE PREFRONTAL CORTEX OF RATS

**R. MYCHASIUK,[a]* H. HEHAR,[a] I. MA,[a] B. KOLB[b] AND M. J. ESSER[a]**

[a] *Alberta Children's Hospital Research Institute, University of Calgary, Faculty of Medicine, Calgary, Canada*

[b] *Canadian Centre for Behavioural Neuroscience, University of Lethbridge, Lethbridge, Canada*

**Abstract—Apart from therapeutic discovery, the study of mild traumatic brain injury (mTBI) has been focused on two challenges: why do a majority of individuals recover with little concern, while a considerable proportion suffer with persistent and often debilitating symptomology; and, how do mild injuries significantly increase risk for an early-onset neurodegeneration? Owing to a lack of observable damage following mTBI, this study was designed to determine if there were changes in neuronal morphology, synaptic connectivity, and epigenetic patterning that could contribute to the manifestation of persistent neurological dysfunction. Prefrontal cortex tissue from male and female rats was used for Golgi-Cox analysis along with the profiling of changes in gene expression (*BDNF*, *DNMT1*, *FGF2*, *IGF1*, *Nogo-A*, *OXYR*, and *TERT*) and telomere length (TL), following a single mTBI or sham injury in the juvenile period. Golgi-Cox analysis of dendritic branch order, dendritic length, and spine density demonstrate that an early mTBI increases complexity of pyramidal neurons in the mPFC. Furthermore, there are also substantial changes in the expression levels of the seven genes of interest and TL following a single mild injury in this brain region. The results from the neuroanatomical measures and changes in gene expression indicate that the mTBI disrupts normal pruning processes that are typically underway at this point in development. In addition, there are significant interactions between the social environment and epigenetic processes that work in concert to perpetuate neurological dysfunction.**
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**Key words: Golgi-Cox, qRT-PCR, telomere, neurodegeneration, pruning, epigenetics.**

*Corresponding author. Address: Alberta Children's Hospital Research Institute, University of Calgary, Faculty of Medicine, Heritage Medical Research Building Room 274, 3330 Hospital Drive NW, Calgary, AB T2N 1N4, Canada. Tel: +1-403-210-7178, fax: +1-403-955-7649.

E-mail address: rmmychas@ucalgary.ca (R. Mychasiuk).

*Abbreviations:* ANOVA, analysis of variance; mTBI, mild traumatic brain injury; TL, telomere length.

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## INTRODUCTION

Mild traumatic brain injuries (mTBI) are relatively common occurrences in childhood with at least 10% of children experiencing an injury by 10 years of age (Faul et al., 2010). While many of these children recover without incidence, a significant proportion suffers from lingering symptomology and present with persistent cognitive and neurological impairments (Barlow et al., 2010). However, research is currently unable to explain how a mild brain injury in the pediatric period is capable of producing enduring impairments and in some situations a prolonged risk for neurological diseases. As there is generally no detectable damage to the brain, mild brain injuries are generally diagnosed based upon their pathological characteristics (e.g. result from forceful biomechanical movement of the head; acceleration/deceleration) and resultant symptomology (e.g. confusion, disorientation, loss of consciousness less than 30 min, etc.) (McCrory et al., 2013). Histological studies tend to corroborate beliefs regarding single mild injuries and often describe very few detectable pathologies; in contrast, individuals with histories of repetitive mTBI often exhibit extensive neurofibrillary tangles, amyloid-ß and tau pathologies, along with brain atrophy and white matter degeneration (for review see (Smith et al., 2013)). Rendering it even more difficult to generate a mechanistic relationship between early mTBIs and longer-term outcomes is a lack of pediatric research. The majority of experimental investigations regarding pathophysiological outcomes associated with mTBI have been conducted on adult brain tissue (e.g. Hamberger et al., 2009; Viano et al., 2009, 2012; Shultz et al., 2011; Jeter et al., 2013). Owing to the great deal of heterogeneity that exists between the developing and adult brain, mild insults early in life may impact disease trajectories in a vastly different manner.

The purpose of this study was to investigate the neuroanatomical changes and modifications to gene expression that occur in the prefrontal cortex following a single mild brain injury in juvenile rats. The prefrontal cortex was the primary area of exploration because it is significantly correlated with the functional impairments generally reported after mTBI and it is undergoing substantial growth and plasticity during childhood and adolescence (Bock et al., 2005; Kolb et al., 2012). mTBIs are often associated with deficits in executive control and

attention (Larson et al., 2011; Golmirzaei et al., 2013; Howell et al., 2013), impaired social skills (Janusz et al., 2002; Mychasiuk et al., 2014b), and diminished short-term/working memory (Malojcic et al., 2008); all of which rely upon the neural circuits of the prefrontal cortex. The examination of the medial prefrontal cortex (mPFC) included Golgi-Cox investigation of pyramidal neuron morphology, analysis of gene expression changes for seven distinct genes, *Nogo-A*, *DNMT1*, *BDNF*, *FGF2*, *IGF1*, *Oxytocin-Receptor*, and *TERT*, and determination of telomere length (TL). The genes that were selected for analysis play important roles in typical brain development, recovery from injury, and modulation of future behaviors. *BDNF*, *IGF1*, and *FGF2* are growth factors important for normal brain development, but are also involved in injury repair and compensation; *DNMT1* and *Nogo*-A (also known as *Rtn4*) are epigenetic modulators of plasticity with *Nogo-A* being particularly involved in preventing neuronal growth after neurological injury; the oxytocin-receptor (*OXY*R) and oxytocin participate in neuroprotection and plasticity with specialized involvement in social interactions; and, although also exhibiting neuroprotective properties, the primary role of *TERT* is elongation of telomeres. Finally, because TL has been recognized as a biomarker for cellular aging and senescence that relates not only to longevity, but also the occurrence and progression of chronic disease (Entringer et al., 2012), changes in mPFC TL may predict the development of long-term degeneration.

## EXPERIMENTAL PROCEDURES

### Animals and mTBI induction

All animals were maintained in a temperature-controlled husbandry room (21 °C), on a 12:12-h light:dark cycle (lights on at 0700), with *ad libitum* access to food and water. All experiments were carried out in accordance with the Canadian Council of Animal Care and approved by the University of Calgary Conjoint Faculties Research Ethics Approval Board. Forty (20 Male: 20 Female) in-house bred animals were used for the experimental procedure. Animals were housed in same-sex groups of four. At postnatal day 30 (P30) half of the pups (2/cage) received an mTBI using the modified weight-drop technique as described elsewhere (Kane et al., 2012; Mychasiuk et al., 2014a), and the other half received a sham injury. In brief, animals were lightly anesthetized and placed chest down on a scored piece of tinfoil that was elevated 10 cm above a foam collection sponge. A 150 g weight was dropped from a height of 0.5 m through a guide tube, at which point it produced a closed-head glancing impact, propelling the rat through the tinfoil. The rat landed on the collection sponge in the supine position after undergoing a 180° vertical rotation that induces acceleration/deceleration and rotational forces on the brain. Immediately after injury induction, topical lidocaine was applied to the rats head and it was placed in a clean, warm cage to recover. Animals experiencing a sham injury were exposed to the same preparation procedure, were quickly placed on the scored tinfoil, removed from the tinfoil, also received administration of topical lidocaine and were then placed in a clean, warm cage to recover. The laboratory administered two tests to validate the presence of a mTBI; time-to-right (immediately post-injury) and beam-walking (24 h post-injury). In addition, the animals underwent play behavior testing and the results from this behavioral testing are published elsewhere (Mychasiuk et al., 2014b).

### Validation of mTBI

*Time-to-right:* Immediately after the mTBI or sham injury, animals are placed in a clean warm cage to recover in the supine position. The time required for each animal to right itself from the supine position to a prone or standing position is recorded as the time-to-right. Previous experiments have demonstrated that animals with a mTBI and post-injury symptomology exhibit significant increases in their time-to-right (Mychasiuk et al., 2014a).

*Beam walking:* Animals are tested in a beam-walking paradigm similar to that described by Schallert et al. (2002) 24-h post-mTBI. Animals traverse a 165-cm tapered beam from a starting position to their home-cage. The beam is equipped with 2-cm wide 'safety' ledges that catch the rat's foot when it slips while negotiating the narrowing walk. The rat is provided with a single learning trial which is followed by four videotaped trials, each separated by 60 s rest periods. The video camera is set up at the starting point and positioned to view the entire length of the beam. A researcher blind to the experimental conditions scored the videos for the number of hind-leg foot slips each rat experiences during the four test trials. Prior experiments have demonstrated that animals with an mTBI display significantly more hind-leg foot slips when compared to sham animals.

### Sacrifice, histological processing, and molecular analysis

*Sacrifice.* At approximately P50 animals were sacrificed and brain tissue was used for histological processing and molecular analysis. Half of the animals (10 Male: 10 Female) were administered an overdose of sodium pentobarbital, weighed, and intracardially perfused with 0.9% saline. The brains were removed and preserved in the dark in Golgi-Cox solution for 14 days. The other half (10 Male: 10 Female) were subjected to isoflourane inhalation, weighed and decapitated. Using the Zilles atlas (Zilles, 1985), tissue from the mPFC was removed, flash frozen on dry ice, and stored at −80 °C.

*Histological processing.* Following 14 days of preservation in Golgi-Cox solution, the brains were transferred to a 30% sucrose solution where they remained for at least 3 days. The brains were then cut at 200 μm on a Vibratome and mounted on gelatin-coated slides. Golgi-Cox staining of the brains was carried out in accordance with the procedures described by Gibb and Kolb (1998).

Neurons selected for analysis were derived from layer III of the Cg3 region of the anterior cingulate cortex (also known as the mPFC) as described by Zilles (1985).

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