Age-related changes in the nigrostriatal pathway following traumatic brain injury

Undergraduate Research Thesis

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Abstract

One of the leading risk factors for the development of a neurodegenerative disease is traumatic brain injury (TBI) through the secondary injury of prolonged inflammation. Aging populations have the most exaggerated immune responses following TBI that often result in hospitalization and/or death. While extensive research has investigated inflammatory mechanisms within the hippocampus that differ as animals age, little is known about the age-related changes in the ascending catecholaminergic systems of the brainstem. The current study investigated the effect of age on the expression of inflammatory proteins, motor learning deficits, and the integrity of the nigrostriatal pathway 28 days following traumatic brain injury. Age- and injury-related impairments in motor coordination and learning were observed 1 month after controlled cortical impact surgery. Interestingly, aged TBI-treated animals had increased tyrosine hydroxylase expression in the striatum and substantia nigra. Combined with low levels of pro- and anti-inflammatory cytokine expression observed across groups, this study points toward a novel compensatory mechanism following injury in the aged brain.

Introduction

Traumatic brain injury (TBI) is one of the leading causes of mortality and disability worldwide. According to the Center for Disease Control and Prevention (CDC), an estimated 1.7 million TBI’s occur every year (Faul et al., 2010). A forceful blow to the head or penetration of the brain that disrupts normal brain functioning characterizes TBI. Normal brain functioning is disrupted by both primary and secondary injury mechanisms.
(Loane and Faden, 2010). Primary injury mechanisms occur through gross damage to brain infrastructure at the time of the injury to neurons, blood vessels, glia, dura mater, and other supporting tissues. Secondary injury mechanisms manifest later and can persist for weeks and months after the initial injury. Among many others, proposed mechanisms for secondary injury include excitotoxicity from the excessive release of glutamate (Faden et al., 1989;), axonal injury (Sharp et al., 2014), apoptosis of neurons (Yakovlev et al., 1997), and overactivation of the immune system in the brain (Morganti-Kossman et al., 2007). These small molecular changes have a huge impact on how the brain processes information and coordinates movement inducing deleterious changes in cognitive and motor abilities.

Children 0-4 years, young adults 15-19 years, and adults over 65 are most likely to sustain traumatic brain injury, and between these three age groups, adults aged 75 years and older have the highest rates of TBI-related hospitalization and death (Faul et al., 2010). The aged brain has demonstrated more susceptibility than younger brains to the damaging effects of injuries such as stroke (Yager et al., 1997) and chronic inflammation (Godbout et al., 2005; Brothers et al., 2013b). Beyond the acute primary injury mechanisms of traumatic brain injury, secondary injuries predispose the brain towards neurodegenerative diseases such as Parkinson’s disease (PD) and Alzheimer’s disease (AD) (Sivanandam and Thakur, 2012). The dysregulation of the immune system in vulnerable regions of the brain, such as the brainstem and hippocampus, has been proposed as a viable mechanism for the widespread degeneration of neurons in these diseases (Smith et al., 2012; Wenk and Hauss-Wegrzyniak, 2001; Wenk et al., 2003; Bardou et al., 2014). Microglia are the immune cells of the brain and their long-term
activation has been recorded after TBI in both human patients and animal subjects (Carbonell and Grady, 1999; Ojo et al., 2013). Prolonged microglial activation is damaging to neurons and perpetuates disease states (Block et al., 2007). The present study investigated the effect of age on the long-term recovery of motor learning deficits, the expression of inflammatory proteins, and the integrity of ascending catecholaminergic systems following traumatic brain injury.

**Background**

A plethora of research has been conducted on the effects of neuroinflammation following TBI (Finnie 2013; Ou et al., 2014; Silleisen et al., 2014; Roth et al., 2014) and age-related differences in microglial activation (Lucin and Wyss-Coray 2009; Sandhir et al., 2008; Fenn et al., 2013). Exaggerated microglial responses to injury have been implicated in the poor recovery of elderly patients and aged mice after TBI (Pennings et al., 1993; Kumar and Loane 2012). However, studies on the detrimental effects of inflammation following brain trauma have mainly focused on the hippocampus (Rosi et al., 2005; Yeung et al., 2014; Bedi et al., 2013; Sun et al., 2013; Belarbi and Rosi 2013). As a critical structure for learning and memory formation, the hippocampus is essential to studying the cognitive and learning deficits after TBI.

While important to the progression of neurodegenerative diseases, studies on the hippocampus alone fail to account for other crucial areas in the brain where extreme cell loss occurs. In patients with PD and AD, significant early cell loss occurs in the substantia nigra, locus coeruleus, and raphe nucleus of the brainstem (Halliday et al., 2006; Heneka et al., 2010; Szot et al., 2006). These catecholaminergic nuclei synapse on
key centers for executive functions, like attention and goal-oriented behavior, such as the nucleus basalis of Meynert in the forebrain. Catecholaminergic neurons within the brainstem are sensitive to global inflammation induced by lipopolysaccharide (LPS) brain infusions and have an age-related increase in cell death and inflammatory markers (Bardou et al., 2014). Decreased dopamine production and motor deficits following TBI have been enumerated in the brainstem of young male rats (Huang et al., 2014). However to date, no one has studied the differential effects of age within the brainstem systems following TBI. Identifying key differences in inflammatory mechanisms between young and aged animals will help tailor treatment following TBI to more accurately target the main progenitors of harmful inflammation and prevent significant cell loss, therefore ameliorating major cognitive and motor deficits.

**Methods**

*Study Design*

Young (3 month, n = 24) and aged (24 month, n = 24) C57/B6 were divided into two cohorts: one young cohort and one aged cohort. Mice of each age were randomly assigned into groups of 12 that received either control or TBI surgeries. Of the twelve animals in each group, half were assigned to histology and half were assigned to biochemical analysis. Behavioral paradigms were assessed at 28 days after injury, and all mice were euthanized a day following the last behavioral task. All surgeries, behavioral paradigms, and brain extractions were conducted at the University of California, San Francisco. All histology, biochemical assays, and analysis were conducted at The Ohio State University.
Subjects

All mice were placed on a reverse 12/12-hours light-dark cycle in a temperature-controlled room (23°C) with access to water and food *ad libitum*. The mice were monitored closely for four weeks following surgery. Mice in each cohort were sacrificed on the same day during the dark cycle. The experiment was conducted in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals (NIH Publications No. 80-23) and the Institutional Animal Care and Use Committee. Approval for the experiment was obtained from the animal subjects review board at the University of California, San Francisco.

Controlled Cortical Impact

Mice were anesthetized with 2.5% isoflurane and placed in a stereotax for controlled respiration and head placement. A heating pad was placed underneath the bodies of the mice to maintain normal body temperature during surgery. Skin was sanitized with betadine solution, and a vertical, midline incision was made through the scalp. A circular craniotomy (3.5 mm in diameter) was made in the left parietal skull between bregma and lambda and 0.5 mm to the left of the midline. The skull was removed carefully without disruption of the underlying dura mater. All subjects underwent this procedure. The mice randomly selected for TBI were then given a controlled cortical contusion to the parietal cortex (Rosi et. al. 2012b). The lesion was produced via an impact device with a 3-mm diameter convex tip. To induce a moderate traumatic brain injury, the contact velocity was set to 4.5 m/s for a deformation of 1.5mm
below the dura mater. Following this procedure, the scalp was closed with sutures and the mice were given an injection of warm saline (0.5 ml) to prevent dehydration.

*Elevated Plus Maze*

On day 28 post-surgery, mice were placed in the middle of a lighted plus maze, facing an open end. Movements in closed and open arms were recorded during a period of 5 minutes with Ethovision tracking software. The elevated plus maze was used to assess anxiety behavior. Mice with greater anxiety spend a greater portion of the 5 minute test in the closed arms.

*Rotarod*

The rotarod task was used to measure motor coordination on Day 29 post-surgery and motor learning on Day 30 post-surgery. Mice were placed five at a time on an accelerating rotarod (Ugo Basile) at a fall height of 30”. The speed of rotation gradually increased from 5 m/s to 40 m/s over 5 minutes. Latency to fall was recorded over 2 days at 4 trials per day.

*Tissue Collection*

Mice assigned for histological analysis were lethally overdosed with ketamine (15 mg/kg) and were transcardially perfused with 50 mL of 0.9% saline containing 50 U/mL heparin, 120mL of 4% paraformaldehyde in 0.1M phosphate buffered saline (PBS) at pH 7.4. Brains were post-fixed in paraformaldehyde for four days and then stored in PBS at
4°C. Mice assigned for protein analysis were rapidly decapitated. Brains were microdissected and stored in an -80°C freezer.

**Histology**

Perfused brains were sliced on a vibratome in 40 µm thick coronal sections. Four slices per animal were first blocked in 5% NGS for 1 hour, following incubation with a primary antibody against tyrosine hydroxylase (TH, 1:1000, Millipore, anti-TH rabbit) overnight at 4°C on a shaker. Free-floating sections were washed in 1M TBST and the secondary antibody (anti-rabbit goat) was applied for 2 hours at room temperature. Brain slices were coverslipped and imaged with a confocal microscope and Nikon elements software. Thresholds of 110 (substantia nigra) and 225 (striatum) were utilized as a cut off for TH detection. Data is expressed as TH+ area fraction.

**Multiplex Protein Assay**

Protein quantification was conducted on fresh midbrain and striatum tissue. Samples were homogenized in cell lysis buffer (Bio-Rad, Hercules CA) and total protein quantified via Bradford assay. Levels of the inflammatory cytokines interleukin (IL-1α, IL-1β, IL-2, IL-3, IL-5, IL-6, IL-9, IL-10, IL-12, IL-13, IL-17a), tumor necrosis factor (TNF-α), Eotaxin, GM-CSF, IFN-γ, KC, MCP-1, MIP-1α, and MIP-1β were analyzed with Bio-Plex Pro™ Mouse Cytokine Group I magnetic bead immunoassays (Bio-Rad). Briefly, coupled beads were added to the bottom of a 96-well plate. Standards and samples were added in duplicate for 30 minutes. Pre-packaged detection antibodies
incubated with the samples for 30 minutes. 100x streptavidin-phycoerythrin (SA-PE), a conjugate reporter dye, was added for 10 min, after which samples were suspended in 100 µl of assay buffer. The plate was read in a dual detection multiplexing machine (Bio-Rad MAGPIX multiplex reader) where a laser detected each protein by the color of its antigen-specific bead and a reporter laser quantified each molecule based on the fluorescence of the antigen-specific streptavidin-phycoerythrin dye. Results are reported in pg/mg protein.

Statistics

All data was analyzed with 2-way ANOVAs, followed by post-hoc Tukey tests (p < 0.05) (Sigma Plot 12.5). When data did not exhibit normality, data was first normalized with a box-cox transformation. Data is expressed as mean +/- standard error of the mean (SEM). * represents p < 0.05, ** represents p < 0.001.

Results

Elevated Plus Maze

There were no significant differences between groups in the time spent in the open or closed arms (Figure 1).

Rotarod

On the first day of rotarod (Figure 2), there was an age-related impairment in motor coordination within sham and TBI groups (p < 0.001). Aged mice with TBI performed significantly worse than their aged sham counterparts (p < 0.05).
On the second day of rotarod (Figure 3), there was an age-related deficit in motor learning within TBI, as well as a significant difference in motor learning between aged sham and aged TBI mice (p < 0.001). Interestingly, young TBI mice performed significantly better than young shams on Day 2 of rotarod (p < 0.05).

**TH Expression in the Substantia Nigra and Striatum**

As expected, an age-related decrease in TH expression in the substantia nigra (Figure 4) was observed in aged shams compared to young shams (p < 0.001). Unexpectedly, aged TBI-treated mice had greater TH+ stain than aged controls (p < 0.05). In the striatum (Figure 5), there was an age-related decrease in TH expression within controls (p < 0.001). Mirroring the substantia nigra TH expression patterns, aged TBI-treated mice had greater TH expression than aged controls (p < 0.001). In addition, aged TBI-treated animals a greater TH+ area than young TBI-treated mice (p < 0.05).

**Cytokine Protein Expression**

There was no significant difference in the cytokine expression of interleukins (IL-1α, IL-1β, IL-2, IL-3, IL-5, IL-6, IL-9, IL-10, IL-12, IL-13, IL-17a), tumor necrosis factor (TNF-α), Eotaxin, GM-CSF, IFN-γ, KC, MCP-1, and MIP-1β between groups. There was an age-related increase in MIP-1α (p < 0.001). Macrophage inflammatory protein 1α (MIP-1α) is a chemokine produced by macrophages that recruits neutrophils to sites of injury or infection and can also induce the release of pro-inflammatory cytokines IL-1β, IL-6, and TNF-α. While this age-related increase in MIP-1α was statistically significant, we caution that it may not be physiologically relevant. Average MIP-1α
protein content ranged from a mere 1.66 to 4.56 pg/mg. Overall, these results indicate that inflammation had resolved by 28-days post-surgery in both the midbrain (Figure 6) and striatum (Figure 7).

Discussion

Traumatic brain injury can cause motor deficits in both young and aged animals; however, aged animals are more susceptible to injury than young animals (Yager et al., 1997; Godbout et al., 2005; Brothers et al., 2013b). We replicated this age-related vulnerability in this study, as we observed an injury-related deficit in motor coordination and learning in aged mice alone. At 28 days post injury, the performance of young TBI-treated mice was not significantly impaired compared to young controls, indicating that the young TBI-mice were able to recover motor ability and resolve secondary injury before aged mice.

We also replicated the age-related decrease in TH activity previously observed with age. Intriguingly, TBI-treated aged mice had a greater TH+ area in both the striatum and substantia nigra than aged controls. To our knowledge, this phenomenon has not been previously observed in aged mice. Recently, young rats were observed to have a greater TH+ expression in the ipsilateral substantia nigra 60 days following TBI (Acosta et. al., 2015). In parallel to the increase in TH expression, Acosta et. al. found an accumulation of α-synuclein and MHCII+ cells in the ipsilateral SN. According to these authors, microglia infiltrating into the substantia nigra altered the inflammatory
environment and therefore increased the build up of α-synuclein, predisposing the rats towards dopaminergic cell death later in life. The increase in TH expression in our models could be due to a compensatory mechanism for cell survival after traumatic brain injury.

Neurons in the substantia nigra could upregulate TH in response to an increase in brain derived neurotrophic factor (BDNF) or glial cell derived neurotrophic factor (GDNF). BDNF elevation has been linked to better performance in motor learning tasks sensitive to the motor cortex (Zhao et. al., 2015; Mang et. al., 1985). GDNF is especially promising as a potential regulator of the increase in dopaminergic synthesis we observed. Many studies have observed the positive effect of GDNF on the survival of dopaminergic cells in the substantia nigra through increases in synaptic excitability (Bourque and Trudeau, 2000), retrograde transport (Tomac et. al., 1995) and the inhibition of apoptosis (Burke et. al., 1998).

All of these studies, however, were conducted in young animals. In aged animals, the brain’s immune system is in a primed state where microglia and macrophages are easily activated and produce a more robust response to insult. We have previously shown that the inflammatory response to injury is dysregulated in aged rats (Hopp et. al., 2014); aged rats were more likely than young rats to translate cytokine mRNA into protein and had an elevated and mixed pro- and anti-inflammatory cytokine profile that outlasted young rats by days. While inflammatory protein levels were not significantly higher in aged TBI-treated mice at 28 days post injury, the increase in TH+ area could be a relic of a robust immune response at earlier time points. Inflammation may indirectly increase TH expression in young mice, too; however, as in our previous study, this inflammation
may have resolved sooner, allowing the young mice to return to baseline functioning before our end point. More work is necessary to elucidate the mechanism by which aged mice are more vulnerable to TBI than young mice.

Tailored treatments for aged individuals after TBI are needed to help reduce the risk of dementia in these individuals later in life. A recent retrospective cohort study of adults with various levels of traumatic brain injury found that adults 65 years or older had an increased risk of developing dementia following mild TBI (Gardner et. al., 2014). In an age where the prevalence of dementia is rising dramatically each year and is currently costing our economy $225 billion in AD alone (Alzheimer’s Association, 2015), it is imperative that new treatments are developed to combat the heightened risk of dementia following traumatic brain injury in aged adults.

References


Figures

Figure 1. The amount of time spent in open and closed arms of the elevated plus maze was recorded during single trials of five minutes each. No significant differences were observed.
Figure 2. Latency to fall was recorded on an accelerating rotarod. Significant age-related impairments in motor coordination were observed within sham and TBI groups (p < 0.001). Aged TBI mice fell off of the rotating rod significantly faster than aged shams (p < 0.05).
A. Latency to fall was recorded on an accelerating rotarod on Day 2. Aged TBI mice performed significantly worse than aged shams and young TBI-treated mice ($p < 0.001$). Intriguingly, young TBI mice performed better than young shams ($p < 0.05$). B.
Figure 4. Tyrosine hydroxylase positive area was recorded as a fraction of total substantia nigra area. Aged shams had less tyrosine hydroxylase (TH) expression than young shams (p < 0.001). Unexpectedly, aged TBI-treated mice expressed more TH than aged shams (p < 0.05).
Figure 5. Tyrosine hydroxylase positive area was recorded over total striatum area. Aged controls had less TH expression than young controls (p < 0.001). Aged TBI-treated mice had greater TH expression than aged shams (p < 0.001) and young TBI-treated mice (p < 0.05).
Figure 6. Cytokine protein expression in the midbrain. Data is expressed as fold change over young controls. Values less than 1 indicate a decrease in expression compared to young controls. MIP-1α was significantly increased in aged sham and TBI-treated mice (p < 0.001).

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Figure 7. Cytokine protein expression in the striatum. Data is expressed as fold change over young controls. Values less than 1 indicate a decrease in expression compared to young controls. MIP-1α was significantly increased in aged sham and TBI-treated mice (p < 0.001).