ASSESSMENT OF A NUTRITIONAL SUPPLEMENT CONTAINING RESVERATROL, PREBIOTIC FIBER, AND OMEGA-3 FATTY ACIDS FOR THE PREVENTION AND TREATMENT OF MILD TRAUMATIC BRAIN INJURY IN RATS

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Abstract—Children and adolescents have the highest rates of traumatic brain injury (TBI), with mild TBI (mTBI) accounting for most of these injuries. Adolescents are particularly vulnerable and often suffer from post-injury symptomologies that may persist for months. We hypothesized that dietary manipulations modify premorbid characteristics. 3S treatment altered the behavioral performance of sham animals indicating that diet and lifestyle are associated with mTBI outcomes in the developing brain. Adolescent male and female Sprague-Dawley rats were randomly assigned to the supplement (3S) or control condition, which was followed by a mTBI or sham insult. A behavioral test battery designed to examine symptomologies commonly associated with mTBI was administered. Following the test battery, tissue was collected from the prefrontal cortex (PFC) and primary auditory cortex for Golgi-Cox analysis of spine density, and for changes in expression of 6 genes (Aqp4, Gfap, Igf1, Nfl, Sirt1, and Tau). 3S treatment altered the behavioral performance of sham animals indicating that dietary manipulations modify premorbid characteristics. 3S treatment prevented injury-related deficits in the longer-term behavior measures, medial prefrontal cortex (mPFC) spine density, and levels of Aqp4, Gfap, Igf1, Nfl, and Sirt1 expression in the PFC. Although not fully protective, treatment with the supplement significantly improved post-mTBI function and warrants further investigation. © 2017 IBRO. Published by Elsevier Ltd. All rights reserved.

Key words: adolescence, concussion, RT-qPCR, Golgi-Cox, prefrontal cortex, primary auditory cortex.

INTRODUCTION

Children and adolescents have the highest rates of traumatic brain injury (TBI) resulting in hospital visits, with mild TBI (mTBI) accounting for 80% of these injuries (Halstead and Walter, 2010; Yeates, 2010; Prins et al., 2013). Adolescents are at particularly high risk to experience chronic post-injury deficits, with 15–20% suffering from post-concussive symptomologies that may persist for months to even decades (Barlow et al., 2010, 2015). Post concussive syndrome (PCS), especially during this critical period of development, often interferes with school, social, and family relationships, further exacerbating risk for poor outcomes (Yeates et al., 2005; Yeates, 2010; Rassovsky et al., 2015). It is currently unknown why many adolescents recover, whereas a significant proportion go on to suffer from PCS. This ambiguity in symptom presentation and prognosis is likely a reflection of the complexity of concussion pathophysiology and the heterogeneity of premorbid characteristics typically observed in patient populations (Cloots et al., 2008; Garden and Sullivan, 2010; Barkhoudarian et al., 2011). Understanding how heterogeneity in premorbid characteristics contributes to PCS presentation may allow us to generate effective therapeutics or preventative strategies that will eliminate PCS completely.

Research has demonstrated that dietary manipulations influence neurological health, cognition, brain evolution, and psychological well-being (For review see (Gomez-Pinilla, 2008)). Growing evidence indicates that excessive caloric intake and high-fat diets modulate brain plasticity, reducing cognitive functioning, recovery from injury, and increasing risk for neurodegeneration (Molteni et al., 2002; Pistell et al., 2010; Langdon et al., 2011; Mychasiuk et al., 2015a,b,c). Conversely, dietary restriction and low caloric intake have been linked to improved brain health, increased synaptic plasticity, and extended lifespan (Eckless-Smith et al., 2000; Wang et al., 2005; Colman et al., 2009; Rich et al., 2010). Given that significant changes to diet and lifestyle are often difficult to maintain, with just over half of patients adhering to recommended treatment regimens for medical conditions (Martin et al., 2005), dietary supplements may be a more effective strategy for manipulating neuroplasticity.

From a system-level approach, an ideal strategy to combat mTBI and promote overall health would be the development of a ‘single pill’, that when taken daily,
could increase neurological resiliency prior to an injury, attenuate the brain's response to the trauma, and mitigate symptomology following the injury. However, single drug-to-target approaches for the treatment of TBI have all failed (Zhang et al., 2010), indicating a need for novel (but empirically supported) strategies that challenge current practices. Therapeutic supplements that build cognitive reserve, attenuate or modify signaling cascades in the brain and other organ systems such as the enteric nervous system, and support neural compensation and repair, would be ideal for mTBI patients. Based upon these propositions and considering the developing adolescent brain, we hypothesized that the combination of resveratrol (RES), prebiotic fiber (PBF), and omega-3 fatty acids (also known as docosahexaenoic acid (DHA)) could be an effective therapeutic for mTBI. Although each of these supplements has shown promise with respect to the prevention and/or treatment of mTBI following independent examination, none have demonstrated the power to induce meaningful change from a systemic perspective. However, given their intrinsic abilities, combining RES, PBF, and DHA into a daily supplement has the potential to be a potent moderator of mTBI outcomes.

RES is a natural polyphenol found in grapes and wine that has confirmed efficacy as a moderator of neuroprotection, reducing damage following ischemic and traumatic brain injury (Della-Morte et al., 2009). RES retards potent anti-inflammatory activity that can act on all organ systems, but also decreases the generation of oxygen free radicals in the brain when administered after a neurological insult (Ates et al., 2007; Das and Das, 2007; Gatson et al., 2013). PBFs are non-digestible carbohydrates that promote the growth of beneficial bacteria in the gut, which are required for normal functioning of the intimate signaling network that links the brain and enteric nervous system (Parnell and Reimer, 2012). GI dysfunction is a known complication of TBI; neurological insults alter the natural bacterial flora of the gut and initiate cascades of intestinal cytokines that increase systemic inflammation (Jin et al., 2008; Parnell and Reimer, 2012). Additionally, alteration of the bacterial composition of the gut, and consequently communication along the gut-brain axis, has been associated with psychological illnesses such as anxiety and depression (Diaz Heijtz et al., 2011). Finally, DHA has been shown to increase axonal repair, increase neurogenesis and dendritic arborization, and foster neuroprotection following biomechanically induced brain injury, dramatically reducing the risk for neurodegenerative diseases such as Alzheimer’s and dementia (Cole et al., 2009; Mills et al., 2011). In addition to its powerful neurological properties, DHA has demonstrated pleiotropic abilities whereby protective effects have also been identified in the cardiovascular and gastrointestinal systems (Hudert et al., 2006).

This study sought to determine if daily administration of a dietary supplement that combined RES, PBF, and DHA could promote resiliency to mTBI and mitigate the fundamental neuropathology of the injury cascades, in turn reducing the risk for PCS. To test this, both male and female Sprague Dawley rats were randomly assigned to the supplemental treatment (3S) or control condition, which was followed by administration of a mTBI or sham insult. A complete behavioral test battery designed to examine many aspects of PCS was administered. Although the exact mechanism underlying persistent symptomology following mTBI is currently unknown, recent research has implicated changes in gene expression and dendritic morphology (Mychasiuk et al., 2015a,b,c). Therefore, synaptic density of pyramidal neurons in the medial prefrontal cortex (mPFC), orbital frontal cortex (OFC), and primary auditory cortex (TE1), was investigated with Golgi-Cox staining. In addition, analysis of changes in Aqp4, Gfap, Igf1, Nfl, Sirt1, and Tau expression were examined in the prefrontal cortex (PFC) and TE1.

**EXPERIMENTAL PROCEDURES**

**Dietary manipulation and injury induction**

All experiments were approved by the University of Calgary Conjoint Faculties Research Ethics board and were carried out in accordance with the Canadian Council of Animal Care. All animals were maintained on a 12:12-h light:dark cycle in a temperature controlled (21°C) animal housing facility. All animals in the control and treatment groups had ad libitum access to food and water. At post-natal day 21 (p21) the animals were weaned from their mothers, and both male and female Sprague Dawley rats were randomly assigned to one of 4 experimental conditions: 3S + mTBI (8M,8F); 3S + Sham Injury (8M,8F); Control + mTBI (7M,7F); Control + Sham Injury (7M,6F). The 3S supplement, which consisted of prebiotics, omega3 fatty acids, and resveratrol, began on P21 and was maintained for the entirety of the experiment. Resveratrol (Sigma Aldrich, St. Louis, MO) was administered via drinking water, with 500 mg of resveratrol being dissolved in 10 mL of 95% Ethanol. 1 mL of the mixture was then added to 1 L of water for a final concentration of 50 mg/L. The omega3 fatty acids and prebiotics were administered via powdered food, with a compilation of 100 g of prebiotic, 300 g of DHA, and 600 g of standard diet per 1 kg of food. DHA powder (MEG-3 30% Powder; DSM Bright Science, Parsippany, NJ) contained 60% a minimum of 60% fish oil, of which 30% was total Omega-3 polyunsaturated fatty acids as triglycerides, including eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA). The prebiotic fiber added to the diet was 10% Oligofructose (Orafti P95, BENEO-Orafti Inc). Animals in the control group had 95% ethanol (1 ml/L) added to their drinking water to control for possible taste differences and were maintained on their standard diet of rat chow.

At p43, rats were lightly anesthetized with isofluorane and placed in a prone position on the lateral impact device designed to promote structural damage to the skull. The impact to the left temporal lobe propels the rat into
acceleration and a 180° rotation in the horizontal plane. Lidocaine was administered to the impact area and the rat was placed in a supine position in a warm empty cage. Time-to-right was measured from the moment the mass hit the rat’s head to the moment the rat righted itself in the empty cage. Animals in the sham condition were lightly anesthetized, placed in a prone position on the lateral impact device, but were then removed without insult.

**Behavioral testing**

Following injury induction, animals were subjected to a behavioral test battery designed and ordered to measure symptoms consistent with the clinical presentation of PCS (Mychasiuk et al., 2014a,b). See Fig. 1 for experimental time line.

**Beam walk.** The beam walk task was administered 24 h post injury (p44) as a measure of balance and motor coordination. Similar to Schallert et al. (2002), the beam is tapered, narrowing toward the home cage, which is used as a reinforcement cue (Schallert et al., 2002). The 165-cm beam was raised 1 m above the ground and equipped with 2-cm safety ledges on either side, used to catch the rat’s foot if it slipped. The number of hind-leg foot slips was measured by a research technician blinded to the experimental conditions.

**Open field.** The open field task, which is used as a measure of locomotor and exploratory behavior, was performed at p45. The rats were placed in a circular arena (135-cm diameter) and were permitted to explore for 9 min. An overhead camera tracked the rat’s movement and scored the distance traveled throughout the trial.

**Elevated Plus Maze (EPM).** The EPM task was used as a measure of anxiety-like behavior (Whishaw and Kolb, 2005) and was administered at p46. The EPM consists of 2 closed arms and 2 open arms, which intersect at the center. The apparatus is raised 55 cm above the ground and is made of dark Plexiglas®. The rat was placed in the center of the EPM and their behavior was videotaped for 5 min. A researcher blinded to the experimental conditions scored the time spent in the open and closed arms.

**Novel context mismatch (NCM).** The NCM task was administered over a 4-day period (p49-p52) and was used to measure short term working memory which is a function of the mPFC. In the first three days, the rat learns two distinct contexts: A and B. They were placed in context A for 5 min and then moved to context B for 5 min before being placed back in their home cage. Context A was comprised of an opaque circular enclosure with two identical objects inside, while context B was a clear rectangular box with two different but identical objects inside. The fourth day was used as the probe day, in which the animal was placed in context A for 5 min, then context B for 5 min, followed by their home cage for 5 min. Lastly, they were placed in the probe context C for 5 min, which was comprised of either box A or B, as well as one object from each box. This context C was videotaped and time spent with the object novel to this context and the object familiar to this context was measured by a researcher blinded to experimental conditions (Spanswick and Sutherland, 2010).

**Forced swim.** The forced swim task was the last to be administered on p58 or p59 and was used as a measure of depressive-like behavior (Yadid et al., 2001). Rats were placed in a cylindrical container (30 cm diameter × 60 cm...
high) filled with warm water \(\sim 25^\circ C\) so that the rat’s tail could not touch the bottom for 7 min. The trial was videotaped and a researcher blinded to the experimental conditions recorded the time spent immobile; determined by no hind leg movement.

**Neuroanatomical analysis**

Following completion of the behavioral test battery, half of the animals (16 male: 16 female) were administered an overdose of sodium pentobarbital, weighed, and intracardially perfused with 0.9% saline. The brain from each animal was removed and preserved in the dark in Golgi-Cox solution for 14 days. Following 14 days of preservation, 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. Maintaining procedural conditions as described by Gibb and Kolb (1998), Golgi-Cox staining of the brains was completed. A research technician blinded to all experimental conditions drew the neurons under investigation. See Fig. 2 for illustrative representation of Golgi-Cox staining quality.

Neurons selected for analysis were derived from layer III of Cg3 (mPFC), AID (Orbitofrontal Cortex (OFC)), and the primary auditory cortex (TE1) as described by Zilles (1985). Individual Golgi-Cox stained dendrites were traced at 1000x using a camera lucida mounted on a microscope. A total of 10 dendrites (5 per hemisphere, from 5 different neurons) were traced from the mPFC, OFC, and TE1 of each animal. The mean of cells from each hemisphere comprised the data points for each of the statistical analyses, i.e. average spine density from the 5 neurons in the left hemisphere of animal 1 comprised 1 data point. Spine density, which is a measure of synapse prevalence, was calculated by counting the number of spine protrusions. The exact length of the dendrite was obtained and spine density was calculated as the number of spine protrusions per 10 μm.

**Molecular analysis**

The other half of the animals (14 male: 13 female) were anesthetized with Isoflourane before being sacrificed via decapitation. Brains were removed and quickly weighed. PFC and TE1 were removed, flash frozen on dry ice, and stored at \(-80^\circ C\). Total RNA was isolated from the frozen brain tissue samples using the Allprep RNA/DNA Mini kit (Qiagen) according to the manufacturer’s protocol. The concentration and purity of RNA was measured with a Nanodrop 2000 (Thermo Fisher Scientific, Waltham, MA). Two micrograms of purified RNA was reverse transcribed to cDNA according to manufacturer protocols using oligo(dT)\(_{20}\) of the Superscript III First Strand Synthesis Supermix kit (Invitrogen, Carlsbad CA). All primers for target and reference genes were designed in house using Primer3 (http://bioinfo.ut.ee/primer3) and purchased from IDT (Coralville, IA). The \(2^{-ΔΔCt}\) method, as described by Pfaffl (Pfaffl, 2001), normalized against two housekeeping genes (Ywhaz and CycA) was used (Bonefeld et al., 2008). Ten nanograms of cDNA with 0.5 μM of each of the forward and reverse primers and 1× SYBR Green FastMix with Rox was used for qRT-PCR analysis on the CFX Connect-Real-Time PCR Detection system (BioRad, Hercules, CA). Each sample was tested in duplicate and PCR efficiency was between 94.5% and 106.2%.

**Statistical analysis**

All statistical analyses were carried out with SPSS 22.0 for Mac. One-way ANOVAs were performed for each of the behavioral, anatomical, and molecular measures examined with Sex (Male, Female), Injury (mTBI, Sham) and Treatment (3S, Control) as factors. Given that the lateral impact device produces a glancing blow to the temporal cortex, we analyzed anatomical changes in TE1 to determine if spine density differed in the contralateral and ipsilateral hemispheres. No differences were identified so neurons from each hemisphere were combined. A \(p < .05\) was considered statistically significant.

**RESULTS**

 Body Mass Index (BMI) was obtained using the weight and length of each animal. Males had higher BMIs than females, and animals fed the 3S had lower BMIs when compared to control animals. The three-way ANOVA demonstrated a main effect of sex, \(F(1, 57) = 49.30, p < .01\), a main effect of injury, a main effect of...
treatment, $F(1, 57) = 39.71$, $p < .01$, a significant sex × injury interaction, $F(1, 57) = 4.69$, $p = .03$, and significant sex × treatment interaction, $F(1, 57) = 16.47$, $p < .01$. In addition, females had lower brain weights than males, and the 3S treatment reduced overall brain weight as measured at the time of sacrifice (Table 1). The three-way ANOVA demonstrated a main effect of sex, $F(1, 57) = 14.69$ $p < .01$, and main effect of treatment, $F(1, 57) = 13.35$ $p < .01$. See Fig. 3.

Behavioral analyses
Animals from the control group that received a mTBI exhibited behavioral impairments similar to previous studies carried out in our laboratory, demonstrating balance and motor deficits, reduced exploratory behavior, impaired short-term working memory and increased depressive-like behaviors (Hehar and Mychasiuk, 2016; Mychasiuk et al., 2016a,b,c). As hypothesized, the initial behavioral phenotype for animals in the 3S group differed from control animals, and although they displayed acute symptomology in the beam-walking and open field tasks, the 3S prevented TBI-induced deficits in the NCM (working memory) task and the forced swim paradigm. See Fig. 3.

Neuroanatomical analyses

Primary Auditory Cortex (TE1). Analysis of apical dendrites in TE1 demonstrated that females had significantly more spines than males, and the 3S treatment further increased spine density in females, but decreased spines in males. The three-way ANOVA revealed a main effect of sex, $F(1, 52) = 14.49$, $p < .01$, and a significant sex × treatment interaction, $F(1, 52) = 4.19$, $p = .05$; See Fig. 2. Analysis of basilar dendrites in TE1 found that females had more spine than males, the mTBI decreased spine density, and the 3S treatment increased spine density when compared to controls. The three-way ANOVA revealed a main effect of sex, $F(1, 52) = 13.01$, $p < .01$, a main effect of injury, $F(1, 52) = 5.83$, $p = .02$, a main effect of treatment, $F(1, 52) = 16.92$, $p < .01$ and a significant sex × treatment interaction, $F(1, 52) = 8.54$, $p < .01$; See Fig. 4.

mPFC (Cg3). Examination of apical dendrites in Cg3 found that again females exhibited higher spine density compared to males, and the mTBI was associated with increased spine density in control animals but not 3S animals. The three-way ANOVA demonstrated a main effect of sex, $F(1, 52) = 15.19$, $p < .01$, a main effect of injury, $F(1, 52) = 7.84$, $p < .01$, a main effect of treatment, $F(1, 52) = 7.41$, $p < .01$ and a significant injury × treatment interaction, $F(1, 52) = 3.86$, $p = .05$; See Fig. 2. The three-way ANOVA for basilar dendrites in Cg3 also demonstrated a main effect of sex, $F(1, 52) = 5.47$, $p = .02$, a main effect of injury, $F(1, 52) = 6.44$, $p = .01$, a main effect of treatment, $F(1, 52) = 5.81$, $p = .02$, a significant sex × treatment interaction, $F(1, 52) = 11.85$, $p < .01$, and a significant three-way interaction between sex, injury, and treatment, $F(1, 52) = 12.12$, $p < .01$; See Fig. 4.

OFC (AID). Contrary to TE1 and Cg3, spine density of apical dendrites in AID was lower for females when compared to males, and in response to 3S treatment. The three-way ANOVA demonstrated a main effect of sex, $F(1, 52) = 52.91$, $p < .01$, a main effect of treatment, $F(1, 52) = 84.82$, $p < .01$ and a significant sex × treatment interaction, $F(1, 52) = 15.01$, $p < .01$; See Fig. 2. For basilar dendrites, control females exhibited higher spine density in AID when compared to control males. However, females in the 3S treatment group were found to have significantly reduced spine densities while spine density for males in the 3S group increased. The three-way ANOVA demonstrated a main effect of treatment, $F(1, 52) = 35.93$, $p < .01$, and a significant sex × treatment interaction, $F(1, 52) = 85.97$, $p < .01$; See Fig. 4.

Gene expression analyses
Changes in PFC gene expression were often dependent upon the interaction between 3S treatment and the mTBI. Aqp4 and GFAP were reduced following mTBI in control animals, but increased in 3S mTBI animals, whereas Igf1 and Nfl were increased following mTBI in control animals but unaltered in 3S animals with a mTBI. See Fig. 5. Conversely, gene expression changes in TE1 were driven by the injury or the treatment, but not an interaction between the two.

Table 1. Summary of statistical results from the three-way ANOVAs for the behavioral measures in question. Red text indicates significant main effects, blue text indicates a trend toward significance.

<table>
<thead>
<tr>
<th>Behavioural Test</th>
<th>Effect of Sex F (p)</th>
<th>Effect of Injury F (p)</th>
<th>Effect of Treatment F (p)</th>
<th>Significant Interactions</th>
</tr>
</thead>
<tbody>
<tr>
<td>Time-to-Right</td>
<td>1.41 (.24)</td>
<td>23.04 (&lt; .01)</td>
<td>0.33 (.58)</td>
<td>Sex x Treatment = 6.45 (.01)</td>
</tr>
<tr>
<td>Beam Walk</td>
<td>0.66 (.42)</td>
<td>15.07 (&lt; .01)</td>
<td>15.59 (&lt; .01)</td>
<td>N/A</td>
</tr>
<tr>
<td>Open Field</td>
<td>9.15 (&lt; .01)</td>
<td>11.90 (&lt; .01)</td>
<td>29.34 (&lt; .01)</td>
<td>N/A</td>
</tr>
<tr>
<td>Elevated Plus Maze</td>
<td>0.36 (.56)</td>
<td>3.68 (.06)</td>
<td>24.01 (&lt; .01)</td>
<td>N/A</td>
</tr>
<tr>
<td>Novel Context Mismatch</td>
<td>3.93 (.05)</td>
<td>2.87 (.03)</td>
<td>7.34 (.01)</td>
<td>Injury x Treatment = 7.62 (&lt; .01)</td>
</tr>
<tr>
<td>Force Swim</td>
<td>0.28 (.60)</td>
<td>4.79 (.03)</td>
<td>139.77 (&lt; .01)</td>
<td>Injury x Treatment = 4.21 (.04)</td>
</tr>
</tbody>
</table>
Expression of Aqp4 and Igf1 mRNA were reduced in animals that received 3S supplementation, while expression of GFAP and Nfl were increased in animals that experienced an mTBI. See Fig. 6.

(Table 2).
The mPFC is believed to possess heightened plasticity, characterized by substantial pruning, during childhood and adolescence; two critical windows for PFC maturation (Crews et al., 2007). This study replicated a prior investigation by our laboratory that demonstrated increased spine density in the mPFC following a mTBI for control rats (Mychasiuk et al., 2015a,b,c). Interestingly, 3S treatment prevented the injury-induced increase in apical spine density for both males and females, but only provided protection for males when examining the basilar dendrites. It is possible that the 3S treatment prevented the mTBI-induced disruption or delay in this normal pruning process. Spine density in the OFC was unaffected by the mTBI but significantly altered by the 3S treatment. Apical and basilar spine density was reduced in females on the diet, whereas males demonstrated opposing changes in apical and basilar spine density (reduced in the apical field; increased in the basilar field) following 3S consumption. The differential changes in spine density in the OFC and mPFC following mTBI and 3S treatment are however consistent with abundant literature that demonstrates two areas of the PFC often exhibit distinctive outcomes in response to the same experience (Kolb et al., 2004, 2012; Crombag et al., 2005; Mychasiuk et al., 2011, 2012, 2016a,b,c). When compared to the mPFC, spine density in the OFC appears to be more resistant to the effects of mTBI. Given that TE1 projects to the mPFC and not the OFC (Kolb, 1990), it is possible that injury to TE (the impact site in this model) had a greater effect on spine density in the connected brain regions. Examination of spine density in TE demonstrated no differences in spine density between ipsilateral and contralateral hemispheres, providing further support that the model is inducing diffuse axonal damage associated with acceleration/deceleration and rotational forces (Viano et al., 2007, 2009; Hamberger et al., 2009; Wright et al., 2017) rather than the production of a lesion or focal injury at the impact site. The reduction in basilar synapse number in both hemispheres of the TE was identified in basilar dendrites of TE1. The direction of the head rotation and acceleration are known to affect the vulnerability of a given fiber tract (Eucker et al., 2011), as axons lying in the plane of the shear force are most susceptible to tensile strain (Maxwell et al., 1993). It therefore stands to reason, that damage would differ in distinct neural pathways as they are subjected to different acceleration and rotational forces during the insult.

Although the mechanisms driving the association between the neurological and anatomical effects of mTBI and pre-morbid characteristics have not been fully elucidated, epigenetic responses that lead to changes in gene expression are likely involved. In this study, changes in mRNA expression in the PFC were often dependent upon the interaction between 3S treatment and mTBI.
and the mTBI. Aqp4 and GFAP were reduced following mTBI in control animals, but increased in 3S mTBI animals, whereas Igf1 and Nfl were increased following mTBI in control animals but unaltered in 3S animals with a mTBI. GFAP is commonly used as an estimate of astrocyte activation (Kamphuis et al., 2012) and serum levels of GFAP have been examined and shown to be elevated in a proportion of individuals following mTBI (Metting et al., 2012; Welch et al., 2016). Although unchanged in control animals following a mTBI, 3S animals exhibited significant increases in GFAP mRNA suggesting recruitment of astrocytes to facilitate cortical recovery and tissue reconstruction following the injury (Suzuki et al., 2012). Aquaporin channels play an important role in water transport; increased Aqp4 reduces edema following brain injury and is necessary for functional recovery (Zhao et al., 2005). Improved recovery in 3S animals with a mTBI may therefore be associated with the increased Aqp4 identified in these animals that was absent in control animals. Neurofilaments are found exclusively in neurons and are involved in maintaining neuronal shape and function (Van Geel et al., 2005; Neselius et al., 2013). The prevention of post-mTBI accumulation of Nfl mRNA in the 3S rats is significant given that Nfl concentrations are significantly increased in CSF obtained from boxers days after a fight and have been used as an indicator neuronal damage (Neselius et al., 2013). Similarly, Igf1 plays important roles in brain development, repair, and response to CNS damage, exhibiting an up-regulation in expression following TBI (Mangiola et al., 2015). In conjunction with Nfl, the lack of Igf1 up-regulation following mTBI in 3S

![Gene expression changes in the prefrontal cortex (PFC) at the time of sacrifice (p60) for male and female animals that received the 3S or control diet and a mTBI or sham injury (mTBI effect * p < .05; Treatment effect * p < .05; Sex effect * p < .05). Aqp4 and GFAP were reduced following mTBI in control animals, but increased in 3S mTBI animals, whereas Igf1 and Nfl were increased following mTBI in control animals but unaltered in 3S animals with a mTBI.](image)

**Table 2.** Summary of the statistical results for the three-way ANOVAs for the 6 genes of interest in PFC and TE1. Text in red indicates significant main effects.

<table>
<thead>
<tr>
<th>Brain Region</th>
<th>Gene</th>
<th>Effect of Sex F (p)</th>
<th>Effect of Injury F (p)</th>
<th>Effect of Treatment F (p)</th>
<th>Significant Interactions</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>PFC</strong></td>
<td>Aqp4</td>
<td>0.01 (.93)</td>
<td>1.65 (.21)</td>
<td>0.40 (.53)</td>
<td>Injury x Treatment = 8.78 (.01)</td>
</tr>
<tr>
<td></td>
<td>Igf1</td>
<td>2.23 (.16)</td>
<td>6.64 (.01)</td>
<td>0.41 (.53)</td>
<td>Injury x Treatment = 6.23 (.03)</td>
</tr>
<tr>
<td></td>
<td>Nfl</td>
<td>0.75 (.41)</td>
<td>15.14 (&lt;.01)</td>
<td>1.74 (.21)</td>
<td>Injury x Treatment = 19.87 (&lt;.01)</td>
</tr>
<tr>
<td></td>
<td>Sirt1</td>
<td>0.03 (.87)</td>
<td>0.04 (.95)</td>
<td>0.15 (.70)</td>
<td>Sex x Injury = 4.48 (.04)</td>
</tr>
<tr>
<td></td>
<td>Tau</td>
<td>4.42 (.05)</td>
<td>0.13 (.79)</td>
<td>0.58 (.45)</td>
<td>Sex x Injury x Treatment = 7.90 (.01)</td>
</tr>
<tr>
<td><strong>TE1</strong></td>
<td>Aqp4</td>
<td>0.32 (.39)</td>
<td>0.00 (.99)</td>
<td>4.74 (.05)</td>
<td>N/A</td>
</tr>
<tr>
<td></td>
<td>Igf1</td>
<td>0.08 (.79)</td>
<td>5.10 (.04)</td>
<td>1.66 (.22)</td>
<td>N/A</td>
</tr>
<tr>
<td></td>
<td>Nfl</td>
<td>0.55 (.47)</td>
<td>2.06 (.17)</td>
<td>16.64 (&lt;.01)</td>
<td>N/A</td>
</tr>
<tr>
<td></td>
<td>Sirt1</td>
<td>0.10 (.76)</td>
<td>10.80 (&lt;.01)</td>
<td>4.14 (.06)</td>
<td>Sex x Treatment = 8.00 (.01)</td>
</tr>
<tr>
<td></td>
<td>Tau</td>
<td>0.18 (.68)</td>
<td>0.03 (.86)</td>
<td>1.04 (.33)</td>
<td>N/A</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.17 (.74)</td>
<td>0.04 (.85)</td>
<td>0.20 (.66)</td>
<td>N/A</td>
</tr>
</tbody>
</table>
animals suggests that treatment with the supplement, (before, after, or both) may have reduced the pathophysiological damage associated with the mTBI incident.

Conversely, gene expression changes in TE1 were driven by the injury or the treatment, but not an interaction between the two. Expression of Aqp4 and Igf1 mRNA was reduced in animals that received 3S supplementation, while expression of GFAP and Nfl were increased in animals that experienced an mTBI. Increased GFAP and Nfl expression may indicate neurological damage (Kumar and Loane, 2012; Metting et al., 2012) and is consistent with the anatomical findings demonstrating a loss of dendritic spines. The differential patterns of recovery identified in the primary auditory cortex and the PFC (i.e. increased GFAP expression in 3S mTBI animals in the PFC and increased GFAP expression in control mTBI animals in TE1) are likely associated with the timing of the injury as a function of region-specific rates of brain maturation and region-specific rates of recovery; both of which could have been additionally affected by the 3S treatment. This complex interaction between injury, brain development, and dietary supplementation makes it difficult ascertain causal relationships.

Although a loss of basilar spines was identified in the TE1 brain region, the 3S treatment was likely still protective. Increased Aqp4 has been linked to pro-inflammatory processes, oxidative stress, and arachidonic acid release, but DHA treatment has been shown to prevent these elevations in Aqp4 expression and subsequent neurological damage (Collins et al., 2013). In addition, a portion of resveratrol’s anticarcinogenic effects are believed to be associated with its ability to reduce levels of Igf1 (Shukla and Singh, 2011). As the neuroprotective properties of resveratrol are believed to be associated with activation of Sirt1 (Ates et al., 2007; Sonmez et al., 2007; Della-Morte et al., 2009), Sirt1 mRNA expression was examined in both the PFC and TE1. Interestingly, treatment with 3S modified Sirt1 expression in a sex-dependent manner in the PFC, but did not alter levels of mRNA expression in TE. In the PFC, males exhibited an increase in Sirt1 expression if they were maintained on the 3S, while females on this supplement demonstrated a reduction in expression. The differential response between males and females and between brain regions is not surprising; many studies have demonstrated sex-dependent susceptibility to epigenetic modification (McCarthy et al., 2009).

In summary, this study demonstrated that a therapeutic supplement chosen to build cognitive reserve, attenuate signaling cascades in the brain, and support neural compensation and repair, was able to mitigate some of the behavioral, genetic, and anatomical impairments associated with mTBI. The combination of RES, PBF and DHA was able to alter baseline function and recovery from a single mTBI. As the 3S treatment was administered both pre- and post-injury, future studies should determine if this supplement would produce the same level of efficacy if provided only post-injury. Moreover, the supplement was administered to adolescent rats and was designed to improve aspects of normal brain development. Additional studies that examine the efficacy of 3S treatment in adult rats would also be beneficial. However, given the extensive literature demonstrating the neuroprotective properties of RES, PBF, and DHA,
in conjunction with this study suggest that dietary supplementation for individuals at high risk for TBI may be beneficial.

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