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Prebiotic Inulin-type Fructans as a Potential Therapy On Functional Recovery In Juvenile Rats Following Traumatic Brain Injury

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PREBIOTIC INULIN-TYPE FRUCTANS AS A POTENTIAL THERAPY ON FUNCTIONAL RECOVERY IN JUVENILE RATS FOLLOWING TRAUMATIC BRAIN INJURY

by

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TABLE OF CONTENTS

CHAPTER 1

INTRODUCTION

Traumatic brain injury (TBI) is the leading cause of death and disability in younger populations in the United States (Thurman et al., 1999; Faul et al., 2010; Taylor et al., 2017). To date, no known effective medical interventions have been found to combat TBI sequela for clinical trials; therefore, more preclinical research must be performed to add to the existing empirical literature. Therefore, research that focuses on different approaches that aid the recovery of function after TBI is of interest. The proposed research will build on recent findings that show processes that can influence the brain and the gut. The gut-brain axis encompasses vast amounts of microorganisms that influence brain functions through multiple processes (Bercik et al., 2011; Neufeld et al., 2011; Savignac et al., 2013; Savignac et al., 2016; Izumi et al., 2018; Neufeld et al., 2018). Given the demonstrated influence of gut microbiota on brain function, researching the effects of prebiotics on the recovery of function following experimental TBI may prove useful for informing clinical interventions. Moreover, given the prevalence of TBI related disability in children and young adults, experimental research on developing brains may be an important endeavor. In the proposed research, the efficacy of inulin-type fructans, a prebiotic, on the recovery of function following juvenile TBI (jTBI) will be assessed.

The high incidence rate of TBI among those 0-24 years old should make studying this population and finding safe and effective treatments in this population a priority. TBI is known as the leading cause of disability among children and adolescents between 14 years old or younger (Potts et al., 2006; Dewan et al., 2016; Taylor et al., 2017). The most common causes of traumatic brain injury are falls, blunt force trauma, and motor-vehicle accidents, where motor-vehicle accidents had the highest rate of incidence of TBI in children and young adults ages 0-19 years old (Taylor et al., 2017). Also, rates of TBI-related emergency department visits due to

blunt force trauma were highest among those 0-24 years old (Taylor et al., 2017). The most challenging task for TBI researchers has been finding effective treatment strategies and therapies that mitigate deficits and produce positive outcomes. To this day, the disappointment lingers that there are no therapies that have shown to be effective in improving functional outcomes in clinical trials, and few have shown potential in pre-clinical models.

Some have suggested that the gut microbiota may influence cognitive and behavioral processes, and therefore, may be of interest to the neurotrauma research community (Savignac et al., 2013; Liang et al., 2015; Appleton, 2018). The ability of the gut microbiota to influence behavior has enabled others to discover that processes in the brain can influence the gut microbiota; this gives this system a bidirectional function. The current understanding of this complex bidirectional relationship is that brain functions can influence processes in the gut and that processes in the gut can affect brain functions. Recent findings suggest targeting the complex bidirectional relationship between the gut microbiota and the brain may prove useful for identifying treatments for TBI as they have been shown to influence changes in the brain (Savignac et al., 2013; Savignac et al., 2016; Williams et al., 2016) and assessments of recovery from injury (Kigerl et al., 2016; Nicholson et al., 2019). This relationship, called the gut-brain axis, has a positive influence on gastrointestinal, cognitive, and physiological functions (Kao et al., 2016; Sundman et al., 2017). Treatments used for gastrointestinal dysfunction and diseases have also shown to influence behavioral functions that have the potential to ameliorate dysfunction and impairment, particularly with TBI associated symptomology (Kleessen et al., 2001; Savignac et al., 2013; Savignac et al., 2016; Kao et al., 2016; Yang et al., 2018).

Recent literature findings suggest that manipulating the gut-brain axis with dietary supplements, such as prebiotics, may be promising, as they have shown to exert effects on cognitive function and modulate plasticity (Gomez-Pinilla, 2008; Savignac et al., 2013; Savignac

et al., 2016; Williams et al., 2016; Yang et al., 2018). One particular prebiotic, inulin-type fructans, are well-established (Campbell et al., 1997; Kleessen et al., 2001; Roberfroid et al., 2010; Kao et al., 2016; Matt et al., 2018) in gastrointestinal disease and may be a promising candidate for experimental treatment following TBI. A review of the available literature suggests these research questions should be pursued to advance our understanding of recovery of function following TBI : 1) if daily administration of inulin-type fructans improves recovery of function after unilateral controlled cortical impact (CCI) contusion in a juvenile model of TBI; 2) if recovery of function following inulin-type fructans (100, 500, 1000 mg/mL) is dose-dependent; and 3) if administration of inulin-type fructans shows evidence of neuroprotective or anti-neuroinflammation effects upon histological examination.

CHAPTER 2

LITERATURE REVIEW

Introduction of Traumatic Brain Injury

In 1999, there were approximately five million people in the United States living with the long-term consequences of TBI (Thurman et al., 1999). Although traumatic brain injuries affect many across the lifespan, the incidence rates of TBIs occur at higher rates in the oldest and youngest age groups, which warrants the need to study developmental and aging TBI models (Thurman et al., 1999; Taylor et al., 2017). In 2010, approximately 470,000 children between 0-14 years old had sustained a TBI, and of those 2,174 were fatalities (Faul et al., 2010). In 2013, 280,000 people who sustained a TBI and survived, were hospitalized and left with physical and cognitive impairments (Taylor et al., 2017). The impairments and additional complications of TBI progress into long-term physical and cognitive deficits that affect a person's daily living activities and interfere with home and occupational roles (Crowe et al., 2012; Rabinowitz & Levin, 2014). Brain injuries that cause severe physical and neurologic impairments can be problematic for children and hinder their ability to reach developmental milestones and to live independently.

This literature review aims to address the mechanical and physiological changes that occur after brain injury using rodent studies to identify potential treatment interventions with prebiotics. This review will cover the etiology and pathophysiology of TBI by discussing the mechanism of injury and the effects it has on the developing brain in children and young adults, the role of animal models used to study TBI, the important relationship between the gastrointestinal tract and the brain as avenues for treatment interventions through the use of prebiotics to promote recovery and plasticity of the central nervous system. Further, some have now considered TBI as a progressive disease, as chronic inflammation can cause long-term

detrimental effects (Masel & DeWitt, 2010). As brain injury is considered a disease, the discussion of etiology and pathophysiology is necessary.

Etiology and Pathophysiology of Traumatic Brain Injury

Traumatic brain injury (TBI) is any damage to the head that causes damage to the underlying cortical tissue that may lead to cognitive and physical impairments (Silver et al., 2005; Faul et al., 2010). The pathophysiology of TBI occurs in two stages: primary and secondary injury (Silver et al., 2005; Thurman et al., 2007). Primary injury is the physical event that causes the injury via strong mechanical force such as a motor vehicle accident, fall, blunt force, or penetrating projectiles (Silver et al., 2005; Faul et al., 2010; Taylor et al., 2017). Primary injury causes direct damage to cortical tissue, including neurons, glial cells, axon fibers, and cerebral vasculature, via mechanical forces (Silver et al., 2005). The mechanical forces of primary injury often result in a coup-counter coup (acceleration-deacceleration) or rotational forces (causing axonal shearing), which increases the severity of the TBI (Silver et al., 2005; Loane & Faden., 2010).

Mechanical compression of cortical tissue cause swelling (edema) and stretching of axons. This stretching of axons induces severe damage to the structural components of axons (Meaney et al., 1994). The mechanical stretching causes changes in structural integrity, which leads to the breakdown of neurofilaments and microtubules within the axon. Also, the structural breakdown may cause dysregulation of calcium and damage to the mitochondria within the axon terminal (Smith & Meaney, 2000). Damage to these structures and other cellular functions may be one of the causal events of disrupting neuronal pathways and synaptic connections.

The primary injury lays the foundation for secondary injury to occur through a cascade of events. The mechanical compression of cortical tissue initiates an inflammatory response that can cause further damage to the brain (Meaney et al., 1994; Smith & Meaney, 2000; Silver et al.,

2005). Global and local activation of cellular activity during the inflammatory response can persist for days to weeks following primary injury. This persistent inflammation causes lasting effects that may present as common neurologic, cognitive, and physical deficits. This cellular activation is called neuroinflammation and is thought to be the complex mechanism and reason for cognitive deficits (Woodroofe et al., 1991; Ajao et al., 2012).

Neuroinflammation marks the beginning of the immune response in the brain through the activation of glial cells called microglia. Microglia activation is the immune response to injury in the brain that initiates phagocytotic properties and the release of cytokines (Streit et al., 2004; Silver et al., 2005; Lull & Block, 2010). Microglia also can function as macrophages that aid in wound healing and debriding damaged or dead cellular tissue (Silver et al., 2005). These glial cells can be used as a biomarker for neuroinflammation and to assess the persistence of an inflammatory processes following brain injury (Loane et al., 2014).

Microglia contribute to the onset of processes that lead to cell dysfunction and death when pro-inflammatory cytokines are elevated (Lull & Block, 2010). The most common pro-inflammatory cytokines secreted by microglia and neurons are interleukin-1β (IL-1β), interleukin-6 (IL-6), and tumor necrosis factor-alpha (TNF-α) (Silver et al., 2005). These pro-inflammatory cytokines are significantly elevated within a 48-hour time period after brain injury, suggesting massive damage to tissue in an acute period (Woodroofe et al., 1991; Hutchinson et al., 2007). Microglia also secrete anti-inflammatory cytokines during injury, including interleukin-10 (IL-10) and transforming growth factor-beta (TGF-β) as a mechanism to reduce the pro-inflammatory immune response (Silver et al., 2005). The increase in pro-inflammatory factors can cause neuron cell death, which may contribute to cognitive impairments observed after brain injury (Woodroofe et al., 1991; Barrientos et al., 2009; Ajao et al., 2012; Romo-Azaiza et al., 2018).

TBI is known to cause massive changes in excitatory amino acids in addition to neuroinflammation. Glutamate is a specific amino acid involved with excitatory neurotransmission (Silver et al., 2005; Zhou & Baudry, 2006). Most neurons have receptors that allow for glutamate binding; these receptors are α-amino-3-hydroxy-5-methyl-4-isoxazolepropionate (AMPA) and N-methyl-D-aspartate (NMDA) (Silver et al., 2005). Glutamate binds to and activates these receptors to regulate cellular membrane potential. TBI causes massive levels of glutamate to exit the neuron, which leads to increased exposure and binding of glutamate to NMDA receptors, triggering neuron excitability (Silver et al., 2005; Vizi et al., 2013). Excessive glutamate binding to NMDA receptors triggers neuron cell death. Studies show specific NMDA subunits are involved in the increased release of glutamate, which causes excitotoxicity (Zhou & Baudry, 2006; Vizi et al., 2016). NMDA subunits have been implicated in neuronal development, neuron survival, and neural cell death (Zhou & Baudry, 2006; Giza et al., 2009; Vizi et al., 2016) This suggests they contribute differently to neuronal functions, such as synaptic plasticity and excitotoxicity. NMDA subunits NR2A, NR2B, GluN2A, and GluN2B are sensitive to glutamate and calcium (Zhou & Baudry, 2006; Vizi et al., 2016). *In vitro* experiments using juvenile rats were done to determine which NMDA subunits were responsible for excitotoxicity and neuroprotective properties by examining hippocampal slices. The study found that increased expression of NR2B subunits was involved with excitotoxicity, and NR2A was involved in neuroprotective properties (Zhou & Baudry, 2006).

NR2A subunits are involved in neuroprotective properties as they appear to be elevated in the presence of growth factors and neuron survival activity (Zhou & Braudry, 2006; Savignac et al., 2013). Further research was done to record the changes in the concentration of NMDA receptors within the rat brain at different life stages. In the first weeks of life, NR2B receptor subunits were found to have increased expression when compared to NR2A subunits and showed

a reduction around three months of age (Zhou & Braudry, 2006). The significance of the higher concentration of NR2B subunits at younger ages relates to their sensitivity to glutamate and calcium, which suggests that the younger population is more vulnerable to excitotoxic events from TBI (Zhou & Braudry, 2006; Giza et al., 2009).

GluN2A and GluN2B are two additional NMDA receptor subunits that are involved with neuroprotection and excitotoxicity properties, respectively (Vizi et al., 2016; Williams et al., 2016). These subunits are more sensitive to calcium entry, which provides a mechanism during injury as there are excessive levels of glutamate and calcium, which depolarizing the neuron cell membrane, causing NMDA subunit activation of GluN2B. This GluN2B subunit is thought to exert excitotoxic effects by trigger downstream effects that inhibit synaptic growth pathways. Specifically, inhibiting the cAMP response element-binding (CREB) protein pathway and contributing to neurodegeneration (Vizi et al., 2016). The other GluN2A subunit has the opposite effect. When glutamate is released, and calcium enters the cell membrane, this triggers the GluN2A subunit. Activated GluN2A could stimulate phosphorylated CREB, which could encode the expression and increase of brain-derived neurotrophic factor (BDNF). The increase in BDNF is involved in neuroprotective properties and synaptic plasticity (Williams et al., 2016).

The brain regions that show changes in NMDA receptor subunits are the hippocampus and frontal cortex (Zhou & Baudry, 2006; Savignac et al., 2016), which indicates NMDA receptors have a role in impacting learning and memory processes (Vizi et al., 2016). Some postulate that Hippocampal NMDA-mediated excitotoxicity results in cognitive deficits (Silver et al., 2005; Zhou & Baudry, 2006; Giza et al., 2009). Others have observed massive hippocampal cell loss and loss of synaptic connections after TBI, which also contributes to impairments in cognitive learning tasks (Scheff et al., 2005). These TBI-induced excitotoxic events may help explain the vulnerability of younger brains and cognitive deficits associated

with TBI.

Brain injury can cause increases in calcium after the glutamate-induced excitotoxicity, and axonal damage can cause the mitochondria to respond by storing excess calcium (Xiong et al., 1997; Maciel et al., 2001). When mitochondria have increased calcium stores and impaired oxygen availability, this causes a process that promotes the production of reactive oxygen species (ROS), which cause damage at high levels (Maciel et al., 2001). Mitochondrial ROS production, as seen within an hour after brain injury in a rodent model where mitochondrial dysfunction was observed (Xiong et al., 1997; Silver et al., 2005). The production of ROS is a normal part of cellular physiology that is regulated by mitochondria antioxidant defense systems. The function of the defense system aids in the reduction of oxidative stress (Giza et al., 2009). This antioxidant defense system is not fully developed in younger brains, as young brains have been observed to have higher levels of oxidative stress when compared to adult brains (Silver et al., 2005; Giza et al., 2009). ROS can also be produced by activated microglia, which are active during neuroinflammatory states. TBI causes activated microglia and oxygen-deprived mitochondria to produce ROS, increasing oxidative stress (Lull & Block, 2010). Younger brains are sensitive to ROS production and oxidative stress, rendering them more vulnerable after TBI (Giza et al., 2009). Finding treatments that can attenuate oxidative stress would be beneficial candidates for juvenile models of TBI.

Secondary injury can involve disruption to the blood-brain barrier (BBB) (Woodroofe et al., 1991; Meaney et al., 1994; Banks et al., 2015). BBB protects the cortical tissues from larger compounds and organisms by acting as a vascular barrier preventing the entry of foreign pathogens and immune mediators from the capillaries (Fukuda et al., 1995; Silver et al., 2005; Pan et al., 2008). Brain injury can disrupt the BBB within an hour and causes increased permeability and allowing infiltration of foreign pathogens, activated cellular organisms, and

ionic compounds into the cortical tissues (Fukuda et al., 1995; Pan et al., 2008). The release of cytokines by either neuron or microglia can exacerbate BBB permeability, exposing cortical tissue to an increased risk of inflammatory action (Woodroofe et al., 1991). Other events can cause further inflammation after TBI, including pathogens that enter the bloodstream and permeate through the disrupted BBB, such as lipopolysaccharide (LPS) (Pan et al., 2008; Banks et al., 2015). In a healthy functioning brain, this does not occur; the barrier is there to protect the cortical tissues from macro compounds and organisms from permeating the barrier. Therefore, BBB disruption is involved with progressive neuroinflammation that contributes to secondary injury cascades. Primary and secondary injuries can profoundly impact brain function that leaves little hope for the recovery of those affected. The younger population is heavily impacted following TBI because they are still undergoing periods of physical and cognitive growth and that damage during this period inhibits functions necessary to lead a successful life (Giza et al., 2009; Crowe et al., 2012).

Traumatic Brain Injury in the Developing Brain

Traumatic brain injury in children and adolescents often hinders their ability to thrive academically, as it causes long-term deficits in physiological, cognitive, neuropsychological, and behavioral functions (Benz et al., 1999; Crowe et al., 2012). Academic performances in children with TBI are often deficient in the domains of arithmetic, (Ewing-Cobbs et al., 1998) spelling, and reading (Jaffe et al., 1992). The more common cognitive deficits are found in tasks that require working memory and visual-spatial skills (Gorman et al., 2012; Gorman et al., 2017). The severity of these cognitive deficits tends to be correlated with the severity of the injury (Jaffe et al., 1992; Ewing-Cobbs et al., 2004). Jaffe and colleagues (1992) followed a cohort of children with brain injuries in the age range 6-15 years old and used the Wide Range Achievement Test-revised (WRAT-R) to assess reading, spelling, and arithmetic. Approximately 26% of their

sample had moderate TBI, and 20% had severe TBI. These authors found there were significant deficits in these academic domains in children with more severe TBI compared to the performances of age, sex, and grade level matched controls. Duval and colleagues (2007) examined the recovery in early-life brain injuries and found that there was a significantly worse recovery associated with early brain injury. Data from this study showed decreased recovery at younger ages, larger lesions or more severe brain injuries show reductions in the IQ measurement in these children, confirming the negative outcome found by others (Jaffe et al., 1992; Duval et al., 2007).

These human studies show opposite results than that of an experiment by Margaret Kennard that led to investigations about early brain injury that results in better functional recovery later in life (Kennard, 1936; Kolb & Tees, 1990). Kennard (1936) investigated brain injuries in two monkeys, one infant and one adult. This study examined the effects of brain injury to the motor cortex in monkeys as it either occurred earlier (day 10) or later (day 40) in life and how these subjects showed signs of recovery. The findings from this study suggested that younger animals exhibited fewer motor deficits compared to the aged counterpart (Kennard, 1936). Though this view of better recovery in young animals would be optimistic, some animal studies may not translate properly to human conditions as other human studies suggest that recovery from TBI and the developing brain does not show better functional recovery compared to older adults (Jaffe et al., 1992; Ewing-Cobbs et al., 1998; Benz et al., 1999; Ewing-Cobbs et al., 2004; Duval et al., 2007). Thus, additional research of TBI in animal models is necessary to determine the extent of continuity between outcomes with nonhuman animals and humans.

TBI acquired in early childhood disrupts developmental during critical periods and causes long-term persisting neuropsychological, psychosocial, and educational/learning deficits (Anderson & Moore, 1995). More so, these deficits impact the ability to have a functional

independent life (Thurman et al., 1999). These persisting deficits create challenges for children in their educational endeavors (Anderson et al., 2011; Crowe et al., 2012) and can create a heavy burden of care for the family and caregivers (Thurman et al., 1999; Selassie et al., 2008).

Within the population of those with brain injury, decreased performance in school is a problem that cannot be ignored. Therefore, more studies of any current treatments for TBI should be continuously examined and reported. Multiple factors of life are affected by TBI and can diminish the quality of life for survivors and their families (Andriessen et al., 2011). Thus, research on juvenile TBI (jTBI) is necessary to understand the dynamics of recovery in this population. By using animal studies to model the effects of TBI, different treatment approaches can be analyzed. Specifically using rodent models to examine the impacts of TBI on cognitive tasks.

There should be a distinction made when examining TBI across different age groups. An adolescent brain injury will have different outcomes than an adult brain injury, mostly because of the incomplete development of the brain in this period (Potts et al., 2006; Giza et al., 2009). In the early stages of human development, the entire body goes through a series of growth spurts and continues to grow and mature. The same happens in the brain, which undergoes several plastic changes early in life to form important synaptic connections and complex networks (Kolb, 1995; Crowe et al., 2012). There are many challenges to advancing our understanding of recovery of function following jTBI. First, results from studies with adult subjects may not generalize to juvenile subjects because of the unique vulnerabilities of the developing brain. The unique vulnerabilities of the immature brain may create difficulties in reproducing the outcomes of studies with adult subjects (Griesbach et al., 2002; Giza et al., 2009). Another challenge in the developmental model is the possibility that therapeutic interventions may produce different outcomes depending on the age when compared to adult studies. (Giza et al., 2009; Crowe et al.,

2012). One difficulty of studying jTBI is that the injury produces different deficits depending on the age of the person or animal (Kolb, 1987; Kolb & Tomie, 1988; Prins & Hovda, 2003; Duval et al., 2007; Giza et al., 2009).

There are age-related differences when discussing TBI; some have examined that the immature brain has different mechanisms when responding to injury than compared to an adult brain (Potts et al., 2006; Griesbach et al., 2002; Zhu et al., 2005; Duval et al., 2007; Semple et al., 2013). Further, some have started to consider that TBI is a progressive disease. The impairments caused by TBI can hinder one's life across the lifespan (Masel & DeWitt, 2010).

Animal Model of TBI

Rodent studies have been used in research to model traumatic brain injuries and are the key to understanding what injury does to the brain and how to treat it. Laboratory animal studies of TBI allow for the study of mammalian brain functions, the examination of treatments to improve functional outcomes in a controlled environment, and translation of findings to human neurological disorders (Kolb & Tees, 1990; Dixon et al., 1991). Researchers currently use rats in animal models because of their similarity to humans with regard to structure and response to injury (Hoffman et al., 1994; O'Connor et al., 2011). Devices such as the controlled cortical impact device (CCI) have been manufactured over the years to replicate certain aspects of human brain injury and the mechanism that lead to dysfunction (Dixon et al., 1991; Meaney et al., 1994). The development of this device helped expand the field of TBI research and the neurotrauma community. The CCI is one of the most widely used devices to model traumatic brain injuries and will be the relevant device for this literature review (Dixon et al., 1991; Hoffman et al., 1994; Meaney et al., 1994; Scheff et al., 2005; Smith et al., 2019).

The CCI device was developed as a model to replicate focal and diffuse trauma to produce motor and cognitive deficits (Dixon et al., 1991; Meaney et al., 1994; O'Connor et al.,

2011; Smith et al., 2019). This model also produces histological pathology that allows researchers to study TBI at the cellular level to analyze the effects of damaged cortical regions on behavior. The device is an electromagnetic or pneumatically driven impactor with a blunt tip used to deform cortical tissue after a craniotomy. The electromagnetic feature allows researchers to adjust the velocity, depth, and dwell time of the blunt tip that will make contact with and deform the cortical tissue. The adjustment setting on this device allows researchers to model mild, moderate, and severe brain injuries. Additionally, allowing for more controlled and reproducible brain injuries between animals.

The CCI device produces a contusion to an area of cortex that causes cortical damage, neuronal cell loss, cerebral edema, and axonal damage (Meaney et al., 1994; Scheff et al., 2005; Yu et al., 2009). The damage from the impactor tip alters the anatomical organization of the rat brain, causing motor and cognitive deficits. These deficits include a loss of general motor abilities, sensory-motor control, and spatial orientation, and memory (Hoffman et al., 1994; Yu et al., 2009; Smith et al., 2019).

The CCI device has been developed as a relevant model of brain injury and is used in similar studies (Dixon et al., 1991; Dash et al., 2016). In an applied model of TBI, CCI injured rats showed significantly fewer exploratory behaviors post-injury day one compared to uninjured sham animals (Hoffman et al., 1994; Smith et al., 2000; Hylin et al., 2018). Others have found that CCI and other head injury models can induce anxiety and depressive-like behavioral phenotypes in rats and mice marked by the subject's performance in behavior tasks (Pandey et al., 2009; Tucker et al., 2017). For this proposed study, the CCI, as described in (Meaney et al., 1994; Smith et al., 2019), will be used to create a moderate brain injury. This model has been shown to produce impairments in cognitive performance on spatial learning tasks and secondary pathologies that impair the juvenile rat brain (Smith et al., 2019).

The detrimental effects of traumatic brain injury on the young population has negative impacts on cognitive skills long-term (Duval et al., 2007; Crowe et al., 2012; Semple et al., 2014). Animal models to study jTBI allows for new treatment approaches in the field that will be useful to treat brain injury. Studying animal models will help mitigate primary and secondary damage sustained after a brain injury in juveniles and help find treatments for recovery of function (Ajao et al., 2012). As TBI has been described to induce profound deficits to learning functions in children and young adults, there are vulnerable areas to consider. Therefore, particular brain structures that are associated with learning functions, such as the hippocampus (Bashir & Collingridge, 1992; Giza et al., 2009; Rezaeiasl et al., 2019), are of interest.

Vulnerable Brain Region

The hippocampus is a structure that, through experimentation, has shown to be vulnerable to brain injury (Hicks et al., 1996; Scheff et al., 2005). The hippocampus is known to be involved with learning and memory as marked by changes in the number of neurons and synapses when stimulation to this area occurs (Bashir & Collingridge, 1992; Hicks et al., 1996; Scheff et al., 2005; Rezaeiasl et al., 2019). The hippocampus has been implicated in studies that examine structural changes that impact synaptic transmissions, such as the induction of long-term potentiation (LTP) (Bashir & Collingridge, 1992; Rezaeiasl et al., 2019).

LTP, which involves the up-regulation of the NMDA receptors, which are thought to be the molecular basis for learning and memory, as studied by synaptic changes in the hippocampus by high-frequency stimuli. This stimulation strengthens the neurotransmission in the synapse between neurons (Bashir & Collingridge, 1992; Giza et al., 2009; Rezaeiasl et al., 2019). These structural changes in postsynaptic neurons are due to increases in NMDA receptors, which are associated with synaptic plasticity and learning (Bashir & Collingridge, 1992; Kolb, 1995; Vizi et al., 2016). Regions of the hippocampus such as the dentate gyrus (DG) and cornu ammonis 1

and 3 (CA1 and CA3), contain neurons and glia important for hippocampal activity (Rezaeiasl et al., 2019).

Hippocampal activity is important for learning, which is disrupted following brain injury. Many have found brain injury causes a massive loss of neurons and synapses in the hippocampus in rats, and this may contribute to functional deficits in learning tasks (Hicks et al., 1996; Smith et al., 1997; Sato et al., 2001; Scheff et al., 2005). Following a brain injury, the CA1 region has a severe reduction in synaptic connections (Scheff et al., 2005). This reduction significantly impairs performance on behavior, such as spatial learning and memory tasks (Scheff et al., 2005).

Some have suggested other factors such as cholinergic activity is involved with disrupting hippocampal and synaptic function (Bashir & Collingridge, 1992). This indicates there is increased cholinergic activity from the basal forebrain, such as the up regulation of acetylcholinesterase (AChE), which may contribute to hippocampal and cellular dysfunction (Bashir & Collingridge, 1992; Muralidhara, 2015; Paban et al., 2010; Paban et al., 2011; Krishna et al., 2015). Neurons in the hippocampus would benefit from the reduction of enzymatic activity in choline acetyltransferase (ChAT), the enzyme that synthesizes acetylcholine (Paban et al., 2011). Damage to the neurons and synapses located in the hippocampus has been suggested to contribute significantly to the cognitive deficits after injury (Hicks et al., 1996; Scheff et al., 2005).

The hippocampus has pyramidal neurons that synthesize and release growth factors such as brain-derived neurotrophic factor (BDNF) (Kolb, 1995). Increased expression of BDNF is associated with neuronal survival and synaptic plasticity following injury (Griesbach et al., 2002; Follesa et al., 2007; Paban et al., 2011). Cell loss in the hippocampus can be offset by the presence of BDNF (Kolb, 1995). An increase in BDNF in the hippocampus was observed after

cholinergic activity decreased, which may reduce damage in this region (Paban et al., 2011).

As changes in the hippocampus that involve increases in BDNF and aid in neuron cell survival and cognitive performance are important factors, they should be considering when searching for potential therapies following injuries to this region (Mizuno et al., 2000; Griesbach et al., 2002; Heldt et al., 2007). A current understanding of how the brain and the gastrointestinal tract form a link of communication has been an area to focus on regarding potential therapies for brain injury. Several animal studies have examined this link as a promising area of focus that could be useful for the neurotrauma community (Atarashi et al., 2011; Bercik et al., 2011; Savignac et al., 2014; Liang et al., 2015; Neufeld et al., 2018). This link has potential therapeutic implications for jTBI and is known as the gut-brain axis (GBA) (Savignac et al., 2013; Liang et al., 2015; Krigerl et al., 2016; Savignac et al., 2016; Neufeld et al., 2018; Treangen et al., 2018; Nicholson et al., 2019).

The Gut-Brain Axis

The GBA has various forms of communication between the central nervous system (CNS) and the peripheral organ systems. Communication between the GBA and the CNS is bidirectional, meaning processes in the gut can signal and influence brain functions, while brain processes can cause changes in the gut. The GBA influences change through multiple communication pathways involving vagal nerve stimulation (Borovikova et al., 2000; Follesa et al., 2007; Bercik et al., 2011), immune responses (Atarashi et al., 2011; Ito et al., 2011; Liang et al., 2015; Izumi et al., 2018), and hormonal expression (Bansal et al., 2010; Savignac et al., 2013; Liang et al., 2015; Neufeld et al., 2018; Izumi et al., 2018).

Vagal nerve afferent and efferent fibers have been shown to signal the gut and influence behavior, neurotransmitter expression, and gut-induced inflammation (Borovikova et al., 2000; Follesa et al., 2007; Bercik et al., 2010; Bercik et al., 2011). Vagal nerve stimulation plays a role

in communicating visceral changes of the gut to the CNS to respond appropriately by controlling other systems (Borovikova et al., 2000; Follesa et al., 2007; Bercik et al., 2011). Acetylcholine and norepinephrine are key neurotransmitters that aid in this communication process (Borovikova et al., 2000; Follesa et al., 2007). Disruption in the communication between the vagus nerve and the CNS can negatively impact inflammatory responses in the body leading to secondary cellular damage as well as negative changes in behavior (Borovikova et al., 2000; Bercik et al., 2011). Another communication pathway the GBA utilizes to connect with the CNS involves the immune system.

The GBA has connections that allow communication through interactions with the immune system (Vogt et al., 2015). There are anatomical structures that are crucial for the maintenance of the gut, and one area is the gut-associated lymphoid tissue (GALT). The GALT includes the mucosa layer, the intestinal epithelium layer, and the lamina propria (Shier et al., 2002; Vogt et al., 2015). These structures contribute to the immune and hormonal signaling component of the GBA. The epithelium layer has B and T- lymphocytes that are meant to combat inflammation and pathogens in the gut milieu (Atarashi et al., 2011; Vogt et al., 2015). Other cells in the epithelium layer aid in hormonal signaling and intestinal barrier integrity (Ito et al., 2011; Vogt et al., 2015). The intestinal epithelium provides a barrier from the outside world that remains intact through the stable integrity of this barrier (Shier et al., 2002). The intestinal epithelium layer utilizes tight junction proteins and Toll-like receptors (TLRs) to maintain gut integrity and to recognize pathogens (Vogt et al., 2013; Vogt et al., 2014; Wu et al., 2017). Gut barrier function and integrity are crucial in the body's response to stress, inflammation, and injury. The gastrointestinal barrier is similar to the function of the BBB mentioned earlier as it provides protection from the infiltration of substances and pathogenic cells that cause an immune response.

Gut tissue is also involved in the movement of luminal content through the gastrointestinal tract (GIT) and with the production of neurotransmitters. Enterochromaffin cells (ECs) that are located in the epithelial layer are responsible for the peristaltic reflex and the synthesis of serotonin (5-HT) (Grider & Piland, 2007; Reigstad et al., 2015). The caecum and colon GALT also is where the mucosa produces immunoglobulin A (IgA) in the presence of pathogenic substances (Ito et al., 2011). T lymphocytes are also found in the lamina propria, which include $CD4^+$ T regulatory cells that express Treg and Th17, and aid IgA in fighting off pathogens when the immune response is active (Atarashi et al., 2011). These structures are integral to the function and continuous communication between the gut and the brain.

There is evidence that gut inflammation and stress-related hormones such as corticosterone (CORT) and adrenocorticotropic (ACTH) can influence anxiety-like behavior (Bercik et al., 2010; Savignac et al., 2014; Liang et al., 2015). Thus, the GBA may also communicate through the hypothalamic-pituitary-adrenal (HPA) axis (Borovikova et al., 2000; Savignac et al., 2014; Liang et al., 2015). Other studies show changes in gut hormones such as proglucagons, which are also involved with HPA axis signaling and physiological changes in the gut and the brain (Cani et al., 2004; Delzenne et al., 2005). Some have suggested that targeting the HPA axis may be useful in the treatment of mood disorders such as anxiety and depression (Neufeld et al., 2018). The GBA has many forms of communication to the CNS, and more studies suggest the endogenous gut microbiota (EGM), bacteria within the cecum and colon, are most involved with this interaction, and therefore should be the likely target for mitigating GBA disruption (Desbonnet et al., 2009; Savignac et al., 2014; Liang et al., 2015).

The Gut Microbiota

Knowing how the EGM interacts with the BGA is not fully understood. Studies that examine inflammatory processes in the GIT have shown convincing data as to their potent

abilities, and the negative consequences on behavior, causing stress, anxiety, and depression (Savignac et al., 2014; Liang et al., 2015; Savignac et al., 2015; Neufeld et al., 2018). Preclinical animal models are the optimal source for understanding the BGA, processes in the GIT, and it's impact on behavior, and future implications for research (Matt et al., 2018; Yang et al., 2018). Studies involving inflammation-mediated stress, causing anxiety and depression-like behaviors, found that administering certain strains of exogenous microbes, known as probiotics, in the gut could mitigate the symptoms of these negative mood behaviors (Desbonnet et al., 2009; Desbonnet et al., 2010; Liang et al., 2015; Neufeld et al., 2018; Savignac et al., 2014; Savignac et al., 2015).

As studies found certain microbes that reduce the symptoms of mood behaviors, many suggest these strains are beneficial and necessary to maintain healthy function in the gut and the brain (Savignac et al., 2014; Savignac et al., 2015). The positive changes in behavior may be due to increasing the beneficial microbiota in the gut. These animal studies give insight into the role that specific strains of microbiota play in health and function. The vast majority of the EGM are concentrated in the large intestine, specifically in the caecum and colon, making these the intuitive section to target for future therapies.

Although there are several thousand types of EGM, the ones that reside in the caecum and colon are of interest to this review. The more prominent and concentrated phyla that make up the gut microbiota include Firmicutes, Bacteroidetes, and Actinobacteria (Krigerl et al., 2016; Treangen et al., 2018; Waligora-Dupriet et al., 2018). Firmicutes include *Lactobacilli*, *Ruminococcus,* and *Clostridium,* while the most prominent phyla in the large intestine region are Actinobacteria includes *Bifidobacteria* (Underwood et al., 2015). These EGM are predominantly anaerobic and lactic acid-producing bacteria, which are thought to be their main functions (Roberfroid et al., 2010; Underwood et al., 2015). The ability of these gut microbiota to possess

properties that aid in gastrointestinal barrier function (Izumi et al., 2018) aid in immune response (Atarashi et al., 2011; Izumi et al., 2018), and have shown to influence behavior through the GBA. Thus, any properties that increase the total number of these species in the caecum and colon may prove to be useful.

Manipulations of beneficial lactic acid bacteria (LAB) can be a useful tool for understanding their effects of gut microbiota during inflammatory and chronic stress in subjects (Desbonnet et al., 2009). This would give insight into the functions of specific EGM strains (Desbonnet et al., 2010; Bercik et al., 2011; Savignac et al., 2014). The exogenous strains relevant to this discussion are LAB species, such as *Lactobacilli* and *Bifidobacteria* (Gareau et al., 2007; Desbonnet et al., 2009; Desbonnet et al., 2010; Bercik et al., 2011; O'Sullivan et al., 2011; Savignac et al., 2014; Liang et al., 2015; Barrera-Bugueño et al., 2017). The composition of these *Bifidobacteria* and *Lactobacilli* in the gut and their interaction with the brain are important to consider for effects on behavior.

Chronic Stress and Inflammation

Stress and inflammation can have negative lasting impacts on the GBA and EGM. Preclinical studies show a wide range of effects that suggest that stress and inflammation can persist long-term and cause anxiety and depressive-like phenotypes. Chronic stress and inflammation have been shown to significantly increase stress hormones, reduce neurotransmitter levels, increase mood disorders, and reduce gene expressions in the brain (Krigerl et al., 2016; Liang et al., 2015; Savignac et al., 2014; Savignac et al., 2016). Additionally, chronic stress and inflammation reduce the amount of beneficial EGM which allows for the damage to persist long-term. Studies have investigated the benefits of administering a mixture of *Bifidobacteria* and *Lactobacilli* show to off-set stress and inflammation-related ailments (Barrera-Bugueno et al., 2017; Bercik et al., 2011; Izumi et al.,

2018; Messaoudi et al., 2011).

Early life stress models such as maternal separation studies show altered levels of anxiety and depressive-like behaviors, which change the overall composition of EGM in rats and mice (Desbonnet et al., 2010; Gareau et al., 2007). This early and chronic exposure to stress is thought to lead to increased anxiety and depression later in life, suggesting that signaling the HPA axis is related to this response. After maternal separation-induced stress, subjects were supplemented with exogenous strains of *Lactobacillus* such as *L. rhamnosus* and *L. helveticus,* and high corticosterone (CORT) levels were mitigated after maternal separation (Gareau et al., 2007).

In another model of maternal separation, the effects of *Bifidobacterium infantis* (*B. infantis)* found no changes in plasma CORT or pro-inflammatory cytokine levels (Desbonnet et al., 2010). However, *B. infantis* supplementation was able to increase the time spent swimming on the forced swim test, which indicates reduced depressive-like behavior, which demonstrated a potential antidepressant property (Desbonnet et al., 2010). *B. infantis* resulted in increased expression of corticotrophin-releasing factor (CRF), which explain the increase in CORT levels, indicating it stimulates the HPA axis. The presence of pro-inflammatory cytokines shows *B. infantis* does not influence the immune system but is useful for other systems.

There are different mechanisms in which *B. infantis* reduces depressive-like behavior (Desbonnet et al., 2010). In a rodent model of chronic stress, reductions in BDNF expression in the hippocampus were observed at early, and later life stages. This long-term administration of *Bifidobacterium breve* (*B. breve*) had no effect on neurotrophic gene expression, which suggests the strain may have a specific function for influencing the GBA (O'Sullivan et al., 2011). These findings show that EGM, particularly LAB, can interact with the GBA through different pathways (Liang et al., 2015; O'Sullivan et al., 2011). The beneficial effects of *Bifidobacterium* and *Lactobacillus* on the GBA have been shown to be widespread, allowing for different strains

to produce various effects showing potential pluripotent treatment effects (Liang et al., 2015; Barrera-Bugueño et al., 2017).

Chronic stress-induced anxious behavioral phenotypes in adult rats was attenuated by the administration of *L. helveticus* (Liang et al., 2015). *L. helveticus* significantly reduced stress hormones CORT and adrenocorticotropic (ACTH) and showed anti-inflammatory properties marked by increased IL-10 (Liang et al., 2015). In a model of colonic inflammation *L. rhamnosus* had no effect on reducing anxiety-like phenotypes and hormone levels were found were not assessed to determine the cause of behavior (Bercik et al., 2010). These results are different from those using these strains in models of early maternal stress suggesting that using single strains may not be an effective approach to mitigating behavioral, immune, and hormonal systems (Gareau et al., 2007; Desbonnet et al., 2009; Desbonnet et al., 2010). These findings show that *L. helveticus* can interact with the GBA through hormone and immune pathways (Liang et al., 2015).

The difference between these studies was that vagal nerve fibers were intact in the maternal stress models, which suggests some gut microbiota can only exert their effects via vagal nerve stimulation (Bercik et al., 2011). Stress had a negative impact on BDNF mRNA expression, norepinephrine (NE), and serotonin (5-HT) in the prefrontal cortex and hippocampus (Liang et al., 2015). *L. helveticus* was able to stimulate increased expression of BDNF, NE, and 5-HT in these regions (Liang et al., 2015). *L. rhamnosus* was unable to increase expression of BDNF or decrease pro-inflammatory cytokines when the subdiaphragmatic vagal fibers were dissected (Bercik et al., 2010; Liang et al., 2015). These findings show that particular strains of *Lactobacillus* can have multifaceted effects on the GBA.

In a study that examined the effects of specific strains of *Bifidobacterium* on stress and inflammation, part of one of the more concentrated phylas in the caecum and colon exhibited

specific effects (Desbonnet et al., 2009; Bercik et al., 2010; Desbonnet et al., 2010; Bercik et al., 2011; O'Sullivan et al., 2011; Savignac et al., 2014). *B. breve* administered to young rats had a significant increase in the total cell counts of *Bifidobacterium and B. breve* (Izumi et al., 2018). This in an important feature for *B. breve* to increase to concentration of other *Bifidobacterium spp,* this implies it has the potential to stimulate the growth of other EGM that may exert their own benefits. Additionally, this feature of *B. breve* may have the potential to shift the composition of EGM to increase the species in the Actinobacteria phyla (Izumi et al., 2018).

In other studies that used LAB such as *Bifidobacterium* species, show some strains exhibited the ability to reduce anxiety-like behavioral phenotypes in adult mice when in non-inflammatory states (Bercik et al., 2010; Bercik et al., 2011; Liang et al., 2015; Savignac et al., 2015). To compare their treatment with a widely used SSRI, *Bifidobacterium longum* (*B. longum*) and *B. breve* were used (Savignac et al., 2014). *B. longum* was able to attenuate the anxiety-like behavioral phenotype (Bercik et al., 2010; Savignac et al., 2014). However, when the vagal nerve fibers were dissected, *B. longum* showed no effect (Bercik et al., 2011).

B. breve produced an anxiolytic effect by subject's performance on the elevated plus maze (EPM), but *B. breve* and *B. longum* had no effect on CORT hormone levels which are usually lower when anxiety is decreased (Savignac et al., 2014). Other strains of *Bifidobacterium,* such as *B. infantis,* were not effective in alleviating depressive-like behaviors during forced swim test in adult rats demonstrating reduced immobility, suggesting that *B. infantis* does not interact with the HPA axis (Desbonnet et al., 2009). However, *B. infantis* was found to significantly reduced pro-inflammatory cytokines INF-γ, TNF-α, and IL-6 suggesting effects on the immune response (Desbonnet et al., 2009). These effects were not observed in the maternal stress model which demonstrates how this strain differs in an age-dependent manner (Desbonnet et al., 2010).

The gut microbiota are key players that can interact with the GBA to influence behaviors and aid in mitigating the effects of gut-derived stress and inflammation (Liang et al., 2015). Other studies show that these LAB strains aid in learning behaviors as they bolster hippocampus function (Liang et al., 2015). The hippocampus function can be disrupted by chronic stress and brain injury (Hicks et al., 1996; Scheff et al., 2005; Liang et al., 2015). When finding promising treatments for recovery of function following TBI, certain LAB species such as *Bifidobacterium* and *Lactobacillus* are crucial to influencing hippocampal function (Rahmati et al., 2019; Rezaeiasl et al., 2019). Evidence implicates *Lactobacillus* and *Bifidobacterium* species have a positive influence on learning behavior (Rezaeiasl et al., 2019). Further research of their role in hippocampal function, spatial learning and object recognition tasks should continue to be investigated (O'Hagan et al., 2017; Rahmati et al., 2019; Rezaeiasl et al., 2019).

Gut Microbiota Influence Learning

Lactobacillus and *Bifidobacterium* strains have the ability to increase metabolites derived from LAB, LTP induction, and reduced neuronal death and cell loss in the hippocampus (O'Hagan et al., 2017; Rahmati et al., 2019; Rezaeiasl et al., 2019). Other factors involve LAB and their influence on the GBA is by reducing inflammation in the periphery and the brain that may be crucial for hippocampal function (Desbonnet et al., 2009; Atarashi et al., 2011). Many have shown pathogenic bacteria can increased pro-inflammatory cytokines in the hippocampus and can impair learning (Wan et al., 2007; Bilbo et al., 2008; Barrientos et al., 2009). LAB play a role in inhibiting the growth of these pathogens that cause this effect (Campbell et al., 1997; Carabin & Flamm 1999; Pan et al., 2009). This is an important feature for some LAB to reduce inflammation-induced learning impairments can aid in hippocampal function and learning tasks. These changes in the hippocampus were more pronounced than the animal's performance on spatial learning tasks, more research in this area needs to be considered as these results on

improved performance are inconclusive.

This particular mixture of LAB strains *(L. casei*, L. *acidophilus*, *L. rhamnosus*, *B. breve*, and *B. longum*) showed a significant reduction in neurons positive for apoptosis and neuron cell loss in three regions of the hippocampus, CA1, CA3, and DG (Rahmati et al., 2019). This same study investigated the induced LTP activity in the CA1 of the hippocampus, these strains of bacteria also resulted in a significant increase of field excitatory postsynaptic potentials (fEPSPs) under *in vivo* conditions suggesting they are involved with improved synaptic activity (Rezaeiasl et al., 2019). Though this study did examine the metabolite profiles of these strains in the frontal cortex and hippocampus (O'Hagan et al., 2017). Metabolites are byproducts of microbes fermenting nutritional supplements (O'Hagan et al., 2017). This study found high concentrations of lactate in the groups given LAB strains, which is an important gut microbiota-derived metabolite which may be involved with the modest performance of rats (O'Hagan et al., 2017).

LPS-induced inflammation can also significantly impair learning performance in the young rat by increased pro-inflammatory cytokines impacting hippocampal function (Bilbo et al., 2008). An increase of IL-1β in the brains of young show significant increases in the hippocampus and parietal cortex compared to aged rats and vehicle group within a 24-hour period. The young rat's behavior on the contextual fear conditioning, which measures hippocampal-dependent memory, was significantly impaired in young rats when there is increased IL-1β (Barrientos et al., 2009). Similar learning impairments were observed by others where reduced BDNF expression and prolonged increase pro-inflammatory cytokines were measured in the hippocampus (Wan et al., 2007; Bilbo et al., 2008). In the context of TBI, finding treatments that increase BDNF and reduce inflammation in the hippocampus may be necessary for the recovery of function and learning (Romo-Araiz et al., 2018).

Pro-inflammatory cytokines that are present after injury have also shown impair

hippocampal functions, reduce BDNF expression, and increase cell death markers, that aid in additional cognitive impairments (Wan et al., 2007; Bilbo et al., 2008; Barrientos et al., 2009). Different LAB strains seem to produce different effects on cognitive tasks, as well as others that are dependent on hippocampal function (Liang et al., 2015; O'Hagan et al., 2017; Rahmati et al., 2019; Rezaeiasl et al., 2019). Though the effects of exogenous strains yield weak results as to their ability to improve performance on these cognitive tasks, they do offer insight as to their potential to do so. *B. longum* was used to measure learning behaviors in mice and made modest improvement with object recognition task but had no effect on spatial learning (Savignac et al., 2015). This gives insight into *B. longum's* potential to produce greater effects on learning tasks gives insight as to the potential of this strain. Others have found that *L. helveticus* improved cognitive performance on object recognition test and object placement test. Rats given *L. helveticus* significantly increased time spent exploring objects and object location (Liang et al., 2015).

Male rats administered a mixture of *L. acidophilus*, *B. Bifidum*, and *B. longum* were used to assess spatial learning and hippocampal function (Rezaeiasl et al., 2019). Using the Morris water maze (MWM) to test spatial navigation, learning and memory, rats given the exogenous bacteria mixture had significantly reduced latency to locate the escape platform (Rezaeiasl et al., 2019). These results indicate that several LAB administered together improve spatial learning and hippocampal functioning. Further research is needed to explore spatial learning and the effects of EGM (Rezaeiasl et al., 2019).

In a stroke model to assess spatial memory dysfunction and neuron cell death in the hippocampus, a combined mixture of *Lactobacillus and Bifidobacterium sp. (L. casei*, L. *acidophilus*, *L. rhamnosus*, *B. breve*, and *B. longum*) strains and three dosages (10^7, 10^8, and 10^9 CFU/mL) were administered to mice (Rahmati et al., 2019). There was a significant

improvement on the radial arm water maze performance only with the highest dosage (10^9 CFU/mL), while the lower dosages had no effect (Rahmati et al., 2019). These findings show beneficial effects of all three dosages in the hippocampus at the cellular level, but higher concentrations may be needed to see improvement at the behavioral level (Rahmati et al., 2019).

Another study examined the effects of four separate strains of *Lactobacillus* and Bifidobacterium (*L. acidophilus* CUL60, *L. acidophilus* CUL21, *B. bifidum* and *B. lactis*) at a dose of (10^8 CFU/mL) to assess spatial learning performance and object recognition, both long-term and short-term memory (O'Hagan et al., 2017). When given as separate strains, there were no significant effects in the acquisition, probe trial, or reversal performance in the Morris water maze (MWM) or object recognition in any group. The rats given the exogenous strain had more interaction with the novel object, but these data did not reach statistical significance. These findings suggest that if given in higher concentrations, these strains may aid in improvements in spatial learning and object recognition tasks (O'Hagan et al., 2017).

The use of several strains of LAB has shown to exhibit beneficial effects on the gut and the brain, more importantly, their interactions with both. However, the efficacy of exogenous strains may not produce robust results or fail to show an effect, these findings should not discourage future studies. These studies discussed above demonstrate that the single strain of exogenous LAB may produce mixed on behavior tasks, but what is interesting is some results show LAB to influence animal behavior. Some have suggested several factors that could explain these results, such as failure of exogenous strains to adhere and colonize properly (Gareau et al., 2007; Savignac et al., 2013; Grimm et al., 2015), increased stress or inflammation (Desbonnet et al., 2010; Bercik et al., 2010; Savignac et al., 2014; Liang et al., 2015) or that a single strain may not be enough to elicit a potent effect, and perhaps increasing multiple LAB strains may be a better approach (O'Hagan et al., 2017; Savignac et al., 2015; Rahmati et al., 2019; Rezaeiasl et

al., 2019). Though the effects may of these exogenous strains may not yield strong results (Bercik et al., 2010; Barrera-Bugueño et al., 2017), the importance of animal studies have led to their use in humans, and what is interesting is, the results on behavior translate with what is found in human studies (Messaoudi et al., 2011). These studies have shown the therapeutic potential and importance of the EGM and their interactions with the GBA however, any profound decrease in EGM is detrimental to the host, which has been observed following CNS injury (Houlden et al., 2016; Krigerl et al., 2016; Treangen et al., 2018; Waligora-Dupriet et al., 2018; Nicholson et al., 2019).

CNS Injury and Gut-Brain Axis

As the above studies have examined the benefits several exogenous strains can do to alleviate systematic inflammation and stress and illustrates their role in maintaining a healthy composition within the caecum and colon. There is now evidence that when a CNS injury occurs, there is no longer profound damage to just brain or spinal cord, but also to the gut (Zhang & Jaing, 2015; Houlden et al., 2016; Krigerl et al., 2016; Treangen et al., 2018; Waligora-Dupriet et al., 2018; Nicholson et al., 2019). This damage is deleterious to the GBA and the host, as the damage is no longer localized to the CNS, but is better described as holistic. Recently, evidence showed that CNS injury causes a massive shift in the composition of EGM mainly impacting Firmicutes, Bacteroidetes, Actinobacteria, and Proteobacteria. This shift in the composition of EGM leads the GIT toward a dysfunctional state, called dysbiosis (Krigerl et al., 2016; Treangen et al., 2018). Gut dysbiosis has been reported using 16S rRNA sequencing techniques, which observed the number of beneficial phyla and sub-classes of EGM within the host organism decrease and pathogenic microbes increase (Krigerl et al., 2016).

After CNS injury the host at an increased risk of opportunistic inflammatory-inducing pathogenic phyla such as Proteobacteria, specifically *Enterobacteriaceae* and

Pseudomonafaceae that are opportunistic pathogens that could negatively impact recovery (Houlden et al., 2016; Kigerl et al., 2016; Nicholson et al., 2019). Different CNS injury models include damage from stroke (Houlden et al., 2016), fluid percussion (Waligora-Dupriet, et al., 2018), a weight drop (Zhang & Jaing, 2015; Houlden et al., 2016), CCI (Treangen et al., 2018; Nicholson et al., 2019), and spinal cord injury (SCI) (Krigerl et al., 2016) and were found to produce a variety of changes in the composition of EGM in the caecum and colon. Suggesting that not all CNS injuries produce the same damage but there are many similar findings between them. CNS injury also causes direct damage to GIT tissue, which increased gut permeability and inflammatory susceptibility (Bansal et al., 2010; Zhang & Jaing, 2015). This gut tissue damage and shift in several phyla following CNS injury have shown to heavily impact the GBA composition (Treangen et al., 2018). Although this damage is detrimental to the host, the composition of the EGM has been the target for other models that involved disruptions to the GBA, it is possible that EGM in the caecum and colon are the key factors to consider when searching for treatments following CNS injury (Krigerl et al., 2016).

Several CNS injury models have noted various changes in phyla, specifically Firmicutes, Bacteroidetes, and Actinobacteria, that when decreased in composition, causes dysbiosis in the caecum and colon regions (Houlden et al., 2016; Treangen et al., 2018; Waligora-Dupriet et al., 2018; Nicholson et al., 2019). SCI observed changes in *Bateroidales, Clostidiales,* and *Lactobacillales* (Krigerl et al., 2016). Fluid percussion injury resulted in similar decreases in species in the *Bacteroides/Prevotella* group, *Clostridium* clusters, *Bifidobacteria,* and *Lactobacilli,* whereas the most profound decrease in *Bifidobacterium* species was observed (Waligora-Dupriet et al., 2018). In an experimental stroke model, there were decreases in Firmicutes and Bacteroidetes but in a closed head weight drop model a decrease in *Prevotellaceae* was not observed (Houlden et al., 2016). CCI models observed significant

decreases in Firmicutes and Bacteroidetes, others using the same model show decreases in *Lactobacilli* and an increase in *Clostridiales* (Treangen et al., 2018; Nicholson et al., 2019). Although there is noticeable variability seen in how CNS injury causes dysbiosis of EGM, there are similar patterns, Firmicutes, Bacteriodetes, and Actinobacteria all seem to be the phyla that decrease and Proteobacteria increase following injury. The EGM species within these phyla are likely to be involved with the progressive damage associated with CNS injury but also involved with recovery and therefore, should be targeted as treatment sources (Houlden et al., 2016; Waligora-Dupriet et al., 2018; Nicholson et al., 2019).

There may be an acute window of opportunity in providing treatment following CNS injury that may aid in the recovery of dysbiosis, GIT dysfunction, and brain pathology which may explain the mixed results in some studies (Nicholson et al., 2019; Treangen et al., 2018; Waligora-Dupriet et al., 2018). Dysbiosis has shown to occur in the seven-day time frame following CNS injury, implicating the short time necessary to aid the host in recovery (Nicholson et al., 2019). There was a significant increase in pathogenicity following a stroke, but not a closed head injury (Houlden et al., 2016). Others found that CNS injury caused significant increases in *E. coli* exacerbates the pro-inflammatory response in the gut and brain, causing systemic disruption (Bilbo et al., 2008; Barrientos et al., 2009; Zhang & Jaing, 2015; Waligora-Dupriet et al., 2018).

These findings show that specific microbes seem to play an important role in the normal functioning of the GBA but are altered to pathogenic states when their numbers fluctuate and cause dysfunction, more so, following CNS injury (Treangen et al., 2018). There are structural changes that occur in the gut and CNS tissue following head injury (Bansal et al., 2010; Zhang & Jaing, 2015; Krigerl et al., 2016; Nicholson et al., 2019). CNS injury-induced dysbiosis is correlated with increases in lesion volumes in CNS tissues (Houlden et al., 2016; Krigerl et al.,

2016; Nicholson et al., 2019). These data implicate that certain EGM are associated with the recovery process and, therefore, should be the target for recovery of function following brain injury. CNS injury has also been observed to cause intestinal barrier structural integrity and permeability dysfunction. Increased barrier permeability by disruption of epithelial villi, tight-junction proteins, and the lamina propria layer allows for cells and other microorganisms to infiltrate, causing infections and immune responses (Bansal et al., 2010; Vogt et al., 2014; Vogt et al., 2015; Zhang & Jaing, 2015).

The induction of dysbiosis and increased lesion volume had led some to suggest that the change or shift in EGM species after brain injury may be a useful biomarker to determine the severity and possible outcomes, whereas current clinical assessments such as the Glasgow Coma Scale (GCS) has limited ability to assess and predict outcomes after TBI (Krigerl et al., 2016; Nicholson et al., 2019). The disruption in intestinal barrier structure and integrity contribute to the increased lesion volume and is likely the harbinger to the persistent pro-inflammatory response that follows CNS injury. When there is increased intestinal barrier permeability and disruption, it causes activation of the immune response and GALT (Bansal et al., 2010; Krigerl et al., 2016).

During the first seven days following CNS injury, there is a significant increase in IL-1β, INF-γ, and TNF-α and activation of GALT (Bansal et al., 2010; Krigerl et al., 2016). Activation of GALT is cellular activation of B and T lymphocytes, $CD4^+$ T cells, and $CD11b^+$ macrophages in response to inflammation (Krigerl et al., 2016). This pro-inflammatory response persists in left uncheck, some have seen increased INF-γ, and TNF-α up to 28 days post-injury (Krigerl et al., 2016). The GALT tissue combats the persistent inflammation by having immune cells increase the expression of tumor growth factor-beta (TGF-β) and $CD4^+$ T cells (Vogt et al., 2015). The detrimental CNS injury and negative impact on the GBA may exhaust the resources of the

immune system has to combat pro-inflammation. Therefore, additional aid will be necessary to combat the persistence of the CNS-induced gastrointestinal inflammation. However, the increased pro-inflammatory cytokine activity in the gut can affect the CNS and, in turn, affect vulnerable brain regions (Krigerl et al., 2016; Zhang & Jaing, 2015).

As mentioned earlier, head trauma causes primary and secondary injury cascades, which causes BBB dysfunction and increases its permeability, intestinal permeability can also increase following CNS injury and can cause a systemic inflammation response (Peng et al., 2007; Zhang & Jaing, 2015). Other mechanisms following systemic inflammation have been reported such as gut-derived IL-1β and TNF-α that can stimulate vagal nerve fibers and cross the permeable BBB and exacerbate microglia-induced neuroinflammation (Woodroofe et al., 1991; Wan et al., 2007). These data represent the complex bidirectional damage CNS injury has on the GBA, little is known about the treating the EGM with various supplements has following TBI, which warrants the need for more research to be completed in this field.

CNS injury can vastly impact the large intestine that encompasses the caecum and the colon by dysbiosis (Houlden et al., 2016; Krigerl et al., 2016; Treangen et al., 2018; Waligora-Dupriet et al., 2018; Nicholson et al., 2019). This shift in EGM is thought to increase the detrimental effects observed in TBI and cause further damage. One particular study examined treatment post-injury VSL#3, which used a mixture of eight LAB strains (Krigerl et al., 2016). When VSL#3 was given, there was a significant reduction of the total lesion volume and degeneration of white matter on the spinal cord (Krigerl et al., 2016). When fecal samples were analyzed, they confirmed these effects were from significant increases in LAB strains, specifically *Lactobacillales* and *Bifidobacteriales* (Kigerl et al., 2016). As others have found multiple strains of LAB to influence behaviors (O'Hagan et al., 2017; Savignac et al., 2015; Rahmati et al., 2019; Rezaeiasl et al., 2019). Some have shown increase in the total number of

these specific gut microbiota can influence the composition in the gut. Targeting the growth of these LAB species to aid in recovery of function following TBI is an approach that may be more reasonable given the vast majority of EGM affected by CNS injury. A different way to augment the growth of LAB would be to use prebiotic supplements, which are compounds found in our diet that have shown to selectively stimulate the growth of several LAB species, such as *Bifidobacteria* and *Lactobacilli* (Blatchford et al., 2013; Burokas et al., 2017; Roberfroid et al., 2010; Savignac et al., 2013).

Prebiotic Supplementation

Prebiotics are dietary ingredients that are important nutrients that aid in select EGM cellular processes. They are resistant to metabolic breakdown from enzymes in the stomach and small intestine and undergo fermentation in the caecum and colon by specific EGM including *Bifidobacteria* and *Lactobacilli* (Blatchford et al., 2013; Gibson & Roberfroid, 1995; Kaplan & Hutkins, 2000; Mu et al., 2016; Roberfroid et al., 2010). Prebiotics are carbon sources obtained from the diet in which EGM act on and stimulate the growth of EGM in the caecum and colon. These prebiotic supplements occur naturally and are found in onion, garlic, chicory root, lentils, and beans (Kao et al., 2016).

When prebiotic compounds are ingested and reach the caecum and colon, they will undergo fermentation by specific EGM strains which begins the process that will allow for to production of byproducts that have beneficial health effects (Blatchford et al., 2013; Ito et al., 2011; Kao et al., 2016; Roberfroid et al., 2010). Several phyla have been known to ferment prebiotics, including Bacteroides, Clostridium, and Enterobacteriaceae (Le Blay et al., 1999). Fermentation of prebiotics by EGM are used as energy sources to produce metabolites such as lactic acid, short chain fatty acids (SCFAs), and neurotransmitters, which aid in immune system modulation, and additionally allow for increased growth of several EGM species, notably lactic

acid producing bacteria (Blatchford et al., 2013; Kigerl et al., 2016). As findings have observed increases in lactic acid-producing bacterial species has shown to increase immune markers and offer mechanisms of neuroprotection, specifically the recovery of neurological function (Krigerl et al., 2016). The use of prebiotics in a jTBI rodent model may prove to effectively target many pathologies that following injury, potentially offering multifaceted treatment mechanisms using one prebiotic supplement.

Indeed, our understanding of how dietary supplements can influence our bodies is widely built upon empirical research to help reach an even better understanding about their function. Further investigation in how prebiotics offer benefits to the host, aid in disease prevention and recovery following TBI may provide additional understanding of the influence of the GBA. Additionally, understanding the actions of prebiotics supplements on gut microbiota occurring in the caecum and large intestine may identify another path to influence the GBA (Le Blay et al., 1999; Treangen et al., 2018). While introducing exogenous gut microbiota shows benefits, the microbiota are being introduced into an environment that may not optimally support microbiota. These microorganisms need food or energy sources and certain pH levels to allow for growth. Thus, introducing prebiotics may be a better approach to enhancing the influence of EGM on the GBA (Burokas et al., 2017; Ito et al., 2011; Kao et al., 2016; Kleessen et al., 2001). Certain strains of bacteria can thrive under increased acidic conditions in the gut, whereas other pathogenic gut microbiota proliferation is inhibited by low pH levels (Gibson & Roberfroid, 1995; Le Blay et al., 1999). The changes in the gut environment could be a reason why many probiotic supplements do not thrive and produce significant results. This could be a reason why prebiotics may be a better supplement to give than probiotics.

There are multiple prebiotic supplements that produce a wide range of changes in the GBA that may need to be further explored in the field of neurotrauma (Blatchford et al., 2013;

Ito et al., 2011; Kao et al., 2016). Galacto-oligosaccharides (GOS), fructo-oligosaccharides (FOS) and inulin-type fructans (INF) are the most commonly studied prebiotics and have shown to proliferate select *Bifidobacteria* and *Lactobacillus* taxa, increase the production of SCFAs, and influence behavior, which are important features when considering the shift in the composition of the gut microbiota (Blatchford et al., 2013; Kao et al., 2016; Roberfroid et al., 2010). The prolonged daily administration of these supplements has shown to be of particular benefit within the peripheral enteric system and central nervous system.

Prebiotic supplements GOS and FOS have been observed to increase the concentration of LAB species, they selectively augment the *Bifidobacteria* and *Lactobacillus* taxa (Campbell et al., 1997; Kaplan & Hutkins, 2000; Muralidhara, 2015; Pan et al., 2009; Reigstad et al., 2015; Savignac et al., 2013; Savignac et al., 2016; Yang et al., 2018). Pan and colleagues observed significant decreases in opportunistic pathogens such as *Enterobacteriaceae* and *Enterococcus* when the concentration of *Bifidobacteria* and *Lactobacilli* increase, potentially being a mechanism for changing the optimal environmental conditions for of bacterial species. Thus, other important concentrations to measure when these species increase are the metabolites that are produced and released into the environment.

These prebiotics have been reported to increase many SCFAs such as butyrate, acetate, and propionate which all have found to have widespread systemic influences in the surrounding environment (Kaplan & Hutkins, 2000; Le Blay et al., 1999; O'Hagan et al. 2017; Sakata, 1987; Savignac et al., 2013). When chronic administration of GOS or FOS is used, the effects are observed for up to 27 weeks, increases of SCFAs with in the caecum and colon are commonly reported (Roberfroid et al., 2010). Le Blay and colleagues (1999) found that when certain acetate and butyrate increase in concentration this allowed some LAB to thrive and reduce metabolites such as lactate to hinder the growth of pathogenic bacterial species. However, some have

observed increases in lactate in the caecum after administration of a mixture of GOS and FOS, indicating there may be more complex interaction between the EGM and prebiotics (Pan et al., 2009). Le Blay and colleagues suggested the increased concentration of SCFAs was related to the reduced levels of lactate. The need for more research on the concentrations of LAB and their metabolites needs further research to gain a solid conclusion explaining the change in the composition of the gut.

Prebiotic supplements that augment particular EGM produce significant beneficial changes in the brain (Savignac et al., 2013; Savignac et al., 2016; Williams et al., 2016). Articles have discussed the presence of SCFAs in cortical tissue and suggest it may be important to brain function. GOS and FOS supplementation has shown exciting results of modulation of periphery and CNS immune response, hormonal changes, and influential CNS cellular processes (Izumi et al., 2018; Qiu et al., 2016; Romo-Araiza et al., 2018; Savignac et al., 2013; Savignac et al., 2016; Yang et al., 2018; Williams et al., 2016). While little information is available to how probiotics and prebiotics may influence recovery after TBI, there is compelling evidence to suggest that they aid in functional recovery, such as reduce stress and anxiety behaviors (Bercik et al., 2011; Savignac et al., 2014; Savignac et al., 2015; Neufeld et al., 2011), increase nerve growth factors such as BDNF and synaptophysin (Romo-Araiza et al., 2018; Savignac et al., 2013; Williams et al., 2016), and increase gene expression associated with the synaptic regulation of NMDA and 5-HT subunits (Savignac et al., 2013; Savignac et al., 2016; Williams et al., 2016). The up regulation of these changes in the brain led by prebiotics would potentially be useful in head injury models to maintain basal levels of these proteins, as they are depleted following injury.

One study examined the daily administration of FOS, GOS and water control group and their effects on augmenting the growth of beneficial EGM and changes in the concentration of proteins associated with neuronal survival and glutamate neurotransmission, specifically, BDNF

and NMDA subunits in the hippocampus, specifically increased expression of NR2A subunits, while no change was found in NR2B subunits in this cortical structure (Savignac et al., 2013; Williams et al., 2016). Others have found increased levels of GluN2A subunit expression while no change was found on the expression of GLuN2B receptors, which showed the same affect in both young and adult rats (Williams et al., 2016). Additionally, GOS was observed to reduce the number of NR2B subunit containing NMDA receptors (Zhou & Baudry, 2006). Interestingly, the role prebiotic play in upregulating certain receptor subunits also increase the expression of potent nerve growth factors.

The increase of BDNF and synaptophysin following prebiotic administration have been extremely important findings for recovery of function and must be further investigated (Romo-Araiza et al., 2018; Savignac et al., 2013; Williams et al., 2016) These data indicate that prebiotic supplements upregulate NMDA subunits that are not associated with neuronal death-derived excitotoxicity and may have the potential to activate the phosphorylation of CREB, which is indicated by the increased BDNF expression after supplementation (Williams et al., 2016; Vizi et al., 2016). Others has seen a significant increase of BDNF in the hippocampus when the compound FOS was administered in rodents (Savignac et al., 2013). This could suggest that there a common beneficial feature to the application of prebiotics, regardless of the compound. Changes in expression of BDNF and NMDA receptor subtypes without prebiotics has shown to impact rodent performance on cognitive tasks, which may prove to be useful for recovery of function following TBI (Mizuno et al., 2000; Heldt et al., 2007). Changes in cortical areas, specifically, the hippocampus, make these compounds strong candidates treatments following TBI. The hippocampus is a vulnerable brain region in developing brains and these supplements are non-invasive treatment options. Together these findings show evidence that prebiotic supplements and their metabolites have the potential to influence animal performance on assays

that aid in recovery of function.

BDNF is crucial for spatial learning tasks such as Morris water maze, radial arm maze and novel object recognition assays (Heldt et al., 2007). Behavioral data from this study shows BDNF depletion disrupts MWM acquisition and the average latency to locate the escape platform was significantly higher in the BDNF deletion group. The probe trial also suggests BDNF deletion impairs long-term memory performance on spatial learning tasks. On the novel object task, the BDNF deletion group exhibited no recognition of objects as there was no difference in the percent time exploring a novel or familiar object (Heldt et al., 2007). Similar results were found that when rats were administered BDNF antisense oligonucleotides to block the effects of BDNF mRNA expression and impaired their performance on the radial arm maze (Mizuno et al., 2000). However, some have observed GOS to significantly increased the time rodents spent with novel object compared to an injury group (Yang et al., 2018). Which may indicate that the dosage of the compound could be an important variable to consider reporting behavioral effects, as some have concluded high doses had no adverse side effects (Anthony et al., 2006; Kobayash et al., 2014; Videla et al., 2001; Zhou et al., 2017). If administration of prebiotics can upregulate concentrations of BDNF, then it would be a suitable treatment to jTBI models using these assays to conclude if they improve the animal's performance on these measures.

Additional properties of prebiotics have been found to reduce gut inflammation-induced cognitive deficits and neuroinflammation. Yang and colleagues (2018) found GOS to significantly reduce the expression of microglia in the CA1 region of the hippocampus in rats. Where others have found prebiotics to significantly reduce neurotoxic-induced ROS levels in both adult and the developing cortical tissue (Krishna et al., 2015) and in peripheral plasma (Yen et al., 2017). Both increased microglia and ROS levels occur when the brain is injured, which

indicates prebiotics could potentially mitigate these processes following TBI. The increased expression of microglia may also explain the up regulation of inflammatory cytokines found in the gut and the brain.

There is evidence to suggest that prebiotics act to reduce the amount of pro-inflammatory cytokines, namely, IL-1β, TNF-α and interleukin-6 (IL-6) (Romo-Araiza et al., 2018). Following CNS injury increases pro-inflammatory cytokines, such as IL-1β. IL-1β can enter vulnerable brain regions such as the hippocampus and impair performance on tasks associated with learning and memory within 24 hours (Barrientos et al., 2009; Wan et al., 2007). When these inflammatory cytokines (IL-1β) enter the hippocampus, they can cause a reduction of BDNF expression further hindering animal performance on behavior tasks (Bilbo et al., 2008).

As GOS and FOS have been observed to produce widespread changes in animal behavior and physiology, the more extensively studied prebiotic of interest is inulin-type fructans (INF). INFs have been shown the produce changes in animal behavior and physiology, the mechanisms are still unknown as to how EGM influences behavior and cellular processes, more so in the context of TBI. Therefore, more research should be conducted to understand what beneficial effects can come about through stimulation of the endogenous microbiota within the gut with INF and other prebiotics. Thus, research in this area is warranted. The potential benefits to implementing prebiotics in a young population such as a jTBI model would be that it is a non-invasive treatment strategy that has shown to ameliorate recovery of inflammatory states in CNS disease (Matt et al., 2018; Song et al., 2013; Yen et al., 2017).

Inulin-Type Fructans

INFs like other prebiotics have shown to ability to influence behavior and cellular processes, potentially having a crucial role in the recovery of TBI (Reigstad et al., 2015). INFs have been widely studied in the field of gastroenterology for their therapeutic effects of

maintaining health and mitigating risk of disease (Roberfroid, 2005; Roberfroid et al., 2010). As GOS and FOS appear to have similar effects on the GBA, INFs closely resemble FOS, and therefore, could have incredible potential for aiding the recovery of function following experimental TBI. To date, INFs have not been assessed in a jTBI study using cognitive measures that may show their influence on recovery of function.

The chemical structure of these prebiotics may be the key to their function exerted to the gut microbiota as their effects can be similar on a broad scale, they also exhibit specific changes that aid in their known mechanistic action. Their degree of polymerization (PD) or the number of saccharide chains and linkage bonds between those saccharide chains may be key to understanding their therapeutic potential. GOS has various β-glycosidic linkages which are broken down by different enzymes that the inulin-type fructans (INF) and FOS groups can resist these enzymes (Kao et al., 2016; Roberfroid et al., 2010). INF have β-(2, 1)-fructosyl-fructose linkages that allow them to be broken down into these other subsequent saccharides commonly extracted from the *Compositae* family that includes the chicory root (Roberfroid, 2005). Most INFs are mixutres that have a PD between 2-8 and 10-60 saccharide chains (Roberfroid et al, 2010), and these can be hydrolyzed into fructo-oligosaccharides (FOS), which are considered INF with shorter chains and therefore, explain why FOS and INF have similar properties (Ito et al., 2011; Kao et al., 2016; Vogt et al., 2014; Wu et al., 2017). Ito and colleagues suggested the differences between the DP and linkages of these prebiotics may be the reason for smaller physiological changes when they are administered.

The data of INF have shown to have beneficial influences on the periphery system and CNS (Campbell et al., 1997; Ito et al., 2011; Izumi et al., 2018; Man et al., 2020; Muralidhara, 2015; Wu et al., 2017). Prebiotic supplements in additional to INFs seem to produce slightly different results because of their structure, which suggest the study and proper labeling of these

supplements is an important factor to consider. INFs are a supplement of interest as it has been shown to increase total number of anaerobes and beneficial LAB that are depleted following injury (Carabin & Flamm, 1999; Ito et al., 2011; Man et al., 2020; Treangen et al., 2018), influence immune processes (Atarashi et al., 2011; Ito et al., 2011; Vogt et al., 2013), and influence hormone levels (Delzenne et al., 2005; Qiu et al., 2016), and produce changes in the brain (Muralidhara, 2015; Romo-Araiza et al., 2018).

INFs and other prebiotic supplements when administered daily have the ability to significantly change the composition of the EGM, specifically *Bifidobacteria* and *Lactobacilli* in the caecum and colon while resisting metabolic breakdown (Carabin & Flamm, 1999; Kleessen et al., 2001). INFs have shown to change the composition of the EGM may off-set the shift caused by CNS injury such as TBI and may contribute to recovery of function. As CNS injury has shown to significantly decrease the amount of several phyla including Firmicutes, Bacteroidetes, and Actinobacteria, prebiotics have shown to significantly increase many EGM species under these phyla (Carabin & Flamm, 1999; Ito et al., 2011; Treangen et al., 2018). This may be one of many mechanisms in which prebiotics such as INF can aid in the recovery after TBI. Another potential benefit provided by administration of INFs are the immune modulatory and endocrine system effects.

INFs have also shown to elicit changes in the immune response in the gut, which aid in reduced pro-inflammatory cytokines, act on Toll-like receptors (TLRs), and up regulate tight junction proteins for intestinal barrier function. Following injury to the gut, pathogens infiltrate the GIT, TLRs are used by EGM to detect and recognize these pathogens. The EGM can act on INFs to produce and release of immunoglobulin A to target the infiltrated pathogens (Ito et al., 2011). Additionally, when fermented INF aid in up regulating TLR type 2 receptors (TLR2) which may aid in intestinal epithelial barrier function and integrity, both reducing intestinal

permeability and the infiltration of pathogens (Vogt et al., 2013; Vogt et al., 2014; Wu et al 2017). Though, the fermentation of INFs by local EGM has direct benefits, the production of metabolites and SCFAs also aid in immune modulation.

When fermented by EGM, INF produce high levels of SCFAs, such as butyrate and acetate. Additionally, INFs significantly elevate lactate levels (Ito et al., 2011). This study found that levels of acetate were positively correlated with the proportion of T lymphocytes with Treg expression which is necessary to fight pathogenic-derived immune responses (Atarashi et al., 2011). Thus, suggesting that acetate is an important short-chain fatty acid for immune regulatory function (Atarashi et al., 2011; Izumi et al., 2018). Some have found the benefits of SCFAs change with the concentration. In *in vitro* model, higher levels of butyrate have been reported to increase intestinal barrier permeability at (8mM) in a model of neonatal immature intestinal barrier function, which suggests that SCFAs derived from prebiotics may have age-dependent effects on intestinal barrier function, sometimes at higher doses of certain SCFAs can be detrimental (Peng et al., 2007). Increased intestinal permeability may be caused by decreased expression of tight junction proteins, which aid in structural integrity and prevent transepithelial infiltration of foreign substances when butyrate is present (Peng et al., 2007). Indeed, the presence of SCFAs are integral for structural integrity and immune response however, the concentration may cause dysfunction in the GIT.

Lactate is another metabolite produced when INF are fermented and are useful for changing the environmental conditions for EGM to thrive. The main phyla that thrive in lactate rich conditions include Bacteroides, Clostridium, and Enterobacteriaceae (Le Blay et al., 1999). Lactate has been observed to decrease the pH levels in the lumen of the gut which could be a factor in preventing the growth of pathogenic microbiota (Pan et al., 2008; Sakata et al., 1987; Vogt et al., 2015). Elevated levels of Clostridium in the colon following INF supplementation

have shown to increase expression of IL-10, Treg, Th17, and TGF-β that aid in immune response to inflammation (Atarashi et al., 2011). The indirect immune effects in INF are impressive when examining the effect of one compound. The influence of INF on endocrine response and behavior provides evidence of this particular prebiotic useful for TBI studies.

Hormone peptide changes have been observed when animals are administered INF, that aid in the management of unhealthy dietary lifestyles (Delzenne et al., 2005). Following administration of INFs, higher levels of GLP-1 and PYY were observed in the caecum, proximal and middle colon. In addition, significant increases in serum levels of GLP-1 and PYY and decreases in serum levels of ghrelin were also observed in rats (Delzenne et al., 2005). Serum concentrations of GLP-1 and GLP-2 were also reported by others, suggesting prebiotics can heavily impact serum hormone levels (Izumi et al., 2018; Savignac et al., 2013). These data replicated findings in another study using the same strain of rat (Cani et al., 2004). Hormone peptide changes may be the mechanism by which the gut microbiome affects vagal nerve stimulation, as these hormones modulate appetite, possibly by signaling the hypothalamic regions that secrete hormones in response to food consumption. Delzenne and colleagues (2005) suggested that INF given at high doses are able to produce increases expressions of GLP-1 and PYY, which may explain why some studies did not observe these effects. Delzenne and colleagues (2005) found that lower levels of ghrelin correlated with decreased appetite when animals were fed inulin-type fructans. Although lower levels of ghrelin may be beneficial, in an experimental TBI mouse model, injections of ghrelin one hour after a severe weight drop TBI model, showed significantly reduced intestinal permeability in the gut (Bansal et al., 2010), an area that was not investigated by (Cani et al., 2004; Delzenne et al., 2005). Ghrelin treated animals had significantly reduced TNF-α levels at six hours post-injury, suggesting hormones such as ghrelin may have anti-inflammatory properties (Bansal et al., 2010). Thus, the endocrine

and immune systems are influenced by prebiotic supplementation and therefore can be used to target disease pathologies that negatively impact these systems.

Interestingly, when EGM were absent INF was found still elicit an effect on the GIT structure and activated protein kinase C (PKC) (Wu et al., 2017). Though some have found activated kinases that have harmful effects (Vizi et al., 2016), some have found activation of kinases, specifically by INFs, to possess beneficial health effects (Stoyanova et al., 2011; Wu et al., 2017). The mechanism in which prebiotics activate kinases and elicit their effect needs further examination to determine the possible harmful or beneficial effects they have and avoid inconclusive findings (Vizi et al., 2016). Activation of protein kinases can also activate downstream pathways such as mitogen-activated protein kinases (MAPKs). The activation of MAPKs could have implications of activating the transcription factor CREB, which targets the transcription and upregulation of BDNF mRNA (Griesbach et al., 2004), which has been shown to be upregulated in the hippocampus when FOS and GOS supplements were given (Savignac et al., 2013; Williams et al., 2016). Where others have found INF to increase BDNA and NMDA receptor subunits which correlate to learning and memory processes (Romo-Araiza et al., 2018).

The administration of INF shows some evidence that consumption and fermentation of INF by EGM may have potential ameliorating neuroinflammatory processes and reactive oxygen species (Muralidhara, 2015; Romo-Araiza et al., 2018; Stoyanova et al., 2011). When INF were administered to middle-aged rats, there were significantly decreased pro-inflammatory cytokines IL-1β and TNF-α in the hippocampus, which was correlated with improved animal performance on MWM (Romo-Araiza et al., 2018). Matt and colleagues 2018 observed INF to significantly decrease inflammatory gene expression IL-1β, IL-6, and TNFα in the brain but not the periphery, confirming the findings from Romo-Araiza and colleagues (2018). An interesting study by Muralidhara (2015) examined the effects INF has on the development of the brain and how it

protected both maternal and fetal rat brains from neurotoxicity-induced oxidative dysfunction. The prebiotic supplement was given during gestation and the fetal brains were later examined and showed significantly reduced reactive oxygen species in the cortex. An earlier study found INF to elicit similar antioxidant properties and OH and O_2 scavenging ability (Stoyanova et al., 2011). Together these studies provide evidence that INF fermentation can provide indirect effects in the brain that aid in reduced inflammatory response. These cellular mechanisms are similar to those involved following TBI which is why INF are an excellent candidate for treatment of these pathologies.

Just as FOS and GOS have shown to improve animal performance on behavioral assays (Savignac et al., 2016; Yang et al., 2018; Yen et al., 2017), the need for more research investigating the influence INFs have on behavior is warranted. Few studies have shown robust effects on changes in behavior using INF, however, there are studies that have observe improved performance on spatial learning (Romo-Araiza et al., 2018) and anti-anxiety-like behaviors (Qiu et al., 2016; Videla et al., 2001). Spatial learning was examined by performance on the MWM from rats treated with a combination of INF with probiotic *E. faecalis* and INF alone. Findings concluded both treatments significantly reduced neuroinflammation in the hippocampus, but not the LAB species alone. The reduction of IL-1β was correlated with improved time to reach the escape platform on the MWM. When the data provided information that probiotics alone did not result in this behavior improvement Romo-Araiza and colleagues (2018) suggested INFs have unique properties that aid the function of this microbe and potentially others. Another study using *B. breve* and a mixture of prebiotics found the probiotic supplement alone was not sufficient to produce changes in behavior (Izumi et al., 2018). In an animal model of post-traumatic stress disorder (PTSD) the administration of INF derived from *Morinda officinalis* was investigated to elicit anti-anxiety effects (Qiu et al., 2016). The INF compound ameliorated stress

associated with PTSD-like behavioral phenotypes in rats by reduced freezing time and decreased time and entries in open arms in the elevated plus maze (EPM) (Qiu et al., 2016). This performance of INF treated animals was evaluated by greater percentage of time spent exploring and entering the open arms, which indicates reduced anxiety-like behaviors. Interesting, in this same study PTSD-like behavior was examined as a measure of conditioned associative fear memory and higher dosages of INF were able to produce anti-PTSD-like effects (Qiu et al., 2016). Additional models have found INFs to reduce anxiety-like behaviors, indicating stronger evidence of anxiolytic effects (Videla et al., 2001). The need for more studies to investigate how INF administration can influence animal performance on spatial learning and other cognitive tasks may provide better understanding of their effect on behavior.

Introducing prebiotic supplements may have more applications in the context of neurological sequela such as treating symptoms of TBI and need to be further explored in that regard. There is compelling evidence that the prebiotic approach has high clinical relevance to neurological functions and diseases, as they may target various secondary injury cascades involved with TBI. The effects of inulin-type fructans have been well-established as exerting beneficial effects to both the periphery (Vogt et al., 2015; Romo-Araiza et al., 2018), and the CNS (Muralidhara, 2015; Matt et al., 2018; Qiu et al., 2016; Romo-Araiza et al., 2018). At the time of writing, there are no preclinical studies that have examined INF supplementation and juvenile traumatic brain injury has been done. In this proposal, sufficient evidence has been presented to establish that specific prebiotic supplements that are safe and produce no known negative health consequences, should be studied to examine the effect of inulin-type fructans on functional recovery after juvenile traumatic brain injury.

CHAPTER 3

METHODS

The Proposed Research

Given the many health benefits that have been reviewed in the previous section, assessing the effects of inulin-type fructans on promoting recovery of function following TBI in developing brains is warranted. Below I describe a potential study which may provide data on the efficacy of administering prebiotic inulin-type fructans in promoting recovery of function following experimental TBI in juvenile rats. Such translation research may inform the development of treatment for people who incur a TBI, sparing them of at least some of the costs that the sequalae of brain injury incurs.

Subjects

This study will take place in the Neurotrauma and Rehabilitation Laboratory at Southern Illinois University. The experimental procedures for this prospective study will be approved by the Southern Illinois University Institutional Animal Care and Use Committee before this experiment begins. Animals will be aged to post-natal day 28 (PND-28) for the beginning of this experiment. Fifty male Sprague-Dawley rats will be used in this proposed experiment and housed in cages, paired with no more than three littermates per cage, and will be given earmarks for subject identification. Subjects will be bred in our colony from breeders ordered from Harlan Laboratories (Indianapolis, IN). Subjects will be on a 12-hour light/dark cycle (7:00-19:00/19:00-7:00) and given a standard rodent chow diet and water *ad libitum* throughout the study.

Surgical Procedure

The controlled cortical impact (CCI) device (Leica, St. Louis, MO, USA) has been an established and relevant model of brain injury and used in similar studies (Dixon et al., 1991;

Meaney et al., 1994; Dash et al., 2016; Smith et al., 2019). Prior to surgery, surgical equipment will be placed in a 70% EtOH solution and then will be placed in a dry glass bead sterilizer for 20 seconds. Rats will be weighed and then placed in a large plexiglass induction chamber and will be anesthetized with 5% isoflurane that will be diluted in oxygen (0.8L/min). A tail pinch and pedal reflex will be used to assess adequate sedation of the subject prior to the surgical procedure. Once the animal has attained the proper level of sedation, the subject will be removed from the induction chamber; the scalp will be shaved with electric clippers. Rats will then be secured into a stereotaxic apparatus via ear bars. The subject will be fitted into a nose cone attachment on this device and will be administered isoflurane at 2-3% for maintenance. Proper levels of sedation will be assessed again by a tail pinch and pedal reflex. Once the subject is secured and sedated, betadine (Iodine) will be applied to the shaved skin using a cotton-tip applicator.

A single midline incision will be made using a surgical scalpel to expose landmarks bregma and lambda on the skull. The skin will be retracted away from these landmarks by four bulldog clamps. Markings will be made on both sides of the skull +3.5mm lateral from the longitudinal fissure and center between bregma and lambda to be used as a guide. Two $6mm^2$ in diameter craniotomies, one on each side of the skull, will be drilled using an electric Dremel tool. Drilling will allow for the removal of a portion of the skull to expose dura with an injury. Once the dura is exposed, a $5mm^2$ diameter impactor tip attached to an electromagnetically activated piston (Leica, St. Louis, MO, USA) positioned and centered above the subject's right hemisphere craniotomy. The impactor tip will be extended and lowered manually until there is contact with the dura surface. The electromagnetic probe will be retracted from the dura and lowered 2.0mm in order to deform the dura. The parameters for this injury model will be set to strike the exposed dura at a velocity of 5.5m/s and a 200-millisecond dwell time to induce a

moderate CCI injury to the parietal lobe, the same injury as described in (Smith et al., 2019). After the impact, the bulldog clamps will be removed, and the skin repositioned to the midline and closed using 9mm surgical staples. Once the scalp has been stapled, a triple antibiotic ointment cream (Kroger®) to prevent the risk of infection will be applied at the incision site with a cotton tip applicator, a similar method described in a CCI rat model (Dunkerson et al., 2014) The subject will then be removed from the stereotaxic device and placed on their side in a cage with a heating pad for recovery. The stereotaxic device will be wiped down using the 70% EtOH solution after each subject. Latency to observe a pain reflex assessed by pedal reflex and latency for the subject to correct their posture from the side position to laying on their trunk (righting time) will be recorded. Subjects that will undergo a sham surgical procedure will consist of similar surgical preparation as CCI subjects. This sham procedure will include isoflurane induction, placement in the stereotaxic device, midline incision, but exclude craniotomy and CCI contusion. The sham procedure will also include the closure of the midline incision with surgical staples and placement in a recovery cage. Once all subject's recovery and pain reflex and righting latencies are recorded, they will be returned to their home cage.

Treatments

Following surgery, subjects will be separated into groups and receive one of the three treatments, Orafti®Synergy1 oligofructose-enriched inulin (INF), which is commercially available in purified form by BENEO (Mannheim, Germany), vehicle (tap water), and sham control. Experimental subjects will be assigned into five groups, sham (n = 10), TBI/vehicle (n = 10), TBI/INF 100mg/mL/day (n = 10), TBI/INF 500mg/mL/day (n = 10), and TBI/INF 1000mg/mL/day (n = 10). Experimental subjects will receive treatment supplementation through sterile oral gavage needles one hour after injury. Subjects will then receive treatment at dose levels of (100mg/mL, 500mg/mL, 1000mg/mL, or tap water (vehicle) and will be given daily via

oral gavage in the morning prior to behavior testing, for the remainder of the study. The group sizes and dosage parameters in this study will resemble those used in previous rat studies using similar prebiotic supplements (Carabin & Flamm, 1999; Videla et al., 2001; Anthony et al., 2006; Yang et al., 2018). The oral gavage and dosage parameters proposed in this study will be modified from Videla and colleagues (2001). The gavage tip will be placed in a glass bead sterilizer between animals to ensure there is no contamination between cages and groups of animals. Sham subjects will not receive an oral gavage in order to serve as an uninjured control group.

Foot Fault Task

In order to assess gross motor performance after injury, the foot fault task will be used. The foot fault task is comprised of a wire mesh grid with dimensions 48cm (L) x 93cm (W) x 7cm (H). The foot fault task will commence post-op days 1, 3, 5, and consist of a five-minute trial per animal on the day of testing. Subjects will be placed on the grid surface to observation of proper placement of limbs will be assessed. When the subject moves around the grid, there may be an improper placement of the forelimb, and the forelimb falls through the grid opening. This improper forelimb placement is considered a foot fault, and quantification of the total amount of foot faults made by the subject will be quantified and analyzed by the two separate observers to ensure inter-observer agreement. Trials will be recorded using a digital video camera (Sony®), and the number of foot faults will be manually counted. The amount of improper forelimb placement on a wire grid or when a limb falls through the grid opening, this will be considered a foot fault. The use of this task has been for assessing motor deficits in traumatic brain injury (Smith et al., 2019). The quantification of total foot faults will be used to address any motor deficit a subject may have, to rule out poor performance in subsequent behavior tasks.

Novel Object Recognition

The novel object recognition (NOR) task is used to assess non-spatial memory and object preference behavior. The NOR is comprised of a clear-plexiglass bin with dimensions 58cm (L) x 36cm (W) x 38cm (H). NOR task will commence post-op days 7, 8, 9, and will consist of habituation, training, and object recognition phases. During the habituation phase, subjects will be placed in the apparatus without objects for 20 min. The next day the training phase will include two identical objects (blue and green plastic blocks) that will be fixed to the apparatus so they will not be displaced by subjects during exploration. Training will be for 10 min, and subjects will then be returned to their home cage for one hour. The chamber and objects will be wiped down with 65% EtOH to avoid giving olfactory cues to the next subject testing. After the one hour, subjects will be placed into the apparatus, which will contain one object from the training phase and one new object (orange and yellow plastic block) to assess short-term memory. Short-term memory will consist of a five min trial in which the subjects explore both objects. Day three of NOR will consist of one object used in the training phase, and a new object (red glass bottle) for the subjects to explore for five min to assess long-term memory. The NOR has been used to assess how prebiotic supplementation influences memory performance in rats (Yang et al., 2018). Subjects will be tracked using AnyMaze® video tracking software and a video camera mounted above the apparatus. The primary dependent variables for this task will include the total amount of time spent with each object and the total number of interactions with each object.

Morris Water Maze

Morris water maze (MWM) will be utilized to examine spatial learning performance. The Morris water maze is commonly utilized for assessing spatial navigation, cognitive flexibility, learning, and memory deficits recovery of function for TBI (Hylin et al., 2018; Scheff et al.,

2005; Smith et al., 2019). MWM consists of a large pool approximately 200cm diameter and 74cm in height. A platform with dimensions 10cm in diameter and 64 cm in height will be submerged 2cm below the water level for all trials. The pool will have non-toxic black paint mixed into the. The pool will be filled with cold water ranging in temperatures 21-26 degrees Celsius, and non-toxic black paint will be added to the water to allow for better video software tracking. The MWM will commence post-op days 14-21 to quantify spatial learning and navigation of the subjects.

On days 1-5 of training, there will be four trials with a total ceiling time of 60 seconds each day. For each trial, rats will be trained to find the platform using visual cues around the room and start each trial from a different location on the side of the MWM. Each subject will be given 60 seconds to locate the escape platform. Once the subject is on the platform, they will stay there for 30 seconds to orient to the surroundings as visual cues. If the subjects fail to locate the escape platform, they will be guided by the experimenter's hand to the escape platform. After each trial, the subject will be placed in a dry heated holding cage. After the fourth trial, subjects will be placed back into their home cage. On the sixth day of MWM, the platform will be removed from the pool for probe testing. The latency to enter the area where the platform originated, and the number of entries will be used to assess long-term memory. Only one 60 second trial will be used for probe testing. Day seven of MWM will consist of a reversal trial, in which the submerged platform will be placed to the adjacent area of the pool from training trials. Subjects will be given four trials with a ceiling time of 60 seconds to find the new location of the submerged platform to assess their ability to locate the novel location of the platform, a form of cognitive flexibility (Smith et al., 2019). Subjects will be tracked using AnyMaze® video tracking software. The primary dependent measure will be a latency to locate the escape platform.

Radial Arm Maze

The radial arm maze will be used to assess spatial working and reference memory (Smith et al., 2019). This apparatus consists of eight arms equal length and distance apart, which attach to a center octagon platform. The maze is approximately 50cm in height and 157cm total wide, measuring from one end of an arm to the adjacent arm. Inside the maze center, there will be internal spatial cues consisting of different shapes to aid in the subject search strategy. On the walls outside of the maze, there will be external spatial cues consisting of large shapes to aid in the subject search strategy in addition to the internal cues. Each arm measures 71 cm in length. The day before testing, subjects will be put on food-restriction 15 grams of rodent chow per animal. Radial arm maze will commence post-op days 22-31. At the end of each arm, there are small, submerged cups. Four of the eight cups at the end of the arms will be baited with a piece of cereal as a reinforcer (i.e., Apple Zings^{TM}). The first three days will consist of habituating subjects to the maze.

Day one of habituation up to six subjects will be placed in the maze with Apple Zings^{TM} covering the surface area of the center platform and all arms to allow rats to explore and consume pieces of cereal for 20 minutes. Day two of habituation Apple Zings^{TM} will only be placed on the farther half of all eight arms, and up to six subjects will be placed in the maze at a time for 10 minutes. Day three of habituation one cage of subjects will be placed in the maze, and Apple Zings^{TM} will only be inside the cups at the end of each arm for 5 minutes. Days 4-10 will consist of testing where each subject will be given one trial per day with a ceiling time of five minutes to learn the location of four arms, baited with Apple Zings^{TM} in the cup. To assess working memory errors during testing, the number of arm re-entries a rat makes into a previously baited arm after they have already consumed the reinforcer in the cup will count as a working memory error. To assess reference memory errors during testing, the number of entries a rat

makes into a non-baited arm will be used to quantify a reference error. Once the trial is over, the subject will be returned to the home cage, and the apparatus is wiped down with 65% EtOH in between animal testing. Dependent measures on radial arm maze will include latency to locate all four baited arms within the five-minute trial, the total number of working and reference errors made during each trial.

Euthanasia and Perfusion

Subjects will be sacrificed at day 33 post-op after the radial arm maze. Rats will be given a lethal 1.5mL intraperitoneal injection of Urethane (50% solution) and will undergo the transcardial perfusion procedure (Smith et al., 2019). A transcardial procedure will begin with the opening of the chest cavity with surgical scissors. The use of a 26-gauge needle will need to be inserted into the left ventricle and clamped into place to avoid any fluid leakage. Before administering any fluid to the subject, the right atrium will be cut, and the pressure will then be released from the heart. Approximately 300-400mL of phosphate-buffered saline (PBS) will be pumped through the body using a peristaltic pump until color indication of lungs (reddish to white color) and liver (from a dark marron to light brown) is sufficient. A solution of 4% paraformaldehyde will follow until approximately 300mL has entered the body.

Histology

Basic Histological methods will be used to examine the lesion and extent of brain damage following similar procedures, as seen in (Scheff et al., 2005; Smith et al, 2019). Histological examination and cortical tissue and cortical volume estimation The stereotaxic depths that will be utilized in order to estimate cortical volume within the site of injury will be depths (-1.40mm, -1.80mm, -2.12mm, -2.56mm, -3.14mm, -3.60mm, -3.80mm, -4.30mm, -4.52mm, -4.80mm, -5.30mm, and -5.80mm posterior to bregma. A cresyl-violet Nissle stain will be used to stain 40-micron (40 μm) thick coronal sections of brain tissue, and these sections will be used for lesion

analysis. Cresyl-violet will stain the tissue a shade a purple, which will allow for cortical structures to be visible underneath the microscope. The tissue will be mounted on 1% gelatin-subbed microscope slides. This tissue will be placed in additional baths to be rehydrated and to remove any residue that may be present. It will then undergo serial dilutions of EtOH in order to get stained with the cresyl-violet. The steps begin with a bath of xylene, 95% EtOH, 70% EtOH, 50% EtOH, and DI-water. The slides will then be placed into the boat containing cresyl-violet stain, and once staining of the tissue has taken place, it will be dehydrated. Dehydration of tissue will begin with a bath of 50% EtOH, 70% EtOH, 95%EtOH, 100% EtOH, then placed in a final xylene bath. Afterword, dehydrated tissue will be coverslipped with a histomount solution and left to dry overnight.

Remaining Cortex Analysis

It will be necessary to examine if the different dosage of inulin would result in a lesion effect of the treatment. Examining the remaining amount of cortex will also aid in concluding the brain injury produced the deficits in the behavioral assays. The Cavalieri method would be used to estimate remaining volume of cortical tissue. This method has been adopted in other studies as a means of estimating the remaining cortical volume. This method uses the area of twelve slices averaged together then multiplied by the thickness of the sliced section to estimate remaining cortical volume.

Statistical Analysis

In order to interpret behavioral results, all data will be calculated with the mean and standard error of the mean. Then all data will undergo one-way analysis of variance (ANOVA) utilizing the general linear models in software program SPSS version 27 for Windows. Repeated measures ANOVA will be used for examining the subject's performance across days. A one-way ANOVA between-subjects design will be used to examine the difference in performance by

groups on NOR short-term and long-term object recognition testing and probe trial on day 6 of the Morris water maze. The behavioral data from the foot fault, Morris water maze, and radial arm maze, will be analyzed using repeated-measures ANOVA.

CHAPTER 4

HYPOTHESIZED RESULTS AND CONCLUSIONS

The proposed research would properly examine the daily administration of inulin-type fructans in a model of juvenile traumatic brain injury. To evaluate the efficacy of inulin-type fructans on recovery of function, a battery of behavior assays would be required to measure gross motor, non-spatial and spatial learning performance. The proposed behavioral assays include the foot fault task, novel object recognition, Morris water maze, and finally the radial arm maze. In order to perform lesion analysis following injury, cresyl-violet will be used to stain cortical tissues and estimates of cortical volume of the remaining cortex will be analyzed. All data will be analyzed with one-way ANOVA using general linear models and Sidak post-hoc where appropriate.

Foot Fault

A gross motor assessment will be measured by the foot fault task. Subjects will be placed on the grid surface to observation of improper placement of limbs. The subject will be allowed to transverse the grid plane, when a paw or forelimb falls through the grid opening this will be scored as a foot fault. Each foot fault will be quantified for total number of foot faults made by the subject. A repeated measures ANOVA will be used to analyze within-subjects effects across days. It is hypothesized that the sham group will perform better on the foot fault task than TBI injured animals. Specifically, sham animals will make significantly less foot faults on the grid compared to TBI injured groups on all three days. No differences are hypothesized to be found between TBI/vehicle vs. TBI/INF groups.

Novel Object Recognition

A non-spatial cognitive task for short-term memory will be measured using the novel object recognition task. The day after habituation phase will begin the assessment of short-term

memory, starting with the training phase. Once animals end their ten-minute trial with two identical objects, they will be placed in their home cage for one hour before the short-term memory test. During short-term memory tests animals will be given five minutes to interact with one familiar object and one novel object, the total time spent and interactions with each object will be used to score the animal's preference of either object. One-way ANOVA will be used to analyze differences between groups. It is hypothesized that TBI/INF groups perform similar to the sham group. Specifically, the TBI/INF 100, 500, and 1000mg/mL/day groups will spend significantly more time and interact more with the novel object compared to the TBI/vehicle group, where no differences will be seen when compared to sham group. Additionally, it is hypothesized post-hoc analysis will be necessary to reveal TBI/INF 1000mg/mL/day will spend significantly more time with the novel object compared to other TBI/INF groups.

Morris Water Maze

A cognitive measure used to test spatial learning will involve the Morris water maze and will commence 14 days post-injury. The first five days of the maze will begin the acquisition phase, where animals will be given four trials to locate the submerged platform within 60 seconds. This sixth day (probe trial) of the water maze will be used to assess the animal's ability to locate the area where the platform was the day prior. The probe trial on the sixth day will be used to assess long-term memory. The seventh day of testing will have the submerged platform on the adjacent area of the maze to test the animal's ability to locate the novel location of the platform. The seventh day will be used to measure the subject's cognitive flexibility. It is hypothesized the TBI/INF groups will show no difference in the average time the locate the escape platform compared to the sham group on days four and five of the acquisition phase. It is hypothesized the TBI/INF groups spend significantly less time locating the escape platform compared to the TBI/vehicle group. Specifically, the TBI/INF 1000mg/mL/day group will reach

the submerged platform in significantly less time compared to other TBI/INF groups.

Radial Arm Maze

An additional cognitive measure will be used to measure spatial working and reference memory and will involve the radial arm maze apparatus. Once the first three days of habituation conclude the fourth day will begin working and reference memory testing. Days 4-10 each subject will be given one trial with five minutes to locate the baited arms within the maze. Working memory will be quantified during testing by the number of re-entries the animal makes in a baited arm, each time they re-enter a baited arm will be scored as a working memory error. Reference memory will be quantified during testing by the number of entries the animal makes in non-baited arms. Each time an animal enters a non-baited arm during testing, a reference error will be scored. The overall latency to locate and enter the four baited arms will also be collected during testing. It is hypothesized that there will be no difference in latency between the sham group and TBI/INF groups. It is hypothesized that the TBI/INF groups significantly make fewer working and reference errors when compared to the TBI/vehicle group. Additional post-hoc analysis will be required to reveal the lowest dose (INF 100mg/mL/day) will significantly make fewer working and reference errors compared to TBI/INF 500 and 1000mg/mL/day groups.

Remaining Cortex

Histological examination of cortical tissue will be used to assess the extent of the lesion site of the brain injury on both hemispheres. After cresyl-violet staining is completed, twelve slices from each animal subject will be imaged and traced for total remaining cortical volume. A one-way ANOVA will be used to analyze the estimated mean total remaining cortical volume of the ipsilateral and contralateral hemispheres between subjects. It is hypothesized that the TBI/INF groups have significantly smaller lesions on the contralateral hemisphere compared to the TBI/vehicle group. Specifically, the TBI/INF highest dose will have a significantly smaller

lesion on the contralateral hemisphere after post-hoc analysis compared to other TBI/INF groups. It is hypothesized the highest dose TBI/INF group has a significantly smaller lesion on the ipsilateral hemisphere compared to other TBI/INF groups and the TBI/vehicle group.

Conclusion

Presently there are few proven medical interventions to aid those who incur a TBI. Mitigating the secondary effects that lead to additional loss of tissue and adaptive function in the hours or days following the initial injury remains an intriguing target for the development of medical interventions. Researching the effects of the gut brain axis on recovery of function may yield effective technologies to aid recovery of function following TBI. This proposed study would examine INF as a potential therapy on functional recovery. The evaluation of the different dosages of INF and other prebiotic supplements would have important implications to consider further dose-response effects in clinically relevant timeframes. In animal studies of several prebiotic oral administration for prolonged periods of time have shown no negative results or inclination of toxicity and have be classified as generally recognized as safe (Anthony et al., 2006; Carabin & Flamm, 1999; Kobayash et al., 2014; Zhou et al., 2017). Whereas the treatment in younger children with TBIs are more vulnerable and using pharmacological agents chronically may have toxicity effects over time making a prebiotic supplement such as INF a suitable candidate for treatment in traumatic brain injury studies (Giza et al., 2009). The evaluation of the cortical tissue remaining volume would have significant medical relevance to TBI patients, so in rodent studies it is imperative to investigate cortical volume measures when prebiotic supplements are administered. The length of daily administration after injury is 32 days, which will allow for the measures to assess prolonged treatment to avoid early treatment effects. This proposed study aims to evaluate the effectiveness of prebiotics in three different dosages in order to examine influences on behavioral deficits and cortical tissue. Although none of the preclinical

research informing interventions for TBI have produced positive results to date, the profound and lasting impacts that TBI has on those who survive them and on those who care for them should stand as continuing motivation to extend the research endeavor.

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