

PRECLINICAL DEVELOPMENT OF A NEURAL STEM CELL THERAPY FOR STROKE
AND TRAUMATIC BRAIN INJURY

by

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(Under the Direction of Franklin D. West)

ABSTRACT

Stroke and traumatic brain injury (TBI) are leading causes of death and long-term disability worldwide. Despite the socioeconomic burden of these neural injuries, there are no restorative therapies. Induced pluripotent stem cell-derived neural stem cells (iNSCs) may be the critically needed therapy to restore neurological function after brain injury. Preclinical studies demonstrate that iNSCs are a multimodal therapeutic that improves recovery after neural injury. However, the enthusiasm of these results has been stifled by the hundreds of therapeutics shown to be effective in preclinical neural injury models but have failed in clinical trials, thus suggesting a lack of translatability of preclinical animal models to human patients. Compared to the rodent, the sole preclinical model iNSC therapy has been tested in, the pig possesses striking similarities in brain anatomy and physiology compared to the human and may be an ideal animal model to bridge the gap between preclinical rodent models and human patients. However, injury models and outcome measures are not well characterized in the pig, and more studies in the pig are needed to determine if large animals truly carry greater predictive power of clinical efficacy. The objectives of these studies were to 1) develop a translational pig model to study TBI pathophysiology and recovery mechanisms, and 2) assess whether iNSC therapy improves recovery in a pig stroke model. These studies strengthen current preclinical

therapeutic development strategies for neural injury by improving the translational capacity of animal models and provide further insight into the potential of iNSC therapy for human patients.

INDEX WORDS: Stroke, Traumatic Brain Injury, Neural Stem Cells, Induced Pluripotent Stem Cells, Cell Therapy, Neural Repair, Magnetic Resonance Imaging, Metabolomics

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CHAPTER 1

INTRODUCTION

For many years it was believed that the brain possessed no regenerative potential and neurons that are lost due to disease or injury could never be replaced [1]. In the 1990s the pioneering research of Eriksson et al. and Kirschenbaum et al. demonstrated that the adult human brain possessed a population of neural stem cells (NSCs) that undergo neurogenesis in the dentate gyrus [1] and subventricular zone [2], respectively. These seminal studies along with others demonstrated that these dividing progenitor cells have the capacity to migrate to damaged brain regions and differentiate into site-specific neurons [3]. However, the regenerative capacity of these cells are limited with about 80% of new neurons dying prior to integration [3], leaving victims of neurological conditions with lifelong deficits in motor function and/or cognition. This led to further investigation of enhancing neural repair mechanisms through the transplantation of cultured NSCs in preclinical animal models of neural injury with evidence of robust therapeutic benefit, thus bolstering tissue repair mechanisms more effectively than endogenous NSCs alone [4-6]. Furthermore, more recent ground-breaking advancements in stem cell engineering led to the development of induced pluripotent stem cells (iPSC), which are pluripotent stem cells that can be derived from the patient's own cells and differentiated into neural stem cells (iNSCs), thus serving as an autologous cellular therapeutic for neurological diseases such as stroke and traumatic brain injury (TBI) [7, 8].

Stroke and TBI both carry a heavy socioeconomic burden worldwide, and victims suffering these diseases could greatly benefit from iNSC treatment [9-11]. In both cases, the

primary neurological insult coupled with the ensuing cascade of excitotoxic and inflammatory insults lead to a significant loss in brain tissue accompanied by functional deficits [12, 13]. Preclinical studies have shown that transplanted iNSCs improve stroke and TBI recovery by 1) augmenting neurogenic activity, 2) protecting neurons from undergoing cell death, 3) promoting new vessel formation in damaged tissues and 4) acting as a cellular replacement thereby improving functional outcome [14-18]. However, the enthusiasm of these promising results has been dampened by the hundreds of therapeutics for stroke and TBI that have shown efficacy in preclinical animal models but have failed in clinical trials [19]. Clinicians and researchers have repeatedly expressed that a major limitation behind the plethora of unsuccessful clinical trials in stroke and TBI is the lack of translatability of preclinical animal models to human patients [20, 21]. Indeed, to date, all preclinical studies testing iNSC efficacy have been performed in rodents, which possesses many brain anatomical and physiological differences compared to humans [22]. Thus, confirming therapeutic efficacy of iNSCs in a large animal model, such as the pig, before moving to clinical trials may better predict the probability of success of iNSC therapy in human patients.

The use of pigs in neuroscience research has increased in recent years due to the striking similarities in anatomy and physiology compared to the human. The pig brain possesses a similar size, gyral pattern, gray-to-white matter ratio, and neurodevelopmental sequence compared to the human brain. These factors are important because brain cytoarchitecture and composition is directly correlated to connectivity and complexity, thus environmental-specific mechanisms of iNSC repair should be observed in a brain more similar to humans [23-25]. Furthermore, technical considerations of iNSC therapy such as number of cells transplanted and number of transplant sites can be more adequately optimized in animals with a similar brain size to humans.

Therefore, the pig model could potentially bridge the gap between preclinical rodent models and human patients thus potentially increasing the probability of success in clinical trials.

The studies that comprise this dissertation sought to 1) develop a novel pig TBI model with quantitative indicators of pathophysiology and recovery mechanisms, and 2) test the therapeutic efficacy of iNSCs in a pig stroke model. First, we develop a novel controlled cortical impact (CCI) TBI model in the pig and show that 1) scaling CCI parameters correlate to increased pathological severity at the cellular, tissue, and functional level after TBI while 2) illustrating that CCI results in unique metabolic signatures dependent injury severity, brain tissue type, and time after injury. In addition, CCI results in spatiotemporal pathological features that can be longitudinally tracked utilizing magnetic resonance imaging (MRI) and confirmed with histology. These results suggest that our pig CCI models serves as an ideal translational platform for testing iNSCs and other restorative therapies. Next, we show that iNSC therapy promotes brain tissue recovery through neuroprotective, regenerative, and cell replacement mechanisms in a pig stroke model. Together, these studies bolster preclinical therapeutic development strategies by building the framework for a translational large animal TBI model with quantifiable deficits at the cellular, tissue, and functional level while also confirming that iNSCs may be an effective therapeutic in human stroke patients.

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CHAPTER 2

LITERATURE REVIEW:

PRECLINICAL TRAUMATIC BRAIN INJURY MODELS:

CURRENT ANIMAL MODELS, MAJOR CHALLENGES, AND CONSIDERATIONS FOR

FUTURE TRANSLATIONAL IMPACT ¹

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Abstract

Traumatic brain injury (TBI) is a leading cause of disability with survivors often exhibiting long-term deficits in cognition, sensorimotor function, and/or personality. Despite the socioeconomic burden of TBI, there is no effective treatment to restore neurological function. Animal models of focal cortical contusion, diffuse axonal injury, or a combination of both are commonly used to recapitulate the TBI sequelae to understand pathophysiologic mechanisms and identify potential therapeutic targets. In this review, we will discuss the advantages and shortcomings of current animal models with respect to animal species, injury type, and the translatability of histopathological features and functional outcome assessments to the clinical setting. Improving our current preclinical animal models may increase their translational capacity and lead to a therapeutic breakthrough for TBI.

Introduction

Traumatic brain injury (TBI) is a leading cause of long-term disability and equates to one third of all injury-related deaths [1]. Even in the case of mild TBI, survivors often exhibit alterations in cognition, sensorimotor function, and/or personality which creates a great socioeconomic burden [2]. In fact, approximately 2% of the United States population is living with long-term disability associated with a TBI [3]. Despite the prevalence of TBI, clinical treatment is limited to supportive care in order to mitigate secondary injury (i.e. fluid therapy to maintain blood pressure and thus brain oxygenation, controlling body temperature, and decompressive craniectomy [4]). There is currently no effective treatment that restores neurological function [5]. Over the past few decades, animal models have been developed to reproduce TBI pathophysiology and resulting functional deficits in order to develop and optimize potential therapeutics. Although numerous neuroprotective pharmacologic agents designed to limit excitotoxicity and/or inflammation through multiple mechanisms [6-12] have demonstrated therapeutic efficacy in animal models of TBI, all have failed in Phase II or III clinical trials [2, 5, 13]. Therefore, the clinical translatability of established animal models and their corresponding therapeutic efficacy indicators must be reevaluated.

In this review, we will discuss the various animal models currently utilized in preclinical TBI therapeutic development with respect to animal species and injury type. Furthermore, the major advantages and disadvantages of each model within the scope of common preclinical endpoints, such as histopathological and functional outcome assessments, will be addressed. Understanding the strengths and limitations of preclinical animal models is essential to optimize current preclinical strategies in order to maximize the predictive power of therapeutic efficacy in human TBI patients.

Animal Models of Traumatic Brain Injury

Animal models are essential in understanding the biomechanical and pathophysiological aspects of TBI that otherwise could not be addressed in the clinical setting [2]. Due to the heterogeneous nature of TBI, a number of animal models have been developed to replicate different types of injury such as rapid acceleration/deceleration, blast waves, or focal impact [2]. However, animal models are inherently more homogenous than what is seen in the clinical setting and do not account for variable injury locations, severities, and comorbidities that often complicate the treatment of clinical TBI [14]. To date, rodents are the primary species utilized in preclinical TBI studies due to their low cost and standardized outcome measures despite anatomical and physiological dissimilarities between the rodent and human brain [15, 16]. Although large animal models more closely mimic human TBI pathogenesis, financial and ethical burdens limit their preclinical use [2]. Therefore, it is important to weigh the strengths and weaknesses of each preclinical animal model with respect to model type, species, budget constraints.

Species of Animal Models

Rodents

As previously mentioned, rodents (i.e. rats and mice) are the most commonly used animal in preclinical TBI modeling. This is due to a number of technical advantages including low cost, accessibility, a variety of genetic backgrounds to assess co-morbidities (e.g. diabetic strains), molecular tools for genetic manipulation and analysis, and highly characterized outcome measures [17]. Indeed, a plethora of previous studies in rodents document measurable histological and functional deficits in multiple TBI models including fluid percussion injury

(FPI) [18, 19], controlled cortical impact (CCI) [20, 21], blast injury [22, 23], and weight-drop injury [24, 25]. Furthermore, numerous therapeutic agents (e.g. progesterone [26], dexamethasone [27], recombinant human erythropoietin [28], and cyclosporine [29]) and strategies (e.g. decompressive craniectomy [30] and hypothermia [31]) tested in clinical trials for TBI were first developed and deemed efficacious in rodent models; however, the clinical trial results have varied [13]. Although rodent models have an essential role in therapeutic development, there are many disadvantages associated with translating preclinical findings in rodents to human patients. First of all, the rodent brain is substantially smaller than the human brain (approximately 2 grams for rats [32] and 1300 grams for adult humans [33]), so it is often difficult to scale up treatment regimens with respect to dose and duration appropriately [34]. As a gyrencephalic species, the human brain exhibits convulsions of the cerebral cortex thereby enabling the expansion of cortical volume within the confines of the skull, the rodent brain possesses a smooth, lissencephalic cortical surface [35]. This is important because the extent of gyral folding dictates brain connectivity and is correlated with a species' cognitive and sensorimotor abilities [35-37]. Furthermore, the rodent brain contains about 86% less of the relative white matter volume than the human brain [15]. Gray and white matter compartments exhibit differences in blood flow, injury susceptibility, metabolism, and repair mechanisms, suggesting that brain composition is a major variable dictating therapeutic efficacy after TBI [16, 38-40]. Taken together, the dissimilarities in brain anatomy and physiology, coupled with lack of heterogeneity in rodent models, are likely the major reasons underlying the failed translatability of therapeutics to the clinical setting. These differences between rodent and human indicate that higher order mammals may be needed to bridge the gap between the rodent model and human patients.

Pigs

The use of pigs in neuroscience, as well as other areas of research, has increased over the past couple of decades due to numerous anatomical and physiological similarities to humans [41]. Pigs have a more similar brain size and cytoarchitecture to humans compared to the widely-used rodent model, making them an excellent model species for TBI. The pig brain is only about 9 times smaller than the human brain (approximately 130 grams vs. 1300 grams) [33, 42]. Furthermore, in parallel with humans, pigs have a gyrencephalic brain comprised of >60% white matter [43]. Finally, pigs exhibit a similar neurodevelopmental sequence compared to humans, making them an intriguing candidate for studying the effect of TBI in the developing juvenile brain [44]. In addition to biological motives behind utilizing pigs as preclinical TBI models, the pig is widely available due to commercialized food production practices and presents many ethical and economic advantages compared to other large animal species such as the non-human primate or dog [41]. Agricultural breeds such as the Landrace have been utilized in preclinical TBI experiments; however, lack of breed standards result in poorly defined bloodlines thus genetic heterogeneity [41]. While this potentially makes experimental reproducibility more difficult, on the other hand, this addresses one of the major pitfalls of traditional rodent systems in that treatments are tested in only one homogeneous genetic pool [45]. Conversely, a considerable disadvantage with the use of commercial pig breeds is the size of mature animals, which can reach upwards of 300kg, which makes the planning of long-term studies more difficult [41]. To overcome the issue of size, miniature pig breeds such as the Yucatan and Göttingen are becoming more commonly used in biomedical applications. These breeds are also more inbred compared to their agricultural counterparts, thus reducing outcome variability [41]. Many preclinical pig TBI models have been developed in recent years including CCI [46-48],

FPI [49, 50], and blast injury [50, 51] with distinct histopathological changes as well as measurable deficits in cognitive function. Perhaps the most well-published pig TBI models are those of Duhaime and Margulies who have developed CCI [47] and non-impact rotational injury [52] models, respectively, in juvenile pigs. These models have provided insight to differences in physiological response after TBI based on developmental stage [47], direction [53] and acceleration [54] of rotational injury, as well as TBI outcomes associated pharmacologic therapy [55-57]. Thus, the pig model could have utility in serving as an intermediate between rodent models and clinical trials in lieu of expensive and technically challenging primate models.

Other Species

In an effort to understand TBI pathology mechanisms in a gyrencephalic species, studies have also been performed in the non-human primate [58, 59], cat [60], dog [61], sheep [62], and ferret [63]. However, these studies are limited compared to those in rodent due to a number of technical challenges such as the need for complex surgical facilities, experienced veterinary staff, and significantly more funding [64]. Together, these factors usually result in smaller sample sizes, higher data variability, and more difficulty drawing scientific conclusions. Despite these challenges, some argue that the dog demonstrates unique utility as a suitable model for naturally occurring brain trauma, which may better replicate some aspects of TBI that experimental models often lack [65]. For example, owners often seek high-quality veterinary care after their dog has experienced a traumatic event, making it feasible to obtain a reasonable number of research subjects. Furthermore, dogs present with similar post-TBI characteristics as humans [65]. For example, post-traumatic epilepsy (PTE) demonstrates a similar prevalence and

phenomenology between dog and human patients; therefore, PTE in the dog may provide a translational platform for human PTE [66].

Categories of Traumatic Brain Injury Models

Aside from the translatability of animal species to human patients, another challenge in developing effective treatments for TBI is the heterogeneity of the disease. As previously mentioned, TBI can occur through a focal impact resulting in cerebral contusion, a rapid acceleration/deceleration or blast event that induces a more diffuse brain injury, or oftentimes a combination of many injury mechanisms [67]. Each of these mechanical insults produces a unique brain pathology with varying neurological outcome (**Table 2.1**). In an effort to understand the pathoanatomic alterations associated with each injury type, several animal models have been developed. Here we will discuss common models of cerebral contusion (including CCI, FPI, and weight-drop), non-impact diffuse axonal injury (DAI), and blast-induced injury (**Figure 2.1**).

Cerebral Contusion Models

Cerebral contusion, defined as a bruise to the brain, falls under the category of “focal TBI” along with intracerebral and intracranial hemorrhage. In the laboratory, models of focal TBI are produced from a blow to the cortex which may or may not be accompanied with more widespread neuropathology [67]. There are three main techniques utilized to generate a focal cortical contusion in animal models: controlled cortical impact (CCI), fluid percussion (FP), and weight-drop (WD). In each of these models, the severity of the TBI is dependent on the impact forces applied directly to the skull or brain [67].

In CCI models, a specialized device is employed to produce focal TBI with high reproducibility. The CCI device is comprised of a beveled impactor tip attached to a piston, which is usually accelerated by a pneumatic cylinder or an electromechanical actuator [68]. Impact parameters including the velocity at which the impactor tip strikes the brain, the depth of depression from the dural surface, and the time at which the tip dwells on the dural surface can be discretely adjusted to regulate TBI severity. Because of its scalability, the CCI model has been employed in a number of different species including rodents [69, 70], ferrets [63], pigs [44, 46, 47], and non-human primates [59]. The main histopathologic feature of the CCI-damaged brain is focal contusion of the cortex, which is initially hemorrhagic before becoming a necrotic cavity over time [47, 69]. In addition, blood-brain barrier (BBB) disruption [71, 72] resulting in vasogenic edema as well as oxidative stress [73, 74] and inflammation [71, 75] is common, which results in neurological impairments including deficits in cognitive [74] and motor [75] function. In addition to scalability, an advantage to utilizing the CCI model is that the methodology can be easily applied toward models of closed head injury (CHI) since a higher velocity of impact can be achieved with CCI compared to the WD model, thus a stronger force can resonate through the exposed skull [76, 77] or even a “helmeted” head [78, 79]. However, a disadvantage to the CCI model is the limited degree of diffuse TBI pathology because the head is often fixed in space upon impact, although certain surgical setups allow for some movement of the head and thus more diffuse injury [77].

Another common model of cortical contusion, deemed FPI, induces injury through the rapid propagation of a fluid-driven pressure wave onto the dural surface of the exposed brain [67]. Similar to the other common TBI models, injury severity can be graded by adjusting the force of the pressure pulse [80]. There are two adaptations of this model which depend on the

location of the craniectomy site. For central fluid percussion (CFP), the craniectomy is performed over the sagittal suture of the skull midway between the bregma and lambda and thus typically leads to bilateral neuropathology [81]. In contrast, lateral fluid percussion (LFP) injuries are performed over the left or right parietal cortex, in a location lateral to the sagittal suture, which produces an ipsilateral lesion [82]. This model has been employed in a number of species including the rodent [81, 82], rabbit [83], cat [60], pig [84, 85], dog and sheep [61]. In addition to cortical contusion [86, 87], hemorrhage [81, 86], and cytotoxic and/or vasogenic edema [88], studies have also documented associated excitotoxicity [89], gliosis [18], diffuse axonal injury [18, 90, 91], and inflammation [18, 19, 92]. These pathological hallmarks are routinely accompanied by cognitive deficits regardless of injury location [18, 86, 93, 94] .

Weight-Drop Models

As the name suggests, the WD model induces injury simply through the gravitational force of a weight guided through a cylindrical apparatus that makes impact on a disc mounted to the skull (to prevent skull fracture) or the exposed dura through a craniectomy [67]. Injury severity is determined by the weight of the free-falling object as well as the height from which the weight is dropped [95]. An advantage of the WD model is that the experimental setup can be modified to produce a focal or diffuse brain injury. The type of injury is determined by whether the skull is open or closed and how the head is secured upon impact. For focal TBI, Feeney et al. developed a WD model in which the weight is dropped onto the exposed dura of a rat whose head is securely fixed on a stereotaxic apparatus. This version results in graded cortical contusion with injury severity depending on impact force (weight x distance dropped) [96]. Conversely, in an adaptation by Marmarou et al., the animal is fitted with a disc mounted to the

exposed skull and placed on a foam bed which allows for head movement post-impact, thus producing histological evidence of diffuse brain injury [95, 97]. Building on this design, Kane et al. sought to design a model that would allow for repetitive mild TBI by placing an anesthetized mouse on a slit piece of aluminum foil instead of a foam bed. Upon impact, the mouse falls through the foil onto a sponge cushion 10cm below. This results in an unrestrained rapid acceleration event, which is a key characteristic of concussive injury in humans [98]. Despite its utility for both focal and diffuse TBI models, a disadvantage of the WD model is that impact depends on gravity alone; therefore, the forces generated may not be strong enough to produce measurable injury in larger animals especially in the context of closed head injuries.

Non-impact Diffuse Axonal Injury Models

In the event of a fall or motor vehicle accident, which are two of the most common causes of TBI [99], the rapid acceleration/deceleration of the head literally pulls apart the axons in white matter compartments resulting in amyloid precursor protein (APP) accumulation and myelin damage [100]. Although the resulting DAI is thought to be one of the most common pathologic features among all TBI cases, relevant animal models of DAI are lacking in comparison to models of focal contusion [67, 95]. Marmarou's WD model in rats remains to be the most widely-used DAI model despite rodents having <10% white matter and thus potentially incompletely recapitulating the effects of DAI in the human brain. To address this, a couple of DAI models have been developed in the non-human primate [58] and the pig [54, 101], both of which have gyrencephalic brains with white matter composition more comparable to humans. In these models, a specialized apparatus rapidly rotates the head in the sagittal, coronal, or horizontal plane [102]. These studies result in human-like DAI pathologies including β -APP+

axonal varicosities and bulbs and intracranial hemorrhage [54, 103-105] . Interestingly, these studies have also shown that the plane of head rotation is a critical determinant in the distinct DAI-associated pathologies [58], the persistence of axonal damage [53], probability of lost consciousness [106, 107], and behavioral outcome [53].

Blast-induced Injury Models

Blast-induced TBIs are prevalent in military engagements worldwide, and these complex events can induce multiple types of brain injury including cerebral penetration by a foreign object, focal injury after being thrown or struck by objects, and/or DAI by the propagation of rapid pressure waves [67]. Although blast-induced injury is quite heterogeneous in the real-world setting, laboratory animal models aim to replicate the primary or “pure” blast injury resulting from blast overpressure (BOP) transmission [67]. The effect of BOP on brain pathology has been studied in rats [22], non-human primates [108], and pigs [51]. In these studies, animals are directly exposed to explosive material in either an open or confined space in order to study the effect of pressure transmission on brain injury in these divergent conditions. Previous studies have demonstrated that blast exposure results in multiple brain pathologies including oxidative stress, inflammation, BBB breakdown, axonal injury, astrogliosis, and neuronal loss with associated cognitive deficits [22, 50, 109]. Furthermore, Elder et al. documented post-traumatic stress disorder (PTSD)-like behaviors after blast-induced mild TBI, providing a platform to understand the complex mechanisms behind PTSD in veterans returning from the battlefield [110].

Model Considerations for Translational Impact

There are a number of pitfalls in the aforementioned preclinical models that likely hinder translatability to the clinical setting. Species-specific factors including the differences in inflammatory sequelae time course, brain tissue composition, and functional assessments may attribute to the lack of successful clinical translation from preclinical animal studies. These pathological elements must be addressed in order to transition TBI therapies from bench to bedside.

Variable Time Course of Neuroinflammation

After TBI induction, a cascade of inflammatory insults lead to secondary brain injury that is augmented, in part, by the upregulation and secretion of pro-inflammatory cytokines and chemokines such as interleukin (IL)-1 β , IL-6, IL-8, tumor necrosis factor (TNF)- α , and monocyte chemoattractant protein-1 [111]. These pro-inflammatory mediators propagate secondary injury mechanisms such as the activation of microglia, astrogliosis, and the infiltration of circulating neutrophils, monocytes, and lymphocytes into the brain parenchyma in the hours/days following the initial traumatic insult [111, 112]. Since the primary TBI-induced injury occurs at the moment of the external force, there is no way to mitigate initial brain damage other than to prevent the trauma altogether [113]. Conversely, the more delayed time course of the inflammatory cascade provides a window for therapeutic intervention to limit secondary brain damage. Animal models of TBI have been instrumental in the discovery and characterization of anti-inflammatory therapeutics for TBI and have resulted in several clinical trials. However, as previously mentioned in this review, the outcome of these clinical trials have been disappointing. This may be partially due to the differences in inflammatory time course

between preclinical animal models and human TBI patients. Indeed, previous studies have suggested that specific pro-inflammatory cytokines peak at varying intervals between rodent and human and is also dependent on the type of injury [70, 114, 115]. For example, these studies suggest that CXCL1 and TNF- α peaks within 2 hours after injury in humans but remains heightened in rodents 4 hours after injury. The spatiotemporal inflammatory sequela is important in optimizing a time window for therapeutic intervention; therefore, more studies are needed to determine what species demonstrates a similar inflammatory time course for translational studies.

Distinct Histological Changes In White Matter Compartments

It is well established that although underdiagnosed, axonal injury is a common occurrence of both diffuse and focal TBI; furthermore, axonal injury is highly correlated to poor neurological outcome [113]. While gray matter is composed of neuronal cell bodies, white matter consists of myelinating oligodendrocytes as well as myelinated and unmyelinated axons that foster communication between different gray matter regions [15]. Previous studies have shown that white matter displays different patterns of deterioration than gray matter after TBI [116, 117]. At the cellular level, while excitotoxic and apoptotic mechanisms lead to neuronal cell death after TBI in gray matter compartments, axonal membranes in white matter become “leaky” and allow the influx of calcium leading to mitochondrial dysfunction, axonal swelling, and ultimately disconnection [113]. Furthermore, while neuronal cell death is associated with focal cortical contusion in gray matter, death of oligodendrocytes may be a unique feature of axonal injury specifically in white matter compartments in both focal and diffuse injuries [90, 117, 118]. On the tissue level, recent diffusion tensor imaging (DTI) studies have illustrated

impaired white matter integrity and connectivity after TBI in both humans and animal models [18, 119-121]. Taken together, this indicates that it is essential to study TBI injury mechanisms in an animal model with white matter anatomy similar to humans. Despite only having 14% of the relative amount of white amount to humans [15], the majority of preclinical TBI studies assessing changes in white matter are performed in the rodent. To address this issue, models of axonal injury through non-impact rotation of the head have been developed in the pig [52, 53]. These studies may hold greater translational value for white matter-specific injuries and are deserving of further study.

Sensitivity of Functional Assessments

The major goal of TBI therapeutic development is to restore the detrimental deficits in cognition and sensorimotor function to improve quality of life for patients. Thus, the development of robust functional assessments in animal models to monitor recovery is critical to preclinical TBI research. Although the establishment of reliable functional assessments in large animal models is still a work in progress, a number of cognitive (e.g. Morris Water Maze, radial arm maze) and sensorimotor (e.g. rotarod, beam walking, tape removal) tests have been well-characterized in the rodent that routinely detect deficits after focal or diffuse TBI [20, 122-124]. In addition to the number of established functional tests, an advantage of the rodent over large animal models is their utility in measuring fine motor control. For example, grip strength is easily tested after TBI in rodents by measuring the time an animal can support their body weight by grasping a string or wire [79, 122]. In addition, the staircase test measures the ability of a rodent to retrieve food pellets on a staircase apparatus [125], which can assess a gradient of fine motor coordination by measuring pellets eaten versus pellets simply retrieved as well as

unilateral deficits [126]. Conversely, large, quadruped animal models such as the pig lack fine motor control and are limited to gross sensorimotor assessments such as balance beam walking [127], which may lack sensitivity.

Furthermore, it is unclear how these functional tests in all animal models will translate to human patient recovery. Previous studies have suggested that long-term functional deficits after TBI can be achieved in preclinical rodent models although cognitive deficits seem to be more persistent than sensorimotor deficits [2]. However, a review of TBI literature by Gold et al. reported that less than 10% of preclinical TBI models demonstrate functional deficits in the chronic phase of TBI (2 months post-TBI or more) [128]. It is possible that the lack of long-term functional assessments is due to the inadequate sensitivity of current tests which may not detect subtle changes over time especially in the case of mild TBI. Indeed, a study by Turner et al. documented no change in functional performance in a battery of cognitive and motor tests utilizing the Marmarou WD model despite evidence of histopathological changes, which questions the sensitivity and translatability of these tests in the context of concussive injury [129]. How can researchers translate preclinical findings based on histologic evaluation alone when clinical concussion diagnosis is dependent on cognitive and neuropsychologic events? Developing a battery of robust neurological assessments and/or biomarkers that are common between preclinical animal models and human TBI patients will lead to more reasonable translation of therapeutics to the clinical setting.

Conclusion

The numerous preclinical TBI animal models developed in the last few decades have increased our understanding of TBI biomechanics and pathophysiology. However, the inherent

limitations of current animal models have proved to be a major challenge in developing effective TBI treatments. Anatomical and physiological differences between small animal models and human patients combined with the financial and ethical issues associated with large animal models makes choosing an ideal model species difficult. Next, although human TBI is often heterogeneous with respect to injury types, experimental TBI models aim to replicate specific types of injury and lack real-world biomechanics which diminishes translatability. Taken together, this suggests that after initial development in economical and validated rodent studies, the efficacy of potential therapeutics may need to be confirmed in large animals with more similar TBI pathophysiology to humans as well as models with varying injury types in order to increase the success of future clinical trials. In addition, preclinical cognitive and sensorimotor studies in the months following TBI should be implemented more often to gain a deeper understanding of long-term functional recovery and feasible therapeutic windows. Thus, improving and refining our current preclinical development strategy through the use of multiple models, rather than seeking the “ideal” animal model, may be the key to a therapeutic breakthrough for TBI.

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Table 2.1: Preclinical Animal Models of Traumatic Brain Injury

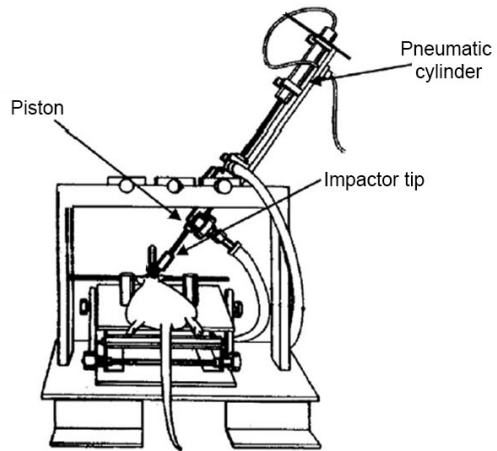
NOTE: BBB = blood-brain barrier, DAI = diffuse axonal injury. Adapted from Xiong et al. [2].

TBI Model	Injury Type	Advantages	Disadvantages	Documented Histopathological Features	Species Studied	References
Controlled Cortical Impact	Focal	• Highly reproducible • Scalable • Strong force can be used • Low mortality rate	• Traditionally requires craniectomy • Head is fixed in space	• Cortical contusion • BBB disruption • Vasogenic edema • Oxidative stress • Inflammation	• Rat • Mouse • Ferret • Pig • Primate	[47, 59, 63, 69, 70]
Fluid Percussion Injury	Mainly focal (with evidence of some diffuse)	• Highly reproducible • Scalable	• Requires craniectomy • High mortality rate	• Cortical contusion • Hemorrhage (intracranial and intracerebral) • Both vasogenic and cytotoxic edema • Excitotoxicity • Inflammation/Gliosis • Axonal injury	• Rat • Mouse • Pig • Cat • Dog • Sheep • Rabbit	[18, 60, 61, 83, 86, 88, 89, 130]
Feeney Weight Drop	Focal	• Inexpensive • Easy to operate • Scalable	• Required craniectomy • Head is fixed in space • Force limited to gravity • High variability	• Cortical contusion • White matter hemorrhage • BBB disruption • Edema • Inflammation	• Rat	[96, 131]
Marmarou Weight Drop	Diffuse	• Inexpensive • Easy to operate • Scalable • Well-characterized	• Force limited to gravity • High mortality • High variability	• Intracranial hemorrhage • Vasogenic edema • Axonal injury • Widespread white matter damage • Cortical neuron damage	• Rat • Mouse	[95, 97, 98]
Non-impact Rotational Injury	Diffuse	• Rotation on multiple planes • Replicates key features of DAI • Low mortality	• Not widely replicated in different laboratories	• Axonal injury • Amyloid precursor protein accumulation • Intracranial hemorrhage	• Primate • Pig	[53, 54, 58]
Blast-induced Injury	Diffuse	• Biomechanics similar to blast overexposure in human	• Needs standardization	• Oxidative stress • Inflammation • BBB breakdown • Axonal injury • Gliosis • Neuronal loss	• Rat • Primate • Pig	[22, 50, 51, 108]

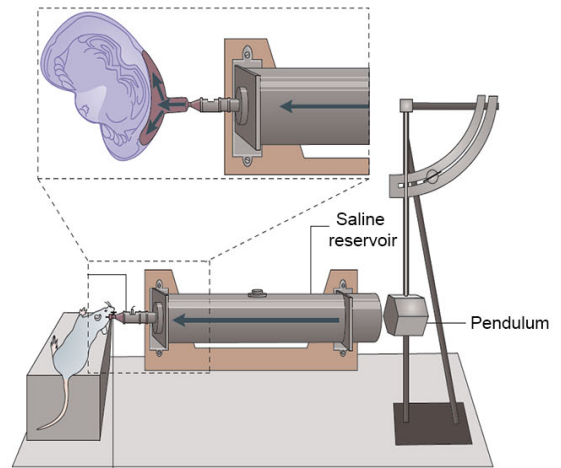
Figure 2.1: Experimental Setups for Animal Models of Traumatic Brain Injury

(A) The controlled cortical impact (CCI) device consists of an impactor tip attached to a piston, which is driven by a pneumatic (or electromagnetic) cylinder. The impactor tip strikes the exposed dura at a known velocity, depth of depression, and dwell time while the animal is secured on a rigid platform. **(B)** The fluid percussion injury (FPI) device utilizes the rapid propagation of a fluid pulse onto the exposed dura. A pendulum is dropped from a predetermined angle which strikes a saline reservoir that is attached to a shaft mounted on the skull. **(C)** In Feeney's weight-drop (WD) model, a free weight is dropped down a hollow cylinder which strikes the exposed dura of an animal secured to a stereotaxic device. The force is determined by the weight of the object and the height at which its dropped. **(D)** In Marmarou's WD model, a metal disc is attached to the animal's skull to prevent bone fracture before the weight is dropped down a hollow cylinder. The animal is placed on a foam bed to allow for movement of the head post-impact. **(E)** In the non-impact rotational injury model, the animal's snout is securely fastened onto a biteplate, which is attached to a sidearm and driven by a pneumatic actuator. The inertia created by the driving sidearm rapidly rotates the head without impact. **(F)** In the blast-induced injury model, the animal is placed in a confined (or open) space, and pressure waves originating from a detonation diffusely injures the brain. NOTE: Animals are anesthetized in all experimental setups. (Reproduced from Xiong et al., Ibrahim et al., and O'Connor et al. with modifications [2, 54, 132]).

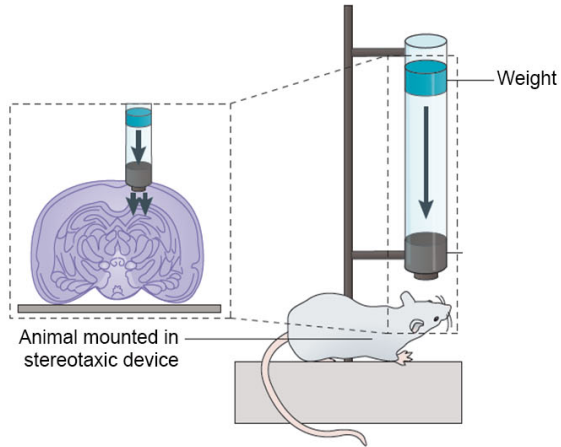
A: Controlled Cortical Impact



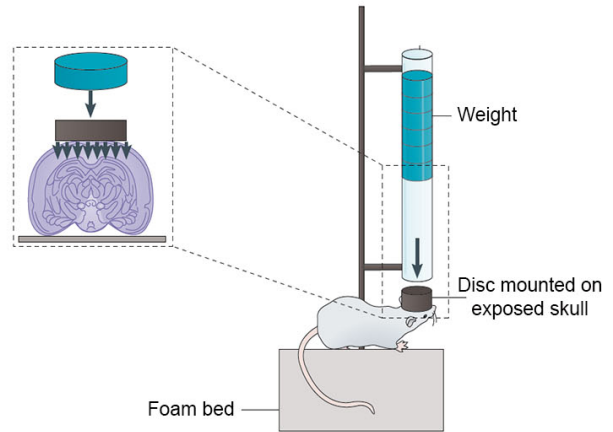
B: Fluid Percussion Injury



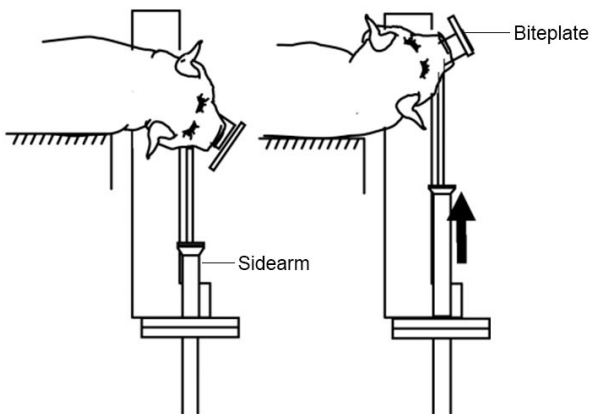
C: Feeney Weight-Drop



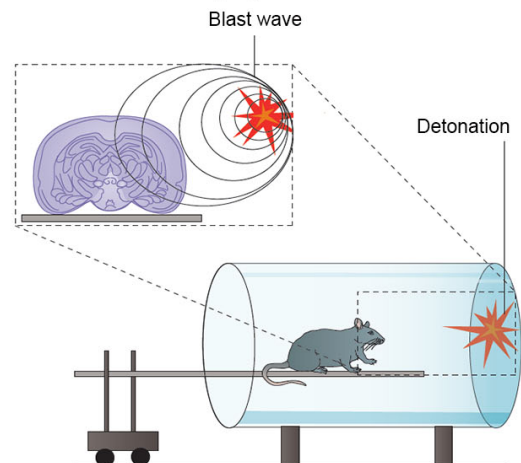
D: Marmarou Weight-Drop



E: Non-impact Rotational Injury



F: Blast-induced Injury



CHAPTER 3

LITERATURE REVIEW:

NEURAL STEM CELL THERAPY FOR STROKE:

CELL SOURCES, MECHANISMS OF THERAPEUTIC ACTION, AND TECHNICAL
CONSIDERATIONS¹

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Abstract

Neural stem cells (NSCs) have demonstrated multimodal therapeutic function for stroke, which is the leading cause of long-term disability and the second leading cause of death worldwide. In preclinical stroke models, NSCs have been shown to modulate inflammation, foster neuroplasticity and neural reorganization, promote angiogenesis, and act as a cellular replacement by differentiating to mature neural cell types. However, there are several key technical questions to address before NSC therapy can be applied to the clinical setting on a large scale. In this review, we will discuss the various sources of NSCs, their therapeutic modes of action to enhance stroke recovery, and technical considerations for the clinical use of NSC therapy. Understanding the key factors involved in NSC-mediated tissue recovery and addressing the current translational barriers may lead to clinical success of NSC therapy and a first-in-class restorative therapy for stroke patients.

Introduction

Although stroke is the leading cause of long-term disability and the second leading cause of death worldwide, there is only one Food and Drug Administration (FDA)-approved pharmacologic therapy [1, 2]. Most therapies recently tested in clinical trials have focused on mitigating secondary injury by diminishing excitotoxicity [3-6], immune and inflammatory response [7], or apoptosis [8], all of which have failed. The Stroke Therapy Academic Industry Roundtable (STAIR) group was formed to bring together leaders in the stroke field to assess the plethora of failed clinical trials and how to best proceed with future therapeutic development. One of the major recommendations for future research is to develop restorative therapies that would enhance tissue and functional recovery beyond limiting secondary damage [9]. Recent developments in stem cell technology has enabled researchers to develop therapeutic cellular products that have multi-modal therapeutic function. Stem cell therapy after stroke in preclinical models has been shown to not only promote neural cell survivability and remodeling, but also replace lost neural cells by integrating into host neural circuits, making stem cells a promising restorative stroke therapeutic.

Due to the encouraging preclinical data of stem cell treatment after stroke, the Stem Cell Therapies as an Emerging Paradigm for Stroke (STEPS) group was formulated to develop guidelines to address critical issues in stem cell therapeutic development for stroke [10]. Since the initial recognition of the potential of stem cell therapy, multiple types of stem cells are being developed and optimized both in preclinical models and clinical trials for stroke including adult stem cells (ASCs) from adipose or hematopoietic sources [11-16] and neural stem cells (NSCs) (**Table 1**), with promising results documented for both cell types. Since NSCs are multipotent and specifically differentiate to neural cell types, they are likely to hold the greatest potential of

cellular replacement therapy after stroke. While much progress has been made over the past several years to understand and augment NSC-mediated tissue recovery mechanisms after stroke, there still remains to be several key unanswered questions to be resolved before NSC therapy can be applied to the clinical setting at a large scale. In this review, we will discuss the sources of NSCs currently being studied, their mode of action in the context of stroke treatment, and technical considerations for optimizing NSC treatment strategies for clinical application.

Sources of Neural Stem Cells

During the 1990's, novel protocols were developed to generate immortalized human cell lines capable of differentiating to mature neurons on a large scale, a feat which was nearly impossible with primary fetal tissue cultures [17]. Since then, multiple types of NSC lines that generate mature neural cell types have been developed and characterized including NSCs derived from fetal tissues, embryonic stem cells (ESCs) and induced pluripotent stem cells (iPSCs), all of which have been shown to enhance recovery after stroke and other neurological disorders **(Figure 3.1)**.

Fetal-Derived Neural Stem Cells

Fetal-derived NSC lines (fetal-NSC) were one of the first potential cell sources developed that had significant potential as a stroke cell therapy. Fetal-NSCs were generated by dissociating human fetal cortex or mesencephalon or spinal cord tissues (between 7 and 21 days post-conception) [17, 18]. These cells proved to be capable of long-term expansion when cultured in mitogens such as EGF and FGF-2, even without genetic modification, while maintaining their neurogenic and gliogenic multipotent differentiation potential. Fetal-NSCs have shown

therapeutic potential for a number of neurological diseases such as stroke [18, 19], traumatic brain injury [20, 21], spinal cord injury [20, 22], and amyotrophic lateral sclerosis [23, 24]. However, previous studies have shown that fetal-NSCs undergo senescence earlier than other NSC lines, which would make large-scale manufacturing more difficult [25]. To address this, some fetal-NSC lines have undergone genetic modification to enhance immortality during expansion. A prominent example of this is the CTX0E03 cell line, which is derived from fetal cortical tissue and transduced with a *c-mycER*^{TAM} construct [18]. Previous studies have demonstrated that CTX0E03 has multimodal therapeutic function in preclinical animal models; furthermore, no cell-related adverse events were documented in a recent Phase 1 clinical trial for stroke [19, 26-28].

Embryonic Stem Cell-Derived Neural Stem Cells

After the establishment of cultured embryonic stem cell (ESC) lines [29], several groups have successfully developed ESC-derived long-term expandable NSCs (ESC-NSCs) from multiple species including human [30], mouse [31], and primate [32]. The therapeutic use of ESC-NSCs could be advantageous over NSCs of fetal origin due to the theoretically limitless source of immortal ESCs in which to scale-up cell manufacturing to meet the clinical demand [33]. To date there have been no clinical trials testing ESC-NSC for stroke as the field has been slowed by ethical concerns associated with their embryonic origin and potential tumorigenicity. However, a Phase 1 clinical trial testing ESC-NSCs was recently completed for spinal cord injury (clinicaltrials.gov ID: NCT01217008), overcoming ethical and regulatory challenges and paving the way for future ESC-NSC stroke clinical trials [34].

Induced Pluripotent Stem Cell-Derived Neural Stem Cells

More recently, the research of Yamanaka and others have shown that adult somatic cells can be reprogrammed to a pluripotent state through the overexpression of transcription factors, and these reprogrammed cells have similar plasticity and neural differentiation potency as ESCs [35-37]. These cells have been deemed induced pluripotent stem cells (iPSCs), and their NSC progeny (iPSC-NSCs) possess unprecedented therapeutic potential for neurological disease as they can be generated from the patient's own somatic cells, avoiding the risk of immune rejection associated with allogeneic transplants [38, 39]. Since the in vitro characterization of iPSC-NSCs, several studies have documented improved tissue and functional recovery after transplantation into preclinical stroke models [40, 41] (**Table 3.1**). Although further progress is needed for clinical application of iPSC technology for stroke, with shortened reprogramming duration being critical, these preclinical studies show that iPSC-NSCs are a promising future treatment option for stroke [42].

Neural Stem Cell Modes of Action

NSCs have demonstrated multimodal therapeutic function after transplantation into preclinical animal models of stroke (documented NSC modes of action in preclinical animal models are summarized in Table 3.1). Depending on the treatment protocol, NSCs are able to protect at-risk neural cells, promote endogenous NSC proliferation and migration, foster synaptic remodeling, stimulate new vessel formation, and/or integrate into host neural circuits, which have been associated with improvements in cognitive and sensorimotor function [43, 44] (**Figure 3.1**). However, further studies are needed to determine which tissue recovery mechanism is most effective in restoring neurological function.

Immunomodulation

Stroke injury is thought to be propagated by a strong immune and inflammatory response through the activation of microglia, the resident immune cells of the brain, which produce pro-inflammatory cytokines such as interleukin (IL)-1 β , IL-6, and tumor necrosis factor-alpha (TNF- α) in response to damage associated molecular patterns (DAMPS) within minutes after onset of ischemia [45-47]. Furthermore, the release of cytokines by activated microglia upregulates the expression of chemokines such as monocyte chemoattractant protein-1 (MCP-1) and chemokine ligand 1 (CXCL1) on endothelial cells, which promotes the infiltration of peripheral monocytes/macrophages across the blood-brain barrier (BBB) which exacerbates inflammatory injury [48]. The modulation of this inflammatory cascade may be the most widely characterized NSC mode of action in preclinical animal models of stroke. The immunomodulatory mechanisms of transplanted NSCs are likely carried out through what is known as the “bystander effect,” in which NSCs release neurotrophic factors such as glial-derived neurotrophic factor (GDNF), nerve growth factor (NGF), and brain-derived neurotrophic factor (BDNF), which have been shown to inhibit mechanisms induced by inflammatory actions [49-51]. Multiple studies in preclinical stroke models have documented decreased immune cell activation after intracranial transplantation of fetal- and iPSC-NSCs [43, 44, 52, 53]. Ultimately, reduced inflammation mediated by NSC transplantation is neuroprotective because the secondary injury cascade is curtailed; thus, reports of reduced inflammation in the stroked brain is often correlated with reductions in final infarct volume [54-56].

More studies are needed to assess whether one NSC type possesses heightened immunomodulatory properties over another. Two similar experiments performed by the same group documented decreases in the expression of pro-inflammatory cytokines TNF- α , IL-6, IL-

1 β , cell adhesion molecules ICAM-1 and VCAM-1, and chemokines MCP-1 and MIP-1 α after intraparenchymal transplantation of fetal- and iPSC-NSCs in a rodent stroke model. This decrease in pro-inflammatory cytokines and chemokines was attributed, at least partially, to NSC-mediated increases in BDNF expression in the ipsilateral hemisphere [52, 55]. This data indicates that neurotrophic factor signaling by transplanted NSCs of both iPSC and fetal origin diminishes upstream inflammatory activity in the early stages of ischemic injury by reducing pro-inflammatory signaling by activated microglia, which in turn inhibits subsequent peripheral immune cell extravasation. Indeed, the documented changes in gene expression correlated to reduced expression of the microglia marker Iba1 and reduced BBB leakage. However, fetal-NSCs were more effective at reducing final infarct volume compared to iPSC-NSCs, which may be due to differences in the neurotrophic signaling capacity of the two cell types.

Although NSC immunomodulatory behavior in the stroked brain is often documented after a intraparenchymal transplantation approach, there is evidence that intravenous delivery of NSCs after stroke also reduces the expression of pro-inflammatory mediators, immune cell activation, and apoptosis in brain tissue [54, 56, 57]. Whether it is necessary for these cells to accumulate in the brain parenchyma after intravenous delivery in order to carry out their anti-inflammatory effects is largely unknown. However, a study by Song et al. demonstrated that intravenously delivered, magnetically targeted fetal-NSCs migrated and accumulated in the stroked brain tissue and correlated to reduced infarct size compared to non-targeted NSCs [56]. Taken together, this data indicates that targeting systemically infused NSCs to the brain parenchyma augments their immunomodulatory capacity after stroke. Therefore, direct intraparenchymal transplantation may be advantageous over intravenous delivery to enhance NSC-mediated tissue repair.

Neuroplasticity and Reorganization

Neurogenesis

For many years it was widely accepted that the brain possessed no regenerative potential and lost neurons could not be replaced after injury or disease. However, more recently, it has been demonstrated that endogenous NSCs are present in the subventricular zone (SVZ) [58, 59] and the dentate gyrus (DG) [60, 61] of the adult mammalian brain, and neurogenesis in these regions is increased after stroke albeit at a level lower than what is necessary to restore tissue function [62]. Indeed, despite evidence of functional integration of these nascent neurons [63, 64], about 80% of the new neuroblasts and neurons die within the first two weeks after formation [62]. The potential to enhance endogenous neurogenesis mechanisms to replace lost neuronal cells has been an exciting therapeutic target for stroke. Previous studies have shown that stroke-induced neurogenic behavior is augmented after NSC transplantation in preclinical stroke models by increasing the proliferation of endogenous NSCs at the SVZ and DG as well as promoting the migration of endogenous neuroblasts to the damaged brain region which differentiate to mature neurons [26, 28, 43, 44, 65-67]. A study by Mine et al. illustrated that intraparenchymal transplantation of fetal-NSCs 2 days after stroke increased both the number of proliferating cells in the SVZ and the number of endogenous neuroblasts in the injured striatum up to 14 weeks after stroke [43]. Furthermore, these newly produced endogenous neuronal cells co-labeled with the mature neuron marker Fox-3, which indicates that NSC transplantation augments several steps of striatal neurogenesis after stroke. Interestingly, in this study the neurogenic activity seemed to be enhanced when NSCs were transplanted 2 days after stroke compared to 3 weeks after stroke. This suggests that timing of transplantation after injury plays an important role in the enhancement of endogenous neurogenesis by transplanted NSCs. However, there are

discrepancies among preclinical studies with respect to duration of enhanced neurogenesis, which may be due to differences in the stroke models, transplantation location, and neurogenic potency of the cell line tested. The mechanism by which transplanted NSCs augment endogenous neurogenic behavior after stroke is not well known, but is likely mediated by the secretion of neurotrophic and regenerative growth factors that suppress immune and inflammatory responses while promoting tissue repair [50, 68, 69].

Neural Reorganization

Many patients show some spontaneous recovery in limb function, language, and other cognitive measures within the first month after stroke onset [70]. This phenomenon is largely attributed to the rewiring of neuronal circuitry in which motor and sensory circuit activity is increased in other brain regions remote from the infarcted area [71]. This reorganization is modulated by structural changes in axons, dendrites, and synapses as well as increased activation of endogenous NSCs. More recently, preclinical animal models have documented that synaptic reorganization is augmented by NSC transplantation after stroke. In a study by Patkar et al., intraparenchymal transplantation of a murine NSC line led to increased expression of the synaptogenesis marker synaptophysin and the axonal growth cone protein GAP-43 in the stroked brain which was partially expressed by the transplanted cells themselves [72]. Furthermore, other studies have demonstrated that intraparenchymal transplantation of fetal- and ESC-NSCs augments dendritic plasticity (including increased dendritic length and branching) in the cortex and axonal sprouting in the cortex, striatum, thalamic nuclei, and corpus callosum after stroke which was associated with recovery in motor function [73, 74]. The observed synaptic reorganization events were associated with transplanted NSC-induced increases in the expression

genes involved in neurite outgrowth including thrombospondins (TSPs) 1 and 2, vascular endothelial growth factor (VEGF), glial-derived neurotrophic factor (GDNF), neurturin, and insulin growth factor-1 (IGF-1). Furthermore, immunodepletion studies of NSCs in vitro demonstrated that a subset of these factors, TSP1, TSP2, and VEGF, were specifically secreted by NSCs and were responsible for NSC-induced effects on dendritic and axonal plasticity [73]. Other studies in preclinical stroke models have demonstrated that transplanted NSCs promote oligodendrocyte proliferation and myelination of new neuronal circuits [28, 33, 44, 74]. These results show that NSC transplantation augment key reorganization of axons, dendrites, and synapses across multiple brain regions that lead to improved recovery.

Angiogenesis

Surrounding the ischemic core, a region of unsalvageable damaged tissue, is an area of mild-to-moderately hypoperfused tissue known as the penumbra [75, 76]. The penumbra is impacted by the ischemic event but still viable due to collateral blood flow and thus potentially salvageable. Increasing microvascularization to the penumbra region is a therapeutic target that could increase survival of the tissue surrounding the infarct and improve functional outcome after stroke [77]. After stroke, vascular regrowth is stimulated by the exchange of neurotrophic and regenerative growth factors between local endothelial cells and endogenous NSCs which have migrated to the site of ischemic insult. This crosstalk between neural and vascular compartments has been shown to promote angiogenesis in preclinical stroke models and human stroke patients leading to improved functional outcome [76, 78-82]. Previous studies in rodents have documented that transplanted NSCs increase endothelial cell proliferation, microvessel density, and angiogenic receptor expression in the penumbra region of stroked brain tissue

indicative of enhanced angiogenesis [28, 56, 66, 67, 82]. It is well-characterized that NSC-mediated angiogenic behavior is largely modulated via vascular endothelial growth factor (VEGF) signaling, secreted either by NSCs themselves or enhanced expression by host tissue [40, 75, 82, 83]. These studies have shown strong correlations between increases in VEGF signaling to increased penumbra vascularization and functional recovery. In addition to VEGF, Hicks et al. demonstrated that fetal-NSCs express the angiogenesis signaling factor Ang1 and lead to increased numbers of microvessels after transplantation in the stroked brain [27]. The formation of robust nascent vessel networks in the penumbra require the creation of functional neurovascular units (NVU) that maintain cerebral blood perfusion, regulate homeostasis, and are selectively permeable [75]. Horie et al. demonstrated that NSC treatment lead to enhanced NVU formation as indicated by increased expression of tight junction proteins (claudin, occludin, and ZO1) and dystroglycan, a protein involved in binding astrocytic endfeet to endothelial cells, and reduced BBB leakage post-stroke in penumbral tissue [82]. Together, these studies indicate that neovascularization is a major mechanism of NSC-mediated tissue and functional recovery after stroke.

Cell Replacement

In addition to the many mechanisms in which NSC-mediated trophic factor signaling protects and promotes tissue recovery after stroke, NSCs themselves can act as a cellular replacement. After transplantation into the stroke-damaged brain, grafted NSCs have been shown to stop proliferating between 2-8 weeks post-transplant and express the neuroblast marker doublecortin by 2 months post-transplant, which eventually diminishes as cells terminally differentiate into neurons or glia [40, 65, 84]. Many studies have documented terminal

differentiation of NSCs into neurons in the stroke damaged brain that express neuron markers such as NeuN, HuD, MAP2, and β III-Tubulin [40, 43, 44, 56, 84-86]. Grafted NSC-derived neurons were capable of differentiating into a wide array of mature neuron sub-types expressing dopaminergic neuron [41, 44, 86], GABAergic interneuron [40, 41, 44, 84], medium spiny striatal projection neuron [40, 41, 44, 86], and cortical neuron markers [40, 84, 85]. Furthermore, some of these studies demonstrate that grafted NSC-derived neurons are electrically active, project axons to appropriate target regions, and form synapses [40, 41, 43, 85, 86]. Glial differentiation (astrocytes and oligodendrocytes) has also been documented with a similar time course to neuronal cells [28, 40, 44, 53, 56, 73, 84]. However, there is much debate whether these cells are truly integrated into the host brain, thus improving functional outcome of the animal. Previous studies have shown that marginal functional recovery occurs earlier than the time it would take to achieve functional integration of the transplanted NSCs, so other NSC-mediated repair mechanisms such as trophic factor support may play a larger role in functional recovery than cell replacement [40]. However, functional integration of the grafted NSCs could lead to further neurological improvements albeit several months after transplantation [85].

It remains unclear whether the differentiation fate of grafted NSCs are driven by intrinsic mechanisms or environmental cues of the host tissue. To assess this question, Oki et al. transplanted NSCs into the cortex and striatum of stroked brains. After 4 months, these cells expressed the specific striatal projection neuron marker DARPP-32 in both the cortex and striatum, indicating that spontaneous neuronal differentiation of grafted NSCs is driven by intrinsic mechanisms [40]. However, Snyder et al. demonstrated that selective layer II/III photolysis in the mouse neocortex prompted transplanted NSCs to differentiate into pyramidal neurons in a cortical layer-appropriate manner while establishing appropriate synaptic contacts,

indicating that external cues facilitated region-specific cortical differentiation [87]. It is possible that effective neuronal replacement in various brain regions will require directed in vitro differentiation of NSCs to site-specific precursors prior to transplantation [40]. Indeed, Tornero et al. found that cortically-fated NSCs more readily differentiate to a cortical phenotype with pyramidal morphology than non-fated NSCs [85]. More studies are needed to understand the mechanism and factors involved in region-specific differentiation of grafted NSCs in vivo to augment the potential of cellular replacement strategies in the stroke-damaged brain.

Technical Considerations for Clinical Translation

There are several challenges to address before NSC therapies can be widely adopted for clinical stroke treatment. First of all, safety concerns associated with transplantation of allogeneic cell lines and the use of immunosuppressive drugs in stroke patients should be addressed [33]. These concerns could potentially be resolved through the use of autologous iPSC-NSCs. However, the amount of time needed to generate and safety test iPSC-NSCs with current technologies is likely beyond the treatment window of therapeutic efficacy. Furthermore, the optimal route of administration and treatment window must be further characterized. These two clinical factors largely depend on the NSC mode of action that will be employed to improve functional outcome.

Route of Administration and Dosing

A variety of NSC administration routes have been described for stroke including intraparenchymal (IP) and intravascular (IV) transplantation. In preclinical animal models of stroke, IP injections are the most commonly reported which is performed through a transcranial

approach (**Table 3.1**). The popularity of IP transplantation is likely due to the numerous advantages of direct transplantation including site specificity, eliminating the need for cells to transverse the BBB and guaranteed delivery of large cell numbers to the injury site, and excluding the potential for off target effects (e.g. clogging microvasculature leading to ischemic events) [42]. However, IP transplantation is a highly invasive procedure requiring a craniectomy surgery. Two recent clinical trials testing the safety and efficacy of IP transplantation of NSCs [19] and ASCs [16] demonstrated that transcranial surgery resulted in minimal adverse events and was generally safe and well tolerated, indicating that IP delivery of NSCs is deserving of further clinical study. However, there are advantages to other routes of administration making them worthy of consideration in the clinical setting. IV administration of cellular products is another popular route since it is generally the least invasive and is less technically challenging [42]. Indeed, IV administration could potentially allow NSCs to be injected during the acute stage of stroke when patients may be too unstable to undergo transcranial IP NSC treatment, or enable NSCs to be administered by healthcare professionals that are not capable of performing a craniotomy. Thus, IV NSC treatment could be employed by paramedics during the ambulance ride to the hospital or utilized in rural areas where neurosurgery expertise is limited. However, IV transplantation results in less cell engraftment in the brain due to the propensity of cells to accumulate systemically in non-target organs such as the lungs and liver [88, 89]. Previous studies have shown that IV NSC delivery results in neuroprotection and improved neurological performance without evidence of cell engraftment in the stroked brain, indicating that anti-inflammatory and regenerative trophic factor release is the main mechanism behind this treatment strategy [57]. Taken together, safety considerations along with intended NSC

therapeutic action should be considered when developing effective, translatable NSC therapies for stroke.

Treatment Window and Dosing

A wide range of NSC treatment windows have been assessed in rodent models, ranging from immediately after reperfusion to 4 weeks post-stroke (**Table 3.1**). In these preclinical studies, the treatment window seems to be dictated by the predicted mode of therapeutic action. If the main objective was to maximize the neuroprotective and immunomodulatory roles of transplanted NSCs, then transplantation often occurred within the acute stage of stroke theoretically before infarct volume is finalized [90]. However, a recent clinical trial of ASC transplantation in the chronic stroke patients with stable neurological function scores demonstrated significant improvement in mean scale scores likely through ASC-mediated neuroprotective and regenerative mechanisms, indicating that tissue remodeling may still be active several months post-stroke in which stem cell therapy can augment tissue recovery through trophic factor signaling [16]. Conversely, for cellular replacement strategies, a transplantation time point in the less cytotoxic subacute to chronic stages may be more advantageous to enhance long-term survival of the grafted cells. Another technical consideration that warrants further study for clinical translation is the optimal NSC dose (i.e. number of cells and number of transplantations). In rodent studies, cell dosing ranges from 1.0×10^5 to 4.5×10^5 cells in one to three transplantation sites for IP and a single dose of 3.0×10^6 to 4.0×10^6 cells for IV with no indication of outcome discrepancies between transplantation protocols. Performing multiple transplantations over time, or the use of other anti-inflammatory therapeutics in conjunction with NSC therapy, to utilize multiple modes of action should be considered.

Conclusion

NSCs provide the unique opportunity to mitigate stroke pathology through multimodal therapeutic action. A number of preclinical studies in rodent stroke models have demonstrated promising evidence that NSCs are able to act as a neuroprotectant by limiting secondary injury through anti-inflammatory mechanisms, promote endogenous neurogenesis and synaptic remodeling, and even act as a cell replacement thereby promoting tissue and functional recovery. However, the vast majority of preclinical studies have tested NSC efficacy in rodent models which possess many neuroanatomical differences compared to the human. Performing these studies in a large animal model with more similar brain anatomy to humans may provide greater predictive power of NSC efficacy in humans. This improvement in translational potential in preclinical studies could significantly close the gap between preclinical NSC testing and the successful implementation of NSC therapies in patients.

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Table 3.1: Preclinical Rodent Ischemic Stroke Models Testing Human Neural Stem Cell

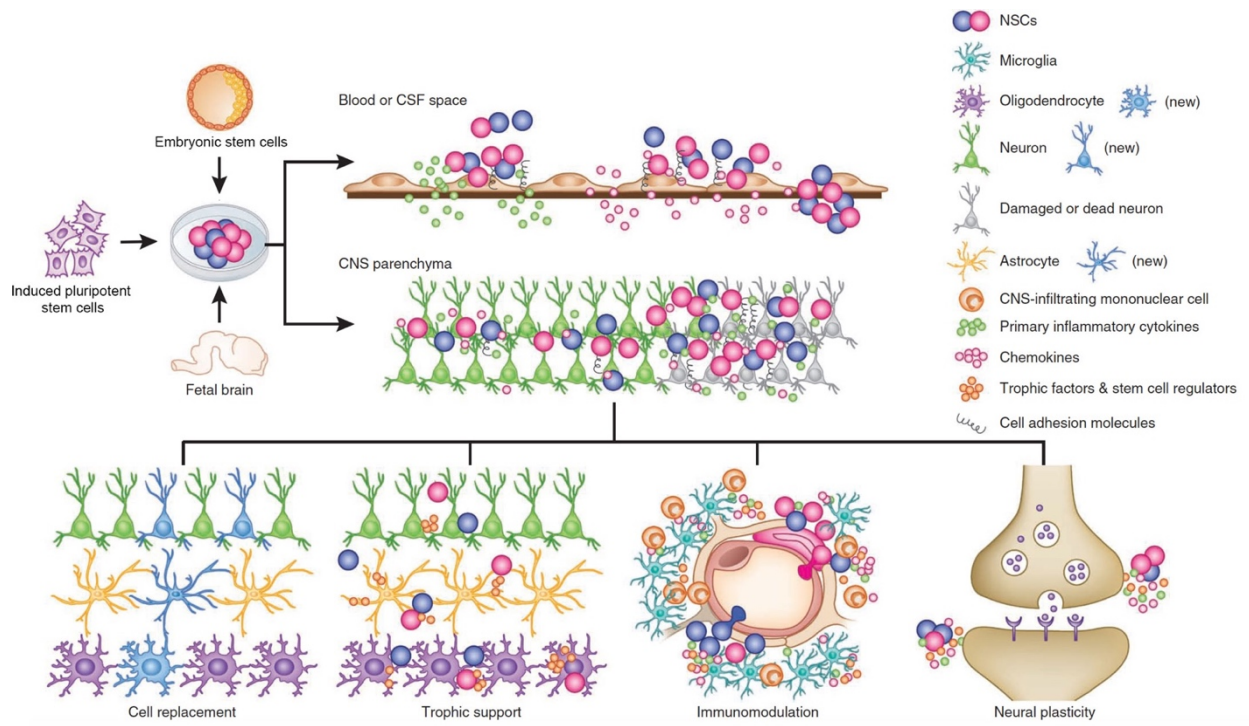
Therapy

NSC = neural stem cell, hESC = human embryonic stem cell, iPSC = induced pluripotent stem cell, IP = intraparenchymal, IV = intravenous, ICV = intracerebroventricular. * indicates that two separate experiments were performed. Cell dosing nomenclature is as follows: [number of injection sites] x [number of NSCs per injection]. For each experiment, all cell injections were performed on the same day.

NSC Type	Transplantation Time Point Post-Stroke	Route of Administration	Cell Dose	Modes of Action Identified	Reference
Fetal-derived	1 week	IP	3 x 100,000	Cell Replacement Synaptic Reorganization	[73]
Fetal-derived	6 hours	IV	1 x 3,000,000	Immunomodulation	[57]
Fetal-derived	1 day	IP	1 x 100,000	Immunomodulation	[55]
Fetal-derived	1-2 weeks	IP	2 x 150,000	Cell Replacement	[84]
Fetal-derived	1 day	IV	1 x 4,000,000	Cell Replacement Neuroprotection Angiogenesis	[56]
Fetal-derived	1 week	IP	3 x 100,000	Cell Replacement Immunomodulation	[53]
Fetal-derived	4 weeks	IP	2 x 225,000; 1 x 4.5x10 ³ , 4.5x10 ⁴ , or 4.5x10 ⁵ *	Neurogenesis Angiogenesis	[26-28]
Fetal-derived	3 weeks, 2 days*	IP	2 x 100,000	Cell Replacement Neurogenesis Immunomodulation	[43]
Fetal-derived	1 day	ICV	1 x 120,000	Cell Replacement Neuroprotection Neurogenesis Angiogenesis	[66]
hESC-derived	1 day	IP	1 x 50,000	Neurogenesis Angiogenesis	[67]
hESC-derived	1 week	IP	1 x 200,000	Cell Replacement Immunomodulation	[86]
hESC-derived	2 weeks	IP	1 x 120,000	Cell Replacement Neurogenesis	[65]
iPSC-derived	Immediately after stroke reperfusion	IP	1 x 1,000,000	Cell Replacement	[91]
iPSC-derived	1 week	IP	Mouse: 1 x 100,000 Rat: 2 x 200,000 or 2 x 150,000*	Cell Replacement Angiogenesis	[40]
iPSC-derived	1 week	IP	1 x 100,000	Cell Replacement Neuroprotection	[41]
iPSC-derived	2 days	IP	2 x 150,000	Cell Replacement	[85]
iPSC-derived	1 week	IP	1 x 200,000	Cell Replacement Immunomodulation Neurogenesis	[44]
iPSC-derived	1 day	IP	1 x 100,000	Immunomodulation	[52]

Figure 3.1: Multimodal Repair Mechanisms of Neural Stem Cells from Different Sources

Transplanted NSCs derived from fetal brain, ESC, or iPSC have demonstrated multimodal therapeutic action through intravenous, intracerebroventricular, intraparenchymal routes of administration. After transplantation, NSCs exhibit pathotropism through the expression of cell adhesion molecules and chemokine receptors that respond to proinflammatory cytokines and chemokines secreted by microglia and infiltrating peripheral immune cells. After reaching the injury site, NSCs interact with the microenvironment depending on the specific needs of the damaged brain tissue, thus cross-talk between transplanted NSCs and endogenous neural and immune cells shape the therapeutic action of NSCs. These mechanisms vary from cell replacement, tropic support, immunomodulation, neural plasticity (i.e. synaptic remodeling or neurogenesis), and angiogenesis (not shown), which are not mutually exclusive from one another. The prepotency for one mechanism to promote tissue repair over another is not well known. Regardless of therapeutic action, the final outcome after NSC transplantation is improved tissue and functional recovery (Modified from Kokaia et al. [68]).



CHAPTER 4

CONTROLLED CORTICAL IMPACT RESULTS IN SCALABLE INJURY IN A PIGLET TRAUMATIC BRAIN INJURY MODEL ¹

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Abstract

Traumatic brain injury (TBI) is a major cause of death and long-term disability in the United States, and children who sustain TBI at a young age may suffer long-term deficits in cognition, behavior, and/or motor function. A number of preclinical animal models have been developed in an effort to recapitulate injury mechanisms and identify potential therapeutics. However, the lack of restorative treatments for TBI has led to considerable criticism of current preclinical therapeutic development strategies, namely the translatability of widely-used rodent models to human patients. The use of large animal models, such as the pig, with more comparable brain anatomy and physiology to humans may enhance the translational capacity of current preclinical animal models. The objective of this study was to develop and characterize a scaled piglet TBI model with quantitative pathological features at the cellular, tissue, and functional level that become more prominent with increasing TBI severity. TBI was produced by controlled cortical impact (CCI) in toddler-aged Landrace piglets with impact velocity and depth of depression set to 2m/s;6mm, 4m/s;6mm, 4m/s;12mm, or 4m/s;15mm resulting in scaled neural injury. Quantitative gait analysis was performed pre-TBI and 1, 3, and 7 days post-TBI, and piglets were sacrificed 7 days post-TBI. Increasing impact parameters correlated to increases in lesion size and the extent of neuronal loss, intracerebral hemorrhage, gliosis, and white matter damage as well as motor function deficits. The piglet TBI model described here could serve as a translational platform for studying TBI sequelae across injury severities and identifying novel therapeutics.

Introduction

In the United States, traumatic brain injury (TBI) results in an estimated 2,174 deaths and 473,947 emergency department visits among children aged 0-14 years annually [1]. Falls account for approximately 50.2% of all sustained TBIs for children, and the rate of fall-related TBIs is highest among children aged 0-4 year (839 per 100,000) [1]. Further, pediatric TBI accounts for more than \$1 billion in total hospital charges annually [2]. Although adolescent brains are believed to be more “plastic” and thus more adept to recover and restore function after injury, recent evidence has shown that children who sustain a TBI at a younger age may suffer long-term neurological consequences such as impairments in cognition, behavior, and motor function [3-7]. At present, a number of management strategies are utilized to monitor and alter critical aspects affecting TBI outcomes including intracranial pressure, cerebral oxygenation, cerebral edema, and cerebrovascular injury after pediatric TBI, which may substantially decrease hospital stays and mortality rates [8]. Treatment options are restricted to techniques such as hyperosmolar therapy, temperature control, cerebrospinal fluid drainage, barbiturate therapy, decompressive craniectomy, and antiseizure prophylaxis that aim to reduce secondary brain insults [9]. However these treatments have little influence on reducing long-term functional deficits [10-12].

Numerous experimental animal models have been developed in an effort to recapitulate the primary and secondary injury aspects of a human TBI and, in turn, have helped delineate many of the biomechanical, cellular, molecular, and functional responses of TBI that cannot be studied in a clinical setting [13]. In rodents, one of the most widely used and studied TBI models is the controlled cortical impact (CCI) model. CCI allows for precise, quantitative control over parameters that affect injury severity such as velocity, depth of depression, and tip diameter [14]. CCI can be manipulated to produce injuries ranging from mild to severe and is highly

reproducible [15]. The ability to control injury severity allows researchers to produce gradable tissue damage, functional impairments, provide a means to better account for the variation in pathologies observed in TBI patients, and most importantly, develop appropriate treatment strategies [14]. Magnetic resonance imaging (MRI) studies in pediatric TBI patients have revealed that lesion size, gray and white matter disruption, and brain atrophy can be influenced by injury severity [16]. Functional deficits, such as cognition and motor function, have been documented to vary according to injury severity in children after TBI [17, 18]. To address this, scaled CCI in rodent models has been used to characterize the effects of injury severity on histopathological changes and behavioral and motor responses after injury and consequently, to identify potential therapeutic targets [19-23]. Despite promising preclinical results in rodent studies utilizing a wide range of therapeutics, there is no Food and Drug Administrative (FDA) approved treatment that promotes functional recovery in humans [24-26]. Given the significant impact of TBI, particularly in children who are at a critical period of brain development, the lack of effective treatments has led to considerable criticism of the use of rodents as a translational model [27, 28].

The pig, having similarities in brain anatomy and physiology to humans, may provide a significant advantage in modeling TBI and testing the safety and efficacy of novel therapeutics. The human brain is gyrencephalic and is comprised of >60% white matter [29, 30]. The gyrencephalic piglet brain has been found to mirror the pediatric brain in terms of size, development, and neuroinflammatory response, and is also comprised of >60% white matter [31-34]. However, the lissencephalic rodent brain is 650 times smaller than the human brain and is composed of only 14% of the relative white matter volume of humans [30, 35-37]. Given the propensity for TBI to contribute to widespread white matter damage in human patients, the use

of a large animal model with comparable white matter composition is critical to more closely recapitulate the human pathology [38]. Despite pressure to pursue more large animal models of TBI, limited progress has been made in the field. No study has been performed to directly assess the effect of scaled changes in velocity and depth of depression on cellular dynamics and motor function in a CCI piglet model [39, 40].

The purpose of this study was to develop and characterize a scaled piglet TBI model by manipulating CCI severity and measuring corresponding changes at the cellular, tissue and functional levels. CCI severity was controlled by manipulating either velocity, depth of depression, or both. Lesion size and histological analysis were assessed to measure changes at the tissue and cellular level and were found to correlate with injury severity. Finally, spatial-temporal gait analysis was performed to assess changes in motor function and was found to respond as a function of increasing injury severity. The development of a piglet TBI model with pathological and functional changes that mirror human TBI patients can be used in future studies to serve as a platform for the testing of novel therapeutics.

Materials and Methods

All work performed in this study was done in accordance with the University of Georgia Institutional Animal Care and Use Committee guidelines.

Animals

Sixteen commercially bred Landrace-cross piglets of mixed sex and 3 weeks of age were obtained from the University of Georgia Swine Unit 1 week prior to surgery. Piglets were group

housed and fed a nutritionally complete starter diet ad libitum. Room temperature was maintained at 26°C with a 12h light/dark cycle and an overhead heat lamp provided supplemental heat. All animals were feed restricted overnight prior to surgery but had free access to water.

Surgical Preparation

Piglets underwent surgery at 4 weeks of age. Anesthesia was induced using 5% vaporized isoflurane in oxygen utilizing a surgical mask. After induction, anesthesia was maintained at 2-3% vaporized isoflurane in oxygen. Continuous physiological monitoring of heart rate, respiration rate, and rectal temperature was performed every 5 minutes after induction of anesthesia. The skin was prepared in a routine manner for sterile surgery using Betadine and 70% alcohol. The surgical site was draped in a standard fashion. A left-sided skin incision approximately 4cm in length was made over the top of the cranium. An air drill (Brasseler, USA) was used to produce a circular craniectomy approximately 20mm in diameter at the left posterior junction of the coronal and sagittal sutures. Care was taken to avoid disruption of the dura mater and underlying cortical surface.

Controlled Cortical Impact

Concussive TBI was generated through the use of a specialized controlled cortical impactor (CCI) device designed by our lab in collaboration with the University of Georgia Instrument Design and Fabrication Shop (Athens, GA). Impactor design was modeled after the design of Manley et al. [40]. Briefly, the impactor is comprised of a small-bore, double-acting, pneumatic cylinder with a piston. The cylinder is mounted to an adjustable frame that can be

angled to the curvature of the piglet's skull. A 15-mm impactor tip with beveled edges is attached to the lower end of the threaded rod. The upper end of the rod is attached to the transducer core of a linear voltage differential transducer (LVDT). Fine-tuning the flow of pressurized gas to the pneumatic piston controls the velocity of the impactor. Velocity is calculated from the time/displacement curve measured by the LVDT and recorded by the computerized data acquisition system DAQ Central. In addition, a CLICK® Series Programmable Logic Controller (PLC) is used to regulate the dwell time of the impactor rod.

Following surgical preparation, the piglet was secured to the CCI frame and the impactor tip was positioned over the craniectomy site. For this initial study we chose to assess the effects of a scaled injury by manipulating either the velocity or depth of depression, or both. Piglets were divided into four groups defined by the impact parameters in which TBI was induced: 2m/s impact velocity with 6mm depth of depression (n=4), 4m/s impact velocity with 6mm depth of depression (n=4), 4m/s impact velocity with 12mm depth of depression (n=4), and 4m/s impact velocity with 15mm depth of depression (n=4). The dwell time was held constant at 400ms for all animals.

After injury, the surgery site was flushed with sterile saline and the skin was re-apposed with surgical staples. Banamine (1.1 mg/kg IM) and Butorphanol (0.2 mg/kg IM) was administered postoperatively for pain management, and Ceftiofur Sodium (Naxcel®; 4 mg/kg IM) was administered as an antibiotic. Anesthesia was discontinued and piglets were allowed to recover until ambulatory before being returned to their home pens. Piglets were monitored every 8 hours for the next 24 hours and any abnormal neurological behaviors were recorded.

Gait Analysis

One week prior to surgery, piglets were individually placed in a semi-circular gait analysis track in a separate climate-controlled room for gait track familiarization and training. Piglets were familiarized to the testing room and trained to move down the track for 3 days prior to data collection. After training, each piglet was video recorded on 3 separate days prior to TBI surgery to obtain pre-TBI gait measurements. Piglets were also evaluated on days 1, 3, and 7 post-TBI. Gait analysis was performed as previously described [41]. Briefly, 2 high speed GigEye Ethernet Cameras (IDS Imaging Development Systems, Obersulm, Germany) set to capture footage (70 frames per second) of each side of the pig in profile view were synchronized through an IDS computer driver. Piglets were recorded as they walked through the straight portion of the semi-circular track, where they were video recorded as they moved perpendicular to the 2 synchronized cameras recording each side of the piglet. Piglets were moved through the track until 5 usable repetitions were obtained. Video data was analyzed using Kinovea software (Kinovea open source project, www.kinovea.org) to obtain swing time, stance time, stride velocity, and 2-limb versus 3-limb support measurements.

Lesion Size Assessment

One week post-TBI, piglets were deeply anesthetized using 5% vaporized isoflurane in oxygen and euthanized via CO₂ inhalation. After euthanasia, brains were removed, sectioned into 5mm coronal slices, and fixed in 10% buffered formalin for lesion size measurement and histology.

For lesion size measurement, serial 5mm coronal sections were scanned with a Canon CanoScan 8800F. Lesion size was measured using ImagePro Plus software (Media Cybernetics,

Rockville, MD). The value obtained from each coronal section was multiplied by 5 to account for brain section depth, and measurements for each section were combined to obtain total lesion volume. Total lesion volumes were compared to the contralateral cerebral hemisphere.

Histology

Representative sections from each animal were collected, routinely processed, embedded in paraffin, sectioned at 4 μ m, and stained with hematoxylin and eosin to evaluate the lesion microscopically. Additional sections were stained by immunohistochemistry for glial fibrillary acidic protein (GFAP; 1:4000; mouse; Biogenex), Olig2 (1:400; rabbit; Genetex), and neuronal nuclei (NeuN; 1:500, guinea pig, Millipore) as previously described [42]. . Heat induced antigen retrieval was performed for GFAP, Olig2, and NeuN using citrate buffer at pH6 (DAKO). Detection was performed using biotinylated anti-mouse (GFAP; Biocare), anti-guinea pig (NeuN; Biogenex), or anti-rabbit (Olig2; Biocare) antibodies, and a streptavidin label (4plus Streptavidin HRP or AP label; Biocare), and 3,3'-Diaminobenzidine (DAB) as the chromogen (DAKO). All sections were counterstained with hematoxylin. For quantification, a peri-lesional ROI was defined, which included dorsal and dorsolateral gyri (cingulate, marginal, suprasylvian and sylvian and/or ectosylvian), and 5 fields were imaged per section using an Olympus BX41 light microscope (Center Valley, PA). Utilizing ImagePro Plus software, the percentage of cells positive for NeuN and Olig2 were manually counted and GFAP was quantified by relative threshold value.

Statistics

Data was analyzed with SAS version 9.3 (Cary, NC) and statistical significances between groups were determined by two-way analysis of variance followed by post-hoc Tukey's Pair-Wise comparisons. Treatments where p-values were ≤ 0.05 were considered to be significantly different.

Results

Lesion volume is correlated to increased impact velocity and depth of depression

To determine if impact velocity and depth of depression had an effect on lesion size, piglet brain tissue underwent volumetric assessment after injury. Piglets in the 2m/s; 6mm group (velocity and depth, respectively) had minor brain lesions. However, when impact velocity was increased to 4m/s while maintaining a 6mm depth of depression, there was a greater than 50-fold increase in brain lesion volume ($2.29\text{mm}^3 \pm 2.29\text{mm}^3$ vs. $124.70\text{mm}^3 \pm 47.18\text{mm}^3$, respectively) and a significant ($p < 0.05$) increase in lesion volume compared to the contralateral hemisphere (**Figure 4.1A-B, E**). Furthermore, when depth of depression was increased to 12mm and 15mm while maintaining the impact velocity at 4m/s, lesion volume continued to increase ($944.0\text{mm}^3 \pm 417.49\text{mm}^3$ and $1236.15\text{mm}^3 \pm 240.94\text{mm}^3$, respectively) and remained significantly ($p < 0.05$) larger than the contralateral side (**Figure 4.1C-D, E**). Taken together, these results indicate there was a clear positive correlation between lesion volume and velocity and depth of depression.

Histopathology reveals correlative increase in lesion size in response to scaled injury

In the 2m/s; 6mm group, microscopic changes were subtle. Tips of gyri (marginal, suprasylvian, and/or sylvian) at the lesion site had increased cellularity (gliosis) in both cortex

and white matter and rare shrunken necrotic cortical neurons. Mild fibrosis of the leptomeninges overlying the lesion site was present (**Figure 4.2A**). Immunohistochemistry for GFAP revealed mild multifocal astrocytosis/astrogliosis in the involved cortex and underlying white matter (**Figure 4.3A**), but no subjective change in oligodendrocytes were present on Olig2 staining (**Figure 4.4I**). The 4m/s; 6mm group had more extensive changes, although similar to the 2m/s; 6mm group in that changes were primarily at the tips of gyri (marginal, suprasylvian, and sylvian) at the lesion site. This was characterized by more extensive neuronal necrosis and loss, reactive vessels, sometimes with mild perivascular cuffing by lymphocytes and plasma cells, and hypercellularity of both cortex and white matter in the involved area due to gliosis and infiltrating gitter cells. Unlike the 2m/s; 6mm group, some animals had shallow tracks filled with hemorrhage and/or gitter cells in the area of impact. In addition, there were hemorrhage, hemosiderin laden macrophages, mild perivascular cuffing by lymphocytes and plasma cells, and fibrosis in the overlying leptomeninges (**Figure 4.2B**). By immunohistochemistry, there was increased staining for GFAP around the lesion site (**Figure 4.3B**), but no discernable change in oligodendrocytes were present in Olig2 staining (**Figure 4.4I**). In the 4m/s; 12mm group, changes were more severe and were present in all the dorsolateral gyri (marginal, suprasylvian, and sylvian) and sometimes the cingulate gyrus. The immediate area of impact was disrupted deep into the white matter and mostly replaced with hemorrhage and macrophages containing hemosiderin. Surrounding the impact tract, adjacent cortex and white matter was hypercellular and the white matter, down to the lateral ventricle, had extensive degenerative changes consisting of axonal and myelin degeneration. The overlying leptomeninges were thickened by hemorrhage and an infiltration of macrophages, lymphocytes, neutrophils, and fibroblasts (**Figure 4.2C**). There was a diffuse increase in staining for GFAP (astrocytosis/astrogliosis)

surrounding the impact tract (**Figure 4.3C**), but numbers of cells staining for Olig2 did not appear increased (**Figure 4.4I**). The 4m/s; 15mm group had the most severe changes also involving the dorsolateral gyri (marginal, suprasylvian, and sylvian) and the cingulate gyrus. The lesion site was characterized by a large, deep tract of tissue necrosis and loss, often extending to the lateral ventricle and disrupting the ependymal lining, which was filled with hemorrhage and gitter cells. The tract was immediately surrounded by hemosiderin/hematoidin-laden gitter cells and a layer of gliosis with reactive vessels. Severe gliosis extended into the surrounding gyri and white matter, and the white matter had degenerative changes consisting of swollen axons and myelin degeneration. The overlying meninges were thickened by hemorrhage, infiltration of hemosiderin-laden macrophages, lymphocytes, plasma cells, and fibrosis. In one animal, the contralateral marginal gyrus had a large deep tract that extended to the ventricle and was filled with hemorrhage (**Figure 4.2D**). Surrounding the impact tract there was severe, diffuse staining for GFAP (astrocytosis/astrogliosis, **Figure 4.3D**), and slightly increased numbers of Olig2 stained cells in the white matter (**Figure 4.4I**).

Immunohistochemistry reveals correlative loss of neurons and increased gliosis in response to scaled injury

Immunohistochemical analysis was performed on the peri-lesional region to assess changes in the number of neurons, astrocytes, and oligodendrocytes as a result of increasing TBI severity. A similar anatomical region on the contralateral hemisphere was utilized as the control to account for individual variability of basal neural cell numbers. First, staining for the neuron marker (NeuN) was used to quantify the number of mature neurons around the site of impact compared to the contralateral side. In the 2m/s; 6mm group, the lesion site was comprised of

37.76% $\pm$ 3.58% NeuN+ cells compared to 45.37% $\pm$ 2.69% NeuN+ cells on the contralateral hemisphere, indicating a decrease in NeuN+ cells around the lesion site, although not statistically significant (**Figure 4.4C**). The disparity of percent NeuN+ cells between the ipsilateral and contralateral hemispheres became more pronounced when impact velocity was increased to 4m/s and furthermore when depth of depression was increased. In the 4m/s; 6mm group, there was a significant ($p<0.01$) decrease in NeuN+ cells around the lesion site compared to the corresponding contralateral side (32.38% $\pm$ 1.59% vs. 42.05% $\pm$ 1.95%, **Figure 4.4C**). There was also a significant ($p<0.01$) reduction in NeuN+ cells around the lesion site compared to the contralateral hemisphere in the 4m/s; 12mm group (29.33% $\pm$ 3.51% vs. 46.41% $\pm$ 1.62%, **Figure 4.4A-C**). Lastly, the most discernable decrease in NeuN reactivity around the lesion site was evident in the 4m/s; 15mm group where there were only 24.75% $\pm$ 2.72% NeuN+ cells in contrast to 45.01% $\pm$ 0.69% NeuN+ cells on the contralateral side ($p<0.0001$, **Figure 4.4C**). Taken together, there was a negative correlation in percentage of mature neurons around the lesion site as velocity and depth of depression was increased.

The dynamic changes in astrocyte numbers around the lesion site was determined by staining for GFAP and compared to the corresponding area on the contralateral hemisphere. A similar pattern observed for NeuN reactivity emerged for GFAP in the mildest, 2m/s; 6mm group; a minimal increase in GFAP reactivity was present around the lesion site comparable to the contralateral side (threshold value 1079.8 $\pm$ 454 vs. 1012.45 $\pm$ 359.38, **Figure 4.4F**). However, as injury severity increased, the disparity between GFAP reactivity around the lesion site compared to the contralateral side became more distinct. The 4m/s;6mm group had a significant ($p<0.05$) increase in GFAP reactivity around the lesion site compared to the same anatomical region on the contralateral hemisphere (threshold value 1530.55 $\pm$ 224.28 vs. 690.65

± 102.32 , **Figure 4.4F**). The 4m/s; 12mm group also had a significant ($p<0.01$) upregulation of GFAP around the lesion site compared to the contralateral side (threshold value 1592.1 ± 177.51 vs. 803.85 ± 63.81 , **Figure 4.4E-F**). Lastly, the 4m/s; 15mm group, the most severe group, had the strongest statistical difference ($p<0.001$) between the ipsilateral and the contralateral sides (1511.47 ± 31.17 vs. 913.8 ± 104.42 , **Figure 4.4F**). Therefore, activation of astrocytes, and thus astrogliosis/astrocytosis, becomes more pronounced as velocity and depth of depression is increased.

Lastly, we quantified the number of oligodendrocytes around the lesion site compared to the contralateral side utilizing staining for Olig2, which labels both mature oligodendrocytes and oligodendrocyte precursor cells. There was no significant difference in Olig2 positive cells around the lesion site compared to the contralateral side in the 2m/s; 6mm group ($29.64\% \pm 6.54\%$ vs. $26.1\% \pm 1.80\%$, **Figure 4.4I**), the 4m/s; 6mm group ($22.86\% \pm 3.31\%$ vs. $34.39\% \pm 2.31\%$, **Figure 4.4I**), the 4m/s; 12mm group ($29.14\% \pm 3.98\%$ vs. $35.16\% \pm 4.49\%$, **Figure 4.4G-I**), or the 4m/s; 15mm group ($26.17\% \pm 2.38\%$ vs. $33.15\% \pm 6.51\%$, **Figure 4.4G**). Thus oligodendrocyte numbers did not vary in response to changes in injury severity compared to the contralateral hemisphere.

Motor deficits become more pronounced with increasing impact velocity and depth of depression

To measure potential deficits in motor function, gait analysis was performed first before TBI to serve as a baseline and then 1, 3, and 7 days post-TBI. Changes in swing time, stance time, stride velocity, 2-limb support, and 3-limb support were assessed as a function of increasing impact velocity and depth of depression using Kinovea gait analysis software (**Figure 4.5A**). Stance time, represented as the percentage of time spent in the stance phase compared to

the swing phase for one full stride cycle, was used to assess whether a shift occurred in the amount of time spent in stance phase compared to swing phase after TBI. For both the 2m/s; 6mm and 4m/s; 6mm groups, animals spent approximately half their stride in stance phase pre-TBI ($47.6\% \pm 1.7\%$ and $47.18\% \pm 0.63\%$, **Figure 4.5B**). Stance time remained unchanged at all time-points after TBI, indicating consistent stance:swing ratio throughout the study. However, the 4m/s; 12mm group demonstrated a significant ($p < 0.001$) increase in percent stance time and spent almost 20% more time in stance phase 1 day post-TBI as compared to pre-TBI ($62.07\% \pm 2.93\%$ vs. $43.04\% \pm 1.1\%$, **Figure 4.5B**). A significant ($p < 0.01$) increase in percent stance time persisted through both 3 and 7 days post-TBI ($61.62\% \pm 1.58\%$ and $56.07\% \pm 3.22\%$, **Figure 4.5B**). Further, the 4m/s; 15mm group also displayed significant ($p < 0.0001$) increases in percent stance time and spent almost 18% more time in stance phase 1 day post-TBI as compared to pre-TBI ($62.765\% \pm 0.40\%$ vs. $45.25\% \pm 0.65\%$, **Figure 4.5B**). Animals maintained a significantly ($p < 0.001$) increased percent stance time at both 3 and 7 days post-TBI ($58.59\% \pm 2.10\%$ and $59.36\% \pm 1.70\%$, **Figure 4.5B**). Taken together, this data suggests that increasing both velocity and depth of depression to 12 or 15mm results in a shift in the stance:swing ratio such that animals spend more time in stance phase during one stride cycle.

Stride velocity, or the time to complete one stride cycle, was used as a measure to detect changes in rate of movement after TBI. Stride velocity remained largely unaffected through all post-TBI time points for both the 2m/s; 6mm and 4m/s; 6mm groups compared to pre-TBI assessment ($1.76\text{m/s} \pm 0.11\text{m/s}$ and $1.58\text{m/s} \pm 0.04\text{m/s}$, **Figure 4.5C**). However, stride velocity was significantly ($p < 0.001$) slower for the 4m/s; 12mm group 1 day post-TBI as compared to pre-TBI ($0.68\text{m/s} \pm 0.14\text{m/s}$ vs. $1.77\text{m/s} \pm 0.10\text{m/s}$), and remained slower both 3 and 7 days post-TBI ($1.05\text{m/s} \pm 0.36\text{m/s}$, $1.02\text{m/s} \pm 0.24\text{m/s}$), although day 3 and 7 results did not reach significance

(**Figure 4.5C**). However, stride velocity was significantly ($p<0.001$) slower for the 4m/s;15mm group 1, 3 and 7 days post-TBI ($0.69\text{m/s} \pm 0.05\text{m/s}$, $0.95\text{m/s} \pm 0.12\text{m/s}$, and $0.87\text{m/s} \pm 0.01\text{m/s}$, respectively) as compared to pre-TBI ($1.64\text{m/s} \pm 0.02\text{m/s}$, **Figure 4.5C**). These results indicate that stride velocity was reduced as a result of increasing TBI severity.

2-limb support, or the time spent on two limbs during a stride cycle, was assessed to determine if pigs shift to 3-limb support which would suggest a decrease in balance and a need for additional support post-TBI. Percent of time spent in 2-limb support remained relatively unchanged for both the 2m/s; 6mm and 4m/s; 6mm groups after TBI for all time points as compared to pre-TBI ($100\% \pm 0.11\%$, $100\% \pm 0.30\%$, **Figure 4.5D**). For the 4m/s; 12mm group, percent time spent in 2-limb support decreased 1 and 3 days post-TBI as compared to pre-TBI ($73.79\% \pm 9.79\%$ and $68.65\% \pm 9.79\%$ vs. $100\% \pm 9.79\%$, respectively), although these results did not reach significance. However, percent 2-limb support significantly ($p<0.05$) decreased by almost 46% 7 days post-TBI as compared to pre-TBI ($54.04\% \pm 9.79\%$ vs. $100\% \pm 9.79\%$, **Figure 4.5D**). The 4m/s; 15mm group's percent time spent in 2-limb support significantly ($p<0.001$) decreased by almost 34% 1 day post-TBI as compared to pre-TBI ($66.39\% \pm 5.82\%$ vs. $100\% \pm 5.82\%$, **Figure 4.5D**). Interestingly, time spent in 2-limb support continued to decrease over time, as opposed to stance time and stride velocity where small increments of improvement were noted at 3 and 7 days post-TBI. Percent 2-limb support significantly decreased by almost 38% 3 days post-TBI and by almost 54% 7 days post-TBI compared to pre-TBI ($61.86 \pm 5.82\%$ and $46.51 \pm 5.82\%$ vs. $100\% \pm 5.82\%$ respectively, $p<0.05$, **Figure 4.5D**). Taken together, this data suggests that uninjured animals and injured animals with a more mild TBI typically relied on only 2-limb support, but that increasing velocity and depth of depression resulted in a shift from 2-limb-only support to a combination of 2-and 3-limb support.

Discussion

In this study, we characterize a scaled piglet TBI model generated using an open skull CCI approach in which the histopathological and functional response to injury increases as a function of cortical impact speed and depth of depression. Increasing the velocity and depth of depression of impact contributed to significant increases in lesion volume 1 week post-TBI. Affected regions demonstrated macro- and microcellular changes in the brain, such as mild damage, mostly in the cortex at the tips of gyri, characterized by neuronal necrosis and mild astrocytosis/astrogliosis in more mildly affected groups to large tracts of necrosis, neuroparenchymal loss, and often hemorrhage extending from the meningeal surface to the ventricle with degeneration in the surrounding white matter and intense astrocytosis/astrogliosis in the more severely affected groups. Most importantly, functional changes were observed that corresponded with TBI severity. Gait biomechanic analysis revealed progressive motor function deficits in stance time, stride velocity, and limb support as CCI parameters increased. These changes are consistent with human TBI outcomes. These results suggest that this piglet TBI model may be an ideal model for use in future studies aimed at further elucidating the complexities of TBI pathophysiology, time course, and functional responses, and may have greater predictive power of the efficacy of potential therapeutics.

We chose to utilize a CCI to generate a TBI as this approach has proven to be reliable and repeatable in both rodent and pig models [43]. The surgical approach is relatively quick and takes less than 1 hour from initial skin incision to final closure. This procedure resulted in no deaths, even in our most severe group despite all pigs exhibiting significant neurological impairments. We developed our CCI device after the design of Manley et al. using a gas driven pneumatic piston to allow for the precise control of the impact parameters, thus giving us the

ability to manipulate injury severity [40]. It has been well established that manipulation of velocity, depth of depression, and dwell time either individually or in combination can affect injury severity.

We took several factors into account when selecting each combination of impact parameters that would best produce features of human TBI. First, a dwell time of 400ms was selected for all groups, similar to previous reports [39, 40, 44, 45]. Duhaime et al. developed a piglet TBI model at three different maturational stages at 5 days, 1 month, and 4 months of age [39]. Depth of depression for 5-day, 1-month, and 4-month old animals varied from 5mm, 6mm, and 7mm, respectively, and velocity was held constant at 1.7m/s for all maturation stages [39]. Similarly, in this study, we used 1 month old piglets and selected a velocity of 2m/s and a depth of depression of 6mm as our initial CCI parameters to achieve a small focal contusion. Interestingly, despite a slightly faster velocity and a larger impactor tip diameter, we observed a smaller lesion than Duhaime et al. However, this may be attributed to their slightly different CCI approach in which injury does not occur as a result of the force of the impactor tip striking the brain, but instead from displacement of the tip resting on the cortical surface. Similar to Duhaime et al., the 2m/s; 6mm group exhibited no gait abnormalities in our study. We next increased velocity to 4m/s similar to the velocity of a CCI model reported by Jin et al. [46]. Again, no motor deficits were observed in the 4m/s; 6mm group; however, we did observe a significant focal contusion with many histopathological hallmarks of TBI. Next we held velocity constant at 4m/s but increased depth of depression to 12mm and 15mm similar to Manley et al. who developed a scaled CCI model in adult pigs (38-42kg) by fixing velocity at 3.5m/s and varied depth of depression from 9mm, 10mm, 11mm, or 12mm [40]. In addition, Meissner et al. measured neuromonitoring parameters and histological changes in an adult pig CCI model

produced with a 2.5m/s velocity and a 9mm depth of depression and Maytar et al. assessed autoregulatory responses in a piglet CCI model with a 3m/s velocity a 6mm and 10mm depth of depression which they classified as moderate and severe, respectively [47, 48]. These previous studies influenced our decision to manipulate depth of depression to a much higher value in order to achieve histopathological and functional deficits with increased severity. Manley et al. did not report any functional deficits, however if they were present they cite lack of functional neurological outcome measures as an explanation [40]. As such, we present the first piglet CCI study to report and quantify functional motor deficits.

Given that there is no common scoring system that has been adopted to assess injury severity for any experimental animal species, like the Glasgow Coma Scale used in human TBI patients, we hesitate to classify our injury outcomes as either mild, moderate, or severe [13]. However, in this study we have achieved a range of injuries in which histopathological and functional changes correlate with severity, such that comparatively among groups outcomes range from mild to severe, similar to scaled studies in rodents [19, 22, 49].

In the present study we assessed changes in lesion size in order to determine the extent of tissue damage as a result of increasing TBI severity. Human MRI studies have found that lesion volume is a critical predictor of outcome after TBI and that lesion volume directly correlates with long-term functional outcomes using multiple clinical scales [50, 51]. In addition, lesion size, characterized by the number of affected brain regions, correlates with the extent of motor deficits and affects the time of recovery [7]. We found a direct relationship between TBI severity and lesion size such that for each increase in CCI parameters, lesion volume was increased. It can also be noted that in this study, similar to humans, we observed a clear correlation between lesion size and motor deficits. The 4m/s; 12mm and 4m/s; 15mm groups with the largest lesion

volumes exhibited the greatest impairments in gait. Previous studies have outlined a similar approach in pigs where serial sections were used to assess lesion volume [39, 52]. Previous reports of lesion analysis using histology and MRI and found that although not exact, histological assessment of lesion volume was comparable to MRI volumetric analysis [42, 53]. Future studies will benefit from MRI analyses to assess more specific changes within the lesion site such as cytotoxic or vasogenic edema, swelling, white matter disruption, and reduced cerebral blood flow after brain trauma.

In this report we provide both a qualitative and quantitative assessment of histological changes across groups. H&E stained brain slices were reviewed by a board certified veterinary pathologist and assessed for specific pathological hallmarks of TBI commonly observed in humans [54]. Histological assessment revealed increasing damage with increasing velocity and depth of impact. Low velocity and depth of depression resulted in a small superficial cortical focus of subtle damage characterized by neuronal necrosis and reactive gliosis. As velocity increased cortical damage and gliosis became more widespread, although still relatively superficial, with associated hemorrhage, necrosis, and vascular reaction, as well as underlying white matter changes. As the depth of depression increased, associated lesions became large tracts of necrosis, tissue loss, and hemorrhage extending deep into the cerebrum often down to the ventricle. These larger lesions were surrounded by a more intense gliosis and vascular reaction, and more severe white matter degeneration. Similarly, scaled CCI in adult pigs has been shown to result in increased presence of vascular congestion and decreased loss of normal cytoarchitecture that corresponded with depth of depression [40]. In addition, TBI in the immature pig has revealed similar pathological features such as the presence of necrosis, hemorrhage, neuronal loss, and reactive gliosis at the site of injury [39, 52, 55].

Histological changes have been quantitatively evaluated using a numerical scoring system that ranks the severity of specific pathological features of TBI [44, 45]. However, a limited number of studies provide a direct quantification of changes of the relative number of specific cells types in the brain after CCI in pigs. Costine et al. evaluated the relative number of migrating DCX+ neuroblasts in the white matter after scaled cortical impact in piglets at different maturational stages, and Taylor et al., in a follow-up study, measured the distribution of DCX+, BrdU+ and DCX+/BrdU+ neuroblasts in the white matter from the subventricular zone to the site of injury. However, quantification of changes in neurons, astrocytes, and oligodendrocytes, three major cell types of the brain, after TBI in a pig brain is lacking. Therefore, in the current study, immunohistological staining was performed with the well-established markers NeuN, GFAP, and Olig2 to assess of the relative number of neurons, astrocytes, and oligodendrocytes, respectively, in the peri-lesion area 1 week post-TBI.

NeuN is a DNA-binding, neuron-specific protein expressed in post-mitotic neurons [56]. NeuN staining of human TBI-affected brain tissue has been shown to decrease at or near the site of injury as a result of neuronal cell death [57]. In addition, rodent TBI studies reveal similar loss of neurons in response to injury and have been found to be sensitive to injury severity [19, 58, 59]. In our piglet TBI model, based on NeuN staining, the percent of mature neurons was significantly reduced for all groups except for the most mild, 2m/s; 6mm group, suggesting that neurons are sensitive to increasing TBI severity in this model.

GFAP, a cytoskeletal astrocytic marker, is well documented to measure astrocyte proliferation (astrocytosis) and hypertrophy (astrogliosis) after TBI [60, 61]. Reactive astrocytes grow in size, proliferate, and migrate to the site of injury where they release neurotrophic factors, promoting tissue repair [62]. However, astrocytosis and astrogliosis can also contribute to glial

scar formation which can be detrimental to axonal regrowth and, ultimately, can contribute to neurodegeneration and the development of functional deficits [63]. GFAP expression is typically most highly upregulated at the site of the lesion and becomes less prominent in the peri-lesioned area in humans [57, 64]. Scaled injury in rodent models has shown that GFAP expression corresponds with injury severity [20]. Globally, we observed substantial astrogliosis and astrocytosis at and near the site of the lesion for the 4m/s; 6mm, 4m/s; 12mm, and 4m/s; 15mm groups and, in accordance, noted a significant upregulation of GFAP expression for these groups.

Lastly, Olig2, a basic helix loop helix (bHLH) transcription factor, is expressed in a number of different cell types including oligodendrocyte precursor cells and mature oligodendrocytes [65]. Oligodendrocytes are responsible for the myelination of axons. TBI in humans results in the death of mature oligodendrocytes followed by increases in oligodendrocyte precursor cells [66]. These dynamic changes may influence white matter function following TBI. Rodent TBI studies report similar changes in oligodendrocyte numbers in response to injury [67, 68]. Interestingly, the present study did not observe either increases or decreases in oligodendrocytes in response to injury; however, there has been limited research on oligodendrocyte activity in the highly myelinated pig brain after TBI. Future studies may benefit from looking at changes in myelin basic protein (MBP) to assess direct changes in white matter composition as a result of TBI.

Children who sustain a TBI may develop impairments in motor proficiency. Several studies have evaluated motor deficits after TBI in children using quantitative gait analysis and found that children may exhibit deficits in spatiotemporal gait parameters [7, 18, 69]. Our group has previously developed a highly sensitive approach to assess changes in spatiotemporal gait parameters in a middle cerebral artery occlusion (MCAO) ischemic stroke pig model using high

speed video cameras [70]. We present for the first time gait analysis performed in a piglet CCI model to assess impairments in motor function. We measured the response of stance percent, stride velocity, and limb support as a function of TBI severity. 4m/s; 12mm and 4m/s; 15mm groups exhibited significant deficits in stance percent at all time-points post-TBI, similar to TBI-affected children [71]. Increased overall stance percent may be explained by two reasons. First, the amount of time the pig spends in swing time may be reduced in an effort to hasten ground contact to begin the stance phase during which the pig is better able to stabilize its weight distribution. Second, limb weakness on the affected right side may limit the amount of propulsive forces needed to initiate and maintain the swing phase, resulting in a reduction of the time the hoof spends off the ground. Reduced stride velocity is one of the most common motor deficits observed as a result of moderate or severe TBI in children [71, 72]. Stride velocity was significantly reduced for the 4m/s; 12mm group 1 day post-TBI, and at all time-points for the 4m/s; 15mm group. Similar to human studies, pigs were given the option to move at a self-selected pace. We observed that normal piglets natural gait of choice is a run, during which 2 limbs support the entire stride cycle. However, post-TBI pigs that sustained a more severe TBI exhibited a preference to travel at either a slow run or even walk during which 3 limbs support a portion of the stride cycle. Therefore, we sought to measure the shift from 2-limb support to 3-limb support as a result of TBI. We found that there was a significant shift in 2-limb support to 3-limb support for the 4m/s; 12mm group 7 days post-TBI, and at all time-points for the 4m/s; 15mm group. This data suggests that the pigs altered both their speed of travel and their gait support as a result of TBI. Similar results were observed in rodent TBI studies that evaluated changes in gait [73, 74]. These findings indicate injury severity influences functional outcomes

and that, similar to children, more moderately to severely affected animals develop motor deficits.

Conclusions

In this study, we developed and characterized a piglet CCI traumatic brain injury model. The piglet has several key similarities to children in both neuroanatomy and brain development making it more translational than traditional rodent models. Manipulating CCI parameters resulted in a reproducible, scalable injury with increasing lesion volume. The lesion site increased from a small superficial focus of neuronal necrosis and gliosis in the mildest impact to a large deep tract of necrosis and tissue loss with marked astrogliosis/astrocytosis and more extensive white matter changes in the more severe impacts. TBI produced motor impairments in gait that correlated with injury severity. This piglet TBI model is ideal to study the short and long-term responses of TBI in a more human-like model and to study the potential of novel diagnostics and therapeutics to translate successfully to human patients.

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Figure 4.1: Lesion Volume is Correlated to Increased Impact Velocity and Depth of Depression

Piglet brains were coronally sectioned and imaged for lesion size measurement. Brains that received a CCI of 2m/s; 6mm exhibited little to no visible lesion (A) while measurable lesions were seen in the 4m/s; 6mm, 4m/s; 12mm, and 4m/s; 15mm groups (outlined by dotted line, B-D, respectively). Lesion size of each brain was quantified and average lesion volume of each group showed that piglets receiving 4m/s; 6mm, 4m/s; 12mm, and 4m/s; 15mm impact velocity; depth of depression exhibited significant brain lesion 1 week post-TBI that was scaled in severity (E). * indicates significant difference compared to contralateral side at $p<0.05$.

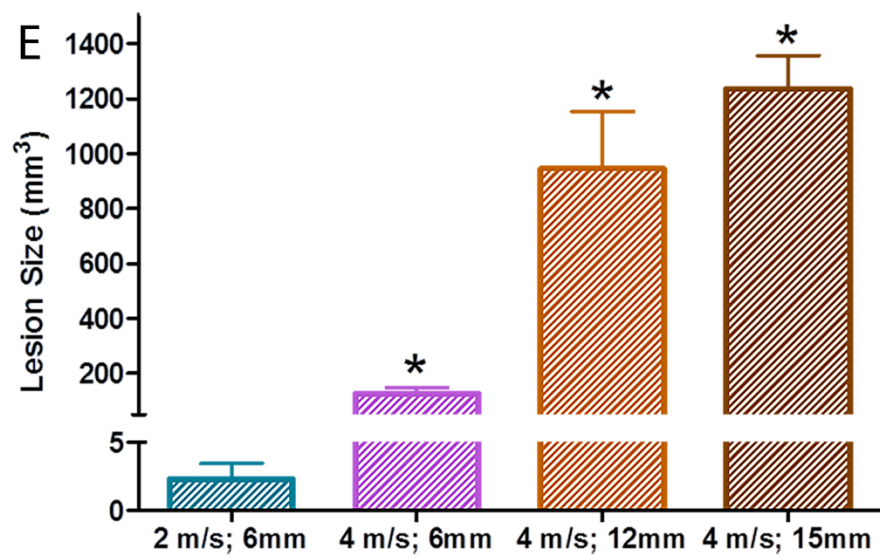
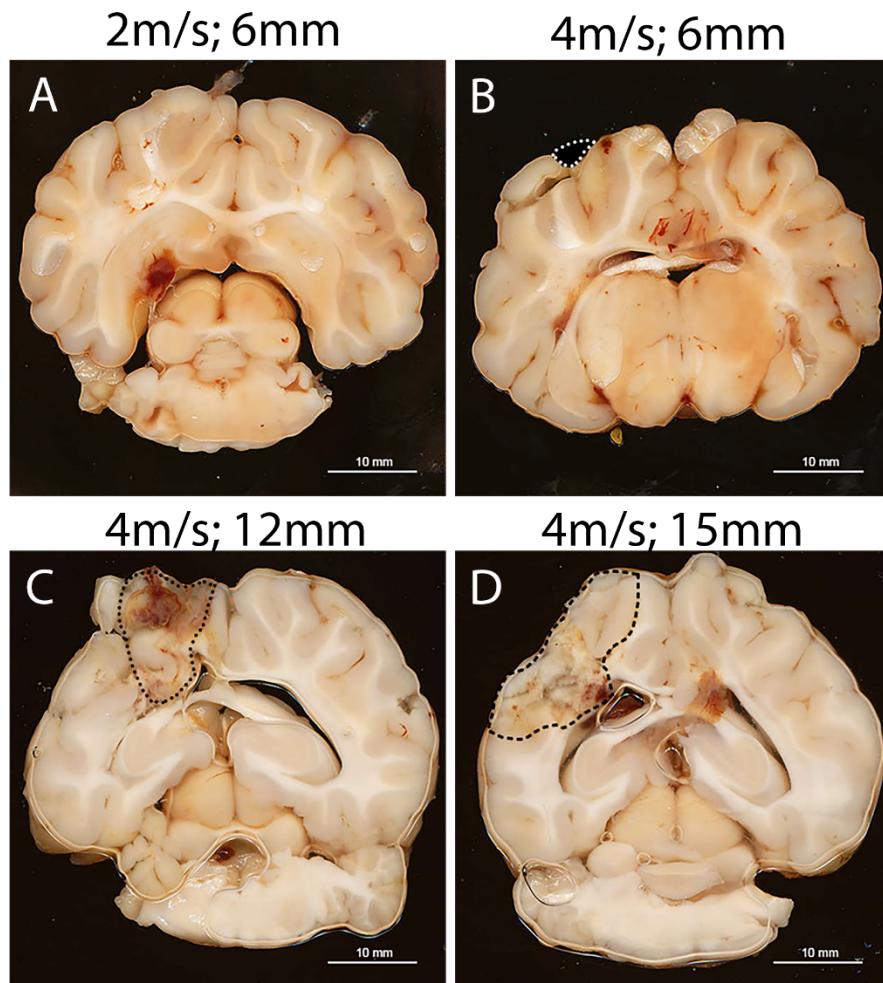


Figure 4.2: Histopathology Reveals Correlative Increase in Lesion Size in Response to Scaled Injury

Hematoxylin and eosin staining of piglets impacted at 2m/s; 6mm (**A**), 4m/s; 6mm (**B**), 4m/s; 12mm (**C**), and 4m/s; 15mm (**D**). For 2m/s; 6mm, impact lesion is subtle and corresponds to focus of increased GFAP staining in Figure 4.3A at arrow. Note area of decreased cellularity in the cortex under the slightly fibrotic leptomeninges (F) and roughly between the *s. Decreased cellularity is due to shrunken eosinophilic necrotic neurons (see inset). Inset: Higher magnification showing necrotic neurons (arrows mark some but not all) in area of decreased cellularity (**A**). For 4m/s; 6mm, impact lesion is more extensive spanning multiple gyri but still relatively superficial. Note the expansion and severe cellularity due to gliosis of the cortex (C) of two gyri. There are also tracts (T) of tissue loss that contain small numbers of gitter cells. Hemorrhage (H) is present in the leptomeninges in the sulcus between the gyri (**B**). For 4m/s; 12 mm, impact lesion is severe and extends deep into the cerebrum almost through the ependyma lining the lateral ventricle (E). The large tract (delineated by double headed arrow) is filled with necrotic debris and hemorrhage (H). The surrounding neuroparenchyma is highly cellular due to gliosis (G) (**C**). For 4m/s; 15mm, impact lesions is severe and extends deep into the cerebrum disrupting the ependymal and into the ventricle (V). The large tract (delineated by thicker double headed arrow) is filled with necrotic debris and hemorrhage (H) surrounded by a thick layer of infiltration gitter cells (*s with thinner double headed arrows denoting thickness). Inset: Higher magnification showing infiltrating gitter cells at *s (**D**). Scale bars: **A**, 1mm; **B-D**, 2mm. Insets taken at 400x.

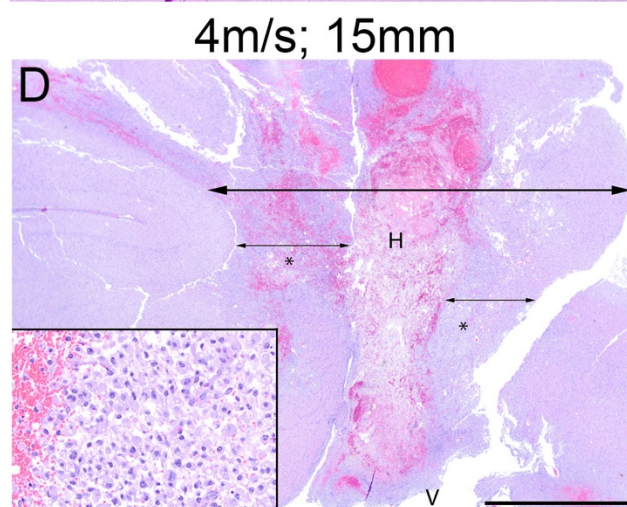
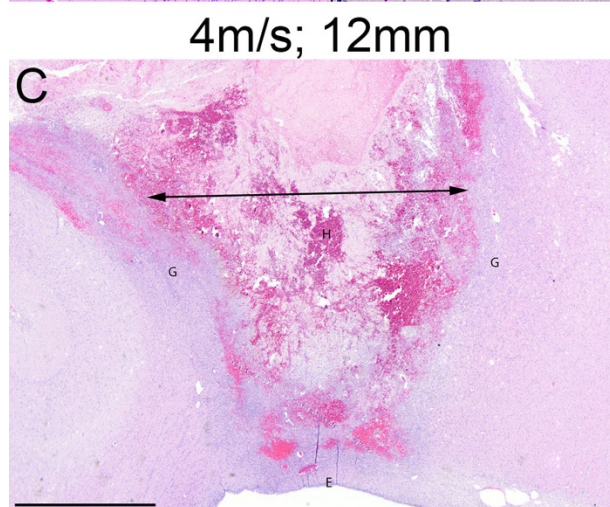
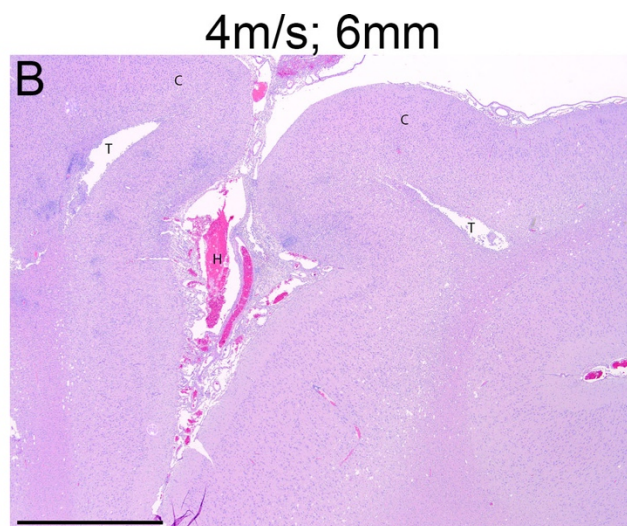
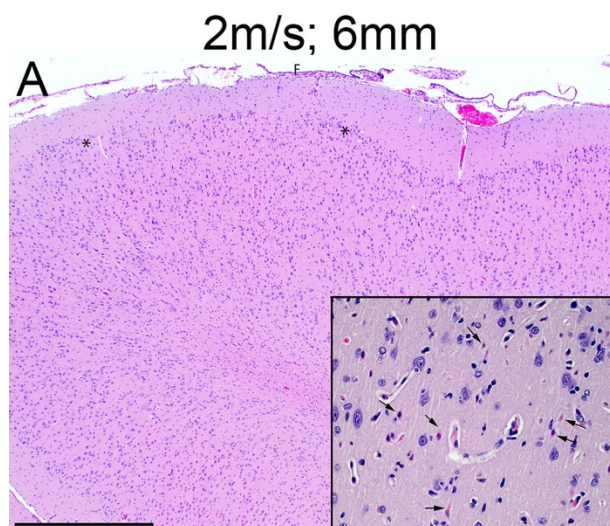
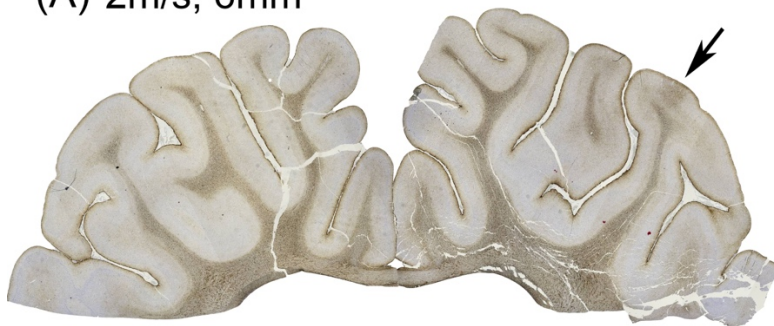


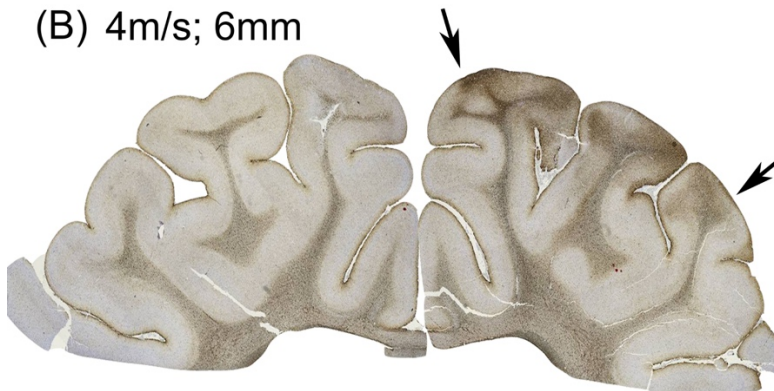
Figure 4.3: TBI Severity Corresponds to Increased Astrocytosis and Astrogliosis

Staining of GFAP+ reactive astrocytes in coronal sections of piglets impacted at 2m/s; 6mm (**A**), 4m/s; 6mm (**B**), 4m/s; 12mm (**C**), and 4m/s; 15mm (**D**). For 2m/s; 6mm, the cerebral cortex at the tip of one dorsolateral gyrus has a small focal area of mildly increased staining for GFAP at the lesion site, (arrow, **A**). For 4m/s; 6mm, the cerebral cortex at the tips of several dorsolateral gyri (marginal, suprasylvian, sylvian) has more extensive and moderately intense staining for GFAP at the lesion site (between arrows, **B**). For 4m/s; 12 mm, there is a large non-stained area of neuroparenchymal damage (D) at the lesion site that is surrounded by marked intense staining for GFAP of both cortical and white matter of the surrounding gyri, including the cingulate gyrus (**C**). For 4m/s; 15mm, there is an extensive area of neuroparenchymal damage and loss (D and L, respectively) at the lesion site with markedly intense staining for GFAP in the surrounding cortical and white matter (**D**). Scale bar is 10mm.

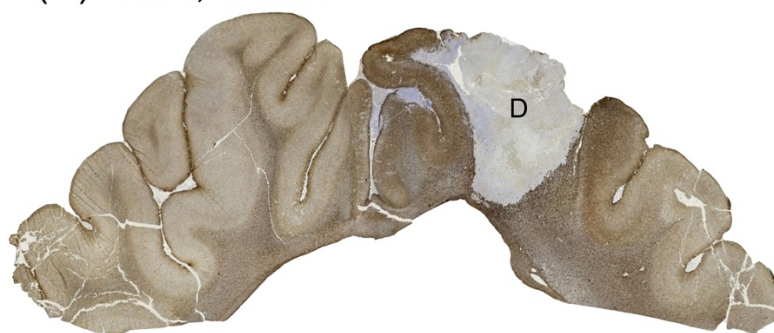
(A) 2m/s; 6mm



(B) 4m/s; 6mm



(C) 4m/s; 12mm



(D) 4m/s; 15mm



Figure 4.4: TBI Severity Corresponds to Loss of NeuN+ Neurons and Upregulation of GFAP+ Astrocytes

Representative images of NeuN expression in 4m/s;12mm uninjured (A) and injured (B) hemispheres. NeuN expression was significantly reduced in the ipsilateral hemisphere compared to the contralateral hemisphere in the 4m/s; 6mm, 4m/s; 12mm, and 4m/s; 15mm groups 7 days post-TBI (C). Representative image of GFAP expression in 4m/s;12mm uninjured (D) injured (E) hemispheres. GFAP expression was significantly increased in the ipsilateral hemisphere compared to the contralateral hemisphere in the 4m/s; 6mm, 4m/s; 12mm, and 4m/s; 15mm groups 7 days post-TBI (F). Representative image of Olig2 expression in 4m/s; 12mm uninjured (G) and injured (H) hemispheres. Olig2 expression was unchanged in the ipsilateral hemisphere compared to the contralateral hemisphere in the for all groups 7 days post-TBI (I). * indicates significant difference compared to contralateral side at $p<0.05$.

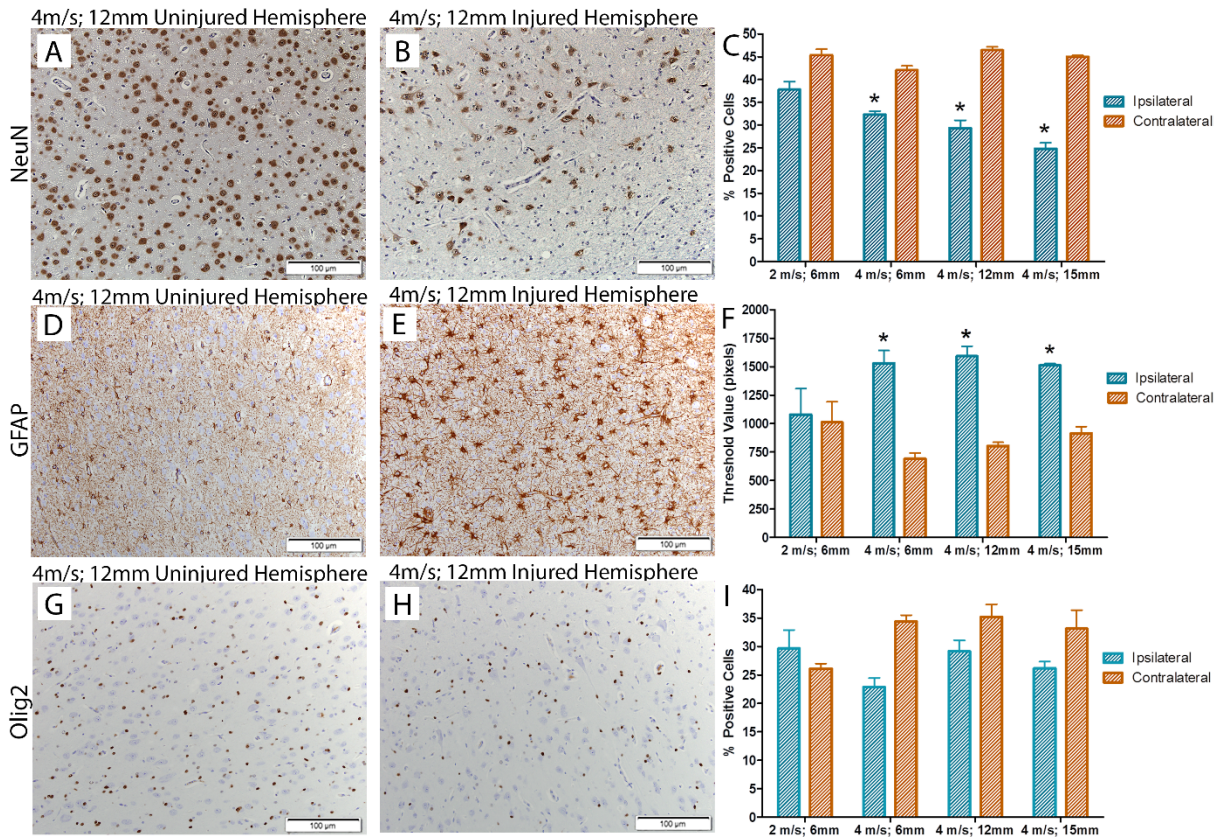
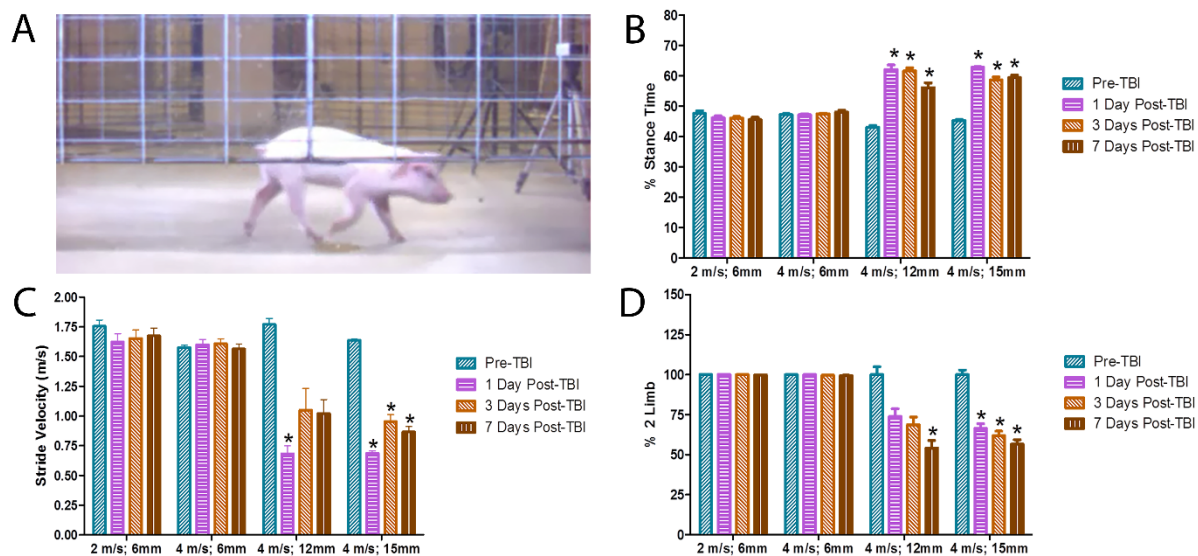


Figure 4.5: Piglets that Received a TBI with a Depth of Depression of 12mm or 15mm
Demonstrate Significant Motor Deficits that Persisted 7 days Post-TBI.

Gait videos were analyzed in Kinovea software frame by frame for changes in stance time, swing time, stride velocity, 2-limb support, and 3-limb support **(A)**. Piglets that received a TBI with a 4m/s impact velocity and either 12mm or 15mm depth of depression demonstrate a significant increase in stance time at 1, 3, and 7 days post-TBI **(B)**. Piglets that received a TBI with a 12mm depth of depression showed a significant decrease in stride velocity 1 day post-TBI, and piglets that received a TBI with a 15mm depth of depression showed a significant decrease in stride velocity 1, 3, and 7 days post-TBI **(C)**. Piglets that received a TBI with a 12mm depth of depression demonstrated a significant decrease in 2-limb support at 7 days post-TBI, while piglets that received a TBI with a 15mm TBI demonstrated a significant decrease in 2-limb support at 1, 3, and 7 days post-TBI **(D)**. No significant changes in motor function were observed for any gait parameters in piglets that received a TBI with a depth of depression of 6mm at either 2m/s or 4m/s velocity. % stance time is represented as the percent of time spent in stance phase of the stride compared to the swing phase of the stride, and % 2-limb support is represented as the percent of time spent in 2-limb support compared to 3-limb support during a full stride cycle.

* indicates significant difference compared to pre-TBI at $p<0.05$.



CHAPTER 5

SCALED TRAUMATIC BRAIN INJURY RESULTS IN UNIQUE METABOLOMIC SIGNATURES BETWEEN GRAY MATTER, WHITE MATTER, AND SERUM IN A PIGLET MODEL ¹

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Abstract

Traumatic brain injury (TBI) is a leading cause of death and long-term disability in the United States. The heterogeneity of the disease coupled with the lack of comprehensive, standardized scales to adequately characterize multiple types of TBI remain to be major challenges facing effective therapeutic development. A systems level approach to TBI diagnosis through the use of metabolomics could lead to a better understanding of cellular changes post-TBI and potential therapeutic targets. In the current study, we utilize a GC-MS untargeted metabolomics approach to demonstrate altered metabolism in response to TBI in a translational pig model, which possesses many neuroanatomical and pathophysiologic similarities to humans. TBI was produced by controlled cortical impact (CCI) in Landrace piglets with impact velocity and depth of depression set to 2m/s;6mm, 4m/s;6mm, 4m/s;12mm, or 4m/s;15mm resulting in graded neural injury. Serum samples were collected pre-TBI, 24 hours post-TBI, and 7 days post-TBI. Multivariate partial least squares-discriminant analysis (PLS-DA) revealed that each impact parameter uniquely influenced the metabolomic profile after TBI, and gray and white matter may respond differently to TBI on the metabolic level with evidence of white matter displaying greater metabolic change. Furthermore, pathway analysis revealed unique metabolic signatures that were dependent on injury severity and brain tissue type. Metabolic signatures were also detected in serum samples which captures both time after injury and injury severity. These findings provide a platform for the development of a more accurate TBI classification scale based unique metabolic signatures.

Introduction

Traumatic brain injury (TBI) is a major public health issue with approximately 1.7 million people sustaining a TBI annually in the United States and one third of survivors experiencing long-term disability [1, 2]. Despite advancements in understanding the underlying mechanisms of TBI, there has been no significant progress in clinical treatment [3]. While most patients that visit the emergency department have sustained mild TBI, a large subset have moderate to severe TBI with persistent deficits in cognition, language, and motor function [4-7]. In clinical trials, it is common for TBI cases to be broadly classified as mild, moderate, or severe based on the Glasgow Coma Scale (GCS) which measures the level of consciousness after traumatic insult [8]. However, the GCS is a gross assessment that does not account for the pathoanatomic features responsible for the measured neurological deficits; therefore two TBI cases with divergent injury characteristics and outcomes could be identically classified [3]. To combat this, attempts have been made to develop scales based on other non-invasive modalities such as computed topography (CT) to identify pathoanatomic characteristics (e.g. midline shift, lesion size, the presence of hemorrhage, and cistern alterations) to better classify TBI [5, 9, 10]. However, it is possible for brain images to appear normal despite metabolic abnormality and the presence of clinical symptoms [11]. Thus, the heterogeneity of the disease coupled with the lack of standardized scales to properly categorize multiple types of TBI remains to be a major challenge facing the development of effective TBI treatments.

Development of more comprehensive diagnostic tools that measure deficits at the cellular level may enable better classification of patients based on TBI type and severity, potentially providing a predictive tool of outcome and ultimately aid in the development of therapeutic treatment plans. While the quantification of TBI biomarkers such as glutamate, glucose, and

S100-B in serum, plasma, or cerebrospinal fluid (CSF) provide greater insight to the physiological alterations resulting from TBI, the clinical management and assessment of these metabolites remains to be inconsistent and unreliable especially in the case of mild TBI [12-14]. Furthermore, the use of currently limited biomarker panels that focus on discrete molecules like glutamate often does not accounting for complex cellular and tissue level interactions between numerous pathways leading to limited predictive potential with respect to injury type, severity and potential therapeutic targets [15, 16]. Emerging tools in the field of metabolomics provides an excellent platform to obtain more robust and comprehensive information about injury status or likelihood of therapeutic benefit based on a signature of markers rather than a single biomarker [12, 16, 17]. This systems level approach to TBI diagnosis could lead to a better understanding of the complex cascades of interacting metabolic changes after TBI and potential therapeutic targets.

Metabolomics is the study of the metabolites and metabolism within a biological system, which is a product of both the organism's genome and the influence of its environment [17]. Analytical chemistry techniques such as gas chromatography mass spectrometry (GC-MS) are used to identify the components of complex biological mixtures including tissues and biofluids with a high degree of sensitivity [17]. A major advantage to MS-based metabolomics is that an untargeted, "bottom-up" approach can be utilized in an attempt to quantify all biomolecules within a sample rather than quantifying a predetermined list of metabolites; therefore, it is possible to obtain the metabolomic signature for a particular patient or discover novel biomarkers for injury or disease [16, 18]. Microarray analysis in rodent TBI models have shown that the brain undergoes abundant changes in gene expression that are dependent on experimental conditions such as brain tissue type, time after injury, and injury severity, providing

further evidence that the heterogenic nature of TBI results in a unique metabolomic signature for each TBI case [19-22]. Thus, metabolomics offers the possibility integrating “personalized medicine” into clinical TBI management by customizing a patient’s medical treatment based on their specific biomarker profile [17]. A major component of the pathophysiologic response to TBI is brain region dependent and more studies are urgently needed to compare the metabolic response of gray and white matter after TBI. Gray and white matter compartments have distinctive cellular composition and cytoarchitecture leading to differing responses to neural injury with respect to structural vulnerability, susceptibility to excitotoxic and ischemic insults, and edema accumulation [23-28]. Preclinical animal models of TBI that possess a similar gray to white matter ratio as humans, such as the pig, will enable the assessment and understanding of large scale region-specific metabolic responses to neural injury that are likely to be more clinically relevant.

Preclinical animal models are an excellent tool to study metabolic alterations in tissue and biofluids after TBI. A metabolomic study of serum across multiple species by Li et al. demonstrated that the pig and the rat have greater metabolic similarity to humans compared to other common laboratory animals such as the guinea pig and mouse, making them suitable animal models for translational metabolomics research [29]. However, compared to the rat, the pig possesses many brain anatomical similarities to humans including gray to white matter ratio and gyral patterning [30, 31]. While the rat brain is composed of approximately 10% white matter, both the human and the pig brain contains >60% white matter [31-33]. Therefore, metabolic alterations after TBI in gray and white matter compartments can be easily measured in the pig model. Together, this evidence suggests that a pig TBI model could lead to a better

understanding of TBI-induced, region-specific metabolic alterations in the human brain compared to traditional rodent systems.

In the current study, we utilize an untargeted GC-MS metabolomics approach to demonstrate variable alterations in brain metabolism after scaled TBI in a pig model. Pathway analysis reveals that gray and white matter are differentially affected by TBI in a severity-dependent manner. Furthermore, metabolomic analysis performed on serum 24 hours and 7 days post-TBI demonstrates a distinct time course of metabolic alteration that is also dependent on TBI severity but independent of alterations observed directly in brain tissue. This proof-of-concept study illustrates that untargeted metabolomic methodology captures unique metabolic signatures that are contingent on sample type (i.e. gray matter, white matter, or biofluid), TBI severity, and time after injury in a translational pig TBI model. These findings will potentially lead to a greater understanding of the intricate, heterogenic cellular changes after TBI, resulting in a more comprehensive and accurate classification system for a personalized medicine approach to TBI treatment.

Materials and Methods

Animals

All work in this study was performed in adherence to the University of Georgia Institutional Animal Care and Use Committee guidelines. Sixteen commercially bred American Landrace piglets of mixed sex and three weeks of age were obtained from the University of Georgia Swine Unit one week prior to surgery. Piglets were group housed and fed a pelleted starter diet ad libitum. The room temperature was maintained at 26°C with a 12 hour light/dark cycle. An overhead heat lamp provided supplemental heat to a portion of the enclosure. Animals

were feed restricted overnight prior to surgery but were permitted free access to water at all times.

Controlled Cortical Impact

Concussive TBI was generated through the use of a specialized controlled cortical impactor (CCI) device that was designed by our lab in collaboration with the University of Georgia Instrument Shop (Athens, GA) and was modeled after the design of Manley et al [34]. The CCI device is designed to generate a focal, reproducible injury to the cerebral cortex with defined impact parameters including impact velocity, depth of cortical depression, and dwell time. At four weeks of age, piglets underwent surgery to induce concussive TBI. Anesthesia was induced via inhalation of 5% vaporized isoflurane in oxygen utilizing a nose cone. After induction, anesthesia was maintained at 2-3% vaporized isoflurane in oxygen. Heart rate, respiration rate, and rectal temperature was measured every five minutes throughout the duration of surgery and post-operative recovery. The skin over the cranium was prepared in a routine manner for sterile surgery using Betadine and 70% alcohol, and the surgical site was draped in a standard fashion. A sagittal left-sided skin incision approximately four centimeters in length was made over the top of the cranium, and the periosteal layers were resected to expose the cranial bone. A dental air drill (Brasseler USA) was used to drill a circular craniectomy approximately 20mm in diameter at the left posterior junction of the coronal and sagittal sutures. The underlying dura mater was left intact. Anesthetized piglets were then secured to the CCI frame, and the impactor tip was positioned over the site of the craniectomy. Piglets were divided into four treatment groups depending on the impact parameters in which TBI was induced, including: 2 m/s impact velocity with 6mm depth of depression (2m/s; 6mm, n=4), 4 m/s impact velocity

with 6mm depth of depression (4m/s; 6mm, n=4), 4 m/s impact velocity with 12mm depth of depression (4m/s; 12mm, n=4), 4 m/s impact velocity with 15mm depth of depression (4m/s; 6mm, n=4). The dwell time, defined as the amount of time the impactor tip makes contact with the cortex before retraction, was held constant at 400 ms for all treatment groups. After TBI induction, the craniectomy site was flushed with sterile saline and the skin was re-apposed with sterile surgical staples. Piglets were then removed from anesthesia and continuously monitored until ambulatory. Banamine (1.1 mg/kg IM) and butorphanol (0.2 mg/kg IM) was administered postoperatively for analgesia, and Ceftiofur sodium (Naxcel®; 4 mg/kg IM) was administered postoperatively as an antibiotic.

Serum and Tissue Sample Preparation

Blood samples were collected pre-TBI, 24 hours post-TBI, and 7 days post-TBI and allowed to coagulate in serum separator tubes (BD Vacutainer®). Blood samples were then centrifuged at 2,000 rpm for 20 minutes, and the serum supernatant was collected, snap frozen in liquid nitrogen, and stored at -80°C. 7 days post-TBI, piglets were deeply anesthetized through inhaled 5% vaporized isoflurane in oxygen and immediately euthanized via CO₂ asphyxiation following AVMA guidelines. The piglets were decapitated and the brain was removed. Brain samples were collected from the peri-lesional area of the ipsilateral cortex and subcortical white matter, snap frozen in liquid nitrogen, and stored at -80°C until use.

Metabolite Extraction

For metabolite extraction, serum and tissue samples were thawed on ice, and tissues were dissected to obtain separate gray and white matter samples weighing approximately 65mg each.

For serum, a volume of ~70 μ L was used for extraction. Metabolites were extracted from the serum and tissue samples in centrifuge tubes using methanol, deionized water, and chloroform (ratio of 1.4:1:1.8). A 3.2-mm stainless steel bead (Biospec Products, Inc.) was added to each centrifuge tube and samples were homogenized using a Tissue lyser (Qiagen). As described previously [35], 400 μ L of methanol, 285 μ L of deionized water, and 500 μ L chloroform was added stepwise to the samples. After centrifugation, the polar metabolite phase was removed, aliquoted into GC vials, and lyophilized in a Savant Speed Vac Concentrator (Thermo Scientific).

Data Acquisition and Analysis of Metabolites

Tissue samples were analyzed on a LECO Pegasus 4D GC-ToF/MS (St. Joseph, MI) interfaced to an Agilent 7890B gas chromatograph. Chromatographic separation was achieved on a DB-5ms (30m, 0.25 μ m thickness, and 0.25 mm ID, Agilent Technologies, CA). All injections were made in the splitless mode with 2 μ L. The injector, transfer line and ion source were held at 275 $^{\circ}$ C, 280 $^{\circ}$ C and 225 $^{\circ}$ C respectively. Samples were injected in splitless mode (2 μ L) and helium maintained at a constant flow of 0.8 mL/min. The initial oven temperature was held for 2 min at 60 $^{\circ}$ C and ramped at 8 $^{\circ}$ C/min to 300 $^{\circ}$ C and held for 5 min. Mass spectra were acquired from 50-650 m/z at an acquisition rate of 20 spectra/second. Serum samples were analyzed similarly on a Waters Autospec mass spectrometer using the same column and temperature profiles. For serum analysis, the trap was set at 350 μ A and the detector at 300 V. All chromatograms were then exported as netcdf files using the instrument specific software and imported into MetAlign® for data alignment using recommended parameters for time of flight mass spectrometers [36]. After spectral alignment, data was filtered in Excel as described

previously [37]. Student's t-test filtered chromatograms comparing the response of TBI groups to control were constructed in Excel by subtracting the average response of control samples from TBI samples at each retention time (R.T.) m/z pair. SIMCA-P+ (Umetrics, Sweden) was used for multivariate analysis, and MetaboAnalyst 3.0 used for ANOVA and pathway analysis as described below [38]. Metabolites were identified by comparing the acquired spectra to commercially available databases (e.g. NIST 2014) and considered a putative identification with a similarity score greater than 700.

Metabolic Pathway Analysis

As described in previous studies [36], the Pathway Analysis module on Metaboanalyst (<http://www.metaboanalyst.ca>) was used to identify affected metabolic pathways based on metabolites identified by GC-MS to be upregulated or downregulated after TBI. A Sus scrofa-specific library is not available at this time, so we selected the Homo sapiens library and used the default “Hypergeometric Test” and “Relative-betweenness Centrality” algorithms for over representation analysis and pathway topology analysis, respectively. In order to report the most relevant metabolic pathways, the impact value threshold was set to 0.1.

Results

Increasing TBI severity results in correlative PLS-DA group separation with distinct responses between gray and white matter

Multivariate partial least squares-discriminant analysis (PLS-DA) was utilized to compare the metabolomic profile of normal control brain tissue and brain tissue isolated from the peri-lesional brain region of pigs that sustained TBI with impact parameters of 2m/s; 6mm, 4m/s;

6mm, 4m/s; 12mm, and 4m/s; 15mm. We first assessed the influence of impact speed and depth of depression on group separation from normal control tissues and within TBI groups, and whether gray and white matter responded differently to changes in these impact parameters. While holding depth of depression constant at 6mm, pigs that sustained a TBI at 2m/s demonstrated a metabolomic profile more similar to normal control tissue compared to pigs that sustained a TBI at 4m/s in both gray and white matter along principal component 1 (PC1), which is illustrated by separation along the X axis, and this distance from control samples potentially represents the largest biological changes in the system (**Figure 5.1A, B**). Similarly, while holding impact speed constant at 4m/s, pigs that sustained a TBI with 6mm depth of depression were more similar to normal controls along PC1 compared to pigs that sustained a TBI at 12mm or 15mm in both gray and white matter; however, the 12mm and 15mm groups displayed less separation in white matter compared to the response in gray matter (**Figure 5.1C, D**). PLS-DA of total TBI effect demonstrated correlative group separation in gray and white matter with increasing TBI severity along PC1 (**Figure 5.1E, F**). However, while a stepwise increase in group separation between TBI groups along PC1 was documented in gray matter, group clustering was apparent in white matter TBI groups in which the 2m/s; 6mm and 4m/s; 6mm groups displayed a more similar metabolomic profile than pigs in the 4m/s; 12mm and 4m/s; 15mm groups (**Figure 5.1F**). Taken together, this data indicates that both impact velocity and depth of depression play a role in changing the metabolomic profile after TBI, and gray and white matter may respond differently to TBI on the metabolic level.

White matter displays a greater number of metabolomic alterations than gray matter after TBI

Univariate analysis was performed to quantify significant changes in metabolite abundance in TBI tissue compared to normal control tissue in both gray and white matter. In this visualization (**Figure 5.2A, B**), peaks in the negative direction (less than 0) represent metabolites that are downregulated (i.e. higher in control samples) after TBI while peaks in the positive direction (more than 0) represent metabolites that are upregulated after TBI. Overall, there was a positive correlation between TBI severity and the total number of peaks (**Figure 5.2C, D**) as well as the total peak amplitude (data not shown) in both gray and white matter. Furthermore, the total number of peaks as represented by R.T./*m/z* pairs was higher in white matter compared to gray matter in the 2m/s; 6mm, 4m/s; 6mm, and 4m/s; 12mm groups (1915 vs. 1348, 2558 vs. 1312, and 1825 vs. 2992, respectively), while the total number of peaks was higher in gray matter compared to white matter in the 4m/s; 15mm group (3051 vs. 5152, respectively, **Figure 5.2C, D**). Peak identification demonstrated that multiple classes of metabolites were either upregulated or downregulated by TBI including biological acids, alcohols, amino acids, fatty acids, and sugars (**Table 5.1**). This data further supports that increasing TBI severity results in a greater change in brain metabolism, and gray and white matter respond differently to TBI with white matter demonstrating more metabolic alterations compared to gray matter in all TBI groups with the exception of the most severe group.

Pathway analysis reveals unique metabolomic signatures dependent on TBI severity and brain tissue type

Metaboanalyst was used to identify impaired metabolic pathways after TBI in gray and white matter. As expected, there were generally more affected metabolic pathways with

increasing TBI severity (**Table 5.2**). Metabolic pathways demonstrated a distinct pattern of susceptibility across TBI severity and brain tissue type; some pathways (i.e. glycolysis, methane metabolism) were preferentially affected in gray matter while other pathways (i.e. cysteine and methionine metabolism, lysine degradation) were preferentially affected in white matter. Additionally, some pathways were only affected in more severe TBI groups (i.e. butanoate metabolism) while some pathways were affected across all TBI severities or brain tissue types (i.e. arginine and proline metabolism, galactose metabolism, etc). This data indicates that each TBI group demonstrates unique changes in metabolomic signature that is dependent on brain tissue type (i.e. gray or white matter).

PLS-DA score plots display distinct group separation in serum samples based on TBI severity and time after injury

Next, we visually assessed the changes in the metabolomic profile of serum collected from piglets 24 hours and 7 days post-TBI in order to 1) determine the extent the metabolomic profile changes over time after TBI and 2) whether TBI-induced metabolic alterations differed between serum and brain tissue. When assessing the effect of time after injury, PLS-DA score plots revealed distinct group separation between the pre-TBI, 24 hours post-TBI, and 7 days post-TBI time points with the 7 days post-TBI metabolomic profile being further separated from pre-TBI compared to 24 hours post-TBI along PC1 (**Figure 5.3A**). When assessing how metabolism is influenced by impact speed over time while holding depth of depression constant at 6mm, we found that serum collected 24 hours post-TBI was more similar to pre-TBI along PC1 for both 2m/s and 4m/s groups than either group 7 days post-TBI, which indicates that time post-TBI has a greater influence on the metabolomic profile of serum than impact speed (**Figure**

5.3B). When assessing the influence of depth of depression over time while holding impact speed constant at 4m/s, we found a stepwise increase in group separation along PC1 in serum collected both 24 hours and 7 days post-TBI as TBI severity was increased (**Figure 5.3C**). Furthermore, distinct group separation was present when assessing total TBI effect across all TBI severities and time points. Interestingly, there was greater group separation across PC1 in serum from pigs in the 2m/s; 6mm and 4m/s; 6mm groups 7 days post-TBI compared to the serum collected from pigs at the same time point that sustained a more severe TBI, albeit less group separation was present along PC2 (Y axis) 7 days post-TBI in the 2m/s; 6mm and 4m/s; 6mm groups (**Figure 5.3D**). Together, this data indicates that time point, impact speed, and depth of depression all contribute to the altered metabolomic signature of serum collected post-TBI.

Serum demonstrates dynamic changes in metabolomic profile in all TBI severities and over time

Univariate analysis was performed on serum collected pre-TBI, 24 hours post-TBI, and 7 days post-TBI across TBI groups to quantify changes in the metabolomic profile between TBI groups and over time. In **Figures 5.4A** and **5.4B**, peaks in the negative direction (less than 0) represent metabolites that are downregulated 24 hours and 7 days post-TBI, respectively, compared to pre-TBI while peaks in the positive direction (more than 0) represent metabolites that are upregulated after TBI compared to pre-TBI. In **Figure 5.4C**, peaks in the negative direction (less than 0) represent metabolites that are downregulated 7 days post-TBI compared to 24 hours post-TBI while peaks in the positive direction (more than 0) represent metabolites that are upregulated 7 days post-TBI compared to 24 hours post-TBI. At 24 hours post-TBI, there was a correlative increase in total number of peaks and the total peak amplitude (data not shown) as TBI severity was increased (**Figure 5.4D**). However, at 7 days post-TBI, there was a

correlative decrease in the total number of peaks and total peak amplitude (data not shown) as TBI severity was increased (**Figure 5.4E**). The differences in correlation to TBI severity between the two time points are indicative of distinct metabolic alterations between 24 hours and 7 days post-TBI. Indeed, univariate analysis comparing 7 days post-TBI and 24 hours post-TBI revealed many peaks above/below baseline indicative of altered metabolite abundance between the two time points in the 2m/s; 6mm, 4m/s; 6mm, 4m/s; 12mm, and 4m/s; 15mm groups (174, 170, 127, and 240, respectively, **Figure 5.4F**). Furthermore, the total number of peaks was declined between 24 hours and 7 days post-TBI when both are compared to pre-TBI in the 2m/s; 6mm, 4m/s; 6mm, 4m/s; 12mm, and 4m/s; 15mm groups (220 vs. 209, 186 vs. 148, 236 vs. 129, and 271 vs. 164, respectively, **Figure 5.4D, E**). Serum metabolite classes similar to brain tissue where determined by peak identification to be upregulated or downregulated by TBI including acids, alcohols, amino acids, fatty acids, and sugars (**Table 5.3**). This data indicates that serum undergoes dynamic changes in metabolism over time evident by altered peak abundance between pre-TBI, 24 hours post-TBI, and 7 days post-TBI.

Pathway analysis of serum supports observed severity and time-dependent metabolomic alterations

Metabolic pathways that were affected by TBI were identified using Metaboanalyst in serum collected 24 hours and 7 days post-TBI. As shown in **Table 5.4**, a distinct pattern of pathway alterations was present based on TBI severity and time post-TBI. Glycerophospholipid metabolism was affected 24 hours post-TBI across all TBI severities while cysteine and methionine metabolism, phenylalanine metabolism, and aminoacyl-tRNA biosynthesis was affected exclusively 7 days post-TBI. Furthermore, a subset of pathways (i.e. citrate cycle,

inositol phosphate metabolism, and butanoate metabolism) were affected 7 days post-TBI in only the more severe TBI groups. In contrast, another subset of pathways (i.e. alanine, aspartate, and glutamate metabolism and arginine and proline metabolism) were affected in nearly all TBI groups and time points. Taken together, the data indicates that serum collected post-TBI, like brain tissue, possesses a unique metabolomic signature dependent on both TBI severity and time post-TBI.

Discussion

Despite advancements in understanding the cellular mechanisms of TBI, there are no reliable classification scales to accurately categorize this heterogenic disease to predict patient outcomes or develop potential therapeutics. Here we utilize an untargeted GC-MS metabolomics approach to show that distinct metabolic alterations occur after TBI which are dependent on injury severity, sample type, and time after injury. PLSDA score plots of brain tissue isolated from the peri-lesional area demonstrated distinct group separation correlative to TBI severity, and these trajectories were different between gray and white matter. Interestingly, the total number of peaks quantified from *t*-test filtered difference chromatograms revealed that more metabolic alterations occur in white matter than gray matter, which provides further justification to study TBI pathophysiology in preclinical animal models with comparable white matter to humans. To our knowledge, we are the first to distinguish between the divergent metabolic alterations in gray and white matter after TBI. Furthermore, metabolic changes measured in serum after TBI revealed dynamic alterations in metabolic pathways with a distinct time course. These results provide evidence that a TBI classification scale based on the global metabolome

could be developed to predict TBI severity and brain structures affected in order to more accurately predict patient outcomes and identify therapeutic targets in a personalized manner.

Untargeted metabolomic assessment of TBI-affected brain tissue demonstrated unique alterations in multiple metabolic pathways based on injury severity and tissue type. Previous metabolomics studies utilizing magnetic resonance approaches have quantified specific metabolites in tissue, cerebrospinal fluid (CSF), and plasma after TBI in order to identify predictors for cellular mechanisms or other clinical measures [37-41]. However, since these studies only target a predetermined list of metabolites, they may not identify novel markers or account for important interactions between molecules at the cellular level [15]. To overcome this limitation, previous studies have performed untargeted metabolomics on serum samples in order to classify TBI patients based on injury severity and the presence of cognitive impairment based on altered metabolomic signature [36, 42]. However, to our knowledge no studies have analyzed the effect of TBI on the global metabolome directly on brain tissue nor assessed differences in metabolic alteration between gray and white matter as we have done in this study. We utilized the Pathway Analysis module on Metaboanalyst, which uses the KEGG metabolic database, to identify metabolic pathways altered by TBI [43]. In order to identify the most relevant alterations, the impact value threshold was set to 0.1. Pathway analysis revealed that gray and white matter responded differently to TBI across all injury severities, a result likely due to the divergent cellular composition of the two compartments. While neuronal cell bodies, dendrites, and synapses are mostly restricted to gray matter compartments, white matter consists of myelinating oligodendrocytes and axon bundles that foster communication between neurons [31, 44, 45]. We found that the metabolic pathways related to cysteine and methionine metabolism, lysine degradation, pantothenate and CoA biosynthesis, and phenylalanine metabolism are

specifically impaired in white matter after TBI, while showing no changes in gray matter.

Furthermore, the citrate cycle, glycolysis/gluconeogenesis, methane metabolism, and glyoxylate and dicarboxylate metabolism were only affected in more severely impaired gray matter while pyruvate metabolism was affected in gray matter regardless of TBI severity. This suggests that gray and white matter undergo different metabolic processes in response to TBI, indicating the need to develop treatment interventions specific to the brain region being affected.

The pathways shown to be altered in white matter directly involve the amino acids serine, methionine, cysteine, alanine, lysine, phenylalanine, and tyrosine along with the amino acid derivative cystathionine. Previous studies have measured the abundance of these amino acids in brain tissue, serum, plasma, and CSF after TBI with variable findings dependent on sample type and time after injury [36-38, 40, 41, 46]. However, to our knowledge, no studies have elucidated the differences in the response of these amino acids between gray and white matter after TBI. Similar to our study, Irie et al. showed a significant reduction in lysine, methionine, phenylalanine, and tyrosine 24 hours post-stroke in both the striatum and cortex utilizing LC-MS in a rat model while an ex vivo NMR study showed elevated levels of lysine, phenylalanine, and tyrosine in CSF collected 7 days post-TBI [40, 47]. These studies support our finding that phenylalanine and tyrosine levels are decreased in tissue but elevated in biofluid collected 7 days post-TBI. This suggests that in the event of TBI, intracellular amino acids are released into the extracellular space and diffuse into circulating biofluids. Furthermore, the observed amino acid response to TBI was different between gray and white matter samples suggestive of a divergent time course or susceptibility to amino acid alterations between the two tissues. Indeed, a study by Shimada et al. found that amino acids diffuse slowly from gray matter compartments into adjacent white matter and later into the CSF [48]. While we documented a reduction in alanine

across all TBI severities and a reduction in serine in more severe TBI groups, previous studies have shown no difference in alanine and serine levels in tissue or CSF after TBI compared to controls at <24 hours and 7 days post-TBI [37, 38, 41]. The difference in the measured alanine and serine response may be due to injury severity or the sensitivity of the analytical method as these studies were collected using less sensitive magnetic resonance approaches rather than GC-MS [17].

The pathways specifically altered in gray matter are implicated in cellular respiration and involve the metabolites pyruvic acid, succinic acid, acetylaldehyde, ethanol, lactic acid, glucose, acetic acid, formic acid, glycine, serine, and oxalic acid, which were almost exclusively downregulated after TBI. Cellular respiration occurs in mitochondria which are highly mobile in both anterograde and retrograde directions in neurons and tend to accumulate in segments with high metabolic demand [49-51]. Mitochondria are abundant in the neuronal cell bodies, dendrites, and axon terminals where they produce ATP to maintain the ionic gradient, and thus neuronal excitability, while also facilitating presynaptic vesicle packaging and postsynaptic protein turnover which exclusively occurs in gray matter compartments [45, 50-52]. Therefore, our finding of altered cellular respiration function in gray matter is indicative of decreased ATP synthesis in neurons and suggests impaired synaptic activity. Reductions in glucose consumption and cerebral energy state (measured by ATP or lactate/pyruvate ratio) are hallmarks of TBI and are directly correlated to patient outcome [53]. The lactate/pyruvate ratio (LPR) has been used extensively in microdialysis monitoring of TBI patients and reflects energy crisis with increases in LPR indicating loss of oxygen in tissue and the switch from aerobic to anaerobic respiration [54, 55]. Although we did not directly quantify LPR in the present study, we documented deficits in metabolites implicated in both aerobic and anaerobic respiration (including lactic acid) in the

peri-lesional gray matter by 7 days post-TBI. However, serum lactic acid levels were elevated 24 hours post-TBI in a subset of TBI groups. This finding supports previous clinical studies which report high lactate levels due to cerebral and corporal hyperglycolysis in the initial post-injury stage that become slightly depressed in the days following TBI [56-58]. Taken together, this suggests that TBI with gray matter involvement may be more likely to respond to therapies targeted at restoring cerebral energy homeostasis.

Pathway analysis also illustrated graded metabolic response correlative to injury severity in a subset of metabolic pathways. Butanoate metabolism was impaired specifically in the 4m/s; 12mm and 4m/s; 15mm groups in both gray and white matter, partly due to decreased levels of butyric acid. Butyric acid has been identified as a potential therapeutic for neurological disease through its ability to serve as a histone deacetylase inhibitor, energy substrate, and G protein-coupled receptor activator; therefore, deficits in butyric acid abundance could be detrimental in the context of brain injury [59]. Interestingly, in the 2m/s; 6mm and 4m/s; 6mm groups, glycerolipid metabolism was only altered in white matter while arachidonic acid metabolism was only affected in gray matter; however, both metabolic pathways were affected in both tissue types in the more severe TBI groups. This suggests that white and gray matter are more susceptible to alterations in glycerolipid and arachidonic acid metabolism, respectively. Alterations in glycerolipid metabolism were driven by the altered abundance of glycerol, which is a component of the myelin sheaths found in white matter compartments [60]. These findings suggest that glycerol levels, and thus glycerolipid metabolism, are altered at a lower threshold in white matter compared to gray matter due to the disruption of myelin sheaths. Furthermore, arachidonic acid has been shown to play a role in the production of reactive oxygen species (ROS) after TBI [61-63]. Since mitochondria are more abundant in gray matter and are a major

source of ROS, this finding indicates that arachidonic acid metabolism is affected at a lower injury severity in gray matter compared to white matter [45, 51, 62]. Together, this indicates that metabolomic analysis in our pig TBI model is able to delineate the individual metabolic vulnerabilities of gray and white matter through changes in compartment-specific cellular processes.

Serum samples demonstrated dynamic alterations in metabolism between 24 hours and 7 days post-TBI. Glycerophospholipid metabolism was shown to be significantly altered in serum collected 24 hours post-TBI in all TBI groups while there was no change compared to controls by 7 days post-TBI in serum or tissue samples. Glycerophospholipids are a major component of neural cell membranes and have been shown to be altered in a number of neurological disorders including TBI due to the disruption of these membranes [64, 65]. In the acute stage of TBI, excessive release of glutamate initiates an excitotoxic cascade, and the resulting buildup of intracellular Ca^{2+} activates phospholipases which degrade neuronal cellular membranes [66]. Therefore, our data provides evidence of excitotoxicity-induced neuronal membrane degradation by 24 hours post-TBI that becomes diminished by 7 days post-TBI when excitotoxicity has largely subsided [67]. Conversely, a number of metabolic pathways were disturbed in serum collected 7 days post-TBI that were not found at 24 hours, which coincided with documented metabolic deficits in tissue collected at the same time point. However, the injury severity at which these pathways were affected varied between tissue and serum. For example, aminoacyltRNA biosynthesis was found to be altered in serum collected from the 2m/s;6mm group but no change was detected in tissue from this TBI group. AminoacyltRNAs are produced by aminoacyl tRNA synthases, and the expression of the pro-inflammatory mediator macromolecular tRNA synthase complex-associated protein (MSC^{p43}) has been shown to be

biomarker in brain injury to delineate between traumatic and ischemic brain injuries [68, 69]. Therefore, this finding indicates that alterations in this pathway detected in serum could be utilized as a marker for brain injury in the context of mild TBI. Furthermore, two metabolic pathways were shown to be significantly affected in serum collected both 24 hours and 7 days post-TBI including arginine and proline metabolism. The documented deficits in arginine and proline metabolism were due in part to alterations in proline levels as well as glutamine and ornithine which are metabolites of arginine [70]. Arginine is a precursor to the free radical nitric oxide (NO), and it has been shown that arginine levels in plasma and CSF are inversely correlated to NO levels; therefore, plasma levels of arginine have been proposed as a biomarker for ischemic brain damage [71, 72]. Similar to our results, Louin et al. and others documented no change in arginine levels but decreased proline levels after TBI in a rat model which was correlative to increased NO levels, indicating that proline may be a better biomarker than arginine in the context of TBI [70, 73]. However, proline can be a precursor for arginine and may be utilized to maintain arginine levels in brain tissue; therefore, it may be more beneficial to assess the entire metabolic pathway rather than the level of a single component [70]. Our results indicate that this metabolic pathway as a whole could be used as a biomarker for TBI of all severities from 24 hours post-TBI to 7 days post-TBI. Together, pathway analysis of serum illustrates the presence of a unique metabolic signature that captures both the time after injury and injury severity.

In conclusion, we utilized an untargeted metabolomics approach to show that distinct alterations in metabolism occur after TBI that are dependent on injury severity, sample type, and time after injury in a translational pig model. We show for the first time that divergent changes in metabolism occur between gray and white matter after TBI with evidence of white matter

displaying a greater number of metabolic changes. These findings provide a platform for the development of a more accurate and comprehensive TBI classification scale based unique metabolic signatures, creating a new avenue for personalized medicine in the management and treatment of TBI.

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Figure 5.1: PLS-DA Score Plots Illustrate Distinct Metabolomic Separation Based on TBI Severity in Gray and White Matter

PLS-DA score plots from analysis of the GC/MS chromatograms of gray (A, C, E) and white (B, D, F) brain matter 7 days post-TBI. Severity-dependent trajectories based on impact speed effect (A, B), depth of depression effect (C, D), and total TBI effect (E, F) are illustrated in each panel.

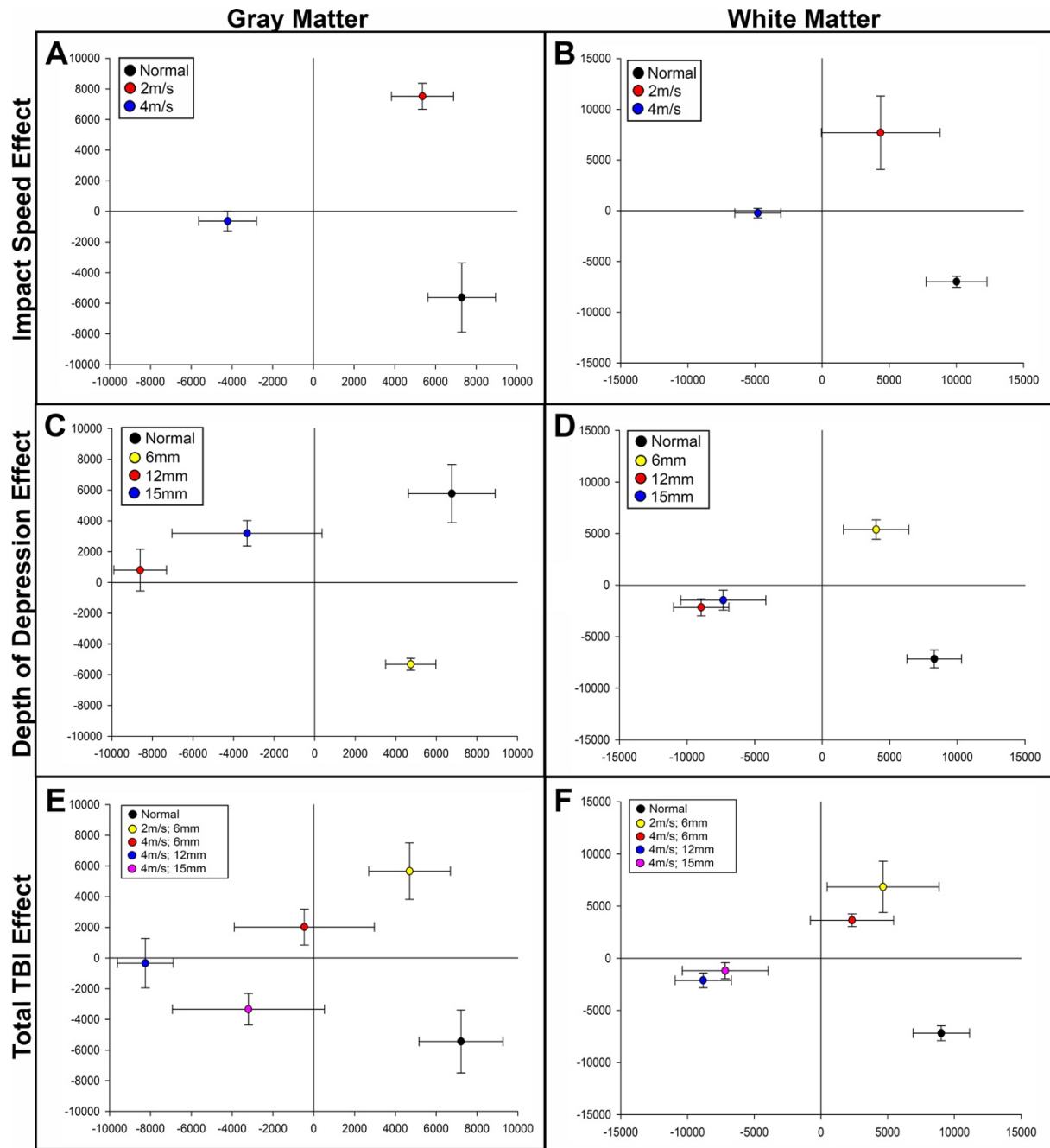


Figure 5.2: White Matter Displays Greater Metabolomic Alterations than Gray Matter after TBI

T-test filtered difference chromatograms ($p \leq 0.05$) illustrating the metabolomic response of gray (A) and white (B) brain matter 7 days post-TBI compared to normal control brain tissue. Peaks above baseline correspond to metabolites that were significantly increased following TBI compared to normal controls, and peaks below baseline correspond to metabolites that were significantly decreased following TBI compared to normal controls (A, B). After peak alignment of each individual animal, the total number of peaks above/below baseline were calculated for each TBI group in gray (C) and white (D) matter.

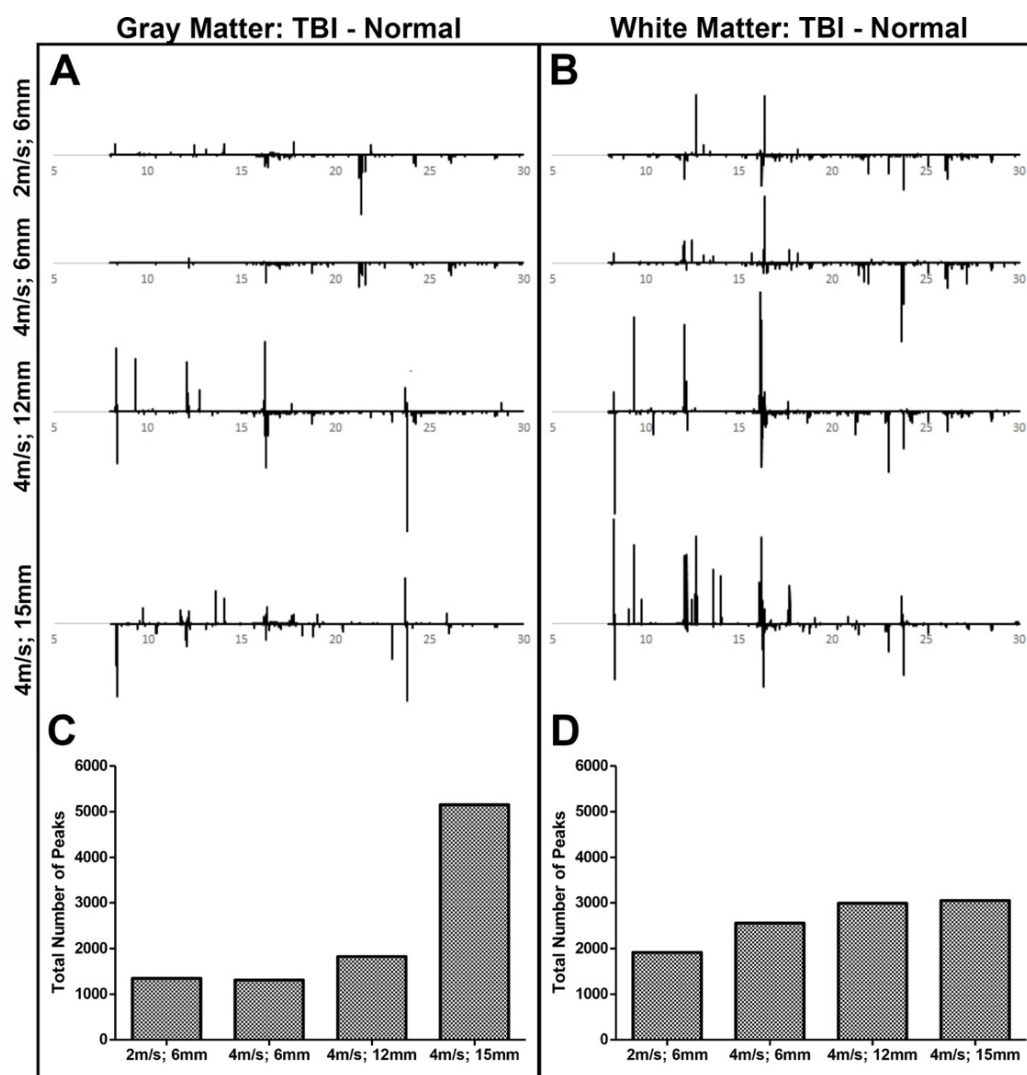


Table 5.1: Metabolites Identified as Significantly Changed after TBI in Gray and White

Brain Matter

Note: ↓ = metabolite decreased after TBI; ↑ = metabolite increased after TBI

Metabolite	GM 2m/s; 6mm	WM 2m/s; 6mm	GM 4m/s; 6mm	WM 4m/s; 6mm	GM 4m/s; 12mm	WM 4m/s; 12mm	GM 4m/s; 15mm	WM 4m/s; 15mm
Acids								
1,2,3-Propanetricarboxylic acid						↓		↓
3-Hydroxydodecanedioic acid			↓		↓		↓	
3,4-Dimethylbenzoic acid		↓		↓		↓		↓
4-Deoxyerythronic acid						↓		↓
Acetic acid	↓	↓	↓	↓	↓	↓	↓	↓
Acetylaspartylglutamic acid								↓
Aminoadipic acid	↓		↓		↓		↓	
Aminobutyric acid		↓	↓	↓	↓	↓		↓
Arachidonic acid	↓		↓		↓	↓	↓	↓
Benzoic acid		↑		↑				
Butyric acid	↓		↓	↓	↓	↓	↑	↓
Formic acid					↓		↓	
Gluconic acid	↓		↓		↓		↓	
Glutaric acid	↑		↑	↓	↓			
Hydroxypyruvic acid	↓	↓	↓	↓	↓	↓	↓	↓
Lactic acid	↓				↓		↓	
Maleic acid		↓		↓				
Malonic acid		↓				↓		
Oxalic acid					↑			
Phosphoric acid		↓		↓	↑	↓	↓	↓

Picolinic acid						↓		↓
Pipecolic acid					↓		↓	
Propionic acid	↓	↓	↓	↓	↓	↑	↓	↑
Pyrrole-2-carboxylic acid		↑		↑	↓	↑	↓	↑
Succinic acid			↓		↓			
Terephthalic acid					↓		↓	
Threonic acid				↓	↓	↓	↓	↓
Valeric acid							↓	
Alcohols								
1-Butanol	↓							
Arabitol				↓	↓	↓	↓	↓
Erythritol			↓		↓	↓		↓
Ethanol	↓		↓		↓	↑	↓	↑
Glycerol		↑		↑	↓	↓	↓	↓
Mannitol	↓		↓		↓		↓	
Myoinositol	↓	↓	↓	↓	↓	↓	↓	↓
Propyl alcohol				↓			↓	↓
Ribitol	↓	↓	↓	↓	↓	↓	↓	↓
Threitol	↓		↑		↑		↓	
Amino Acids and Derivatives								
Acetyllysine		↓		↓				↓
Acetyltyrosine		↓		↓	↓		↓	↓
Alanine	↓		↓	↓	↓	↓	↓	↓
Asparagine	↓		↓		↓		↓	

Aspartic acid	↓	↓	↓	↓	↓	↓	↓	↓
Cystathionine				↓		↓		↓
Cysteine						↓		↓
Glutamic acid	↓	↓	↓	↓	↓	↓	↓	↓
Glutamine	↓	↓	↓	↓	↓			
Glycine	↓		↓		↓		↓	
Homocysteine	↓		↓		↓	↓	↑	↓
Isoleucine		↓	↑	↓	↑	↓	↓	↓
Leucine	↓		↓		↓	↓	↑	↓
Lysine		↓		↓		↓		↓
Methionine						↓		↓
Norvaline	↑	↓		↓	↑	↓		↓
Ornithine		↑		↑				
Phenylalanine		↓		↓		↓		↓
Proline	↓	↓		↓	↓	↓	↓	↓
Pyroglutamic acid					↓	↑	↓	↑
Sarcosine								↓
Serine				↓	↓		↓	↓
Succinylacetone	↓		↓					
Threonine		↓		↓		↓		↓
Tryptophan		↓		↓		↓		↓
Tyrosine		↓	↓	↓	↓	↓	↓	↓
Valine						↓		↓
Fatty Acids								

Linoelaidic acid			↓		↓		↓	
Oleic acid	↓				↑		↓	
Palmitic acid							↓	
Stearic acid			↓		↓		↓	
Sugars								
Acetylglucosamine	↑	↓	↓	↓	↓		↓	↓
Arabinose				↓		↓		↓
Erythrose	↑		↑		↑		↑	
Fructose		↓		↓	↓	↓	↓	↓
Galactose	↓	↓	↓	↓	↓	↓	↑	↓
Glucose	↓	↓	↓	↓	↓	↓	↓	↓
Maltose	↓		↓	↓	↓	↓	↓	↓
Mannobiose	↓				↓		↓	
Mannose		↓		↓		↓		↓
Methylglucoside	↓	↓	↓	↓	↓	↓	↓	↓
Ribose	↓	↑	↓	↑	↓	↓	↓	↓
Sedoheptulose	↓	↓		↓	↓	↓	↓	↓
Sorbose			↑		↑		↑	
Tagatose		↓		↓		↓		↓
Turanose	↓				↓		↓	
Xylose		↓		↓		↓		↓
Other								
1-Propanethiol		↓		↓		↓		↓
1H-Indole-3-acetamide		↓						

2-Hydroxyphenethylamine				↓		↓		↓
Acetaldehyde			↓		↓		↓	
Adenosine	↓	↓		↓	↓	↓	↓	↓
Benzene			↓					
Butanal					↓	↓		↓
Cadaverine				↓	↓	↓	↓	↓
Cholesterol					↓			
Decanal					↑		↑	
Formamide		↓		↓		↓		↓
Guanosine						↓		
Hexane				↑		↑		↑
Homolanthionine		↓		↓	↓	↓	↓	↓
Inosine	↓		↓		↓	↓		
Niacinamide				↓		↓		↓
Oleamide					↓		↓	
Pantothenic acid		↓		↓		↓		
Pectin					↓		↓	
Prolylglycine		↑		↑				
Propanal	↓			↓	↓	↓		↓
Purine						↓		↓
Putrescine		↓		↓	↓	↓	↓	↓
Pyridine	↑		↓		↓	↓	↓	↓
Pyrimidine		↓		↓	↑	↓	↓	↓
Pyruvic acid					↓		↓	

Quinoline	↓		↓		↓		↓	
Ribonolactone	↓					↓	↑	↓
Thiourea	↓				↓		↓	
Triethylamine		↓		↓		↓		↓
Urea		↑		↑		↑		↑
Uridine		↓		↓				

Table 5.2: Altered Metabolic Pathways after TBI in Gray and White Brain Matter

Note: + = significantly altered after TBI based on impact-value threshold set to 0.1

Pathway	GM 2m/s; 6mm	WM 2m/s; 6mm	GM 4m/s; 6mm	WM 4m/s; 6mm	GM 4m/s; 12mm	WM 4m/s; 12mm	GM 4m/s; 15mm	WM 4m/s; 15mm
Citrate cycle (TCA cycle)					+			
Glycolysis or Gluconeogenesis					+		+	
Methane metabolism					+		+	
Glyoxylate and dicarboxylate metabolism					+		+	
Pyruvate metabolism	+		+		+		+	
Cysteine and methionine metabolism				+		+		+
Lysine degradation		+		+		+		+
Pantothenate and CoA biosynthesis		+		+		+		
Phenylalanine metabolism		+		+		+		
Starch and sucrose metabolism				+		+		+
Butanoate metabolism					+	+	+	+
Glycerolipid metabolism		+		+	+	+	+	+
Arachidonic acid metabolism	+		+		+	+	+	+
Aminoacyl-tRNA biosynthesis				+				+
Alanine, aspartate and glutamate metabolism	+	+	+	+	+			
Arginine and proline metabolism	+	+	+	+	+	+	+	+
Galactose metabolism	+	+	+	+	+	+	+	+
Glycine, serine and threonine metabolism	+	+	+	+	+	+	+	+
Inositol phosphate metabolism	+	+	+	+	+	+	+	+

Figure 5.3: PLS-DA Score Plots Displays Distinct Metabolomic Separation in Serum Based on TBI Severity and Day Post-TBI

PLS-DA score plots from analysis of the GC/MS chromatograms of serum collected 24 hours and 7 days post-TBI. Trajectories based on day post-TBI (A), impact speed effect (B), depth of depression effect (C), and total TBI effect (D) are illustrated in each panel.

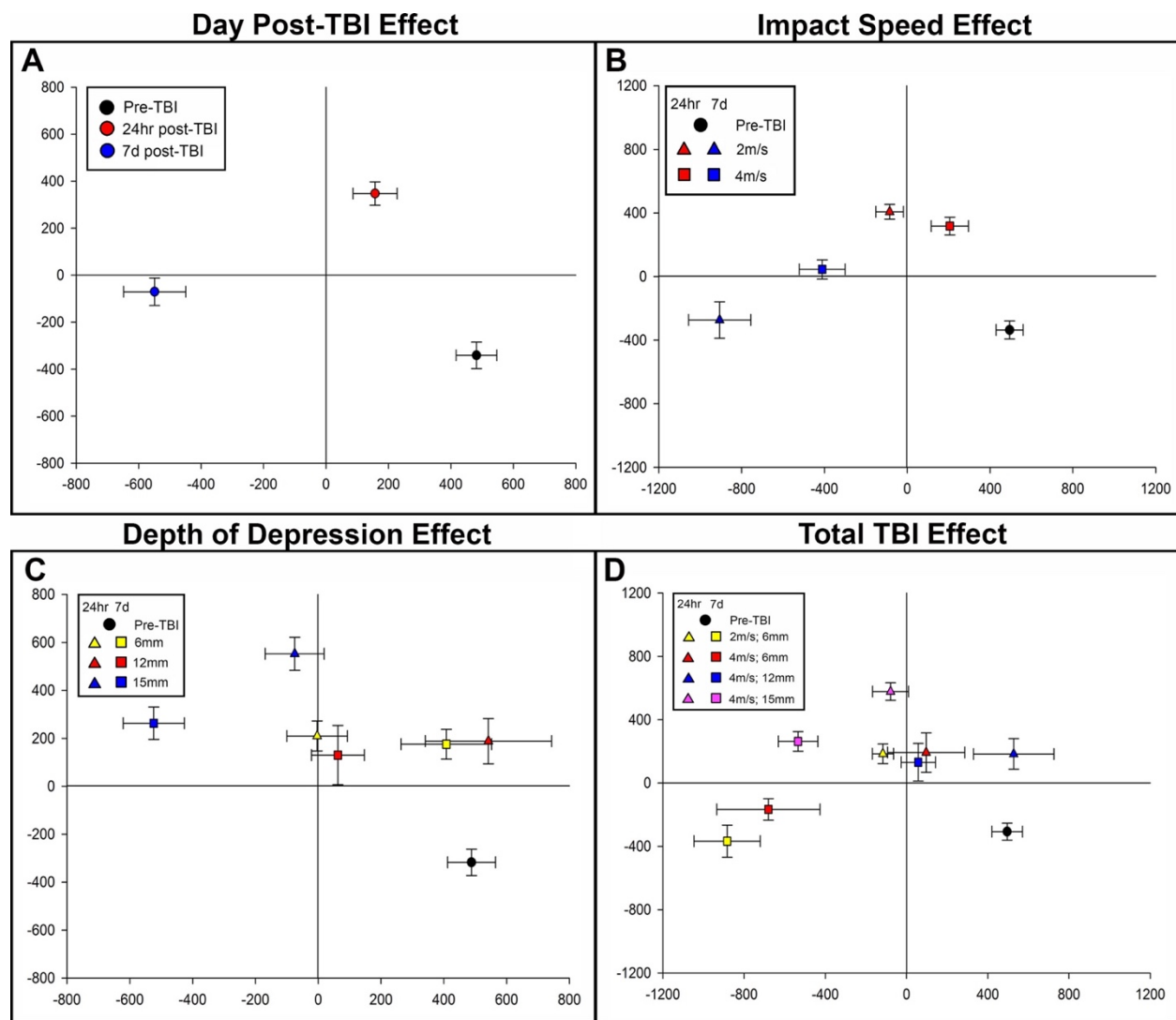


Figure 5.4: Serum Demonstrates Dynamic Changes in Metabolomic Profile Across TBI

Severities and Over Time

T-test filtered difference chromatograms ($p \leq 0.05$) illustrating the metabolomic differences 24 hours post-TBI compared to pre-TBI (A), 7 days post-TBI compared to pre-TBI (B), and 7 days post-TBI compared to 24 hours post-TBI (C). For A and B, peaks above baseline correspond to metabolites that were significantly increased following TBI compared to pre-TBI, and peaks below baseline correspond to metabolites that were significantly decreased following TBI compared to pre-TBI. For C, peaks above baseline correspond to metabolites that were significantly increased 7 days post-TBI compared to 24 hours post-TBI, and peaks below baseline correspond to metabolites that were significantly decreased 7 days post-TBI compared to 24 hours post-TBI (A-C). After peak alignment of each individual animal, the total number of peaks above/below baseline were calculated for each TBI group within the 24 hours post-TBI vs. pre-TBI (D), 7 days post-TBI vs. pre-TBI (E), and 7 days post-TBI vs. 24 hours post-TBI (F) comparisons.

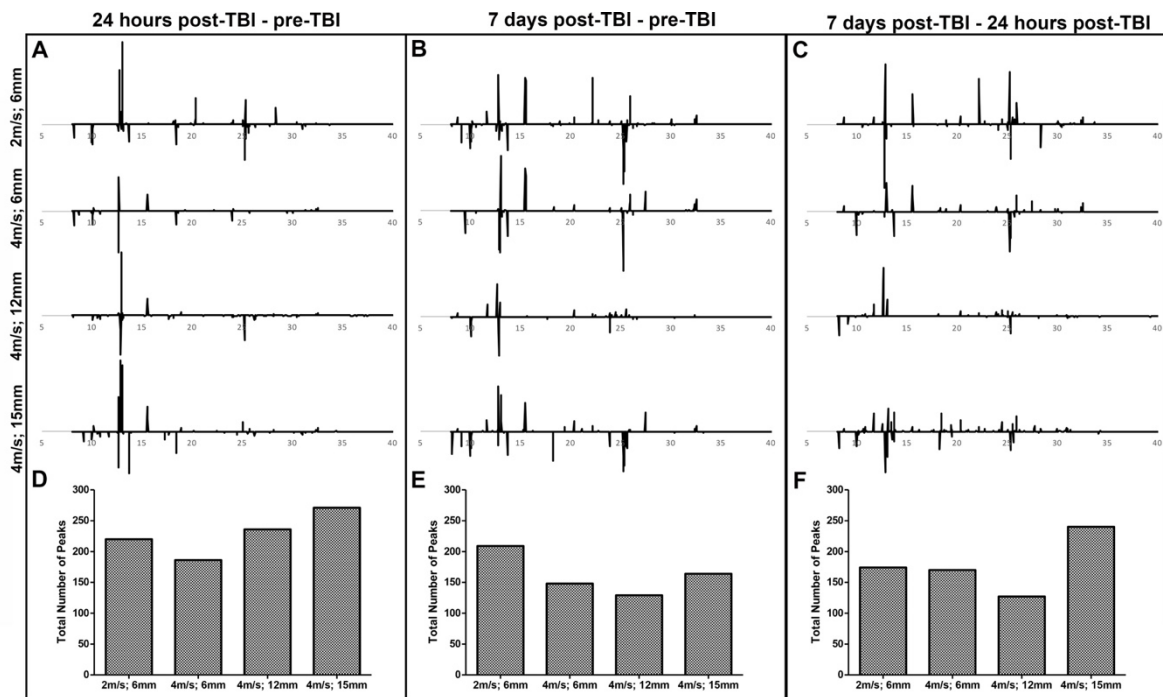


Table 5.3: Metabolites Identified as Significantly Changed in Serum 24 Hours and 7 Days

Post-TBI

Note: ↓ = metabolite decreased after TBI; ↑ = metabolite increased after TBI

Metabolite	2m/s; 6mm 24 hours	2m/s; 6mm 7 days	4m/s; 6mm 24 hours	4m/s; 6mm 7 days	4m/s; 12mm 24 hours	4m/s; 12mm 7 days	4m/s; 15mm 24 hours	4m/s; 15mm 7 days
Acids								
2,4-Dihydroxybutanoic acid		↑	↑	↑	↑		↑	
Aminooctanoic acid			↑				↑	
Citric acid						↓		
Homovanillic acid					↓		↓	
Lactic acid	↑		↑					
Malic acid	↑			↑			↑	↑
Malonic acid	↓	↓	↓		↓	↓		↓
p-Hydroxymandelic acid			↓	↓			↑	
Phosphoric acid	↓		↓		↓		↓	
Succinic acid					↓		↓	↓
Valeric acid		↑	↑	↑	↑		↑	↑
Alcohols								
1,3-Butanediol	↓		↓		↓		↓	
Galactitol		↑	↑		↓	↑		↑
Myoinositol				↑				↑
Amino Acids and Derivatives								
Alanine		↓						↓
Asparagine					↑		↑	
Aspartic acid	↓	↑	↑	↑		↑		↑

Cysteine		↓				↑		↑
Glutamine	↑				↑			
Glycine	↓	↓		↓			↓	↓
Isoleucine	↑	↓		↓			↑	↓
Leucine	↑	↓		↓	↑			↓
Lysine		↓		↓	↓		↓	
Ornithine		↑	↓	↑	↓	↑		
Phenylalanine		↑		↑				↑
Proline	↓		↓		↓		↓	
Threonine	↑				↓			
Tyrosine		↑		↑				
Valine		↑						↑
Creatinine			↑		↑		↑	↑
Ketoleucine					↓		↓	
N-Acetyl-L-aspartic acid			↓	↓	↓	↓		↓
Pyroglutamic acid				↑				
Fatty acids								
4-Hydroxybutyric acid			↑				↑	
Palmitic acid	↓						↓	
Sugars								
D-arabino-Hexulose 6-phosphate	↓		↑		↓		↓	
Deoxyribose	↑		↑					
Fructose	↓	↓			↓	↓	↓	↓

Galactose	↓						↓	
Glucose	↓	↓		↓	↓	↓		↓
Ribose	↑				↓			
Other								
2-Phenylacetamide	↓						↓	
Cytosine					↓		↓	
Guanine					↓		↓	
Octanoylcarnitine			↓				↓	
Phosphate	↑	↓		↓			↑	
Pyruvic acid		↓				↓		↓
Uracil			↓				↓	
Urea	↑	↓	↑	↓	↑	↓	↑	

Table 5.4: Altered Metabolic Pathways After TBI in Serum 24 Hours and 7 Days Post-TBI

Note: + = significantly altered after TBI based on impact-value threshold set to 0.1

Pathway	2m/s; 6mm 24 hours	2m/s; 6mm 7 days	4m/s; 6mm 24 hours	4m/s; 6mm 7 days	4m/s; 12mm 24 hours	4m/s; 12mm 7 days	4m/s; 15mm 24 hours	4m/s; 15mm 7 days
Glycerophospholipid metabolism	+		+		+		+	
Cysteine and methionine metabolism		+				+		+
Phenylalanine metabolism		+		+				+
Aminoacyl-tRNA biosynthesis		+		+				
Pyruvate metabolism	+	+	+			+		+
Glycine, serine, and threonine metabolism	+	+		+			+	+
Pyrimidine metabolism			+					
Galactose metabolism	+						+	
Lysine degradation		+		+	+		+	
Citrate cycle (TCA cycle)						+		+
Inositol phosphate metabolism								+
Butanoate metabolism								+
Alanine, aspartate, and glutamate metabolism	+	+	+	+	+	+		+
Arginine and proline metabolism	+	+	+	+	+	+	+	

CHAPTER 6

LONGITUDINAL MAGNETIC RESONANCE IMAGING AND HISTOLOGICAL ASSESSMENT OF TRAUMATIC BRAIN INJURY PATHOLOGY IN A PIGLET MODEL ¹

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Abstract

Approximately 1.7 million people sustain a traumatic brain injury (TBI) in the United States each year. Despite the socioeconomic burden and hundreds of clinical trials, there remains to be no treatment to restore neurological function after TBI. Experts have repeatedly emphasized that the lack of translatability of preclinical models to human patients remains to be a major challenge facing TBI therapeutic development. Compared to the widely-used rodent model, pigs may be an ideal large animal model of TBI as they share key neuroanatomical similarities with humans. The objective of this study was to longitudinally assess TBI pathophysiology using multiparametric magnetic resonance imaging (MRI) and histological approaches in a piglet model. TBI was produced by controlled cortical impact (CCI) in Landrace piglets. MRI was performed 24 hours and 12 weeks post-TBI, and a subset of animals were sacrificed 24 hours, 1 week, 4 weeks, and 12 weeks post-TBI for histological analysis. TBI resulted in temporal changes in lesion size, white matter integrity, cerebral blood flow, and metabolism measured through T2-weighted imaging, diffusion weighted imaging (DWI), diffusion tensor imaging (DTI), arterial spin labeling (ASL), and proton magnetic resonance spectroscopy (^1H MRS), respectively, which correlated with histological evidence of neuronal loss, astrogliosis, microglia activation, and oligodendrocyte pathology. Taken together, this study illustrates the distinct time course of TBI pathoanatomic features at the cellular and tissue level up to 12 weeks post-TBI in a translational piglet TBI model, which can be used to study TBI injury and recovery mechanisms for application to the clinical setting.

Introduction

In the United States, of the approximately 1.7 million people who sustain a traumatic brain injury (TBI) each year, almost one third occur among children aged 0 to 14 [1]. The initial TBI insult causes immediate physical damage to neurons, glial cells, white matter tracts, and the blood brain barrier (BBB) [2]. A complex secondary injury sequela rapidly ensues post-primary injury characterized by the release of pro-inflammatory cytokines, chemokines, and free-radicals leading to vasogenic and cytotoxic edema, and ultimately, additional cell death and tissue necrosis [3]. Despite a vast number of promising neuroprotective therapies developed in rodent TBI models, there are currently no Food and Drug Administration (FDA)-approved treatments for TBI [4]. The heterogeneous and complex nature of TBI coupled with the use of small animal models may be a major contributor of the current translational gap from pre-clinical to clinical outcomes [5]. Improved characterization of TBI sequela in a more translational large animal model may yield promising insight in potential therapeutic targets and may provide a platform with which to test the efficacy of potential therapeutics.

Juvenile pigs may be an ideal large animal model of TBI as they share key similarities to human adolescents. Piglets exhibit a similar pattern of brain development and growth [6-8]. As a result, piglets have been used as a model to study neurodevelopmental outcomes influenced by pediatric nutrition as well as models of cognition and brain disorders [9-11]. Furthermore, the piglet brain follows a similar pattern of neurogenesis and migration of neuroblasts from the subventricular zone as human infants [12, 13], a similar time course of brain myelination [14], and possesses a comparable innate immunity [15]. These critical developmental parallels make piglets the ideal model to study the effects of TBI on neurodevelopment and the potential of novel therapeutic interventions [16].

Magnetic resonance imaging (MRI) has emerged as a powerful clinical and preclinical tool with high sensitivity and specificity for detection of complex pathoanatomic and pathophysiologic changes [17]. The use of noninvasive MRI modalities is highly advantageous as it allows for longitudinal assessment of TBI progression [17]. MRI is capable of providing highly detailed information about the spatiotemporal progression of structural changes resulting from TBI, such as lesion size, tissue volumetrics, and midline shift [18-20]. A major hallmark of focal TBI in the acute and sub-acute stages is the accumulation of cerebral edema. MRI is sensitive to microstructural changes and can thus identify predominate vasogenic or cytotoxic edema, which may indicate BBB compromise or ischemia, respectively [21, 22]. Cerebral edema accumulation can induce a series of secondary injury cascades, including decreased cerebral blood flow and neuronal dysfunction. MRI can create quantitative blood flow maps to track changes in cerebral blood flow [23], and localized *in vivo* proton magnetic resonance spectroscopy (^1H MRS) provides highly specific information regarding dynamic changes in cell metabolism by measuring small fluctuations in neurometabolite concentrations, including N-acetylaspartate (NAA), choline (Cho), creatine (Cr), myo-inositol (Ins), glutamate-glutamine (Glx), and lactate (Lac) after TBI [24, 25]. Furthermore, TBI primary or secondary injury mechanisms can contribute to widespread white matter damage. MRI provides an excellent measure of axonal damage and reflects changes in white matter integrity over time [26]. Experimental animal models of TBI have demonstrated the utility of correlating changes in MR signal and histological outcome [27-29]. However, there are few MRI studies with complementary histological changes that track the time course of TBI pathophysiology in a piglet TBI model [30]. Therefore, the combined use of multiparametric MRI and histopathological assessment in a piglet TBI model to study key TBI pathoanatomic and

pathophysiologic changes may lead to improved clinical translatability and may augment the predictive potential of novel therapeutic interventions.

The purpose of this study was to longitudinally assess the pathophysiology of TBI using a multiparametric quantitative MRI and histology approach in a piglet TBI model. Here we show evidence of spatio-temporal changes in lesion size, diffusion, white matter integrity, cerebral blood flow, and metabolism after TBI using T2-weighted, DWI, DTI, ASL, and ^1H MRS imaging, respectively. Complementary histological results show evidence of neuronal loss, increases in astrogliosis and microglia activity, and transient changes in oligodendrocytes. These results demonstrate the utility of measuring TBI sequelae in a more human-like piglet TBI model as a means to identify potential treatment targets and to test the efficacy of therapeutic interventions.

Materials and Methods

Animals

All work in this study was performed in accordance with the University of Georgia Institutional Animal Care and Use Committee guidelines. Eighteen commercially bred, castrated male pigs were born at the Large Animal Research Unit at the University of Georgia. Piglets were weaned at 3 weeks of age, group housed, and fed a nutritionally complete starter diet ad libitum. After 4 weeks, pigs were fed a nutritionally complete grower diet ad libitum. All animals underwent surgical procedures between 4-5 weeks of age. Room temperature was maintained at 26°C with a 12h light/dark cycle and an overhead heat lamp provided supplemental heat until 4 weeks of age. Feed was restricted the night prior to surgery but all animals had free access to water at all times.

Surgical Preparation and Controlled Cortical Impact

Piglets were anesthetized with 5% vaporized isoflurane mixed with oxygen with a surgical mask and maintained between 2.5-3% isoflurane through all surgical procedures. Temperature, heart rate, and respiration rate were monitored every 5-10 minutes for the duration of surgery. The surgical site was prepared in a routine manner using Betadine and 70% ethanol and covered with a sterile drape. A left-sided incision approximately 4cm in length was made at the top of the cranium to expose the underlying skull. A craniectomy was performed using an air drill (Brassler, USA) at the left anterior junction of the coronal and sagittal sutures, approximately 20mm in diameter, to expose the underlying dura.

The piglet was secured in a controlled cortical impactor (CCI) device (University of Georgia Instrument Design and Fabrication Shop; Athens, GA), previously described [31]. A 15mm impactor tip was positioned over the intact dura and a TBI was induced with the following parameters: 4m/s velocity, 9mm depth of depression, 400ms dwell time. After injury, the surgical site was flushed with sterile saline and the skin was re-apposed with surgical suture. Banamine (2.2mg/kg) and Butorphanol (0.2mg/kg) were administered for analgesia and Oxytetracycline (19.8mg/kg) was administered as an antibiotic for 5 days. Anesthesia was discontinued and piglets were allowed to breath oxygen until recovered. Once ambulatory, piglets were placed back in their home pens and monitored daily for health or signs of abnormal neurological behaviors.

For this study, we aimed to assess the time-course of cellular and tissue changes after TBI. Therefore, piglets were separated into 4 groups based on sacrifice date: 1 day (n=4), 1 week (n=4), 4 weeks (n=4), and 12 weeks (n=6) post-TBI.

Magnetic Resonance Imaging

Magnetic resonance imaging (MRI) was performed 24 hours and 12 weeks post-TBI on a Siemens TIM Trio 3.0 Tesla whole-body MR scanner using a 12-channel phased array head coil. Under the same anesthesia protocol used for CCI, MRI of the cranium was performed with the animal positioned in dorsal recumbency. Standard multiplanar MR brain imaging series at the coronal, horizontal, and sagittal planes were acquired including T2-weighted, T2-weighted fluid attenuated inversion recovery (FLAIR), diffusion-weighted imaging (DWI), diffusion tensor imaging (DTI), arterial spin labeling (ASL), and magnetic resonance spectroscopy (^1H MRS).

T2-weighted images were analyzed using OsiriX software (Pixmeo, Bernex, Switzerland). For volumetric analysis, T2-weighted images were utilized to measure the area of the lesion, ipsilateral (injured) hemisphere, and contralateral (uninjured) hemisphere at the levels containing abnormal tissue. Area and slice thickness were combined to obtain total lesion and hemisphere volumes. T2-weighted images were also used to measure midline shift. Midline shift was defined as the maximal horizontal displacement of the septum pellucidum in relation to the midline [32, 33]. The distance from the septum pellucidum to the outer border of the cortex was measured for each hemisphere, and midline shift was calculated using the formula “midline shift = (total diameter/2) – contralateral diameter.”

Apparent diffusion coefficient (ADC) maps were obtained for each slice from the DW images. Mean ADC values were calculated with NIH ImageJ software at the lesion site by manually outlining the region of interest (ROI) based on corresponding T2-weighted images for each slice with apparent lesion. Corresponding ROIs were drawn in a symmetrical site in the contralateral hemisphere to serve as an internal control. Mean ADC was obtained by calculating the average signal intensity across all slices. All ADC values are reported as $10^{-3} \text{ mm}^2/\text{s}$.

Fractional anisotropy (FA) maps and color coded FA maps were generated from DT images. ROIs were drawn at the internal capsule and corpus callosum on one representative slice per animal using NIH ImageJ software at the ipsilateral and contralateral side of the injury. FA values are expressed as percent decrease of signal intensity compared to the contralateral side for each animal.

For ASL, two dimensional images were obtained from each brain slice, and cerebral blood flow (CBF) was calculated with NIH ImageJ software at the lesion site by manually outlining an ROI based on T2-weighted images. Corresponding ROIs were drawn in a symmetrical site in the contralateral hemisphere to serve as an internal control. All values were expressed as a percentage decrease of signal intensity compared to the contralateral hemisphere for each animal.

For ^1H MRS, a point-resolved spectroscopy (PRESS) sequence was used to acquire single-voxel spectra in the ipsilateral and contralateral hemispheres with the following parameters (repetition time (TR)=1800 ms; echo time (TE)=136 ms; averages=160; complex data points = 1024; bandwidth=1200 Hz; voxel size= 12x12x12 mm³). ^1H MRS data were processed with LCModel (version 6.3-1L) [34]. Spectra were analyzed from 4 to 0.2 ppm, and metabolite concentrations were determined in the frequency domain with an 18 metabolite gamma-simulated basis set. Due to variations in total creatine concentration between hemispheres and over time, metabolite concentrations were compared qualitatively within scan session to control for inter-scan session variations. A number of limitations including small cerebral volume, increased lipid layer near the skull, and substantial hemorrhage at the lesion site prevented quantitative comparisons between ipsilateral and contralateral hemispheres and between time points. However, a case study was performed using a single animal at 24 hours

post-TBI to demonstrate that changes in neurometabolites can be measured in a piglet TBI model using ^1H MRS.

Histology

Pigs were euthanized 24 hours, 1 week, 4 weeks, and 12 weeks post-TBI. After euthanasia, the brains were removed, coronally sectioned, and fixed in 10% buffered formalin for immunohistochemical analysis. After fixation, a section of the ipsilateral hemisphere from each animal at the level of the lesion site was routinely processed, embedded in paraffin, and stained with antibodies specific to NeuN (Millipore, 1:500), GFAP (Biogenex, 1:4000), Olig2 (Genetex, 1:400), and Iba1 (Wako, 1:8000). Heat induced antigen retrieval was performed for all antibodies using citrate buffer at pH6 (DAKO). Detection was performed utilizing biotinylated antibodies and a streptavidin label (4plus Streptavidin HRP, Biocare) followed by an HRP label and DAB chromagen (DAKO). All sections were lightly counterstained with hematoxylin. Four healthy animals of a similar age that received no CCI surgery were processed as described above to serve as a baseline for quantification of cellular changes compared to normal control animals.

Microscopic inspection and imaging was performed on a light microscope utilizing SPOT Imaging v5.2 software (Sterling Heights, MI). For quantification, an ROI in the perilesional area was drawn including the dorsal and dorsolateral gyri (cingulate, marginal, suprasylvian and sylvian and/or ectosylvian), and five fields were imaged per animal. For NeuN and Olig2, positive and negative cells were manually counted using the cell counter plugin on ImageJ 2.0 and the percentage of NeuN+ and Olig2+ cells was determined [35]. For GFAP and Iba1, the total area of immunoreactivity corresponding to increased optical density was determined by

ImageJ 2.0 software. All histological data was expressed as a percentage of normal control animals.

Statistics

Quantitative data was analyzed by two-way analysis of variance (ANOVA) and post-hoc Tukey's Pair-Wise comparisons to determine statistical significance between groups using SAS version 9.4 (Cary, NC). Groups where p-values were ≤ 0.05 were considered to be significantly different.

Results

CCI results in cerebral lesioning, significant parenchymal swelling followed by atrophy

First, we assessed cerebral lesioning and changes in hemisphere volume at 24 hours and 12 weeks post-TBI. At 24 hours post-TBI, distinct parenchymal lesioning comprised of both hyper- and hypointense regions was present on T2-weighted images (**Figure 6.1A**). At 12 weeks post-TBI, the lesion site was hyperintense on T2-weighted images, and the ipsilateral hemisphere had undergone parenchymal tissue atrophy resulting in an enlarged and distorted lateral ventricle (**Figure 6.1B**). There was no significant change in lesion size between 24 hours post-TBI and 12 weeks post-TBI ($3.44 \pm 0.52 \text{ cm}^3$ and $1.95 \pm 0.44 \text{ cm}^3$, respectively) (**Figure 6.1C**). Since the pigs increase approximately 7-fold in size over the course of the study, the percentage of the total volume of tissue that contained lesion was calculated to account for changes in brain size. The percentage of tissue volume comprised of lesion was significantly ($p < 0.05$) decreased 12 weeks post-TBI compared to 24 hours post-TBI ($9.40\% \pm 2.21\%$ vs. $18.58\% \pm 1.83\%$, respectively) (**Figure 6.1D**). In addition, we assessed the volumetric ratio of the ipsilateral hemisphere (left

hemisphere, LH) and the contralateral hemisphere (right hemisphere, RH) where a normal brain with equal volume between hemispheres would exhibit a LH:RH ratio of 1.0. At 24 hours post-TBI, the LH:RH ratio was significantly ($p<0.001$) increased compared to normal which indicates substantial parenchymal swelling in the ipsilateral hemisphere. At 12 weeks post-TBI, the LH:RH ratio was significantly ($p<0.0001$) decreased compared to normal indicating that the ipsilateral hemisphere has undergone parenchymal atrophy. Furthermore, there was a significant ($p<0.0001$) decrease in LH:RH ratio at 12 weeks post-TBI compared to 24 hours post-TBI (0.90 ± 0.01 vs. 1.17 ± 0.03 , respectively) (**Figure 6.1E**). Together, this data illustrates a correlative decrease in both lesion size and ipsilateral hemisphere volume by 12 weeks post-TBI.

CCI results in cerebral midline shift indicative of brain swelling and atrophy

In human patients, the magnitude of midline shift is correlated with poor outcome after TBI [36]. The degree of midline shift was calculated at the level of the septum pellucidum at 24 hours and 12 weeks post-TBI. At 24 hours post-TBI, the midline was shifted 1.22 ± 0.68 mm toward the contralateral hemisphere which is indicative of parenchymal tissue swelling in the ipsilateral hemisphere (**Figure 6.2A, C**). Conversely, midline was significantly ($p<0.0001$) shifted 2.98 ± 0.29 mm toward the ipsilateral hemisphere at 12 weeks post-TBI (**Figure 6.2B, C**). Furthermore, there was a significant ($p<0.0001$) change in midline shift between 24 hours and 12 weeks post-TBI (1.22 ± 0.68 mm and -2.98 ± 0.29 mm, respectively) (**Figure 6.2C**). Taken together, this data demonstrates that changes in midline shift supports the observed hemisphere volumetric changes and further corroborates parenchymal swelling at 24 hours post-TBI followed by substantial tissue atrophy by 12 weeks post-TBI.

TBI leads to vasogenic and cytotoxic edema at the lesion site and disruption of white matter integrity

ADC maps were generated from diffusion weighted (DW) images to measure diffusivity changes at the lesion site and to distinguish between vasogenic and cytotoxic edema accumulation. A representative T2-weighted image demonstrates the presence of a focal lesion (**Figure 6.3A**) and a corresponding ADC map shows heterogeneous contrast of hyperintense vasogenic edema and hypointense cytotoxic edema accumulation at the lesion site (**Figure 6.3B**) at 24 hours post-TBI. Despite the heterogeneous nature of TBI, mean ADC at the lesion site was significantly ($p < 0.001$) increased 24 hours post-TBI compared to the contralateral side ($0.968 \pm .179$ vs. 0.862 ± 0.113 , respectively) (**Figure 6.3I**). At 12 weeks post-TBI a representative T2-weighted image shows a substantial reduction in lesion size (**Figure 6.3C**). However, a corresponding ADC map shows a focal region with a significant ($p < 0.0001$) increase in ADC hyperintensity compared to the contralateral side as well as compared to the ADC of the ipsilateral side 24 hours post-TBI ($1.563 \pm .613$ vs. $0.951.38 \pm 0.008$ and 0.862 ± 0.113 , respectively) (**Figure 6.3D, I**), likely as a result of gliosis or cyst formation [27, 37]. These results indicate that both vasogenic and cytotoxic edema occur together in traumatized tissue, but mean ADC reflects that vasogenic edema is most prominent 24 hours post-TBI. However, late ADC increases at 12 weeks post-TBI can be attributed to gliosis and/or cystic formation as a result of tissue necrosis.

Diffusion tensor imaging (DTI) was performed to assess changes in white matter integrity after TBI. Representative fractional anisotropy (FA) maps demonstrate disruption of white matter tracts 24 hours (**Figure 6.3E**) and 12 weeks post-TBI (**Figure 6.3G**). Representative color coded FA maps show directional changes of white matter fibers 24 hours

(**Figure 6.3F**) and 12 weeks post-TBI (**Figure 6.3H**). FA values at the ipsilateral internal capsule were significantly ($p < 0.01$) decreased compared to the contralateral side at 24 hours and 12 weeks post-TBI ($-15.19 \pm 3.48\%$ and $-15.74 \pm 0.881\%$, respectively) (**Figure 6.3J**). Similarly, FA values at the ipsilateral corpus callosum were significantly ($p < 0.05$) decreased compared to the contralateral side at 24 hours and 12 weeks post-TBI ($-10.51 \pm 2.12\%$ and $-11.056 \pm 4.149\%$, respectively; **Figure 6.3J**). These results suggest that TBI can lead to rapid loss of white matter integrity in the internal capsule and corpus callosum at 24 hours post-TBI that is sustained for up to 12 weeks post-TBI.

Cerebral blood flow is decreased at the lesion site post-CCI

Arterial spin labeling (ASL) was performed to assess whether CCI affected cerebral blood flow (CBF) at the lesion site. CBF was calculated at both the lesion site and a comparable area on the contralateral hemisphere. CBF at the lesion site was expressed as a percent change compared to CBF on the contralateral hemisphere with changes closer to 0% being more similar to normal tissue. There was a significant ($p < 0.005$) decrease in CBF at both 24 hours and 12 weeks post-TBI at the lesion site compared to the contralateral hemisphere ($-54.17\% \pm 7.12\%$ and $-30.73\% \pm 6.38\%$, respectively) (**Figure 6.4A-C**). However, there was a significant ($p < 0.05$) recovery in CBF by 12 weeks post-TBI compared to 24 hours post-TBI ($-30.73\% \pm 6.38\%$ vs. $-54.17\% \pm 7.12\%$; **Figure 6.4C**). This data indicates that although deficits in CBF are sustained through 12 weeks post-TBI compared to normal tissue, initial impairments in CBF at 24 hours post-TBI are improved by 12 weeks.

TBI leads to changes in neurometabolite concentration 24 hours post-TBI

Magnetic resonance spectroscopy (^1H MRS) has the ability to identify changes in brain metabolites enabling characterization of injury and recovery. However, it has yet to be attempted in a pig neural injury model. As this is a novel assessment tool in pigs, we attempted a ^1H MRS case study in a single pig at 24 hours post-TBI. Sample ^1H MRS spectra were acquired from ipsilateral and contralateral voxels in a single subject. Changes in the healthy neuron marker NAA, the cellularity marker Cho, the energy marker Cr, the glial cell marker Ins, the amino acid marker Glx and the inflammatory marker Lac were assessed 24 hours post-TBI. Relative concentrations of NAA, Cho, Cr, Ins, and Glx were within a normal range in the contralateral hemisphere 24 hours post-TBI (**Figure 6.5A**). However, decreases in NAA, Cho, Cr, Ins, and Glx as well as an increase in Lac were observed in the ipsilateral hemisphere (**Figure 6.5B**). These results suggest that in a single animal, ^1H MRS can detect a number of neurometabolic changes that accompany TBI after 24 hours.

Histological analysis reveals neuronal loss, gliosis, and neuroinflammation correlative to observed tissue-level deficits

Parenchymal tissues underwent histological assessment to characterize cellular changes in the perilesional cortex after TBI including neuronal loss, astrogliosis, oligodendrocyte loss/proliferation, and microglial activation. To this end, we quantified NeuN+ neurons, GFAP+ astrocytes, Olig2+ oligodendrocytes, and Iba1+ microglia at 24 hours, 1 week, 4 weeks, and 12 weeks post-TBI and data was expressed as a percentage compared to normal where values closer to 100% being more similar to normal control animals. There was no difference in the amount of neurons in the perilesional cortex 24 hours post-TBI compared to normal control animals.

However, there was a significant ($p<0.01$) decrease in neurons at 1 week post-TBI compared to 24 hours post-TBI ($72.49\% \pm 4.08\%$ vs. $95.47\% \pm 2.47\%$, respectively) and normal control animals. Significant ($p<0.005$) neuronal loss was sustained through 4 weeks and 12 weeks post-TBI ($64.26\% \pm 8.42\%$ and $62.31\% \pm 0.48\%$, respectively) (**Figure 6.6A-D**). Conversely, we found a significant ($p<0.0005$) increase in astrocytes from 24 hours to 12 weeks post-TBI compared to normal control animals. Furthermore, there was a significant ($p<0.005$) increase in astrocyte reactivity at 1 week post-TBI compared to 24 hours post-TBI ($204.87\% \pm 0.58\%$ vs. $123.79\% \pm 3.68\%$, respectively), 4 weeks post-TBI compared to 1 week post-TBI ($255.09\% \pm 9.79\%$ vs. $204.87\% \pm 0.58\%$, respectively), and 12 weeks post-TBI compared to 4 weeks post-TBI ($966.75\% \pm 7.98\%$ vs. $255.09\% \pm 9.79\%$, respectively; **Figure 6.6E-H**). This demonstrates progressive increases in astrogliosis beginning 24 hours post-TBI through 12 weeks post-TBI. There was no difference in oligodendrocyte number at 24 hours post-TBI compared to normal control animals. However, there was a significant ($p<0.05$) decline in the number of oligodendrocytes at 1 week and 4 weeks post-TBI compared to normal control animals ($67.62\% \pm 11.32\%$ and $66.51\% \pm 9.13\%$, respectively). In contrast, there was a significant ($p<0.005$) increase in the number of oligodendrocytes in the perilesional cortex at 12 weeks post-TBI compared to both 4 weeks post-TBI ($112.89\% \pm 1.94\%$ vs. $66.51\% \pm 9.13\%$, respectively) and normal control animals, which indicates that oligodendrocyte proliferation occurs between 4 weeks and 12 weeks post-TBI (**Figure 6.6I-L**). Initially there was a significant ($p<0.05$) decline in the number of microglia in the perilesional cortex 24 hours post-TBI compared to normal control animals. However, there was a significant ($p<0.05$) increase in microglia number at 1 week and 4 weeks post-TBI compared to normal control animals ($130.74\% \pm 13.11\%$ and $153.64\% \pm 13.98\%$, respectively). Furthermore, there was a significant ($p<0.0001$) increase in

microglia abundance at 12 weeks post-TBI compared to both 4 weeks post-TBI ($861.46\% \pm 15.45\%$ vs. $153.64 \pm 13.98\%$, respectively) and normal control animals, demonstrating sustained microglia activation beginning 1 week post-TBI through 12 weeks post-TBI (**Figure 6.6M-P**).

Discussion

Despite the prevalence of TBI and numerous clinical trials, there are few therapeutic options available to combat the tissue loss that leads to devastating functional deficits [4]. This may be due in part to the lack of translational animal models to optimize treatment strategies for clinical application. Here we combine translational multiparametric MRI and ^1H MRS approaches with histology to characterize TBI pathology in a piglet model. At 24 hours post-TBI, substantial parenchymal swelling due to both vasogenic and cytotoxic edema accumulation lead to increased ipsilateral hemisphere volume and midline shift with evidence of disturbed CBF and brain metabolism. Despite the edema heterogeneity present at 24 hours post-TBI, increased mean ADC suggests that vasogenic edema is more prominent over cytotoxic edema in our model. At 12 weeks post-TBI, we observed parenchymal atrophy correlating with a directional change in midline shift, increased mean ADC likely due to cystic formation and/or gliosis, and improved albeit abnormal CBF compared to baseline. Furthermore, TBI lead to a sustained disruption in white matter integrity. These tissue-level deficits measured through MRI correlated to progressive neuronal loss, neuroinflammation, gliosis, and oligodendrocyte pathology. Taken together, the injury indicators outlined here could be applied to the clinical setting as a baseline for treatment efficacy indicators in the future, augmenting the translational capacity of current preclinical models as well as the probability of a successful clinical trial.

Human clinical studies have consistently demonstrated that midline shift is a strong indicator of abnormal intracranial pressure, and shift magnitude is associated with poor patient outcomes [36, 38]. T2-weighted images revealed parenchymal swelling that shifted the cerebral midline approximately 1.2 mm at 24 hours post-TBI which, when the difference in brain size is considered, equates to approximately a 4.5 mm shift in the human brain. In both the Marshall and Rotterdam CT classifications, a midline shift of 5 mm is considered the major turning point of increased mortality rate after TBI [39]. However, clinical studies of TBI have concluded that there is no distinct cut-off value for the degree of midline shift in relation to clinical outcome, and the authors recommended that midline shift should rather be viewed as a continuous variable for outcome prediction [32, 40]. Jacobs et al. found that patients with favorable outcomes had an average midline shift of 4.9 mm, which correlates with our study in which pigs demonstrated 0% mortality and 100% favorable outcome long term [32].

We demonstrate that TBI in our piglet model leads to early disruptions in white matter integrity that is sustained for 12 weeks post-TBI. White matter integrity was quantified through fractional anisotropy (FA) which measures the degree of anisotropic diffusion, or the directionality of water molecules in specific regions of the cerebrum [41, 42]. Areas with a high anisotropic diffusion like the corpus callosum or internal capsule are characterized by high FA values and appear bright on FA maps whereas areas with low anisotropic diffusion like the CSF or gray matter are characterized by low FA values and are dark on FA maps [43]. White matter is comprised of myelinated and unmyelinated axon bundles which foster communication between neurons in different gray matter compartments, and white matter disruption has been shown to lead to motor dysfunction and cognitive impairment [44]. Preclinical animal models have documented white matter disruption after TBI through DTI and/or histological measures;

however, the majority of these studies are carried out in the rodent which only has about 14% of the relative white matter volume of humans thus may not be directly translatable to white matter injury in the human [28, 29, 44-48]. Clinical studies have documented decreased FA in the corpus callosum in the chronic stages after TBI with focal lesions having a lower FA than non-focal lesions, which correlates with our finding that FA impairment is sustained between 24 hours and 12 weeks post-TBI [26, 49, 50]. Although deficits in white matter integrity were documented 12 weeks post-TBI, histological analysis revealed an increase in oligodendrocyte number at this time point. Oligodendrocytes are the myelinating cells of the CNS and after initial oxidative stress-induced apoptosis, their progenitors have been shown to proliferate in response to neural injury [51-53]. Similarly, we demonstrate reduced oligodendrocyte numbers 1 and 4 weeks post-TBI followed by oligodendrocyte proliferation 12 weeks post-TBI, which indicates that oligodendrocyte lineage precursors may be facilitating endogenous repair processes to remyelinate damaged axons and restore white matter integrity.

ADC is a measure of the overall magnitude of the apparent water diffusion, thus areas with a high rate of diffusion will appear hyperintense and have a high ADC value and areas with restricted diffusion will appear hypointense and have a low ADC value [43]. As a consequence, the assessment of ADC changes in a cerebral lesion can be used as a marker of tissue damage and can help delineate between the formation of vasogenic and cytotoxic edema accumulation, respectively [22]. In the acute phase of TBI, it is widely accepted that both cytotoxic and vasogenic edema accumulate in the damaged territory, often leading to signal heterogeneity on ADC maps [21, 22, 28, 54]. We found increased mean ADC 24 hours post-TBI, indicating that vasogenic edema is prominent over cytotoxic edema in our model, with progressive increases in ADC by 12 weeks post-TBI. Similar to our finding, other preclinical models of focal injury

documented an increase in mean ADC by 24 hours post-TBI indicative of BBB breach and thus vasogenic edema [27, 28, 47, 55, 56]. However, there is discrepancy in the longitudinal pattern with some studies reporting a progressive increase in ADC and others finding ADC values returning to baseline within a few days. A clinical study by Marmarou et al. documented increased mean ADC in patients presenting with focal TBI containing a contusion core and surrounding hyperintensity comparable to our injury model [57]. In contrast, other studies found only transient increases in ADC with decreased values by 24 hours post-TBI indicative of prominent cytotoxic edema, which eventually return to baseline levels [20, 22, 24]. Progressive increases in ADC between 24 hours and 12 weeks post-TBI, as documented here, can be initially attributed to vasogenic edema followed by neuron collapse, dissolved lesioned tissue, and/or gliosis [27, 56]. Indeed, we found a 7-fold increase in astrogliosis between 24 hours and 12 weeks post-TBI and significant tissue atrophy, which is consistent with these previous observations. Similarly, Mac Donald et al. documented sustained increases in GFAP+ astrocytes post-TBI which correlated to increased mean ADC one month post-TBI [47]. Taken together, changes in ADC after TBI over time seems to be highly variable depending on TBI type and severity.

Successful acquisition of MR spectra demonstrated that it is feasible to monitor cellular TBI changes utilizing ^1H MRS in our porcine model. However, without an accurate internal standard combined with varying total creatine concentrations, relative concentration changes were the most reliable from our ^1H MRS data. Given the additional challenges of ^1H MRS acquisition in the porcine model of TBI including smaller cerebral volume and subsequently lower metabolite concentrations relative to humans, increased lipid layer near the skull, and respiration during scanning, further studies are needed to optimize ^1H MRS in this model. The

preliminary results from this case study involving one animal revealed that neurometabolism was impaired 24 hours post-TBI with deficits in the abundance of NAA, Cho, Cr, Ins, and Glx along with increased Lac which correlates to the observed cerebral lesioning on T2-weighted images. Similar to our findings, previous studies have documented decreased NAA abundance within 24 hours post-TBI which has been attributed to impaired NAA synthesis in neuronal mitochondria [24, 25, 58, 59]. However, there was a temporal discrepancy between decreased abundance of the neuronal marker NAA at 24 hours post-TBI and a loss of NeuN⁺ neurons at 1 week post-TBI. Therefore, our findings correlate with others in that deficits in NAA does not directly correlate to neuronal death but rather metabolically endangered neurons, providing a potential indicator of at-risk tissue that could benefit from therapeutics that enhance mitochondria function [24, 25, 58, 59].

We demonstrated that TBI resulted in decreased CBF utilizing ASL, which is a contrast-free MRI method that employs magnetically labeled blood water as a flow tracer to quantify CBF on a voxel-by-voxel basis [60]. CBF did not return to normal levels in our study likely due to substantial tissue necrosis, we found that CBF was significantly recovered 12 weeks post-TBI compared to 24 hours post-TBI. To our knowledge, this is the first study to longitudinally monitor CBF utilizing ASL in a large animal TBI model with substantial white matter structures, which have been shown to possess different microvasculature anatomy compared to gray matter [61]. Although further optimization of ASL collection in a porcine model is needed, these results demonstrate that ASL could be a valuable tool in assessing CBF recovery longitudinally after therapeutic intervention in a translational large animal model.

In conclusion, we correlate longitudinal multiparametric MRI and ¹H MRS approaches with distinct histological changes to characterize long-term TBI pathology in a translational large

animal model. TBI pathology illustrated a distinct time course with TBI-induced tissue and cellular level deficits up to 12 weeks after injury. These findings can be used as a platform to further study critical TBI injury mechanisms, deficits and recovery for application to the clinical setting.

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Figure 6.1: CCI Results in Significant Brain Lesioning Characterized by Parenchymal Swelling Followed by Atrophy

Representative T2-weighted images collected 24 hours (A) and 12 weeks (B) post-TBI displaying distinct brain lesion (arrows). Lesion size was quantified both 24 hours and 12 weeks post-TBI, and there was no difference in lesion size between the two time points (C). The percentage of measured tissue that was comprised of lesion was decreased 12 weeks post-TBI compared to 24 hours post-TBI (D). The volume of the left hemisphere (LH) and right hemisphere (RH) was calculated and expressed as a LH:RH ratio, which indicated parenchymal swelling 24 hours post-TBI followed by atrophy at 12 weeks post-TBI. Data are expressed as mean $\pm$ s.e.m. # indicates significance between time points ($p < 0.05$). * indicates significance from a normal ratio of 1.0 ($p < 0.05$).

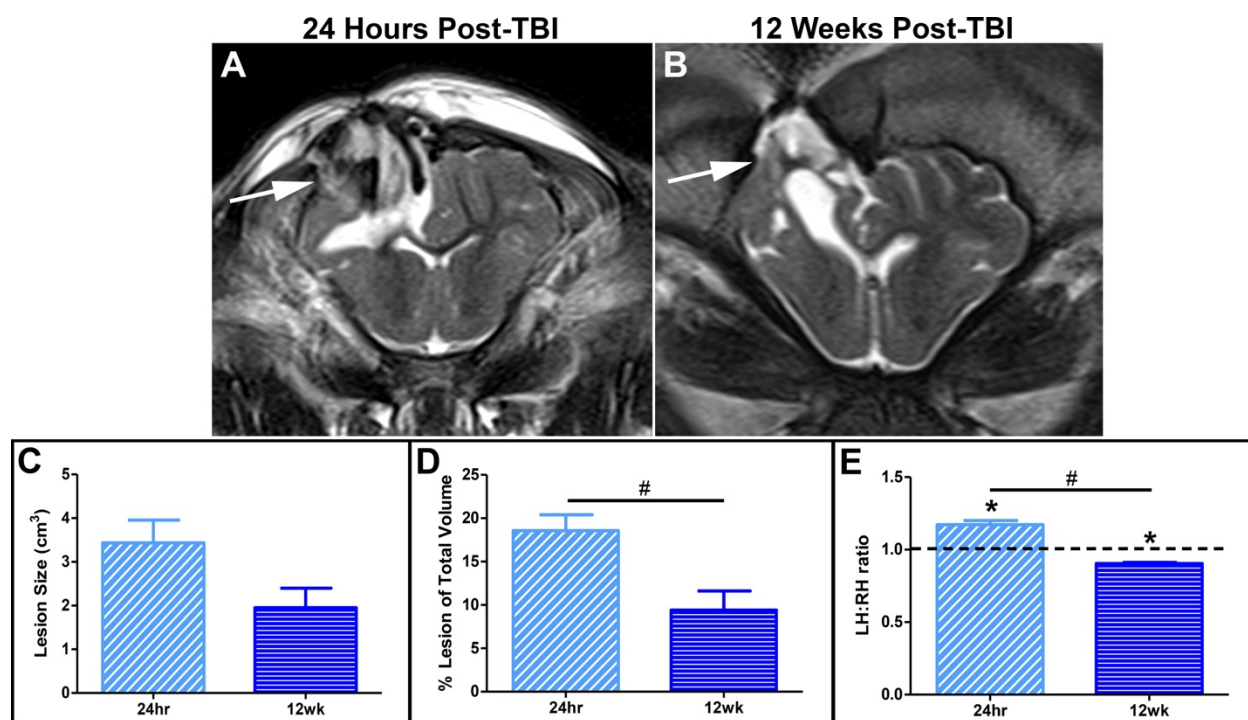


Figure 6.2: Directional Change in Midline Shift Supports Observed Brain Atrophy at the Lesion Site

Representative T2-weighted images collected 24 hours (A) and 12 weeks (B) post-TBI displaying distinct midline shift by quantifying the width of the left hemisphere (red line) and right hemisphere (blue line) at the level of the septum pellucidum (yellow line). Midline was shifted away from the ipsilateral hemisphere 24 hours post-TBI while midline was shifted toward the ipsilateral hemisphere 12 weeks post-TBI (C). Data are expressed as mean $\pm$ s.e.m. # indicates significance between time points ($p < 0.05$). * indicates significance from a normal midline (0.0) ($p < 0.05$).

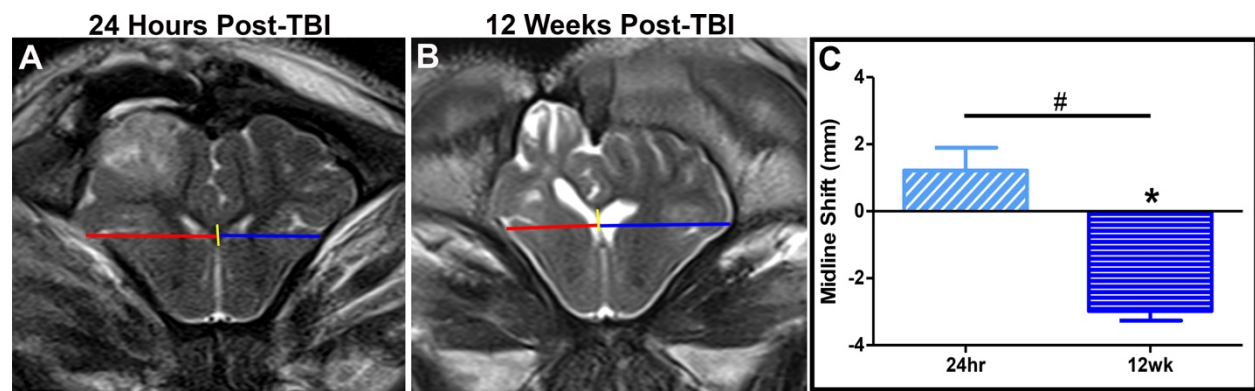


Figure 6.3: CCI Leads to the Accumulation of Edema and Disruption of White Matter Tracts.

Representative T2-weighted images and corresponding ADC maps collected at 24 hours (A, B) and 12 weeks (C, D) post-TBI. The ADC maps show heterogeneous contrast indicative of cytotoxic and vasogenic edema, but overall significant increases in mean ADC values were observed at 24 hours and 12 weeks post-TBI at the site of the lesion compared to the contralateral side and between time points (I). Representative FA maps and corresponding colored coded FA maps collected at 24 hours (E, F) and 12 weeks (G, H) post-TBI. A significant reduction in FA values were observed in the internal capsule and corpus callosum 24 hours and 12 weeks post-TBI indicative of white matter disruption (J). Data are expressed as mean $\pm$ s.e.m. # indicates significance between time points ($p < 0.0001$). * indicates significance from a contralateral side ($p < 0.05$).

24 Hours Post-TBI

12 Weeks Post-TBI

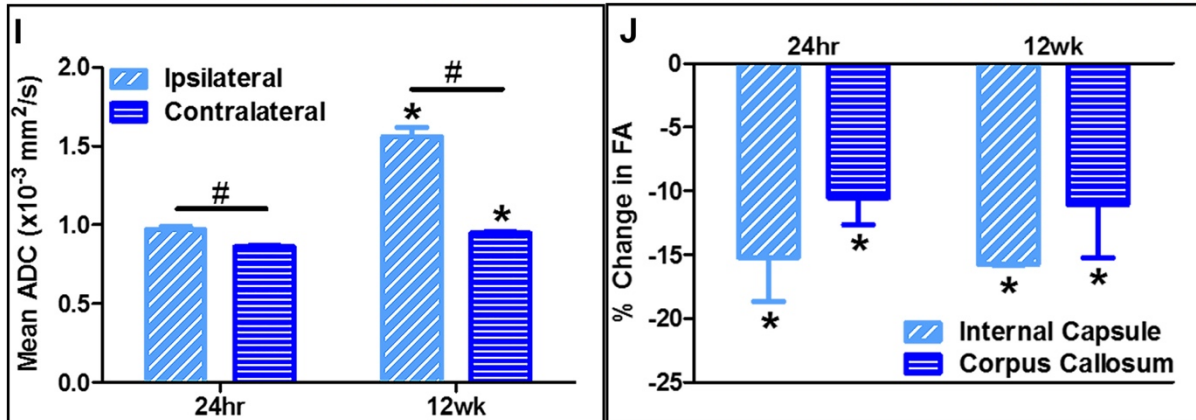
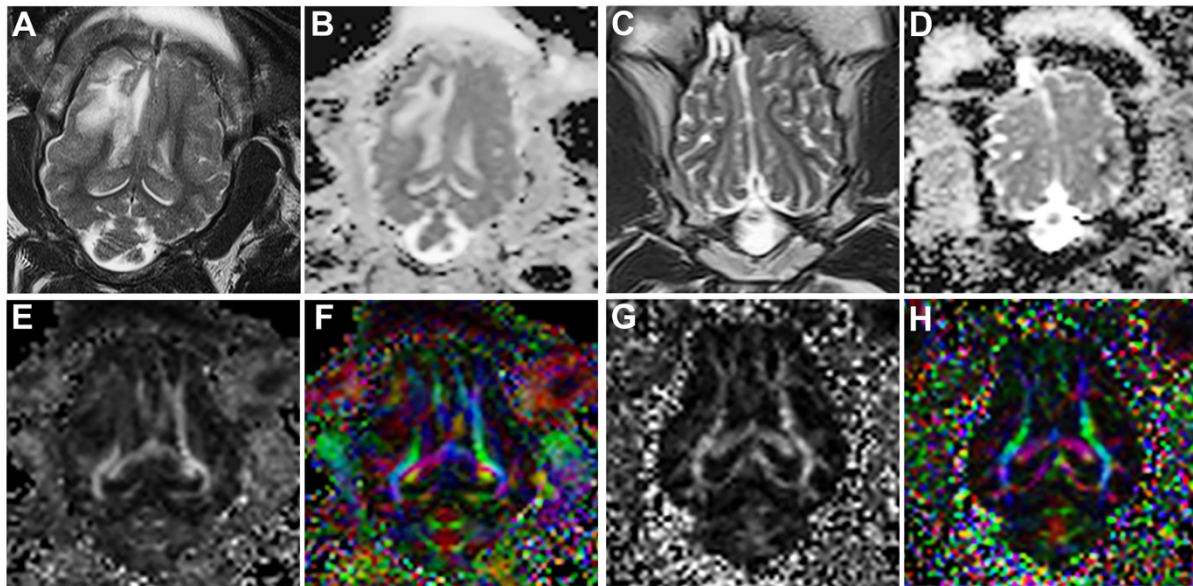


Figure 6.4: CCI Results in Impaired Cerebral Blood Flow

Representative ASL images taken 24 hours post-TBI illustrating hypointensity (arrows) at the lesion site indicative of restricted blood flow (A, color coded in B). A significant decrease in cerebral blood flow was observed both 24 hours and 12 weeks post-TBI while a significant improvement was documented at 12 weeks post-TBI compared to 24 hours post-TBI (C). Data are expressed as mean $\pm$ s.e.m. # indicates significance between time points. * indicates significance from the contralateral side.

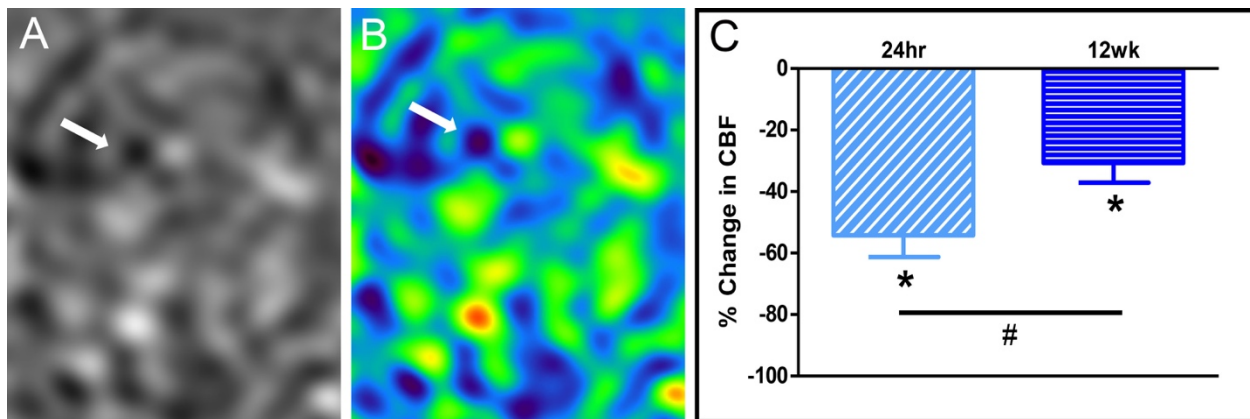


Figure 6.5: MRS Can Be Utilized to Track Changes in Brain Metabolites after CCI in a Piglet Model

Sample MR spectra acquired from contralateral (A) and ipsilateral (B) voxels in a single subject at 24 hours post-TBI. Compared to the contralateral side, the ipsilateral side demonstrates decreased Ins, NAA, Cho, Cr, and Glx as well as increased Lac at 24 hours post-TBI (B).

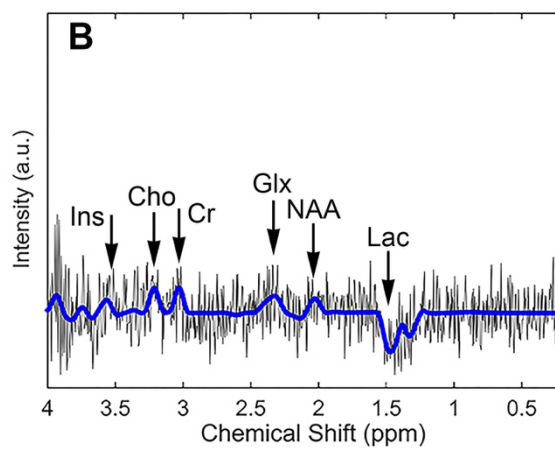
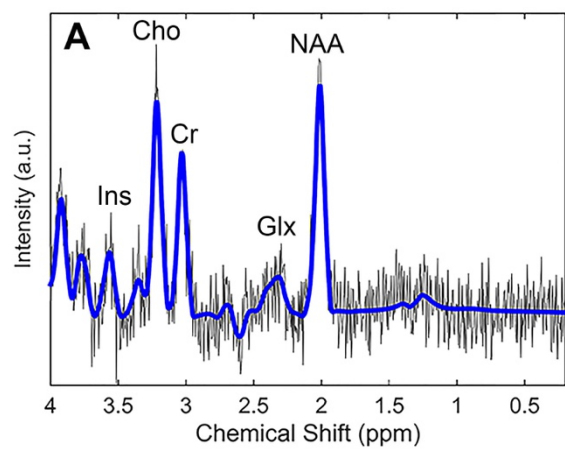
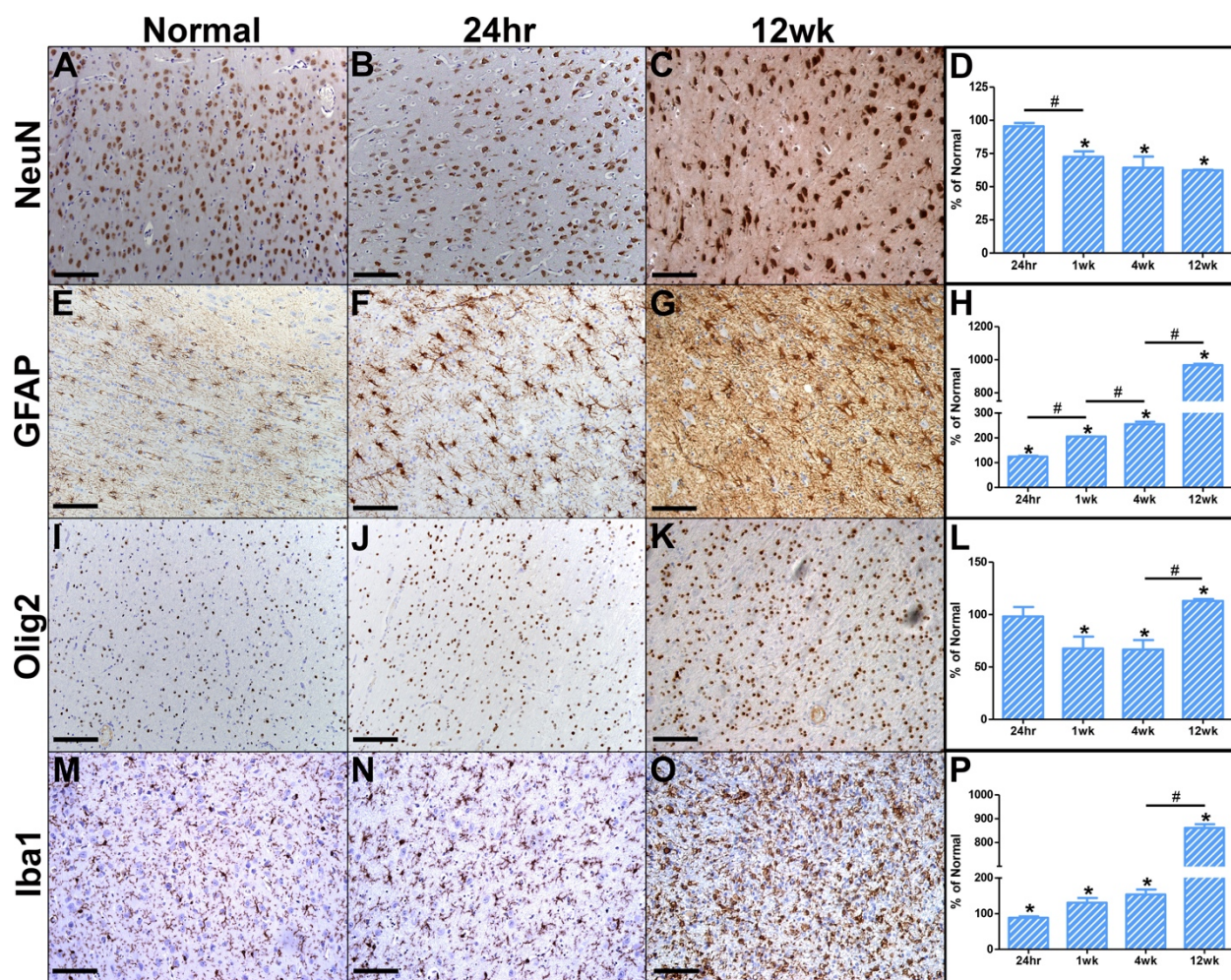


Figure 6.6: CCI Results in Longitudinal Changes in Neurons, Astrocytes, Oligodendrocytes, and Microglia in the Perilesional Area

Representative image of NeuN+ neurons in the perilesional area in normal control animals (A), 24 hours post-TBI (B), and 12 weeks post-TBI (C). The percentage of NeuN+ neurons relative to normal control animals at 24 hours, 1, 4, and 12 weeks-post-TBI (D). Representative image of GFAP+ astrocytes in the perilesional area in normal control animals (E), 24 hours post-TBI (F), and 12 weeks post-TBI (G). The percentage of GFAP+ area relative to normal control animals at 24 hours, 1, 4, and 12 weeks-post-TBI (H). Representative image of Olig2+ oligodendrocytes in the perilesional area in normal control animals (I), 24 hours post-TBI (J), and 12 weeks post-TBI (K). The percentage of Olig2+ oligodendrocytes relative to normal control animals at 24 hours, 1 week, 4 weeks, and 12 weeks post-TBI (L). Representative image Iba1+ microglia in the perilesional area in normal control animals (M), 24 hours post-TBI (N), and 12 weeks post-TBI (O). The percentage of Iba1+ area relative to normal control animals (P). Data are expressed as mean $\pm$ s.e.m. # indicates significance between time points ($p < 0.05$). * indicates significance from normal control animals ($p < 0.05$).



CHAPTER 7

INDUCED PLURIPOTENT STEM CELL-DERIVED NEURAL STEM CELL THERAPY

ENHANCES RECOVERY IN AN ISCHEMIC STROKE PIG

MODEL ¹

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Abstract

Induced pluripotent stem cell-derived neural stem cells (iNSCs) have significant potential as an autologous, multifunctional cell therapy for stroke, which is the primary cause of long term disability in the United States and the second leading cause of death worldwide. Here we show that iNSC transplantation improves recovery through neuroprotective, regenerative, and cell replacement mechanisms in a novel ischemic pig stroke model. Longitudinal multiparametric magnetic resonance imaging (MRI) following iNSC therapy showed delayed tissue loss while improving white matter integrity, cerebral blood perfusion, and brain metabolism in the infarcted tissue. The observed tissue level recovery strongly correlated with decreased immune response, enhanced neuronal protection, and increased neurogenesis. iNSCs differentiated into neurons and oligodendrocytes with indication of long term integration. The robust recovery response to iNSC therapy in a translational pig stroke model with increased predictive potential strongly supports that iNSCs may be the critically needed therapeutic for human stroke patients.

Introduction

Stroke is the leading cause of long-term disability in the United States and the second leading cause of death worldwide [1]. Due to the epidemic magnitude of stroke, hundreds of clinical trials have been performed to assess therapeutics that mitigate the sequela of stroke injury [2]. Unfortunately, these treatments have all failed in clinical trials with the exception of tissue plasminogen activator (tPA), the only Food and Drug Administration (FDA) approved agent for treating stroke. Despite the benefits of tPA, < 5% of patients receive this treatment due to its narrow therapeutic window and its effectiveness in only ischemic stroke [1, 3, 4]. The mechanism of tPA and the majority of other tested therapeutics focus on limiting ischemic damage by promoting reperfusion of brain tissue or acting as neuroprotectants. However, their functions have no direct restorative or regenerative action that would enhance replacement of brain tissue leading to improved functional recovery [4, 5]. An assessment of the litany of failed clinical trials by the Stem Cell Emerging Paradigm in Stroke (STEPS) Consortium resulted in identifying key therapeutic development criteria including 1) the development of a regenerative cell therapy that will not only protect cells from ischemic injury but also replace lost and damaged tissues and 2) testing of novel therapeutics in translational animal models that would be more reflective of human pathology with improved predictability [6, 7]. Recent breakthroughs enabling the robust generation of neural stem cells from autologous sources and the development of a robust pig stroke model make it possible to address both of these major criteria [8-10].

Recent pioneering research has shown that induced pluripotent stem cells (iPSCs) can be generated from a patient's own somatic cells. These iPSCs have similar plasticity to embryonic stem cells and are able to differentiate into neural stem cells (iNSC) which are used to effectively treat ischemic stroke in rodent models [11, 12]. These studies have demonstrated that upon

transplantation, iNSCs have a dual mode of therapeutic action: 1) serving as a cell replacement therapy by differentiating into neurons and glia that integrate into surrounding tissues and 2) producing neuroprotective and regenerative growth factors that stabilize and promote healing of damaged parenchyma [13-16]. In rodent stroke models, transplanted iNSCs reduce microglia activation, glial scar formation, promote angiogenesis, reduce tissue atrophy, and differentiate to mature neuronal cell types that engraft into the host brain [13-15, 17]. However, iNSCs were tested in a rodent brain which, when compared to the human, possesses many cerebral anatomical and physiological differences; therefore, these results may not be directly translatable to clinical applications. Compared to the rodent, the pig brain has greater anatomical and physiological similarities to humans with respect to gray to white matter composition, blood flow, gyral patterning, metabolism, and size - key factors that directly affect injury evolution, tissue recovery and treatment development [19-26]. Therefore, the use of the pig model to develop stroke therapies, particularly iNSC based therapy, could bridge the gap between preclinical rodent models and human clinical trials.

In this study, we report for the first time that intraparenchymal transplantation of human iNSCs improves tissue recovery in the stroke-damaged brain in a pig model. Non-invasive longitudinal magnetic resonance imaging (MRI) of stroked animals provided evidence of recovery in brain metabolism, white matter integrity, and cerebral blood perfusion after iNSC therapy. Histologic assessment showed that iNSC therapy also resulted in neural protection, decreased microglial activation, and stimulated endogenous neurogenesis. Transplanted iNSCs survived for 12 weeks in the pig brain and differentiated into neuronal and glial cells. These results demonstrate that iNSCs have significant potential as a regenerative and cell replacement

therapy in a robust translational large animal model, providing further insight into the potential of these cells as a therapeutic for patients.

Materials and Methods

Human Induced Pluripotent Stem Cell-Derived Neural Stem Cell (iNSC) culture

Human induced pluripotent stem cell-derived neural stem cells (HIPTM hNSC BC1, GlobalStem[®], Rockville, MD; hereafter “iNSC”) were used for transplantation into the pig brain. iNSCs were maintained on Matrigel-coated (BD Bioscience) tissue culture plates and maintained in neural stem cell media composed of Neurobasal medium (Thermo Fisher Scientific), 2% B-27 Supplement (Thermo Fisher Scientific), 1% non-essential amino acids (Life Technologies) 2 mM L-glutamine (Life Technologies), 1% penicillin/streptomycin (Invitrogen), and 20 ng/mL bFGF (R&D Systems). A complete media change was performed every other day. When iNSCs reached confluence, cells were enzymatically passaged using Accutase (Innovative Cell Technologies) and removed from the dish using a cell scraper. After passaging, the cells were replated at a ratio of 1:4.

iNSC immunocytochemistry

iNSCs were plated onto Matrigel-coated glass four-well chamber slides and fixed with 4% paraformaldehyde (PFA; Electron Microscopy Sciences) for 15 minutes. Cells were permeabilized in blocking solution containing 0.1% Triton X-100, 1% Polyvinylpyrrolidone (PVP; Sigma-Aldrich), and 3% serum. Primary antibodies were diluted in blocking solution and incubated for one hour at room temperature. Primary antibodies used were Nestin (Neuromics, 1:200) and Sox1 (R&D Systems, 1:20). Primary antibodies were detected using a fluorescently

conjugated secondary antibody, Alexa Fluor (Invitrogen; 1:1000) and incubated for one hour at room temperature before washing and mounting with Prolong Gold with DAPI (Life Technologies). Cells were imaged on an IX81 microscope with Disc-Spinning Unit (Olympus, Inc.) using Slide Book Software (Intelligent Imaging Innovations).

Labeling of iNSCs

Immediately prior to transplantation, low passage (passage <15) iNSCs were labeled with the lipophilic tracer 1,1'-dioctadecyl-3,3',3'-tetramethylindotricarbocyanine iodide (DiR; Invitrogen) for in vivo tracking at a concentration of 2 µg/mL diluted in phosphate buffered saline (PBS). After incubation at 37°C for 5 minutes, iNSCs were washed twice with PBS via centrifugation at 1500rpm. Cells were then resuspended in PBS at a final concentration 150,000 cells/µL for transplantation.

Animals

All work performed in this study was done in accordance with the University of Georgia Institutional Animal Care and Use Committee guidelines. Eight commercially bred, male castrated Landrace pigs aged 6 months and weighing approximately 180 lbs were obtained from the University of Georgia Swine Farm two weeks prior to surgery. Pigs were individually housed and fed a nutritionally complete diet. Room temperature was maintained at 26°C with a 12 h light/dark cycle.

Middle Cerebral Artery Occlusion (MCAO) Surgery

The pig stroke model was prepared based on the procedure described previously and permanent middle cerebral artery occlusion (MCAO) was performed in all animals [9]. All animals were administered antibiotics daily for 3 days prior to surgery (Naxcel[®]; 4mg/kg IM). Pre-induction analgesia and sedation was obtained using xylazine (5 mg/kg IM), butorphanol (0.2 mg/kg IM), and midazolam (0.2 mg/kg IM). Anesthesia was induced with intravenous propofol to effect and 0.5-1.0 mL of 2% lidocaine was topically applied to the laryngeal folds to facilitate intubation and was maintained with 1.5% inhaled isoflurane (Abbott Laboratories) in oxygen. Artificial ventilation was utilized at a rate of 8-12 breaths per minute with a tidal volume of 5-10 mL/kg. During surgery, lactated ringers solution was administered intravenously in the ear vein at a rate of 5 mL/kg/hour. A Doppler probe placed on the ventral tail artery monitored heart rate continuously throughout surgery, and a digital thermometer was used to check rectal temperature every 15 minutes. While the pig was positioned in sternal recumbency, the head was angled 45 degrees to its left with the mouth in an open position and secured to facilitate a right sided craniectomy approach. This position utilizes gravity to pull the cerebrum away from the skull during intracranial surgery. Hair between the eye and ear was shaved and the skin was prepared in a routine manner for sterile surgery using alternating applications of Betadine and 70% alcohol. The surgical site was draped in a standard fashion.

As previously described [9], injury was induced by performing a frontotemporal craniectomy with orbital rim osteotomy and zygomatic arch resection. A curvilinear skin incision was performed that extended from slightly above the right orbit to an area rostral to the ear canal. The temporal fascia and muscle were incised and reflected along with the skin flap. The branches of the superficial temporal artery and the associated vein were exposed and occluded using high

frequency bipolar cautery forceps. Next, the insertion of the temporalis muscle was incised from below the zygomatic arch and elevated away from the parietal bone. The periosteum overlying the zygomatic arch was incised and bluntly elevated away. The zygomatic arch was partially resected, which exposed the ventral aspect of the calvaria to the level of the orbital fissure. A surgical defect was generated in the exposed calvaria using a pneumatic drill and burr and extended using Kerrison rongeurs; the lateral portion of the roof of the orbit was rongeured away while the orbital contents were protected with a hand held surgical retractor. The visible dura mater was incised and reflected dorsally as a flap. The middle cerebral artery (MCA) was located just distal to the Circle of Willis at its origin and was permanently occluded utilizing the bipolar electrocautery forceps. After occlusion, the exposed portion of the brain was covered with a sterile biograft made of porcine small intestine submucosa (MatriStem, ACell). The temporalis muscle was routinely reattached and the skin was routinely re-apposed. Analgesia (Banamine[®], 2 mg/kg IM) and antibiotic (Naxcel[®], 4 mg/kg IM) was administered for 3 days postoperatively. Anesthesia was discontinued and pigs were returned to their pens upon extubation and monitored every 4 hours for the next 12 hours. Heart rate, respiratory rate, and temperature were recorded at each time point.

Magnetic Resonance Imaging

Magnetic resonance imaging (MRI) was performed 24 hours post-stroke and 1, 4, and 12 weeks post-transplantation on a Siemens 1.5 Tesla MRI system (Magnetom Avanto, Siemens Healthcare System). Under the same anesthesia protocol utilized for surgery, MRI of the cranium was performed using an 8-channel head coil with the animal positioned in dorsal recumbency. Standard multiplanar MR brain imaging series at the coronal plane were acquired, including T1-

weighted, T2-weighted, T2-weighted fluid attenuated inversion recovery (FLAIR), diffusion-weighted imaging (DWI), diffusion tensor imaging (DTI), dynamic susceptibility contrast perfusion-weighted imaging (PWI), and magnetic resonance spectroscopy (MRS). For PWI, the contrast agent (Magnevist[®], 20 mL, IV) was administered at a rate of 2 mL/second. T1-weighted, DWI, and DWI-derived apparent diffusion coefficient (ADC) maps were analyzed using Osirix software. For brain tissue volumetric analysis, T1-weighted images were utilized to measure the areas of the ipsilateral (injured) and contralateral (normal) hemispheres. Hemisphere area and slice thickness were combined to obtain total hemisphere volume, and changes in the volume of the ipsilateral hemisphere were expressed as a percentage change relative to the contralateral hemisphere. Fractional anisotropy (FA), a measurement for white matter integrity, was calculated using DTI data in 3 different areas of the peri-infarct region while 3 comparable anatomical regions on the contralateral hemisphere were used as a control. The average change in FA at the area of the infarct was calculated relative to each respective area on the contralateral hemisphere. MRS was utilized to assess the level of selected neurometabolites in 3 separate regions in the peri-infarct area while 3 separate measurements were taken at a comparable area on the contralateral hemisphere. The integral of each peak was obtained from the magnetic resonance spectra for each neurometabolite of interest, and the change in peak integral of the ipsilateral region relative to the contralateral region was calculated. Mean transit time, time to peak, and cerebral blood volume in the peri-infarct area and at a comparable region on the contralateral hemisphere were calculated from PWI. The change of cerebral perfusion was expressed as a percentage change in the ipsilateral hemisphere relative to the comparable area on the contralateral hemisphere.

iNSC Transplantation

Five days after MCAO surgery, all pigs were randomly assigned to either iNSC treated or non-treated (vehicle only) groups and were anesthetized under the same protocol utilized for the surgery. The surgery site was then reopened for iNSC transplantation into the brain parenchyma. Transplantation surgeries were performed utilizing a large animal stereotaxic frame (David Kopf Instruments) with modifications to meet our needs with pigs. The frame is equipped with a mounted Quintessential stereotaxic injector (Stoelting Co., Wood Dale, IL) to regulate cell injection location and parameters. Immediately prior to injection, iNSCs suspended in PBS were loaded into a glass micro-pipette syringe attached to a specialized 24 gauge needle (Hamilton Co, Reno, NV) and attached to the stereotaxic apparatus. Two separate injections of 6.0×10^6 iNSCs or vehicle only (n=4 per treatment group) were transplanted into two sites of the penumbra region at a depth of 6mm, which was defined as the interface between the cortex and subcortical white matter, at a rate of 2 μ L/minute to prevent backflow. The location of the penumbra region was determined by 24hr post-stroke MRI scans of each animal. After transplantation was complete, the needle was retracted at a rate of 1 mm/minute to prevent backflow. Anesthesia was discontinued and the pigs were returned to their pens upon extubation.

Brain Tissue Collection and Processing

Twelve weeks after MCAO surgery, all eight pigs were sedated utilizing the pre-operative drug protocol and immediately euthanized by intravenous pentobarbital overdose (1 mL/4.5kg). Brains were removed and coronally sectioned using a brain slicer specific to pig (Zivic Instruments, Pittsburgh, PA). For iNSC transplantation analysis, brain sections were imaged using an IVIS Lumina II System (PerkinElmer, Waltham, MA). DiR fluorescence was

visualized using an excitation wavelength of 745 nm and emission wavelength set to Indocyanine Green filter. Tissue areas of high DiR fluorescence were collected, fixed in 4% PFA (Electron Microscopy Sciences), cryoprotected in 30% sucrose, embedded in Tissue-Tek OCT compound (Sakura), and stored at -80°C. For gene expression, brain tissue samples were collected from the peri-lesion area, dissected to separate gray and white matter, snap frozen in liquid nitrogen, and stored at -80°C. For tissue immunohistochemical analysis, whole representative sections at the level of the infarct were immersed in 10% buffered formalin.

Immunofluorescence of iNSC Transplantation Sites

Brain tissue samples were cryosectioned (Leica CM1950) at a thickness of 10 µm and collected on gelatin-coated microscopy slides for immunofluorescence analysis. Primary antibodies used were human nuclear antigen (HNA; Millipore, 1:50) neuronal nuclei (NeuN; Millipore, 1:500), oligodendrocyte lineage transcription factor 2 (Olig2; Genetex, 1:250), and glial fibrillary acidic protein (GFAP; Abcam, 1:500). After rehydration with PBS, tissues were blocked in 10% normal donkey serum and 0.3% Triton X-100 for one hour. Primary and secondary antibodies were diluted in PBS with 1% normal donkey serum and 0.3% Triton X-100. Primary antibodies were incubated overnight at 4°C and were detected using a species-specific fluorescently conjugated secondary antibody by Alexa Fluor (Invitrogen; 1:1000 for HNA, NeuN, and Olig2, 1:500 for GFAP) which was incubated for one hour at room temperature. Slides were mounted in Prolong Gold with DAPI (Invitrogen) and allowed to dry overnight before microscopic inspection. Imaging was performed on a IX81 microscope with Disc-Spinning Unit (Olympus, Inc.) utilizing the Z-stack capabilities of Slide Book Software

(Intellegent Imaging Innovations). Quantification was performed by imaging 5 random fields for each animal and antibody and utilizing the cell counter plugin on ImageJ 2.0 software.

Immunohistochemistry of Endogenous Tissues

Formalin-fixed whole coronal sections were routinely processed, embedded in paraffin, and stained with antibodies specific to NeuN (Millipore, 1:500), doublecortin (DCX; Abcam, 1:2000), GFAP (Biogenex, 1:4000), and Iba1 (Wako, 1:8000). Heat induced antigen retrieval was performed for all antibodies using citrate buffer at pH6 (DAKO). Detection was performed utilizing biotinylated antibodies and a streptavidin label (4plus Streptavidin HRP, Biocare). A HRP label and DAB chromagen (DAKO) was used and all sections were lightly counterstained with hematoxylin. Microscopic inspection and imaging was performed on a Nikon Eclipse TE300 inverted microscope. All quantification was carried out on ImageJ 2.0 software. For NeuN, GFAP, and Iba1, images were taken at the lesion border ranging from the ectomarginal gyrus to the piriform lobe. For quantification of NeuN, positive cells were manually counted using the cell counter plugin on ImageJ 2.0 and expressed as cells/mm². For semi-quantitative analysis of GFAP and Iba1, the total area of immunoreactivity corresponding to increased optical density was determined by ImageJ software. For DCX, as described in previous studies [28], DCX+ neuroblasts in three separate anatomical regions were analyzed at the level of the caudate nucleus: 1) the neuroblasts positioned directly adjacent to the ependymal cells lining the lateral ventricle (ventricular subventricular zone; vSVZ), 2) the neuroblasts organized laterally into chains adjacent to but distinct from the vSVZ (abventricular SVZ; aSVZ), and 3) the neuroblasts that have migrated laterally to the lesion border. DCX+ cells were manually counted using the cell counter plugin on ImageJ and expressed as cells/mm². Four healthy pigs of the

same age that received no MCAO surgery, iNSC injection, or vehicle injection were sacrificed and brains were processed as described above to serve as normal control animals.

Gene Expression

Brain tissue samples allocated for gene expression analysis were weighed, and approximately 150mg of tissue was transferred to cold RNAlater-ICE (Thermo Fisher Scientific) and allowed to thaw overnight at -20°C. Total RNA was extracted using an RNeasy mini kit (Qiagen) according to the manufacturer's protocol. The quality and quantity of the isolated RNA was measured on a NanoDrop 8000 (Thermo Fisher Scientific). Nine hundred nanograms of RNA from each sample was used for reverse transcription using the High Capacity cDNA Reverse Transcription Kit (Thermo Fisher Scientific) according to the manufacturer's instructions. Quantitative PCR was performed using a customized TaqMan Array Card (Thermo Fisher Scientific) on an Applied Biosystems 7900HT Real-Time PCR System (Thermo Fisher Scientific) using SDS v2.4 software. Data was processed using ExpressionSuite Software v1.0.3 to obtain C_T values. $\Delta\Delta C_T$ and Relative Quantification values were calculated manually using *Sus scrofa*-specific GAPDH as a housekeeping control, and data was expressed as a fold change in iNSC treated samples relative to non-treated samples.

Statistics

All quantitative data was analyzed with SAS version 9.3 (Cary, NC) and statistical significances between groups were determined by two-way analysis of variance and post-hoc Tukey's Pair-Wise comparisons. Treatments where p-values were ≤ 0.05 were considered to be significantly different.

Results

iNSC treatment inhibits post-stroke cerebral atrophy

MRI performed 24 hours post-stroke confirmed the presence of cytotoxic ischemia and cerebral infarction. DWI showed abnormal territorial hyperintensity in the right cerebral hemisphere in all 8 pigs indicating the presence of edema (**Figure 7.2A**). ADC maps demonstrated distinct hypointensity at the same region indicative of a bona fide cerebral infarction rather than vasogenic edema (**Figure 7.2B**). A hyperintense territory on T2-weighted FLAIR images 24 hours post-stroke suggested edema formation and resulted in brain swelling in the ipsilateral (injured) hemisphere that shifted the midline towards the contralateral (uninjured) hemisphere, causing the parenchyma and ventricles to be compressed and distorted (**Figure 7.2C**). The ipsilateral hemisphere was significantly ($p<0.0001$) larger than the contralateral hemisphere in both iNSC treated and non-treated animals ($4.99\% \pm 0.71\%$ and $6.22\% \pm 0.46\%$, respectively, **Figure 7.2G**). Five days post-stroke, 6 million iNSCs expressing Sox1 and Nestin were transplanted in two injections (for a total of 12 million cells) into the stroke penumbra region (**Figure 7.1**). The hyperintense lesion persisted 1 week post-transplant with observations of midline shift towards the ipsilateral hemisphere and an enlarged right ventricle due to parenchymal tissue atrophy (**Figure 7.2D**). The ipsilateral hemisphere of the non-treated group was significantly ($p<0.01$) smaller than the contralateral hemisphere while there was no statistical difference between the two hemispheres in iNSC treated animals ($2.04\% \pm 0.66\%$ and $0.07\% \pm 0.36\%$, respectively, **Figure 7.2G**). The lesion site became hypointense on T2-FLAIR by 4 weeks post-transplant due to parenchymal tissue atrophy, and there was significantly ($p=0.0005$) less tissue atrophy in the ipsilateral hemisphere of iNSC treated pigs compared to non-treated pigs ($8.83\% \pm 0.14\%$ vs. $13.80\% \pm 0.83\%$, respectively, **Figure 7.2E, G**). The

hypointense signal persisted through 12 weeks post-transplant, and the ipsilateral hemispheres of both iNSC treated and non-treated groups displayed significant ($p < 0.0001$) atrophy with a $13.06\% \pm 0.51\%$ and $15.57\% \pm 0.68\%$ decrease in volume, respectively (**Figure 7.2F, G**). Although a smaller degree of atrophy was observed in iNSC treated animals, there was no statistical difference between the two treatment groups (**Figure 7.2G**). Taken together, this data indicates that iNSC treatment delays tissue atrophy.

iNSC treatment results in recovered white matter integrity in the chronic stages of stroke recovery

DTI was used to determine whether iNSC treatment recovered white matter integrity after stroke. Changes in FA, a measure of white matter integrity, in the ipsilateral hemisphere were compared to the contralateral hemisphere with calculated changes closer to 0 being more similar to normal tissue (**Figure 7.3**). FA values were increased in selected regions in iNSC-treated animals 12 weeks after the iNSC transplant (**Figure 7.3A, B**). There was significant ($p < 0.01$) recovery in FA in iNSC treated pigs at 12 weeks post-transplant compared to 24 hours post-stroke (32.25 ± 4.20 vs. 197.17 ± 35.83 , respectively), while there was no statistical difference between the two time points in non-treated animals (107.33 ± 30.18 vs. 124.93 ± 14.77 , respectively, **Figure 7.3C**). This indicates that iNSC treatment leads to significant recovery in white matter integrity by 12 weeks post-transplant in the ipsilateral hemisphere.

iNSC treatment significantly improves recovery of brain metabolism post-stroke

The levels of neurochemicals N-acetylaspartate (NAA, neuronal marker), choline (Cho, marker of cellularity), and creatine (Cr, a cell energy metabolite) are indicative of brain

metabolism and can be longitudinally measured by MRS to assess tissue damage and the effect of iNSC treatment (**Figure 7.4**). MR spectra obtained from iNSC treated and non-treated groups showed distinct changes in the levels of NAA, Cho, and Cr (**Figure 7.4A, B**). Peak integrals for each metabolite were measured in both hemispheres and the difference in the ipsilateral hemisphere relative to the contralateral hemisphere was calculated to determine the injury and recovery induced changes with changes closer to 0 being more similar to the normal tissue. iNSC treated animals showed significant ($p<0.01$) improvement in NAA levels relative to non-treated animals at 1 (5.14 ± 0.11 vs. 6.44 ± 0.08 , respectively), 4 (3.41 ± 0.13 vs. 5.17 ± 0.064 , respectively), and 12 weeks post-transplant of iNSCs (1.75 ± 0.38 vs. 3.80 ± 0.28 , respectively). Additionally, the change in NAA was significantly ($p<0.0001$) decreased at both 4 weeks and 12 weeks post-transplant in iNSC treated animals compared to 24 hours post-stroke (3.41 ± 0.13 and 1.75 ± 0.38 vs. 5.81 ± 0.27 , respectively), which was not the case for non-treated animals (**Figure 7.4C**).

Cho levels also showed significant ($p<0.05$) improvement in iNSC treated pigs relative to non-treated at 4 weeks post-transplant (-0.61 ± 0.30 vs. 2.96 ± 0.15 , respectively) and 12 weeks post-transplant (2.15 ± 0.25 vs. 4.63 ± 0.57 , respectively). Similar to NAA, there was also a significant ($p<0.05$) reduction in the change in Cho at 4 weeks and 12 weeks post-transplant in iNSC treated animals compared to 24 hours post-stroke (-0.61 ± 0.30 and 2.15 ± 0.25 vs. 3.81 ± 0.34 , respectively), which was not observed in non-treated pigs (**Figure 7.4D**).

The cell energy metabolite marker Cr demonstrated a similar neurometabolic improvement with a significant ($p<0.05$) reduction in the change in Cr in iNSC treated animals compared to non-treated animals at 1 week post-transplant (3.88 ± 0.36 vs. 8.35 ± 1.15 , respectively) and 12 weeks post-transplant (1.00 ± 0.06 vs. 3.61 ± 0.31 , respectively). In

addition, there was a significant ($p<0.05$) reduction in the change in Cr in iNSC treated animals at 4 weeks and 12 weeks post-transplant compared to 24 hours post-stroke (0.77 ± 0.62 and 1.00 ± 0.06 vs. 5.77 ± 1.26 , respectively), which was not observed in non-treated animals (**Figure 7.4E**). Taken together, this data suggests recovery in brain metabolism in iNSC treated animals.

iNSC treatment significantly improves cerebral blood perfusion in the ipsilateral hemisphere

Perfusion weighted imaging (PWI) was performed to determine if iNSCs could normalize blood perfusion to the brain. Color-coded cerebral perfusion maps taken at 12 weeks post-transplant indicate increased cerebral blood flow at the area of the infarct in iNSC treated pigs compared to non-treated pigs (**Figure 7.5A, B**). Mean transit time (MTT), time to peak (TTP), and cerebral blood volume (CBV) were calculated in both hemispheres, and the measurement of the affected ipsilateral hemisphere was expressed as a percentage of the normal contralateral hemisphere for each parameter. MTT is defined as the time it takes for blood to move through a particular volume of tissue. An increase in MTT suggests blood flow is decreased in injured tissue [29, 30]. At 12 weeks post-transplant, there was a significant ($p<0.01$) reduction in MTT in iNSC treated pigs compared to non-treated pigs ($92.29\% \pm 3.26\%$ vs. $132.99\% \pm 8.04\%$, respectively) (**Figure 7.5C**). TTP is the time it takes for injected contrast to reach threshold maximum, and an increase in TTP suggests that the arrival of the injected contrast bolus is delayed due to reduced cerebral blood flow [29]. Similarly, there was significant ($p<0.01$) decrease in TTP in iNSC treated animals compared to non-treated animals ($102.94\% \pm 3.70\%$ vs. $137.60\% \pm 5.67\%$, respectively) at 12 weeks post-transplant which indicates improved cerebral blood flow in iNSC treated animals (**Figure 7.5D**). CBV quantifies the volume of blood within a particular volume of brain tissue; a decrease in CBV indicates decreased perfusion within a

volume of brain tissue [29]. CBV was found to be significantly ($p<0.05$) improved in iNSC treated animals relative to non-treated animals at 12 weeks post-transplant with increased CBV in iNSC treated animals compared to 24 hours post-stroke ($58.95\% \pm 9.23\%$ vs. $18.31\% \pm 4.43\%$, respectively), which was not observed in non-treated animals (**Figure 7.5E**). Taken together, this data indicates that iNSC treatment enhances cerebral blood perfusion in the ipsilateral hemisphere by 12 weeks post-transplant.

iNSC treatment leads to neuronal protection and reduced microglial activation at the lesion border

Next, we sought to determine the effect of iNSC transplantation on neuronal survival and immune response after stroke. We quantified NeuN+ neurons at the lesion border as well as the polymorphic layer of the dentate gyrus (DG; **Figure 7.6A-H**). At 12 weeks post-transplant, there was a significant ($p<0.01$) decline in the number of NeuN+ neurons in non-treated animals at the lesion border compared to normal control animals (161 ± 35 cells/mm² vs. 290 ± 12 cells/mm², respectively). However, there was no significant difference in the number of NeuN+ neurons between iNSC treated and normal control animals (248 ± 32 cells/mm² vs. 290 ± 12 cells/mm², respectively) at the lesion border, indicating that iNSC treatment leads to neuronal protection at the lesion site post-stroke (**Figure 7.6A-D**). Similarly, we observed a significant ($p<0.01$) decline in the percentage of NeuN+ neurons in the DG of non-treated animals compared to normal control animals ($62\% \pm 2\%$ vs. $83\% \pm 3\%$, respectively) while there was no significant difference in the percentage of NeuN+ neurons between iNSC treated animals and normal control animals ($66\% \pm 9\%$ vs. $83\% \pm 3\%$, respectively), suggesting that iNSC treatment also protected neurons in the DG after stroke (**Figure 7.6E-H**).

In addition, we assessed immune response by quantifying microglia activation and astrogliosis at the lesion border through Iba1 and GFAP antibody staining, respectively (**Figure 7.6I-P**). 12 weeks post-transplant, there was a significant ($p<0.005$) increase in Iba1+ area in both iNSC treated and non-treated animals compared to normal control animals ($6.47\% \pm 1.06\%$ and $15.15\% \pm 2.22\%$ vs. $0.85\% \pm 0.08\%$, respectively). Remarkably, there was a significant ($p<0.05$) reduction in Iba1+ area in iNSC treated animals compared to non-treated animals ($6.47\% \pm 1.06\%$ vs. $15.15\% \pm 2.22\%$, respectively) demonstrating that iNSC treatment mitigates microglia activation after stroke at the lesion border (**Figure 7.6I-L**). Astroglial activity at the lesion border was significantly ($p<0.05$) upregulated as indicated by the GFAP+ area in both iNSC and non-treated pigs compared to normal control animals ($46.78\% \pm 2.68\%$ and $51.81\% \pm 1.34\%$ vs. $36.27\% \pm 0.49\%$, respectively). However, there was no significant difference in GFAP+ area between iNSC and non-treated treated groups suggesting that iNSC treatment does not have an effect on astrogliosis after stroke at the lesion border (**Figure 7.6M-P**). Taken together, this data indicates that iNSC treatment likely has a neuroprotective effect leading to decreased neuronal cell death in cortical and DG regions of the brain and suppressed microglia activation.

iNSC treatment promotes endogenous neuroblast activation and migration to the lesion border

After stroke, endogenous neural stem cells located in the subventricular zone (SVZ) have the ability to differentiate into immature neuronal cells, or neuroblasts, which migrate toward the site of injury replacing damaged and lost cells (albeit at a low level) and have a neuroprotective effect through the release of neurotrophic factors [31]. Specifically in the pig, Costine et al. has characterized a process in which neuroblasts in the SVZ proliferate at the ventricular lining of

the SVZ (vSVZ) before forming migratory chains directly adjacent to the vSVZ, deemed the abventricular SVZ (aSVZ) [28]. From the aSVZ, neuroblasts migrate toward the site of neural injury. We wanted to determine if neuroblasts in our pig MCAO model demonstrated this same migratory pattern, and if iNSC treatment promoted neuroblast migratory behavior from the SVZ. 12 weeks post-transplant, there was a significant ($p<0.001$) increase in DCX+ neuroblasts at the level of the vSVZ in non-treated animals compared to normal control animals (5.43 ± 0.23 cells/mm² vs. 3.40 ± 0.17 cells/mm², respectively). Furthermore, there was a significant ($p<0.01$) increase of DCX+ neuroblasts in the vSVZ in iNSC treated animals compared to non-treated animals (11.52 ± 1.19 cells/mm² vs. 5.43 ± 0.23 cells/mm², respectively) (**Figure 7.7A-D**).

When assessing the amount of DCX+ neuroblasts in the aSVZ, we found no significant difference between non-treated and normal control animals (1.87 ± 0.05 cells/mm² vs. 1.90 ± 0.80 cells/mm², respectively). However, there was a significant ($p<0.01$) increase in DCX+ neuroblasts in the aSVZ in iNSC treated animals compared to non-treated animals (4.83 ± 0.50 cells/mm² vs. 1.87 ± 0.05 cells/mm², respectively) (**Figure 7.7E-H**). Last, we compared the amount of DCX+ neuroblasts between treatment groups at the lesion border and found no significant difference in the number of DCX+ neuroblasts between non-treated animals and normal control animals (0.031 ± 0.013 cells/mm² vs. 0.003 ± 0.003 cells/mm², respectively). However, we found significant ($p<0.05$) increase in the amount of DCX+ neuroblasts at the lesion border in iNSC treated animals compared to non-treated animals (0.095 ± 0.011 cells/mm² vs. 0.031 ± 0.013 cells/mm², respectively) (**Figure 7.7I-L**). These results indicate that stroke alone stimulates neurogenesis at the vSVZ; however, iNSC treatment significantly augments this neurogenic activity and promotes neuroblast migration to the aSVZ and the lesion border.

Transplanted iNSCs survive long-term and differentiate into neurons and oligodendrocytes

Transplanted human iNSC differentiation in the post-stroke pig brain was assessed by immunohistochemistry (**Figure 7.8**). Transplanted human cells were identified by expression of the human nuclear antigen (HNA, **Figure 7.8A**). HNA⁺ cells were co-labeled with either NeuN, Olig2, or GFAP to identify transplanted cells that differentiated to neurons, oligodendrocytes, or astrocytes, respectively. Co-labeling HNA⁺/NeuN⁺ cells made up the majority of the HNA⁺ cells (**Figure 7.8A-G**). A subset of HNA⁺ cells also co-localized with Olig2 (**Figure 7.8H-J**). The percentage of HNA⁺ cells that co-localized with either NeuN or Olig2 was $77.15\% \pm 18.8\%$ and $26.45\% \pm 11.54\%$, respectively (**Figure 7.8D**). Due to the nature of the GFAP label, it was difficult to confidently identify HNA⁺/GFAP⁺ cells; therefore, we did not include HNA⁺/GFAP⁺ cells in our quantification. This data shows that transplanted iNSCs differentiate predominantly into neurons. Furthermore, we show evidence of substantial iNSC differentiation into oligodendrocytes, likely the result of utilizing an animal model with a high percentage of white matter.

iNSC treatment leads to distinct changes in gene expression related to tissue recovery in gray and white matter

Many studies have shown that transplanted iPS- and fetal-derived NSCs produce regenerative trophic factors that decrease inflammation, promote angiogenesis, and aid in tissue regeneration after stroke [13, 14, 17, 32, 33]. We performed quantitative RT-PCR to determine if iNSC treatment resulted in changes in gene expression related to tissue recovery (**Figure 7.9**). We analyzed gray and white brain matter separately to determine whether the two tissue types responded differently to signaling from transplanted iNSCs. After 12 weeks post-transplant,

several *Sus scrofa*-specific genes related to angiogenesis, neurotrophism, and inflammation were significantly ($p < 0.05$) changed in iNSC treated brain tissue relative to non-treated brain tissue. Furthermore, gray and white matter exhibited distinct changes in gene expression compared to one another. In gray matter, the angiogenesis marker Ang1, neurotrophic factors BDNF and GDNF, anti-inflammatory cytokine IL-10, growth factors NOG and NTF3, and neurite branching mediator Rtn4 were significantly ($p < 0.05$) upregulated in iNSC treated animals by 3.01 ± 0.82 , 4.68 ± 1.49 , 2.92 ± 0.81 , 2.31 ± 0.43 , 1.90 ± 0.31 , 1.32 ± 0.09 , and 1.98 ± 0.30 fold, respectively, relative to non-treated animals. In white matter, the genes encoding colony stimulating factor 2 (CSF2) and inflammatory cytokines IL-1 β and IL-6 were significantly downregulated in iNSC treated animals by 234.33 ± 105.85 , 21.19 ± 10.38 , and 71.74 ± 25.21 fold, respectively, relative to non-treated animals. Taken together, this data indicates that iNSC treatment leads to changes in gene expression that decreases inflammation and promotes tissue recovery. Interestingly, the changes in gene expression were dissimilar between gray and white matter indicating that gray and white matter responds differently to iNSC trophic factor signaling.

Discussion

The potential to perform autologous or allogeneic iNSC transplantation to replace and protect brain tissue has significant implications for the treatment of stroke, with promising rodent studies demonstrating drastic improvements in recovery. However, the enthusiasm of the preclinical success of iNSC therapy has been tempered with the hundreds of failed clinical trials of therapeutics previously developed in rodents and a need for a deeper understanding of the underlying recovery mechanisms [5]. For the first time, we demonstrate in a pig stroke model utilizing translational approaches (MRI and MRS) that iNSC cell therapy leads to significant

tissue recovery and cell replacement. This tissue level recovery strongly correlated with decreased immune (microglia) response and increased neuroblast migration, demonstrating that the iNSC recovery mechanism is both neuroprotective and regenerative. In addition, iNSCs demonstrated long term integration with robust cell differentiation into both neurons and oligodendrocytes. These results demonstrate that iNSCs are a multi-modal therapeutic with neuroprotective, regenerative, and cell replacement capabilities.

Interestingly, we found that iNSC treatment promotes neurogenesis and the migration of DCX⁺ neuroblasts from the SVZ to the lesion border. Previous reports have shown that in addition to the SVZ proper (vSVZ), pigs possess an extensive aSVZ containing neuroblast chains extending laterally similar to human infants and other mammalian species which migrate toward the lesion after neural injury [28, 34]. Neurogenesis after NSC transplantation has been well documented in rodent stroke models. These studies showed that hESC- and fetal-derived NSC treatment enhances cell proliferation and migration in the SVZ, DG, and striatum, and a subset of these cells co-label with the neuroblast marker DCX and the mature neuron marker Fox3, indicating endogenous neurogenesis mediated by NSC transplantation may contribute to neuronal replacement in the stroke-damaged brain [35-41]. In the current study we provide evidence that iNSCs exhibit similar neurogenic behavior in the stroke-damaged pig brain. To our knowledge, we are the first to provide evidence of how stroke and iNSC therapy effects neurogenic activity in the SVZ in a gyrencephalic species. Stroke alone stimulated neurogenesis at the vSVZ, but iNSC treatment augmented the neurogenic activity at the vSVZ and was important for increased neuroblast migration to the aSVZ and lesion border, likely through enhanced trophic factor signaling mediated by transplanted iNSCs. Indeed, BDNF and NOG, the gene encoding Noggin, expression was upregulated in brain tissue of iNSC treated animals and

have both been shown to stimulate neurogenesis from progenitor cells in the SVZ [31, 42, 43]. These results support a strong paracrine-mediated endogenous neurogenesis role of the transplanted iNSCs.

We found a higher number of neurons at the lesion border and dentate gyrus in iNSC treated animals compared to non-treated animals 12 weeks post-transplantation. Due to the limited number of identified engrafted iNSCs and migratory endogenous neuroblasts, it is unlikely that neuronal replacement would have such a profound effect on neuronal number by this time point. Therefore, we hypothesize that the observed increase in neurons at these sites is probably due to neuronal protection by iNSC trophic factor signaling. Polentes et al. demonstrated TH⁺ neuronal protection in the substantia nigra one month post-iNSC transplantation, an effect attributed to unknown iNSC-derived trophic factors acting on host substantia nigra afferents [14]. In the current study, we found increased expression of the neurotrophic factors BDNF, GDNF, and NTF3 in the brain tissue of iNSC treated animals, which have been shown to protect neurons from mechanisms related to ischemic insult [44-46]. Furthermore, we found increased expression of the gene encoding neurite branching mediator Rtn4 in iNSC treated brain tissue, which further indicates that iNSCs may foster neural repair mechanisms after stroke.

Neuroinflammation is a hallmark of stroke pathology and is a component of the secondary injury cascade in both human and preclinical animal models [47-49]. Microglia, the resident macrophages of the brain, proliferate and migrate to the lesion where they produce cytotoxic substances that exacerbate brain injury after an ischemic event [50]. Clinical studies have shown that the pharmacologic amelioration of microglia activity improves outcome after stroke [51, 52]. Our data indicates that iNSC treatment significantly reduced the number of

microglia at the lesion border by 12 weeks post-transplant, thereby diminishing pro-inflammatory signaling. Indeed, we found a significant decrease in the expression of pro-inflammatory cytokines CSF2, IL-1 β , and IL-6 in the brain tissue of iNSC treated animals [53, 54]. Similar to our findings, Huang et al. and Chang et al. documented reduced microglia activity in rodent stroke models after fetal-derived and iPSC-derived NSC treatment, respectively [13, 17, 32]. However, we found no iNSC-mediated effects on astrogliosis, another hallmark of cerebral secondary injury, in our model. Similarly, Oki et al. found that iNSC transplantation did not influence the number of activated astrocytes after stroke [13]. Although reduced astrogliosis after iNSC therapy has been documented in other rodent models of stroke, it is possible that the cell line tested in this study, or other therapeutic factors such as cell dose or treatment window, did not possess the required signaling capabilities to mitigate astrogliosis in the pig brain after stroke [17].

We demonstrate that our transplanted iNSCs differentiate into both neuronal and glial phenotypes by 12 weeks post-transplantation. The majority of the transplanted cells co-localized with the mature neuron marker NeuN while a smaller subset co-localized with the oligodendrocyte marker Olig2. Our data correlates with previous reports in rodents showing that transplanted iNSC preferentially take on a neuronal phenotype [13-15, 17]. Other groups have demonstrated astrocytic differentiation by iNSCs; however, we are the first to show evidence of robust oligodendrocyte differentiation by iNSCs after transplantation in a preclinical large animal stroke model [17, 38, 55-58]. We hypothesize that this finding may be attributed to the environmental signals inherent to a gyrencephalic brain with abundant white matter; external cues specifically mediated in white matter compartments may induce iNSCs to differentiate toward an oligodendrocyte fate [58, 59]. Future studies are needed to elucidate the different

signaling mechanisms needed to induce differentiation to region-specific phenotypes after iNSC transplantation in vivo.

Cerebral blood perfusion status is believed to be the most important factor in the clinical management of stroke because restoration of regional perfusion provides substantial help in salvaging endangered ischemic brain tissue [3]. In the present study, stroke-induced abnormalities in MTT, TTP, and CBV were significantly improved by iNSC therapy by 12 weeks post-transplantation. Previous studies have shown that NSC transplantation promotes the expression of the angiogenesis factor VEGF in endogenous tissues and augments angiogenic activity at the lesion border in rodent stroke models, resulting in improved perfusion [13, 37, 39, 40, 60, 61]. In the present study, we observed a significant increase in the expression of the angiogenesis marker Ang1 12 weeks after iNSC treatment, which may in part explain the observed improvement in cerebral perfusion after iNSC treatment. Previous reports show that transplantation of the fetal NSC cell line CTX0E03, which highly expresses the Ang1 protein, increases the amount of microvessels in the stroked rat brain. A recent Phase I clinical trial testing CTX0E03 (PISCES) noted functional improvement after treatment [62]. Thus, the expression of Ang1 coupled with improved cerebral perfusion in our model suggests increased cerebral microvessel density, which could improve functional recovery.

We also demonstrate that iNSC therapy leads to improved recovery of white matter integrity and brain metabolism by 12 weeks post-transplantation. Disruption of the myelinated and unmyelinated axon bundles present in white matter impairs communication between neurons in gray matter regions, often leading to motor dysfunction, neurobehavioral syndromes, and cognitive impairment [21]. Previous studies in rodent stroke models aimed to recapitulate white matter injury demonstrate that stem cell treatment facilitates white matter reorganization,

enhances connectivity, and improves FA through increases in myelination, axonal sprouting, and white matter bundle thickness in the ischemic boundary [63-68]. The use of the rodent model in these studies possesses certain limitations as the rodent brain contains only 14% white matter, while the human brain contains >60%; therefore white matter repair may be achieved more easily in the rodent than the human [21]. Furthermore, the PISCES trial demonstrated that NSC treatment increased FA value in white matter, a similar result observed in this study [62]. MRS results demonstrated recovery in neurometabolites NAA, Cho and Cr in iNSC treated animals, complementing our histological findings that iNSC treatment enhances neuronal survival and promotes tissue recovery. Previous studies in rodents demonstrate increased NAA abundance in the stroked hemisphere mediated by adult stem cell trophic factor signaling correlated with improved functional outcome [69, 70]. These results demonstrate notable enhancement in white matter structural and total brain metabolic recovery due to iNSC therapy.

In conclusion, we show for the first time in a translational large animal pig stroke model that iNSC therapy improves recovery through neuroprotective, regenerative, and cell replacement mechanisms resulting in improved white matter integrity, cerebral perfusion, and brain metabolism at the tissue level. These macro-level tissue changes in recovery were mechanistically supported with cellular level changes with iNSC treatment leading to reduced immune response, enhanced neurogenesis, and increased neuron survival. Finally, iNSCs survive long-term and differentiate predominantly into neurons. With the increased predictive potential of testing iNSC therapy in a gyrencephalic pig stroke model, these findings support that iNSC therapy may serve as a robust therapeutic in human patients and are deserving of further study.

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Figure 7.1: Induced Pluripotent Stem Cell-Derived Neural Stem Cells Express Sox1 and Nestin In Vitro

iNSCs expressed the neural stem cell markers Sox1 (A) and Nestin (B; co-localization of Sox1, Nestin, and DAPI (C)).

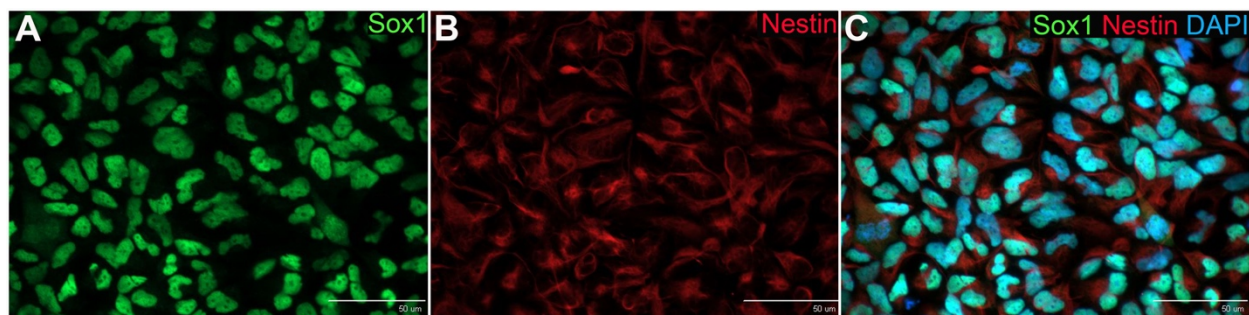


Figure 7.2: Ischemic Stroke Results in Significant Brain Swelling and Atrophy

MRI was performed 24 hours post-stroke and 1, 4, and 12 weeks post-transplant. Diffusion weighted imaging (DWI) demonstrated abnormal territorial hyperintensity in the right cerebral hemisphere 24 hours post-stroke (A). Corresponding ADC maps showed hypointense signal at the lesion site, validating the presence of ischemic stroke (B). T2-weighted FLAIR images displayed initial brain swelling in the ipsilateral hemisphere at 24 hours post-stroke (C) that subsides by 1 week post-transplant (D). T2-weighted FLAIR images taken at 4 weeks (E) and 12 weeks (F) post-transplant showed significant brain atrophy at the area of the infarct. Volumetric measurements on T1-weighted images supported these changes (G). Data are expressed as mean $\pm$ s.e.m. * indicates significance from the volume of the contralateral hemisphere ($p < 0.05$).

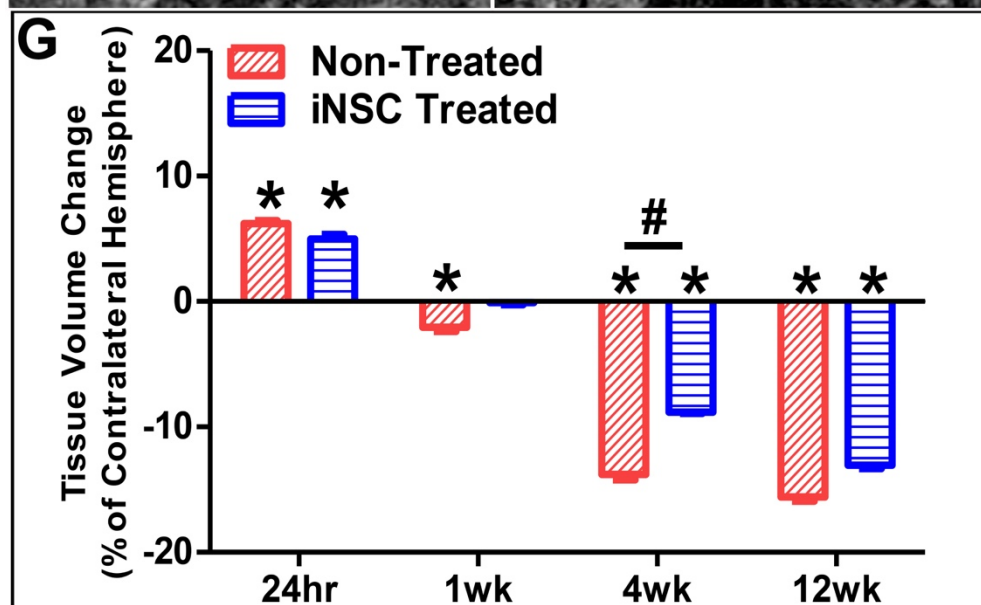
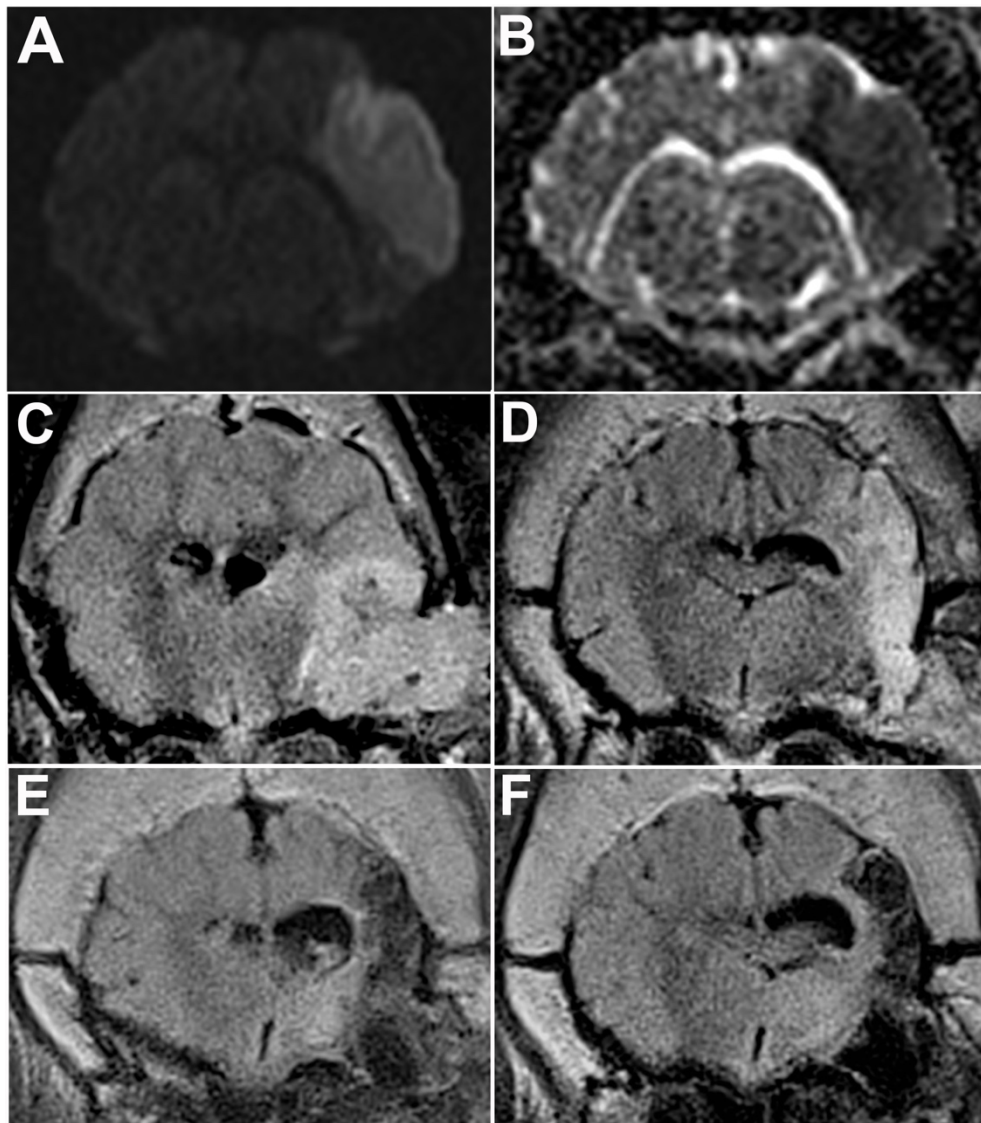


Figure 7.3: iNSC Treatment Results in Improved White Matter Integrity After Stroke

DTI was performed 24 hours post-stroke and 1, 4, and 12 weeks post-transplant, and FA was calculated within the peri-infarct of the ipsilateral hemisphere and a comparable anatomical region on the contralateral hemisphere. Representative FA maps of non-treated (A) and iNSC treated (B) animals. The average change in FA in the abnormal territory relative to the contralateral normal control hemisphere was calculated at each time point, and iNSC treatment resulted in enhanced recovery of FA in the ipsilateral hemisphere 12 weeks post-transplant compared to 24 hours post-stroke (C). Data are expressed as mean $\pm$ s.e.m. * indicates significance in FA from 24 hours post-stroke within treatment ($p < 0.05$).

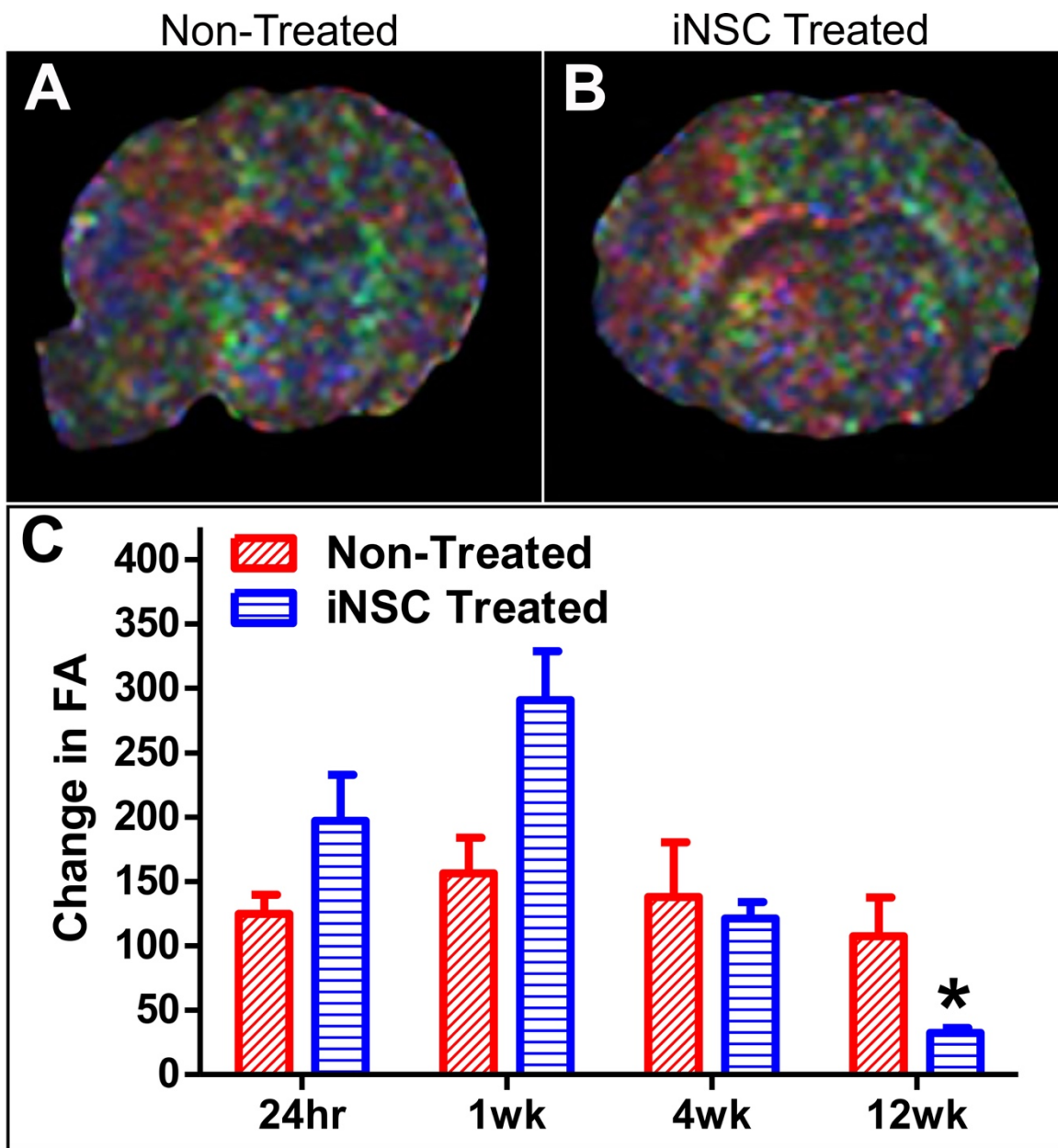


Figure 7.4: iNSC Treatment Leads to Recovery in Neurometabolite Abundance After Stroke

MRS measured the abundance of the neurometabolites NAA, Cho, and Cr 24 hours post-stroke and 1, 4, and 12 weeks post-transplant. Representative MRS spectra of non-treated (A) and iNSC treated (B) animals. The average difference in neurometabolite abundance between the ipsilateral and contralateral hemispheres was calculated and expressed as a change in the ipsilateral hemisphere relative to the contralateral hemisphere. iNSC treatment significantly enhanced NAA abundance in the ipsilateral hemisphere 4 weeks and 12 weeks post-transplant compared to 24 hours post-stroke, and NAA abundance was closer to that of the contralateral hemisphere in the iNSC treated animals compared to non-treated animals at 1, 4, and 12 weeks post-transplant (C). Cho abundance was significantly improved in the ipsilateral hemisphere at 4 and 12 weeks post-transplant compared to 24 hours post-stroke, and Cho levels were recovered in iNSC treated animals compared to non-treated animals both 4 and 12 weeks post-transplant (D). Cr abundance was significantly enhanced in iNSC treated animals compared to non-treated animals at 1 and 12 weeks post-transplant, while Cr levels were significantly improved at 4 weeks post-transplant compared to 24 hours post-stroke (E). Data are expressed as mean $\pm$ s.e.m. * indicates significance between treatment groups within time point ($p < 0.05$). # indicates significance from 24 hours post-stroke within treatment ($p < 0.05$).

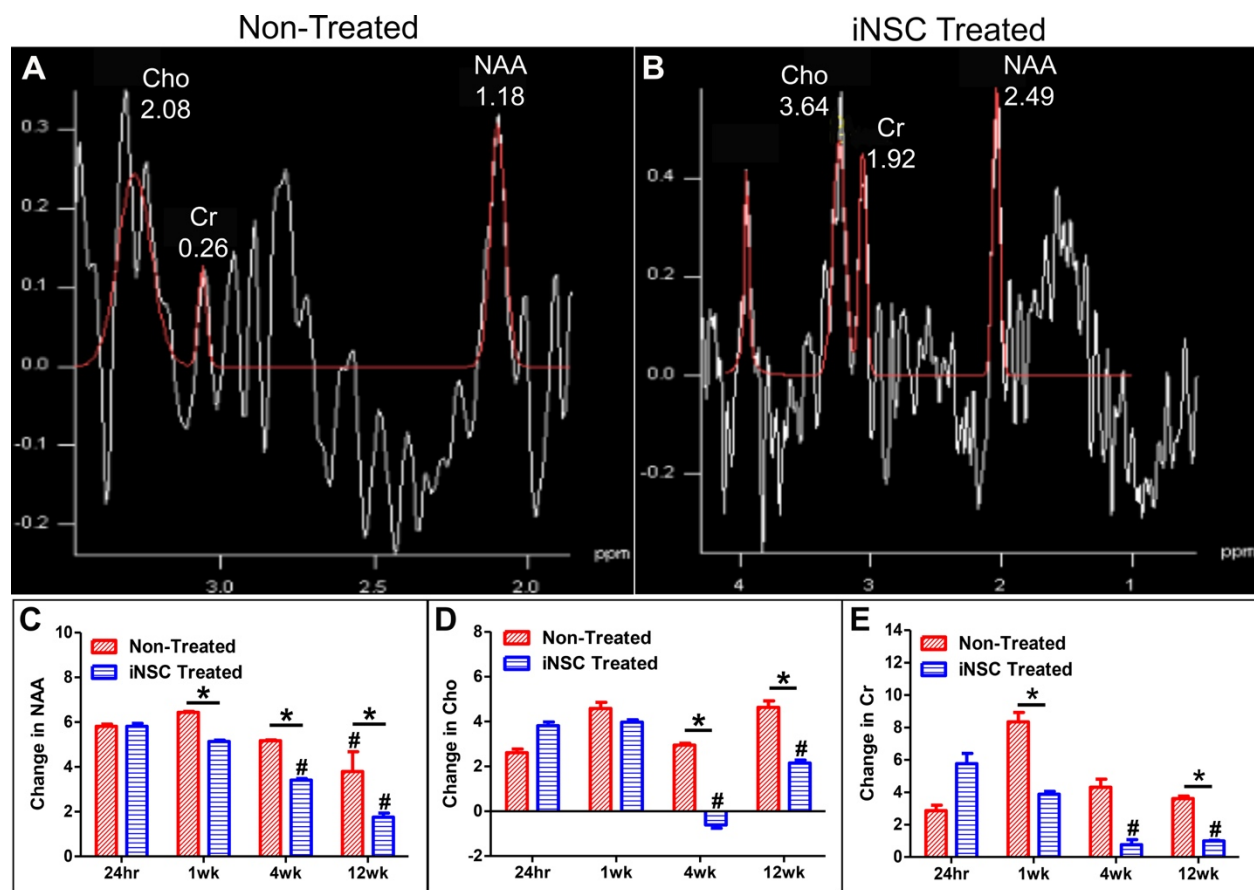


Figure 7.5: iNSC Treatment Improves Cerebral Blood Perfusion in the Infarcted Area

PWI was utilized to measure MTT, TTP, and CBV in the infarct region. Representative perfusion maps of non-treated (A) and iNSC treated (B) animals indicating increased cerebral perfusion in the infarct (white arrows) in iNSC treated animals at 12 weeks. MTT was significantly reduced in iNSC treated animals compared to non-treated animals 12 weeks post-transplant (C). TTP was significantly reduced in iNSC treated animals compared to non-treated animals 12 weeks post-transplant (D). CBV was significantly increased in iNSC treated animals 12 weeks post-transplant compared to 24 hours post-stroke (D). Data are expressed as mean $\pm$ s.e.m. * indicates significance between treatment groups within time point ($p < 0.05$). # indicates significance from 24 hours post-stroke within treatment ($p < 0.05$).

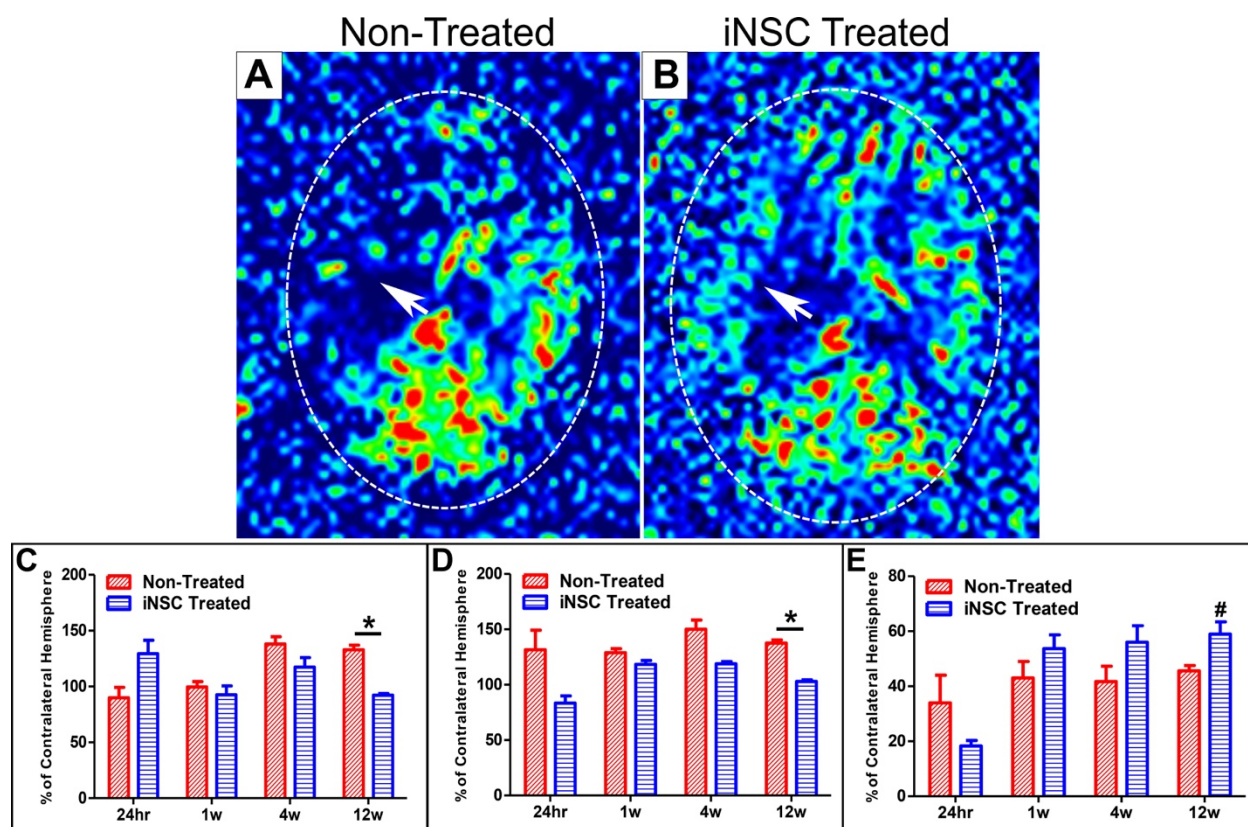


Figure 7.6: iNSC Treatment Aids in the Protection of Neurons and Reduces Microglia

Activation at the Lesion Border

Quantification of NeuN+ cells/mm² at the lesion border shows significant reduction of neurons in non-treated animals but no significant change between normal controls and iNSC treated animals (A). Representative image of NeuN+ neurons at the lesion border in normal control (B), non-treated (C), and iNSC treated (D) animals. Quantification of the percentage of cells that were NeuN+ in the polymorphic layer of the dentate gyrus (DG) demonstrated a significant reduction in neurons in non-treated animals but no significant change between normal control and iNSC treated animals (E). Representative image of NeuN+ neurons in the polymorphic layer of the DG in normal control (F), non-treated (G), and iNSC treated (H) animals. In addition, a larger proportion of NeuN+ cell bodies seemed shrunken in non-treated animals (G).

Quantification of the percentage Iba1+ area at the lesion border shows a significant increase in Iba1+ area in stroked animals compared to normal control animals and a significant reduction in Iba1+ area in iNSC treated animals compared to non-treated animals (I). Representative image of Iba1+ microglia at the lesion border in normal control (J), non-treated (K), and iNSC treated (L) animals. Quantification of the percentage GFAP+ area demonstrates a significant increase in GFAP+ area in both non-treated and iNSC treated groups compared to normal control animals (M). Representative image of GFAP+ astrocytes at the lesion border in normal control (N), non-treated (O), and iNSC treated (P) animals. Data are expressed as mean $\pm$ s.e.m. * indicates significance from normal controls ($p < 0.05$). # indicates significance between treatment groups ($p < 0.05$). Scale bars, B-D, J-L, N-P, 500 μ m; F-H, 100 μ m.

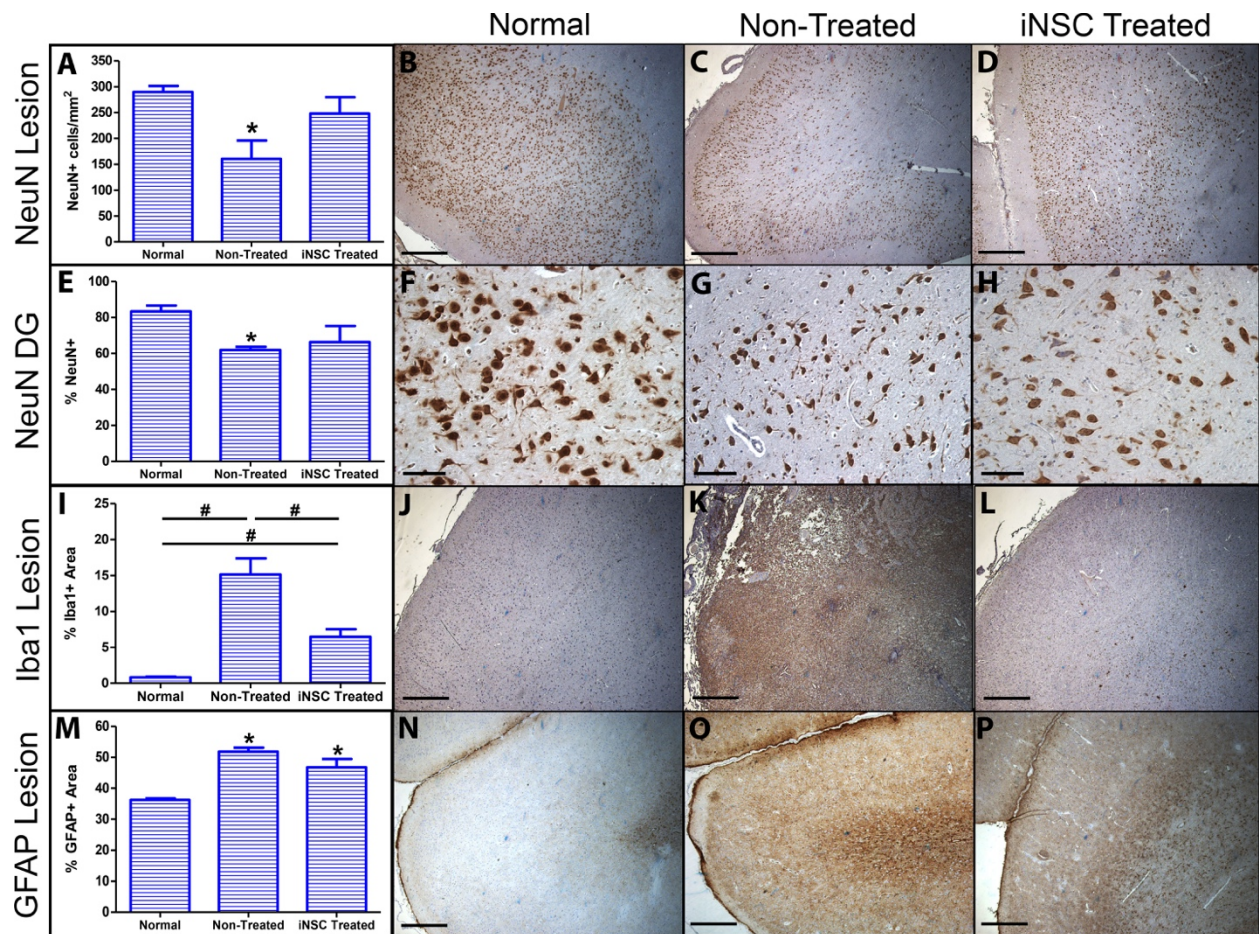


Figure 7.7: iNSC Treatment Promotes Endogenous Neuroblast Proliferation and Migration to the Lesion Border

Quantification of DCX+ cells/mm² in the vSVZ demonstrated a significant increase in DCX+ neuroblasts in non-treated animals compared to normal control animals and a significant increase in DCX+ neuroblasts in iNSC treated animals compared to non-treated animals (A).

Representative image of DCX+ neuroblasts at the vSVZ in normal control (B), non-treated (C), and iNSC treated (D) animals. Quantification of DCX+ cells/mm² in the abventricular subventricular zone (aSVZ) showed a significant increase in DCX+ neuroblasts in iNSC treated animals compared to both non-treated and normal control animals (E). Representative images of DCX+ neuroblasts at the aSVZ in normal control (F), non-treated (G), and iNSC treated (H) animals. Quantification of DCX+ cells/mm² at the lesion border demonstrated a significant increase in DCX+ neuroblasts in iNSC treated animals compared to both non-treated and normal control animals (I). Representative image depicting minimal DCX+ neuroblasts in normal control animals (J). Representative image of DCX+ neuroblasts (black arrows) in non-treated (K) and iNSC treated (L) animals. Black dotted square represents inset in J-K. Data are expressed as mean $\pm$ s.e.m. * indicates significance between treatment groups ($p < 0.05$). Scale bars, B-D, F-H, J-L, 100 μ m.

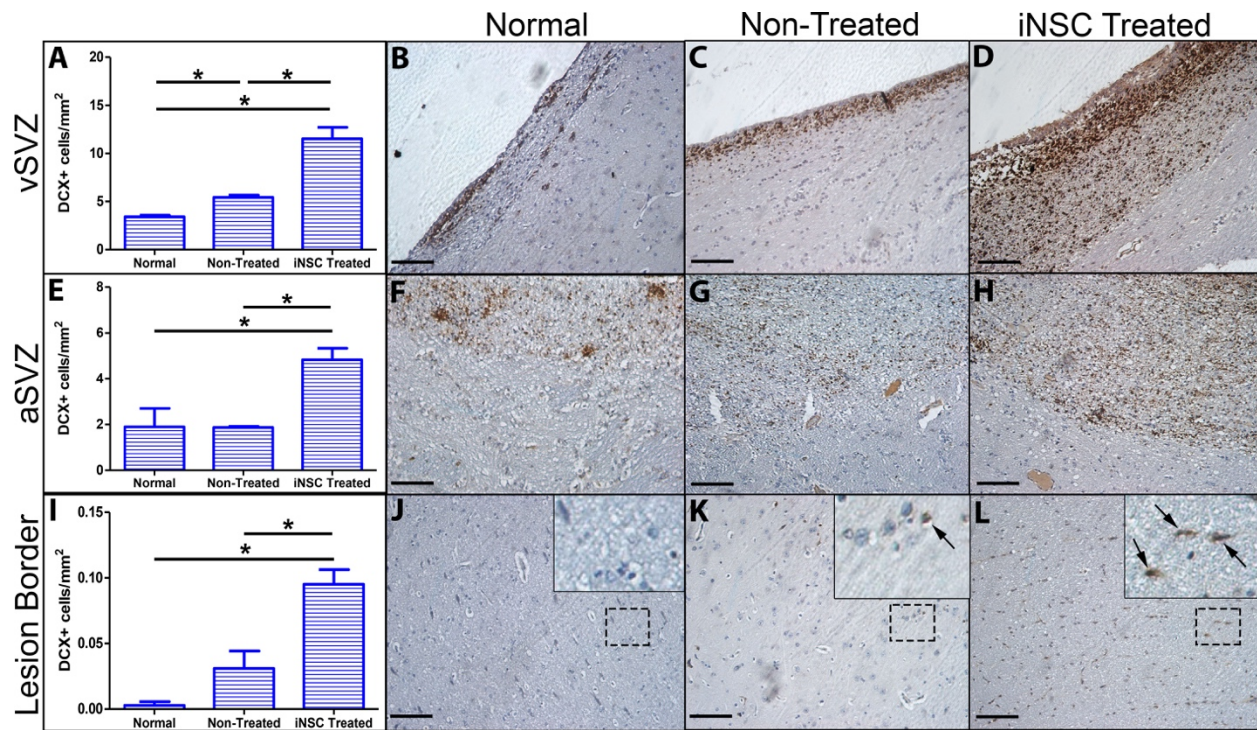


Figure 7.8: iNSCs Survive Long-Term and Differentiate into Neurons and Oligodendrocytes in the Pig Brain After Stroke

Pigs were sacrificed at 12 weeks post-transplant, and transplant sites underwent immunohistochemistry to characterize iNSC differentiation. Representative image of HNA+ human cells in the brain parenchyma co-localizing with the mature neuron marker NeuN (A-C, white arrows). Quantification of HNA+ cells co-localized with either NeuN or Olig2 revealed both neurons and oligodendrocyte populations derived from transplanted iNSCs (D). Representative image of HNA+ cell colocalized with NeuN displaying distinct neuronal morphology (E-G). Representative images of HNA+ cells colocalized with the oligodendrocyte marker Olig2 (H-J). Data are expressed as mean $\pm$ s.e.m.

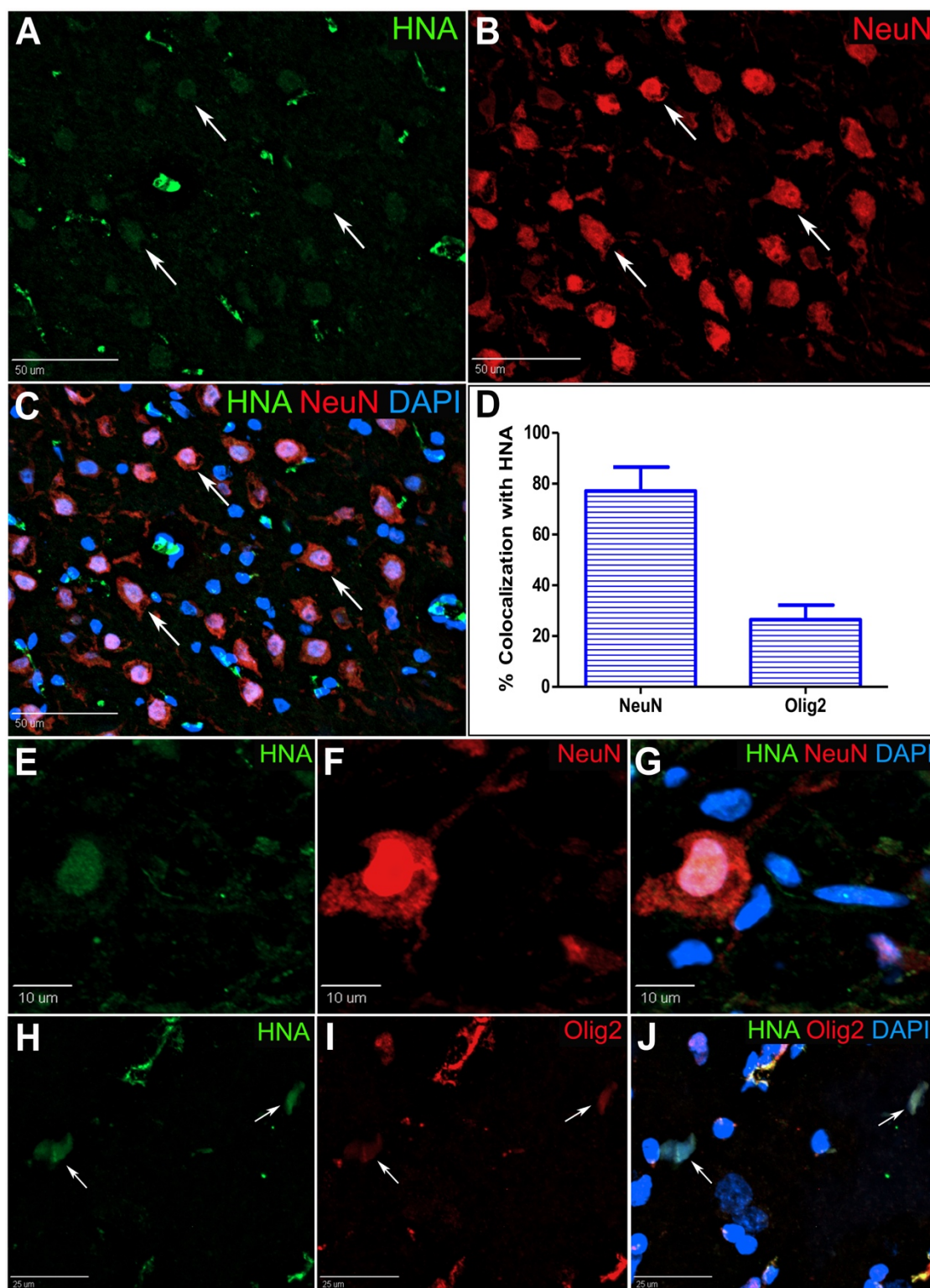
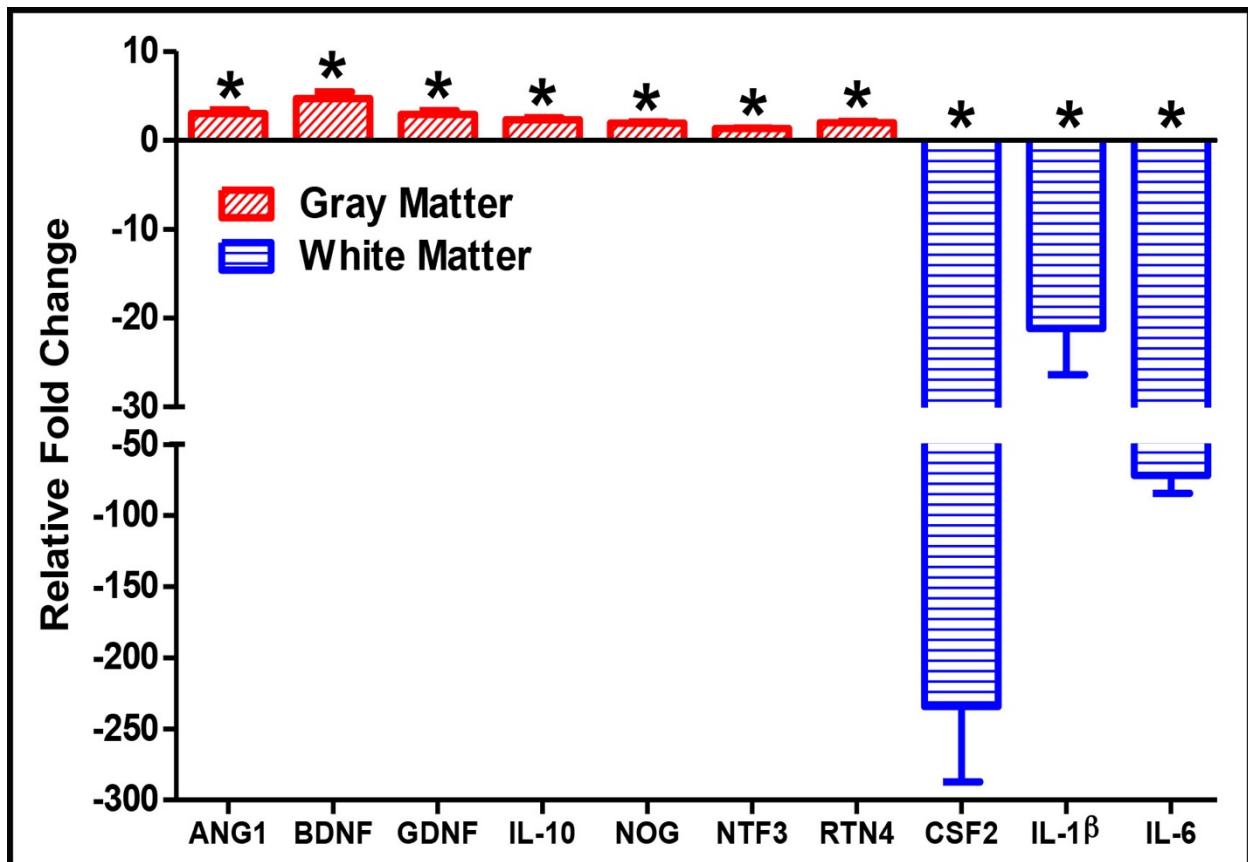


Figure 7.9: iNSC Treatment Leads to Distinct Changes in Gene Expression Related to Tissue Recovery in Gray and White Matter

Quantitative RT-PCR was performed on iNSC treated and non-treated gray and white matter tissue samples. Graph represents relative fold change of iNSC treated gray and white matter relative to non-treated gray and white matter samples. There was a significant upregulation in the expression of the genes ANG1, BDNF, GDNF, IL-10, NOG, NTF3, and RTN4 in iNSC treated gray matter samples relative to non-treated gray matter samples. There was a significant downregulation in the expression of the genes CSF2, IL-1 β , and IL-6 in iNSC treated white matter samples relative to non-treated white matter samples. Data are expressed as mean $\pm$ s.e.m.

* indicates significance from non-treated sample within tissue type ($p < 0.05$).



CHAPTER 8

CONCLUSION

The potential to replace lost neurons and foster neural repair after injury has significant biomedical and socioeconomic implications worldwide. Previous studies have shown that induced pluripotent stem cell-derived neural stem cells (iNSCs) promote tissue recovery after neural injury through neuroprotective, neurogenic, angiogenic, and cell replacement mechanisms [1-4]. To date, these studies have been carried out in rodent models due to their low cost and highly characterized outcome measures [5]. However, it has been noted that hundreds of agents for stroke and TBI appear to be highly effective in preclinical rodent studies but have shown no efficacy in humans [6], which has dampened the enthusiasm of iNSC therapy as an effective therapeutic for human patients. Thus, it is thought that one of the major reasons underlying clinical therapeutic failure for these neural injuries is the lack of translatability from preclinical animal models to human patients. This significant shortcoming suggests that the development of clinically translatable large animal models, such as the pig, could bolster preclinical therapeutic development strategies and make preclinical data more predictive of what will occur in humans.

To address this issue, the use of pigs as preclinical stroke and TBI models has gained more interest due to their similar brain cytoarchitecture and composition to humans. However, injury models and outcome measures are not as well characterized in the pig model compared to the rodent model, and more therapeutic development strategies in the pig are needed to determine if large animals truly carry greater predictive power of clinical efficacy. To this end, the studies in this dissertation consisted of two major aims: 1) to develop a novel controlled

cortical impact (CCI) model in the piglet with quantifiable deficits at the cellular, tissue, and functional levels to serve as a translational platform for restorative cellular therapeutics, and 2) to determine whether iNSC transplantation improves tissue recovery in a preclinical stroke pig model.

The first set of studies developed and characterized a clinically relevant pig controlled cortical impact (CCI) TBI model that captures quantifiable pathoanatomic and functional features across a range of injury severities. In the clinic, while most TBI patients have sustained mild TBI, a large subset of patients have moderate to severe TBI and exhibit long-term deficits in cognition and/or motor function [7-9]. These general classifications (mild, moderate, severe) are largely determined through the Glasgow Coma Scale (GCS), which does not account for pathoanatomic alterations at the tissue level [10]. Thus, the heterogeneity of TBI coupled with the lack of biologically informative and standardized scales to adequately categorize multiple types of TBI remains to be a major challenge facing therapeutic development.

In the present studies, we first show that increasing CCI parameters results in scalable and reproducible focal brain injury. The mildest impact parameters resulted in small albeit measurable neuronal damage and astrogliosis while more severe impact parameters produced large necrotic contusions with extensive white matter degeneration. Quantitative gait analysis revealed that pathoanatomic alterations correlated with increased deficits in motor function. These results suggest that adjusting CCI parameters in our pig model can produce clinically relevant brain pathologies ranging from mild contusion to severe, debilitating brain injury with quantifiable, injury-specific pathological features. Furthermore, utilizing an untargeted metabolomics approach, we show that scaling impact parameters results in unique metabolic signatures dependent on injury severity, time after injury, and brain tissue type. These findings

could potentially bolster current TBI classification scales by categorizing TBI based on the global metabolome to predict injury severity and brain structures affected, thereby gaining integral biological information in order to accurately predict patient outcomes or identify therapeutic targets in a personalized manner.

Next, we expanded on our pig TBI model by assessing cellular and tissue changes over time utilizing advanced, multiparametric magnetic resonance imaging (MRI) and spectroscopy (MRS) approaches. At 24 hours post-TBI, vasogenic and, to a lesser degree, cytotoxic edema accumulation resulted in increased ipsilateral hemisphere volume and corresponding midline shift with evidence of disturbed cerebral blood flow (CBF) and brain metabolism. By 12 weeks post-TBI, parenchymal atrophy resulted in a directional change in midline shift, increased mean apparent diffusion coefficient (ADC) likely due to cyst formation and/or gliosis, and improved albeit abnormal CBF. Furthermore, disruptions in white matter integrity began within 24 hours post-TBI and was sustained through 12 weeks post-TBI. These tissue-level deficits measured by MRI were confirmed histologically with evidence of progressive neuronal loss, immune response, gliosis, and oligodendrocyte pathology. These cellular and tissue level changes closely resemble TBI sequelae in human patients. This study highlights longitudinal pathoanatomic features measured non-invasively, providing a unique translational platform to further study TBI pathophysiology and recovery mechanisms for application to the clinical setting.

In the second set of studies, we explored iNSC-mediated tissue repair mechanisms after stroke in a pig model. For the first time, we demonstrate in a large animal model utilizing clinically translatable approaches (MRI and MRS) that iNSCs promote recovery after stroke through neuroprotective, regenerative, and cell replacement mechanisms. iNSC therapy stimulated endogenous neurogenesis and neuroblast migration to the lesion border, mitigated

neuronal death in the dentate gyrus and at the lesion border, reduced the inflammatory and immune response, improved penumbral perfusion, and improved white matter integrity as well as brain metabolism after stroke likely through the production of regenerative trophic factors. Furthermore, transplanted iNSCs differentiated into neuronal and glial phenotypes in the stroke-damaged brain. These findings confirm previous reports in rodent models by validating iNSC therapeutic mechanisms in a more predictive gyrencephalic large animal stroke model, and further supports that iNSCs may be the critically needed stroke therapeutic.

Pitfalls and Future Studies

Although the implications of these studies significantly progress preclinical strategies behind TBI and stroke therapeutic development, there are a number of concerns that remain to be addressed as well as a number key experiments that will be carried out in the future. A common limitation between both overarching studies was the difficulty in obtaining reliable, consistent, clinical-quality MRI data due to a number of challenges including the presence of hemorrhage, small cerebral volume, increased lipid layer near the skull, and respiration during scanning. These factors limited consistent region of interest (ROI) placement while data was unable to be obtained for a subset of animals.

In agreement with other CCI models, our TBI model produces graded focal injury consisting of cortical contusion, edema accumulation, neuronal loss, and inflammation. Although not directly assessed, it is unlikely that our model produces prominent diffuse axonal injury (DAI) based on these previous reports [11]. Since DAI is a common and important feature of human TBI and could be easily studied in the pig since they possess abundant white matter [12], we would like to examine brains for evidence of diffuse injury in both the contralateral and

ipsilateral hemispheres. However, the presence of diffuse injury would require a new approach to our MRI analysis methodology since the the contralateral hemisphere is currently utilized as a baseline control. Furthermore, we documented improvements in stride velocity in our 4m/s; 12mm TBI group by 3 days post-TBI as well as recovery in cerebral blood flow by 12 weeks post-TBI in our longitudinal study. However, we did not assess the underlying spontaneous recovery mechanisms in these animals. It is important to elucidate these recovery modalities especially when we apply our iNSC transplantation methodology to the pig TBI model and evaluate iNSC-mediated improvements in TBI outcome. To this end, future studies will determine whether tissue and functional recovery can be augmented by iNSC transplantation through similar mechanisms documented in our pig stroke model. Furthermore, it is possible that more sensitive functional tests would delineate long-term motor deficits more robustly after mild to moderate TBI as well as incremental improvements between treated and non-treated animals.

Although our pig stroke model is one of the few of its kind, it has inherent disadvantages related to the methodology of stroke induction. Many rodent stroke models induce ischemia utilized an intraluminal suture approach in which a monofilament is advanced to the base of the middle cerebral artery (MCA) and retracted after several minutes to mimic spontaneous reperfusion [13]. In contrast, due to the vascular anatomy of the pig, ischemic stroke in our model is produced through a transcranial approach and permanent MCA occlusion. This approach has two limitations: 1) the craniectomy site cannot be fully resealed after surgery, which results in parenchymal herniation through the craniectomy site and alterations in intracranial pressure, and 2) no reperfusion occurs after MCA occlusion although this is an important pathological feature in the clinic and occurs in approximately 18% of stroke patients [14, 15]. Furthermore, we were unable to obtain functional data from these animals due to high

variability and lack of sensitivity in the assessments. One of our future goals is develop more sensitive, comprehensive functional assessments to detect subtle changes in recovery after iNSC treatment. We plan to carry out these studies long-term (as performed here) to fill in the current knowledge gap of functional recovery in the chronic stages after neural injury in preclinical animal models [16].

In the future, we will perform a more in-depth assessment of iNSC integration into the host tissue and factors that influence functional integration. Specifically, we will assess the ability of transplanted iNSCs to differentiate into region-appropriate, specialized neuronal cell types. We will evaluate whether these cells undergo synaptogenesis and send axons to the appropriate target locations. From a functional perspective, we will assess whether cell replacement contributes to long-term functional recovery over trophic factor signaling alone. Beyond iNSC integration characteristics, further studies addressing logistical factors such as iNSC treatment window, number and location of injections, and number of cells transplanted will be conducted to maximize treatment efficacy.

In conclusion, we have developed an enhanced translational platform to longitudinally and quantitatively study injury and recovery mechanisms after TBI and stroke, which may hold greater predictive power when testing novel therapeutics for clinical applications. Indeed, previous reports of iNSC-mediated recovery mechanisms were confirmed in our pig stroke model. These studies have major implications to the biomedical community by strengthening current preclinical TBI models and providing further evidence of iNSC efficacy in a translational pig stroke model. Although future studies are needed to further validate the translational capacity of these models, these findings could augment the chances for successful clinical trials of iNSCs and other restorative therapies that improve recovery in stroke and TBI patients.

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