Analysis of Immediate Cellular Changes in Porcine Brains Using an Ex Vivo Model of
Traumatic Brain Injury

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Abstract

Traumatic brain injury (TBI) is one of the most common forms of injury in the world, affecting millions worldwide. Given the significance of TBI, the need for a better understanding of the neural responses that occur during a head impact is of utmost importance. Computer models have provided a wealth of knowledge of the biomechanics of brain movement after a TBI given the closed system nature of the skull. The movement of the head and the resulting inertial forces have been shown to create high amounts of strain within the brain, in particular at the curve in the sulcus. Cellular changes occur after an impact to the head that may begin the progression of neurodegenerative diseases. Given the vulnerability of the sulcus and the damage that occurs there, the traditional rodent model is not a suitable model for translating from animal studies to what occurs in humans. Given the similarities to the human brain, pig brains were used to investigate the biomechanical forces at play and the cellular changes that occur in the sulcus of the brain after an impact. In a normal pig brain, the apex of the sulcus showed a higher density of neurons compared to the adjacent arms of the sulcus. The Golgi-Cox stain was used to visualize neurons within the pig brain in more detail. One hour after an *ex vivo* impact, there was a decrease in MAP2 cell density only in the apex of the sulcus of the impacted tissue. There was no change in overall neuronal density between conditions or changes in neuron size, one hour after impact. The change in MAP2 cell density could reveal an early indicator of cytoskeletal rearrangement following impact. While there was no change in neuron density that accompanied the change in MAP2 levels, it could be an indication of compromised cellular integrity. This could lead to a window of vulnerability if a second impact was to occur.

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Abbreviations

5-HT	Serotonin
A β	Amyloid-Beta
ABC	Avidin-Biotin Complex
aCSF	Artificial Cerebral Spinal Fluid
ALS	Amyotrophic Lateral Sclerosis
APP	Amyloid Precursor Protein
ATP	Adenosine Triphosphate
Ca ²⁺	Calcium
CaCl ₂	Calcium Chloride
CaMKII	Calcium-calmodulin Kinase II
CSF	Cerebral Spinal Fluid
CTE	Chronic Traumatic Encephalopathy
DAB	Diaminobenzene
DAMP	Damage-associated Molecular Patterns
DNA	Deoxyribonucleic Acid
EEG	Electroencephalogram
EPSP	Excitatory Postsynaptic Potential
FDOPA	Fluorodopa
GCS	Glasgow Concussion Scale
GTP	Guanosine-5'-triphosphate
GDP	Guanosine-5'-diphosphate
IL-6	Interleukin-6
K ⁺	Potassium
KCl	Potassium Chloride
MAP	Microtubule Associated Protein
MAP2	Microtubule Associated Protein 2
MARK	Microtubule Affinity Regulating Kinase
MAPK	Mitogen-Activated Protein Kinase
M/s	Meters/Second
MgSO ₄	Magnesium Sulphate
MRI	Magnetic Resonance Imaging
Na ⁺	Sodium
NaHCO ₃	Sodium Bicarbonate
NaCl	Sodium Chloride
NaH ₂ PO ₄	Sodium Phosphate
NMDA	n-methyl-d-aspartate
PBS	Phosphate Buffered Saline

PET	Positron Emission Topography
PFA	Paraformaldehyde
PKA	Protein Kinase A
SNTF	α II-spectrin N-terminal fragment
STEP-61	Striatal-Enriched Protein Tyrosine Phosphatase
TBI	Traumatic Brain Injury

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Introduction

Traumatic Brain Injury

Traumatic brain injuries (TBI) are a serious medical concern with millions of reported cases in North America each year (Taylor et al., 2017). The causes of TBI range from physical sports such as ice hockey, football, and soccer (Kiernan et al., 2015; Omalu et al., 2005; Tuominen et al., 2017), to self-harming behaviours such as head banging (Lee et al., 2017) to soldiers exposed to blast waves in combat zones (Goldstein et al., 2012). TBI due to falling is among the leading cause of injury in the elderly due to their decline in physical abilities such as gait, balance, and coordination (Ghajar, 2000; Taylor et al., 2017). Not only is there an immediate health concern, but the indirect cost ascribed to Canadians suffering from TBI is estimated to be \$7.4 billion, with projected costs reaching \$8.4 billion by 2031 (Bray et al., 2014). This is compared to the indirect cost of \$600 million associated with Canadians suffering from other neurodegenerative diseases such as Alzheimer's Disease and Parkinson's Disease (Bray et al., 2014). Indirect costs are defined as the total amount of money lost due to working-age deaths and disabilities associated with a particular condition. Considering how easily a TBI can occur, it is no surprise that the total indirect costs are considerably high.

In the general population, most hospitalized cases of TBI are the result of motor vehicle accidents, falls, or unexpected blows to the head, with rates being higher in males than females (Blennow et al., 2016; Mortimer et al., 1991; Taylor et al., 2017). TBI's are commonly categorized into 3 types based on the Glasgow Concussion Scale (GCS) diagnostic tool: mild, moderate and severe (Yamamoto et al., 2018). Mild TBI, often referred to as a concussion, is

considered to be the most commonly reported subtype but often goes undiagnosed (Blennow et al., 2016). Diagnosis of mild TBI requires a GCS score between 13-15 and is often accompanied by impaired memory, difficulty concentrating, becoming emotionally unstable, nausea and vomiting. These symptoms usually persist for roughly 7 days (Blennow et al., 2016). Moderate TBI requires a GCS score of between 9-12 with increased severity of previously described symptoms in addition to prolonged loss of consciousness typically leading to hospitalization (Marshall and Riechers, 2012). Diagnosis of severe TBI is based on a GCS of 3-8 and is often accompanied by a skull fracture or close exposure to blast waves (Maas et al., 2008; Yamamoto et al., 2018). Severe TBI's are accompanied by subdural haematomas as well as hemorrhagic lesions and often lead to long term disabilities, vegetative states and death (Bigler and Maxwell, 2011; Ghajar, 2000). These cases of severe TBI make up a very small portion of hospital-admitted TBI cases.

Biomechanics of Traumatic Brain Injury

Advancements in computer engineering have allowed researchers to get a better understanding of the biophysics of a TBI. Because the skull is a closed system, it makes it difficult to study the mechanics and forces involved in head impacts *in vivo*. The ability to simulate TBI accurately allows for a framework to focus in on particular aspects during a head impact. Findings from computer models can then be correlated to experimental results to further improve both simulated and *in vivo* models. One of the most commonly used computer models for TBI is the finite element model (Ghajari et al., 2017; Zhang et al., 2001). Finite element modelling takes into account all factors associated with skull and brain deformation (such as

skull geometry, differences in material response rates, non-linear and time-dependent changes) and makes it possible to simulate various types of head impacts (Ghajari et al., 2017).

Calculations of internal stress, changes in pressure and complex loading conditions are done using a 3-D model of the skull and brain based on images collected from MRI scans. Finite element modelling can take into account all the unique properties of the major internal brain structures, such as ventricles, the corpus callosum, cerebral cortex, cerebral spinal fluid, blood vessels, to predict the outcome of head impacts (Takhounts et al., 2008). Using finite element modelling, Zhang and colleagues (2001) found that lateral impacts to the skull produce greater changes to intracranial pressure compared to frontal impacts due to the shape of the skull and the deflection of forces exerted on the skull.

There are two broad categories that describe the forces resulting in a TBI: contact and inertial forces (Stemper and Pintar, 2012). Contact forces occur at the site of impact and are more likely to result in skull fracture as would occur following moderate and severe TBI. Inertial forces are comprised of linear and rotational acceleration of the head and often result in concussions or mild TBI's (Stemper and Pintar, 2012). In a scenario where there is an impulsive head movement with no contact to the head (i.e. whiplash) only inertial forces are exerted onto the brain as there is no impact to the skull (Stemper and Pintar, 2012). In a scenario where the head is impacted (i.e. falling and hitting the head on the ground), both forces occur one after the other. The skull and focal brain region experience the contact force from the impact, followed by the inertial forces and movement of the brain. The inertial forces lead to increased deformations within the brain as deep brain structures move at a different speed than outer structures and continue to rotate even when the head has stopped moving (Gomez et al., 2018). This

deformation of the tissue with respect to time can be calculated to give the strain rate exerted on structures within the brain. Strain rate can be defined as a material being subjected to a parallel shear without the change to its original volume to produce a single number. This calculated number is the ratio of the initial length to the change in length that the material has experienced (Noyes et al., 1984).

Information on how much force is exerted on to a particular area can be quantified and visualized to represent the biomechanical characteristics of TBI. Ghajari and colleagues (2017) demonstrated that the highest levels of strain can be found within the depths of the sulci of the brain. These high levels of strain map to the pathology seen in human autopsies of American football players diagnosed with chronic traumatic encephalopathy (CTE; McKee et al., 2013). Analysis of impact velocity from in game footage of American football was compared to fall injury impact using silicone surrogates. These data revealed that fall impacts had three times faster acceleration and larger strain in the cortical sulci than impacts experienced during football collisions. However, the duration of impact was greater in football collisions than those seen during a fall (Ghajari et al., 2017). Along with the high strain transmitted to the sulci, another area that receives high strain fields is the corpus callosum (Laksari et al., 2018). These models have predicted areas of damage that are seen *in vivo*, particularly the depths of the sulci. The high amounts of strain exerted on to the sulci results in cellular changes and the development of neurodegenerative diseases.

Animal Models of Traumatic Brain Injury

While computational models have provided a host of knowledge regarding the biophysics and biomechanical forces involved in TBI, *in vitro* and *in vivo* experimentation are still required. The end result of these biomechanical forces exerted on the brain during a TBI culminate into cellular dysfunction and the potential onset of neurodegenerative diseases. To better understand these neurodegenerative diseases as a whole, changes in cellular functioning must first be addressed. To do this, mechanically stretching cultured neurons is a common method used to investigate the cellular response of shearing and stretching that occurs during a TBI.

Cation Changes Following TBI

One of the major aspects of neuronal communication is the propagation of electrical signals through the axon to the synaptic terminal. If the axon is damaged during a TBI, cellular communication can become impaired leading to decreased cellular functioning and viability (Johnson et al., 2016). When the head is impacted, the axons within the brain become compressed and stretched, causing the axons to swell due to axonal microtubules being disrupted (Gennarelli et al., 1982). Axonal stretching increases membrane permeability leading to an influx of cations, such as Ca^{2+} and K^{+} , into the cell (MacFarlane and Glenn, 2015; Tao et al., 2017; von Reyn et al., 2009). The influx of Ca^{2+} initiates various neurometabolic cascades that will be further discussed in a later section. Interestingly, it has been reported that the rapid influx of K^{+} is enough to trigger aberrant depolarization, initiating the release of glutamate into the synaptic cleft (MacFarlane and Glenn, 2015). The excitotoxic effect of excess glutamate release is one of the major contributing factors to the neurometabolic cascade that follows damage to neurons and the pathology associated with TBI.

Calcium Specific Changes and Mitochondrial Dysfunction

As previously mentioned, damage to axons leads to an influx of Ca^{2+} within the cell. Moreover, the binding of glutamate to NMDA receptors promotes additional influx of Ca^{2+} (MacFarlane and Glenn, 2015). This increase in Ca^{2+} occurs immediately following *in vitro* axonal stretch and remains elevated up to 24 hours post injury (Staal et al., 2010). Under normal physiological conditions, the mitochondria play a critical role in the production of energy for the cell through ATP synthesis. Working with the endoplasmic reticulum, the mitochondria also play a role in buffering levels of Ca^{2+} within the cell. The mitochondria become overworked when there is an overload of Ca^{2+} leading to dysfunction of the mitochondria, and the production of reactive oxygen species (Kim et al., 2012; Tortora et al., 2017; Vercesi et al., 2018). This dysfunction of the mitochondria induced by neuronal injury results in decreased amounts of ATP required for proper cellular functioning (Rao et al., 2015). The energy disruption and oxidative stress triggers the release of p53 from the nucleus which targets the mitochondria and begins expression of specific apoptotic inducing factors within the mitochondria (Yang et al., 2017). These factors include but are not limited to: PUMA, p53AIP1, Bid, and Bax. Collectively, these apoptotic-inducing factors release cytochrome c, which travels to the nucleus and begins the cellular apoptotic cascade (Wang et al., 2014). Cellular shrinkage occurs as the internal cytoskeleton begins to break down and the dying cell detaches from surrounding cells, impairing communication (Gulbins et al., 2000).

Calpain activity

Calpain is a family of Ca^{2+} -dependent proteases that play a role in numerous intracellular survival processes such as cytoskeleton remodelling, cell differentiation, and vesicular trafficking. Calpain also regulates cellular death processes involved in apoptosis (Ma, 2013). Within the cell, calpain moderates the dephosphorylation of various mitogen-activated protein kinases (MAPK), including p38 (Wu and Tymianski, 2018). Under excitotoxic conditions, calpain improperly cleaves striatal enriched protein tyrosine phosphatase 61 (STEP-61) into the shortened STEP-33. This shortened STEP-33 no longer has the ability to dephosphorylate p38 resulting in enhanced p38 activity further promoting cellular death (Wu and Tymianski, 2018). Excitotoxicity from TBI directly affects the microtubule integrity through this calpain pathway. Calpain mediates the cleavage of p35 to p25 which dimerizes with cdk5 in response to increased intracellular levels of Ca^{2+} (Yousuf et al., 2016). The cdk5/p25 complex has been shown to phosphorylate microtubules, specifically tau, within the hippocampus of rats that experienced controlled cortical impact (Yousuf et al., 2016). This phosphorylation of tau and increased activity of the cdk5/p25 complex impaired signal transmission between neurons as evident from the reduced field EPSP recordings 24 hours after impact. Taken together, the disrupted microtubule function, increased cdk5/p25 complex activity and decreased synaptic transmission resulted in apoptosis of cells within the hippocampus (Yousuf et al., 2016).

TBI-induced Necrosis

Neuronal damage induced by TBI can also result in the passive process of cell death known as necrosis. Necrosis and apoptosis both result in cell death, however, they have distinct mechanisms that lead to the same end result. The actual process of necrosis, known as oncosis,

occurs when there is an interference with energy transportation throughout the cell or if there is damage to the cellular membrane (Elmore, 2007). Immediate damage to the integrity of the microtubules has been shown to initiate oncosis (Lazar et al., 2016). Cellular membrane damage can result in the rapid movement of Na^+ into the cell and fully rupture the membrane as Na^+ moves from high extracellular concentration to low intracellular concentration (Zhang et al., 2018). This rapid movement of cations can raise the acidity of the cytoplasm and activate pH sensitive enzymes that begin to fragment DNA to stop nuclear growth factors (Koike et al., 2000). These cells begin to swell and burst, releasing their intracellular components into the extracellular space (Kerr et al., 1972). Damage-associated molecular patterns (DAMPs) are released during membrane rupture that have been shown to trigger inflammation. Upregulation of inflammatory factor IL-6 was observed with an increase in necrotic cells in the hippocampus of pigs who received fluid percussion injury (Curvello et al., 2018). The release of intracellular components and debris as a result of TBI signals reactive astrocytes (Blennow et al., 2016). This damage and inflammation at the site of injury impairs cellular signaling and functioning, all of which contribute to the pathological cascade started from a TBI.

Microtubule Dynamics

Within a neuron exists a highway of microtubules that bring organelles needed for communication from the outreaching dendrites, to the soma and into the axon. Along with assisting communication, microtubules make up the internal architecture of the cell in the form of a stable cytoskeleton (Conde and Cáceres, 2009; Sánchez et al., 2000). Needless to say, maintaining the integrity of the microtubules is crucial for proper cellular functioning and ongoing survival of the cell.

On a molecular scale, microtubules are made up of a backbone of α - and β -tubulin heterodimers. (Conde and Cáceres, 2009; Tucker et al., 1989) These $\alpha\beta$ -tubulin dimers polymerize to form a linear filament. It is estimated that 10 to 15 filaments bind together to form a hollow tubular structure with a diameter of 24 nm. (Conde and Cáceres, 2009). The orientation of the $\alpha\beta$ -tubulin dimer makes the microtubules a polar structure, with the faster growing end having a net positive end (Conde and Cáceres, 2009). This net positive end is achieved by the hydrolysis of β -tubulin with guanosine-5'-triphosphate (GTP) to form guanosine-5'-diphosphate (GDP). Once in the GDP state, the $\alpha\beta$ -tubulin dimer becomes stable and remains bound to other $\alpha\beta$ -tubulin dimers until reorganization occurs (Conde and Cáceres, 2009). At the base of the microtubule stalk rest a negatively charged γ -tubulin cap. The function of the γ -tubulin cap is to serve as a template for the correct anchoring and assembly of the microtubule (Conde and Cáceres, 2009).

The construction and deconstruction of microtubules (termed rescue and catastrophe events, respectfully) allows for the cell to be in a persistent state of what is known as dynamic instability. The cell is constantly reacting to spatial and temporal changes that occur within the extracellular space, with the microtubules responding in accordance to these external changes (Atarod et al., 2015). Indeed, it has been shown that mice treated with a drug that over stabilizes microtubules (i.e. keeps the microtubules rigid in a stabilized state) within the hippocampus can interfere with cellular dynamics and negatively affect learning (Atarod et al., 2015; Smith et al., 2017). On the contrary, not enough stability, in the form of dissociating stabilizing proteins (these proteins will be discussed in a further section) has been well documented in the

progression of neurodegenerative diseases such as Alzheimer's disease, Parkinson's disease, and amyotrophic lateral sclerosis (ALS; Baas et al., 2016). These findings highlight the constant changing state of microtubule state to ensure for proper cellular functioning.

Along with providing the internal structure to the cell, microtubules allow for the transport of various proteins, organelles, and genetic information throughout the cell. This is achieved through the use of motor proteins. Within the cell exists two superfamily's of motor proteins: kinesin and dynein (Vale et al., 1992). Kinesin is considered to be a plus-end directed motor protein, moving in an anterograde fashion towards the synapse. Dynein, on the other hand, is considered a negative-end directed motor protein, moving in a retrograde fashion towards the cell body (Vale et al., 1992). It has been theorized that the dynamic instability of the microtubules could be due to the bidirectional movement of these motor proteins along the microtubules (Klinman and Holzbaur, 2018; Vale et al., 1992). Extensive research has been conducted on the function and movement of these motor proteins, so much so that it has been shown that Kinesin-1 takes approximately one hundred 8 nm steps before dissociating from the microtubule (Block et al., 1990). Upon dissociating, Kinesin-1 binds to an adjacent microtubule and begins to walk down until reaching its step limit only to repeat the process (Mudrakola et al., 2009). Dynein has also been observed to jump between microtubules in live axons back towards the cell body (Mudrakola et al., 2009). The rate of transportation depends on what cargo is being brought throughout the cell. Cytoskeletal components and proteins have a slow rate of transportation while organelle and vesicles have a faster rate of transportation (Bercier et al., 2019). In order for these cellular components to be properly transported throughout the cell,

stabilizing proteins are required so that the microtubule does not dissociate, leaving the motor proteins paused or stranded.

Microtubule-associated proteins (MAP) are a superfamily of stabilizing proteins that bind to the microtubules to increase rigidity and stability (Dehmelt and Halpain, 2004). MAPs are rather large proteins, weighing around 200 kDa (Weisshaar and Matus, 1993). Within the MAP superfamily exists a wide range of proteins that play a role in microtubule integrity, development, cell division and transportation: MAP1, MAP2, MAP3/4, MAP6, MAP7, MAP9 and tau (Ramkumar et al., 2018; Weisshaar and Matus, 1993). These proteins have an 18 amino acid binding sequence that binds to tubulin to increase the rigidity of the microtubule (Sánchez et al., 2000; Weisshaar and Matus, 1993). The location of expression varies between the MAP family, for example MAP2 is mainly expressed within the soma and dendrites of the neuron, as well as in oligodendrocytes. Tau, on the other hand, is exclusively expressed in the axon of the neuron while MAP4 is expressed in non-neuronal cells and is absent from neuronal expression (Banks et al., 2017; Conde and Cáceres, 2009; Dehmelt and Halpain, 2004; Tucker et al., 1989). The genes that encode for MAP2 are located on multiple exons and is highly conserved across the animal kingdom (Dehmelt and Halpain, 2004). MAP2 has 4 isoforms that are expressed at different stages of brain development (Ramkumar et al., 2018). MAP2D is mainly expressed during development followed by expression of MAP2C and MAP2B as adulthood approaches. MAP2A is the isoform that is expressed during adulthood and becomes the main isoform that is expressed throughout adulthood (Ramkumar et al., 2018). The dynamic instability of the internal cytoskeleton and the shift between rescue and catastrophe events within the cell body and

extending dendrites is achieved through the dephosphorylation/phosphorylation of MAP2 (Dehmelt and Halpain, 2004).

The regulation of MAP2 phosphorylation is performed by numerous kinases to maintain cytoskeletal stability. Microtubule affinity regulating kinases (MARKs), protein kinase A (PKA), and calcium-calmodulin kinase II (CamKII) are the main kinases that phosphorylate MAP2, mainly at serine residue 136 (Każmierczak-Barańska et al., 2015). Phosphorylation of MAP2 decreases microtubule affinity resulting in the dissociation of MAP2 from the microtubule (Illenberger et al., 1996; Ramkumar et al., 2018). Under normal physiological conditions, this phosphorylation and dissociation of MAP2 from the microtubules promotes dynamic instability of the cytoskeleton and the movement of cellular components to different regions within the cell (Vale et al., 1992). Interestingly, even though phosphorylated MAP2 dissociates from the microtubule, there is evidence to show that it can still interact with the microfilament protein, actin, to some degree. However, this interaction and purpose of phosphorylated MAP2 still having the capability to bind to actin and microfilaments remains unclear (Ramkumar et al., 2018). Drastic changes to the extracellular space and overactivation of these kinases, mainly through increase in Ca^{2+} levels, can lead to hyperphosphorylation of MAP2 and compromise the cytoskeletal integrity within the cell.

Microtubule Dynamics following TBI

Given MAP2's crucial role in maintaining the stability of the microtubules within the cell, alterations to MAP2's activity can greatly impact cell survival. As previously described,

excitotoxicity due to excess amounts of glutamate and increases in intracellular Ca^{2+} levels promote neuronal death (Gulbins et al., 2000). Increased Ca^{2+} results in the initiation of numerous biochemical pathways. One of these pathways is the activation of proteases, in particular calcium-activated neural proteases and calpain-1 (Taft et al., 1992). Calpain-1 has been shown to cleave MAP2 and other neurofilaments. Overactivity of calpain-1, through increased intracellular Ca^{2+} , is present in various pathological conditions as it promotes the progression of cell death by breaking down the cytoskeleton (Taft et al., 1992). Upregulation of NMDA receptors along the cell membrane also occurs following increased levels of Ca^{2+} . These upregulation of NMDA receptors increases the permeability of Ca^{2+} , further promoting Ca^{2+} dependent pathways and the activation of calpain-1 (Wang et al., 2019). Interestingly, the treatment of NMDA receptor agonists on cultured rat hippocampi has been shown to lead to focal swelling of dendritic spines and altered microtubule dynamics (Fontana et al., 2016; Tseng and Firestein, 2011). This effect was reduced with the treatment of NMDA receptor antagonists and reducing excitotoxicity.

Within the context of TBI, the sudden increases in Ca^{2+} is considered to be an early event of TBI damage and possibly the start to the pathological sequelae (Taft et al., 1992). After impact, dendrites show swelling and fragmentation. Along with dendritic damage, unaffected portions of the dendrites undergo synaptic reconstruction, possibly in an attempt to preserve remaining connections and prevent large amounts of synaptic loss (Chuckowree et al., 2018). This morphological change was indicated as an increase in mushroom dendritic spine phenotype, which are more likely to maintain synaptic connectivity (Chuckowree et al., 2018). With altered dendritic integrity, MAP2 levels have also been observed to decrease within the cortex and

hippocampi of rats following impact and sudden increases in intracellular Ca^{2+} (Folkerts et al., 1998; Fontana et al., 2016; Taft et al., 1992). This decrease could be contributed to the increase in kinase activity and phosphorylation of MAP2 leading to the change in protein confirmation and dissociation from microtubules (Basavappa et al., 1999; Mudrakola et al., 2009). This alteration to the microtubules due to MAP2 dissociating can lead to motor protein pauses along the microtubules as well as more jumping between crosslinked microtubules tracks (Mudrakola et al., 2009). These changes in MAP2 levels have been shown to occur as early as one hour after impact in a rat model of TBI, with levels peaking at 24 hours and returning to normal after 72 hours (Folkerts et al., 1998). Interestingly, this decrease in MAP2 levels because of impact is not associated with cell death (Taft et al., 1992). This could potentially indicate a window of vulnerability where if a second impact is to occur, the microtubules are already in a compromised state leading to further damage. After this window of vulnerability passes, the cell begins to reorganize and bundle microtubules together as evident from MAP2 levels returning to normal. This could potentially showcase the usefulness of MAP2 as a biomarker of injury. Indeed, Papa and colleagues (2018) discovered that MAP2 levels within cerebral spinal fluid (CSF) are the reverse to what is observed within the cells after severe TBI in humans. They report an increase in CSF MAP2 levels 6 hours post impact with levels returning to normal after 72 hours. Given the lack of viable biomarkers in determining the severity of TBI in humans, this could be a useful tool in assessing cellular integrity after a head impact.

General anatomy and Cytoarchitecture of the Human Brain

Ever since Brodmann's classification of the anatomical regions and their corresponding function in the early 1900's, the human brain has been characterized in great detail with various

sub-regions and neuronal populations. To the naked eye, the brain gives very little indication as to what specific function takes place in a particular region. However, through histological analysis and staining, the different regions of the brain and neuronal subpopulations are easily established allowing for the understanding of connectivity and function. The evolutionary old, subcortical regions of the brain mainly maintain autonomic functions within the body (Zilles and Amunts, 2010). These functions include, but not limited to, the control of breathing, heart rate, and blood pressure changes depending on feedback from inside and outside the body. The subcortical regions that control these behaviours are highly conserved across species, from rodents to humans. What separates humans, as well as a number of species, from primitive animals is the development of the neocortex and higher order cognitive processes (Marín-Padilla, 1998; Zilles and Amunts, 2010). The neocortex consists of various layers of different neuronal populations designed to integrate more information (i.e. sensory, perceptive, environmental) and form complex connections between them. Brodmann used this layering of the brain, as well as the distribution of different cell types within these layers, to divide the brain into its various regions based on function (Zilles and Amunts, 2010). The development of cortical layering is a highly conserved mechanism, however the ratio of neurons between each layer differs greatly (Kriegstein et al., 2006). This asymmetrical ratio is thought to be one of the main driving factors behind the evolutionary gain of gyrified brains, which is only seen in relatively few species within the animal kingdom.

Conventionally, there are six layers that make up the neocortex (Constantinople and Bruno, 2013; Jelsing et al., 2006; Krieg, 1946; Vogt and Paxinos, 2014). Layer 1, known as the molecular layer, contains a specific type of neuron known as the Cajal-Retzius neuron (Marín-

Padilla, 1998; Peters, 2002). These primitive types of neurons expand the superficial layer of the cortex with large dendritic projections that integrate into adjacent parts of the cortex. While the true nature of these neurons is still not fully understood, it has been shown that they are inhibitory in function, suggesting some type of modulator role to other regions of the cortex (Peters, 2002). The Cajal-Retzius neurons are also one of the first neurons to develop and migrate before the rest of the cortical layers develop (Marín-Padilla, 1998). Interestingly, these neurons within layer 1 of the cortex do not express NeuN, a neuronal nuclei protein, that is expressed by almost every type of neuron throughout the brain (Duan et al., 2016). The Golgi-Cox method of staining is often used to visually identify these neurons and their vast projections, while the Cresyl-Violet histological stain can identify their cell bodies (Marín-Padilla, 1998).

Underneath layer 1 lies layer 2, a dense band of small-bodied stellate neurons that make up the granular layer (Krieg, 1946; Vogt, 2016). This layer receives input from various other cortical regions as well as subcortical regions, such as the amygdala and lateral hypothalamus (Gabbott et al., 2005; Ghashghaei et al., 2007). These neurons then project to adjacent cortical layers (Ghashghaei et al., 2007). This layer contains the highest concentration of neurons (Brody, 1955; Vogt, 2016). Layer 3 consists of smaller pyramidal cells that extend projections into the first and second layers (Balaram and Kaas, 2014). These pyramidal cells have large basal dendritic arbors with high density of spines to form synapses, both inhibitory and excitatory (Elston, 2000; Shepherd, 2005). The dendrite length is also considerably long, allowing for a high degree of interconnectivity between brain regions (Elston, 2000). Due to the high interconnectivity between brain regions, the pyramidal cells of layer 3 have been shown to play a role in learning and memory with connections to the CA1 field of the hippocampus (Lavenex

and Amaral, 2000). Similar to layer 3, layer 5 mainly consists of large pyramidal cells that have large dendritic fields (Vogt and Paxinos, 2014). This layer has a relatively low density of neurons due to the presence of gigantopyramidal cells and vast dendritic field (Vogt, 2016). Layer 5 is broken up into two sections, 5a and 5b respectfully, given the different pyramidal cell sizes, with layer 5b containing clusters of large neurons (Vogt and Paxinos, 2014). The larger pyramidal cells of layer 5b have extensive connections, with evidence to indicate cortical innervation within the spinal cord in rats (Gabbott et al., 2005). Layer 6 is broken up into 3 sections, 6a, 6b, and 6c with extensive projections to thalamic regions (Gabbott et al., 2005). Interestingly, the thalamocortical neurons that innervate the different sections of layer 6 receive the same information but appear to process that information independent of each other (Constantinople and Bruno, 2013).

Between layer 3 and 5 exists the internal granular layer, or layer 4 (Jelsing et al., 2006). These granular cells seem to almost exclusively be found in the medial and posterior cingulate cortex of the brain, with connections to thalamic regions, however further research is needed to fully understand the exact function of layer 4 (Constantinople and Bruno, 2013; Jelsing et al., 2006). There is much debate over the existence and function of layer 4, as it was previously thought to only be in species with complex brain development (Jelsing et al., 2006). Interestingly, given the similarities between the human brain and the porcine brain, Jelsing and colleagues (2006) were not able to identify the presence of the layer 4 granular layer in the Göttingen minipig. Further studies would should be carried out to confirm the lack of a layer 4 within other species, including pigs, and if this layer is exclusively found in non-human primates and humans.

Translational Gap Between Rodents and Humans

While there is a growing body of work using rodent models of TBI, there remains a translational gap between the mechanically stretched neurons, *in vivo* models and what is known in humans. Historically, rodents have been the primary model system in the exploration of the mechanisms behind TBI. The advantages of using rodents for experimental study are that they are inexpensive, can be genotypically altered to suit an experimental design and have an extensive understanding of behaviour and cellular processes. Given that the biomechanical strain exerted on to the brain occurs in the depths of the sulci, the smooth, lissencephalic brain of the rodent makes it a poor model for studying this phenomenon. In the past decade, there has been increased interest in using large animals to better understand the mechanisms behind the neurodegenerative component of TBI (Lind et al., 2007). One large animal of particular interest is pigs.

Pigs have been utilized in a wide array of biomedical and biological research ranging from toxicology, to experimental surgery, to behavioural research (Danek et al., 2017; Richer et al., 1998; Swindle et al., 2012). Bustad and McClellan (1965) were one of the first to review the topic following the first symposium of pig use in biomedical research. Extensive research had already been underway before this symposium, including the demonstration of a high degree of homology between pigs and humans. One major finding was the similarity of pig cardiac sections to those of humans; a finding still used today for valve transplantation (Manji et al., 2014). Other examples include: heat production and loss in piglets is similar to that of newborn humans; formation of spontaneous atherosclerosis lesions in pigs is comparable to humans in the

pre-atheromatous phase of atherosclerosis, and severe protein malnutrition in pigs can lead to biochemical and anatomical changes similar to what is seen in children suffering from kwashiorkor (Bustad and McClellan, 1965). Researchers have used pigs for preclinical testing of invasive neurosurgical therapies such as deep brain stimulation (Gorny et al., 2013; Paek et al., 2015). The larger size of the pig brain allows for the same instruments to be used for those in humans without the need for scaling (Orlowski et al., 2017). This preclinical work allows for the refinement of methods and safety measures to be investigated before implementation in humans.

An extensive atlas, similar to those developed for the human and rodent brain, has been put together for identification of porcine brain structures (Félix et al., 1999). The porcine brain is considered a gyrencephalic neocortex that closely resembles that of a human (Villadsen et al., 2018). Using computer simulation software, the gyration of the brain results from grey matter developing at a faster rate than white matter (Tallinen et al., 2014). This finding has been confirmed using longitudinal MRI scans, with similarities between humans and pigs during the first months of brain development (Conrad et al., 2012; Knickmeyer et al., 2008; Winter et al., 2011). The folding of the brain allows for the increased complexity of neuronal networks and produces similarities in subcortical nuclei between pigs, non-human primates, and humans (Hofman, 1989; Larsen et al., 2004). Indeed, Larsen and colleagues (2004) found similarities in subthalamic nuclei shape and location between porcine brains, non-human primates and humans. The similarities in structures between pigs, non-human primates and humans could be a result of the rapid gyration of the brain pushing down on the subcortical regions to form distinct nuclei not seen in rodents.

The majority of brain growth, composition and myelination occurs around birth in pigs, similar to human brain development (Conrad et al., 2012; Dickerson and Dobbing, 1967) and the white and grey matter densities in the porcine brain are similar to those in humans (Cullen et al., 2016). Sex-specific development is also similar between pigs and humans. In humans, females experience hippocampal development earlier than males but have a smaller maximum volume (Giedd et al., 1996). Conrad and colleagues (2012) found that female pig hippocampal development occurs 5 weeks before males. Moreover, the growth window for female hippocampal development is shorter than males. Typically, pigs live for 12 to 15 years of age, allowing for longitudinal studies to be conducted on the natural pathological development of neurodegenerative diseases.

The size of the pig allows for the use of modern preclinical and clinical imaging techniques used for humans without the need for scaling instrument size (Fang et al., 2005; Jørgensen et al., 2016; Villadsen et al., 2018). While humans do not necessarily need to be anaesthetized for scans, pigs must be put under for accurate readings (Holm et al., 2016). However, protocols can be adjusted to accommodate this need as common drugs used for human anaesthetics, such as isoflurane and Propofol, can be safely used on pigs (Holm et al., 2016). Positron emission tomography (PET) scans have been used extensively in preclinical work on pigs for the development and testing of radioligand effectiveness prior to human use (Jørgensen et al., 2018; for review see Lind et al., 2007). These PET scans have revealed a number of similarities between human monoaminergic systems and pigs. Within the serotonergic system, pigs express numerous forms of 5-HT receptors within the caudate nucleus (5HT4, 5HT6, 5HT1D, 5HT2C) and share a highly homologous 5HT1B to humans (Lind et al., 2007).

Cumming and colleagues (2007) found that pigs and humans share similar serotonin neuron numbers within the raphe nucleus which differ greatly from rats (95,000 and 140,000 to 16,000, respectively). Similarities in the dopaminergic systems have been observed with similar numbers of projections from the substantia nigra pars compacta to the substantia nigra pars reticula seen in pigs, primates and humans (Lind et al., 2007). Along with projections, D1/D2 receptor binding organization has been observed in pig brains that resembles that seen in human brains (Minuzzi et al., 2006). Using FDOPA, an exogenous substrate to analyze DOPA decarboxylase levels during PET scans, it has been shown that pigs and humans share similarities in the regulation and metabolism of dopamine within the brain (Danielsen et al., 1999). Based on the reliability of using pigs in PET scans, further improvement and refinement of radio-labelled ligands used to detect specific pathological markers can occur without the necessary need for scaling from a rodent to a human.

Porcine models of TBI

The similarities between the pig brain and human brain provide a unique opportunity to study the pathological effects of TBI. The development of the HYGE device allows for the analysis of neuropathological changes from rotational acceleration in an experimental setting initially used on non-human primates (Abel et al., 1978). With the ethical issues surrounding testing on non-human primates, there was a shift towards using the device on large animals such as pigs (Cullen et al., 2016). The HYGE device allows for the pathological profile of head trauma while controlling the biomechanical forces similar to what is observed in human TBI (Cullen et al., 2016; Vink, 2018). Compared to other head trauma models adopted from rodent

studies, such as controlled cortical impact, fluid-percussion and weight-drop (reviewed in Xiong et al., 2013), the HYGE rotational model is considered to be the most accurate method to model human head trauma (Cullen et al., 2016).

Using the HYGE device, Smith et al. (1999a) were able to demonstrate axonal swelling in a pig model of TBI 1 day post-injury. This axonal swelling was marked by increased gliosis within the sulci of the injured brains. It is possible that the gliosis observed was due to the presence of astrocyte reactivity to damaged tissue (Levitt and Rakic, 1980), however Smith and colleagues did not measure these levels. Measuring the levels of astrocytes following an impact using the HYGE device is crucial as increased astrocytic levels in the sulci has been observed following TBI and is considered to be a hallmark for the development of CTE (McKee et al., 2013).

Rotational acceleration of the porcine head results in accumulation of A β and hyperphosphorylated tau within the subcortical white matter (Eucker et al., 2011; Smith et al., 1999; Wolf et al., 2017). Electrophysiological recordings from the CA1 field of the hippocampus revealed a significantly greater output in the injured brains, suggesting a possible compensatory mechanism due to decreased signal input from disrupted circuitry (Wolf et al., 2017). Interestingly, Wolf et al. (2017) found that while neuronal circuitry was altered, there was no accumulation of either A β or hyperphosphorylated tau within the hippocampus itself. Hyperphosphorylation of tau disrupts axonal transport and facilitates reactive oxygen species production, eventually leading to cell death (Stamer et al., 2002). This may indicate a slower

time course of cellular degeneration as this subcortical structure only receives secondary-like biomechanical strain rather than focal impact strain (Sarntinoranont et al., 2012).

Johnson and colleagues (2016) performed rotational TBI on six-month old female Hanford pigs and found APP accumulation as early as 6 hrs post injury. Following injury, levels of neurofibrillary subtypes increased and were seen to co-label with APP. These increased levels of neurofibrillary subtypes showed a delayed response following injury, with levels peaking between 48-72 hrs post injury. They did find that in some axons, only one pathological phenotype was found (i.e. only NF-st, or APP, or the SNTF). This could lead to the development and use of clinical biomarkers for these different phenotypes.

Present Study

The development of an experimental *ex vivo* pig brain model was used to explore the cellular changes associated with TBI as well as provide evidence for the usefulness of the pig brain in bridging the gap between rodents and humans. This was done to bridge the gap between what has been previously seen in rodent models and the cellular pathology observed in humans. The present study used high speed x-ray cinematography along with state of the art motion tracking software to map the strain and force loading on to the brain in real time. The real time mapping of strain and force loading allows for the precise analysis of which regions experience the highest amounts of biomechanical forces. Given what has been discovered using computer models of TBI, it was hypothesized that the deep valley of the sulci would experience the highest amount of strain and where cellular changes would most likely be observed. These changes

include microtubule integrity, cellular volume and cellular density. These changes will provide insight on what may occur in a human when experiencing a TBI and highlight the usefulness of pigs as a translational model of TBI.

Materials and Methods

Animals

Whole porcine heads (10-12-month-old Yorkshire Pigs) were sourced from a local abattoir. The heads were collected roughly 15-20 minutes following slaughter and placed on ice for transportation. The pigs were slaughtered by electrocution to the back of the neck and then bled, a common practice of slaughter in the agriculture industry. Since both control and impacted brain slabs would come from the same brain, both conditions received this electrocution. Therefore, any more damage to the brain would be from the result of the impact. Since these pigs were used for food processing, no pigs were slaughtered for the purpose of this experiment. This is in accordance of the 3 R's of animal research (Reduction, Replacement, and Refinement). The brains of these pigs would be disposed of if they were not used for this experiment, however the rest of the body would be used for food production. Time of transportation was approximately 50 minutes. All experimental procedures were completed within 4 hours of removal.

Brain Removal

Upon arrival to the laboratory, the brains were removed from the skull and placed into bubbling ice cold artificial cerebral spinal fluid (aCSF) containing: 124mM NaCl, 3mM KCl, 1.3mM MgSO₄, 1.4mM NaH₂PO₄, 10mM glucose, 26mM NaHCO₃ and 2.5mM CaCl₂. Time of removal took about 20-25 minutes. The brain remained in ice cold aCSF for 1 hour to slow down biochemical cascades of natural degeneration as the brain is not receiving oxygen and blood flow. The brain was then placed into a custom-made stainless-steel brain matrix (Figure 1) that

was placed on an ice pack and sliced into four 5mm coronal slabs. Surrounding dura mater was removed from sections. Coronal slabs were taken from the medial temporal region of the brain (Figure 2). The coronal slabs were then placed into a compartment filled with fresh aCSF that was bubbled with air for 30 minutes prior to impact for acclimation. The compartment had a plastic grate floor with a coiled plastic tube underneath. Holes were poked into the top of the tube to allow air to pass through and bubble the aCSF. The slabs were then placed into individual plastic petri dishes with holes in the bottom and placed on top of the plastic grate (Figure 3). This allowed for the slabs to be submerged in the bubbled aCSF and for proper labelling of which group (control or impact) the slab was assigned.

Figure 1: A) Custom-made pig brain matrix. Each section is 5mm wide. The grooves were set to the width of the blades to allow for precise slicing of the brain. The matrix was placed on an ice pack to keep the brain cold while slicing. B) Pig brain placed into matrix.

Figure 2: A) Representative image of the location where the coronal slabs were taken from. 4 slabs were taken from each brain, two for the control condition, 2 for the impact condition. Each slab was 0.5cm in thickness. The slabs were taken 3.0cm from the front of the brain. B) Coronal section corresponding to the approximate location of the first slab to show internal neuroanatomy. Image taken from the pig brain atlas (Félix et al., 1999).

Figure 3: Bubbler System Setup. A coiled plastic tube with holes in the top sits underneath a plastic grate. The plastic container is insulated to control the temperature of the aCSF. Air is supplied through a tank bubbler.

Marker Insertion

During the acclimation period each brain slab was taken out of the petri dish and placed into a glass dish. An ice pack was placed underneath the glass dish to keep the brain slabs cool so that the markers could be inserted effectively. Using a canula, barium sulfate markers were inserted following the white/grey matter boundaries of the brain. These markers allowed for the visualization of the deformation and analysis of strain fields during impact. The markers were inserted into the first 0.5 millimeter of the tissue leaving the remaining 4.5 mm for tissue processing. When the markers were inserted, the slab was placed back into the bubbling aCSF for the remaining acclimation period. All four slabs had markers inserted into the tissue.

Impact

After acclimation, one slab was placed into a sealed elastic encasement and was dropped from a height of 0.9 m with a velocity of 3.8 meters/second (m/s). Following impact, the impacted slab was placed back into bubbling aCSF for one hour. After one hour, the slab was immersed in 4% paraformaldehyde (PFA; pH 7.4). A control slab from the same brain underwent the same procedure, with the exception that there was no impact. The remaining two slabs from the same brain followed the same procedure (impacted and control) but were flash frozen in absolute ethanol and dry ice. These slabs were immediately placed into a -80°C freezer.

Cresyl-Violet Stain

Whole slices were mounted on to gelatin-dipped slides and left out to dry at room temperature. Once the slides were dry, they were washed with DH₂O to remove any loose debris. Slides were immersed in 2 three-minute washes of 100% ethanol. Following the ethanol washes, slides were placed into 2 seven and a half minute washes of xylene, after which they were placed into a ten-minute wash of 100% ethanol. The slides were rinsed with DH₂O before going into 0.1% Cresyl-Violet stain for eight minutes. To enhance stain penetration, the slides were placed into a 37°C oven during the Cresyl-Violet step. Following the eight minutes, the slides were dipped 3-4 times in 70% ethanol. A large majority of the excess stain was washed off during this step. Slides were differentiated in 95% and 2 drops of glacial acidic acid for one minute. Following the differentiation step, the slides were dehydrated in 2 three-minute changes of 100% ethanol and then cleared for five minutes in xylene. Slides were mounted with Permount and coverslipped.

Immunohistochemistry

One day after being immersed in 4% PFA, the brain slabs were placed in a 30% sucrose/0.1M phosphate buffered saline (PBS) to be cryofixed. Coronal slabs were hemi-sectioned, and one hemisphere was placed back in the sucrose solution. The other hemisphere was sectioned at 60 µm on a cryostat and placed in a 0.1% sodium azide/0.1M PBS solution.

Brain sections underwent three, 5-minute washes in phosphate buffer solution with 0.2% Triton X (PBS-Tx). Following this, sections were placed into 3.0% hydrogen peroxide for 30-minutes. Sections were then placed in a peroxidase blocking solution (BLOXALL, Vector Laboratories) for 10-minutes followed by a one 5-minute wash in PBS-Tx. Sections were placed

into one of two primary antibody solutions for overnight incubation at room temperature (rabbit anti-MAP2 from Abcam, 1:10000; mouse anti-NeuN from Abcam, 1:10000). The following day the sections were washed three times, 10 minutes per wash in PBS-Tx. Sections were placed into the secondary anti-body solution for a 2-hour incubation at room temperature (anti-rabbit biotinylated from Vector Laboratories, anti-mouse biotinylated from Vector Laboratories, 1:1000). Sections were placed in PBS-Tx for three, 10-minute washes. Following the washes, sections were placed into Avidin/Biotin Complex solution (ABC; Vector Laboratories). The sections were then washed in three, 5-minute washes using PBS before being placed in a diaminobenzene solution (DAB; 0.02 g DAB in 10 ml PBS) for 30 seconds. Just before the slices were placed into the DAB solution, 25 μ l of H₂O₂ was added to solution to activate the DAB. Sections were then mounted onto slides and coverslipped.

Golgi-Cox Stain

The Golgi-Cox stain was used to visualize entire neuron dendritic fields and cell body morphology after an impact occurred. Apart from testing the effects of metal cannulas on stain reactivity, careful preparation was used to ensure no other metal touched the tissue. For this reason, a plastic brain matrix and a ceramic knife were used to prepare the 0.5cm brain slabs. All other aspects of the procedure remained as described previously in the *Brain Removal* section. A separate experiment was conducted to test the effects of various types of cannula coatings to observe if the Golgi-Cox stain would react differently during marker insertion. These types of coatings were designed to prevent direct metal from touching the tissue. The types of coatings used were: epoxy, Sylgard, and 2 types of organic nail polishes.

After impact, the brain slabs were placed into their assigned jars filled with prepared Golgi-Cox solution. The slabs remained in the stain for 7 days before undergoing 3 washes in DH₂O (4 hours, 3 hours, overnight). After the DH₂O washes, the brain slabs went through 3 washes of graduated sucrose solutions (10% for 8 hours, 20% overnight, 30% storage). The jars were covered with aluminium to avoid light exposure and kept at room temperature.

After the 7 days, the brain slabs were sectioned at 200 µm on a Vibratome 1500. The cingulate sulcus region was cut away from the rest of the brain and glued on to a plastic cube. The cingulate sulcus was put into a 6% sucrose solution and sectioned with a blade speed of 6 and an amplitude of 8. Brain sections were placed on to a gelatinized slide, blotted dry, and left in a humidified box over night to allow the slide to dry.

On the day of staining procedure, the slides were placed in DH₂O for 1 minute, and then transferred into an ammonium hydroxide (28%) solution for 40 minutes. Following the ammonium hydroxide, the slides were washed in from DH₂O for 1 minute then into a Kodak Film Fix solution for 40 minutes (diluted 1:1 with DH₂O). After the 40 minutes, the slides were again washed with DH₂O for 1 minute before going through graduated ethanol solutions (50%, 70%, 95%; 1 minute each). Next, the slides were placed into three 5-minute washes of desiccated 100% ethanol, after which they were placed into a solution of 33% absolute ethanol, 33% xylene, 33% Chloroform for 10 minutes. The slides were then placed into two 15-minute washes of desiccated xylene, taken out and coverslipped with a liberal amount of Permount and left to dry

in the fume hood for 1 hour before being placed into a desiccated box for storage. Small weights were placed onto the slides to ensure that the coverslip adhered to the slide.

MAP2 Quantification

Quantification of cells was conducted using unbiased stereological measures to estimate the cell density of MAP2 positive-labelled cells in apex and arm of the cingulate sulcus (Figure 4). The region of interest chosen for quantification was the cingulate sulcus located laterally to the corpus callosum. This region was chosen due to evidence suggesting that sulcal regions experience high amounts of biomechanical forces following impact. Visualization of each stained section was done using an Olympus BX51 bright field microscope with an MBF motorized stage. Images of the stained sections were captured on an Olympus U-CMAD3 camera. Unbiased stereology quantification was performed using Stereo Investigator software (MBF Bioscience, Williston, VT). Separately, the arm and apex of the cingulate were traced at 2.5x magnification. MAP2 quantification was performed using parameters to ensure a Gunderson's coefficient of error range (GCE, $m=1$) of 0.12 – 0.14 was achieved. MAP2 quantification was done at 60x magnification. The estimated population using mean section thickness was recorded for each section. The estimated population was divided by the measured volume (μm^3) to get one data point representing the estimated MAP2 cellular density of that section. The data was analyzed with a one-way ANOVA. To ensure unbiased measurements, investigators performing all quantification were blinded from the conditions of the brain slabs.

Figure 4: MAP2 labeled sulcus regions of interest. Resembling the shape of the letter U, the top black box represents the arm of the sulcus, while the bottom box represents the apex of the sulcus. DAB stained image at 4x and 10x magnification.

NeuN Cellular Density Quantification

Quantification of NeuN positive labeled cells was conducted in the same manner as the previously described MAP2 quantification methods. One difference between the two methods was quantification of NeuN cellular density was measured only in the apex of the cingulate sulcus. The data was analyzed with a two-tailed independent samples t-test.

Cellular volume quantification

Because of the way that the tissue was sectioned, it was considered preferentially-oriented, and thus considered biased. Quantification of cellular volume was conducted to measure the distribution of cell volumes in layers 2 and layer 5 within the cingulate sulcus apex using NeuN positive-labelled cells (Figure 5). Visualization of each stained section was done using the same methods as previously described. Cellular volume was done using the Nucleator function in Stereo Investigator (MBF Bioscience, Williston, VT) with 4 rays as previously described in Gundersen (1988). Three to four cells within the grid frame were chosen at random and the outer boundaries of the cell body were marked. Cellular volume was quantified using parameters to ensure a coefficient of error range (CE) of 0.12 – 0.14 was achieved. Because of how cells can exhibit both shrinkage and expansion following impact, taking an average of the volumes would not provide an accurate representation of the cell volume within the apex of the cingulate sulcus. Therefore, cell volumes were considered on a continuum and the individual data points were plotted on a histogram to visualize the spread of cell volumes. A non-parametric Mann-Whitney U test was conducted to analyze the difference in spread of data points between control and impacted tissue.

Figure 5: NeuN labelled cells of whole tissue sectioning. Black boxes indicate the region of interest within the cingulate sulcus. DAB stain at 4x and 10x magnification reveal the cellular densities within the arm and the apex of the sulcus.

Results

Gross Anatomy Between Human and Porcine Brain

One of the main points for establishing the usefulness of using pigs to study neuroscience is the anatomical similarities between the porcine brain and human brain. Examination of coronal porcine brain sections using Cresyl-violet stain makes the similarities between the human brain and porcine brain evident (Figure 6). Structures such as the cingulate gyrus and sulcus, white matter tracks and striatum are easily identifiable and closely located to where they would be found in the human brain. Along with the location of these structures, the gyri and sulcal development of the pig cortex closely resembles that of the human neocortex.

Figure 6: Anatomical comparisons between the porcine brain (left) and the human brain (right). A Cresyl-Violet stain was used to show the grey and white matter of both brains. While the human brain visually contains larger amounts of white matter, the grey matter thickness is similar between both brains. Along with similar grey matter,

the pig and human brain have similar subcortical structures, such as striatum and development of the temporal lobe. Image of human brain taken from Ding et al. (2016). Scale bars = 5mm.

Cytoarchitecture of the porcine brain

The analysis of the cellular makeup and organization of neurons within the cortex closely resembles the human brain. From the NeuN immunohistological stain, the layering of the porcine cortex contains 6 distinct layers in both the arm and apex (Figure 7a and 7b) of the cingulate sulcus. At higher magnification the differences in cell types within the layers become clearly visible (Figure 8). The lack of staining within layer 1 of the cortex confirms the lack of NeuN expression in the Cajal-Retzius cells described by Duan and colleagues (2016). Using the Golgi-Cox staining technique, however, these cells are visible (Figure 8a). As previously described, the Cajal-Retzius cells span across Layer 1, making connections with adjacent cortical regions. Layer 2 of the cortex consists of small cell-bodied neurons. This layer is highly concentrated with neurons, as evident from its dark band appearance compared to the other cortical layers. Jelsing and colleagues (2006) describe layer 3 of the pig cortex to have two sublayers, the high density, darker 3a and the lighter 3b. However, from visual analysis, there does not seem to be a separation of layer 3 based on different density of neurons. Further cell density work should be carried out to determine if layer 3 is heterogenous in neuron density. Layer 5 of the cortex resembles what has been previously described; subdivision into normal sized pyramidal cells and larger pyramidal cells (Figure 8e). The spacing between the layers is fairly consistent, with layer 5 appearing to have the largest spacing between neurons given the large dendritic fields. Layer 6 is also consistent with what has been previously described.

Of interest, there appears to be the presence of an internal granular layer 4, as evident by a band of granular neurons that separate layer 3 from layer 5 (Figure 7). Higher magnification confirms the distinctive small cell bodies of these internal granular cells (Figure 8d). This is of interest, as Jelsing and colleagues (2006) report the lack of a layer 4 within the anterior cingulate cortex of the Göttingen minipig. It could be that this layer 4 develops later on in the porcine brain, as the sections that were used for this experimental model were taken from what would be considered the midcingulate cortex (Vogt, 2016).

Figure 7: Representative image of the porcine brain cortical layering in the apex of the sulcus (A) and along the arm of the cingulate sulcus (B). All 6 layers of the cortex can be seen in both images, however within the apex of the sulcus layers 5a, 5b and 6 become compressed. In the arm of the sulcus, the gaps between the layers increase along with the spacing between the neurons.

Figure 8: High magnification images of the different layers of the cortex. Image A taken at 10x magnification using Golgi-Cox staining while images B-F were taken at 60x magnification using the NeuN that stains neuronal cell bodies. A) The Golgi-Cox staining reveals the Cajal-Retzius cells that make up the layer 1, molecular layer. These cells have long horizontal spanning processes that make connections with adjacent regions of the cortex. B) This dense layer contains mainly granular cell bodies. C) Layer 3 of the cortex. Pyramidal cells mainly make up this layer, with projections and innervations to layer 2. D) Layer 4 of the cortex. This layer is considered the internal granular layer. The granular cells within this layer have very small cell bodies and is fairly densely packed compared to layer 3 and layer 5. This layer is only found from the starting of the midcingulate cortex into the post-cingulate cortex. E) Layer 5 of the cortex. This layer mainly consists of larger pyramidal cells that have innervations that span the different layers. This layer also contains the gigantopyramidal cells. F) Layer 6 of the cortex. Located just above the white matter, these neurons have long projections to both higher layers of the cortex, as well as projections to subcortical structures.

Apex of the cingulate sulcus has a higher neuronal density than the arm of the sulcus

To get a better understanding of the nature of the sulcus for this model, the neuronal densities between the arm and the apex of the cingulate sulcus were compared. The cingulate sulcus was chosen given how it runs adjacent to the corpus collosum for a majority of the brain, making it an easy and reliable landmark to take readings from. Similar to the shape of the letter

U, the arm of the sulcus consisted of the adjacent walls while the apex was considered to be the deep curve at the bottom of the sulcus.

An independent samples t-test on the density of positive NeuN labelled cells revealed a difference between the arm ($M = 2.12$, $SD \pm 0.36$) and apex ($M = 3.77$, $SD \pm 1.36$) of the sulcus; $t = 2.62$, $p < 0.05$ (Figure 9). The layering of the sulcus appears to become more compact as the apex curve begins, with layer's 5a, 5b and 6 becoming almost indistinguishable at the apex (Figure 7a).

Figure 9: Mean cell density (cell/μm³*100,000) of NeuN labelled neurons in the arm and apex of the cingulate sulcus (mean± SEM). The apex of the cingulate sulcus is significantly denser compared to the arm of the sulcus (star = $p < 0.05$).

Application of Golgi-Cox Staining Technique to study the porcine brain

One of the oldest methods for visualizing neuron shape is through the Golgi-Cox method. To summarize, a heavy-metal solution, consisting of mercury and chromate, stains select

neurons, making the entire dendritic projections, soma, axon and synaptic buttons turn black. While the actual mechanism of this reaction is not fully understood, the outcome of this stain provides a detailed look into the structure of the neuron. This stain has been used across numerous tissue types from humans, to rodents, to non-human primates (Gao et al., 2011; Marín-Padilla, 1998; Schmechel and Rakic, 1979). For the purpose of this model, the Golgi-Cox staining method was used to reveal the cellular makeup of the porcine brain (Figure 10). From this stain, large networks of dendrites became visible with enough detail to identify various types of synapses at higher magnifications (Figure 10b). Along with the staining of the neurons, the Golgi-Cox technique also revealed what appear to be astrocytes as described by Schmechel and Rakic (1979; Figure 10c).

Figure 10: Golgi-Cox stain reveals the internetwork of neurons within the cortex of the porcine brain. A) Along with dark, large cell bodies, the network of dendrites and projecting axon. B) High magnified image of different types of synapses (arrow heads) along the pyramidal cell dendrites. C) Along with the staining neurons, the Golgi-Cox stain reveals astrocytes within the cortex.

Along with visualizing neural networks within the cortex of the pig, the Golgi-Cox stain can be applied to other regions of the brain to visualize more complex subcortical structures (Figure 11). Neurons within this region appear to take on non-linear projections, with some neurons having curved projections around certain areas in this region (Figure 11b). As described

by Johannes and colleagues (2003), these neurons are most likely the different types of interneurons of the striatum. These interneurons found in the pig striatum have very similar morphology to those seen in humans (Johannes et al., 2003). In particular, Type 1 and type 2 interneurons can be seen in Fig. 11a and 11b. Type 1 interneurons have a curved, bow-shape with long and small amount of branching on the projections (Johannes et al., 2003). Type 2 interneurons can be seen with their irregular shaped soma's and large amounts of projections (Johannes et al., 2003). These interneurons are more commonly seen in humans and non-human primates than they are in rodents (Graveland and Difiglia, 1985).

Figure 11: Golgi-Cox stain of the striatum of the porcine brain. A) The different types of the interneurons can be seen that make up the striatum. Type 1 neurons appear to wrap around regions. B) At 10x magnification, these different cells types become more evident. The type 1 interneurons (black arrowhead) seem to contour around regions within the striatum. Type 2 interneurons (white arrowheads), with their irregular shaped soma's. These interneurons have numerous splits in their dendritic branches, allowing for a large number of synaptic innervations.

A common warning when performing the Golgi-Cox stain is to not use metal during the procedure. However, this warning has been not described as to why it is important to avoid using metal when handling the tissue. Due to the nature of this experimentation requiring the use of metal cannulas to implant the barium sulfate markers, we sought to explain what occurs when metal is used during the staining procedure. Along with testing the effects of metal, 4 other

coatings were used: nail polish, organic nail polish, epoxy, and Sylgard coating. The different types of coatings were used to try and get around the warning of using metal. Figure 12 represents the effects these various cannula types have on the tissue. The use of metal appeared to lead to the increased levels of astrocytes surrounding the site of implantation. Interestingly, it was the nail polishes and epoxy that resulted in the dense clustering of astrocytes around the insertion site. (Figure 12d, 12e, 12f respectfully). Control, metal, and Sylgard having visually less clustering of astrocytes (Figure 12a, 12b, 12c respectfully). Figure 13 represents higher magnification of the cannula's used and the resulting astrocyte activation.

Figure 12: Representative image of the different types of cannulas used for marker insertion and tissue reactivity. A) Astrocytes from a control brain with no cannula insertion. Large reactive astrocytes are seen sparse throughout the white matter. B) The use of bare metal during the Golgi-Cox stain resulted in the activation of astrocytes surrounding the site where metal made contact with the tissue. Interestingly, in comparison to other cannula types used, metal appeared less reactive to the tissue and visually produced less astrocyte activation. C) The Sylgard coated cannula produced the least amount of astrocytic activation, both around the insertion site as well as the tissue surrounding the site. Non-reactive fibrous astrocytes were also present in the surrounding tissue. D) The epoxy

coated cannula resulted in less activation of astrocytes directly around the site of insertion. However, further away there was a visual increase in the amount of astrocytes present. E/F) Both nail polishes produced the highest amount of astrocyte activation around the site as well as within the surrounding tissue. Interestingly, the nail polishes contained organic materials, which seem to have caused a large reaction in the tissue. The use of barium sulfate markers did not seem to have an effect on the tissue or the Golgi-Cox staining procedure. Images taken at 2.5x magnification.

Figure 13: Representative image of astrocyte morphology at 20x magnification. A/B/C) Astrocytes from control, metal and Sylgard brains. These astrocytes are seen throughout the white matter but are not clustered together. They still contain spiny processes that are seen in reactive astrocytes. D/E/F) Astrocytes from epoxy, and the two nail polishes. Astrocytic clustering can be seen surrounding the site of insertion. With the visual increase in clustering, there are less non-reactive, fibrous astrocytes present.

***Ex vivo* model of traumatic brain injury**

To briefly summarize, the brains used for this study came from food-grade pigs from a local abattoir. Following slaughter, the heads were brought to Carleton University and the brains were removed and placed into waiting ice-cold aCSF for one hour. Following the one hour, brains were sectioned into four 5 mm slabs and had barium sulfate markers placed 1mm into the tissue.

Two of the four slabs were placed into an elastomer and impacted at a speed of 3.8 m/s. The remaining two slabs were placed into separate elastomer encasements; however, they did not undergo impact. After the impact, all four slabs were removed from their encasements and placed into bubbling aCSF for one hour. The purpose behind waiting this one hour was to investigate the immediate result of impact. These changes included: overall neuron density, neuron size changes, and assessment of microtubule and cytoskeletal integrity.

No difference between neuronal density 1 hour after impact

Previous reports have used NeuN as an indirect measure of cell death (Duan et al., 2016). The rationale is since NeuN is expressed in the nucleus and soma of the neuron, a decrease in NeuN levels would indicate damage or disruption to the neuron. Based on this assumption an independent samples t-test on the density of NeuN labeling in the apex of the cingulate sulcus (Figure 14) revealed no difference in neuronal density between control ($M = 1.28$, $SD = .212$) and impact ($M = 1.25$, $SD = .305$) one hour after impact; $t = .333$, $p > 0.05$.

Figure 14: A) Average cell density (mean cell count/ $\mu\text{m}^3 \times 100,000$) of NeuN labelled neurons in layer 5 in the apex of the cingulate sulcus (mean $\pm$ SEM). No significant difference was found between the two groups one-hour after impact. B) Representative image of NeuN labelled neurons within the apex of the cingulate sulcus. Staining and cellular density are almost identical between both groups, indicating no neuronal death following one hour after impact.

No difference between cell volume in layer 2 and 5 of cortex 1 hour after impact

Since it has been reported that cell size decreases during apoptosis but increases during necrosis, it would be considered that cell size is a continuum. Therefore, taking the average of the cell volumes would violate the assumption of normal distribution required for an independent samples t-test. A Mann-Whitney U test was performed to compare the changes in cell volume in layers 2 and 5 of the cortex, 1 hour after impact (Figure 15, Figure 16). There was no change in the distribution of cell volumes between the groups in layer 2 (mean ranks for control and impact were 828.75 and 812.53, respectively; $U = 326652.0$, $z = -0.689$, $p > 0.05$). There was no change in the distribution of cell volumes between the groups in layer 5 (mean ranks for control and impact were 616.59 and 614.60, respectively; $U = 186882.0$, $z = -.098$, $p > 0.05$).

Figure 15: Representative image of layer 2 of the apex of the cingulate sulcus. Cell volume recordings were taken from this area to compare to different layers due to different neuron types found within each layer. Layer 2 mainly consists of small granular cells. A histogram reveals the frequency distribution of cell volumes within layer 2 in the apex of the cingulate sulcus. Following one hour after impact, there was similar distributions of neuron volumes within layer 2 of the cortex.

Figure 16: Representative image of layer 5 of the apex of the cingulate sulcus. Cell volume recordings were taken from this area to compare to different layers due to different neuron types found within each layer. Layer 5 consists of mainly pyramidal neurons and gigantopyramidal neurons. A histogram reveals the frequency distribution of cell volumes within layer 5 in the apex of the cingulate sulcus. Following impact, there was a similar distribution of neuron volumes within layer 5. Of note, the spread of the normal curve visually appears tighter towards smaller cell volumes 1 hour after impact.

Change in MAP2 cellular density 1 hour after impact

A one-way ANOVA comparing the MAP2 cellular density in the cingulate sulcus (Figure 17) revealed a significant difference between control and impact in the apex, 1 hour after impact ($F(1,11) = 14.55, p < 0.05$). There was no significant difference between control and impact in the arm of the sulcus ($F(1,11) = 1.84, p > 0.05$).

Figure 17: A) Average cell density (mean cell/ $\mu\text{m}^3 \times 100,000$) of MAP2 labeled neurons in layer 5 in the apex and arm of the cingulate sulcus (mean $\pm$ SEM). The apex of the impacted groups had significantly less MAP2 staining in

layer 5 of the cingulate sulcus compared to the arm of the impacted groups as well as the sulcus and arm of the control group (star = $p < 0.05$). B) Representative image of MAP2 labeled neurons within layer 5 in the apex and arm of both groups. The apex of the impacted groups had less staining compared to the arm of the impacted group as well as the arm and apex of the control group.

Discussion

The primary objective of this thesis was to establish the usefulness of the pig brain and develop an *ex vivo* model with the pig brain to assess potential immediate changes following impact. By doing so, further investigation can now begin to better understand the immediate neural outcomes that follow when an impact to the brain occurs. The techniques that have been used throughout this thesis emphasize that the pig brain is not only useful for biomedical applications (Danek et al., 2017), but also in studying neurological conditions.

The Need for a Better Translational Model

Considering the cost and ethical debate over using non-human primates to study human neurological conditions, the porcine brain can be an alternative to bridge the translational gap between rodents and humans. To begin with, the neuroanatomy of the pig brain is comparable to that of a human brain (Lind et al., 2007). In particular, the gyrencephalic nature of the brain and the location of subcortical brain regions. In humans, the grey and white matter ratio is roughly the same at birth, however, during development, the white matter greatly increases as myelination increases (Mukherjee et al., 2002). This is due, in part, to how the number of migrating neurons that make up the grey matter is nearly completed at birth (Paredes et al., 2016). As development occurs, the connections between regions expands leading to an increase in white matter development compared to grey matter development (Mukherjee et al., 2002). While the pig brain is nearly fully developed at birth, similar to humans (Conrad et al., 2012), the differences in white and grey matter ratio is not the same. The pig brain does not have as much

white matter development as the human brain (Figure 6). However, what is of interest is that the grey matter thickness is comparable to the human brain. Within the midcingulate sulcus, the pig brain consists of 6 heterogeneous layers. Along with the similar grey matter thickness, several anatomical regions in the pig closely resemble that of the human. Regions such as the anterior limb of the internal capsule, globus pallidus and putamen are located in similar positions as the human brain (Félix et al., 1999). The caudate nucleus and putamen that make up the striatum are separated in the pig brain (Sauleau et al., 2009). This is a major difference from rodent brains, in which the caudate nucleus and putamen are considered to be one region (Schilman et al., 2008).

As previously mentioned, the pig brain contains 6 distinct layers of the cortex that make up the similar grey matter thickness as the human brain (Figure 7). In particular, the midcingulate sulcus of the porcine brain contains the intergranular layer 4. While Jelsing and colleagues (2006) mention that the prefrontal cortex of the Göttingen minipig does not contain this layer 4, in this study it appeared that layer 4 began to develop between the anterior cingulate and midcingulate region in the brain. Layer 4 mainly receives input from the thalamocortical region then disperses information to the upper cortical layers. This layer is fairly conserved across species with it being found in rodents, primates and humans (Moore, 2002; Narayanan et al., 2017; Öngür and Price, 2000). The exact location of this layer, however, is slightly different between species. In rodents, this layer appears to develop in the somatosensory cortex, receiving excitatory stimulation from the whiskers (Narayanan et al., 2017). In nonhuman primates and humans, it appears in the prefrontal cortex and into the primary motor cortex (Öngür and Price, 2000; Sherwood et al., 2007). The development of this layer appears to develop in the

midcingulate region of the brain in the porcine brain, falling in between human and rodent brains.

The interest in using pigs to study neurological conditions has started to increase in the past decade. Because of this, it is important to establish common techniques to visualize neurocellular anatomy. A variety of techniques were used here to show that the same methodology of staining that is used for rodents can also be applied to the pig brain. Classical histological techniques, such as Cresyl-Violet staining, can be applied to visualize gross anatomical structures and basic cell types throughout the brain. Immunohistochemical assays can be used with a wide array of antibodies, allowing for further in-depth analysis of the neurocellular anatomy. With the application of the Golgi-Cox stain, it is possible to get a detailed look into axon and dendrite integrity such as the presence of swollen or beading regions. The detail of the Golgi-Cox stain can identify different types of dendritic spines. Spine classification can be used to see if there is a change towards a particular subtype of dendritic synapse following an insult to the brain, providing evidence of tissue reactivity (Chuckowree et al., 2018). Taken together, these basic techniques have provided a look into how the pig brain has developed and has shown the similarities between the pig brain and the human brain.

An important consideration in the development of an *ex vivo* model is preservation of tissue viability and reactivity. Electrophysiological studies have utilized *ex vivo* models to record the synaptic activity of neurons and astrocytes several hours post-mortem (Kramvis et al., 2018; Testa-Silva et al., 2014). This involves careful preparation of the tissue as soon as it is removed from the skull and maintaining an environment in which the cells can remain viable. Kramvis and colleagues (2018) recorded action potentials in post-mortem human hippocampal pyramidal

cells up to 3 hours post-mortem. These cells continued to produce spontaneous action potentials and inhibitory and excitatory synaptic potentials. As post-mortem delay increased, the ability to record activity decreased as the cells began to deteriorate. This study demonstrates that cellular responses can still occur 3 hours *ex vivo*, suggesting the preparation used in the present thesis used viable cortical neurons. Combined with synaptic density changes revealed by Golgi-Cox reconstruction, electrophysiological recordings of synaptic activity could provide an in-depth analysis of cellular response following impact.

While testing the effects on different types of cannulas, astrocyte activation was seen surrounding the sites of insertion. The highest amount was observed in the nail polish coated cannulas, indicating that something was reacting with the tissue. A common warning when performing the Golgi-Cox staining procedure is to avoid the use of metal or metal touching the brain tissue (Zaqout and Kaindl, 2016). Even though it is well documented, there is no real explanation as to why it should be avoided. We present that metal causes astrocytic activation within the tissue. While the exact reasoning why metal causes the tissue to react is still unknown, it is worth noting that the tissue continues to respond to an external manipulation. While the brain is removed from the skull, the tissue still has the capacity to remain viable and responsive.

Astrocytic activation was seen in all of the different types of cannulas, as well as the control brain. There was, however, a difference in the types of astrocytes within each of the tissues. The control, metal, and Sylgard cannulas produced large-bodied reactive astrocytes within the white matter, but not clustering of these cells. Reactive astrocytes were identified based on their thick spiny processes coming from the cell body (Wilhelmsson et al., 2006). The

remaining three types of cannulas (epoxy, and nail polishes) produced large amounts of clustering surrounding the site of marker insertion. The nail polishes produced the most amount of cellular clustering with astrocytes having thick spiny processes. The epoxy coated cannula had no astrocytic activation immediately surrounding the site of marker insertion, however, extensive clustering was observed outside of this zone. To the best of our knowledge, this is the first-time astrocytic activation, different types of cannula coatings and Golgi-Cox stain have been used together. The reasoning why the epoxy coated cannulas produced this zone of no activation surrounded by extensive activation is not known. Further testing could reveal why some materials (such as nail polish) react so strongly to *ex vivo* tissue while other materials do not lead to extensive activation.

Cellular Changes Following Impact

The model that was developed in this work was used to test the immediate cellular changes that occur following an impact. Based on the data presented here, neurons within the apex of the sulcus appear to undergo some form of cytoskeletal restructuring but do not experience cell death. It is difficult to say what exactly is responsible for this decrease in MAP2 in the apex. Previous computational data indicate the sulcus receives some of the highest amount of strain during an impact (Ghajari et al., 2017). This high strain and sudden change in extracellular environment could compress this area, shearing dendrites and breaking synaptic junctions (Chuckowree et al., 2018).

Biomechanical studies have shown that the apex of the sulcus is the point in which maximum mechanical stress is experienced in gyrencephalic brains (Vink, 2018). Previous research has identified the formation of NFT and phosphorylated tau deposits within the apex of the sulcus (McKee et al., 2013). Here we show within the apex of the sulcus, there is a higher density of neurons compared to the adjacent arms. Hyperphosphorylated tau has been shown to “seed” to neighbouring neurons and lead to aggregation of tau in the otherwise healthy neuron (Katsinelos et al., 2018). Since the neurons in the apex of the sulcus are closer together, this spreading of disease can happen at a faster rate than the sides of the sulcus. Moreover, changes to the extracellular environment could have a more severe effect on the densely packed neurons. There is less space for the spread of biochemical signals between neurons compared to an area of lower neuronal density, such as the arm of the sulcus.

Ca^{2+} changes within the cell have been shown to both phosphorylate MAP2 and activate various proteases that begin the progression of cell death following a TBI (Taft et al., 1992). The resulting conformation change to MAP2’s structure causes it to dissociate from the microtubule (Mudrakola et al., 2009; Ramkumar et al., 2018). This results in decreased levels of MAP2 binding and altered cellular integrity following an impact (Folkerts et al., 1998). Here we show that after an impact of 3.8 m/s, changes to the cytoskeleton occur within the first hour after impact. While there was no change to the overall neuronal density, there was a change in cell soma size in the pyramidal cells of layer 5, as well as a decrease in MAP2 cellular levels. This could indicate a possible early marker of degeneration, and a greater vulnerability to damage from another impact due to the compromised cytoskeleton. Zhao and colleagues (2016) found decreased levels of MAP2, but no cell death, in the cortex of mice after an in vivo TBI. Altered

MAP2 levels were accompanied with swollen and beading dendrites within layer 2/3 of the cortex. As MAP2 levels decrease and microtubules are no longer bundled, motor proteins experience delays in transporting cellular components within the neuron (Mudrakola et al., 2009). The decrease in MAP2 could be an indication of Ca^{2+} changes within the neuron, given how Ca^{2+} promotes calpain cleavage and MAP2 phosphorylation (Taft et al., 1992), however more experimentation would be needed to confirm this change.

The strength of this finding also extends to the fact that the *ex vivo* tissue reacts in a similar way to what is seen in the literature using a different model of TBI. Following death, the cells of the body begin to degrade, and tissue starts to degenerate. The reactivity following impact indicates that the tissue has the capability to respond to an external stimulus and still be similar to what is seen in other models of TBI. Even though the tissue is naturally degrading, the impact accelerates the degradation (Figure 18).

Figure 18: Visual representation of tissue degradation after death. As time increases, under normal circumstances the tissue will naturally degrade, decreasing the viability of the tissue. This degradation increases when subjected to an impact.

Limitations

Even though this model is a step towards translating what is seen in rodents and humans, the experiment has several limitations. First, and foremost, the model developed here is an *ex vivo* model of traumatic brain injury and cannot be fully applied to what is seen in humans. Careful consideration was made to make the cells within the tissue still viable, with the tissue incubated in bubbling aCSF. The tissue, however, did not receive oxygenated blood nor was it under normal intercranial pressure. These variables cannot be controlled for with the use of in-tissue barium sulphate markers. If changes were made to investigate blood flow, as described in Vrselja et al. (2019), the model could be further applied to *in vivo*. Blood brain barrier permeability has been shown to increase after impact, allowing for toxins to enter into the closed blood system of the brain (Blennow et al., 2016). Moreover, one of the hallmarks of the long-term pathology is the formation of tau surrounding blood vessels (McKee et al., 2013).

Another limitation was the time frame following impact. Future studies can investigate ways of keeping these tissue slabs viable for longer periods of time. For the current study, only 1-hour post impact was analyzed. In order to keep the tissue viable, modifications to the procedure will need be done. While the data were not presented in the current work, one of the brains was taken out of the skull and placed in ice cold aCSF for 4 hours after death. These cells had substantially weaker staining in both the NeuN and MAP2 and deteriorating cell structures. It may be possible to achieve longer periods of time outside of the skull and avoid substantial cellular deterioration (Ensign et al., 2012). The current methodology could be modified to allow for this time point analysis to be investigated.

A third limitation would be the damage that the brain experiences from the slaughter. The abattoir where the pigs were collected stun the pigs using an electrical shock to render the pig unconscious. Electrical stunning has been shown to lead to altered neurological functioning. Within 30 minutes of electrical stunning of pigs, an isoelectric EEG was observed which was considered to be brain dead (Stiegler et al., 2012). Correct stunning is intended to disrupt the brain's electrical activity, resulting in an artificially induced epileptic seizure (Terlouw et al., 2016). This procedure is intended to render the pig unconscious before bleeding. Along with neurological dysfunction, stunning induces cardiac fibrillation and eventually cardiac arrest (Terlouw et al., 2016). Without proper energy supplied from the blood, as well as disrupted brain electrical activity, neurons begin to release glutamate and K^+ leading to excitotoxic conditions (Terlouw et al., 2016). However, it has been recently shown that, despite these negative consequences, normal brain activity can be restored to brains from pigs that have been slaughtered hours before (Vrselja et al., 2019). To control for the consequences of electrical stunning, control and impacted brains came from the same brain. Therefore, if any damage happened to the whole brain from stunning, both conditions – impacted and control - would experience this. Any further damage would be then from the impact.

Future Directions

Overall, the development of this model has great potential to bridge the gap between rodent models and human TBI. Considering the novelty of this model, the groundwork had to have been put down before further investigation. There are a number of investigations that can

now take place to further understand what exactly is going on in the folded brain following an impact. For instance, axonal swelling has been reported in pig models of TBI around 6 hours and up to 24 hours post impact (Ibrahim et al., 2010; Johnson et al., 2016). As membrane permeability increases during this time, cations such as Ca^{2+} and K^{+} enter the neuron leading to an increase in intracellular concentrations (Tao et al., 2017). This increase results in the starting of a wide range of biochemical pathways, such as reactive oxygen species production, activation of calpain, phosphorylation of microtubules and cytoskeleton remodelling (Vercesi et al., 2018; Yousuf et al., 2016). Using this ex vivo model of porcine TBI, analyses can be conducted to look at the immediate changes in cation levels following impact. The movement of cations in and out of the cell play a role in the morphology of these cortical neurons. Along with further assessing the immediate changes, long term changes following TBI can be looked at in future studies as phosphorylation of proteins takes time to occur and alter cellular functioning (Yousuf et al., 2016).

One of the major contributors behind the negative effects of TBI is neuroinflammation. There are reports of increased reactive astrocyte activation within minutes and up to several hours following impact in pigs (Lafrenaye et al., 2015; Smith et al., 1999; Wofford et al., 2017). The immediate activation of astrocytes following impact are most likely due to the sudden changes in cations within the extracellular space (Curvello et al., 2018; Lafrenaye et al., 2015). Swollen neurons begin to burst and release DAMPS into the extracellular space, furthering neuroinflammation (Kerr et al., 1972). Considering how the apex of the sulcus shows a higher density of neurons than the wall, this could be a contributing factor to the spread of excitotoxicity and the propagation of cell death. Future directions could look at the activation of astrocytes and

neuroinflammation specifically in the apex. A better biological understanding of why the apex is particularly vulnerable to damage is key in understanding the long-term consequences of head impacts.

Modifying the current procedure could allow for longer post-impact times while still maintaining cellular integrity. Research can be done into what is required to keep brain tissue viable for longer periods of time. Even though there was no change in cellular density in the apex, this could be due to the time needed for cell death to occur. It has been reported that apoptotic processes take several hours to result in substantial cell death (Yousuf et al., 2016). By modifying the current procedure, time point analysis of cell death can be investigated, potentially looking at several hours following impact. It is documented that *ex vivo* studies can last for hours, so adapting to similar procedures can allow for this type of analysis (Ensign et al., 2012). Along with looking at longer time points, the potential for analyzing the electrophysiological properties of the neurons can assure the maintenance of proper cellular functioning. An advantage, however, to the 1-hour time point selected for the present study is investigating the immediate effects of injury. The immediate effects begin the biochemical cascades that exponentially increase as time goes on. These changes that are occurring within the first hour after impact could give an indication as to how the long-term pathology of head trauma begins.

Conclusion

In summary, the need for developing a model that allows for the comparative analysis of TBI is greatly needed. The gyrencephalic nature of the pig brain is a valuable tissue for such analysis. Combining biomechanical strain info and biological changes provides an interdisciplinary look into what exactly is occurring within the sulcus of the brain. In this thesis, we developed an *ex vivo* model to investigate biomechanical and biological changes and bridge the translational gap between rodent and human TBI. Immediately following an impact, cytoskeletal remodelling was noticed in the apex of the sulcus, but not the adjacent arm, indicating a particular vulnerability of the apex. Future directions can set out to investigate this phenomenon in depth by analyzing cation changes, neuroinflammatory markers and protein phosphorylation. Integrating these findings with what is described in biomechanical fields can further advance the understanding of human TBI consequences.

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