The Effect of Hydrostatic Pressure on Neuronal Cell Morphology In Vitro

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THE EFFECT OF HYDROSTATIC PRESSURE ON NEURONAL CELL MORPHOLOGY IN VITRO

A Thesis
Presented to
the Graduate School of
Clemson University

In Partial Fulfillment
of the Requirements for the Degree
Master of Science
Bioengineering

by
Kallie Etten
December 2017

Accepted by:
Dr. Jiro Nagatomi, Committee Chair
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Dr. Delphine Dean
ABSTRACT

Traumatic brain injury (TBI) and subsequent rises in intracranial pressure (ICP) are associated with high mortality and has a number of physical and behavioral consequences to which the specific causes are unknown. We hypothesized that exposure of neuronal cells to elevated pressure can cause neurite retraction and/or apoptosis. Loss of communication between neurons due to these cellular-level events may play a part in complications seen in TBI patients, such as neurodegenerative diseases, increased anxiety, fear, depression, cognitive problems, as well as motor and visual deficits. In the present study, the effect of elevated hydrostatic pressure was evaluated at the cellular level in vitro by examining cell morphology, particularly neurite retraction and extension, as well as apoptosis. Neuro-2A cells (a mouse neuroblastoma cell line) were plated in cell culture dishes, as well as on soft polyacrylamide gel + type I collagen substrates. The cells were imaged using phase contrast microscopy (Nikon) before and after exposure to 25 or 35 mmHg of hydrostatic pressure in a custom-made pressure chamber for 15, 30, 60 min, as well as 4 and 6 hours. Pressure values were chosen to model elevated ICP correlated with moderate to severe TBI. Using the Fiji software, the post-pressure images were compared with the pre-pressure images of the same cells and the data were reported as normalized change in neurite length per cell. Additionally, TUNEL assay was performed using a commercially available kit (TiterTACS™, Trevigen) to quantify apoptotic cells after exposure of Neuro-2A cells to 35 mmHg pressure. Results indicate that neurite length decreased significantly (p<0.05) when Neuro-2A cells were exposed to a pressure of 35 mmHg. When results are compared from 15, 30, 60 minutes and 4 hours
of pressure exposure, it appears that neurite retraction is correlated with exposure time. To further understand these morphological changes, the Rho/ROCK pathway was examined as a potential pathway involved in the mechanotransduction of hydrostatic pressure by neuronal cells. By using Y-27632 as a p160ROCK inhibitor, it was demonstrated that the pressure-induced neurite retraction response was blocked, therefore indicating the pathway’s involvement. The results of TUNEL assay indicated that the number of apoptotic cells were similar between the no pressure control and the cells exposed to 35 mmHg of pressure for 24 hours. The results of the present study provide evidence that elevated hydrostatic pressure causes Neuro-2A cell neurite retraction in a time-dependent manner in vitro. The model highlights the importance of urgent ICP clinical management, as elevated ICP caused by TBI may have a negative impact on neuronal tissue and contribute to further consequences.
DEDICATION

I would like to dedicate this thesis to my family and friends. I am forever grateful to my parents for raising me with love and instilling in me the values for success, as well as for providing me with the wonderful opportunity to grow up as a Third Culture Kid. Words cannot explain how that international experience has molded my character and outlook on life. Thank you for fostering my passion for creativity and curiosity for knowledge, for loving me, guiding me, supporting me, and believing in me. To my wonderful friends, thank you for your endless love, kindness, and encouragement. You made the rough times bearable and the great times the best. Without your support this work would have not been possible.
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CHAPTER ONE
INTRODUCTION AND BACKGROUND

1.1 Introduction

Traumatic brain injury is an immediate insult to the brain due to an external mechanical force that can range from mild to severe, resulting in both immediate and long-term effects (Caeyenberghs et al, 2014). Approximately 1.7 million traumatic brain injuries (TBI), 52,000 of which are fatal, occur every year in the United States, with an associated annual treatment cost of $70 billion (MedGadget, 2016). TBIs can result from a number of events including falls, vehicle accidents, assault, sports impacts, and battlefield blasts, and account for 50% of injuries sustained in war zone conflicts (MedGadget, 2016). The severity of TBI is classified as either mild, moderate, or severe. Concussions are a common type of mild TBI that occur largely due to sports impact injuries, and often go unreported or untreated due to their lack in severity. Moderate and severe TBIs are associated with higher mortality rates, and often result in severe disabilities (Caeyenberghs et al, 2014). Treatments exist to treat some secondary injury mechanisms, such as corticosteroids or calcium channel blockers (Xiong et al, 2009). However, no reliable treatment currently exists to alleviate the effects of TBI as a whole and prevent future consequences.
1.2 Anatomy and Physiology of the Brain

1.2.1 Structures

The brain is the main organ of the nervous system and acts as a control center that receives and processes information, then dictates responses. It is a complex structure with several tissue and cell types (McKinley et al., 2012).

The cerebral cortex, the outermost layer of the brain, is composed of gray matter, which contains motor neurons and interneuron cell bodies, dendrites, and unmyelinated axons (McKinley et al., 2012).

Cerebrospinal fluid (CSF) is an important component of the central nervous system (CNS) that circulates in the ventricles and subarachnoid space, and surrounds all exposed surfaces of the brain and spinal cord. The clear liquid not only protects and supports the brain, but also facilitates the transportation of nutrients, waste, and gases such as carbon dioxide and oxygen. The choroid plexus, a layer of ependymal cells and capillaries that is housed in each ventricle, produces CSF. After circulating in the ventricles, CSF enters the subarachnoid space where it removes waste products, and is then absorbed via arachnoid villi into the venous blood stream for eventual urinary excretion (McKinley et al., 2012).

1.2.2 Cell Types

Brain tissue is composed of several cell types including neurons, astrocytes, microglial cells, oligodendrocytes, and ependymal cells (Pekna et al., 2012). Interneurons are the primary cell type of the central nervous system (CNS) and are responsible for signal transmission between sensory and motor neurons. These specific neurons are multipolar, meaning that they have multiple neurites- namely one axon and several
dendrites. Dendrites receive sensory information from stimuli and send it to the cell body for processing, from which an output signal is transported via the axon to the effector cell. This allows the interneurons to receive, store, and interpret sensory information, then dictate the response. Depending on the complexity of the response, several interneurons may be involved in the transmission process (McKinley et al, 2012). Thus, properly structured interneuron networks are essential to facilitate full functional signal processing and communication.

Astrocytes, microglial cells, oligodendrocytes and ependymal cells are classified as glial cells, which support the function of neurons and are able to undergo mitosis. Astrocytes are the most common type of glial cell. Their star-shaped structure allows them to provide structural support to neurons and organize nervous tissue, as well as contribute to forming the blood-brain barrier (BBB) through their connection with capillaries in the brain. Additionally, astrocytes regulate fluid composition in the brain by controlling molecular diffusion between blood and interstitial fluid. More importantly, astrocytes assist in neuronal development and synapse formation through the release of molecular signals. Ependymal cells are responsible for producing cerebral spinal fluid (CSF) in a structure called the choroid plexus. Microglial cells act as macrophages of the nervous system by migrating through the tissue and phagocytizing debris. Oligodendrocytes wrap around the axons of neurons to form the myelin sheath, which protects the axon while hastening signal transmission (McKinley et al, 2012).
1.2.3 Vasculature and Intracranial Pressure

Intracranial pressure (ICP) is determined by three components including tissue (brain and spinal cord), blood, and cerebrospinal fluid (CSF), which are housed in the skull and intervertebral canal, creating a fixed incompressible system. The Monroe-Kellie hypothesis states that the total intracranial volume remains constant. Therefore, when a volume increase in one of these components occurs, an equal decrease in another accommodates the change to maintain the appropriate intracranial pressure. For a normal healthy adult, the ICP ranges between 5 and 15 mmHg (Rangel-Castillo et al, 2008).

Another factor essential to maintain optimal ICP is the cerebral perfusion pressure (CPP). The CPP controls the cerebral blood flow (CBF) via the paired carotid and vertebral arteries, ensuring adequate blood flow and delivery of oxygen and glucose to brain tissue. More specifically, the blood vessels detect carbon dioxide concentration in the blood (partial pressure), and respond to increased partial pressure by vasodilation in order to increase CBF, and respond to decreased partial pressure by vasoconstriction, resulting in lowered CBF (Snyder, 2012). The CPP also depends on the mean arterial pressure (MAP), which is determined based on the individual’s systolic and diastolic blood pressure. In order to maintain adequate CPP, the ICP must be reduced, or the MAP increased (Rangel-Castillo et al, 2008).

1.3 Traumatic Brain Injury

1.3.1 Types of Traumatic Brain Injury

Depending on the type and location of the injury, certain structures within the brain are affected. Two main types of TBI occur including 1) focal trauma and 2) diffuse
axonal injury (DAI) (Gaetz, 2004). Focal trauma constitutes direct mechanical damage to brain tissue which affects local neurons and cerebrovasculature and leads to ischemia and cytotoxic cascades. It can also result in traumatically induced masses such as hematomas and hemorrhagic contusions. DAI accounts for about 40-50% of dysfunction associated with TBI. It affects superficial layers of brain tissue, particularly neurons and their axons, through acceleration and deceleration forces, then radiates deeper through the white matter (Gaetz, 2004). Specifically, DAI tends to affect certain brain regions including the parasagittal white matter of the cerebral cortex, the corpus callosum, and the pontine-mesencephalic junction adjacent to the superior cerebellar peduncles (Ashley, 2010).

1.3.2 Symptoms and Consequences of TBI

In moderate to severe TBI, there is a primary injury due to the initial impact which typically results in either a) contusions, lacerations, and intracranial hemorrhage, or b) diffuse axonal injury (Werner et al, 2007). Secondary injury results downstream of the primary insult as a consequence of cascading pathological biochemical processes such as disruption of cellular calcium homeostasis, increased free radical presence, lipid peroxidation, inflammation, apoptosis, and mitochondrial dysfunction (Xiong et al, 2009). Several secondary events may occur, including elevated intracranial pressure (ICP) or intracranial hypertension, and cerebral ischemia (Werner et al, 2007).

1.3.2.1 Short-Term Consequences

Upon impact, direct cell damage and death may occur immediately. Neuronal and glial cell death lead to disruption in neuronal circuitry due to synapse and neurite destruction between cells. Additionally, TBI disrupts blood flow and the blood-brain
barrier, CSF production and regulation, as well as cell metabolism and function (Pekna et al., 2012). Traumatic hemorrhagic contusions, as well as epidural hematomas or subdural hematomas may occur upon injury (Rangel-Castillo et al., 2008) and contribute to blood volume increase inside the skull (Alali et al., 2013). Cerebral edema, an increase in brain-water content either intracellularly or extracellularly, may occur and increase fluid volume inside the skull (Alali et al., 2013).

Additionally, patients who suffer non-fatal severe TBI may experience coma or amnesia (Centers for Disease Control and Prevention, 2017). Other potential effects of severe TBI that may be either short or long-term include deficits in cognitive functions (attention and memory), motor functions (weakness, impaired balance and coordination), sensation (hearing, vision, touch), and emotion (depression, anxiety, aggression, impulsivity, personality changes) (Centers for Disease Control and Prevention, 2017).

In DAI, when the initial damage occurs, the local neurons experience cell membrane disruption, which leads to cytotoxic biochemical cascades and decline in cell health and function. More specifically, small ion species are believed to enter the disrupted axons, and inflict damage on the microtubules that compose the cytoskeleton (Gaetz, 2004). These local events eventually extend and affect distant structures as well (Ashley, 2010).

On the cellular level, DAI induces axolemmal permeability, allowing an influx of \( \text{Ca}^{2+} \) in the affected neurons at the primary injury site via calpain activation. This causes proteolytic digestion of a spectrin, which is a key component of the cytoskeleton membrane. These neurofilamentary changes may result in axonal cytoskeleton
mechanical failure. Negative downstream effects may occur such as mitochondrial
migration from the cell body to the axon or axon terminals. Further axonal degeneration
occurs over time. This specific degeneration may occur within days or months and
progresses from initial axonal swelling to axonal bulb swelling, which then results in the
aggregation of microglia. Within the first two years after DAI is sustained, the myelin
sheath ultimately degenerates (Ashley, 2010).

On a macroscopic scale, DAI damages white matter and disrupts tissue organization
in the brain. Previous studies that examined specific brain regions including the superior
longitudinal fasciculus, corpus callosum, and cingulum fibre bundles demonstrated that
TBI associated cognitive impairment correlates with white matter damage (Caeyenberghs
et al, 2014). A study performed by Caeyenberghs et al, examined the effect of DAI on
neural communication networks and found that TBI patients had disrupted topological
organization of white matter networks which could have been responsible for observed
decreased task-switching ability when compared with the control (Caeyenberghs et al,
2014).

1.3.2.2 Long-Term Consequences

Several long-term consequences are observed in TBI patients including those of an
emotional, cognitive, and physical nature. Neuropsychological deficits associated with
cognition include decreased mental flexibility, lack of planning and organizational skills,
poor judgment, impaired attention, trouble with sequencing, speech impairment,
increased impulsive behavior, and deficits with working memory. These effects are
attributed mainly to diffuse axonal injury (DAI, Caeyenberghs et al, 2014). Impaired
memory has been observed in severe TBI patients, however there have been mixed reports in terms of mild and moderate TBI cases. Case study results find that sports impact injury patients sustained memory impairment, whereas veterans who experienced mild battlefield blast TBIs did not. Additionally, a decreased ability to recollect autobiographical details in TBI patients was correlated with increased CSF volumes in the bilateral genual cingulate gyrus, medial temporal lobe, anterior basal ganglia, left posterior cingulate gyrus, right anterior cingulate gyrus, and right posterior temporal, superior parietal, inferior parietal, and middle cingulate regions (Esopenko et al, 2017).

1.3.2.2.1 Neurodegenerative Diseases

Previous studies indicate that moderate to severe TBI serve as a significant risk factor for the eventual development of neurodegenerative diseases such as Parkinson’s disease (Jafari et al, 2013), Alzheimer’s disease (Mortimer et al, 1991; Fleminger et al, 2003) and amyotrophic lateral sclerosis (ALS, Chen et al, 2007), as well as frontotemporal dementia (Rosso et al, 2003; Kalkonde et al, 2012). While a holistic understanding of the all mechanisms responsible for linking the two pathologies remains unclear, several involved components have been elucidated.

Several correlations between traumatic brain injury and neurodegeneration associated with Parkinson’s disease (PD) exist. Animal studies have shown that after brain injury, protein accumulation and aggregation have occurred (Smith et al, 1999; Iwata et al, 2002), as well as proteosomal dysfunction (Yao et al, 2008), and an increase in nitrated α-synuclein (Uryu et al, 2003), as seen in PD patients. In post-mortem human studies, TBI patients exhibited increased levels of α-synuclein. α-synuclein aggregation
is a key indicator for the development of a neurodegenerative disease, as it is the main component present in Lewy bodies—abnormal protein aggregates observed in the neurons of PD dementia patients. It is hypothesized that α-synuclein aggregation may be due to disruption of axonal transport processes or axonal shear stress (Uryu et al, 2003; Siebert et al, 2010; Newell et al, 1999; Gaetz, 2004). Additionally, a study by Goldman et al found that patients with head injuries have overexpression of the alpha-synuclein (SNCA) gene and elevated levels of Rep1, which increases its transcription, suggesting that this is a contributor to the α-synuclein aggregation observed in PD (Goldman et al, 2012). Moreover, α-synuclein aggregation activates microglia, which in turn activates α-synuclein to aggregate when activated, creating a cycle of α-synuclein upregulation (Wilms et al, 2009; Zhang et al, 2005).

1.3.2.2.2. **Glial Scar Formation**

Glial cells become activated as a protective response after neurotrauma in a process termed “reactive gliosis”. This process encompasses several events including the hypertrophy of astrocyte extensions, astrocyte proliferation, and elevated amounts of intermediate filaments. When activated, astrocytes immediately perform beneficial functions to protect surrounding tissue by preserving neuronal synapses, restricting the spread of injury, and aiding in wound repair. However, at a later stage of injury, glial scar activation has been shown to correlate with reduced regeneration of neuronal synapses and axons (Pekna et al, 2012).
1.3.2.2.3 Elevated Intracranial Pressure

Several factors compound to result in an increase in intracranial pressure (ICP) following TBI. As mentioned previously, hemorrhages and hematomas raise blood volume and edema increases fluid volume. Disruptions in CSF absorption can contribute to increased CSF volume throughout the CSF cavities, since the excess liquid cannot be removed and fresh CSF continues to be produced. Foreign bodies acquired during injury, or depressed skull fractures may also be introduced to the space (Rangel-Castillo et al., 2008). With any or all of these increases in volume in the fixed space within the skull, intracranial pressure is forced to rise. Additionally, edema can compress cerebral vasculature, which reduces CBF, increases carbon dioxide concentration and consequently causes vasodilation and further increases ICP. When ICP increases enough, the brain loses the ability to autoregulate CBF (Snyder, 2012). ICP associated with mild to moderate TBI ranges from 20 to 30 mmHg, while an ICP of 40 mmHg or greater is correlated with severe TBI (Rangel-Castillo et al, 2008).

Rising ICP is often reflected in a patient’s mental status. Early on, the patient may exhibit confusion, later developing into a state of irritability, and ultimately transcending into combativeness. Severe headache and vomiting are indicative of subarachnoid hemorrhage and meningeal irritation as well as tissue compression. Unresponsiveness is a sign of the later stages of increased ICP. Additional late symptoms include seizures, a blown pupil, and Cushing reflex as a result of direct compression of the brain cortex tissue from hematomas or other expanding masses or free blood (Snyder, 2012).
Increased ICP is known to be responsible for ischemia—when blood flow is restricted to brain tissue, resulting in cell damage and death, as well as cerebral herniation. Since the pressure increases in the fixed volume of the skull, sometimes the brain itself is pressed unnaturally against structures such as the tentorium cerebelli or the cranial foramen, through which it herniates (Snyder, 2012). Other types of herniation that may occur due to elevated ICP include cingulate herniation, which occurs when the middle lobe of the brain shifts under the falx cerebelli, uncal herniation, which occurs when the uncus is pressed against the tentorium cerebelli and brain tissue is forced through the tentorial hiatus, cerebellotonsillar herniation, in which the cerebellum herniates through the foramen magnum and compresses the brain stem, and finally transcalvarial herniation, in which brain tissue is displaced to the exterior of the skull if a skull fracture exists (Snyder, 2012).

### 1.3.2 Current Treatment for Controlling ICP

The initial stage of severe TBI treatment traditionally take place at the location where the injury occurred, followed by at the emergency department of the hospital to which the patient is transported, and ultimately the intensive care unit (ICU). The early acute care provided is crucial as it could prevent additional consequences and improve patient outcome. Critical steps include stabilization of the patient, prevention of ICP increase, and optimization of cerebral perfusion and oxygenation. To do so, medical personnel monitor the patient using electrocardiography, pulse oxymetry to measure arterial oxygen saturation, arterial blood pressure, electrolytes, osmolality, central venous pressure, and systemic temperature (Haddad et al, 2012).
Since the primary brain injury cannot be repaired in TBI patients, physicians focus on treating the secondary injuries in hopes of eliminating or reducing further damage that may lead to long and short-term functional deficits or death. To date, no therapeutic has been successful in clinical trials to treat TBI secondary injury (Xiong et al, 2009).

Death and disability after severe TBI are most strongly correlated to high ICP, rather than any other TBI symptom (Xiong et al, 2009). Of those who die within the first 48 hours following injury, the most likely cause is an uncontrolled rise in ICP (Alali et al, 2013). Therefore, it seems vital that ICP is monitored closely when TBI patients receive treatment for their injuries (Xiong et al, 2009). However, some physicians argue the benefit from ICP monitoring due to its invasive nature, which could potentially further harm the patient. For example, results from a survey on practicing neurosurgeons in Canada, only 20% reported that they were highly confident in the benefit of invasive ICP monitoring in severe TBI patients (Alali et al, 2013). In a retrospective study performed by Alali et al, it was found that the use of invasive ICP monitoring was associated with lower in-hospital mortality rates for TBI patients, when compared to facilities that did not use invasive ICP monitoring for severe TBI patients (Alali et al, 2013).

ICP monitoring is an invasive technique that is associated with risks such as infection at the catheter placement site and hemorrhage or device malfunction. Therefore, it is only employed when patients exhibit signs of significant risk, which is usually evident on head CT scans. Detectable abnormalities include contusions, hematomas, herniation, edema and compression of basal cisterns (Rangel-Castillo et al, 2009).
Current methods for ICP monitoring include the use of catheters in epidural, subdural, subarachnoid, parenchymal, and ventricular locations, with ventricular catheterization being the most common. Ventricular catheterization is often the most viable method as it is reliable, low-cost, and more accurate than other options. An added benefit is that this same catheter can be used for CSF drainage in the event that ICP needs to be lowered. ICP lowering techniques are employed when an ICP of 20 mmHg or above is detected (Haddad et al, 2012).

Elevated ICP is treated clinically based on the symptoms exhibited at the present time. Treatment is centered on protecting the airway, breathing, and circulation in an effort to maintain sufficient cerebral perfusion and avoid ischemic events. This is accomplished by administering oxygen, assuring adequate ventilation, and possibly hyperventilation in cases of herniation in order to decrease carbon dioxide concentration and induce vasoconstriction to lower ICP (Snyder, 2012).

1.3.2.1 Medical Interventions

A common method to alleviate elevated ICP is the use of non-invasive hyperosmolar therapy (Rangel-Castillo et al, 2009). Osmotic diuretics such as mannitol and hypertonic saline (Rangel-Castillo et al, 2009) may be administered in order to draw excess fluid as a consequence of edema from the brain tissue and into intravascular space for elimination. This method, however, may not be ideal for all patients, such as those with previously existing conditions such as renal failure, hematomas or other blood-related etiologies (Snyder, 2012). It is more successful in lowering ICP in patients who have an intact autoregulation system, as compared to those who do not. Specifically, with
a functional autoregulation system, mannitol can induce cerebral vasoconstriction which in turn maintains stable CBF and significantly lowers ICP. When the patient is unable to autoregulate pressure, mannitol only increases CBF, which leads to a small decrease in ICP (Rangel-Castillo et al, 2009).

Hypothermia (reducing body temperature to 32°C) is another treatment method used to control elevated ICP and improve patient overall outcome. There are several mechanisms through which this technique improves brain condition which include the following: improving cerebral blood flow, reducing metabolic rate, calcium antagonism, preventing excitotoxic cascades, preserving protein synthesis, modulating inflammatory response, protecting white matter, modulating apoptosis, and decreasing edema formation (Xiong et al, 2012). In a study performed by Markgraf et al, rats showed decreased cerebral edema and improved functional outcomes when hypothermia had been administered either immediately or 1 hour after TBI was sustained, as compared to those who had received the same treatment after 90 or 120 minutes where no benefit was observed (Markgraf et al, 2001). In a patient study, it was found that hypothermia sustained for at least 48 hours showed the best outcome, however this also puts the patient at risk of pneumonia. Hypothermia may also be beneficial to traumatic axonal injury, which is commonly observed in severe TBI (Xiong et al, 2012).

A last resort for severe TBI treatment is the administration of barbiturates to induce coma. This method should only be considered if the patient experiences refractory intracranial hypertension (RICH, when intracranial pressure exceeds 25 mmHg for 30 minutes, 30 mmHg for 15 minutes, or 40 mmHg for 1 minute) because it is often
associated with serious complications such as hypotension and immunosuppression (Censullo et al, 2003; Haddad et al, 2012). This treatment method may be selected in order to sedate the patient if their behavior becomes harmful to their condition, if it is beneficial to immediately reduce the metabolic needs of the brain tissue (Snyder, 2012), or to immediately lower ICP by lowering the metabolism, and in turn lowering CBF (Haddad et al, 2012).

1.3.2.2 Surgical Interventions

The primary surgical procedure performed to alleviate elevated ICP is the removal of mass lesions such as acute epidural and subdural hematomas. Secondly, CSF drainage is employed by inserting a catheter into the ventricular space in order to immediately lower intracranial fluid volume and ICP (Rangel-Castillo et al, 2009).

Another last resort treatment for severe TBI and intracranial hypertension is decompressive craniectomy, in the event that medical management fails (Haddad et al, 2012). Surgeons remove a portion of the skull in order to allow the swelling brain tissue to expand without being compressed against the rigid skull, and by opening the fixed volume of the skull, ICP is reduced (Xiong et al, 2009). The favorability of this technique is currently debated since conflicting evidence exists for its effect on patient outcome. Current clinical trials aim to clarify issues associated with the technique in regard to the timing of the operation, its role in secondary injury, and brain edema formation (Xiong et al, 2009). This method is used only as a last-ditch effort when the patient is close to death (Haddad et al, 2012).
1.4 Neuronal Cell Responses to Stress

Brain cells regularly experience stresses and respond to them accordingly. Several environmental stimuli such as drugs, cytokines, hydrostatic pressure, and other mechanical forces can evoke stress responses in cells. One response commonly examined in drug studies on neuronal cells is neurite retraction, since it is easily observable in this cell type.

1.2.1 Neurite Retraction

Neurites are mainly composed of two cytoskeleton elements- microfilaments, and microtubules. Microtubules consist of tubulin and microtubule-associated proteins (MAPs), which when phosphorylated, appear to effect microtubule stability and dynamics. Neurite extension and retraction is controlled by reorganization of these components with the help of molecular motors, which are still not entirely understood (Sayas et al, 2006).

1.2.1.1 Causes and Mechanisms

Neurite retraction has been studied in relation to several disease states. Neurite retraction plays a significant role in the progression of neurodegenerative diseases, as well as CNS injuries and glaucoma. Pathways have been identified as moderators of neurite retraction and extension, however their specific stimuli haven’t been identified. It is also unclear through which pathway hydrostatic pressure as a stimulus affects neuronal cells.

The Rho/ ROCK pathway has been identified as a moderator of actin cytoskeleton and consequentially neurite structure in previous studies (Hirose et al, 1988; Sun et al,
Specifically, activation of the small GTPase Rho and its downstream effector Rho kinase have been demonstrated to play a role in neurite retraction via the disassembly of intermediate filaments and microtubules (Hirose et al., 1988). Additionally, a pyridine derivative Y-27632 has been characterized as a specific inhibitor of p160ROCK, a Rho-associated coiled coil-forming protein kinase within the Rho/ROCK pathway, which was shown to prevent neurite retraction in neuroblastoma N1E-115 cells (Hirose et al., 1988; Monnier et al., 2003). In a study by Hirose et al., N1E-115 cells were serum-starved to induce neurite extension, then exposed to Y-27632. An agonist known to stimulate neurite retraction, lysophosphatidic acid (LPA), was added to the cell culture. Results indicated that p160ROCK was a downstream effector responsible for agonist-induced neurite retraction since the addition of Y-27632 prevented neurite retraction (Hirose et al., 1988). In a study by Sayas, glycogen synthase kinase-3 (GSK-3) was found to be activated by LPA in primary neurons based on GSK-3 activity measurement, while GSK-3 inhibition partially blocked neurite retraction. Additionally, results indicated that Neuro2A cells experience GSK-3 activation is mediated by the small GTPase RhoA (Sayas, 2006).

### 1.2.1.2 Indications and Consequences

Neurites are an important component of the neuronal cell body as they establish synapses with other neurons, and thus create the interconnected neuronal network present in the brain. Neurite outgrowth during neurodevelopment is essential for neuronal connectivity, and a lack thereof can lead to cognitive deficits (Radio et al., 2008). While
several factors may have an influence on neurite retraction, the consequences involve loss of connection and communication in the neuronal network, and thus limited function.

Neurite outgrowth is commonly used to assess neuroInveity of certain chemicals. A reduction in length is interpreted as an interruption of proper development, and indicates some level of neurotoxicity with the assumption that the chemical of concern induces changes in molecular signaling pathways responsible for neurite extension (Radio et al, 2008).

Neurite retraction is also associated with neurodegenerative diseases. Characteristics common to all neurodegenerative diseases include neurite retraction, synapse disruption and destruction, and eventual cell death (Sheinerman et al, 2013).

Additionally, LPA-induced neurite retraction has been associated with Tau phosphorylation, which is seen in the Alzheimer’s disease state. In a study by Sayas et al, it was determined that Tau phosphorylation was modulated by GSK-3 in the Rho pathway. It is possible that activation of GSK-3 involved in neurite retraction may also be linked to Alzheimer’s disease (Sayas et al, 1999).

1.2.2 Neurite Retraction in TBI

Several indicators of neuronal injury exist, including blebbing, neurite retraction, and neurite thinning (Bar-Kochba et al, 2016). Neuronal injury and neurite retraction have been observed in experimental models of TBI that study the effects of mechanical insult from the primary injury.
1.2.2.1 In Vitro Findings

Neurite injury and retraction have been observed in studies using cell culture models of TBI. A study performed by Bar-Kochba et al utilized a 3D compression model, consisting of primary cortical neurons cultured in collagen type I gels, to characterize the effects of compressive impacts with specific strain rates on neuronal morphology. Samples were subjected to a compressive impact with different loading rates, at a compressive strain of 0.38 in quasistatic and dynamic loading. Through the use of confocal time-lapse microscopy, it was discovered that cytoskeleton showed signs of structural degeneration— including neurite retraction, thinning, and blebbing— six hours after experiencing the impact, then proceeding to deteriorate completely within four hours, eventually leading to cell death (Bar-Kochba et al, 2016).

Using micropatterned neuronal cell culture silicone membranes, Tang-Schomer et al modeled high strain rates responsible for white matter deformation in TBI. It was determined that dynamic stretching of neurons induced mechanical failure in axonal microtubules, which was responsible for disruption in axonal transport and swelling due to protein accumulation, leading to the complete axonal degeneration and eventual death of neurons (Tang-Schomer et al, 2010).

A study by Prado et al modeled TBI by using a novel device to apply stress to primary cortical neuronal cultures on a microelectrode array. Results indicated that membrane permeability was increased, along with spontaneous electrical activity perturbations, suggestive of electrophysiological dysfunction in the interconnected neuronal network (Prado et al, 2005).
1.2.2.2 In Vivo Findings

Previous *in vivo* studies have elucidated the effects that TBI has on neuronal and neurite morphology (Wu *et al*, 2012; Gao *et al*, 2011; DiLeanordi *et al*, 2009; Casella *et al*, 2014).

Several histological results following controlled cortical impact (CCI) injury to rodent models have illustrated the effects on tissue components. In a study by Wu *et al*, after CCI to the left hemisphere of Wistar rat subjects, it was discovered that along with neurological functional deficits, rats experienced axonal injury and decreased synaptic density following the injury (Wu *et al*, 2012). In a separate study, dentate gyrus tissue sections revealed that there was a significant decrease in dendritic length and dendritic branching following CCI injury to juvenile rat subjects when compared to the non-injured control (Casella *et al*, 2014). A mouse CCI injury model of mild TBI revealed that while there were no significant tissue lesions or cortex volume changes, there was evidence of some neuronal death and evidence of extensive dendritic degeneration marked by swelling and blebbing. Additionally, the number of apical dendrites and dendrite branches in pyramidal cells was greatly reduced in the mild TBI model, as well as the number of synapses between cells, and consequentially disruption of the neural circuitry. Since synapse degeneration was observed without the occurrence of neuronal death following the injury, it is possible that degeneration and death are separate processes that should be prevented individually (Gao *et al*, 2011).

A study that experimentally induced a diffuse injury TBI to a juvenile rat model demonstrated that axon swelling can occur in the cingulum 6 hours post-injury, followed
by additional axon swelling in the corpus callosum and lateral white matter tracts after 24 hours. At 72 hours post-injury, results indicated axonal degeneration (DiLeonardi et al., 2009).

Past studies have focused on the primary mechanical insult of TBI as the root cause for axonal injury, yet fail to evaluate how elevated ICP may contribute to pathologic mechanotransduction processes as well.

1.3 Effects of Hydrostatic Pressure on Neuronal Cells

The application of hydrostatic pressure on different cell types and tissues is clinically relevant to a multitude of disease states. Hydrostatic pressure can mimic the functions of pressure states found in the body such as hypertension, elevated ICP, and intraocular pressure. By investigating the effect of hydrostatic pressure on a number of cell types and tissues, it may prove helpful in understanding the underlying mechanisms of cellular responses to TBI.

1.3.1 Associated Pathologies

There are several pathologies that result from or are exacerbated by super physiological levels of pressure. As previously discussed, TBI patients experience elevated ICP as a secondary condition, which has a direct effect on the physiology of the brain, and may lead to additional consequences such as neurodegenerative diseases.

Another pathology that involves increased pressure is glaucoma. Glaucoma is associated with elevated intraocular pressure and the progressive degeneration of optic nerves, leading to loss of vision and eventually irreparable blindness. Retinal ganglion
cell death is a major factor involved in the pathophysiology of the disease, and may be wholly or in part due to increased intraocular pressure (IOP) (Almasieh et al, 2012).

1.5.2 Effect on Cells

Several in vitro studies have been performed to determine the effect of hydrostatic pressure on specific cell types to gain understanding of its physiological role in disease states. Cultured retinal ganglion cells (RGCs) are used in glaucoma model studies, as well as PC12 cells, which are also used in neurodegenerative disease studies.

1.5.2.1 Retinal Ganglion Cells

RGCs are a type of Central Nervous System (CNS) neuron whose soma is located in the retina, and whose axons are located in the optic nerve (Almasieh et al, 2012). Studies have been performed on RGCs to determine if the elevated pressure contributes to neuronal apoptosis. In a study using differentiated RGC-5 cells housed in a pressurized live cell imaging chamber, it was discovered that after application of 100 mmHg pressure (a pressure that resembles the intraocular pressure observed in acute angle-closure glaucoma), the cells experienced peaks of intracellular Ca$^{2+}$ elevation, followed by caspase-3/7 activation. Intracellular Ca$^{2+}$ is a known apoptosis regulator, and caspase-3/7 an apoptosis effector, so it was expected that the peaks of intracellular Ca$^{2+}$ would correspond with morphological changes in the cells associated with apoptosis- cell shrinkage, and neurite retraction, which they did. This correlation of Ca$^{2+}$ peaks with the morphological changes indicates that Ca$^{2+}$ is the initiator of apoptosis in RGCs (Lee et al, 2010).
According to a 2013 study, results suggest that elevated IOP (90 mmHg for 1 hour) in a rat model resulted in the upregulation of the P2X$_7$ receptor, and was responsible for RGC death. The P2X$_7$ receptor is likely responsible for the upregulation of tumor necrosis factor (TNF)-α, interleukin (IL)-1β (a mediator in chronic inflammation and neurodegeneration), and IL-6 inflammation responses which contributed to cell death, as well as intracellular Ca$^{2+}$ increase (Sugiyama et al., 2013). As observed in the previous study, elevated intracellular Ca$^{2+}$ levels correlate to apoptosis. Other studies suggest transient receptor potential vanilloid-1 (TRPV1) expression may play a role in the increase of intracellular Ca$^{2+}$ as well. TRPV1 is a channel that mediates multiple Ca$^{2+}$ related cascades that influence the cell’s response to mechanical. In a glaucoma state mouse model, elevated IOP correlated with increased levels of TRPV1 and apoptosis (Sappington et al., 2015).

In addition to Ca$^{2+}$ increase, apoptosis, and inflammatory response, new evidence suggests that elevated IOP induces oxidative stress in RGCs, which leads to neuronal damage. A study performed on both RGC-5 cells in culture and a mouse model concluded that after application of either 30 or 100 mmHg hydrostatic pressure, the oxidation products 4-hydroxy-2-nonenal (HNE) and heme oxygenase-1 (HO-1) were elevated in a dose-dependent manner. Results showed that HNE is toxic to RGC-5 cells, as it killed them in a dose-dependent manner. When pretreated with antioxidant resveratrol, HNE adduct formation was reduced in RGC-5 cells (Liu et al., 2007).
1.5.2.2 PC12 Cells

As an *in vitro* glaucoma model, PC12 cells (a rat adrenal phaeochromocytoma cell line, Westerink *et al.*, 2008) subjected to elevated hydrostatic pressure experienced mitochondrial membrane depolarization that resulted in apoptosis and oxidative stress. PC12 cells in culture were exposed to 0, 15, and 70 mmHg hydrostatic pressure for 1 and 24 hours. Afterwards, several factors were examined including cell viability, lipid peroxidation and intracellular reactive oxygen species production, mitochondrial membrane depolarization, reduced glutathione (GSH) and glutathione peroxidase (GSH-Px), cell apoptosis, and caspase-3 and caspase-9 activities. When hydrostatic pressure was applied at either 15 or 70 mmHg, PC12 cells experienced oxidative cell damage as indicated by decreased GSH and GSH-Px values, as well as increased membrane potential of PC12 mitochondria, suggesting compromised mitochondrial activity. Application of 70 mmHg for 24 hours resulted in apoptosis as indicated by caspase-3 and caspase-9 values (Tök *et al.*, 2014).

Another study indicate that neuronal exposure to increased pressure (as a model of high ICP in TBI patients) plays a role in the development of neurodegenerative disease. Neurodegenerative diseases such as Parkinson’s and Amyotrophic Lateral Sclerosis (ALS) develop as a result of protein aggregation comprised mainly of α-synuclein (AS). PC12 cells in suspension were subjected to either 500 or 1000 bars (375,030 mmHg to 750,051 mmHg) of hydrostatic pressure for a certain period of time, and then allowed 24 hours to recover. Western Blot results indicated that acute increases in hydrostatic pressure caused aggregation of intracellular endogenous AS, a decrease in its binding
partner PLCβ1, and reduced binding between the two in PC12 cells. However, the exact mechanism through which this occurs remains unknown (Golebiewska et al., 2015). Although pressures used in this specific study were far greater than ICP observed in TBI patients, the results may be relevant. If PC12 cell behavior models central nervous system neuronal behavior as predicted, it is possible that increased ICP stemming from TBI contributes to the development of neurodegenerative diseases through the accumulation of AS,.
CHAPTER TWO
RESEARCH RATIONALE

2.1 Rationale

Approximately 1.7 million TBIs ranging from mild to severe occur every year in the United States (MedGadget, 2016). Resulting elevated ICP is managed with treatments such as hyperosmolar therapy, hypothermia, or craniectomy to reduce risk of ischemia (Rangel-Castillo, 2009; Xiong et al., 2012). However, it remains unclear what role elevated ICP has directly on cellular structure, and how it may play a role in pathological mechanotransduction pathways. Axonal degeneration, neurite retraction, and increased plasma membrane permeability are morphological characteristics that have been observed in several TBI models in vitro and in vivo studies. Since neurite retraction is a hallmark characteristic of neuronal cells in neurodegenerative diseases and functional nervous system deficits, it is possible that neurite retraction induced by TBI may manifest into the development of such pathologies. The present study investigates the direct role of elevated hydrostatic pressure on neuronal morphology alteration to elucidate processes that can be targeted for treatment options for TBI patients.

2.2 Hypotheses

The objective of this present research is to test our hypothesis that neuronal cells respond to elevated hydrostatic pressure, and that this response is a facilitated via a specific pathway. To test this hypothesis, using custom experimental setups, mouse Neuro2A cells were subjected to controlled levels of hydrostatic pressure for specific
time periods, and cell morphology and apoptosis activity were examined. The specific aims for this project are as follows:

- **Aim 1:** To quantify changes in Neuro2A cell morphology following exposure to elevated hydrostatic pressure

  *Rationale:* Axons and dendrites are vital to forming connections in the neural network that allow proper CNS function and cell-cell communication. Neurite retraction may lead to limited communication and therefore compromised functionality.

  *Approach:* Neuronal cell morphology was compared before and after exposure to hydrostatic pressure using fluorescent and phase contrast microscopic images. Normalized change in neurite length per cell was determined based on the comparison of “before” and “after” neurite length summations.

- **Aim 2:** To determine whether elevated hydrostatic pressure induces apoptosis in Neuro2A cells

  *Rationale:* While direct injury to brain tissue during TBI can result in cell death, it is unclear whether unrelieved elevated intracranial pressure as a secondary injury associated with TBI contributes to cell death either locally or globally in the brain.

  *Approach:* A colorimetric TUNEL Assay, TiterTACS, was used to determine whether Neuro2A cells underwent apoptosis after 1 hour or 24 hours of exposure to 35mmHg hydrostatic pressure.
• **Aim 3:** To identify the mechanism through which hydrostatic pressure induces neurite retraction in Neuro2A cells

  *Rationale:* Once identified, the specific pathway could serve as a target for therapeutics that would act to reduce or prevent neurite retraction in neuronal cells induced by elevated intracranial pressure sustained after TBI, and limit subsequent consequences.

  *Approach:* Neuro2A cells were exposed to hydrostatic pressure in the presence or absence of Y-27632, an inhibitor to p160ROCK in the Rho/ROCK pathway. Neurite lengths for specific cells were measured and compared to determine the role of Rho/ROCK pathway in pressure-induced neurite retraction.
CHAPTER THREE

MATERIALS AND METHODS

3.1 Pressure System

In the present study a custom-made pressure system was used to expose neuronal cell cultures to hydrostatic pressure. A computer with a custom LabView code (National Instruments, Corporation, Austin, Texas) was used to regulate the pressure inside a sealed pressure chamber, which housed the cell culture dishes. A pressure transducer was used to monitor the pressure inside the pressure chamber and convey the pressure to LabView using voltage output values. Based on the readings, inlet and outlet valves that controlled the flow of 95% air/5% CO₂ through the pressure system were adjusted. The pressure chamber was housed inside an incubator under standard cell culture conditions.

In addition to the previous design of a rectangular chamber (Figure 1), a new pressure chamber (Figure 2) was designed and fabricated in the present study to 1) limit space inside the container for air to fill and therefore reducing pressure pump usage, 2) provide a cylindrical shape to house petri dishes or cell culture dishes, 3) to achieve capacity for higher pressures.
Figure 1. Pressure Chamber 1. Rectangular pressure chamber with box lid mechanism used for low-pressure experiments.

Figure 2. Pressure Chamber 2. Newly fabricated cylindrical pressure chamber with screw-top lid for high-pressure experiments.
3.2 Cell Culture

Neuro2A cells (ATCC), a mouse neuroblastoma cell line, were cultured in Dulbecco’s Modified Eagle Medium (D-MEM) supplemented with 10% fetal bovine serum (FBS) and 1% Penicillin-Streptomycin (Thermo Fisher Scientific) under standard cell culture conditions with a humidified atmosphere of 95% air and 5% CO₂ at 37°C. Media were changed out every 48-72 hours. Cells were passaged when 80% confluency was achieved, approximately once a week using a trypsin-EDTA solution (Corning).

In order to induce neurite extension, cells were serum-starved for 24 hours (Figure 4). This was accomplished by replacing regular growth media (D-MEM, 10% FBS, 1% Penicillin-Streptomycin) with serum-free media (100% D-MEM).

3.3 Soft Substrate Coating

To simulate an environment that mimicked that of brain tissue, a polyacrylamide + type I collagen layered substrate was prepared for Neuro2A cell culture. The protocol in the present study was adapted from that developed by Fischer et al (Fischer et al, 2012).

3.3.1 Coverslip Acid Washing

One-inch circular glass coverslips (Fisher Scientific) were soaked in a beaker containing 20ml 1M HCl in a hot water bath at 50-60°C under a fume hood for 8 hours with occasional agitation. The coverslips were removed and rinsed extensively in distilled water and subsequently in 100% ethanol. Coverslips were left to air-dry overnight.
### 3.3.2 Coverslip Activation

The acid-washed coverslips were carefully placed in a wire rack and soaked in 0.5% (3-aminopropyl)trimethoxysilane (Sigma-Aldrich) in dH₂O for 30 minutes at room temperature with gentle agitation of the rack in solution. After washing in six changes of dH₂O the rack carrying the coverslips was dried on a hot plate and cooled to room temperature. After cooling the coverslips on the rack were immersed in a 0.5% glutaraldehyde (Polysciences)-PBS solution for 30 minutes at room temperature with occasional agitation to prevent bubble accumulation on the coverslip surface. The coverslips were then washed with three changes of distilled H₂O and allowed to air-dry overnight.

### 3.3.3 Polyacrylamide Gel Coating

Glass slides (Fisher Scientific) were polished with a KimWipe soaked in a hydrophobic silicone solution (Rain-X, ITW Global Brands), rinsed well with distilled water, and then allowed to air dry. Two glass slides were placed in each 15cm glass Petri dishes. To prepare acrylamide gel with a shear modulus of 0.43 kPa to mimic that of brain tissue (~0.49 kPa, Rashid et al., 2013), 0.2925 mL acrylamide, 0.1604 mL bis-acrylamide, 2.0471 mL 1M HEPES were mixed together for a total volume of 2.5 mL with the final concentrations of 5% acrylamide and 0.05% bis-acrylamide. To this mixture, 10 µL of TEMED and 15 µL of freshly made 10 % APS were added and mixed briefly. Two 12 µL drops of the whole mixture were placed with 1 inch space apart on each glass slide and one coverslip was carefully placed on each drop, with a small portion of the coverslip overhanging the edge of the glass slide. The acrylamide mixture was
allowed to polymerize at room temperature for 20 minutes. After polymerization was completed, the surface of the slides and coverslips were flooded with freshly made 50 mM HEPES buffer. The coverslips were then removed from the glass slides using forceps by applying lateral pressure to the overhanging edges of the coverslips. The coverslips were placed gel side-up in another Petri dish and rinsed five times with 50 mM HEPES buffer. The polyacrylamide-coated coverslips were stored in fresh 50 mM HEPES buffer at 4°C overnight prior to the collagen coating step.

3.3.4 Type I Collagen Coating of Soft Substrates

Sulfo-SANPAH (Thermo Fisher Scientific) stock in dimethyl sulfoxide (DMSO, ATCC) at a concentration of 25 mg/mL was prepared and flash-frozen in liquid nitrogen for storage at -80°C. Four 40 µL aliquots of sulfo-SANPAH were thawed at room temperature and 960 µL of distilled water was mixed with each aliquot. The type I collagen solution was prepared at a concentration of 200 µg/mL in PBS. The coverslips were placed gel side-up on a Parafilm coated Petri dish. 200 µL sulfo-SANPAH solution was pipetted onto the polyacrylamide surface of each coverslip. The Petri dish supporting the coverslips was placed on top of a heightened support in the cell culture hood so that it was ~10 cm from the UV lamp and left for 10 minutes to induce cross-linking. After excess buffer was removed from coverslips, each coverslip was inverted onto a single collagen droplet so that the polyacrylamide surface was in contact with the droplet. The collagen was allowed to covalently couple with the polyacrylamide overnight at 4°C. The following day, the coverslips were rinsed with sterile PBS in sterile conditions and a grid was drawn on the bottom of each coverslip with a permanent marker. Coverslips were
stored in fresh sterile PBS in cell culture dishes. Coverslips were sterilized by UV light for 3 hours at room temperature. Coverslips remained in their cell culture dishes and were stored at 4°C.

3.3.5 Coomassie Blue Staining

To confirm the immobilization of collagen onto the soft substrates, the coverslips were fixed in fixing solution (50% methanol, 10% glacial acetic acid in dH2O) for 3 hours and stained with Coomassie blue (40% methanol, 60% Coomassie Blue + acetic acid in dH2O) for 20 minutes with gentle agitation. The samples were then destained eight times in destaining solution (40% methanol, 10% glacial acetic acid in dH2O) and stored for imaging in storage solution (5% glacial acetic acid in dH2O) Macroscopic images were taken of each coverslip using a cell phone camera and microscopic images were taken using phase contrast microscopy (10x, Nikon).

3.3.6 Cell Seeding onto Gel Coverslips

Each coverslip was rinsed in warm sterile PBS, then placed gel side up in a 35 mm cell culture dish with 1 mL warm growth media. N2A cells (56,600 cells per dish) in growth media were seeded on each coverslip and cultured for 24 hours.

3.4 Pressure Experiments

Prior to cell seeding, a grid coordinate system was drawn on the bottom of each cell culture dish using a permanent marker (see Figure 3), which allowed for the relocation of individual cells. Neuro2A cells were cultured in 35mm cell culture dishes at an initial seeding density of 56,600 cells per dish in growth media for 24 hours. Growth media was removed and cells were cultured in serum-free media (100% D-MEM) for 24 hours in
order to induce neurite extension. At this point, “Before” images were taken of Neuro2A cells using the microscope (Nikon). The specific cell locations were recorded in order to locate the same cells for the “After” images. Using the custom setup (Section 3.1), the cells were exposed to hydrostatic pressure (either 25 or 35 mmHg) for 30 min, 1 hour, 2 hours, or 4 hours. Control was the cells that were prepared in a similar manner but maintained under atmospheric pressure conditions for the duration of the experiments. Immediately following the pressure application, cells in all dishes were fixed with 10% neutral buffered formalin (NBF, Protocol) for 10 minutes. “After” images of the same cells captured in the “Before” images were taken using phase contrast microscopy (Nikon).

![Figure 3. Cell Culture Dish Grid Schematic.](image-url) A coordinate grid that was drawn on the bottom of each cell culture dish using a permanent marker was used to locate cells.
3.5 Microscopy

Cells were imaged using phase contrast and fluorescence microscopy (Nikon). CellLight Actin-GFP, BacMam 2.0 (Thermo Fisher Scientific) was used to label actin in the Neuro-2A cells with green fluorescent protein for live cell fluorescence imaging.

3.6 Neurite length analysis

3.6.1 Image analysis

Neurite length was measured for individual Neuro2A cells using the Fiji program with the Simple Neurite Tracer plugin (Longair, 2011). The program allows for manual selection of end points for measurement, and reports the length in units of pixels. The number of neurites per cell, as well as the length of each neurite per cell were measured on the “Before” images and the “After” images for all groups.

3.6.2 Calculations

Normalized change in neurite length was calculated using the following equation:

\[
\Delta L = \frac{\sum n \frac{l}{N_o}}{\sum n_o \frac{l_o}{N_o}}
\]

Equation 1. Equation used to determine average normalized change in length per neurite per cell
The variables are defined below:

\[ \Delta L = \text{normalized change in neurite length per cell} \]
\[ l_0 = \text{combined length of neurites for a cell BEFORE} \]
\[ N_0 = \text{number of neurites for a cell BEFORE} \]
\[ l = \text{combined length of neurites for a cell AFTER} \]

Hence, \( \Delta L = 1 \) signifies no change in neurite length per cell, while a value above 1 indicates increase in neurite length, and a value below 1 represents a decrease in neurite length.

**3.7 p160 ROCK Inhibition**

**3.7.1 Part One: Pressure and Y-27632**

To determine the potential mechanism for pressure-induced neurite retraction in N2A cells, the pressure experiments (35 mmHg, 1 hour) were repeated with or without 30 min pre-treatment of cells with a ROCK inhibitor Y-27632 (1 µM, ATCC). Four experimental groups were designated as 1- No Pressure, 2- No Pressure + Y-27632, 3- Pressure, and 4- Pressure + Y-27632.

**3.7.2 Part Two: LPA and Y-28632**

In addition, to confirm the involvement of Rho-Rock pathway in neurite retraction and effect of the inhibitor, a positive control experiment was performed. Lysophosphatidic acid (LPA) is a bioactive phospholipid known to cause neurite retraction in neuronal cells. By using it as a neurite retraction stimulus in place of hydrostatic pressure, LPA was applied to cells in order to provide a comparison for the reaction observed in cells exposed to pressure. Briefly, following serum-starvation for 24
hours, Neuro2A cells were treated with 1 µL Y-27632 solution each for 30 minutes and imaged using phase contrast microscopy (Nikon). The cells were then treated with a lysophosphatidic acid (LPA) solution [0.3mM LPA, Sigma-Aldrich, in PBS, Corning, 0.1% weight/ volume bovine serum albumin (BSA), Sigma-Aldrich] for 10 minutes. Cells in all dishes were fixed with 10% neutral buffered formalin (NBF, Protocol) for 7 minutes and imaged again.

3.8 TUNEL Assay

After pressure experiments (35 mmHg, for 1 hour or 24 hours), TUNEL assay was performed using a commercially available kit (TiterTACS™, Trevigen) and following the manufacturer's instructions (Batch Method) to quantify apoptotic cells. Duplicates for each group (4 groups total) were assayed. The 4 groups included the following: Control group (no pressure), Pressure group, Positive control (nuclease-treated), and Negative control (unlabeled). The Control group, Positive control, and Negative control groups all used cells that were not exposed to pressure.

3.9 Polyacrylamide / Collagen Type I Coating of Substrates

Soft culture substrates for N2A cells were prepared according to a published method (REF), which is summarized in the following subsections.

3.10 Statistical Analysis

A paired $t$ test (two tail) was performed when appropriate. The level of significance used was $p < 0.05$. All data are presented as means ± standard errors of mean.
CHAPTER FOUR

RESULTS

4.1 Neuro2A Cell Neurite Extension

Neuro2A cells were cultured in growth medium containing D-MEM, 10% FBS, 1% Penicillin-Streptomycin for 24 hours. Medium was switched to serum-free media (100% D-MEM) for 24 hours in order to induce neurite extension from the cell body. Phase contrast images exhibited that there were more neurites per cell, and that those that existed were longer in length in the serum-free media group (Figure 4A) than the regular media group (Figure 4B).

![Figure 4. Phase Contrast Images of Neuro2A Cells in Growth Medium and Serum-Free Medium. Neuro2A cells cultured in regular growth media containing D-MEM, 10% FBS, 1% Penicillin-Streptomycin after 24 hours (A) and Neuro2A cells cultured in serum-free growth media containing 100% D-MEM after 24 hours. Scale bars = 100 µm.](image-url)
4.2 Neurite Response to Pressure Exposure

4.2.1 Hard Substrate

To determine elevated hydrostatic pressure on neuronal cell morphology in vitro, Neuro2A cells cultured in 35 mm plastic dishes (Greiner Bio-One) were exposed to pressure (25 or 35 mmHg for up to 60 min) and imaged using phase contrast microscopy (Nikon) (Figure 5).

![Figure 5. Phase Contrast Images of Neuro2A Cells Before and After Pressure Application.](image)

Neurite length of about 30 cells from each dish was measured and normalized length was calculated for both the pressure group and a control group (no pressure). The cells exposed to these two different pressures show similar general trend and exhibited a decrease in neurite length when compared with the control. However,
only application of 35 mmHg induced a significant (p<0.05) decrease in neurite length. Based on these results, 35 mmHg pressure was used in subsequent experiments to evaluate the effect of pressure on neurite length at additional time points.

![Image of bar graph showing average normalized change in neurite length over 1 hour]

**Figure 6. Average Normalized Change in Neurite Length Over 1 Hour.** Average neurite length per cell was normalized over two 30-minute segments for control group (no pressure) and pressure (either 25 mmHg or 35 mmHg) groups of Neuro2A cells. Error bars indicate ± S.E.M. * indicates p < 0.05.

Overall, the results demonstrated that pressure group experiences a decrease in neurite length when compared to the control group. Specifically, neurite length of the Neuro2a cells decreased significantly (p<0.05) after 30 minutes, 60 minutes, and 4 hours of exposure to 35 mmHg hydrostatic pressure. Additionally, it appears that change in neurite length is time dependent up to at least 4 hours.
Figure 7. Average Normalized Change in Neurite Length Over Time at 35 mmHg Pressure. Average neurite length per cell was normalized over time segments for control group (no pressure) and pressure (35 mmHg) group of Neuro2A cells. Error bars indicate ± S.E.M. * indicates p < 0.05.

4.2.2 Soft Substrate

To examine neuronal cell morphology changes due to hydrostatic pressure in an environment similar to that of brain tissue, a soft substrate was prepared based on the published methods (Fischer et al., 2012). Cells seeded on the soft polyacrylamide gel + type I collagen substrate were imaged using phase contrast microscopy (Nikon) before and after exposure to either 35 mmHg pressure or no pressure (control) for 1 hour. Data were compared to that acquired from the hard substrate experiment.

First, to confirm that the collagen was immobilized onto the polyacrylamide gel layer on the coverslip, a Coomassie Blue stain was performed (de Moreno et al., 1986;
Duhamel, 1983). Resulting images show that both coverslips A and B have clear and blue-colored regions, with coverslip B having slightly more blue spots (Figure 8).

**Figure 8. Coomassie Blue Staining of Polyacrylamide Gel Coverslips.** After Coomassie Blue stain was performed, images were taken on a phone camera of the coverslips which had either A) a polyacrylamide gel layer or B) a polyacrylamide gel layer + type I collagen layer.

Unlike Neuro2A cells on the hard substrate, most Neuro2A cells on the soft substrate appear to have a more rounded cell body and to have fewer neurite extensions (Figure 9). Neuro2A cells seeded on the soft substrate appear to experience neurite retraction similar to that of Neuro2A cells plated on the hard substrate when exposed to 35 mmHg hydrostatic pressure for 1 hour (Figure 10). Neurite length decreased significantly on both the hard substrate and the soft substrate (p < 0.05).
Figure 9. Phase Contrast Images of Neuro2A Cells Seeded on Soft Substrate. Phase contrast images were taken of Neuro2A cells (A, C) at time=0, (B) after 1 hour (no pressure), and (D) after 1 hour of exposure to 35 mmHg hydrostatic pressure. Scale bars = 100 µm.
Figure 10. Average Normalized Change in Neurite Length Over 1 Hour on Hard and Soft Substrates. Average neurite length per cell was normalized over 1 hour for control group (no pressure) and pressure (35 mmHg) group of Neuro2A cells. Error bars indicate ± S.E.M. * indicates p < 0.05.

4.3 p160 ROCK Inhibition

To determine whether hydrostatic pressure induced neurite retraction through the Rho/ROCK pathway, experiments were repeated in the presence or absence of the p160 ROCK inhibitor Y-27632 with LPA-treated group of Neuro2A cells as a positive control. Imaging of the cells and calculation of the normalized neurite lengths demonstrated that the control groups and the Y-27632 groups experienced slight
changes, while the others experienced a significant (p<0.05) decrease in length (Figure 11). There was a significant difference between the Y-27632-treated cells and non-treated cells in both the pressure and LPA groups.

Figure 11. Average Normalized Change in Neurite Length Over 1 Hour for Treatment Groups With or Without Exposure to Inhibitor Y-27632. Average change in neurite length per cell was normalized over 1 hour for control group (no pressure), control group (no pressure) + Y-27632, pressure (35 mmHg) group, pressure (35 mmHg) group + Y-27632, LPA group, and LPA + Y-27632 group of Neuro2A cells. Error bars indicate ± S.E.M. * indicates p < 0.05.
4.4 Apoptosis Study

To determine the effect of elevated hydrostatic pressure on Neuro2A cell apoptosis, a commercially available colorimetric TUNEL assay kit (Trevigen), was used to quantify the level of apoptotic Neuro2A cells directly after 24 hours of exposure to pressure (35 mmHg). Positive (nuclease-treated group) and negative (unlabeled group) controls were generated to interpret absorbance values, with high values (0.611-1.159) indicating 100% apoptotic cells, and low values (~0.111-0.211) indicating minimal apoptotic cells present (Figure 12). The average values indicate that minimal apoptosis is detected in both the control and pressure groups with absorbance values of 0.3055 and 0.3500, respectively, which fall between the positive and negative control absorbance values.
Figure 12. Quantification of Apoptosis in Neuro2A Cells Using HT TiterTACS Assay. TiterTACs Assay was performed to quantify apoptosis in Neuro2A cell cultures directly after 24 hours of exposure to 35 mmHg hydrostatic pressure. Error bars indicate ± S.E.M.
CHAPTER FIVE

DISCUSSION

Although numerous studies have previously investigated pathological mechanisms involved in TBI (Bar-Kochba et al, 2016; Casella et al, 2014; DiLeonardi et al, 2009; Gao et al, 2011; Prado et al, 2005; Tang-Schomer et al, 2010; Wu et al, 2012; Xiong et al, 2009), none have studied the direct effect of elevated ICP on neuronal cell mechanobiology. As there is currently no therapeutic treatment available for secondary injury after TBI (which may be partly due to elevated ICP), researchers aim to elucidate potential pathways to target for therapeutic approaches (Xiong et al, 2009). Additionally, an understanding of the mechanisms involved in elevated ICP may provide insight necessary to establish conventional elevated ICP management procedures in TBI patients, which currently remains debated amongst physicians (Alali et al, 2013).

Previous studies have examined changes in neuronal cell morphology and health in vitro, particularly neurite extension/retraction and apoptosis, in order to evaluate cell health in response to drugs and other chemicals (Hirose et al, 1988; Monnier et al, 2003; Sayas, 2006). In the present study, changes in neurite length was measured to determine the effect of elevated hydrostatic pressure on Neuro2A cells as an in vitro model of TBI-associated elevated ICP. It was hypothesized that elevated hydrostatic pressure would induce neurite retraction, and possibly apoptosis, in the Neuro2A cell culture model.

5.1 Neuro2A Cell Differentiation

Neuroblastoma cells are frequently used in in vitro studies to investigate neuronal cell behavior (Seeds et al, 1970; Schubert et al, 1974). A number of methods exist for
differentiating neuroblastoma cell lines and inducing neurite outgrowth, with the simplest being exposure to serum-free medium (Gurwitz et al, 1988; Schubert et al, 1971; Schubert et al, 1974; Seeds et al, 1970). When serum is removed from the environment, the protein synthesis that is required for the microtubule assembly involved in neurite extension, occurs (Seeds et al, 1970). Additionally, it is possible that after serum removal, the cells are able to interact with the plate surface more stably, and thus form neurites on its surface (Seeds et al, 1970; Schubert et al, 1971). In the present study, Neuro2A cells were first cultured in growth medium (D-MEM, 10% FBS, 1% Penicillin-Streptomycin), then switched to serum-free medium (100% D-MEM) after 24 hours, which led to successful differentiation indicated by neurite outgrowth. In the present study, differentiation was necessary in order to achieve a neuronal phenotype as clinically relevant as possible.

5.2 Neurite Response to Pressure Exposure

The present study examined the effects of elevated hydrostatic pressure on Neuro2A cell morphology, particularly neurite retraction/extension. The majority of the experiments were performed in polystyrene cell culture dishes (Greiner Bio-one) while some experiments were performed with a soft substrate layer (Greiner Bio-one, plus polyacrylamide + type I collagen gel) to mimic brain tissue stiffness.

5.2.1 Hard Substrate

Differentiated Neuro2A cells were exposed to pressure (25 mmHg or 35 mmHg) for either 15 minutes, 30 minutes, 60 minutes, 4 hours, or 6 hours. The pressures of 25 mmHg and 35 mmHg were selected to mimic those of ICP observed in moderate and
severe TBI patients, respectively (Rangel-Castillo et al, 2008). Phase contrast images analyzed with the Fiji program with the Simple Neurite Tracer plugin (Longair, 2011) showed that there was neurite retraction and extension in both the control and pressure groups. This is most likely due to the fact that the cells naturally migrate, as well as react to differences in temperature when being removed from the incubator and being transported to the microscope. Thus, the study aimed to compare the amounts of change in neurite length between the control and pressure groups in order to determine whether there was a difference.

The results comparing the 25 mmHg and 35 mmHg pressure groups provided evidence that cells in the 35 mmHg group experienced a more drastic decrease in neurite length than those in the 25 mmHg group when exposed to pressure for 30 and 60 minutes (Figure 6). The trend indicated that neurite retraction may occur at moderate pressures (~25 mmHg), and progress extensively as pressure continues to rise (to at least 35 mmHg).

Since Neuro2A cell exposure to 35 mmHg pressure results indicated that there was significant impact, subsequent studies were performed in order to more closely examine the effect of 35 mmHg pressure on neuronal cell morphology at additional time points. The results provide evidence that exposure to 35 mmHg pressure for 30 minutes or more causes significant (p<0.05) neurite retraction in Neuro2A cells in cell culture dishes. After 6 hours of exposure to 35 mmHg pressure, neurite length did not decrease as much as it did after 4 hours. While it is possible that neurites began extending after the 4 hour time point, the observation in the 6 hour group is most likely due to the fact that
there was greater variability in the data due to more extensive cell migration after such a long period of time.

Together, the results indicate that neuronal cells present in brain tissue exposed to elevated ICP in severe TBI patients may experience neurite retraction. Neurite retraction induced by ICP may lead to disruption in the brain's neuronal network by destroying synapses and cell-to-cell communication (Gao et al, 2011). It is possible that neurite retraction as a result of elevated hydrostatic pressure may play a role in the development of long-term consequences commonly observed in TBI patients such as neurodegenerative diseases (Chen et al, 2007; Fleminger et al, 2003; Jafari et al, 2013; Mortimer et al, 1991) and cognitive deficits (Caeyenberghs et al, 2014) since these are also characterized by neurite retraction (Radio et al, 2008; Sheinerman et al, 2013). Additionally, the presented evidence supports the notion that elevated ICP (~35 mmHg) in severe TBI patients should be relieved as soon as possible, ideally within 30 minutes of elevated ICP detection, in order to avoid neuronal cell damage. Neurite retraction is one form of neuronal cell damage that was observed, however, additional cellular pathologic events may occur that were not observed in the present study.

5.2.2 Soft Substrate

Previous studies have thoroughly documented changes in cell morphology and stem differentiation based on substrate stiffness (Engler et al, 2006; Yeung et al, 2005). The brain is a soft organ, with brain tissue having a shear modulus of about 0.49 kPa (Rashid et al, 2013), which is much lower than that of a rigid plastic cell culture dish.
(~ 1 GPa) (Sayed et al, 2015), thus cells may have behaved differently based on the substrate used.

To confirm the validity of the results from initial experiments using hard polystyrene cell culture dishes, experiments were repeated using a soft substrate that consisted of a coverslip with a polyacrylamide gel layer coated with a type I collagen layer. First, to confirm the presence of collagen on the polyacrylamide gel layer, the specimens were stained with Coomassie Blue. The results indicated that some protein may be present on both the polyacrylamide gel coverslip and the polyacrylamide gel + type I collagen coverslip. This may have been due to unsuccessful destaining procedure, or actual presence of type I collagen or some other protein on both coverslips. Despite Coomassie Blue stain results, cells were seeded on the polyacrylamide gel + type I collagen coverslips. After 24 hours, it was evident that the Neuro2A cells seeded on the polyacrylamide gel + type I collagen coverslips had adhered. Since type I collagen was necessary for cell adherence to the substrate, the results indicate that the type I collagen immobilization was successful. Images showed that Neuro2A cells plated on the soft substrate exhibited little neurite outgrowth following serum-free culture, especially when compared to that of Neuro2A cells in cell culture dishes (Figure 9). However, some cells with neurite extension were identified and the results demonstrate that Neuro2A neurites retract significantly after 1 hour of exposure to 35 mmHg pressure on the soft substrate. This finding supports the view that the hard substrate experiments serve as valid models for studying elevated ICP on neuronal cell morphology in vitro.
5.3 p160 ROCK Inhibition

Previous studies have shown that the Rho/ROCK pathway is responsible for neurite retraction induced by external stimuli such as LPA (Hirose et al., 1988; Monnier et al., 2003). However, none have yet examined how hydrostatic pressure specifically induces neurite retraction. When the p160ROCK inhibitor Y-27632 was administered to neuronal cell cultures prior to pressure exposure (35 mmHg for 1 hour), there was significantly (p<0.05) less neurite retraction when compared to the cultures that were exposed to pressure without prior administration of Y-27632, suggesting that Y-27632 blocked the effects of. Additionally, when LPA was administered to Neuro2A cells in the present study neurite retraction occurred as expected. However, when cells were treated with Y-27632 prior to LPA, the effect of LPA was blocked. These results indicate that hydrostatic pressure is transduced to induce neurite retraction through the same pathway that LPA is and that the pathway responsible is the Rho/ROCK pathway.

5.4 Apoptosis Study

Numerous studies have demonstrated that neuronal cell death occurs as a result of TBI (Pekna et al., 2012; Xiong et al., 2009). Specific causes such as ischemia to brain tissue, diffuse axonal injury, and direct trauma to neuronal cells have been identified as contributors (Gaetz, 2004; Werner et al., 2007). The present study investigated whether elevated hydrostatic pressure contributed to the neuronal cell death and a commercially available TUNEL assay kit (Trevigen) was used to quantify apoptotic cells in a control group and groups exposed to pressure. Results demonstrate that following 24 hours of exposure to 35 mmHg pressure, similar and minimal Neuro2A
cell apoptosis occurred for the control and pressure groups. While the values of the nuclease-treated group were similar to the other groups, it was due to the large variance and, the nuclease-treated absorbance values in two out of three trials were higher (~1.15) than those represented in Figure 12. Although these in vitro results suggest that elevated hydrostatic pressure alone is likely not responsible for neuronal cell death, it remains possible that in the presence of other cell types in the in vivo environment, other mechanisms may be involved that would lead to neuronal cell apoptosis due to elevated ICP.

5.5 Limitations

As previously discussed, the present study used an in vitro system to model TBI-associated elevated ICP. While hydrostatic pressure could be closely monitored and controlled, the scope of the study was limited in regard to several factors.

In the present study, pressure experiments relied on the measurement of extensions from Neuro2A cell bodies, collectively called “neurites”. Neurites include both axons and dendrites, however the two were not distinguished from each other for simplicity of data collection and analysis in the study.

Throughout the study, Neuro2A cells were cultured in an incubator with a humidified atmosphere of 95% air and 5% CO₂ at 37°C. This models hyperventilation, which is not representative of physiological conditions experienced in healthy brain tissue. Normally, neurons present in brain cortex are exposed to low oxygen levels (Ndubuizu et al, 2007). Therefore, oxygen concentration would need to be adjusted to more accurately model physiological conditions in future studies.
In the pressure experiments, Neuro2A cells were first cultured in growth medium, then switched to serum-free medium after 24 hour to induce neurite extension. Pressure experiments were carried out while cells were in serum-free medium, however cells in brain tissue exposed to elevated ICP would have access to additional nutrients in the environment. For a more accurate TBI model, cells should be cultured with complete nutrients available in brain tissue, as cells may behave differently.
CHAPTER SIX
CONCLUSIONS AND RECOMMENDATIONS

In the present study, exposure to elevated hydrostatic pressure (35 mHg) for at least 30 minutes was proven to induce neurite retraction in Neuro2A cells in vitro. However, 24-hour exposure to elevated hydrostatic pressure did not induce neuronal apoptosis. Additionally, the study provides evidence that the pressure-induced neurite retraction response is mediated by the Rho/ROCK pathway. The presented findings may be applied to future TBI pathology studies in order to elucidate potential solutions to treating elevated ICP. Future studies include the following:

- To clarify the effect of hydrostatic pressure on neuronal apoptosis by performing additional experiments that expose Neuro2A cells to 35 mmHg hydrostatic pressure for 24 hours and 48 hours.
  - **Rationale:** Current results suggest that minimal apoptosis occurs after 24 hours of exposure to 35 mmHg hydrostatic pressure, however the positive control values remained low. Additional trials of the experiment should be run. Further, longer exposure to hydrostatic pressure may elucidate apoptotic events later on.

- To identify and examine the components present in the neurite structures using stains for actin and microtubules before and after pressure exposure in Neuro2A cells.
• **Rationale:** Specification of molecular components and their activity may provide information regarding Rho/ROCK pathway mechanisms involved in pressure-induced neurite retraction.

• To establish *in vitro* synapse formation by using a stem cell-derived neuron platform such as induced pluripotent stem cells (Bradford *et al.*, 2015) in order to evaluate the effect of hydrostatic pressure on synapses.
  
  • **Rationale:** Current results suggest hydrostatic pressure induces neurite retraction which may indicate loss in synapses. However, there is no evidence that synapse destruction does occur.

• To investigate the effects of hydrostatic pressure on an animal brain tissue sample.
  
  • **Rationale:** Since all physiological structures and cell types are included in the brain tissue sample, it would provide a highly accurate model for TBI-related ICP studies.

• To combine the application of hydrostatic pressure with another model of TBI pathology in order to produce a more relevant TBI disease model.
  
  • **Rationale:** In order to achieve the most accurate model, all environmental factors should be included. Other studies without ICP elevation considerations may not produce accurate results. Elevated ICP may exacerbate pathological conditions or influence cells.

• To investigate the effects of hydrostatic pressure application on a co-culture composed of multiple cell types naturally present in brain tissue
including primary neurons, astrocytes, oligodendrocytes, and microglia from a rat.

- **Rationale:** Primary neurons are a better representation of cells in the brain than Neuro2A cells, and would therefore serve as better predictors for neuronal behavior to elevated hydrostatic pressure experienced during brain swelling following TBI. While neurons may be the primary functioning cell type in brain tissue, it is important to evaluate the response of the surrounding support cells as well. Elevated hydrostatic pressure may have an effect on these cell types independently, or may induce them to impact neurons in an indirect way.

- To investigate the effects of hydrostatic pressure application on a brain tissue-like construct seeded with multiple cell types naturally present in brain tissue including primary neurons, astrocytes, oligodendrocytes, and microglia.
  - **Rationale:** Cells may respond in a different manner when exposed to hydrostatic pressure while on a compliant soft tissue model as compared to soft substrates in plastic dishes.

- To conduct an animal study in which two mice receive blunt TBIs, followed by the administration of Y-27632 in one mouse, and no treatment in the other. Following the injury and recovery time, mouse behavior would be
evaluated. Extraction and analysis of brain tissue morphology would be examined post-humously.

- **Rationale**: A mouse model would provide evidence as to whether the inhibitor has an effect on the mouse behavior or brain tissue morphology as compared to the control.

- To examine cell morphology in brain tissue samples after rat brain is exposed to elevated hydrostatic pressure, and compare these to brain tissue samples of healthy rat brain.
  - **Rationale**: When investigating cell behavior and response to stimuli, actual tissue samples provide more accurate evidence than do cell culture models.

- To determine through which specific receptor hydrostatic pressure stimulates neurite retraction in neuronal cells.
  - **Rationale**: Another potential target for drug therapy may be the specific receptor involved in pressure-mediated responses.

- To develop a live imaging pressure chamber system to record videos or take time-lapse images of cells in real time.
  - **Rationale**: This would allow investigators to observe neuronal morphological change in real-time and better assess cell behavior, as well as provide clearer evidence to support the conclusion that 35 mmHg pressure causes neurite retraction in Neuro-2A cells.
REFERENCES


