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Investigating The Effects of In-vivo Therapeutics Hypoxia Treatment Paradigms In Neurite Outgrowth Patterns

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INVESTIGATING THE EFFECTS OF *IN-VIVO* THERAPEUTIC HYPOXIA TREATMENT PARADIGMS IN NEURITE OUTGROWTH PATTERNS

THESIS

A thesis submitted in partial fulfillment of the
requirements for the degree of Masters of Science in the
College of Medicine
at the University of Kentucky

By

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Lexington, Kentucky

Director: Dr. Warren J Alilain, Professor of Neuroscience
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2022

ABSTRACT OF THESIS

INVESTIGATING THE EFFECTS OF *IN-VIVO* THERAPEUTIC HYPOXIA TREATMENT PARADIGMS IN NEURITE OUTGROWTH PATTERNS

Spinal cord injury, specifically in the cervical C3-C4 region of the cervical spine, contributes to impaired breathing and a diminished quality of life. Therefore it is important to find effective and safe therapeutics to restore breathing function. Indeed, there are a myriad of research being performed in addition to valuable collaboration amongst different institutions. As such, inspired by a previous experiment, we decided to test our hypothesis that an enriching environment consisting of different hypoxic environments - sustained and intermittent hypoxia alongside normoxia - would result in neurite outgrowth. Moreover, we hypothesized that sustained hypoxia would result in the greatest neurite outgrowth, followed by intermittent hypoxia. Lastly, we predicted that normoxia would result in the smallest amount of neurite outgrowth. We tested this hypothesis first *in-vivo* by placing female, retired breeder rats (n=3) in sustained or intermittent hypoxia or normoxia conditions for a period of 2 hours and 32 minutes. This was followed by dorsal root ganglion (DRG) culturing, plating, and fixing. Notably, because the premise of this experiment is a hybrid of both an *in-vivo* and *in-vitro* (specifically an *ex-plant)* we cannot, with accuracy, state that the growths are of axonal in nature. As such, we will refer them to as neurite. We used NeuronJ to manually trace and measure neurite outgrowth. In addition, we also utilized the Sholl analysis to better understand neurite arborization and their location in relation to the soma. We conclude that, interestingly, normoxia resulted in the greatest neurite outgrowth, followed by intermittent hypoxia and lastly by sustained hypoxia. As such, this experiment augments others experiments that show the promising therapeutic effects of intermittent hypoxia in restoring breathing function whether it is through enhancement of the crossed phrenic pathway or increased serotonergic receptor activation.

KEYWORDS: hypoxia, neurite, outgrowth, sprouting, respiratory function

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April 21, 2022

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CHAPTER 1: INTRODUCTION

The history of spinal cord injuries (SCI) can be traced back as far as ancient Egypt during a time in which physicians believed the injury to be irreversible and untreatable.[1] Indeed, it is reasonable to say that since that time, there have been many advances in medicine and technology allowing for a degree of restoration of function following injury. Yet, with the majority of spinal cord injuries occurring at the cervical level, this can lead to severe impairment of the respiratory system including diaphragm paresis and paralysis.[2] Moreover, it has been shown that the more rostral the level of injury, the more likely it is that the injury will affect breathing function. Indeed, following a traumatic SCI, respiratory dysfunction is the primary cause of death.[3] As of now, there are no cures to alleviate diaphragmatic paralysis and as such, without proper ventilation, patients with cervical SCI must rely on mechanical ventilations.[3] Moreover, while effective, mechanical ventilation is not without faults. Mechanical ventilation increases the risk of infection, pneumonia and death in patients already suffering from SCI.[3] As such, despite the level of SCI (i.e thoracic, lumbar), the main cause of death is a result of respiratory insufficiency.[3]

The circuitry that describes the neural pathway that innervates the diaphragm has been extensively studied and well defined. Figure 1 shows a pictorial description of said pathway. The central nervous system (CNS) is defined as the brain and spinal cord. Importantly, other features of the CNS that are involved in the breathing circuitry includes the brainstem, of which includes the midbrain, pons, and medulla. The respiratory neural circuitry begins at the brainstem with respiratory rhythm generating neurons located inside the pre-Bötzinger complex (PBC) and the para-facial respiratory group (pFGR) located in the ventral lateral medulla. These neurons connect to the propriobulbar neurons as well as to the premotor neurons found in the ventral respiratory group (VRG) found laterally and ventrally to the nucleus ambiguous. The rostral aspect of the VRG, abbreviated rVRG, contains inspiratory bulbospinal neurons. Neurons project out of both the rVRG and innervate the ipsilateral and contralateral phrenic motor nucleus. Moreover, neurons in the phrenic nucleus innervate downward towards the ipsilateral aspect of the hemidiaphragm (i.e left phrenic nerve innervates the left hemidiaphragm.[3]

Clinically, cervical SCI, especially the upper region of the cervical spine leads to impaired neural signaling originating from the brainstem down to the diaphragm; this is the fundamental cause of diaphragmatic paresis, and ultimately impaired respiratory function. In order to further study cervical injury in animal models, scientists performed C2 hemisections (C2Hx) to mimic the human condition. Moreover, a C2Hx, a small incision made to severe one side of the C2 spinal cord, mimics a SCI and disrupts breathing patterns on the ipsilateral aspect

of the diaphragm. As such, this incision interrupts the innervation originating from rVRG to the phrenic motor nucleus. This injury model results in reduced tidal volume (amount of air that moves in/out of the lungs in a respiratory cycle) and increases breathing frequency. Previous extensive studies performed on rat models have led to the discovery of an important phenomena referred to as the cross phrenic pathway. As described earlier, the neurons from the rVRG innervates the ipsilateral phrenic motor nucleus. Indeed, animal studies have shown that there are neurons that arise from the rVRG that decussate and thus innervate the contralateral phrenic motor nucleus. This gave some insight into possible therapeutic avenues that could be taken to rescue respiratory pattern following SCI at the cervical level.[3]

This activation of the crossed phrenic pathway (CPP) has been historically induced by asphyxia, specifically hypoxia or hypercapnia. As such, experiments have sought to target methods in which to enhance the CPP. For example researchers investigating the effects of theophylline, a respiratory stimulant, has shown that it enhanced CPP by modulating adenosine A_1 receptors. Specifically, they discovered that respiratory function improved through activation of the CPP by using an adenosine A_1 antagonist and adenosine A_2 agonist, simultaneously. Indeed, while the mechanism is not yet understood, theophylline induced respiratory recovery lasted for at least 1 month following treatment. In addition, further studies have pointed to the idea that there may be sex differences as it relates to CPP activation. Moreover, it has been shown that female rats underwent CPP activation when compared to male rats. There is also the possibility that the CPP synaptic terminal may be ineffective in innervating the phrenic motor neurons which would not be able to provide the depolarization necessary in order to drive a respiratory response. As it has been shown that the phrenic motor neurons decrease in size following a SCI and are effectively more excitable (i.e Henneman's principle), more CPP innervation and activation could allow for more contraction of the diaphragm and increase respiration.[3]

Aside from the activation of the crossed phrenic pathway, there have been other experimental avenues that researchers investigated in order to elicit an increase in respiratory function following SCI. One of routes taken was to investigate the effects of acute intermittent hypoxia (AIH) on improving respiratory drive. AIH is characterized by short bouts of hypoxia (i.e 5 minutes) followed by bouts of normoxia (i.e 5 minutes). As such, this paradigm led to phrenic long term facilitation (pLTP), a type of neural plasticity. Overall, pLTP has been shown the strengthen synaptic networks that lead to the phrenic motor neurons. Importantly, further studies have elucidated that pLTP involves serotonergic activation alongside brain derived neurotrophic factors (BDNF). Similar to the mechanism underlying long term potentiation (LTP) as it relates to

learning and memory, serotonin permitted strengthening of glutamatergic synapses - a process required for pLTF. Indeed, blocking NMDA receptors have been proven to limit pLTF. Specifically, experimental models have elucidated that the strengthening of the glutamatergic synapses between the descending respiratory neurons and the phrenic motor neurons is serotonin-BDNF dependent as well as activation of $5HT_{2A}$ receptors. As such, it is likely that serotonin dependent-BDNF synthesis leads to the strengthening and remodeling of presynaptic and postsynaptic terminals.[3] As we alluded before, the latent crossed phrenic pathway has been extensively studied as a manner in which we can elicit recovery in respiratory drive following SCI. Moreover, published studies revealed that serotonin (5HT) indeed plays a significant role in the activation of the CPP.

Collectively, the mechanisms described above illustrate the ability to exploit endogenous mechanisms in order to induce plasticity in order to improve respiratory motor output and thereby lead to recovered breathing patterns among individuals suffering from SCI. As such, the highlight of our research explored the possible therapeutic effects of acute intermittent hypoxia, sustained hypoxia and normoxia and how it relates to axonal regeneration in the peripheral nervous system (PNS), specifically dorsal root ganglion (DRG). Previous studies (Fuller et al., 2000; Mitchell et al., 2001; Baker-Herman et al., 2002) have demonstrated that intermittent hypoxia under the following paradigm - 3 5 minutes intervals of a hypoxic challenge - was able to induce phrenic motor output in a serotonin dependent manner. Indeed, a study conducted by Mitchell et al (2003) demonstrated that chronic and acute intermittent hypoxia treatments following chronic SCI (C2Hx) led to spontaneous recovery on the ipsilateral side of injury, evident by EMG recordings that show an increase in amplitude of inspiratory burst as well as a serotonin-dependent plasticity. It is important to note here that these results highlights improved respiratory motor output in chronic, not acute SCI as research shows that intermittent hypoxia is not effective until 4-8 weeks post injury.[4] As such more research needs to be performed in order to find therapeutics to treat patients with acute SCI. Another important limitation of this therapeutic tool centers around the duration of intermittent hypoxia, either acute or chronic, and how it relates to the development of pathophysiology. Simply put, the applications of CIH or AIH can be offset as it has been implicated in the development of side effects such as neurocognitive deficits (likely due to decreased oxygen), hippocampal cell death, and impaired sympathetic chemoreceptor function.[4,5] It is important to also highlight the duration of pLTF following bouts of chronic and acute intermittent hypoxia. Moreover, Fuller et al (2005) points out that while the duration pLTF in acute intermittent hypoxia is unknown, pLTF elicited by chronic intermittent hypoxia (defined as 12h per night for 7 nights) lasted for up to 24hrs (normally 90 minutes). In addition, chronic intermittent hypoxia enhanced CPP for up to 2 weeks following a C2Hx, with an increase in

respiratory motor output for up to 1 week. Together, with the deleterious effects of prolonged exposure to a hypoxic exposure and the unknown during of pLTF in acute intermittent hypoxia, it may be useful to pursue another therapeutic route involving physical therapy to further augment respiratory motor output in order to strengthening respiratory muscles.[4]

Prior research has shown that it is the failure of CNS cells to regenerate that leads to permeant disabilities in patients who suffer from SCI. In this sense, there is a stark contrast between peripheral nervous system (PNS) cells in that these cells have been shown to take part in a regenerative paradigm and as such, it is important to understand the mechanism PNS cells utilize in order to regenerate. While it is not difficult to maintain the integrity of CNS tissue once they have been harvested, the main issue lies in the manner which they are harvested. Moreover, DRGs (figure 2) are pseudounipolar and such, in addition to their peripheral branches, they are also able to extend axons into the spinal cord and/or brainstem. As such, many researchers utilize dorsal root ganglions, sensory neurons that in some way, bridges the gap between the peripheral and central nervous systems.[5]

Interestingly, it has been shown that injury to the peripheral axon leads to neurite outgrowth and thus activation of pro-regenerative gene expression as compared to injury to the central axon branch. Moreover, multiple regeneration associated genes (RAGs) have been identified as it relates to promoting neurite regeneration and outgrowth. Yet, it has been elucidated that it is not just RAG that promotes the regenerative capacity in dorsal root ganglia but the activation of some, in addition to inactivation of other RAGs. Of particular interest to our research was hypoxia inducible factor 1 alpha (HIF1a) and its downstream target gene vascular endothelial growth factor A (VEGF-A). Under normal conditions (i.e normal oxygen levels or normoxia), HIF1a is hydroxylated and this makes it a target for ubiquitination and subsequent degradation via proteasome. However, during hypoxic conditions (i.e low oxygen levels), the rate of HIF1a hydroxylation is reduced, leading to the accumulation and translocation of HIF1a to the nucleus where it will bind to hypoxia response elements. Prior work by Nix et al (2014) has shown that deletion of HIF1a leads to reduced regeneration but this work had not been done on mammals. As such, Cho et al (2015) demonstrated that acute intermittent hypoxia was able to stimulate axonal regeneration *in vivo* in mice. Moreover, AIH (10 minutes at 8% oxygen interspersed with normoxia) led to the upregulation of HIF1a but also downstream target genes such as VEGFA.[6]

Previous research sought to investigate HIF1a and VEFGa expression levels at the end of each treatment paradigm, which included sustained hypoxia, intermittent hypoxia and normoxia.[2]

As alluded to before, it is important to ensure that the level of hypoxia induced does not lead to pathological conditions (i.e neurocognitive disorders). As such, previous studies such as the one described wanted to elucidate the proper level of hypoxia that will lead to the ideal level of HIF1a/VEGFa expression as it relates to regeneration and neurite outgrowth. In order to measure neurite outgrowth, we decided to utilize DRGs as they are easy to harvest and culture and have regenerative capabilities. As such, previous studies conducted by Caroline Devine (Alilain Lab) were inspired by Cho et al (2015) by treating DRGs with sustained hypoxia and normoxia *in vitro*. Further, in order to further augment research highlighted here, my experimental design focused on providing an enriching environment as it relates to sustained and intermittent hypoxia and normoxia *in vivo* and determine how these treatments translate *in vitro* when dorsal root ganglia were cultured on a dish. As with Caroline Devine's study, I utilized DRGs as a way to measure total neurite outgrowth but to see how DRGs how pronounced the treatment paradigms can persist, from an *in vivo* treatment to *in vitro* culturing. We hypothesize that intermittent hypoxia followed by sustained hypoxia and lastly normoxia (**SH > IH > N**) would promote the greatest amount of axonal outgrowth.

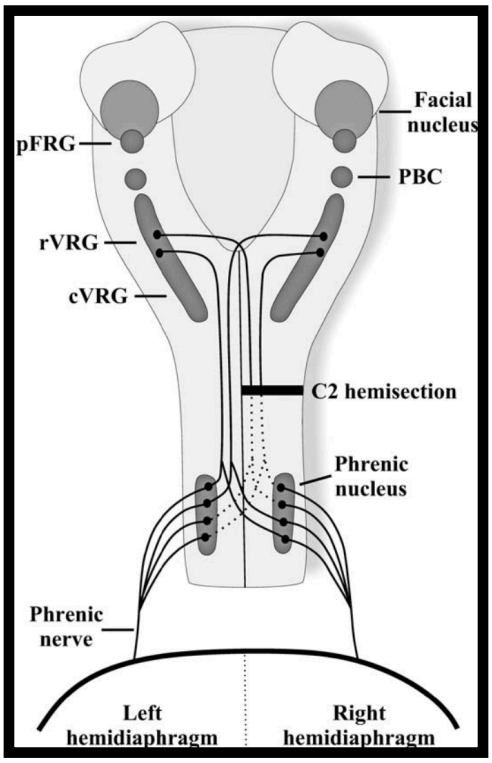


Figure 1. Neural circuitry

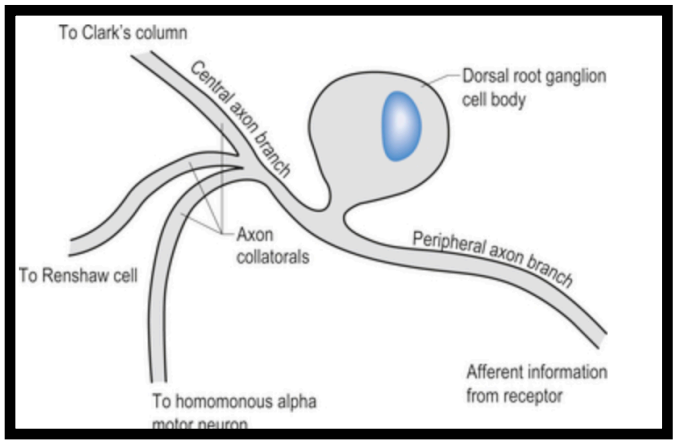


Figure 2: Dorsal root ganglion depicting central and peripheral branches

CHAPTER 2: METHODS

Please note that all animal care/handling were performed under strict compliance with the Institutional Animal Care and Use Committee regulations here at the University of Kentucky

Part 1 of dorsal root ganglion (DRG) harvest: dissection of DRG from rat spinal cords

For this series of experiments, we utilized 3 uninjured, retired breeder female Sprague Dawley rats (Envigo-Harlan, Indianapolis, IN, USA). Each rat was assigned a specific treatment paradigm (i.e intermittent hypoxia, sustained hypoxia, and normoxia) and were subject to said treatment for a duration of 5 consecutive days. For intermittent hypoxia, the rat was treated with 10% oxygen interspersed with normal oxygen concentration or normoxia (20% oxygen) for a period of 2 hours and 32 minutes. For sustained hypoxia, the rat was treated with a constant flow of 10% oxygen for 2 hours and 32 minutes. The hypoxia treatments were performed using the OxyCycler model A84XOV Atmosphere-controlled Chamber (Biospherix, Ltd., New York, USA). Normoxia treatment was subject to a rat at 20% oxygen for 2 hours and 32 minutes as a means of control. In order to maintain a narrow window between treatment and *ex-plant* cultures immediately following treatment, we anesthetized the rats using carbon dioxide under a hood at a rate of 5L/min. After the rats were anesthetized, in a faraday cage, we utilized curved forceps and scissors to cut the skin rostrocaudally (top down) along the midline of the spine. This allowed for the exposure of musculature which was cut through using scissors in order to expose the spinal column. Using heavy dissecting scissors, we cut the rat spinal column at the base of the skull and at the lumbo-sacral level of the spinal cord. Once removed, we carefully cut away the musculature and surrounding ribs as to further expose the spinal column. Importantly, while dissecting away muscle and ribs, it is important to identify the spinous processes. After dissecting away muscle, using the spinal process as a guide, we cut down the midline of the spinal cord and separated the column into the left and right sides.

We pinned one half of the exposed spinal cord onto a rubberized matt (blue) which was laid onto an ice pack to maintain the tissue. In addition, we prepared 3, 1mL microcentrifuge containing 1X calcium-magnesium free Hank's balanced salt solution (CMF-HBSS) which was placed in a bucket of ice; this is the solution we placed the DRGs after extracting them from the cord. To save time, before dissections started we placed 3 aliquot containing 1.5mL of collagenase/dispase in a 37ºC water bath in the cell culture room to allow ample time to thaw; each collection of DRGs from different treatments were placed in their own aliquot. For reference, the aliquots of collagenase/dispase are in neurobasal A media and are placed in the -20ºC freezer. Using a dissection microscope, microscissors, and two small forceps we dissected

out the DRGs from each side of the dissected spinal column. It is important to note here that when the DRGs were removed, their axonal processes were cut, leaving only the soma. The DRGs were placed in the 1mL microcentrifuge tube with CMF-HBSS. It is important to note here that we labelled the microcentrifuge tubes with the respective treatment paradigms each DRG underwent *in-vivo.* The steps listed above were performed on all rats following their respective treatment paradigms.

Upon dissection of the final DRGs from the final rat, in the cell culture room, we removed the 1mL of CMF-HBSS from each microcentrifuge tube and added 1.5mL of collagenase/dispase. Collagenase/dispase is an enzymatic mixture that allows us to digest DRGs prior to culturing. Specifically, collagenase degrades collagen while dispase degrades proteases in a nonspecific manner. We decided to wait until all the DRGs were harvested from all rats before proceeding with this step in order to ensure consistency and to make sure all DRGs were placed in the aliquot of collagenase/dispase at the same time. We added parafilm to the tops of the microcentrifuge tube to prevent evaporation of the solution as well as to prevent spillage. We gently shook the microcentrifuge tubes in order to mix the DRGs with the enzyme. Following, we placed each microcentrifuge tube into a 50 mL conical tube filled with tissue paper. The purpose of the tissue paper was to allow for the microcentrifuge tube to fit inside the conical tube. Because we had 3 microcentrifuge tubes with DRGs, we used 3 conical tubes. The conical tubes containing the microcentrifuge tubes were then placed on top of a rocker/shaker; the rocker was set at 40 rotations per minute (RPM). This allowed the conical tubes to gently shake the DRGs overnight, for approximately 16 hours.

Part 2 of dorsal root ganglion (DRG) harvest: plating neurons onto well plates

We prepared the six well plates by adding coverslips to the bottom of each well.

After approximately 16 hours on the rocker/shaker, remove the 50mL conical tubes from the rocker and allow the cells to settle to the bottom of the microcentrifuge tube. Recall the following steps were performed for three separated microcentrifuge tubes. In the tissue culture hood within the cell culture room, we removed the collagenase/dispase from each microcentrifuge tube into a waste beaker and added 1 mL of chilled 1X CMF-HBSS. Following, we centrifuged them at 2000 RPM for 2 minutes. This wash and centrifuge step was performed 3 times, with specific differences at each step here described. Following the first centrifuge, we used a P1000 micropipet to triturate the cells at the bottom in order to break them up; on average we triturated 10x. Following the second and third triturations, we used fire-polished glass pipet

until the DRGs broke apart and the solution looked homogenous and free of large clumps. Following the third wash, we added 500mL of neurobasal A (NBA; 75.75mL of neurobasal media A, 1.5mL of B27, 750µl of pen/strep, 187.5µl of glutaMAX) complete media.

We used a 1:5 dilution (vs a 1:3 dilation stated in the protocol) in order to enhance our ability to visualize the cells under the microscope. As such, for each condition we used 10µl of cell solution, 10µl of trypan blue (stains dead neuronal cell bodies), and 30µl of NBA and added it to a .5mL microcentrifuge tube. We added 10µl of the cell/trypan blue/NBA mix into the groove of a hemacytometer and placed the coverslip. It is important to have wiped off the coverslip with a kimwipe to ensure that there are no dust particles. We used a microscope to visualize neurons - under a microscope, neurons are circular cells and have a halo surrounding them. The hemacytometer is divided into grids (4) and we counted the number of neurons per grid. On average there should be between 6-9 cells per grid. Following, we determined that we had enough cells in solution to culture 5,000 cells per well. After determining the average number of cells per treatment, we calculated the total volume needed to culture two plates (6 wells each plate for a total of 12 wells; some treatments did not have 12 wells). Following, we calculated the volume of cell suspension we needed to add to the total volume, of which we added 1.5mL into each well. We then placed all the cell culture plates (six total) into the incubator at 37ºC for 24 hours.

We decided to plate each condition on two plates as to see the difference in neurite outgrowth following a one day incubation in NBA media vs a seven day incubation/media exchange with anti-mitotic.

Part 3A dorsal root ganglion (DRG) harvest: cell fixation and media collection

We labeled each 6 well plate from numbers 1-12. As such, we used the well plates labeled 7-12 to serve as the one day incubation cohort. We removed these plates from the incubator and removed 1mL of the media into a new 50mL conical tube. We labeled each conical tube with the corresponding treatment paradigm (IH, SH, normoxia) and put it in the -80ºC freezer for preservation. Following this, we fixed the neurons with 4% paraformaldehyde (PFA). Important to note here at all cell fixation steps took place in a special chemical hood. We prepared the 4% PFA by first adding 10mL of 10X PBS to a 50mL conical tube; ensure that the 10X PBS is at room temperature or the cells will burst. Then we added the 10mL pre-made ampoule of 8% PFA. Using a 10mL syringe and 18 gauge feeding needle, we removed the PFA and added it to the PBS mix. Because we had already removed media and had 0.5mL of media left, we removed the remaining media and added 1mL of 4% PFA to each well. We let this incubate at room

temperature of 30 minutes. After the 30 minutes, we removed the PFA into a PFA only waste beaker and washed each well with PBS three times. After the last wash, we added 1mL of PBS, wrapped the plates in parafilm and placed into the 4ºC refrigerator.

Part 3B of dorsal root ganglion (DRG) harvest: media exchange

Following the 24 hour incubation period at 37ºC, we performed a media exchange on the other 3 well plates. The new media contains anti-mitotic agents as a means to prevent the growth of other cell types. As such, this will make it easier for us to quantify neural outgrowth following seven days of incubation at 37ºC. We calculated the total volume of solution needed for all well plates (calculation for normoxia is different as only 5 wells were plated). We then calculated the necessary amount of Fudr and Ara-C. Each well had 1.5mL of NBA media prior to incubation. As such, we removed 1mL of the old media and kept 0.5mL in each well to prevent the cells from drying out. Following, we added 1mL of the anti-mitotic mix to each well. The well plates were then placed back into the 37ºC incubator.

Part 4 of dorsal root ganglion (DRG) harvest: Primary antibody

After incubating the well plates immersed in anti-mitotic media for seven days, we fixed the cells using 4% PFA. Please refer to the steps listed above for the cell fixation process. Following the cell fixation, we put the well plates into the refrigerator overnight. At the start of the staining process, we removed all wells from the refrigerator and allowed the cells to warm in room temperature. Then we calculated the total volume of blocking solution needed for all 6 well plates and subsequently calculated the amount of normal goat serum (NSG), BSA, and Triton X-100. We gently vacuum aspirated the PBS and added 750µl of the blocking solution to each well. We allowed this to sit at room temperature for 30 minutes. Save the remaining amount of blocking solution to be used in day five or secondary antibody staining.

During the incubation period, we prepared the primary antibody (1:1000 dilution) of mouse anti-rat ßIII tubulin (Sigma T8578). After the 30 minute incubation with blocking solution, we added 250µl of the primary antibody solution into each well. Each well plate was wrapped in parafilm and placed in the refrigerator for overnight incubation.

Part 5 of dorsal root ganglion (DRG) harvest: Secondary antibody - performed in the dark*

This was performed in the dark*:

After overnight incubation, we prepared the secondary antibody, goat anti-mouse IgG Alexa 594 (Invitrogen A11032) at a dilution of 1:1500; we added the appropriate amount of secondary antibody into the saved blocking solution from day four.*

We removed the wells from the refrigerator and allowed the cells to warm to room temperature. We then vacuum aspirated the primary antibody + blocking solution from each well and washed the wells three times (3x) with PBS (room temperature); in between each wash, the wells were incubated at room temperature for 15 minutes with PBS.

This was performed in the dark*:

Then we added 1mL of secondary antibody + blocking solution into each well. Following this, we wrapped each well in aluminum foil and incubated them at room temperature for 2 hours in the dark.* The primary purpose of performed said steps (i.e incubation with 2º antibody and preparation of the 2º antibody) in the dark is to prevent light from reacting with the secondary antibody, a property we will exploit when using the Keyence, an all-in-one fluorescence microscope (Keyence Corporation of America, Itasca, IL, USA). After 2 hours of incubation at room temperature, we washed each well by vacuum aspirating the secondary antibody and adding PBS to each well three times; again, between each wash we incubated the well plates for 15 minutes. We wrapped each well plate in parafilm and aluminum foil and placed them in the refrigerator.

Part 6 of dorsal root ganglion (DRG) harvest: imaging for data analysis

Once all the cells were staining with ßIII tubulin, we imaged them using the BZ-X810 all-in-one fluorescence microscope, or the Keyence (Keyence Corporation of America, Itasca, IL, USA). To limit user bias, we decided the visualize neurons in the outer edges of each well plate. Specifically, we looked at the same general area of each well (i.e upper right quadrant of the well/ coverslip) to ensure consistency without bias.

When we imaged the cells that were incubated for only 24 hours and subsequently stained, we observed that there were no neurite outgrowths and decided not to quantify these images/neurons. We imaged the cells that underwent a media exchange and were incubated for

seven days before being fixed. Once the images were taken, the image files were renamed as letter codes (i.e A, B, C, etc) as to blind the investigator quantifying them. In total, three investigators quantified the neurite outgrowths using NeuronJ (a plug in for the software ImageJ) and all were blinded. Once all quantifications were performed, everyone was unblinded. We utilized the software PRISM to analyze the data following each blinded quantification. For this experiment, we utilized the ANOVA test to determine whether the results obtained were statistically significant (denoted by a *p*-value of 0.05 or less) as there were at least three independent variables (IH, SH, normoxia). In addition, we decided to utilize Sholl analysis to further quantify neurite outgrowth as a way to provide another tool that can be used to quantify neurite outgrowth in a more efficient way. Moreover, Sholl analysis is a more accurate way to quantify dendritic arbors[7] and as such, we decided to utilize this technique to compare to our manual traces of neurite outgrowth using ImageJ.

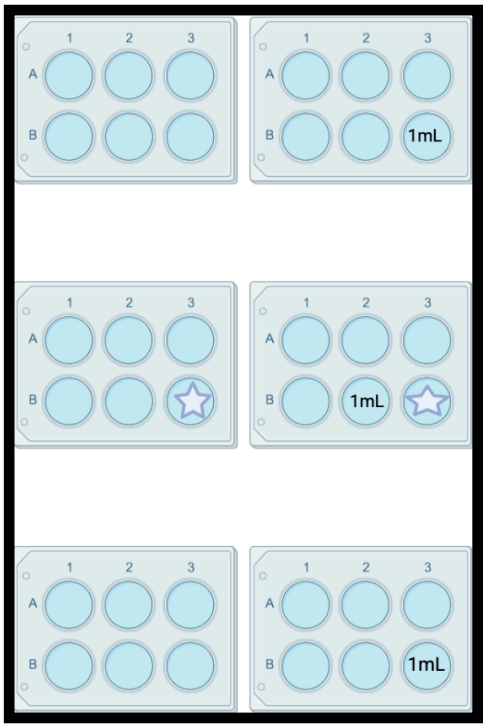


Figure 3: schematic image of well plates

Intermittent hypoxia: note that well 12 has only 1mL of NBA media due to inadequate solution; likely an error in calculation

Normoxia: note that the stars represent lack of cells/media in wells 6 and 12; well 11 only has 1mL of NBA media - again, likely error in calculations

Sustained hypoxia: note that well 12 has only 1mL of NBA media; likely error in calculation

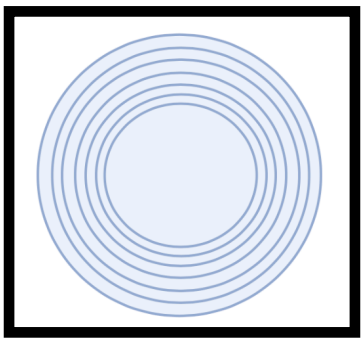


Figure 4: Concentric circles used to measure dendritic arbors from the center (i.e soma) to periphery

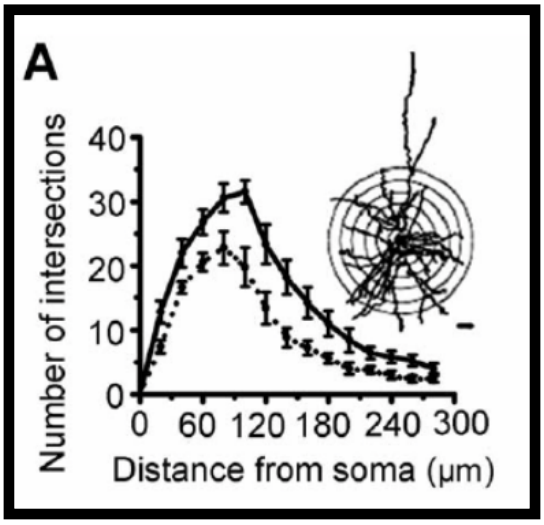


Figure 5: Graph depicting Sholl analysis - as the distance from the soma increases, the number of crossing/intersections increase then decreases[7]

CHAPTER 3: RESULTS

Neurite outgrowth analysis using NeuronJ

The results we obtained for this experimental paradigm did not support the hypothesis. Previously, we hypothesized that sustained hypoxia treatment would result in the greatest neurite outgrowth followed by intermittent hypoxia; normoxia would result in the least amount of outgrowth. Following five days of specific treatment paradigms established previously, rats treated *in-vivo* with sustained hypoxia at moderate oxygen levels (i.e 10%) showed the most resistance to neurite outgrowth. It is important to note here that the results obtained were not statistically significant (p-value = 0.1626). Indeed, the average neurite outgrowth taken across all three quantifiers (see tables 1-3) reveals that normoxia had the greatest total neurite outgrowth when compared to sustained hypoxia and intermittent hypoxia.

Table 1: Each image letter represents an image of a neuron taken with Keyence. The total neurite outgrowth is the total taken across all three quantifiers. The average length is shown in inches for sustained hypoxia treatment

	Total neurite outgrowth (inches)
Images	Sustained hypoxia (n=12)
U	41.57266667
V	23.60483333
W	23.43466667
X	31.94133333
Y	20.34183333
Z	28.787
AA	15.69666667
BB	6.723333333
CC	8.847666667
DD	2.768666667
EE	11.60733333
FF	8.231333333
Average	18.62977778

Table 2: Each image letter represents an image of a neuron taken with Keyence. The total neurite outgrowth is the total taken across all three quantifiers. The average length is shown in inches for intermittent hypoxia treatment

	Total neurite outgrowth (inches)
Images	Intermittent hypoxia (n=11)
A	9.598
B	71.88266667
C	12.654
D	23.95266667
E	13.44633333
F	29.624
G	24.11933333
H	15.71566667
I	29.32666667
J	40.04066667
K	11.43066667
Average	25.61733333

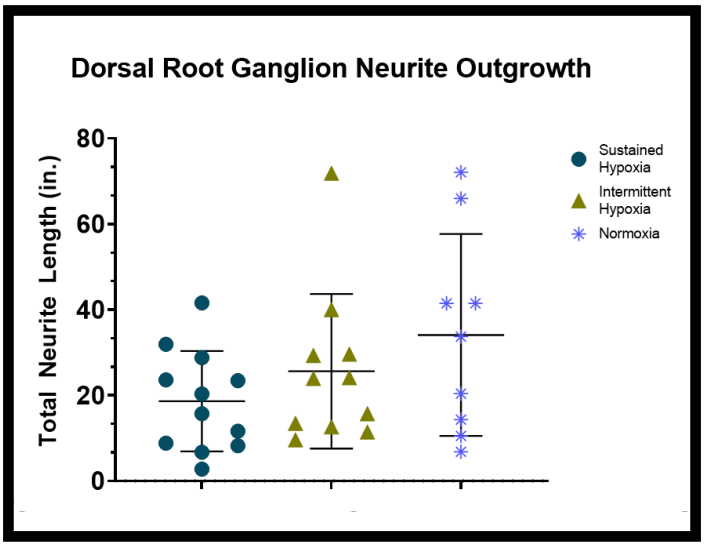
Table 3: Each image letter represents an image of a neuron taken with Keyence. The total neurite outgrowth is the total taken across all three quantifiers. The average length is shown in inches for normoxia treatment

	Total neurite outgrowth (inches)
Image	Normoxia (n=9)
L	14.32283333
M	72.09733333
N	6.832
O	65.96466667
P	10.51133333
Q	20.39733333
R	41.4655
S	33.715
T	41.49633333
Average	34.08914815

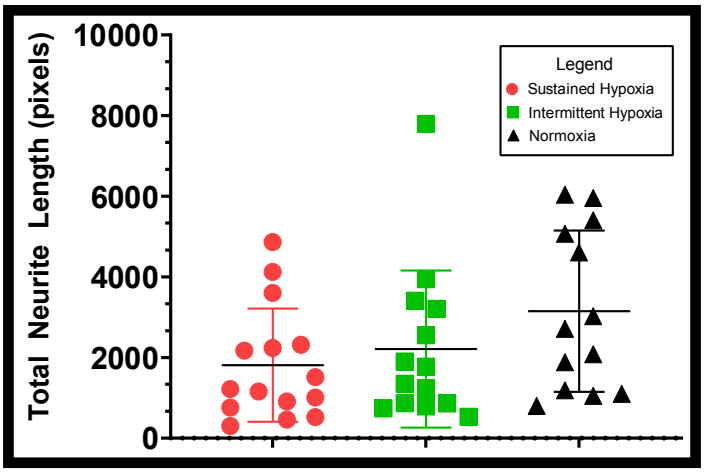
Table 4: One way ANOVA test; *p*-value: 0.1626, not statistically significant

F	1.935
P value	0.1626
P value summary	ns
Significant diff among means (P<0.05)	No
R squared	0.1178

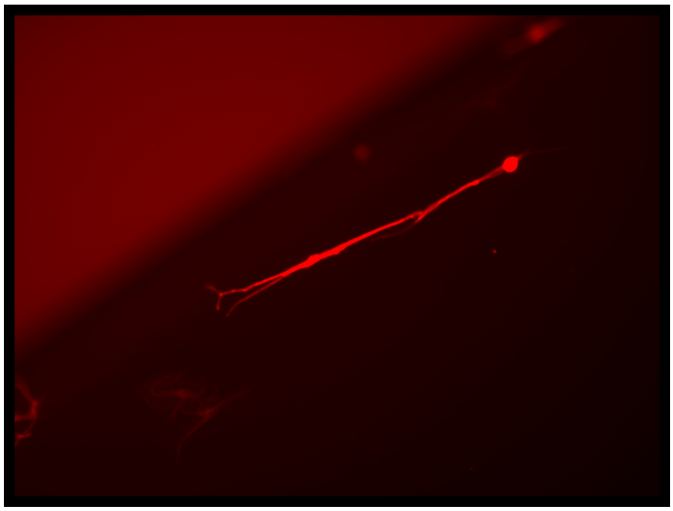
A


Dorsal Root Ganglion Neurite Outgrowth
Total Neurite Length (in.)
80
60
40
20
0
Sustained Hypoxia
Intermittent Hypoxia
Normoxia

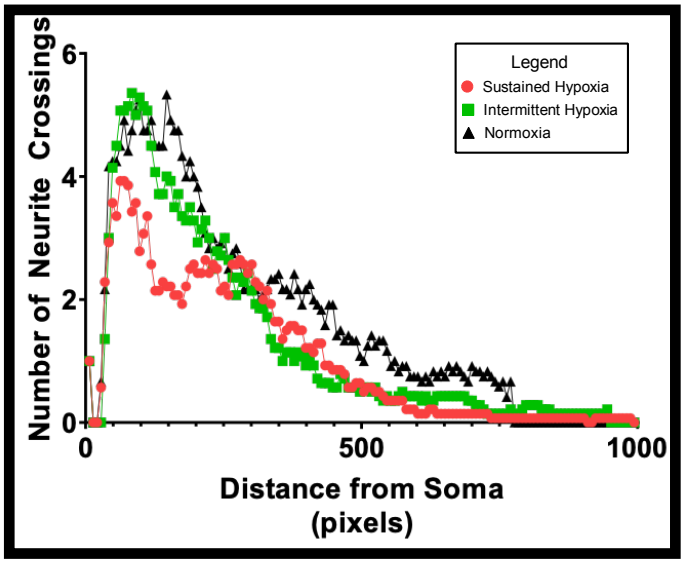
B


Total Neurite Length (pixels)
10000
8000
6000
4000
2000
0
Legend
Sustained Hypoxia
Intermittent Hypoxia
Normoxia

C



D


Number of Neurite Crossings
6
4
2
0
0
500
1000
Distance from Soma
(pixels)
Legend
Sustained Hypoxia
Intermittent Hypoxia
Normoxia

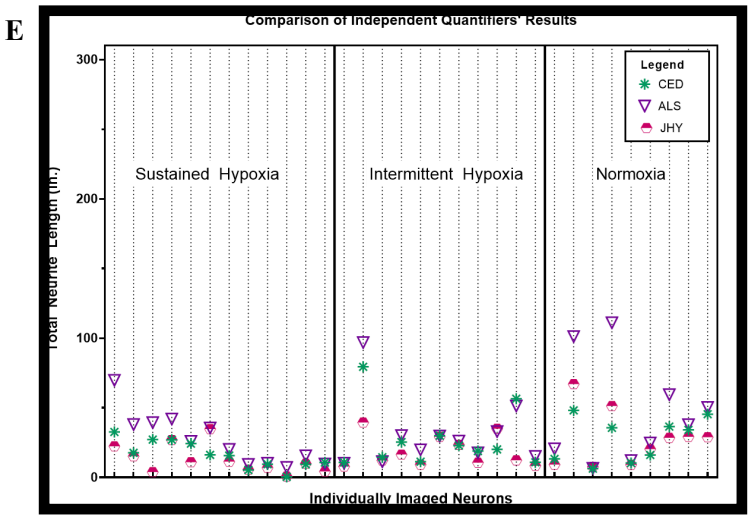


Figure 6: **(A)** This graph shows dorsal root ganglion outgrowth for each treatment paradigm; sustained hypoxia (n=12) is on the left, intermittent hypoxia (n=11) is in the middle and normoxia (n=9) is on the right. **(B)** This image, used with permission by ALS, shows the automated neurite outgrowth following the Sholl analysis. As such, it is shows a similar trend to the manual traces shown in **(A)**. **(C)** This is an image obtained by fluorescent microscopy of a single DRG showing a neurite outgrowth projecting from its soma. **(D)** This image, used with permission by ALS, shows the Sholl analysis which trends towards increasing neurite outgrowth. It is evident that IH treatment and normoxia has more neurite outgrowths as compared to SH but it can also be extrapolated that SH treatment allowed for more sustained outgrowth i.e there are more crossings as distance increased. **(E)** This graph depicts the results following independent quantifications by all three quantifiers (ALS, JHY, CED).

CHAPTER 4: DISCUSSION

There is considerable research that describes the effects of intermittent and sustained hypoxia on neural plasticity following cervical spinal cord injuries (SCI). In particular, a study conducted by Silverstein and Alilain (2021) sought to exploit the difference between fixed duration intermittent hypoxia (12 cycles of 5 minutes of hypoxia interspersed with normoxia for 2hrs and 32min) and varied duration intermittent hypoxia (condition where the hypoxic episode varied from either 2 minutes or 8 minutes) 1 week and 8 weeks post C2Hx. Moreover, this study looked to explore a novel pattern sensitivity in order to determine the best treatment paradigm to generate phrenic long term facilitation (pLTF) in rats as a means to hopefully translate this treatment method to the clinic. Further, this highlights a point mentioned previously regarding LTF. Following a cervical SCI, there are usually spared or latent pathways that can innervate the phrenic motor nucleus. As such, research has been done to potentially exploit this pathway and restore respiratory motor function following injury. One of the mechanism that leads to LTF is the serotonergic pathway, termed the Q pathway, as intermittent hypoxia has been shown to increase serotonergic signaling, and thus LTF[1]. Yet this is determined by the availability of serotonin. Research has shown that following a hemisection, serotonin and serotonergic sprouting is most abundant 8 weeks post injury. From this study, it was determined that FD-IH led to greater respiratory motor recovery when compared to VD-IH 8 weeks post injury.[1]

Indeed, while a lot of research has been performed on the effects of IH and SH in relation to LTF, there haven't been many studies that looked at neurite outgrowth and regeneration. As such, we decided to utilize DRGs as a means to determine their regenerative capabilities. Inspired by an experiment performed by Hutson et al (2019) and Cho et al (2015), our experimental design focused on exposing rats to an enriching environment i.e intermittent and sustained hypoxia and normoxia in order to determine whether these conditions would upregulate certain transcription factors and proteins (i.e HIF1a, VEGFA) *in-vivo* and how these effects would translate once neurons (DRGs) were harvested and plated. Moreover, the idea was to determine if the treatment paradigms performed *in-vivo* would translate once cells (DRGs) were *ex-plant* onto well plates. The experiments carried out by Cho et al (2015) revealed that acute intermittent hypoxia not only upregulated HIF1a in DRGs following injury, but also activated downstream protein VEGFA as a means to further stimulate axonal regeneration on DRGs. The findings from this experiment show that rats exposed to normoxia showed the greatest response and thus resulted in the greatest total neurite outgrowth. This result contradicts our hypothesis that sustained hypoxia would have the greatest neurite outgrowth. Moreover, this shows that there were other factors that led to normoxia having the greatest neurite outgrowth. These results do not point to the idea that

hypoxia treatment does not lead to neurite outgrowth. Indeed, from the experimental data, it can be seen that both intermittent and sustained hypoxia treatments result in outgrowth. As such, our results contribute to other studies showing neurite outgrowth in response to hypoxic treatment paradigms.

HIF1a is induced during exposure to hypoxia and VEGFA is an injury induced HIF1a target gene[6]. The Cho et al (2015) experiment, performed in mice, revealed two important facets. Firstly, during an *in-vitro* experiment using DRGs, continuous (i.e sustained) hypoxia treatment led to the upregulation of HIF1a (1.5 fold increase) in addition to an increase in phosphorylation of protein kinase C (PKC), a key protein usually found downstream of the G_q pathway (i.e adenosine A_2).[6] Specifically, treatment was applied immediately after injury, and it was shown that delayed treatment (i.e 4, 8, 12h post injury) does not result in regeneration. Secondly, acute intermittent hypoxia at 8% oxygen level, which constitutes a severe hypoxic condition, in *in-vivo* injured mice resulted in an upregulation of HIF1a and its downstream target, VEGFA. Further, this treatment paradigm resulted in axonal regeneration when compared to normoxia. Similar to the experiment carried out by Cho et al (2015), Silverstein (2020) sought to investigate the effects intermittent/sustained hypoxia and normoxia treatment paradigms in uninjured adult rats had in HIF1a/VEGF expression. Upon treating uninjured rats (n=9 with 3 cohorts per treatment paradigm), CNS tissue from the brain, brainstem, cerebellum and spinal cord were harvested to measure HIF1a and VEGF. Interestingly, it was revealed that the normoxia condition resulted in the greatest expression of HIF1a while moderate sustained hypoxia resulted in the greatest expression of VEGF. Specifically, HIF1a was found to be at the highest level in the spinal cord following intermittent hypoxia which could point to the idea that intermittent hypoxia and HIF1a could be directly involved in improving respiratory motor function. Furthermore, these results corresponds well with my results in that moderate intermittent hypoxia indeed led to neurite outgrowth. Future directions would then include analyzing the collected media for VEGFA/HIF1a levels, as well as staining for HIF1a/VEGFA, and comparing the results to each treatment paradigm's total neurite outgrowth. It would also be interesting introduce severe intermittent/ sustained hypoxia and their effects on both neurite outgrowth and HIF1a/VEGFA profile in rats. It is important to include different measures of hypoxic exposure as this would give us a better understanding as it relates to giving the proper dose in clinical trials, and beyond. As we know, expression of a protein is not a consequence of one set of actions. As such, it would be interesting to further experiment with other transcription factors such as JUNB, VEGFB, ENO2, etc[6] to determine which factors are upregulated or downregulated, either separately or together, to promote axonal regeneration.

It is important here to address some variables that could have led to our results. For example, upon repeating this experiment, it would be beneficial to decrease the volume of cells being culture. Moreover, for this experiment, we cultures on average 5,000 cells per well plate and this could have led to "overcrowding" of the well plate. This is important as it relates to the manner in which some neurite outgrowths appeared. As seen in figure 8, it is evident that while there were neurite outgrowth projecting from the soma, some of these projections also appear to project back to the soma albeit at a different point. Moreover, we predict that while neurons assist surround cells to project processes outward, it is also very possible that these same neurons also inhibit the outgrowth of other neurons by releasing inhibitory chemicals. Indeed, previous studies working with rat hippocampal tissue revealed that it is neither chemicals nor fibroblasts that provide inhibition to growth. Rather, it appears that it is an innate feature of the scarred surface that, in the absence of oligodendrocytes, resulted in the failure of neurite outgrowth.[8,9] As such, this points to the notion that further research needs to be performed with a lower density cell culture with the addition of conditioned media that could allow for neurite outgrowth. Moreover, the conditioned media here would serve as "neurons" that would help facilitate each other's outgrowth. In addition, it would also bear fruitful to repeat this research paradigm using same density of cells and possibly using a larger well plate, again with conditioned media, to allow for more room for neurite outgrowth.

As we know, neurons send information down the axon to the axon terminal where it will synapse with a dendrite process of another neuron. Thus, it is important for the proper functioning of not only the central nervous system but with the body as a whole. Because of this, it is important to have a way to quantify dendritic arborization in relation to either injury or disease, as changes to dendrites can result in decreased neuronal communication and function.[10] Moreover, it is essential to understand the dendritic arbors and their complexities in order to determine whether we are heading in the right direction. If dendritic processes are critical for the CNS/PNS to properly function, it is important to quantify and study these arborizations as it may give us valuable insight into role of dendritic processes in cervical SCI. Indeed, one of the earlier studies observing dendritic regeneration by Ramon y Cajal in 1928 showed that there were no dendritic regrowth following injury to pyramidal neurons found in the cerebral cortex but contusion in the spinal cord resulted in dendritic regrowth.[12] Juxtaposing this study, Thompson-Peer et al (2016) looked at dendritic regrowth in *Drosophila* larvae and indeed confirmed that PNS neurons have a more robust mechanism in which to regenerate as opposed to CNS neurons. Despite the fact that dendritic regeneration in response to injury had a different morphology as compared to uninjured controls, it still validates the claim that PNS neurons have an elusive regenerative property not found in the CNS. As such, whether it is a cell intrinsic pathway or environmental enrichment that

allows for regeneration, regardless of CNS/PNS neurons warrants further investigation. Thus, in addition to manual traces, we used the Sholl analysis, which measures the neurite outgrowth as it crosses each concentric circle. When we look at *in-vivo* experiments that look to measure dendritic regeneration following injury, it is easy to determine that the outgrowth is indeed a dendritic process. Moreover, when we compare that to our experiment here, which observed *in-vitro* outgrowth, it is difficult to say for certain whether the outgrowth is dendritic or axonal in nature. As such, we can simply refer to it as neurite outgrowth. This analysis would be able to give us a better idea of neurite arborization and its complexity, or the measure at which neurons can interact with other neurons. As such, it provides useful information such as the number of branches each neurite outgrowths could have possibly had, something that cannot be traced manually using NeuronJ. Indeed, based on figure 6D, it is evident that SH leads to a reduced number of neurite outgrowth. If we look at neurite outgrowth following IH treatment and normoxia, there is more neurite outgrowth extending from the soma albeit it is not sustained. We can compare the neurite outgrowth trends for SH and IH and as described previously, while IH and SH do promote neurite, it appears that SH trends towards a more sustained neurite outgrowth pattern. Experiments from *in-vitro* DRG cultures (figure 9D) show the same trend for its Sholl analysis in the sense that SH lead to more neurite outgrowth but also for a sustained period of time. Further research needs to be performed to determine why SH lead to juxtaposing neurite outgrowth but an overall trend that seemingly leads to a a more sustained neurite outgrowth. Additionally, it would be worth performing ELISA of media collected following incubation of cell cultures for 7 days (see methods) to measure HIF1a and VEGFA levels for each treatment paradigm. Moreover, we decided to utilize this experiment as the pilot project in which to test a new MATLAB code that will help us further and, more readily and accurately, quantify neurite arborization in hope of better understanding manner in which regeneration appears, but importantly where the neurite outgrowth is spatially in relation to the soma.

Additionally, because the premise of our experiment looked at three separate independent variables as it would relate to one specific dependent variable, neurite outgrowth, we decided to utilize the ANOVA analysis to analyze statistical significance. Research discussed by Wilson et al (2017)[13] simple, linear model such as the ANOVA does not provide an accurate analysis of results during intra-class correlations, specifically resulting in an inaccurate p value. Moreover, this experiment can be considered an intra-class correlation in the sense that we analyzed sampled multiple neurons from a single animal per treatment paradigm. As such, Wilson et al (2017)[13] showed that a mixed effect model analysis would better account for intra-class correlations and allow for a more accurate standard deviation and thus more accurate p values. In future studies, it would be beneficial to utilize a more accurate system such as the mixed effect models of analysis

for the Sholl analysis and the measurement of total neurite outgrowth, in order to more accurately measure p value.

In a juxtaposing study by Caroline Devine (figure 9A-D), dorsal root ganglion (DRGs) were subject to moderate sustained hypoxia (10% oxygen level; ProOx C21 and incubator sub-chamber (Biospherix, Ltd, New York, USA)) *in-vitro* for five consecutive days, for 2 hours and 32 minutes, alongside a normoxia condition (20% oxygen level). It is important to note here that this particular study did not treat a cohort of DRGs with intermittent hypoxia due to limitations presented by the incubator. The results from that study reveal that the greatest neurite outgrowth resulted in response to sustained hypoxia (mean growth 89.52 inches) when compared to normoxia (mean growth 52.64 inches), contrasting the results from our experiments (figure 6A-E). Moreover, these results were of statistical significance which allows us to ascertain that the results are due to a specific cause and not by chance. Of significance is the Devinney et al (2016) experiment performed that revealed the opposite response to moderate sustained hypoxia. It was shown that moderate intermittent and moderate sustained hypoxia treatment paradigms does not result in phrenic motor facilitation (pMF) nor pLTF due to inhibitory cross-talk between the serotonergic/adenosine pathways.[14] Moreover, it was revealed by Devinney et al (2016) that pharmacologically inhibiting spinal adenosine A_2 receptors resulted in pMF after treatment with moderate sustained hypoxia. Additionally, it was also shown that severe acute sustained hypoxia triggered pMF in an adenosine A_2 dependent manner while simultaneously inhibiting serotonin receptors. As such, further study has to be performed in order to determine the reason why neurite outgrowth was the greatest following moderate sustained hypoxia in this experiment, whether it is due to another underlying mechanism or one that inhibited either adenosine A_2 receptors or serotonin receptors.[14] Furthermore, one of the possible errors described by Caroline Devine is that some of the well plates dried out. As such, this possible error needs to be exploited further as it could explain a greater neurite outgrowth due to less inhibitory factors that could arise from less cell volume in the well plates. In addition, it could bear fruitful to investigate the possible effects of neurite outgrowth with and without conditioned media. Moreover, if sustained hypoxia treatment promoted the greatest neurite outgrowth in less media and possibly less cell volume, it is worth investigating whether conditioned media would bolster the effects of neurite outgrowth and vice versa.

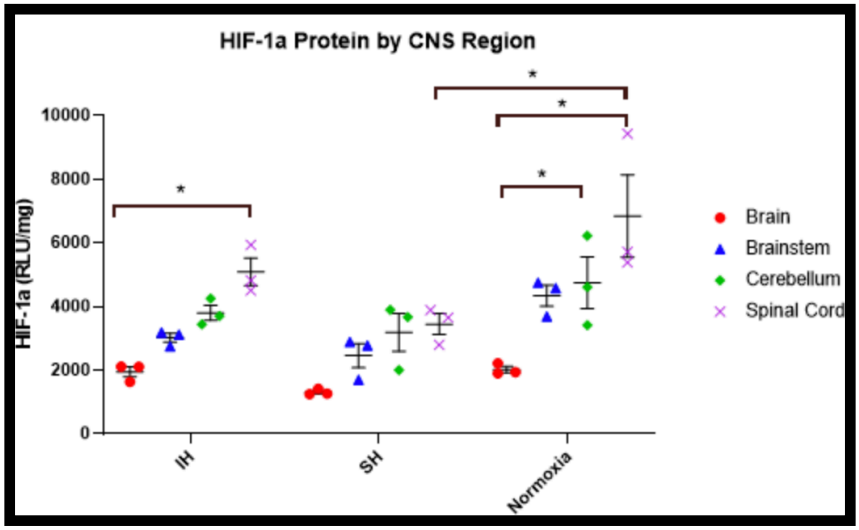


Figure 7**:** This graph, used with permission from ALS, shows the expression of HIF1a across the different treatment paradigms. As you can see, the highest expression of HIF1a occurs in normoxia but within the scope of intermittent hypoxia, HIF1a expression is highest in the spinal cord. This allows us to speculate the effects of HIF1a in respiratory plasticity.

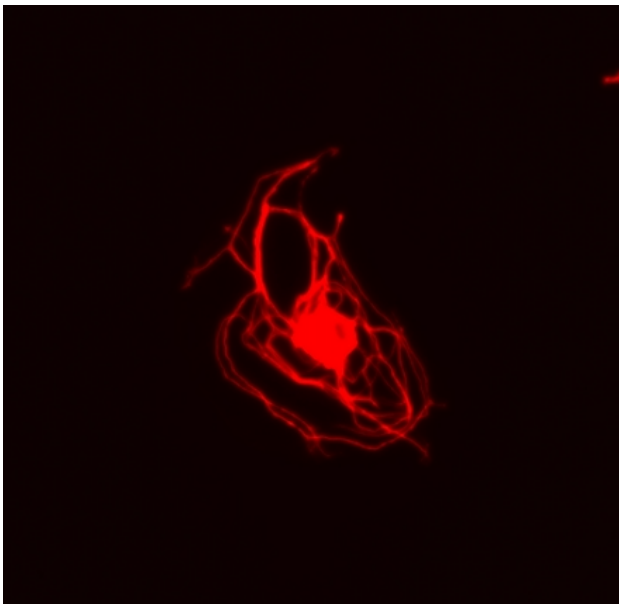
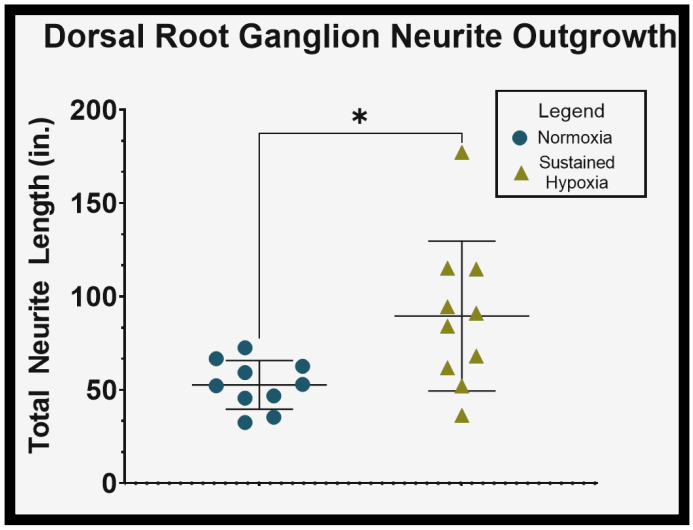
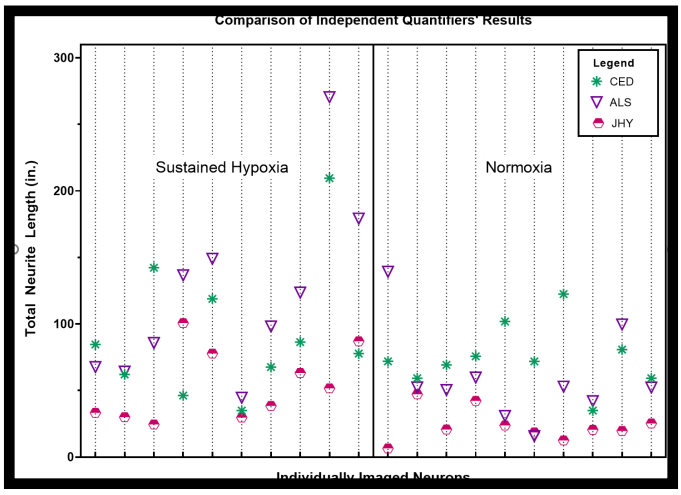


Figure 8: image of stained DRG showing neurite projections leaving soma but warping back to soma at a different point

A


Dorsal Root Ganglion Neurite Outgrowth
Total Neurite Length (in.)
200
150
100
50
0
*
Legend
Normoxia
Sustained Hypoxia

B


Comparison of Independent Quantifiers' Results
Total Neurite Length (in.)
300
200
100
0
Sustained Hypoxia
Normoxia
Legend
CED
ALS
JHY
Individually Imaged Neurons

C

```
Column B    Sustained Hypoxia
vs.   vs.
Column A    Normoxia

Unpaired t test
    P value 0.0127
    P value summary     *
    Significantly different (P < 0.05)?   Yes
    One- or two-tailed P value?     Two-tailed
    t, df   t=2.765, df=18

How big is the difference?
    Mean of column A    52.64
    Mean of column B    89.52
    Difference between means (B - A) ± SEM      36.89 ± 13.34
    95% confidence interval   8.864 to 64.91
    R squared (eta squared)   0.2982

F test to compare variances
    F, DFn, Dfd   9.506, 9, 9
    P value 0.0025
    P value summary     **
    Significantly different (P < 0.05)?   Yes

Data analyzed
    Sample size, column A     10
    Sample size, column B     10
```

D

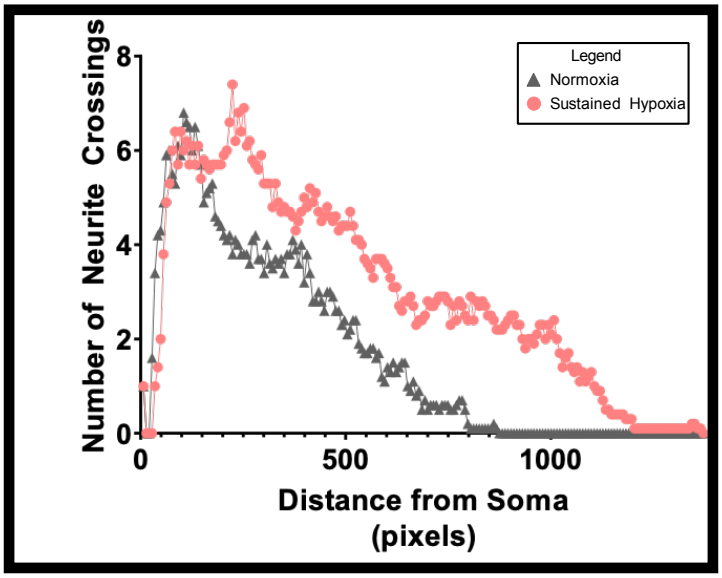


Figure 9: **(A)** This graph, used with permission by CED, depicts statistically significant results showing SH had a profound effect in neurite outgrowth as compared to normoxia. **(B)** This graph, used with permission by CED depicts the results following independent quantifications by all three quantifiers (ALS, JHY, CED) **(C)** This chart, used with permission by Caroline Devine, depicts the unpaired t-test showing statistically signifiant results pointing at the idea that neurite outgrowth following *in-vitro* sustained hypoxia treatment is attributable to a cause. (**D)** This Sholl analysis, used with permission by AS and CED, shows increasing neurite outgrowth as you move away from the soma indicating possible branching patterns in outgrowth. It is evident that SH treatment leads to more sustained outgrowth as compared to normoxia and thus more crossings.

CHAPTER 5: CONCLUSION

This experiment sought to investigate the effects of enriched treatment environments such as intermittent hypoxia, sustained hypoxia and normoxia on neurite outgrowth patterns. Importantly, this study can be considered a hybrid of both *in-vivo* and *in-vitro* experiments. In that sense, we were interested in investigating how well treatment paradigms would translate once cells in rats treated *in-vivo,* have been *ex-planted* and cultured in well plates. This experiment contributes to other experiments that seek to exploit moderate intermittent and sustained hypoxia treatment paradigms as a way to promote axonal regeneration in injury and disease processes. Impairment of breathing function following cervical spinal cord injury is devastating and can ultimately lead to death. As such, this experiment shows that intermittent hypoxia indeed facilities neurite outgrowth and it is our hope this will contribute to other experimental models that can contribute to benefit the quality of life of individuals with not only spinal cord injuries but other devastating neurological disorders.

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VITA

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