The Effects of Oxygen Deprivation on the Movement of Peripheral Neuron Growth Cones Harvested from Chicken *Gallus gallus* Embryos

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Introduction:

Neuron remodeling is a necessity for function of cognitive capacities in higher order organisms (Kandel et al., 2013). The neuron is a unit of the nervous system that acts as the messenger to carry signals to elicit a response by the body (Machens et al., 2003). As an individual unit, the neuron must be able to support its own growth meaning that it must produce its own supply of energy in the form of adenosine triphosphate (ATP) (Nicholls & Budd, 2000). The neuron's dependency on energy means that substrates required for energy production in a neuron are directly connected to health of the neuron (Mattson et al.,2008).

In this study, peripheral neurons harvested from chicken *Gallus gallus* embryos, were examined for growth rate while in an oxygen depleted environment. These model organisms were used because the embryos are relatively easy to maintain and allow for easy extraction of peripheral neurons from their spinal region. For the purposes of this experiment, neuron growth was defined as only the forward advancement of growth cones across the view field. Growth cone motility was observed through transmitted light time lapse microscopy. Creation of ATP in oxygen rich environments allows mitochondria to use an efficient production of energy called aerobic respiration. However, in oxygen depleted environments mitochondria are forced to use less efficient anaerobic respiration to create energy and as a result produce less ATP (Tiwari, 2002). For this reason we hypothesize that growth cones in oxygen depleted environments will exhibit less motility then controls.

Growth cone motility is important for the formation of a myriad of connections between neurons. The growth cone, a sensory-motile tip on the leading edge of axons guides the growth of axons so that specific neurons connect, allowing for signals to be transferred with accuracy (Vitriol et al., 2011). Oxygen deprivation and the resulting loss of energy prevent neurons ability to grow or function, with negative results as seen by hypoxia victims. (Csaba, 1996; Erecińska, 2001) Study of neuron growth in oxygen deprived environments brings us closer to understanding the durability of neurons in hypoxic environments potentially leading to a better healing mechanism to correct hypoxia triggered neuron damage in the future.

Materials & Methods:

Removal of dissolved Oxygen from Growth Medium:

To test the effects of oxygen deprivation on neurons in vitro, dissolved oxygen must be removed from growth medium around the neurons in culture. To accomplish this a BBL 2 Liter vacuum chamber was used to house three petri dishes of growth medium. N_2 Gas was pumped into the vacuum chamber using 1/4 inch tubing at 25 Psi through a hole located in the vacuum chamber lid. To create a range of dissolved oxygen levels the petri dishes filled to a height of 3mm with growth medium were left in the N_2 rich environment for 10, 20 and 30 minutes respectively. One petri dish holding 3mm of growth medium was removed at a time, after having been in the N_2 environment for the desired length. The growth medium from that petri dish was poured into one well of a 6 well plate. This increased the depth of the growth medium so the dissolved oxygen meter would be covered to its required depth. The concentration of dissolved oxygen was then recorded with a ProDo dissolved oxygen meter. The steps to remove the growth medium from the N2 Environment and measure the dissolved oxygen level were repeated once for each time point. The control sample was unperturbed, regular growth medium was measured for its oxygen content with the Prodo meter without use of a vacuum chamber of N_2 gas Using the dissolved oxygen data from the experimental and control sets, approximate predictions of the oxygen levels in the growth medium could be predicted.

Experimental Procedure:

Following the Procedure for primary cell culture (Morris, 2015a) peripheral neuron dorsal root ganglia where harvested from 10 day Gallus gallus embryos. The Ganglia were allowed to grow and adhere to treated coverslips for two days in a Symphony 5.3A VWR incubator (37°C) using the technique explained in (Morris, 2015a). After incubation plates were moved from the incubator and examined with TS 100 Nikon inverted scope to check for the presence of ganglia. The (Morris, 2015b) procedure was used with the exception that no imaging chamber was created or utilized. The plates were then placed into the vacuum chamber (pre heated to 37°C with a space heater) for 0, 10, 20 and 30 min respectively. After sitting in the nitrogen environment for the desired amount of time a dish was then removed from the vacuum chamber and wrapped tightly in Parafilm on the sides to prevent oxygen from returning back into solution. A razor blade was used to remove excess Parafilm from the top and bottom of the petri dish, to create a clear imaging window. The dish was gently moved to the TS 100 Nikon Ellipse inverted microscope (Spot Idea Camera model # 27.2-3.1 MP Color, Diagnostic Instruments 0.5x C clamp) to be imaged again using the (Morris,2015b) procedure without using a coverslip. The stage of the inverted microscope was preheated to 37°C using a Homes space heater on top of a Styrofoam container and a thermometer taped to the stage to monitor temperature. Using the time lapse function on the Spot imaging program (version 5.2), transmitted light images were taken every ten seconds for 15 minutes at 20x pseudo phase 2. The series of images was saved to a flash drive and then opened in the Image J(ver. 1.50a) program in the ICUC on the Taurus computer for analysis.

Quantification of data:

The segmented line tool was used to measure the distance that the distal tip of a growth cone moved between images in the time lapse set. The growth cone was identified as a dark triangular bead located at the very distal tip of an axon. Measurements were repeated for three growth cones in each time lapse set except the 8.10 mg/L where 2 were used. Using the "Analyze" drop down menu in Image J and the Measure display, the distance each growth cone moved was recorded in pixels. To convert the measurement in pixels to mm a micrometer was used at the same exact settings that the experimental time lapses were used. It was found that 330

pix on the computer screen was equivalent to 0.1mm on the sample. Using that conversion factor the distances that the growth cones traveled in pixels was converted to mm. These data were then plotted in excel using a line graph for better visualization of results.

Results:

Using time lapse transmitted light images I monitored the advance of growth cones from peripheral neurons. Figure 1 is the control where no perturbation of the sample occurred resulting in 9.40mg/L dissolved oxygen. Figures 2-3 highlight a representative growth cone of perturbed samples with reduced oxygen concentration of 8.10 mg O_2/L, and 5.90mg O_2/L. All these data were produced and analyzed in collaboration with Emma Hart (Hart, 2015).

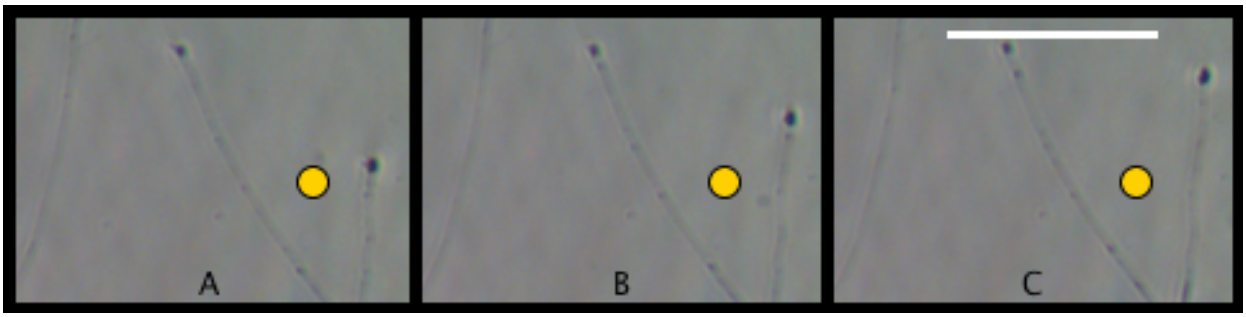

Figure 1) Shows the control environment with 9.40 mg/L dissolved oxygen. Time-lapse transmitted light images of a peripheral nerve axon growth cone taken with at pseudo phase 2, 20x magnification. The orange marker is in the same position on the sample to use as a reference as the growth cone moves over this 15 minute time lapse. Images A, B and C were taken at 0, 7.5 & 15 minutes respectively. The scale bar is 25 micrometers. Notice there is movement of the growth cone from image A to Image C.

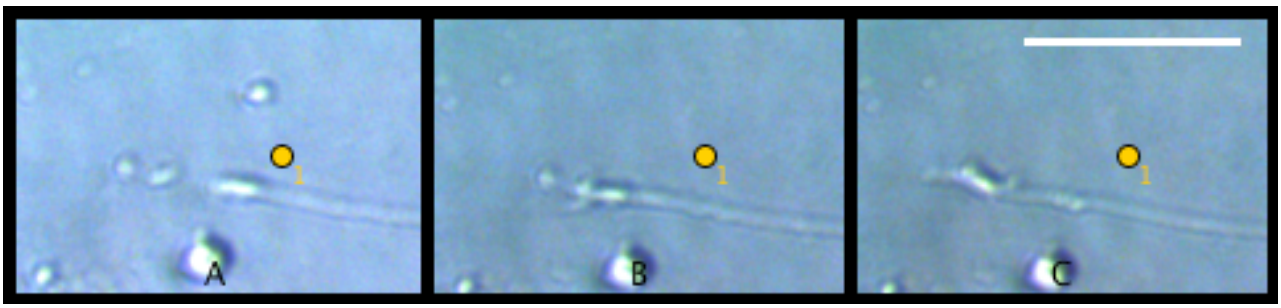

Figure 2) Shows the experimental condition with 8.10 mg/L dissolved oxygen. Time-lapse transmitted light images of a peripheral nerve axon growth cone taken with pseudo phase 2, 20x magnification. The orange marker is in the same position on the sample to use as a reference for the movement of the growth cone over this 15 minute time lapse. Images A, B and C were taken at 0, 7.5 & 15 minutes respectively. The scale bar is 25 micrometers. Notice the growth cone movement when transitioning from image A through to Image C.

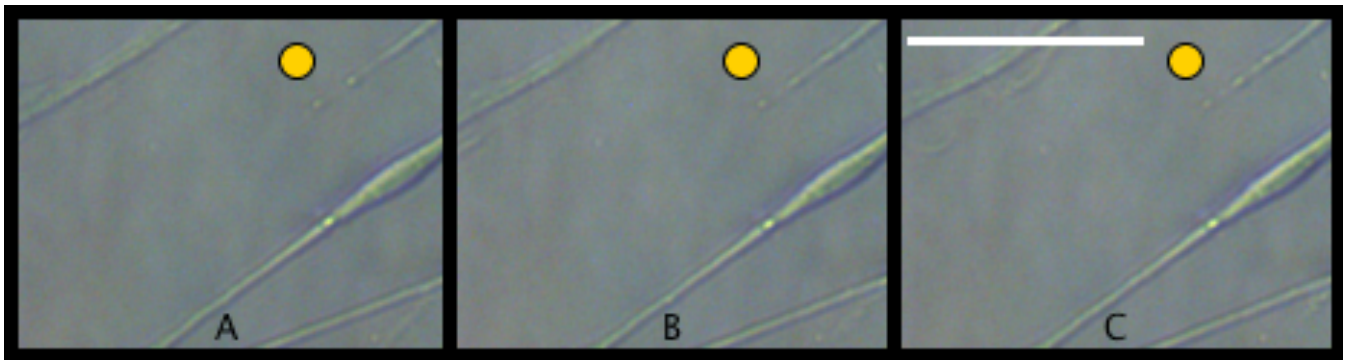

Figure 3) Shows the experimental condition with 5.90 mg/L dissolved oxygen. Time-lapse transmitted light images of a peripheral nerve axon growth cone taken with pseudo phase 2, 20x magnification. The orange marker is in the same position on the sample to use as a reference for the movement of the growth cone over this 15 minute time lapse. Images A, B and C were taken at 0, 7.5 & 15 minutes respectively. The scale bar is 25 micrometers. Notice the lack of movement when transitioning from image A to Image C.

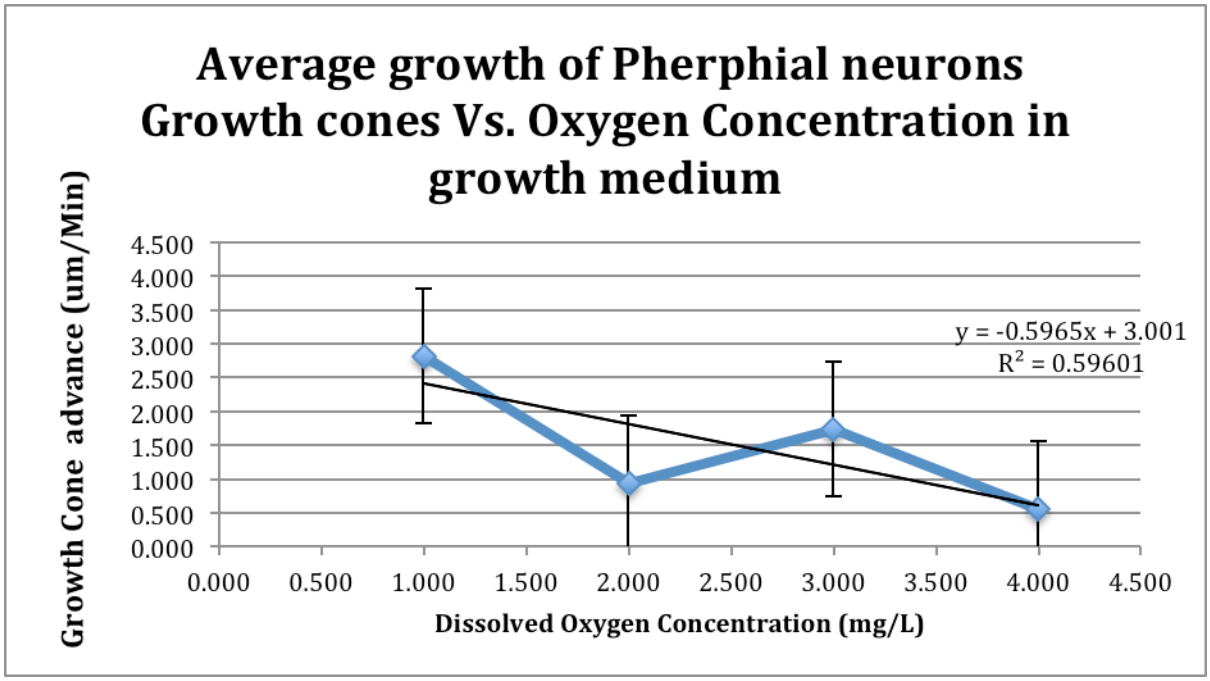

Figure 4) Shows the trend of peripheral growth cone advancement to dissolved oxygen concentration in the growth medium. These data shown are averaged velocities of individual growth cone movements (n= 3 for 9.40 mg O_2/L growth medium, 6.30 mg O_2/L growth medium, 5.90 mg O_2/L growth medium, n=2 for 8.10 mg O_2/L growth medium). The error bars are set for 5% deviation. Notice the general downward trend in velocity as dissolved oxygen concentration decreases.

Discussion:

The hypothesis that neuronal growth cones decrease growth rate as the concentration of dissolved oxygen decreases is supported by these data. These results are similar to previous research done on this subject (Dubinsky, 1995; Erecińska,2001). These data also support previous research that dysfunction of mitochondria has negative effects on the neuron health (Merad-Boudia,1998). All oxygen-depleted samples had a reduced growth cone movement when compared with controls. The sample with 6.30 mg/L dissolved oxygen had a greater average velocity then the sample with 8.1 mg/L leading one to believe that there is a uneven descending trend. From a cellular standpoint, this may imply that after a certain threshold of oxygen deprivation, anaerobic respiration is more prevalent then aerobic and can sustain the energy requirements for translational growth cone advance but with less vigor. This would support research that neurons have some ability to resist hypoxia (Jo, 2012; Lars, 2008; Caltana, 2014). After the oxygen threshold is passed, the neuron can no longer sustain its own energy requirements and growth cone advance drops once again eventually leading to apoptosis (Erecińska, 2001). If this experiment were repeated with similar results, it would imply that neurons have a buffer mechanism for dealing with hypoxia.

It would be beneficial if this experiment were to be refined and repeated. The drawbacks of this experiment are the low n values for the measured values and the narrow range of oxygen levels tested. There may have also been some experimental error arising from the methods used to measure dissolved oxygen in the growth medium. The experimental samples were heated while in the N2 environment while the growth medium used to find the oxygen levels was not. On a cellular level this probably means that there was actually less oxygen in the experimental samples than measured. In future experiments it would be useful to observe a wide range of dissolved oxygen ranges to reinforce the trend and make it statistically significant. It would be exciting to compare growth cone movement in neurons next to glia and compare them to neurons without glia to see the role glia have to play in the hypoxia response. The effect of oxygen deprivation on glia does not follow the same trend as the neurons (Ganat,2002; Hart, 2015). Glia seem

to be less effected by oxygen depletion in their environment and are potentially the reason neurons have any capacity to buffer against hypoxia at all.

References:

Caltana, L,. Rutolo,D,. Luisa Nieto, M,. Brusco, A.(2014). Further evidence for the neuroprotective role of oleanolic acid in a model of focal brain hypoxia in rats, Neurochemistry International, Volume 79, December 2014, Pages 79-87, ISSN 0197-0186,

Csaba Nyakas, Bauke Buwald, Paul G.M. Luiten, Hypoxia and brain development, Progress in Neurobiology,(1996) Volume 49, Issue 1, May 1996, Pages 1-17, 19-23, 25-27, 29-31, 33-39, 41-51, ISSN 0301-0082, http://dx.doi.org/10.1016/0301-0082(96)00007-X

Chen, L.(1988) Mitochondrial membrane potential in living cells. Annu Rev Cell Biol. 155–181

Dubinsky, JM,. Kristal, BS,.and Elizondo-Fournier, M.(1995).An obligate role for oxygen in the early stages of glutamate-induced, delayed neuronal death The Journal of Neuroscience, 1 November 1995, 15(11):7071-7078

Erecińska, M,. Silver, I,.(2001) Tissue oxygen tension and brain sensitivity to hypoxia, Respiration Physiology, Volume 128, Issue 3, 15 November 2001, Pages 263-276, ISSN 0034-5687, http://dx.doi.org/10.1016/S0034-5687(01)00306-1.

Eric A. Vitriol, James Q. Zheng, Growth Cone Travel in Space and Time: the Cellular Ensemble of Cytoskeleton, Adhesion, and Membrane.(2012) Neuron, Volume 73, Issue 6, 22 March 2012, Pages 1068-1081, ISSN 0896-6273

Ganat, Y,. Soni, S,. Chacon, M,. Schwartz, M., Vaccarino, F. (2002) Chronic hypoxia up-regulates fibroblast growth factor ligands in the perinatal brain and induces fibroblast growth factor-responsive radial glial cells in the sub-ependymal zone, Neuroscience, Volume 112, Issue 4, 19 July 2002, Pages 977-991, ISSN 0306-4522,

Hart, E.(2015). Collaborator for data collection

Kandel, E., Schwartz, J., Jessell, T., Siegelbaum, S., Hudspeth, A. (2013) Principles of NeuralSscience, Fifth Edition

Jo, S,. Kalló, I,. Bardoczi, Z,. Drigo, R,. Zeold, A,. Liposits, Z,. Oliva, A,. Lemmon, V,. Bixby, J,. Gereben,B,. Bianco, A. (2012) Neuronal Hypoxia Induces Hsp40-Mediated Nuclear Import of Type 3 Deiodinase As an Adaptive Mechanism to Reduce Cellular Metabolism *The Journal of Neuroscience, 20 June 2012, 32(25):8491-8500; doi:10.1523/JNEUROSCI.6514-11.2012*

Lars P. Folkow, Jan-Marino Ramirez, Stian Ludvigsen, Nicolo Ramirez, Arnoldus S. Blix,(2008) Remarkable neuronal hypoxia tolerance in the deep-diving adult hooded seal (Cystophora cristata), Neuroscience Letters, Volume 446, Issues 2–3, 3, Pages 147-150, ISSN 0304-3940, http://dx.doi.org/10.1016/j.neulet.2008.09.040.

Ly, J. Grubb, D., Lawen, A. (2003) The mitochondrial membrane potential (Δψm) in apoptosis; an update. Apoptosis March 2003, Volume 8, Issue 2, pp 115-128

Machens, C., Schütze1, H., Franz, A., Kolesnikova, O., Stemmler, M. Ronacher, B.,& Herz, A. (2003) Single auditory neurons rapidly discriminate conspecific communication signals. Nature Neuroscience 6, 341 – 342

Mattson, M., Gleichmann, M., Cheng, A. (2008) Mitochondria in Neuroplasticity and Neurological Disorder Neuron.Volume 60, Issue 5, 10 December 2008, Pages 748–766

Merad-Boudia, M,. Nicole, A,. Santiard-Baron, D,. Saillé, C,. Ceballos-Picot, I,. (1998).Mitochondrial impairment as an early event in the process of apoptosis induced by glutathione depletion in neuronal cells: relevance to Parkinson's disease, Biochemical Pharmacology, Volume 56, Issue 5, 1 September 1998, Pages 645-655, ISSN 0006-2952, http://dx.doi.org/10.1016/S0006-2952(97)00647-3.

Morris, R.L. (2015a) Neurobiology Bio324 primary culture of chick embryonic peripheral neurons 1: dissection. Accessed 11/2/2015*. Available at: http://icuc.wheatoncollege.edu/bio324/2015/NBlabPrimaryTissCultureProc1Dissn2015.

Morris, R.L. (2015b) Neurobiology Bio324 primary culture of chick embryonic peripheral neurons 2: observation of live unlabled cells. Accessed 11/2/2015*. Available at: http://icuc.wheatoncollege.edu/bio324/2015/ NBlabPrimaryTissCultureProc2ObservnOfLliveUnlabeledCells2015.htm.

Nicholls, D., Budd, S. (2000) Mitochondria and Neuronal Survival. Physiological Reviews Published Vol. 80 no. 1, 315-360

Tiwari, B., Belenghi, B., Levine, A.(2002) Oxidative Stress Increased Respiration and Generation of Reactive Oxygen Species, Resulting in ATP Depletion, Opening of Mitochondrial Permeability Transition, and Programmed Cell Death. Plant Physiology April 2002 vol. 128 no. 4 1271-1281

I have abided by the Wheaton college honor code in this work.

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