The Effects of Oxygen Deprivation on Embryonic *Gallus gallus* Glial Cell Filopodia Elongation

Emma R. Hart
Neurobiology Short Report
BIO 324/ Neurobiology
Wheaton College, Norton, Massachusetts, USA
November 18th, 2015

Introduction

Glia roots from the Greek word meaning "glue". It was presumed in the nineteenth century that these cells held the nervous system together (Purves et al., 2001). Glial cells serve an important role in the cerebral cortex of all animals in providing this function. Unlike neuronal cells, glial cells do not directly create synaptic interactions and action potentials from electrical signaling (Purves et al., 2001). Instead, glial cells collectively create a support system for neurons and aid in maintaining these signaling pathways (Purves et al., 2001). For every neuron, there are approximately three glial cells in the brain (Purves et al., 2001). Their cellular extensions have proven to be complex, however, they are much smaller than neurons and contain no axons or dendrites (Purves et al., 2001). These extensions are called filopodia. The functions of these cells as a whole include maintenance of ionic milieu of nerve cells, controlling the rate of nerve signal propagation, controlling the uptake of neurotransmitters to regulate synaptic action and providing a scaffolding for the cells within the nervous system (Purves et al., 2001). One of the more important roles, however, is recovery from neural injury (Purves et al., 2001).

Erythropoietin (EPO) is an essential hormone in regulating blood cell production in mammals. The synthesis of EPO is responsive to changes in physiological oxygenation

(Weidemann et al., 2009). In the central nervous system, both neurons and astrocytes (a subset of glial cells) possess the ability to synthesize EPO. Astrocytes make up a majority of the glial cells in the CNS and are a determining factor in recovery after ischemic damage to neurons (Weidemann et al., 2009). In injured and uninjured nerves, Schwann cells possess the EPO receptor. EPO receptor-cell signaling and Schwann cell proliferation are thought to protect injured peripheral nerves as well as aid in regeneration (Li, Gonias, & Campana, 2005). Previous studies have also shown that glial cells produce lactate, an essential aerobic energy substrate post hypoxia (prolonged oxygen deprivation) (Schurr et al., 1997). The lactate is produced anaerobically and is transferred into neuronal cells via a transporter after oxygen deprivation (Schurr et al., 1997). This lactate molecule is a astrocytic signaling molecule in the locus coeruleus in the brain (Tang et al., 2014). This is the principle source of norepinephrine to the frontal brain, the main modulatory center of the brain (Tang et al., 2014). Inhibition of the transporter for lactate reveals that glial cells are the main source of lactate production and the bulk of what is utilized by neurons originates in glial cells (Schurr et al., 1997). The lactate produces by glial cells pools and creates a resource for neurons when high energy conditions are present (Bernaudin et al., 2000).

The actin within the cells are still active during a stroke, but the action of this component is greatly reduced. Actin is the major component in glial cell movement and during hypoxic conditions, it performs at a lesser degree than if it was in normal conditions (Brown & Davis, 2005). Based on these studies, glial cell filopodia should still have mobility post hypoxia due to being anaerobically active and actin retaining its functioning, but movement may be very limited. The aim of this study was to observe movement in *Gallus gallus* glial cell filopodia after these cells were treated in a vacuum chamber with a reduced concentration of oxygen (caused by Nitrogen gas circulation through the growth medium). It was hypothesized that glial cells will have

less movement in their filopodia, therefore, a slower rate of filopodia elongation as the concentration of oxygen decreases in the growth medium surrounding the cells.

Materials and Methods

Dissection:

Dr. Robert L. Morris's protocol was utilized in order to isolate ganglia as well as sympathetic nerve chains (Morris, 2015a), however, the ganglia proved to be more useful than the sympathetic nerve chains. Coverslips were treated with 10 μg/1ml of poly-lysine instead of 1 μg/1ml for 1.5 hours. Coverslips were also treated with laminin for 1 hour instead of 20-30 minutes. The increased concentration of poly-lysine and longer treatment of laminin allowed for better growth and adhesion of cells. The growth medium used in the aforementioned protocol was utilized for this experiment.

Microscopy:

A TS 100 Nikon eclipse inverted scope was used for this experiment. It contained C-W 10xA/22 Nikon oculars, C-HSG Nikon slide glass adapter for a 54mm petri dish, a Diagnostic Instruments 0.50x C-Mount and a halogen 30 watt bulb. The camera used for this experiment was an Idea Camera model #27.2-3.1MP color. The cells were observed using Robert L. Morris's protocol for observation of unlabeled cells, but instead a chip chamber was not made and an inverted scope was used (Morris, 2015b) in pseudo phase 2 on 20x magnification. Spot program on an iMac computer with Mac OS X Version 10.5.8 programming was used to collect images on time lapse for 15 minutes, taking images every 10 seconds.

Nitrogen Application/Oxygen Deprivation

For this experiment, a pressurized nitrogen gas tank was used with tubing coming out for the application of nitrogen to the samples. The nitrogen tank was nearly empty and the pressure coming out of it was approximately 45 psi. A vacuum chamber with the metal insert used for lifting samples out of the chamber, was set up on a surface with the tubing from the tank flowing into the chamber. A space was left between the lid of the chamber and the chamber itself in order to achieve a circulation of nitrogen through the sample. To determine the levels of oxygen in growth medium, a measurement of the height of the growth medium in the small petri dishes was taken. Once this was determined, the same height of growth medium was poured into 4 petri dishes. Three of the petri dishes were then placed in the vacuum chamber stacked on top of one another for 10, 20 and 30 minutes (taking the top out at 10 minutes etc.) exposures. The fourth petri dish was the control.

The levels of dissolved oxygen were measured using a ProDo meter. Calibrations were done per manufacturer's instructions in order to ensure proper readings. The control petri dish was poured into a six-well plate to create a height of liquid for the ProDo meter to measure the oxygen levels. When the reading was complete, it was recorded and the liquid discarded. This was repeated for the petri dishes treated for 10, 20 and 30 minutes. The oxygen concentrations of these petri dishes were 8.10 mg/L, 6.30 mg/L, and 5.90 mg/L, respectively. The control concentration was 9.40 mg/L.

The coverslip dishes were too small to fit into the chamber, so a piece of Styrofoam was taped onto the metal insert to provide a shelf for the petri dish to sit during Nitrogen exposure. The first coverslip was treated with Nitrogen for 10 minutes while the heater was placed facing the vacuum chamber. A thermometer was used to ensure the cells were at optimal temperature (37^{o}C) and would still be moving while under anaerobic conditions. When the time was up, the petri dish

was removed from the chamber and sealed with parafilm. The parafilm encouraged the growth medium would stay under the deoxygenated conditions during observation. The cells were taken to an inverted scope for observations. The cells were observed in pseudo phase 2 under 20x magnification. The cell was found after locating the coverslip on 10x magnification and then moving the slide until the cells appeared. Once the cells were in focus, the transition to 20x magnification was made and the fine adjustment knob created a sharper image. The image was switched into the Spot program and the "restart" button was pressed in order to find the image on the screen. The fine adjustment knob was used again to refine the image and make it clearer for imaging. A time lapse sequence of images was taken for 15 minutes, with pictures taken sequentially every 10 seconds. These steps were repeated for cells treated for 20 minutes and 30 minutes, as well as the control.

Data Analysis:

Images were opened in ImageJ to make measurements of filopodia movement over time (not cell body movement). The cells were measured using the segmented line tool in ImageJ to determine growth in pixels. The tool was placed at the start position and then moved to proceeding positions. When movement ceased, the measurement of pixels was calculated over the amount of time passed (each picture was taken every 10 seconds so the number of time passed equated to number of pictures times 10). This was repeated for several filopodia in each condition. Averages were taken for each concentration.

A micrometer with 0.1mm lines was used to calculate the scale for data collection. Three measurements of the length of the line was measured and averaged. It was found there were 330 pixels/0.1mm on 20x magnification.

Pixels per second was then converted to micrometers per minute using the equation:

$$\frac{0.1\text{mm}}{330\text{ pixels}} \times \frac{\#\text{pixels (average)}}{\#\text{seconds (average)}} \times \frac{60\text{ seconds}}{1\text{ minute}} \times \frac{1000\ \mu\text{m}}{1\text{ mm}} = \text{average } \mu\text{m/min}$$

These data were then plotted on a scatterplot.

Resulting figures were then created on ImageJ. The multipoint tool was used to label each filopodia being observed n=14 filopodia on 11 glial cells. Then pictures were taken at the very beginning, after 5 minutes, 10 minutes, and 15 minutes. These then were made into a sequential order to show movement over time.

Results

Images taken via the inverted scope were utilized for my data collection. It was found there was a difference in filopodia movement, but there was no decrease in movement with each application of nitrogen. The following figures display filopodia of glial cells moving over time.

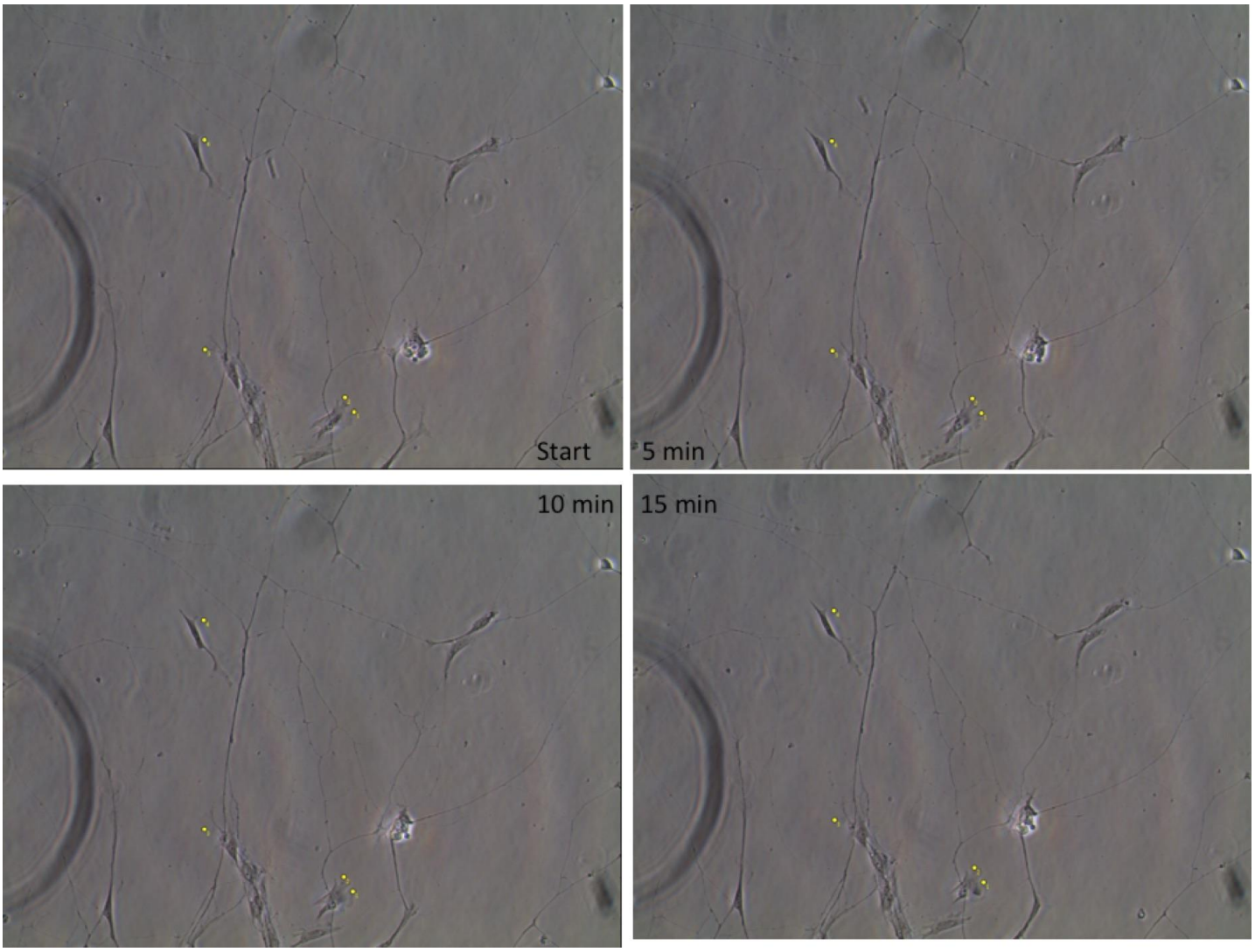

Figure 1. Movement of filopodia of glial cells in oxygen concentration of 9.40 mg/L (control). Yellow dots indicate the filopodia of interest. Notice the movements over time. Compare this image to the figures presented later in this paper. This image was captured with a TS 100 Nikon Eclipse inverted microscope using a Diagnostic Instruments 0.50x C-Mount, and processed using Spot Software. Image was gathered in collaboration of Walker Fuchs.

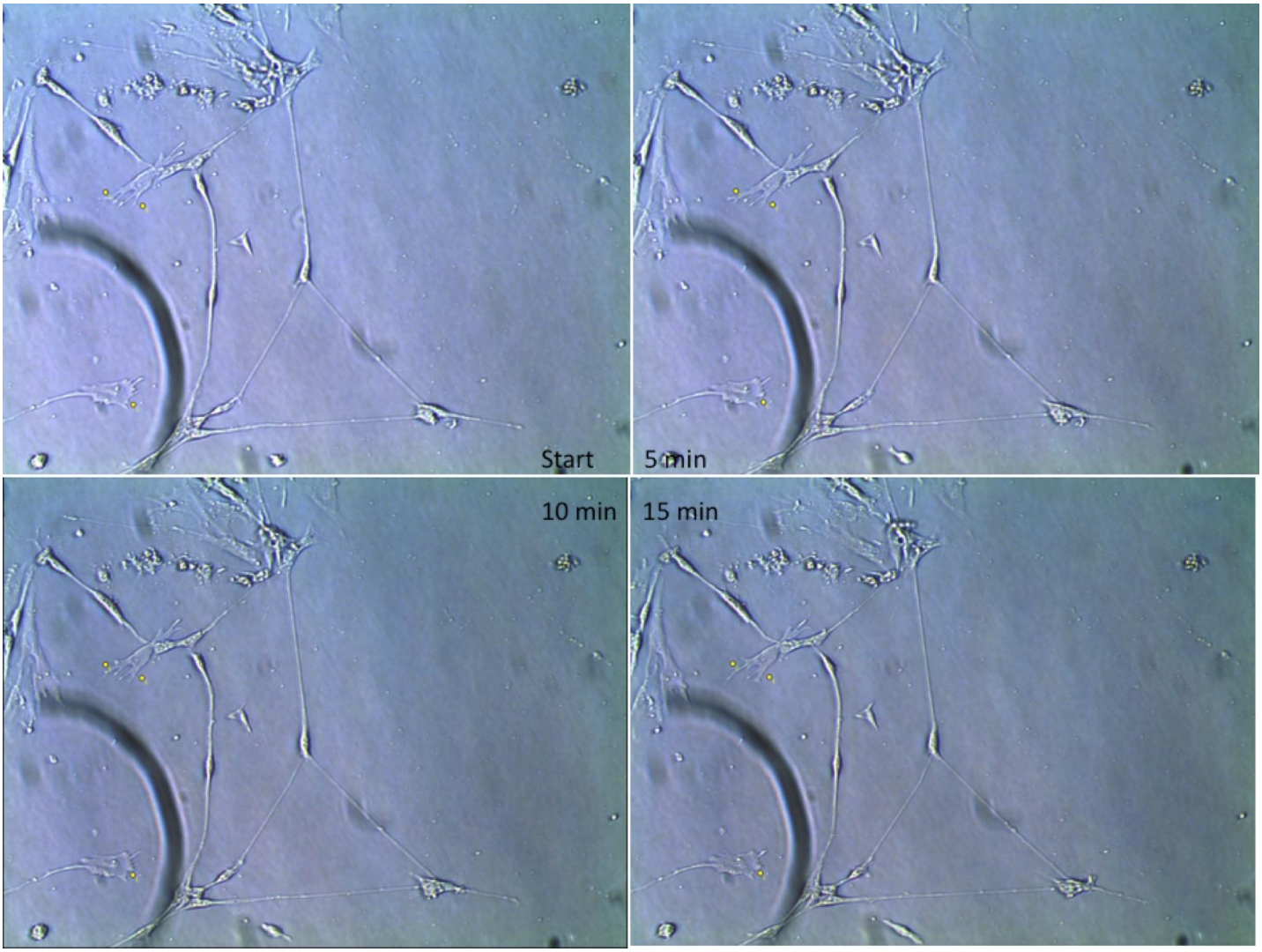

Figure 2. Movement of filopodia of glial cells in oxygen concentration of 8.10 mg/L (10 minute exposure to nitrogen). Yellow dots indicate the filopodia of interest. Notice a small movement of filopodia over time. This image was captured with a TS 100 Nikon Eclipse inverted microscope using a Diagnostic Instruments 0.50x C-Mount, and processed using Spot Software. Image was gathered in collaboration of Walker Fuchs.

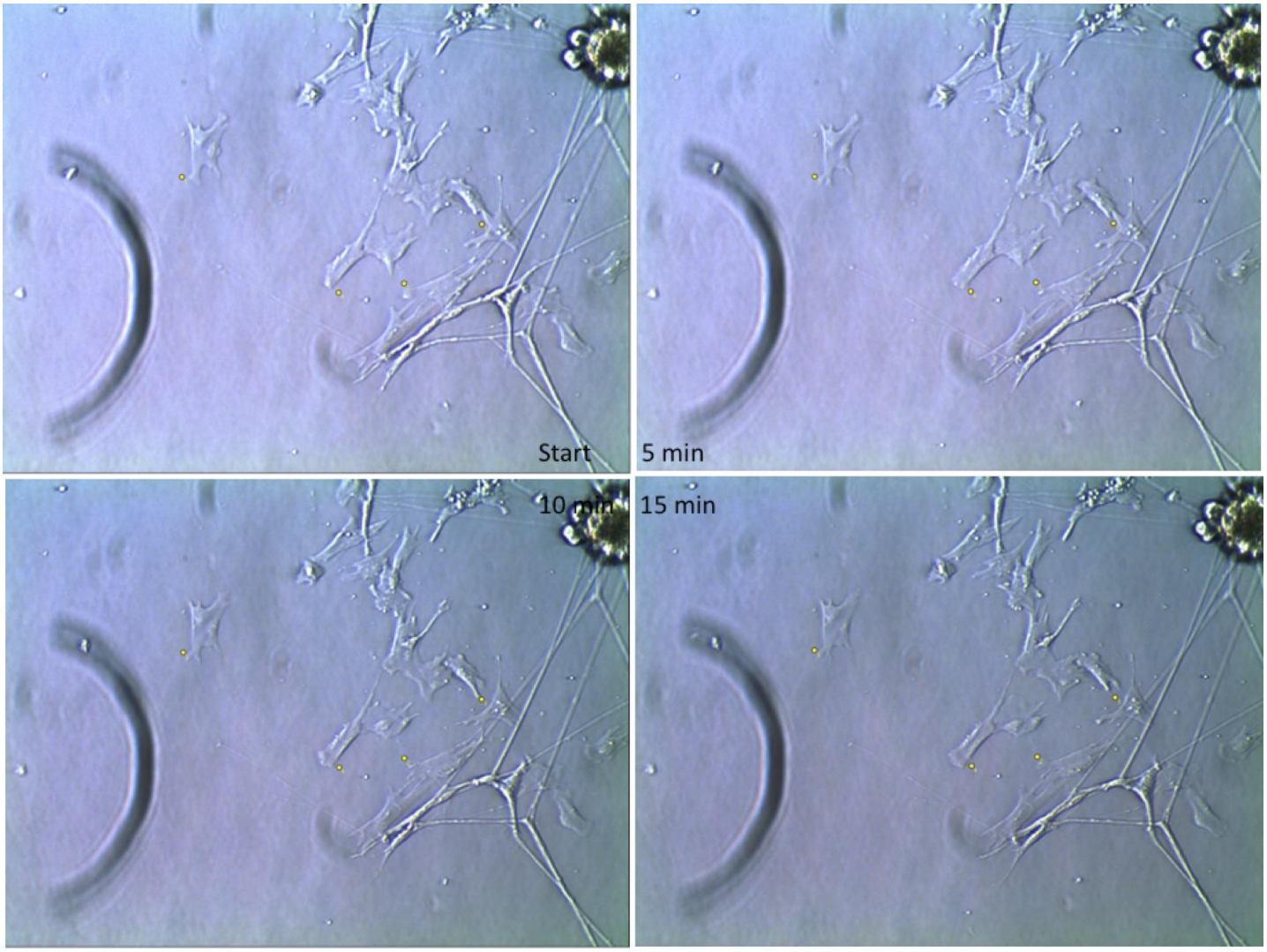

Figure 3. Movement of filopodia of glial cells in oxygen concentration of 6.30 mg/L (20 minute exposure to nitrogen). Yellow dots indicate the filopodia of interest. Notice the drastic movements of some of the filopodia over time as well as smaller movements of other filopodia present in this image. This image was captured with a TS 100 Nikon Eclipse inverted microscope using a Diagnostic Instruments 0.50x C-Mount, and processed using Spot Software. Image was gathered in collaboration of Walker Fuchs.

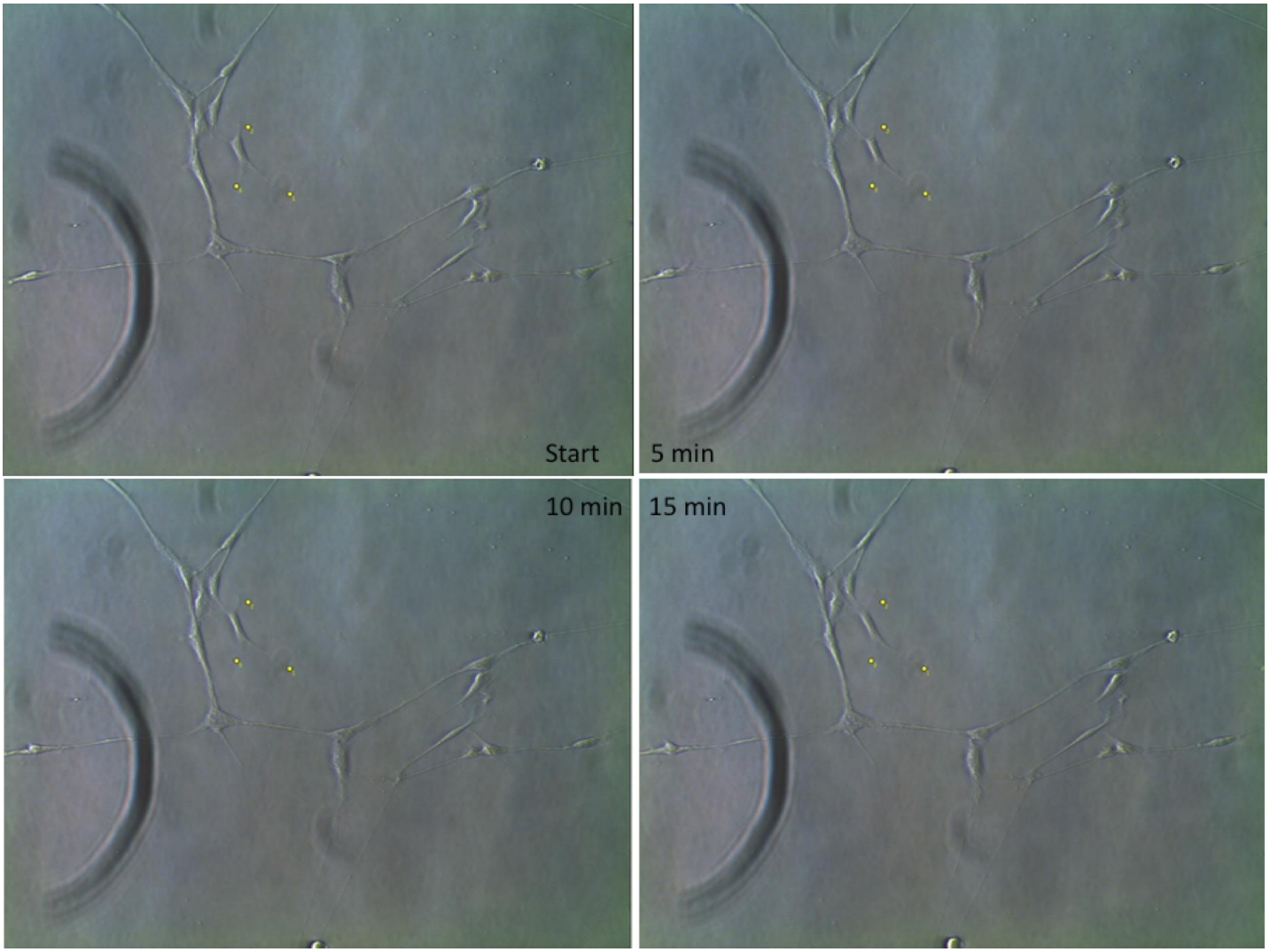

Figure 4. Movement of filopodia of glial cells in oxygen concentration of 5.90 mg/L (30 minute exposure to nitrogen). Yellow dots indicate the filopodia of interest. Notice the lack of movement in one of the filopodia through time. This image was captured with a TS 100 Nikon Eclipse inverted microscope using a Diagnostic Instruments 0.50x C-Mount, and processed using Spot Software. Image was gathered in collaboration of Walker Fuchs.

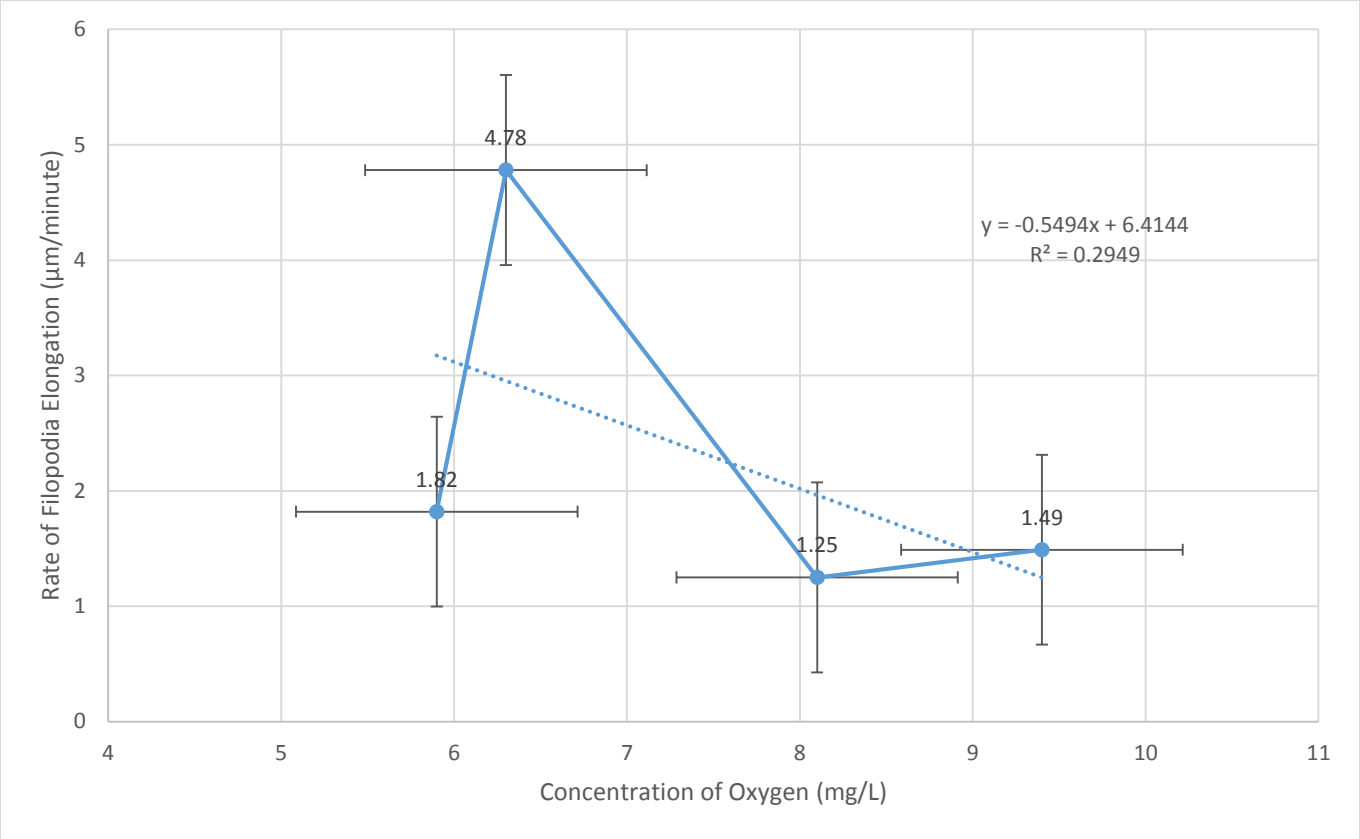

Figure 5. Rate of Elongation of Filopodia in reduced concentrations of oxygen in growth medium (n=14 on 11 glial cells). Concentrations were taken using the ProDo oxygen meter to measure concentration of oxygen in growth medium. Notice the control is the furthest point to the right and concentrations decrease from there.

Discussion

By analyzing the above images, we can see there is a difference in the movement of filopodia of glial cells brought on by decreased oxygen concentrations. In Figures 1 through 4 we see there is a difference in the beginning and ending shapes of the filopodia. Figure 1 displays the control group where movement was noticeable in the top left glial cell as well as significant movement in Figure 3. In Figure 4 and 2, we see there is very little movement of the filopodia. In

the trend line displayed in Figure 5, we can see there is an increase in movement with a decrease in oxygen concentration. There is low predictability (R^2=0.29), indicating the difficulty to predict the movement of filopodia.

Based on the data above, this evidence does not support the hypothesis that filopodia movement of glial cells will decrease as the concentration of oxygen in the growth medium surrounding them decreases. The data show that in higher concentration of oxygen, there is less movement of filopodia. If this experiment were repeated and you got the same results the each time, it would show that in higher concentrations of oxygen, glial cell filopodia have less movement than in lower concentrations. Studies have shown that glial cells do receive hormones (EPO) and also create lactate anaerobically for a nutrient source for neurons (Schurr et al., 1997; Weidemann et al., 2009). This could explain the behavior of the glial cells during a time of deoxygenation. They need to produce lactate as well as act as receptors for EPO, meaning they are still metabolically active. Glial cell filopodia also move via actin and although anaerobic conditions are present, actin can still produce movement (Brown & Davis, 2005). However, another explanation to describe what is happening in the data may root from sources of error.

In this experiment, there are many aspects that could have varied slightly to cause the results above. First, the medium measured for concentrations of oxygen at the start were not heated like the growth medium in the petri dishes during the trials. This could have created a different concentration of oxygen in the nitrogen-infused samples and could have changed the behavior of the filopodia. Another mistake could have appeared in the construction of the vacuum chamber. In the third trial, when the concentration was 5.90 mg/L, the Nitrogen gas ran out and the vacuum chamber was sealed to keep the petri dish in a deoxygenated environment. This could have then

yielded different results if the nitrogen was continuously flowing throughout the time the dish was present.

There are many experiments that could be done to further these results. Since it is known erythropoietin receptors and lactate appear during deoxygenation (Schurr et al., 1997; Weidemann et al., 2009), a study could be made to test the concentrations of these molecules during a time of hypoxia. It would be very interesting to see the glial cell response to deoxygenation on a more molecular level because testing the concentrations of lactate or the receptors for EPO could explain how the cells could protect the brain during hypoxia.

References

Close collaboration with Walker Fuchs.

Bernaudin, M., Bellail, A., Marti, H. H., Yvon, A., Vivien, D., Duchatelle, I., … Petit, E. (2000). Neurons and astrocytes express EPO mRNA: oxygen-sensing mechanisms that involve the redox-state of the brain. *Glia*, *30*(3), 271–8. http://doi.org/10.1002/(SICI)1098-1136(200005)30:3<271::AID-GLIA6>3.0.CO;2-H

Brown, R. C., & Davis, T. P. (2005). Hypoxia/aglycemia alters expression of occludin and actin in brain endothelial cells. *Biochemical and Biophysical Research Communications*, *327*(4), 1114–23. http://doi.org/10.1016/j.bbrc.2004.12.123

Li, X., Gonias, S. L., & Campana, W. M. (2005). Schwann cells express erythropoietin receptor and represent a major target for Epo in peripheral nerve injury. *Glia*, *51*(4), 254–65. http://doi.org/10.1002/glia.20202

Morris, R.L. (2015a) Neurobiology Bio324 primary culture of chick embryonic peripheral neurons 1: dissection. Accessed 11/02/2015. 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 11/02/2015. Available at: http://icuc.wheatoncollege.edu/bio324/2015/ NBlabPrimaryTissCultureProc2ObservnOfLliveUnlabeledCells2015.htm.

Purves, D., Augustine, G. J., Fitzpatrick, D., Katz, L. C., LaMantia, A.-S., McNamara, J. O., & Williams, S. M. (2001). Neuroglial Cells. Sinauer Associates. Retrieved from http://www.ncbi.nlm.nih.gov/books/NBK10869/

Schurr, A., Payne, R. S., Miller, J. J., & Rigor, B. M. (1997). Glia are the main source of lactate utilized by neurons for recovery of function posthypoxia. *Brain Research*, *774*(1-2), 221–224. http://doi.org/10.1016/S0006-8993(97)81708-8

Tang, F., Lane, S., Korsak, A., Paton, J. F. R., Gourine, A. V, Kasparov, S., & Teschemacher, A. G. (2014). Lactate-mediated glia-neuronal signalling in the mammalian brain. *Nature Communications*, *5*, 3284. http://doi.org/10.1038/ncomms4284

Weidemann, A., Kerdiles, Y. M., Knaup, K. X., Rafie, C. A., Boutin, A. T., Stockmann, C., … Johnson, R. S. (2009). The glial cell response is an essential component of hypoxia-induced erythropoiesis in mice. *The Journal of Clinical Investigation*, *119*(11), 3373–83. http://doi.org/10.1172/JCI39378