The Effects of Lithium on Growth Cone Activity in *Gallus gallus* Neurons

Jesse P. Foehl
Neurobiology Short Report
Bio 324/ Neurobiology
Wheaton College, Norton, Massachusetts, USA
November 18, 2015

Introduction

Growth cones are the enlarged tips of axons that are in the process of elongating. From growth cones protrude a matrix of microspikes and other membrane structures. In this experiment, dorsal root ganglia were dissociated from *Gallus gallus* chick embryos and grown in vitro in order to observe growth cones. For many years scientists have used chick embryos due to their rapid development and close similarity to human's molecular and cellular anatomy (Vergara & Canto-Soler, 2012). Growth cones can easily be observed using a light microscope and transmitted light source (Ziv & Smith, 1996). The highly filamentous structures that extend out from the growth cones consist of lamellipodia and filopodia. Filopodia and lamellipodia are responsible for the exploration of their environment and for the movement into those probed areas. Lamellipodia are located at the "leading edge" of an elongating axon. Leading edge refers to the area of the membrane facing the direction of motion (Mattila & Lappalainen, 2008). Lamellipodia are thin complexes of actin that press on the leading edge of the membrane, giving filopodia a structure from which to grow. Filopodia are thin strands of actin that bundle together and protrude the leading edge of the membrane forward. Once extended, polymerization of new membrane is initiated and the growth cone with its lamellipodia and filopodia continue onward (Mattila & Lappalainen, 2008).

Bipolar disorder, or manic-depressive disorder, exists in approximately 4.4 percent of American adults. Bipolar disorder causes patients to constantly fluctuate between hyper behaviors or moods and sad and depressive states (Machado-Vieira et al., 2009). It is not completely clear exactly how bipolar disorder affects the brain, however, many postmortem studies and brain images have shown that bipolar disorder patients have neuronal stress and degradation. When compared to healthy subjects, bipolar patients exhibit significant reduction of brain size in mood regulation areas. There is a significant reduction in neuronal and glial density and size in grey matter of the prefrontal cortex (Machado-Vieira et al 2009).

Lithium is one of the most common treatments for bipolar disorder. Lithium has been known to increase neuron function as well as overall health of the neuron (Machado-Vieira et al., 2009). Grey matter in healthy individuals is the area in which neuronal cell bodies are located. Patients with bipolar disorder have been observed to have reduced regions of grey matter that regulate emotions and other cognitive function. Lithium is used to treat bipolar disorder because it can increase the density of grey matter and neuron health (Sassi et al., 2002). Further, a study conducted by Hong et al (1997) demonstrated that lithium promoted the assembly of microtubules. More specifically, Colombo et al (1991) found that lithium induces the polymerization of actin, which is what makes up growth cones. The increased microtubule assembly, as illustrated by these two studies, lead to increased growth cone activity.

Since it is known that lithium is used to increase neuronal health in bipolar disorder patients and increase microtubule and actin assembly, this study will explore the hypothesis that when *Gallus gallus* neurons are exposed to lithium solution, growth cone activity will increase. In this study growth cone activity was measured before and after lithium exposure. The results

of this experiment are interesting due to the known relationship between lithium and bipolar disorder.

Methods and Materials

Cellular Preparation

The cells used in this experiments were taken from 10 day old chick embryos. All materials used and steps taken for preparation and dissection steps were completed following protocol from primary chick embryo dissection (Morris, 2015b). A flow chamber was made in order to observe the same single growth cones under control and lithium conditions. Flow chambers were made following protocol (Morris, 2015c).

Control and Lithium Solutions

A 10mM concentration of lithium chloride (LiCl) was used for the experimental trials in this study. A 1:100 dilution of a sterile 1 M LiCl stock solution was performed for the desired 10mM LiCl solution. The control solution was created via 1:100 dilution of sterile water into serum free medium.

Microscope and station

A portable heater and digital thermometer were used to keep the microscope stage at a consistent thirty-seven degrees Celsius. A Nikon Eclipse E200 microscope was used for observation. A Sony Digital Interface (DFW-X700) camera with a Nikon diagnostic instrument (1.0x) mount was used to capture images along with BtV 6.061 software. BtV 6.061 software

was downloaded on a Macintosh iMac desktop computer for imaging. Proper Koehler illumination was aligned following protocol (Morris, 2015a). 10x objective and phase 1 (Ph1) were used in order to locate possible subjects for images. Once found 40x objective and phase 2 (Ph2) were used while imaging subject growth cones.

Experiment trials

The timer app on an iPhone was used, at time zero (T_0) control medium was added to the flow chamber via inflow, outflow procedure (Morris, 2015b). Once control medium was added, one photo was captured every three minutes for twenty-four minutes. The focus on the microscope was adjusted constantly to maintain perfect resolution. The same inflow, outflow method was used to inundate the growth cone with the lithium concentrated medium immediately following the twenty-fourth minute (T_{24}) (Morris, 2015b). A second trial was performed. The control medium was added at T_0. This time, the lithium medium was added at T_{30} instead of T_{24}. For all trials, respective medium was added every ten minutes so that cells would not dry out.

Data Analysis

After all trials were complete, a photo of a micrometer slide which had 0.01mm as its smallest increment was taken. ImageJ software was used to analyze photo data. The ruler photo was opened, and using the line tool, a fifty micrometer section of the photo was selected. Then Analyze was clicked, the scale was set to the known distance entered (50 micrometers), and the global box was checked to apply the scale to all photos opened in ImageJ. All photos were taken using command+shift+3 function so that every photo would have the same dimensions. For each trial, control and lithium photos were opened. On control photo 1 (T_0) a set of X and Y

coordinates were chosen as a starting position. The coordinates were chosen at a point on the neurons approximately 20μm from the leading edge of the growth cone, near some sort of land mark. The landmarks, whether it be a fragment of another neuron, a turn the neuron took or a bubble in the solution made it easier to find the general location of the coordinate on a new photo. The same starting coordinate was used to analyze each photo to eliminate experimental error. The exact coordinate was found in each photo using the Image J coordinate system. The segmented line tool was used to draw a line from the (x, y) coordinate to the leading edge of the growth cone. The criterion for choosing the leading edge of a growth cone was to end the segmented line where lamellipodia were present. Thin filopodia extensions were excluded from measurement because the role of filopodia are partially for probing the environment so in a photo, where there is a filopodia extension the neuron might not actually follow that path. In ImageJ the measure function was selected and the length of the segmented line was recorded. The same procedure was repeated for each photo at T_{12}, T_{24}, T_{36} and T_{48} for trial 1 and T_{15}, T_{30}, T_{45} and T_{60} for trial 2. For each trial, more data could be collected when a field of view that contained multiple growth cones was located. Therefore, measurements were recorded for each growth cone, labeled A and B, in all photos for both trials.

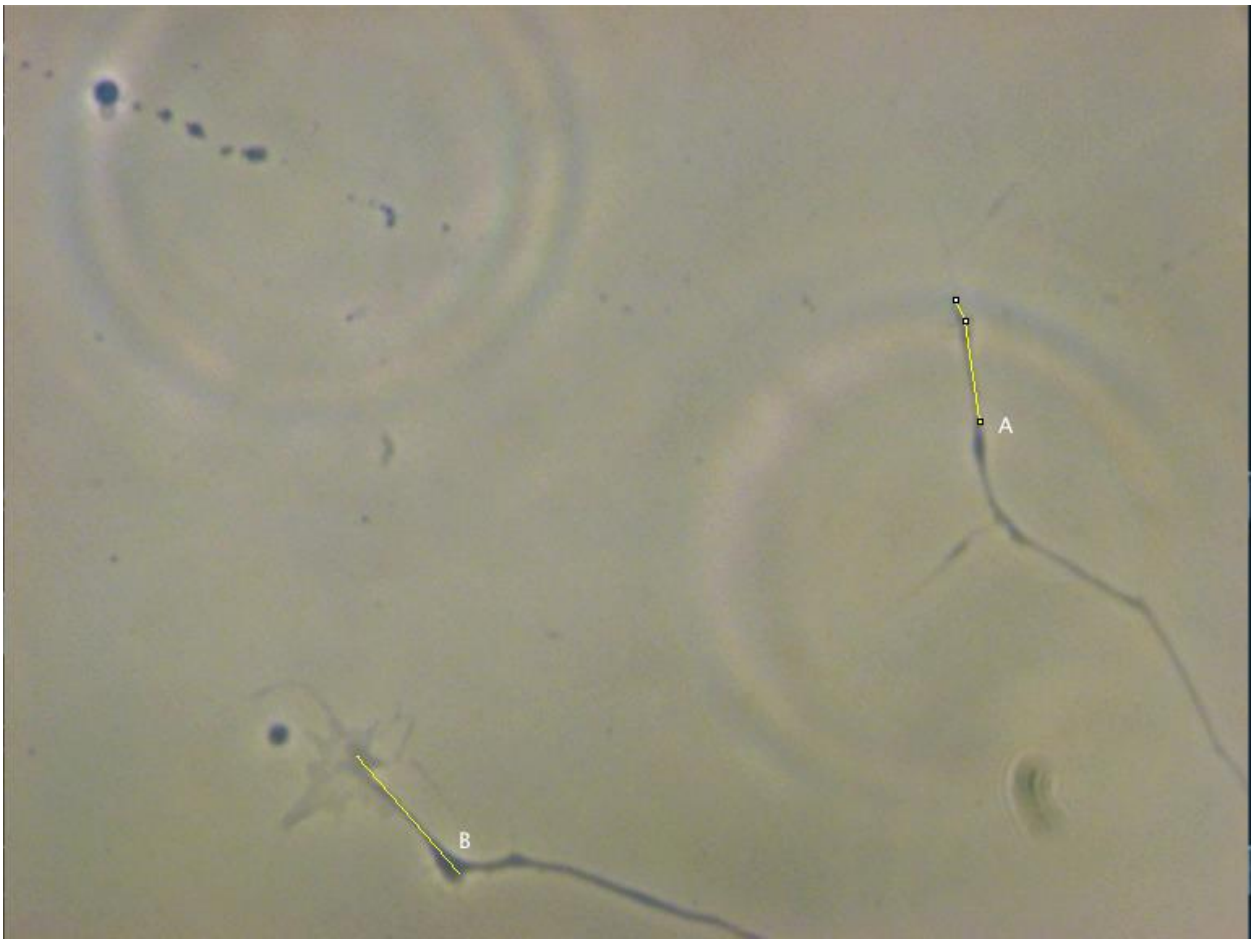
Analysis was completed on data to determine effect of lithium on growth cone activity. For each trial a graph was created, showing distance versus time for growth cone A and growth cone B in control and lithium solutions. For graph from trial one data points were used from T_0, T_{12}, T_{24} T_{36} and T_{48}. For graph from trial two data points were used from T_0, T_{15}, T_{30}, T_{45} and T_{60}. For neurons A and B of trial 1 the length (of segmented line) at the T_0 was subtracted from the length at T_{24} and divided by time to find rate of growth cone activity in control solution. The length at T_{24} was subtracted from the length at T_{48} and divided by time for find rate of growth

cone activity in lithium solution. This procedure was repeated to find control and lithium rates of trial 2 using T_0, T_{30} and T_{60}.

Results

The graph and images below illustrate the rate of growth cone extension in embryonic chick neurons. Rate was found for control and experimental portions of each trial. Rates were higher in the control as compared to Lithium trials. All times are presented with a capital T followed by a minute. For example: T_0 refers to time zero and T_{24} refers to twenty four minutes into the trial. Photos were analyzed at T_0, T_{12}, T_{24}, T_{36}, and T_{48}, to observe the progress of the growth cones in trial 1. Photos were analyzed at T_0, T_{15}, T_{30}, T_{45}, and T_{60} for trial 2, however not shown in images due to similarity to trial 1 results. Photos below show T_0, T_{24}, T_{24} (With addition of lithium) and T_{48} for trial 1.

1)



2)

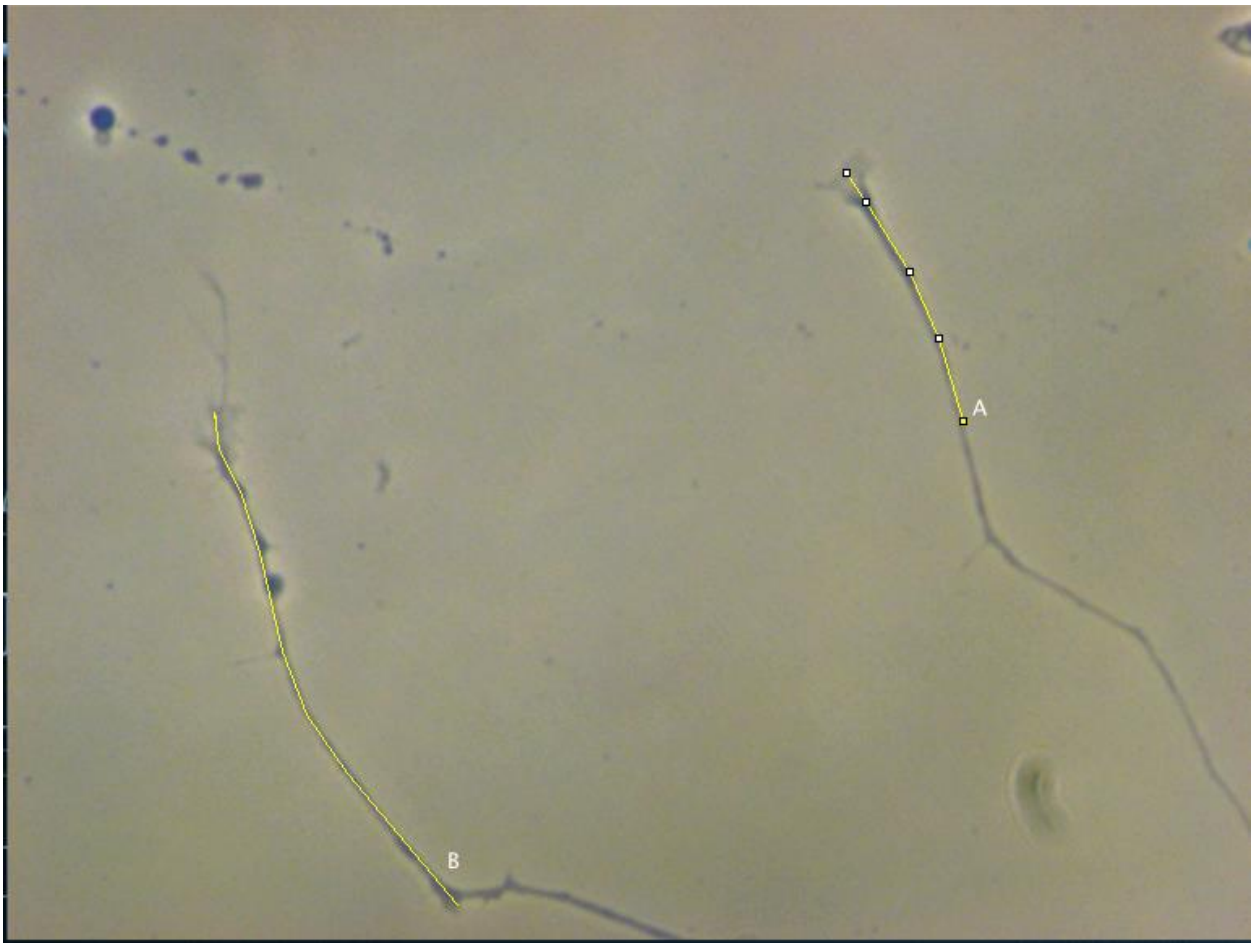
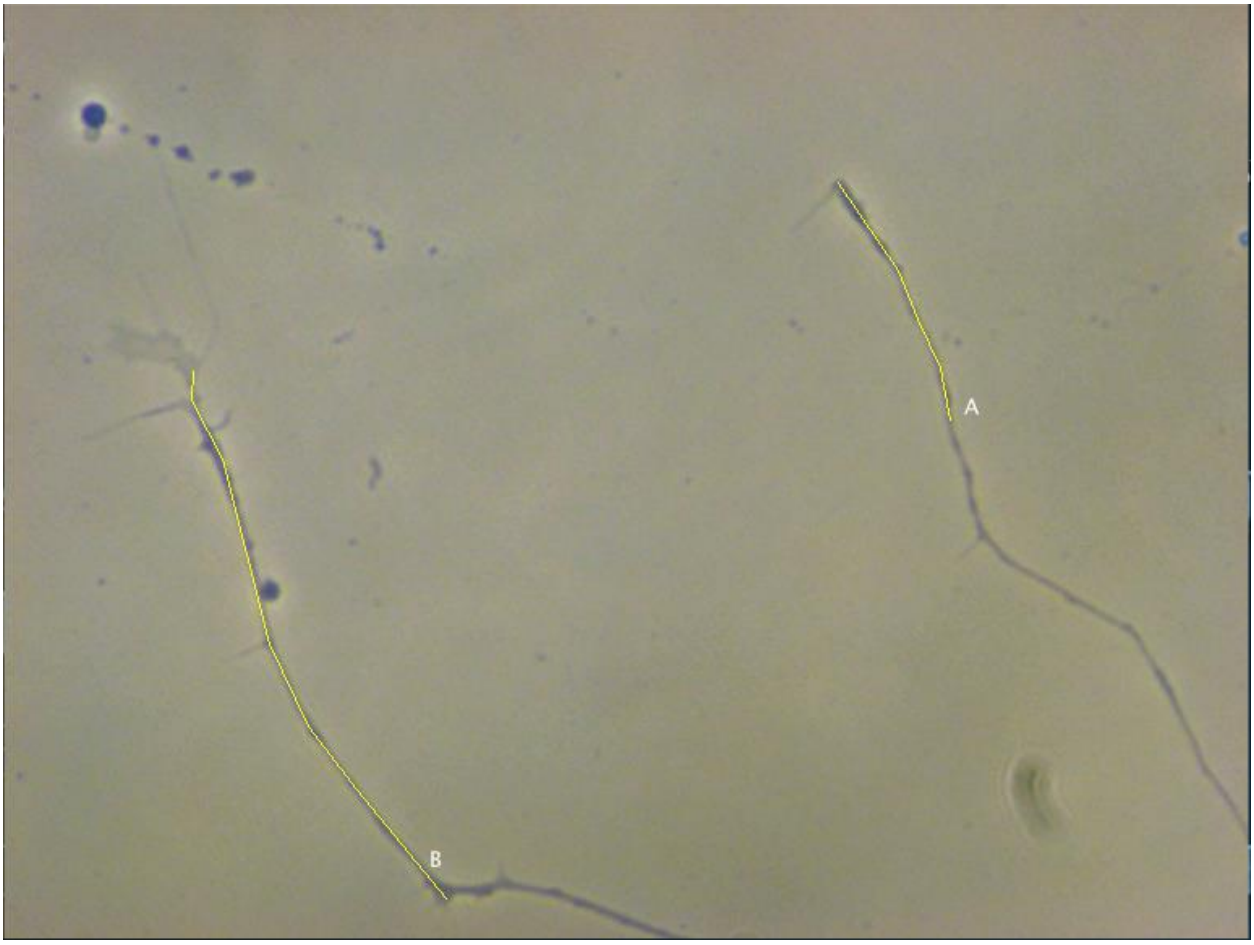


Figure 1. Control trial 1(No Lithium) at T_0 (1) and at T_{24} (2). Images were taken with transmitted light microscopy using Nikon Eclipse E200 at 40x magnification. At T_0 (1) Growth Cone A: 15.76µm, Growth Cone B: 20.73µm. At T_{24} (2) Growth Cone A: 34.92µm, Growth Cone B: 75.58µm.

1)



2)

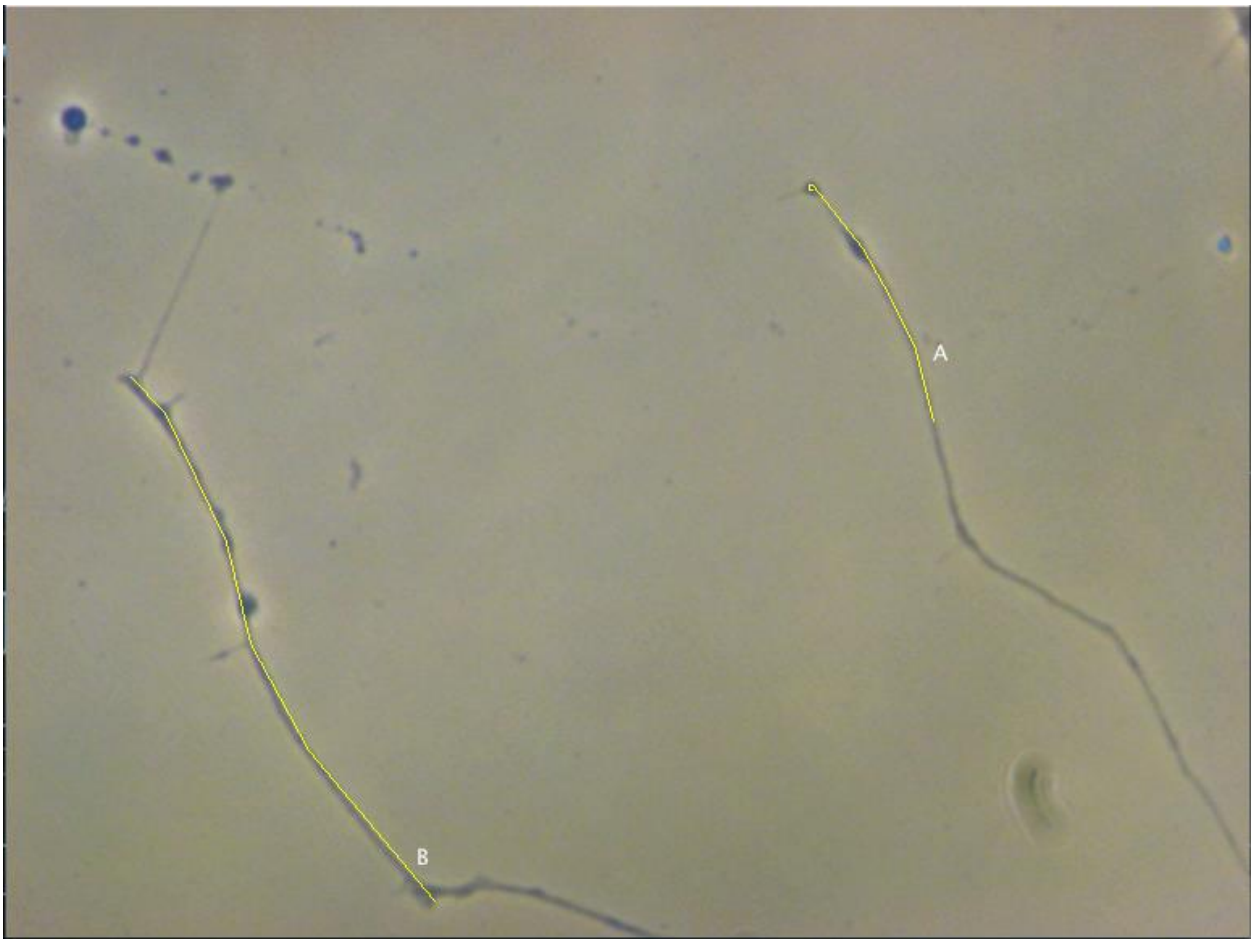


Figure 2.Experimental trial 1(With Lithium) at T_{24} (1) and at T_{48} (2). Images were taken with transmitted light microscopy using Nikon Eclipse E200. At T_{24} (1), which is when Lithium was added, Growth Cone A: 34.92µm, Growth Cone B: 75.58µm. At T_{48} (2), Growth Cone A: 36.56µm, Growth Cone B: 77.55µm.

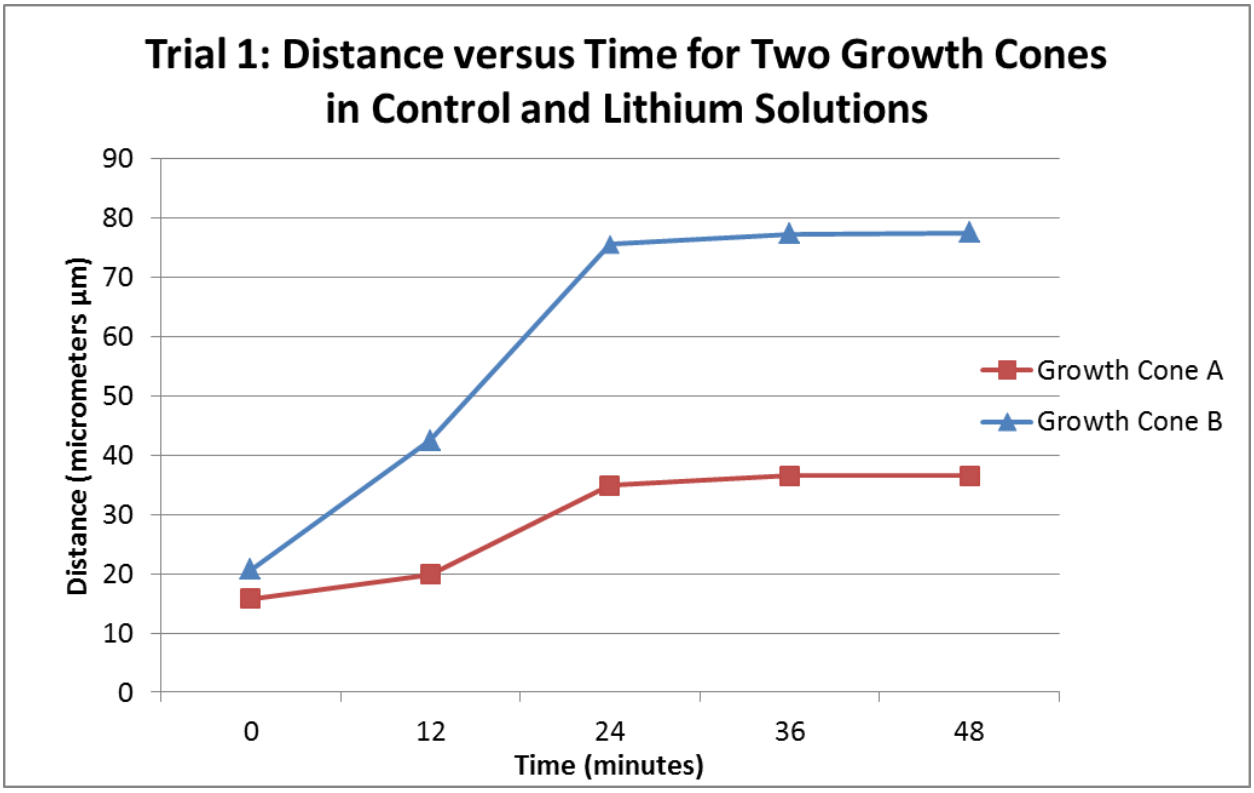


Figure 3. Distances travelled (µm) over a period of 48 minutes for growth cone A and growth cone B for trial 1. Growth cones were exposed to the control solution between T_0 and T_{24}, where the growth cones were then exposed to 10 mM lithium chloride solution from T_{24} and T_{48}. Distance moved by extension was greater in the control solution than the lithium solution.

The slopes of the lines in Figure 3 from T_0 to T_{24} represent rates of growth cone activity while in control solution. The slopes of the lines from T_{24} to T_{48} represent rates of growth cone activity while in lithium solution. For growth cone A, the rates of growth cone activity in the control and lithium solutions were 0.793µm/min and 0.068µm/min respectively. For growth cone B the rates in the control and lithium solutions were 2.29µm/min and 0.082µm/min respectively.

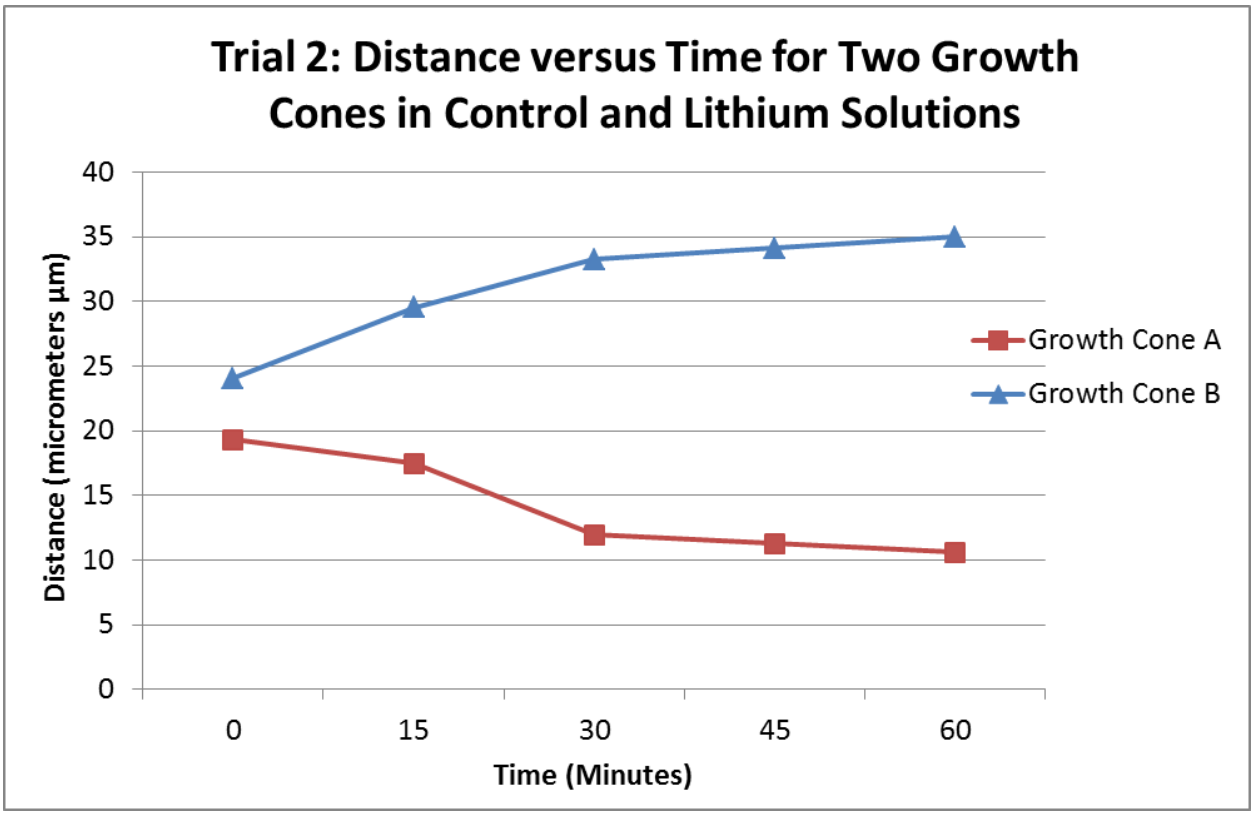


Figure 4. Distances travelled (µm) over a period of 60 minutes for growth cone A and growth cone B for trial 2. Growth cones were exposed to the control solution between T_0 and T_{30} , where the growth cones were then exposed to 10 mM lithium chloride solution from T_{30} and T_{60}. Distance moved, either by extension or retraction, was greater in the control solution than the lithium solution.

The slopes of the lines in Figure 4 from T_0 to T_{30} represent rates of growth cone activity while in control solution. The slopes of the lines from T_{30} to T_{60} represent rates of growth cone activity while in lithium solution. For growth cone A, the rates of growth cone activity in the control and lithium solutions were -0.307µm/min and -0.057µm/min respectively. For growth cone B the rates in the control and lithium solutions were 0.382µm/min and 0.0733µm/min respectively.

Discussion

It was hypothesized that exposing growth cones to LiCl solution would increase growth cone rate of activity. The results of this experiment do not support the original hypothesis; in fact they show the opposite effect. Although previous literature suggested that through increased actin assembly, growth cone activity would increase (Colombo et al., 1991), the activity decreased after exposure to 10mM LiCl solution. The data shows that after lithium was added, growth cones extended more slowly over time, thus moving at a reduced rate. If this experiment was repeated many times to give the same result, it could be concluded that lithium treatment decreases growth cone activity. This result parallels results demonstrated by Takei et al (1998) and Owen & Gorden-Weeks (2003).

Calcium is required for proper growth come activity. Lithium has been shown to act as a competitive inhibitor of a receptor that allows calcium to be released from internal storage sites in the cell. Therefore, there is decreased calcium to be used by the growth cones in the presence of lithium. As a result, growth come activity is decreased (Takei et al, 1998). Further, lithium has been shown to reduce axon elongation due to the inhibition of glycogen synthase kinase. This kinase changes the activity of a protein that regulates microtubule stability during axon genesis. It decreases its activity to cause inhibition of growth cone extension (Owen & Gordon-Weeks, 2003). This decreases growth come rate.

A few sources of error could have affected the results. Growth cones are most efficiently active at thirty-seven degrees Celsius. Since a portable heater was used to heat the stage, it was difficult to maintain the optimal temperature. A collaborator would have been helpful in order to

constantly check temperature and adjust heater accordingly. A collaborator should have also aided during the application of solutions to flow chamber; it was difficult not to knock the table while performing the task alone and any movement of the table skews results because the microscope cameras are so sensitive. A better method for determining what the leading edge of a growth cone would help future experiments be more consistent.

In the future, more trials would be completed in order to gather more data. The duration of each trial would be lengthened to give neurons longer exposure to both control and lithium solutions, and the same duration would be used for each trial. The results of these changes would be observed and analyzed. It would be interesting to use a variety of different concentrations of LiCl solution, to see how lower concentrations affect growth cone activity (Sassi et al., 2002). Results from Takei et al (1998) and Owens and Gordon-Weeks (2003) show that as concentration of lithium increases, growth cone activity decreases. It would be interesting to see if there were a low enough concentration of lithium that could be added that would have no effect on growth cone activity. This could be beneficial to determine proper therapeutic dosages of lithium in bipolar disorder patients. Moving forward more experiments should be completed to further understand the relationship between lithium exposure and single neurons, in order to help explore the reason for lithium use to treat bipolar disorder.

References

Bhattacharyya, B., & Wolff, J. (1976). Stabilization of microtubules by lithium ion. Biochemical and biophysical research communications, 73(2), 383-390.

Colombo, R., Milzani, A., & Dalle Donne, I. (1991). Lithium increases actin polymerization rates by enhancing the nucleation step. *Journal of molecular biology*, *217*(3), 401-404.

Hong, M., Chen, D. C., Klein, P. S., & Lee, V. M. Y. (1997). Lithium reduces tau phosphorylation by inhibition of glycogen synthase kinase-3. Journal of Biological Chemistry, 272(40), 25326-25332.

Machado-Vieira, R., Manji, H. K., & Zarate Jr, C. A. (2009). The role of lithium in the treatment of bipolar disorder: convergent evidence for neurotrophic effects as a unifying hypothesis. Bipolar disorders.

Mattila, P. K., & Lappalainen, P. (2008). Filopodia: molecular architecture and cellular functions. *Nature reviews Molecular cell biology*, *9*(6), 446-454.

Ziv, N. E., & Smith, S. J. (1996). Evidence for a role of dendritic filopodia in synaptogenesis and spine formation. *Neuron*, *17*(1), 91-102.

Morris, R. (2015a). Microscopes & Scale - Lab 2, 2015

Morris, R. (2015b). Primary Culture of Chick Embryonic Peripheral Neurons 1: DISSECTION

Morris, R. (2015c). Primary Culture Of Chick Embryonic Peripheral Neurons 3: STAINING and OBSERVATION of LIVE CELLS

Owen, R., & Gordon-Weeks, P. R. (2003). Inhibition of glycogen synthase kinase 3β in sensory neurons in culture alters filopodia dynamics and microtubule distribution in growth cones. *Molecular and Cellular Neuroscience*,*23*(4), 626-637.

Sassi, R. B., Nicoletti, M., Brambilla, P., Mallinger, A. G., Frank, E., Kupfer, D. J. & Soares, J. C. (2002). Increased gray matter volume in lithium-treated bipolar disorder patients. *Neuroscience letters*, *329*(2), 243-245.

Takei, K., Shin, R. M., Inoue, T., Kato, K., & Mikoshiba, K. (1998). Regulation of nerve growth mediated by inositol 1, 4, 5-trisphosphate receptors in growth cones. *Science*, *282*(5394), 1705-1708.

Vergara, M. N., & Canto-Soler, M. V. (2012). Rediscovering the chick embryo as a model to study retinal development. Neural Dev, 7(1), 22.

I have abided by the Wheaton College honor code in this work.
Jesse Foehl