The Effect of Lithium on Axon Brightness in *Gallus gallus* Neurons

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Introduction

Lithium is the most commonly used treatment for bipolar disorder. Its therapeutic effects are cited across the literature (Goodwin 2001; Geddes et al., 2004; as reviewed by Cipriani et al., 2005. However, the mechanism behind its treatment is poorly understood. Bipolar disorder is a heritable and chronic disorder. It disrupts the normal function of excitable tissues, specifically at the level of membrane ion channel expression (Butler-Munro et al., 2010). Membrane potential in neurons is regulated by the flow of sodium and potassium ions into and out of the cell. These ions enter and leave the neuron via sodium potassium ATPases, voltage-gated sodium channels, voltage-gated potassium channels, and potassium leak channels. Further, the influx of sodium through its voltage-gated channels activates potassium channels (Butler-Munro et al., 2010).

Lithium can act as a substitute for sodium in the generation of an inward current. It also decreases the outward current of potassium through the sodium-activated potassium channels. These channels contribute the largest percentage, about sixty percent, of the potassium outward current. The decrease in potassium current affects resting membrane potentials and action potentials. Specifically, lithium has been shown to depolarize resting membrane potentials and to increase the frequency of action potentials (Butler-Munro et al., 2010). The depolarization was dose dependent; increased concentration resulted in increased depolarization (Grafe et al., 1983).

Chick *Gallus gallus* embryonic neurons were chosen for cell culture in this study. It is advantageous to use chick embryos because their development occurs very quickly and they provide easy accessibility for viewing many different physiological structures. Therefore, chick embryos are effective vertebrate models to study neurons *in vitro.* Further, chick embryos are similar to the human embryo (Vergara, 2012). The cultured neurons were treated with lithium and stained with FM1-43, a lipophilic, cationic dye that inserts itself into the outer leaflet of the plasma membrane. The insertion occurs due to its attraction to the phospholipid bilayer and the negative membrane potential. It cannot cross between the membrane leaflets so it remains inserted in the membrane. As a result, the dye will brightly fluoresce for visualization of the plasma membrane (Meyers et al., 2003).

This study explores the hypothesis that lithium treatment will decrease axonal brightness. It is hypothesized that lithium will do this by reducing FM1-43 uptake as a result of resting membrane potential depolarization. Depolarization from lithium treatment results in a more positive membrane potential, thus decreasing the attraction between the cationic FM1-43 and the membrane and subsequently reducing its insertion into the membrane. This study is significant because bipolar disorder has been recognized to exhibit abnormal expression of ion channels and as such abnormal excitability of neurons (Butler-Munro et al., 2010). The study may provide evidence that lithium's mechanism of treatment is to target the membrane.

Materials and Methods

Primary Tissue Culture

All materials needed for cell culture were obtained from Sigma-Aldrich Corporation. Ten day old chick embryos were dissected following the protocol given by Dr. Robert Morris, Wheaton College Department of Biology (Morris, 2015b). The cultured cells were incubated at thirty-seven degrees Celsius for about twenty-four hours.

Lithium Treatment

A 1M sterile lithium chloride stock solution was made and diluted into serum-free growth medium 100-fold to create a 10mM experimental working solution. The concentration 10 mM was chosen in order to test acute exposure. To create the 0 mM lithium chloride control solution, a 100-fold dilution of sterile water into serum free growth medium was performed.

The lithium perturbation occurred about six hours after the dissection performed by Dr. Robert Morris. Three petri dishes of cultured cells were removed from the incubator and examined under a Nikon TMS inverted microscope to check for viable cells. Dishes with around the same amount of cells were chosen for the experiment. The three dishes were labeled as two lithium experimentals and one control. The existing growth medium in all three dishes was removed with a sterile Pasteur pipette. A sterile pipette was used because the neurons were still growing. About 2 mL of 10 mM lithium chloride was added to each experimental dish. About 2 mL of the control solution was added to the control dish. The three dishes were then returned to the incubator and left to incubate for around nineteen hours at 37 degrees Celsius.

FM1-43 Staining

The FM1-43 was ordered from Sigma-Aldrich Corporation. The working solution was made to be 25 μg/mL. The dye was diluted 200 fold from a 5 mg/ mL stock into calcium and

magnesium free Hanks Balanced Salt Solution (HBSS). The two experimental and one control dish were removed from the incubator. The existing lithium chloride and serum free growth medium were removed with a Pasteur pipette. Around 2 mL of the FM1-43 dye was added to each dish. The dishes were covered with a lid to prevent light exposure and were left to incubate at room temperature for twenty-five minutes. The incubation time was determined according to a successful test incubation period performed by Dr. Robert Morris.

Chip chambers were made for the control and two experimentals following the protocol provided by Dr. Robert Morris (Morris, 2015c). Modifications to the protocol included removing the FM1-43 dye from the dishes to the point of a meniscus, instead of growth medium.

Fluorescence Imaging

Images were taken in collaboration with Corinne Murphy. A Nikon Eclipse E200 microscope was used. The camera was mounted with Nikon c-mount 1.0X. The camera itself was made by Diagnostic Instruments, Model number 18.2 Color Mosaic, Spot Insight FireWire 2. Fluorescence illumination was provided by Chiu Technical Corporation, Mercury- 100W. The Apple IMAC OSX Yosemite Version 10.10.4 computer "Taurus" found in the ICUC at Wheaton College was used. A portable heater was placed adjacent to the microscope to keep the cells at 37 degrees Celsius. Temperature was monitored with a temperature probe. Cells were found at 40X objective and Phase 2. The sample was then aligned for Koehler illumination following the protocol provided by Dr. Robert Morris (Morris, 2015a).

The SPOT Imaging Solutions program was opened to capture both transmitted light and fluorescent images of areas within the control slide and the two experimental slides. All fluorescent images were taken using filter four to emit red light and at a two second exposure time.

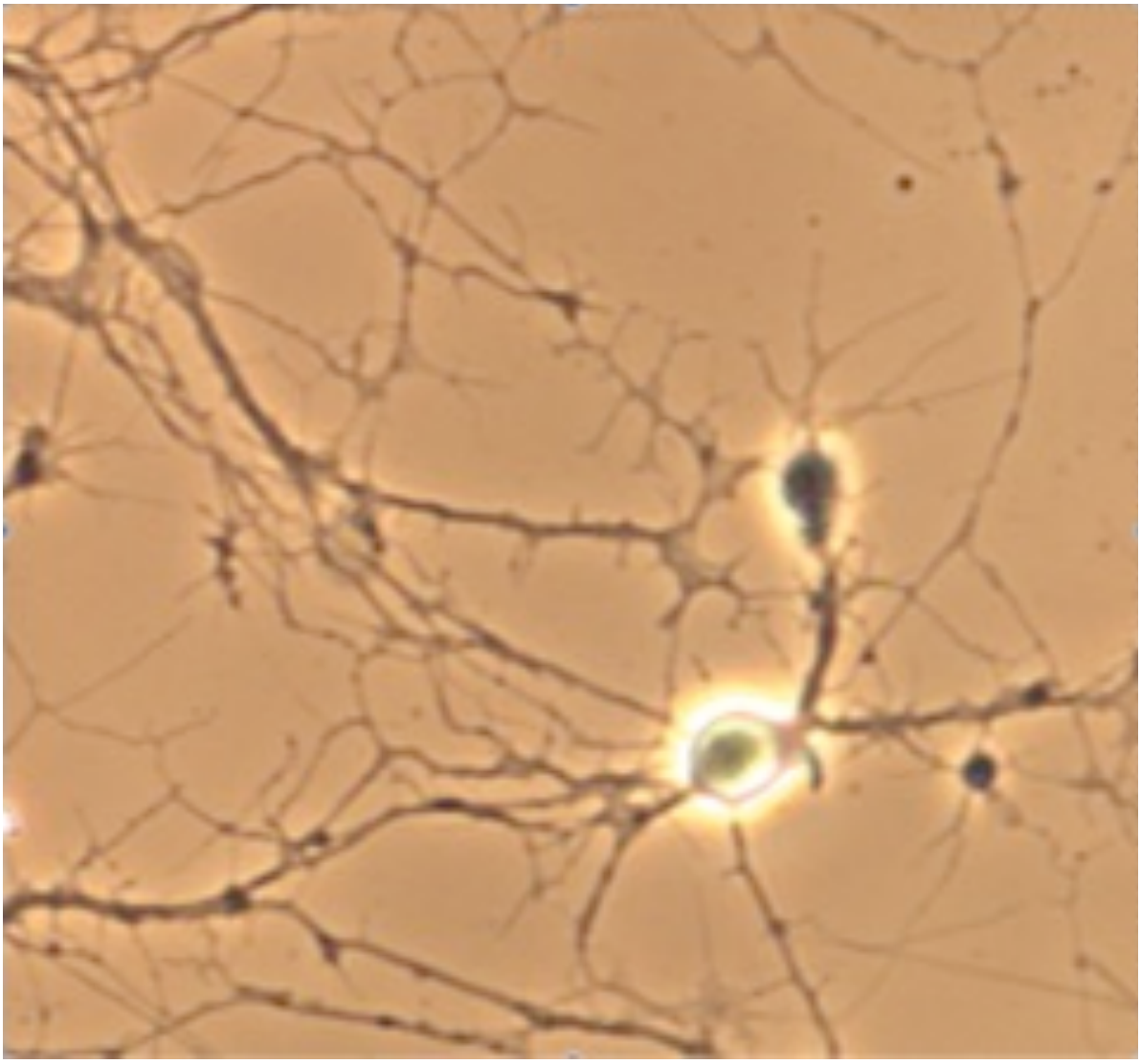
Quantification and Analysis

ImageJ 1.40 software was used to analyze the chosen images. Of the areas of cells that were imaged, the one containing the greatest number of clear axons in both its transmitted light image and more importantly, its fluorescence image, was chosen for the control and the experimental. The images were not selected based on whether or not the axons were branched or unbranched. Also, whether or not the axons were single axons or fasciculated was not a selection measure. The only selection criterion was the greatest number of clear, visible axons. The images were opened in ImageJ and viewed in RGB stack to measure the brightness of the red pixels. Five axons were magnified and selected using the polygon selection tool. The area and the mean brightness of the pixels for each axon were recorded. The polygon selection tool was also used to select the extracellular background adjacent to each axon. The mean brightness was recorded. The actual brightness of each axon was determined to be the brightness measured for the axon subtracted by the brightness of the background directly next to it. The average of the five axons was taken to find the average axon brightness in the control and experimental groups.

Results

The images depicted below show *Gallus gallus* neurons stained red with FM1-43. Uptake of the dye was observed in both the control and experimental groups. However, as illustrated in Figures 1 and 2, the control image is observably more fluorescent than the experimental image.

A.



B.

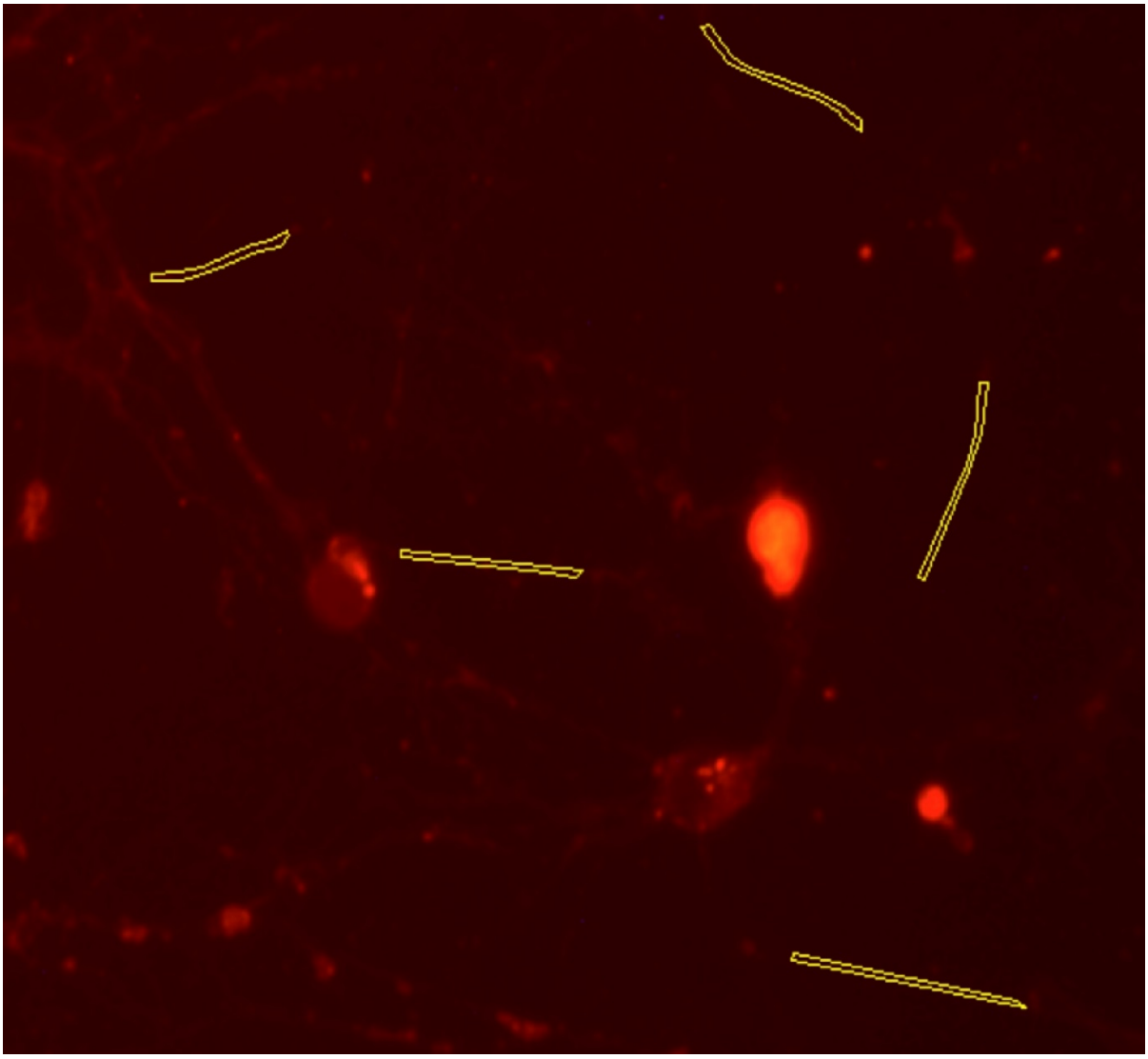
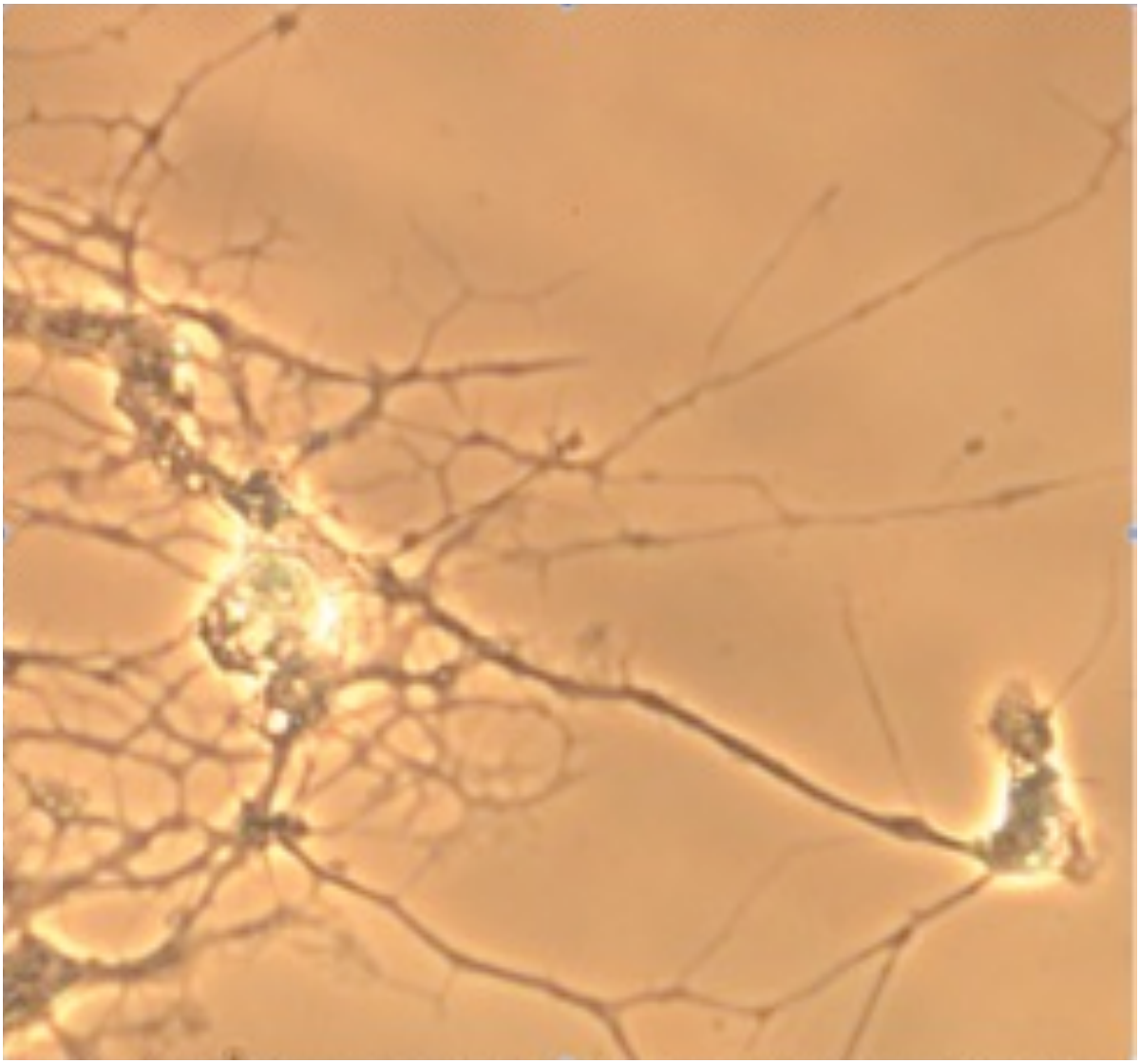


Figure 1: A. Control group under transmitted light exposure and B. Two second fluorescent light exposure at 40X using a Nikon Eclipse E200. Fluorescence is clearly visible in most axons. Axons boxed in yellow (n=5) were used for brightness measurements.

A.



B.

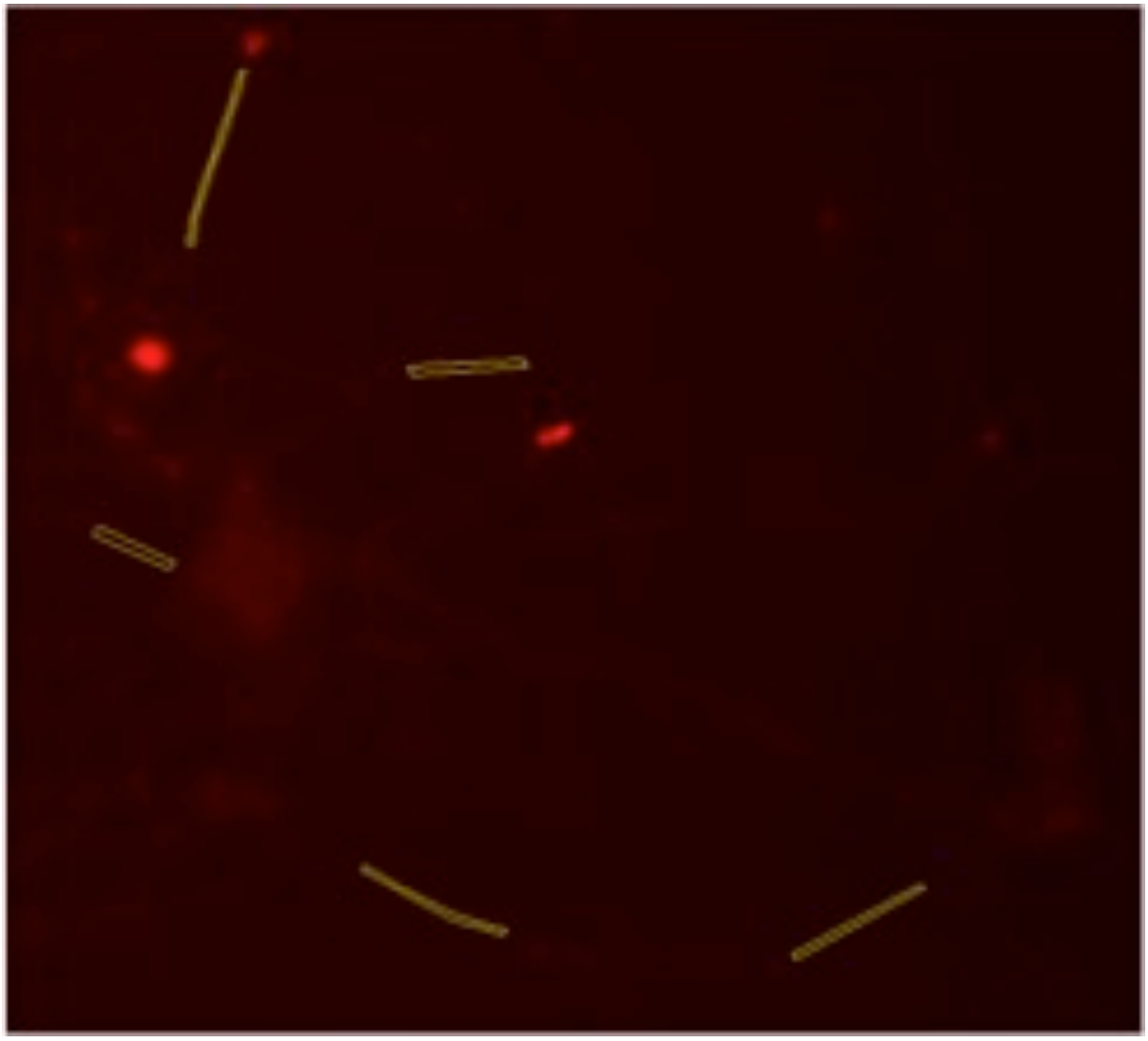


Figure 2: A. Experimental treated with 10 mM lithium chloride under transmitted light exposure and B. Two second fluorescent light exposure at 40X using a Nikon Eclipse E200. Fluorescence is slightly visible in some axons. Axons boxed in yellow (n=5) were used for brightness measurements.

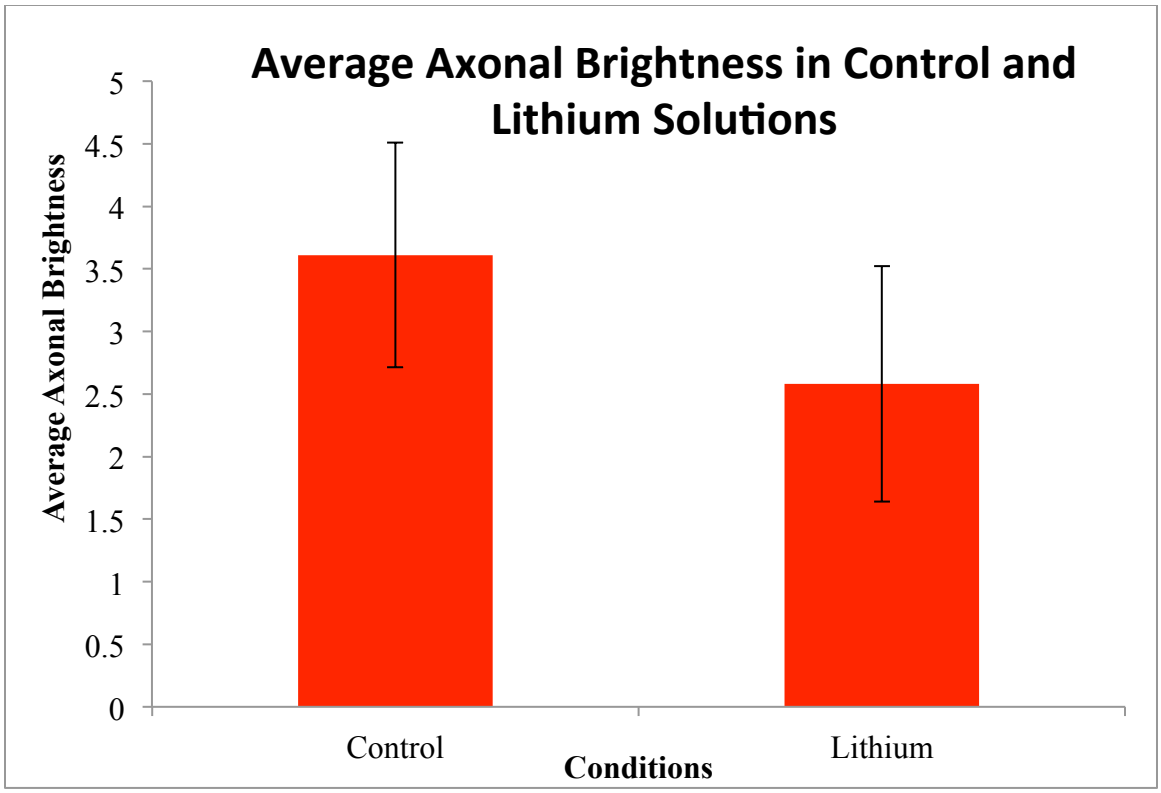


Figure 3: Average brightness of axons in the control solution without lithium and in 10mM lithium chloride. Standard deviation bars are shown. Axons growing under control conditions are brighter than the axons growing in the 10 mM lithium chloride.

Discussion

The hypothesis that lithium treatment decreases axonal brightness was supported by the data. The average brightness of the axons grown in the control condition was greater than the average brightness of the axons grown in 10 mM lithium chloride. The data can be used to conclude that less FM1-43 was inserted into the outer leaflet of the plasma membranes in lithium-treated neurons. This indicates that lithium targets neurons at the level of the membrane, which parallels what is demonstrated by Butler-Munro et al (2010). Their experiment also demonstrated that lithium targets the membrane. Specifically, it was shown that lithium causes resting membrane potential depolarization (Butler-Munro et al., 2010).

It is important to note that membrane potential was not actually measured in this experiment. As a result, the data do not give direct evidence for membrane potential depolarization. However, the data do support the hypothesis. If lithium does cause membrane potential depolarization, the membrane potential is made more positive. Then, the attraction between the cationic FM1-43 and the plasma membrane is decreased to result in less insertion of the dye into the membrane. The data suggests that this is occurring through decreased axonal brightness. The membrane potential of the neurons in the control group does not experience the depolarization that is caused by lithium. Thus FM1-43 uptake into the cell is not decreased by this factor, suggested in the results by increased brightness.

The data in this study represent one trial. Repeated trials are necessary to determine whether these data are truly accurate and significant. A greater n value would also contribute to the significance of the data. If it were the case that many repeated trials resulted in the same result, then decreased axonal brightness means reduced FM1-43 up-take and implies membrane potential depolarization.

In order to refine this experiment, multiple trials must be performed. Also, different concentrations of lithium should be examined. Graffe et al (1983) stated that the depolarization increased as lithium concentration increased. It would be interesting to explore whether there is an evident decrease in axonal brightness as lithium concentration is increased. This experiment would contribute to the idea that axonal brightness is related to membrane potential. Also, it could compare the different concentrations to see which causes the greatest effect, which could possibly be used to determine therapeutic dosages for lithium treatment. The study can also be refined to have the images taken without other researchers in the vicinity. This will allow for clearer pictures to be taken for a more accurate measurement of axon brightness. A future experiment would measure membrane potential. Membrane potential can be measured using whole-cell recording (Butler-Munro et al., 2010). It can then be determined if axon brightness is an accurate measure of membrane potential depolarization.

As stated, data provide evidence to lithium's mechanism of treatment at the membrane and that decreased axonal brightness can possibly be used as an indicator of membrane potential depolarization. Membrane potential is regulated by sodium and potassium channels. Therefore the data suggest that these channels are involved and are being targeted by lithium. The experiment shows that lithium decreases axonal brightness, thus implying a change in membrane potential and a change in the expression of the ion channels. This is significant because patients with bipolar disorder exhibit abnormal expression of ion channels and as such, abnormal excitability of neurons. Lithium treatment can target the membrane, specifically at the sodium and potassium channels that regulate excitability in brain neurons. This results in therapeutic effects for patients with bipolar disorder (Butler-Munro et al., 2010).

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I have abided by the Wheaton Honor Code in this work.

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