Effects of Lithium on Axonal Growth Cone Area

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Introduction

Growth cones are a vital part of nervous system development, navigating growing axons towards their targets, such as other axons or glial cells, where they create a synapse (Purves et al, 2001; Williams et al, 2004). Growth cones are able to do this by detecting and responding to soluble and surface-bound environmental chemical cues (Dongjiang and Goldberg, 2000; Williams *et al,* 2004). Movement of the growth cones occurs with the combined efforts of the actin filaments and microtubule cytoskeleton (Dongjian and Goldberg, 2000; Purves *et al*, 2001; Shaltiel *et al*, 2007). The actin filaments and microtubule cytoskeleton create the lamellipodium, the sheet-like expansion on the growth cone, and the filopodia, which are the finger-like protrusions. Both the lamellipodium and filopodia drag the growth cone towards its synaptic partner destination (Williams *et al,* 2004; Purves *et al*, 2001; Shaltiel *et al*, 2007; Dongjiang and Goldberg, 2000). The behavior of the growth cone movement and growth is important for it has been suggested that by understanding its cellular processes, it could be used as a "cell biological model of neuronal behavior" (Shaltiel *et al*, 2007) reflecting the axonal behavior that leads up to synaptogenesis (Shaltiel *et al*, 2007).

Lithium (Li+) is a neuroprotectant which has been used as a mood stabilizer for illnesses such as bipolar mood disorder since the nineteenth century (Williams *et al*, 2004). However, despite this long term use of the substance, the molecular mechanisms of it are largely unknown (Williams *et al*, 2002; Shaltiel *et al*, 2007). This lack of knowledge is a barrier for developing

improved drug therapy to treat mental disorders like bipolar mood disorder (Williams *et al*, 2004). What is known about the psychotropic drug is that it inhibits glycogen synthase kinase-3 (GSK-3) which can alter microtubule and axonal structure. This inhibition along with the deletion of the coding genes for the proteins tau or MAP1B (which are substrates of GSK-3) can result in abnormalities in growth cones, specifically in their spread size (Williams *et al*, 2002; Williams *et al*, 2004). Multiple studies have shown that the exposure of lithium to neuronal growth cones increase the average spread area of growth cones, which in turn results in reduced axon length. This increase in spread area takes more time and slows down the axonal growth movement (Williams *et al*, 2004; Williams *et al*, 2002; Shaltiel *et al*, 2007).

Inspired by the studies done on axon growth cones and lithium, this independent study tests the hypothesis that a single exposure to lithium increases axon growth cone area. Embryonic chick (*Gallus gallus*) sympathetic neurons were dissected and used, and then were exposed once to 10mM lithium serum-free growth medium for a single four-hour incubation period.

Materials and Methods

Materials

Materials used for the experiment-such as chick (*Gallus gallus*) embryo ganglia, coverslips, and HBSS-were collected and prepared using Professor Bob Morris's Primary Culture of Chick Embryonic Peripheral Neurons 1: DISSECTION protocol (Morris, 2015a). The chick (*Gallus gallus*) embryos were 10 days old and were dissected to collect primary neural cultures using Professor Bob Morris's protocol (Morris, 2015a). Serum-free growth medium was made up to be used with lithium or sterile water for the perturbation incubation portion of the

experiment. Serum-free growth medium consisted of Leibovitz L-15 medium, 0.6% glucose, 2 mM L-glutamine, 100ug/ml streptomycin, 100U/ml penicillin, and 10-50 ng/ml NGF. To create the lithium serum-free growth medium solution, Lithium Chloride was diluted with serum-free growth medium solution from 1M to 10mM, resulting in a 1:100 dilution. For the control, serum-free growth medium was diluted with sterile free water, also resulting in a 1:100 dilution. For each coverslip, 2ml of either the lithium serum-free growth medium (if experimental) or 2ml of the sterile water serum-free growth medium (if control) was used. The reason why serum-free medium was used was because a study done by Tint *et al* in 1998 on neurons and lithium (Tint et al, 1998) suggested that serum-free growth mediums should be used to avoid the potential problems that could arise with the negatively charged proteins in the serum interacting with the positively charged lithium.

Imaging Equipment

To image coverslips with axon growth cones a Nikon E-200 microscope with a Sony DFW-X700 Camera with the Adapter C-mount 1.0x magnification was used along with the software program BTV 6.0B1. To analyze images in order to collect data the software ImageJ 1.50a was used. All programs were on the Wheaton College ICUC Capricorn iMac computer running OS X Yosemite Version 10.10.4.

Methods

Dissection of chick embryonic cells followed Professor Bob Morris's Primary Culture of Chick Embryonic Peripheral Neurons 1: DISSECTION protocol (Morris, 2015a). The viewing, locating of live dissected neuron axon cells followed Professor Bob Morris's Primary Culture of Chick Embryonic Peripheral Neurons 2: OBSERVATION of LIVE UNLABLED CELLS protocol (Morris, 2015b).

To test the hypothesis that neuron axon growth cones increase in area when exposed to lithium, live neuron cells were perturbed with lithium. For both the experimental and control trials, coverslips with dissected neural cells were collected after 48 hours of incubating in the C growth medium and checked for growing axon cells. If axon cells could be found, these coverslips would be used. The growth medium covering the coverslips in each petri dish was pipetted out with a sterile pipette. Avoiding drying out the coverslips, 2ml of the 10mM lithium serum-free medium was then immediately pipetted with a new sterile pipette into the experimental petri dishes (4 petri dishes each receiving 2ml), covering the coverslips with the new growth medium that has lithium in it. For the control coverslips, 2ml of the sterile water serum-free medium solution was immediately pipetted with a fresh sterile pipette into the control petri dishes (2 petri dishes each receiving 2ml). Once in the 10mM lithium serum-free medium or the sterile water serum-free medium solution, the coverslips were placed back in the incubator to be incubated for 4 hours.

Imaging

After incubating for 4 hours, coverslips were processed into chip chambers and imaged using a Nikon E-200 light phase microscope under 40x magnification on phase 3 using Professor Bob Morris's Primary Culture of Chick Embryonic Peripheral Neurons 2: OBSERVATION of LIVE UNLABLED CELLS (Morris, 2015b). The only difference in methods is that the BTV program was not allowing images to be captured through the program so a screenshot had to be taken of the image instead.

Measuring and Quantifying:

To analyze axon growth cone data, images collected were imported into ImageJ 1.50a on the Wheaton College ICUC Capricorn iMac computer running OS X Yosemite Version 10.10.4.

Since ImageJ is automatically calibrated to measure 1024x768 images by pixels, it is necessary to recalibrated it so it instead measures the 1024x768 pixel images in μm to make data collection easier and accurate. To do this, an image of a 0.01mm objective micrometer at 40x magnification was taken at the same microscope used to image coverslips. This image of the objective micrometer was imported into ImageJ. Using the line selection tool in ImageJ, a selection line of the known length was drawn (in our experiment this was 50μm) on the micrometer image. The line selection tool knows how many pixels long the line drawn is. Then Analyze-Set Scale was selected in the menu, this pops up a menu asking for the known distance of the line drawn and the units of measurements. In the "known distance" field 50μm was entered. The units of measurement were set to μm. The "Global" option was checked and turned on, so that all images opened in ImageJ will take on the same new spatial calibration created (White, 2009).

Once ImageJ was calibrated, data measuring and quantification could begin. Opening the image containing the desired axon growth cones in it in ImageJ, the freehand selection tool was selected from the tool bar. The entire axon growth cone perimeter was traced starting at the enlargement of the axon located at the end of the axon indicating the beginning of the growth cone all the way around the filopodia and lamellipodia until the tracing line reached the other side of the axon enlargement. Once the perimeter was traced, "Analyze" and then "Measure" was selected in the menu bar. This popped up the results window which indicated the area in μm^2 of the traced axon growth cone. The areas collected from each control and experimental trial axon growth cones were then averaged and plotted on a bar graph, seen in Fig. 3, for comparison.

Results

It was found that the appearance of the size and shape of the axon growth cones varied widely in both the control and experimental trials. Fig. 1. and Fig. 2. show examples of what these axon growth cones looked like in both the control and experimental trials. This large variance can be seen in comparison of the largest growth cones and the smallest growth cones in both the control and experimental trials. The largest control axon growth cone area imaged was 415.11 μm² and is 10x larger than the smallest control axon growth cone area at 41 μm². The largest experimental axon growth cone area imaged was 257.86 μm² and is 7x larger the smallest experimental axon growth cone area imaged at 33.27 μm². The average area of the control axon growth cones was 139.36 μm² while the average area of the experimental axon growth cones average area was 128. 26 μm². These averages can be seen in Fig. 3.

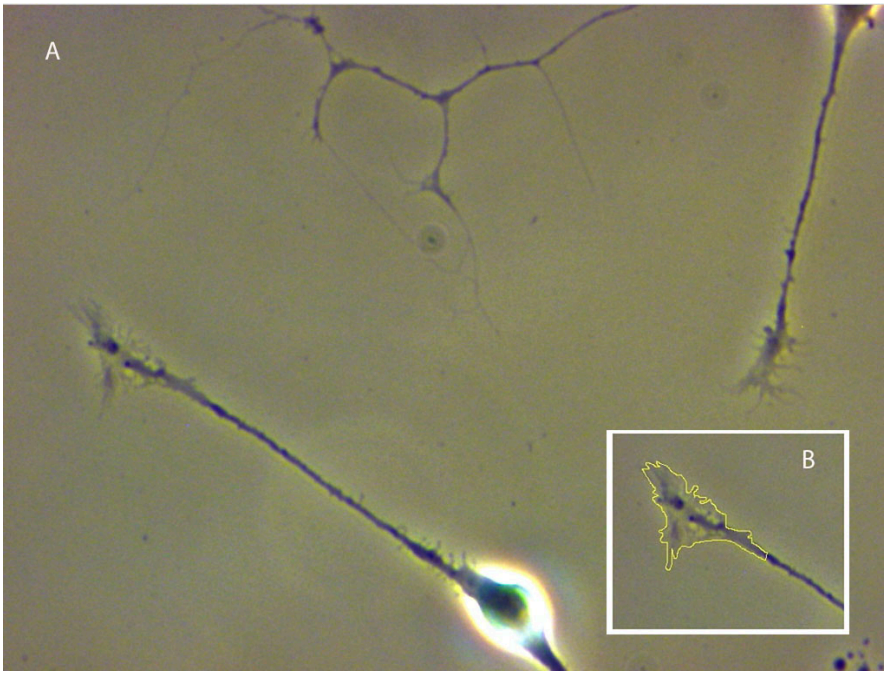

Fig. 1. Image A is the control image of one of the control axon growth cones under 40x magnification after a 4-hour incubation in sterile water and serum-free growth medium solution. Image B is the same axon growth cone with its area traced in yellow.

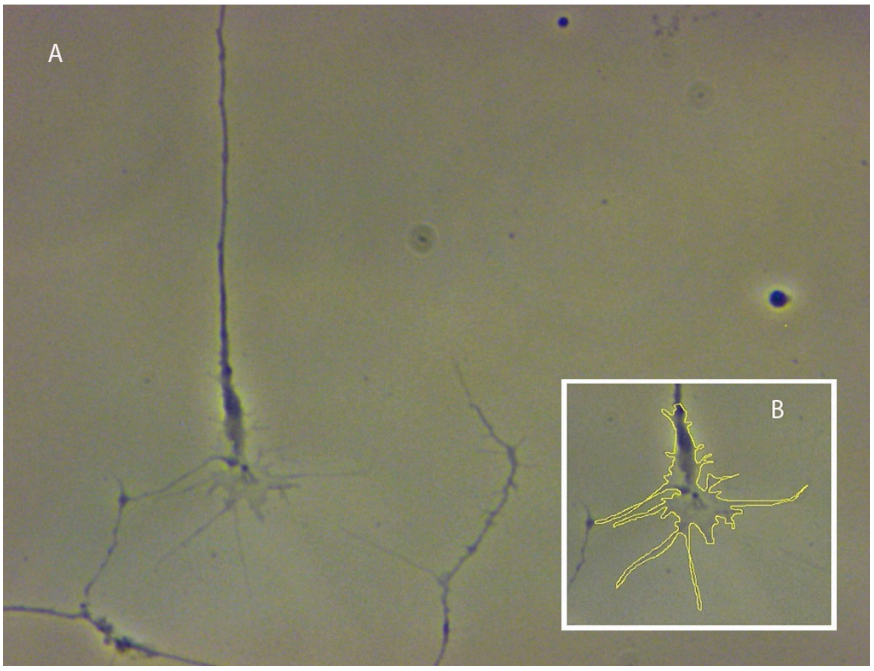

Fig. 2. Image A is the experimental image of one of the experimental axon growth cones under 40x magnification after a 4-hour incubation in 10mM lithium and serum-free growth medium solution. Image B is the same axon growth cone with its area traced in yellow.

Fig. 3. The difference in the average axon growth cone areas in µm² between the control and experimental trials. It was found that the control trials had a larger axon growth cone area average than the experimental trials axon growth cone area average. Control n=18 axon growth cones in control trials. Experimental n=25 axon growth cones in experimental trials.

Discussion

The hypothesis that lithium increases axon growth cone area was not supported in this study. Instead it was found that the average axon growth cone area size was larger in the control trials which had not been exposed to lithium (mean=139.36 μm²) than in the experimental trials which had been exposed to lithium (mean=128.26 μm²), shown in Fig. 3. This result was surprising, seeing that multiple papers on the subject have found the opposite result (Williams *et al*, 2004; Williams *et al*, 2002; Shaltiel *et al*, 2007).

Possibilities for why these results were received could be due to the concentration of the lithium exposed in this experiment. The lithium exposure concentration was 10mM whereas the other studies used concentrations of at most 2mM of lithium (Shaltiel, 2007; Williams, 2002). It could be possible that high concentrations of lithium stunt the growth of the actin filaments and the microtubule cytoskeleton in the growth cones, while low concentrations of lithium enhance the growth. Another possibility is the exposure incubation length of time. Other studies appeared to expose cells for at least 8 hours and in some cases even 24 hours, while this study only exposed the cells to the lithium for 4 hours. Perhaps a 4-hour exposure time is not enough time for the lithium to effect the axonal growth cone spread size.

For future studies, it would be interesting to reattempt this test with a smaller concentration of Lithium and/or a longer lithium exposure incubation time. Comparisons with this study and future studies could confirm if the concentration of Lithium and/or the exposure time is an important aspect in the effects of lithium on axon growth cone area. It also would be interesting to attempt studies with lithium and axon growth cone area size looking at the speed of the growth cone movement, seeing that the study done by Robert Williams *et al* in 2004 states that this exposure to lithium slows down the axon growth cone movement (*Williams* et al, 2004).

Since in this study it was found that the exposure of lithium decreased axon growth cone area size, it might be interesting to see if it also caused the axon growth cone movement to increase in speed.

It is important to continue studies working with lithium and axon growth cones because it could help improve treatment for illnesses such as bipolar mood disorder and post traumatic stress disorder in the future. By better understanding the molecular mechanism of lithium treatments on the behaviors of neurons, specifically the growth cones (which if subtly changed could have large effects on the connectivity of neural networks), it may be possible to develop a better understanding of topics such as neurogenesis in humans (Williams *et al,* 2004).

References

Dongjiang T and Goldberg D.J. (2000). Bundling of Microtubules in the Growth Cone Induced by Laminin. *Molecular and Cellular Neuroscience.* 15: 303-313.

Morris, R.L. (2015a) Neurobiology Bio324 primary culture of chick embryonic peripheral neurons 1: dissection. Accessed 11/04/2015. 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 11/04/2015. Available at: http://icuc.wheatoncollege.edu/bio324/2015/NBlabPrimaryTissCultureProc2ObservnOfLliveUnlabeledCells2015.htm.

Purves D, Augustine GJ, Fitzpatrick D, et al., editors. (2001). Neuroscience. 2nd edition. Sunderland (MA): Sinauer Associates; 2001. The Axonal Growth Cone. Available from: http://www.ncbi.nlm.nih.gov/books/NBK11114/

Shaltiel, G, Dalton EC, Belmaker RH, Harwood AJ, Agam G. (2007). Specificity of mood stabilizer action on neuronal growth cones. *Bipolar Disorders.* 9: 281-289

Tint I, Slaughter T, Fischer I, Black M.M. (1998). Acute Inactiviation of Tau Has No Effect on Dynamics of Microtubules in Growing Axons of Cultured Sympathetic Neurons. *The Journal of Neuroscience.* 18(21): 8660-8673.

White, D.J. (2009, May 28). *SpatialCalibration.* Received from http://imagej.net/SpatialCalibration

Williams R.S.B, Cheng L, Mudge, A.W, Harwood, A.J. (2002).. A common mechanism of action for three mood-stabilizing drugs. *Nature.* 417(6886): 292-295.

Williams R, Ryves W.J, Dalton E.C, Eickholt, B, Shaltiel G, Agam G, Harwood A.J. (2004). A molecular cell biology of lithium. *Biochem Soc Trans. 32*(Pt 5): 799-802.

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

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