The Effects of Lithium on Mitochondrial Activity in *Gallus gallus* Embryo Single Neurons

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**Introduction**

Bipolar disorder is a chronic psychiatric illness that affects people with severe changes between manic and depressive episodes. Recently, there has been extensive research looking into the pathophysiology of this mental disorder. With bipolar disorder, there are implications of mitochondrial dysfunction and oxidative stress, which contribute to neurodegeneration through cell death (Andreazza & Young, 2014).

The mitochondria are where most of the cell’s adenosine triphosphate (ATP), or chemical energy, is generated (Kandel et al., 2012). Dysfunctions of the mitochondria, like those that occur with bipolar disorder, can lead to oxidative stress and apoptosis in neurons (Machado-Vieira et al., 2009).

Lithium, the lightest of all metals, is often used to treat bipolar disorder as a mood stabilizer and has been found to have a neuroprotective effect, that is lithium acts to protect neurons in oxidative stress (Andreazza & Young, 2014). Since the discovery of lithium’s effectiveness against mania in 1949, protective effects have been the most expected and replicated uses of lithium. By enhancing neuroprotection with lithium, researchers intended to slow or halt the progression of neuronal loss to prevent the onset of bipolar disorder or clinical decline. Lithium has this neuroprotective effect by inducing multiple biochemical and molecular effects which have been linked to the activation of neurotrophic pathways involved in the physiological processes of bipolar disorder. Lithium affects neurotransmitter and receptor-mediated signaling, signal transduction cascades, hormonal and circadian


regulation, ion transport and gene expression (Machado-Vieira et al., 2009). Through these processes, lithium is able to reduce apoptosis from oxidative stress, thus carrying out its neuroprotective effects (Andreazza & Young, 2014). Similarly, Tam et al. (2014) found that when compared to the control, lithium-treated C. elegans showed a significantly higher ATP production. Their findings suggest lithium can increase the overall energy output of mitochondria (Tam et al., 2014).

In this experiment, chicken, Gallus gallus, embryo neurons are used. The chicken’s nervous system development is somewhat similar to a human’s, and because chick development occurs within days they are a model organism for dissection. The embryos used in this study were 10 days old by the time of dissection; their nervous system was not yet developed to the point where pain can occur but was developed to the point at which the peripheral nervous system can be identified (JoVe Science Education Database, 2015).

In this study, I hypothesized that lithium would increase mitochondrial activity in Gallus gallus single neurons in comparison to those not treated with lithium. With it's neuroprotective effects, lithium would work on the cell’s mitochondria to increase productivity and ATP production, and therefore increase mitochondrial activity (Andreazza & Young, 2014) (Tam et al., 2014).

To measure mitochondrial activity in single neurons, a vital fluorescent dye was used to stain mitochondria red. This dye, MitoTracker, uses the mitochondria membrane potential to enter the organelles. MitoTracker is chemically reactive and after linking to the thiol groups in mitochondria, it is permanently bound to the mitochondria. The dye becomes brighter to signify higher activity because of this increased membrane potential and charge (Chazotte, 2011). In this study, I focus only on the brightness of the dye signifying the level of mitochondrial activity and the differences between the experimental and control. Because I hypothesized lithium would increase mitochondrial activity in comparison to the control, neurons treated with lithium should show higher brightness than control neurons.
Materials and Methods

Dissection and Cell Culture

Dissection followed Professor Morris’ protocol in Primary Culture of Chick Embryonic Peripheral Neurons 1: Dissection (Morris, 2015a) to harvest 40 dorsal root ganglia and 6 sympathetic nerve chains from three G. gallus embryos.

Control and Experimental Procedures

Control and experimental procedures followed parts A and B of Primary Culture of Chick Embryonic Peripheral Neurons 2: Observation of Live Unlabeled Cells (Morris, 2015b) and part II of Primary Culture of Chick Embryonic Peripheral Neurons 3: Staining and Observation of Live Cells (Morris, 2015c) with a modification. The modification was having an observation chamber rather than a flow chamber, so all four sides were sealed with VALAP; this was because only one treatment of lithium was used so a flow chamber was not needed to continue to add lithium. A simplified version of the procedure is as follows:

Control: Obtain two dishes of cells and remove growth medium. Add 1.5ml of 1:100 water into serum free medium (SFM) into each dish. Incubate 4 hours at 37°C. Wash out water in SFM with Hank’s Balanced Salt Solution (HBSS) three times, the last time with SFM. Remove SFM, add 100nM MitoTracker vital dye in SFM into each dish. Incubate 10 minutes at 37°C. Wash three times with HBSS and replace with original medium. Create observation chamber for each coverslip with cells.

Experimental: Obtain four dishes of cells and remove growth medium. Add 1.5ml of 10mM LiCl in water into each dish. Incubate 4 hours at 37°C. Wash out LiCl in water with HBSS three times, the last time with SFM. Remove SFM, add 100nM MitoTracker vital dye in SFM into each dish. Incubate 10 minutes at 37°C. Wash three times with HBSS and replace with original medium. Create observation chamber for each coverslip with cells.
For this, I collaborated with Kara Coraccio, Shiqi Liu, Angie Mirabella, Madeline Parker and Kaitlyn Solano. Incubation in 10mM LiCl was for 4 hours because Kim and Thayer (2008) found that as early as 4 hours of exposure to 5mM lithium, there was significant change in cell structure (Kim & Thayer, 2008). However, my collaborators and I decided that with one control and one level of dosage for our experimental, 4 hours with a high dose of 10mM LiCl would show amplified results and contrast the presence and absence of lithium’s effects between the control and experimental trials.

**Equipment**

Throughout data collection and analysis in this experiment, we used a Nikon Eclipse E200 fluorescent microscope with a SPOT Insight 2 camera and a Nikon 1.0X camera mount, with one iMac computer (Scorpio) complete with Mac OS X Yosemite 10.10.4 software. We used SPOT software for image collection and ImageJ 1.49 software for data analysis. All equipment was provided by Wheaton College in Norton, Massachusetts in the Imaging Center for Undergraduate Collaboration, ICUC, of the Mars Center for Science and Technology.

**Data and Image Analysis**

These data were calculated from six neurons, two control neurons and four lithium-treated neurons (n=6). A fluorescent light image and a transmitted light image still for each single neuron were selected based off clarity and quality of the transmitted light image. The neurons chosen had to be single neurons, not interacting with other neurons. These images were taken using SPOT to capture live image stills of single neurons at 40x objective lens. All fluorescent images were at 500ms exposures. Data analysis of the images was based off Data Analysis in The Effects of Mercury on Mitochondria Abundance in *Gallus gallus* Embryo Glial Cells with some modifications (Crespo, 2014). The analysis was completed as follows: all six images of neurons were opened in ImageJ on the Scorpio Mac computer in the ICUC. Background hot spots, 150x150 pixel squares of the background near the cell, were selected and brightness was measured using the “Measure” option under the “Analyze” tab. The
darkest image’s brightness level, 2.191, was used with the other image’s brightness level to calculate a divisor with the following equation:

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\frac{\text{Lighter image hot spot brightness level}}{2.191} = \text{divisor}
\]

The divisor was then used to create a baseline background brightness through ImageJ using the the “Process” tab >> “Math” option >> “Divide” option and entering the divisor in. Once the baseline background brightness of 2.191 was applied to all of the images, images could be compared.

In ImageJ, the “Freehand” tool was used to draw around the perimeter of the neurons in the transmitted light images to ensure the entire neuron would be included when calculating brightness. This selection shape was then copied and put on the corresponding fluorescent image. From this selection, the “Measure” tool was again used to calculate brightness level within the neuron. For both conditions, the brightness levels were averaged to find a mean brightness level for both the control neurons and the lithium-treated neurons.

**Results**

All captured fluorescent images of single neurons (n=6) showed mitochondrial activity, indicated in red, because the MitoTracker dye was both visible and bright within the mitochondria. It was found that lithium-treated neurons showed a higher brightness than control neurons. Comparison could be made between all of images because images were altered to have a baseline background brightness of 2.191. The following images show one example of a control neuron and one example of a lithium-treated neuron.

**Figure 1:** A 75% zoomed image of a single neuron without lithium treatment at 40x magnification seen with transmitted light microscopy. The region outlined with the yellow line indicates the perimeter of the neuron, this shape was then used on the fluorescent light microscopy image, Figure 2, to indicate the outline of the neuron and where mitochondria may be. Image generated in collaboration with Kaitlyn Solano, Angie Mirabella, Madeline Parker, Kara Coraccio, and Shiqi Liu.
Figure 2: A single neuron without lithium treatment and stained with MitoTracker Orange at 40x magnification after a 500ms exposure of fluorescent light. The region outlined with the yellow line indicates where mitochondria are, this outline was drawn on the transmitted light microscopy image, Figure 1, and the selection was copied onto the fluorescent image. Image generated in collaboration with Kaitlyn Solano, Angie Mirabella, Madeline Parker, Kara Coraccio, and Shiqi Liu.
**Figure 3:** A single neuron with lithium treatment at 40x magnification seen with transmitted light microscopy. The region outlined with the yellow line indicates the perimeter of the neuron, this shape was then used on the fluorescent light microscopy image, Figure 4, to indicate the outline of the neuron and where mitochondria may be. Image generated in collaboration with Kaitlyn Solano, Angie Mirabella, Madeline Parker, Kara Coraccio, and Shiqi Liu.
Figure 4: A single neuron with lithium treatment and stained with MitoTracker Orange at 40x magnification after a 500ms exposure of fluorescent light. The region outlined with the yellow line indicates where mitochondria are, this outline was drawn on the transmitted light microscopy image, Figure 3, and the selection was copied onto the fluorescent image. Image generated in collaboration with Kaitlyn Solano, Angie Mirabella, Madeline Parker, Kara Coraccio, and Shiqi Liu.
Figure 5: Comparison of the mean brightness of the outlined areas, as shown in Figures 2 and 4, of the two control neurons and four lithium-treated neurons (n=6). The mean brightness of the control cells was 4.068 and the mean brightness of lithium-treated cells was 10.979. Comparison could be made between images because they both have a baseline background brightness of 2.191.

Discussion and Conclusions

The results of this experiment support my hypothesis that lithium would increase mitochondrial activity in *Gallus gallus* single neurons. The single neurons that were exposed to lithium showed much higher brightness than control neurons implying higher mitochondrial activity because of the evident increased membrane potential and charge. However, due to my limited data (n=6), these results merely suggest this effect of lithium, more research is necessary for a just conclusion.

If this experiment had enough of the same result for statistical significance, the conclusion could be drawn that lithium has a mitochondrial productivity increasing effect not only on neurons in oxidative stress from bipolar disorder as documented in the literature, but also on neurons not in oxidative stress like those of the *Gallus gallus* embryo we dissected (Machado-Vieira et al., 2009).
Although Tam et al. (2014) focused on lifespan they also looked at effects of 10mM LiCl exposure on mitochondrial activity (Tam et al., 2014). But instead of Gallus gallus single neurons, they studied whole C. elegans. Tam et al. found that control worms showed an age-related decrease in ATP production while lithium-treated worms showed a significantly higher ATP production (Tam et al., 2014). Their findings that suggest lithium can increase the overall energy of mitochondria in C. elegans are similar to our findings of lithium’s effects of increasing mitochondrial activity in single neurons of Gallus gallus.

The main limitation in this study could be the limited data collected. Data analysis was only conducted on two control neurons and four lithium-treated neurons, which restricts our findings and cannot imply statistical significance or lack there of. One source of error could have been the negative effects of possible bleaching. If the MitoTracker dye is exposed to too much light it can bleach and no longer be active. During the procedures of washes and time spent under the microscope, the dye could have been bleaching more or less for one slide than another. This would have a large effect on the fluorescent images and therefore on the brightness measurements for analysis.

To better improve this study and for future experiments, more neurons could be imaged and analyzed to create a larger set of data. Lithium could also be given at varying doses ranging between our control and our high dose of 10mM. Different doses could show at what dose lithium’s effects begin to take place. It would be interesting to expand this even more and look at differences in exposure time of lithium and dosage to see how these effects interact.
References Cited


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