The effects of lithium on mitochondrial size and shape in axons from *Gallus gallus*

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I. Introduction

*Gallus gallus* are frequently used in biological research for their easy accessibility in the scientific community and due to being a well-understood biological model for studying mechanisms of early development. The easy accessibility of *Gallus gallus* is critical to studies ranging from watching normal development versus perturbed development at a microscopic to a plainly visible level. In this experiment, *Gallus gallus* embryos were used to study growing neurons, specifically their axons, to better understand the differences of size and shape in mitochondria in a normal state versus mitochondria in a lithium-perturbed state.

Mitochondria are vital to biological research for their role in the production of adenosine-5’-triphosphate (ATP) and carbon dioxide by breaking down fatty acids, amino acids, and carbohydrates in eukaryotic cells (Lodish et al., 2000). Mutations in mitochondrial respiratory chains resulting in dysfunction have been known to be associated with oxidative phosphorylation disorders and mood disorders (Hance & Larsson, 2005). Dysfunction in mitochondria can be found in mood disorders, bipolar disorder specifically, and is visible in patterns of brain metabolism and the effects of mood stabilizers in these individuals (Hroudová et al., 2013). In order to observe
mitochondria, fluorescent microscopy and MitoTracker Orange CM-TMRos were utilized. MitoTracker is a lipophilic cationic mitochondrial dye, which passively diffuses across the plasma membrane and accumulates in active mitochondria (Molecular Probes, 2008).

Unlike methyl mercury, lithium, a known mood stabilizer, is not a tool commonly used for perturbation in cell cultures, therefore not much is known on its effects at the cellular or neural level. Little is understood about the effects of lithium on mitochondrial size, shape, abundance, and activity. Studies that have been done using lithium as a perturbation have promising results, specifically for the treatment of bipolar disorder and other diseases (Chuang et al., 2002; Maucer et al., 2009). In addition to lithium being used to treat for bipolar disorder, mitochondrial abnormalities have been shown to also correlate with a bipolar disorder diagnosis (Kato & Kato, 2000), which may suggest that lithium could impact the physical appearance, in the form of its size or shape, of mitochondria. In addition, when lithium is added to saline treated WT and saline treated G931, it was shown to decrease the size of mitochondria of Mus musculus’ cervical and lumbar spinal cord, while subsequently increasing the number of mitochondria (Fornai et al., 2008).

The following studies suggest that lithium being used as a tool for perturbation for neurons could show promising results. In order to progress our knowledge of how lithium can be used to help treat bipolar disorder, it will be useful to know its effects on mitochondria. Therefore, studying the effects of lithium on mitochondrial size and shape in axons from Gallus gallus could prove to be useful in understanding lithium’s practical uses, in addition to its effects at the cellular level. In our study, we tested the effects of
embryonic exposure to lithium on *Gallus gallus* axons to test the hypothesis that the size and shape of mitochondria are impacted by the presence of lithium, resulting in a quantifiable decrease in size and a more rounded oval appearance of these mitochondria.

II. Materials and Methods

*Coverslip Cleaning, Dissection, and Plating*

Coverslips were cleaned and prepared to function as viable and sustainable environments for the dissected *Gallus gallus* embryos. This procedure was originally designed by Peter J. Hollenbeck at Purdue University in Indiana, and was modified for this study by Robert L. Morris at Wheaton College in Massachusetts (Morris, 2015a). No changes were made to this procedure, although it is important to note that the type of alcohol utilized for cleaning these coverslips was 100% concentrated ethanol. Forty dorsal root ganglia and six sympathetic nerve chains were harvested from the three 10-day-old *Gallus gallus* embryos dissected for use in this experiment. Each were partially dissociated with trypsin and plated on Poly-Lysine and laminin treated coverslips.

*Lithium Perturbation*

Once the *Gallus gallus* embryos’ dorsal root ganglia and sympathetic nerve chains were removed, they were kept on the treated coverslips in growth medium, and were left in an incubator at 37 °Celsius. The growth medium used was discarded and 1.5 mL of 10 mM lithium chloride, diluted from 1 M lithium in a water stock solution (1:100 dilution of this stock solution in serum-free medium), was quickly added to the experimental cultures so the perturbation could take place. This lithium perturbation only
occurred in the four experimental petri dishes. The two remaining control coverslips did not receive lithium, but the growth medium was extracted and re-added to provide the same environmental change. The experimental and control conditions were both kept in a small incubator for four hours at 37 ° Celsius.

After the four hours, the lithium solution was removed; 1.5 mL of Hanks’ Balanced Salt Solution (HBSS) was quickly added to each culture to avoid drying. HBSS was also added to the control conditions. HBSS was kept on the coverslips for five minutes at a time, and was replaced with new HBSS. These washes took place simultaneously after one another three times, lasting about fifteen minutes total, to ensure that the lithium perturbation only lasted the four hours. Once the washes were complete, all the coverslips were placed in serum-free medium, which was used as a buffer. The leftover lithium solution, which had been previously set aside upon being discarded, was added to a waste container.

**Addition of MitoTracker**

The serum-free medium was removed to add the 100 nM of MitoTracker Orange CM-TMRos in DMSO for a 1:100 dilution, and 1.5 mL of this MitoTracker solution was used in each condition. The addition of MitoTracker was based off of a procedure for staining and observing cells (Morris, 2015c). Each coverslip culture was exposed to the MitoTracker for fifteen minutes after an initial exposure of two minutes was not long enough. During exposure, the petri dishes were kept in the incubator at 37 ° Celsius. After the fifteen minutes had passed, we conducted three additional washes of HBSS, and then re-added the serum-free medium, following a similar procedure that had been utilized after the lithium solution was removed. Once the washes were completed, forceps
were used to remove the coverslip from the serum-free medium, and a kimwip was carefully side along the side of the coverslip without the neurons, and placed on the slide with the chip chamber procedure.

**Observation of Mitochondria in Axons and Data Analysis**

By utilizing microscopes in the ICUC lab, located at Wheaton College in Norton Massachusetts, images of each neuron found in the experimental coverslip cultures and control coverslip cultures were taken using a SPOT imaging camera 1X with Spot software the same day that the perturbation occurred. These images were taken in both transmitted light microscopy in addition to fluorescent microscopy at 40x magnification in Phase 2 on Nikon-E-200 microscopes to study each individual mitochondrion in the present axons. The typical exposure time for the fluorescent microscopy was 2.5 seconds in the control group. The exposures were as short as 0.5 seconds in the experimental group. The differences in exposure times were accounted for by using ImageJ’s Analyze -> Histogram feature to get the average brightness value for each image and comparing it against one another by using the Process -> Math feature of ImageJ. This was done in collaboration with Walker Fuchs and Angela Mirabella.

The coverslips of both the experimental and control conditions were utilized to create chip and was sealed on all sides with VALAP to prevent the culture from drying out while being observed. To provide as accurate of an environment to these neurons as possible, a heater set to 37 °Celsius and a thermometer were placed next to the chip chambers while they were being observed under the microscope (Morris, 2015b). The chip chambers and images taken were done in collaboration with Shiqi Liu, Angela Mirabella, Kara Coraccio, Kaitlyn Solano, and Ellen Fosset.
The microscope was adjusted for Koehler Illumination, and was connected to an iMacintosh 2013 desktop running on OS X Yosemite Version 10.10.4, which was nicknamed “Virgo”. The images taken using the SPOT cameras were used to analyze the size and shapes of the individual mitochondrion in the axons in both the control and experimental conditions. In order to use these observations to create and analyze data, shape and size of the mitochondria were quantified using a decoding sheet. This decoding sheet differentiated between the following: the shape of the mitochondria (rounded oval shape or elongated oval shape) in addition to the size of the mitochondria (small or large). Once the individual images were decoded (as described below), the relative sizes and shapes were totaled and used to create two bar graphs in Microsoft Excel. Having the control condition was necessary to see if there was a significant difference in the size and shape of the mitochondria in axons of the coverslip cultures treated with lithium and the coverslip cultures that were not treated with the lithium.

The brightness and size of the mitochondria in the axons from the fluorescent images were analyzed using ImageJ 1.50a and Adobe Photoshop Elements 6. To quantify individual mitochondria in this experiment, any area lit up from MitoTracker that had brightness value fewer than 30 lumens in ImageJ was not considered to be mitochondria. Brightness was also utilized to determine if more elongated mitochondrion were overlapping with other mitochondrion by looking at the edges of individual mitochondrion. If there appeared to be two rounded edges touching, they would be considered separate mitochondrion. Pixel width was measured and accounted for in Adobe Photoshop Elements 6, and converted into micrometers. The average size for the mitochondria was 0.7 micrometers. Therefore, any mitochondria that was less than 0.7
micrometers was put in the small category, and anything above 0.7 micrometers was put in the large category. Mitochondria that were unevenly distributed through length and width were considered elongated, while mitochondria that had an equal width to length ratio were put in the rounded category for size.

Mitochondria shape and size of both the lithium perturbed experimental conditions and the control conditions were analyzed separately, and then were analyzed together in this experiment. Four fluorescent microscopy images for the control condition containing 10 axons with 145 total mitochondria \((n = 145)\) and four fluorescent microscopy images for the experimental condition containing 12 axons with 115 total mitochondria \((n = 115)\) were utilized. The total of the mitochondria for each condition, control and experimental, in each category of size and shape, were divided by the amount of total axons, to account for the difference in total mitochondria between groups. The fluorescent images for both the experimental and control conditions are those that have a black background with lit up red areas, which are the result of the exposure of MitoTracker under the red filter on the fluorescent microscope. These fluorescent images were compared and overlaid at 50% opacity with their respective light microscopy images to be sure only mitochondria in the axons were accounted for in the data analysis.
III. Results

In the control group, 10 different axons containing 145 mitochondria were utilized to study size and shape of each individual mitochondria (Figure 2a-d). In the experimental group, 12 different axons containing 115 mitochondria were utilized to study size and shape of each perturbed mitochondria (Figure 3a-d). Shape and size of mitochondria for this experiment was investigated using a decoding sheet (Figure 1). While each mitochondrion was being analyzed, this decoding sheet was utilized to be sure each was being treated with the same attention to detail.

<table>
<thead>
<tr>
<th>Size</th>
<th>Shape</th>
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<tbody>
<tr>
<td>Small</td>
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<tr>
<td></td>
<td><img src="image" alt="Small Mitochondrion" /></td>
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<tr>
<td>Large</td>
<td>Elongated</td>
</tr>
<tr>
<td></td>
<td><img src="image" alt="Large Mitochondrion" /></td>
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*Figure 1.* Decoding sheet showing the various average sample sizes of small mitochondria and large mitochondria for size on the left-hand side. On the right-hand side are average sample shapes of rounded oval shaped mitochondria and elongated oval shaped mitochondria.
Figure 2a. Fluorescent microscopy image of one axon in control group at 40x magnification. Image taken in collaboration with Angela Mirabella.

Figure 2b. Transmitted microscopy image of control group at 40x magnification. Image taken in collaboration with Angela Mirabella.
Figure 2c. Transmitted microscopy image of control group overlaid on fluorescent microscopy of image at 50% opacity.

Figure 2d. Each mitochondrion was labeled in this fluorescent microscopy image of control group at 40x magnification.
Figure 3a. Fluorescent microscopy image of two axons in experimental group at 40x magnification. Image taken in collaboration with Kaitlyn Solano and Ellen Fosset.

Figure 3b. Transmitted microscopy image of experimental group at 40x magnification. Image taken in collaboration with Kaitlyn Solano and Ellen Fosset.
Figure 3c. Transmitted microscopy image of experimental group overlaid on fluorescent microscopy of image at 50% opacity.

Figure 3d. Each mitochondrion was labeled in this fluorescent microscopy image of experimental group at 40x magnification.
Mean percentage values were used for both the mitochondria of the experimental and control conditions to account for the different sample sizes. These mean percentage values for the mitochondria in each condition type were utilized in each category for quantified size and shape.

**Figure 4a.** Graph showing the average percentage of mitochondria in each condition type, control and experimental, with respect to quantified size, small or large, while also accounting for standard deviation between each condition type in each category.
Figure 4b. Graph showing the average percentage of mitochondria in each condition type, control and experimental, with respect to quantified shape, rounded or elongated, while also accounting for standard deviation between each condition type in each category.
IV. Discussion

Due to the commonality of small, rounded mitochondria in both the control and not the experimental condition, our hypothesis that the effects of exposure to lithium on *Gallus gallus* neurons would show a significant decrease in size and more rounded shape of mitochondria in axons due to the presence of lithium was refuted. The mitochondria in the control condition type were more likely to be smaller in size than larger in size, while also accounting for standard deviation. This observation was not found in the experimental group. The mean percentage of mitochondria in the control condition was more frequently rounded in shape and smaller in size, while the mean percentage of mitochondria in the experimental condition were approximately the same between rounded or elongated in shape and smaller or larger in size. Further experimentation would need to be conducted to come to a conclusion on whether more rounded and smaller mitochondria are more typically found in unperturbed axons in comparison to lithium-perturbed axons.

There were a few setbacks in this experiment. The sample size of mitochondria drastically ranged between conditions, even when utilizing four fluorescent images with almost the same amount of total axons. Parts of the axons were also cut off in some images. While these setbacks likely did not skew the results, having consistency in the total number of axons and mitochondria would have insightful. In future research, it would be useful to have more consistency in the number of mitochondria and their visibility in axons. Another shortcoming of this study was that another experimental group with a longer exposure to lithium could be been used to check for an effect in roundness of shape and smallness of size. This longer exposure time could be compared
against the four hour long exposure to see if other variations of lithium perturbation have an effect on size and shape in a quantifiable way.

While decoding the mitochondria, it was difficult to account for overlapping areas, as the microscope and SPOT software turned three-dimensional objects into two-dimensional images. Brightness was quantified in ImageJ to account for what was mitochondrion and what was not, as described in the Methods section. This same technique was also utilized to determine if elongated mitochondrion were overlapping with other mitochondrion by looking at the brightness with respect to the mitochondrion’s edges. Unfortunately, this technique could not ensure complete accuracy. In addition to potential overlap error, there may have been a source of error while accounting for the differences in exposure times for the fluorescence microscopy. While normalizing the background, mitochondria that may have been previously above 30 lumens in brightness may not have been accounted for in this study.

It would be worth looking into how various lithium concentrations effect mitochondria, since little is understood about the effects of lithium perturbation on mitochondria. Testing various lithium concentrations would show whether or not the same observations would be found if the experiment was done repeatedly. Understanding lithium as a tool for perturbation is important in understanding how to treat bipolar disorder, as mitochondria abnormalities and lithium have been observed in patients with bipolar disorder (Chuang et al., 2002). More experimentation will need to be conducted to account for the shortcomings of this experiment, in addition to better understanding the role lithium and mitochondria play in bipolar disorder to come to conclusions about how lithium can be utilized in a practical setting.
V. References


