The Effects of Lithium on Mitochondria Membrane Potential of Glia Interacting with Neurons of *Gallus gallus* Embryonic Peripheral Neurons Growing *in vitro* Using Fluorescence Light Microscopy

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Introduction

Mitochondria are essential for eukaryotic cell metabolism due to their role in the production of metabolic energy from the breakdown of lipids and carbohydrates and the subsequent conversion of this energy into ATP via oxidative phosphorylation (Cooper, 2000). Mitochondria are unique organelles in part due to their double-membrane system. The cristae or inner membrane folds extend into the matrix which contains mitochondrial genetic information and the enzymes that catalyze oxidative metabolism (Cooper, 2000). The proton gradient that drives ATP synthesis is maintained by the impermeability of the inner membrane to most ions and small molecules, as opposed to the freely permeable outer membrane which is studded with porins that allow for small molecules to be transported in and out (Cooper, 2000). The proton gradient is comprised of a membrane potential and a pH gradient. Thus in order to draw conclusions on cell metabolism and how it is affected by foreign molecules, the activity of the mitochondrial membrane potential (MMP) is a good cog in the system to focus on.

In this study we dissected 10 day old *Gallus gallus* embryos in order to isolate and grow primary cultures of sympathetic neurons, and to perturb these cells with an experimental

concentration of lithium to see the effect of this ion on the cellular level, by staining and labeling cell cultures with MitoTracker® Orange and imaging the results using fluorescent light microscopy and comparing the amount of cell fluorescence between the experimental and control cultures as a representation of MMP.

MitoTracker® Orange CMTMRos is the means by which we will be able to analyze MMP in this study (Johnson et. al., 2010). MitoTracker® Orange is a fixable mitochodrion-selective, membrane-potential-dependent probe, comprised of tetramethylrosamine, an orange-fluorescent dye, and mildly thiol-reactive chloromethyl moiety, that can diffuse passively through the plasma membrane and mitochondrial membrane to accumulate in and stain mitochondria of living cells (Johnson et. al., 2010). The ability for the dye to remain associated with the mitochondria after fixation is attributed to the chloromethyl group. Standard mitochondrial fluorescent stains, rhodamine 123 and tetramethylrosamine lack this ability and tend to be washed out of the cells once MMP is lost (Johnson et. al., 2010). The dye is excited by green fluorescent light (554nm) and emits in the red spectrum (576nm) (Johnson et. al., 2010). The stable retention allows for MitoTracker® Orange and other MitoTracker® reagents to be used in studies like this and those more complex that may require live cells during immynocytochemistry, *in situ* hybridization, or electron microscopy (Johnston et. al. 2010).

While the effects of lithium as a mood-stabilizer, including as a treatment for bipolar disorder, have been documented for many years and in abundance, the mechanism of the action of lithium is not known (Kim and Thayer, 2009). A study has been conducted within the last 10 years researching the underlying effects of lithium in patients with bipolar disorder on the number of synaptic connections between hippocampal neurons in culture (Kim and Thayer, 2009). The researchers developed their own imaging-based assay able to detect small

concentrations of green fluorescence produced by the fusion of postsynaptic density protein 95 to green fluorescent protein (Kim and Thayer, 2009). The hypothesis that lithium increased these synaptic connections was supported. Another study supported the hypothesis that lithium alters the morphology of neurites to promote regeneration and functional recovery in a differential, concentration-dependent fashion (Shah et al., 2013). Both of these studies broadly show a stimulatory relationship between lithium and neurons, therefore in this study it is proposed that there will be a stimulatory relationship between lithium and the *Gallus gallus* sympathetic neurons.

This study tested the hypothesis that the perturbation of *Gallus gallus* embryonic sympathetic neurons with Lithium will show an increase of the membrane potential of mitochondria of glial cells that are interacting with neurons.

Materials and Methods

Dissection

Sympathetic nerve chains and dorsal root ganglia were dissected from 10 day old *Gallus gallus*, or chick, embryos and plated on poly-L-lysine and laminin treated coverslips as per the protocol in "Primary Culture of Chick Embryonic Peripheral Neurons 1: Dissection" (Morris, 2015a).

Treatment

For the coverslips undergoing Lithium perturbation, the F+ medium used to culture the neurons following the dissection was replaced with 1.5mL of 10mM Li in Serum Free Medium, SFM, (diluted 1:100 from a 1M LiCl in sterile water stock solution) (adapted from Machado-Vieira et. al., 2010). The coverslips were incubated at 37°C for four hours (adapted from Kim &

Thayer, 2009), after which the lithium solution was removed and the coverslips underwent two washes with HBSS and one wash with SFM with 5 minute incubations. The control coverslips were treated identically, except for that they received a perturbation of a 1:100 dilution of sterile water in SFM.

Fluorescent Labeling

The SFM was then removed from the coverslips, both the experimental and control, and saved for later use. The cells were stained according to the protocol in part II of "Primary Culture of Chick Embryonic Peripheral Neurons 3: Staining and Observation of Live Cells" (Morris, 2015c) with a few specifications. The vital dye used was 1.5 mL of 100mM MitoTracker® Orange CMTMRos in DMSO. Instead of using DMEM for the washes, the coverslips were washed three times with HBSS and once with the reserved SFM with 5 minute incubations. Tin foil was used to cover the sample as much as possible during these steps in order to prevent light exposure and subsequent bleaching of the samples. A chip chamber was created for each of the coverslips according to the protocol in "Primary Culture of Chick Embryonic Peripheral Neurons 2: Observation of Live Unlabeled Cells" (Morris, 2015b). All methods up to this point were collaborative with Kara Coraccio, Ellen Fossett, Shiqi Liu, Angela Mirabella and Madeline Parker, all of whom are also testing hypotheses involving lithium perturbation and mitochondria characteristics.

Imaging

Imaging of cells using transmitted light was performed according the protocol in "Primary Culture of Chick Embryonic Peripheral Neurons 2: Observation of Live Unlabeled Cells" (Morris, 2015b). After taking a transmitted light photograph of the cell in question, the transmitted light was blocked, the fluorescent microscope was set to excite the sample with green

fluorescent light and capture the image of the subsequent red emission, and images were captured by quickly exposing the sample to the fluorescent beams at a manually set exposure time. A Nikon Eclipse E200 fluorescent microscope with a Spot Insight 2 camera, a 1.0x c-mount, a 40x Nikon Objection with a Phase 2 condenser setting hooked up to the "Scorpio" computer in the Imaging Center for Undergraduate Collaboration of Wheaton College, MA was used to collect all images in Spot Basic 5.2 running on OS X Yosemite 10.10.4. Of the images taken three sets of images (one transmitted and one fluorescent microscopy image in each set) of glia interacting with neurons were selected from the control group and four similar sets of images of glia interacting with neurons were selected from the experimental group. Therefore n=3 for the control group and n=4 for the experimental group. These images were taken in collaboration with Ellen Fossett, Madeline Parker, and Angela Mirabella. Due to a lack of comparable images between the control and experiment sets in the first round of experimentation, a second set of control data was performed in collaboration with Ellen the following week, following the exact same protocols as described above. The control group image sets are a mix between week one and week two data, however, no difference is expected between the images.

Data Analysis

Image J 1.40 software for Windows was used to analyze the area (in pixels), mean gray value (mean pixel intensity), and integrated pixel density (area multiplied by the mean gray value) of the fluorescent images for both the control and experimental images. The fluorescent images were converted to 8-bit grayscale for quantitative comparison. All of the experimental images and one of the control images were taken with an exposure time of 0.5 seconds. Two of the control images were taken with an exposure time of 1.0 second. Because these two images

were taken at a different exposure time, background normalization needed to be performed. This was done by adapting the protocol of Hazie Crespo and Michael Cummings (2014). The experimental images were taken at the lower exposure with the darker background; therefore, the two control images were adjusted to match those references. The transmitted light images were overlaid on the fluorescent images in ImageJ so that it would be clear to see where the structures of the cells were and where the background was. Three areas of the same size were measured around each experimental glial cell in the fluorescent image and an average mean gray value for the background was calculated for each image. An average was then taken between all three images and was found to be 2.24. The same area box was used to measure three areas around each control glial cell in question and the average mean values for the background were found to be 3.84 and 2.61. These numbers were divided by 2.24, and multiplied by the ratio of the control exposure time to the experimental exposure time (1.0s/ 0.5s = 2) to yield 3.44 and 2.34 respectively. Therefore the value of every pixel of the control image was divided by 3.44 and 2.34 respectively for the two controls with the 1.0s exposure time in order to correct for the effect on the brightness of the image created by the difference in exposure time. This was done using the commands “Process” > “Math” > “Divide” and resulted in the brightness of the fluorescence being reduced to more accurately reflect the simulated effect of uniform exposure.

The corrected total cell fluorescence, CTCF, was then calculated for each cell in order to remove the background from the measured cell fluorescence following the protocol of Amrita University (2012) and Burgess et. al. (2010). Each cell was selected by tracing the periphery of the cell using the freehand selection tool in ImageJ on the transmitted light image overlaid on the fluorescent image. The overlay was removed and the area, mean gray value, and integrated density were calculated for the selected cell. The mean gray value of the background (calculated

as described in the previous paragraph) multiplied by the area of the cell was subtracted from the integrated density of the cell (the area of the cell multiplied by the mean gray value of the cell). This calculation removed the background from the total cell fluorescence measured in order to normalize for differences in background brightness between the images not attributed to differences in fluorescent light exposure time.

Results

The amount of fluorescent light emitted by the glial cells that are interacting with neurons in the samples treated with 10 mM Li in serum free media (the experimental group) and in the control group was able to be quantified.

The following three figures show one of the three glia cells found in the control samples under transmitted light, excited under green fluorescent light with an exposure of 0.5s, and converted to 8-bit grayscale with the glial cell to be analyzed outlined. The isolation and juxtaposition of the glial cell in relationship to the axon it's interacting with and other nearby cells show that the calculation of total cell fluorescence will not be skewed by the overlapping of another cell's mitochondria.

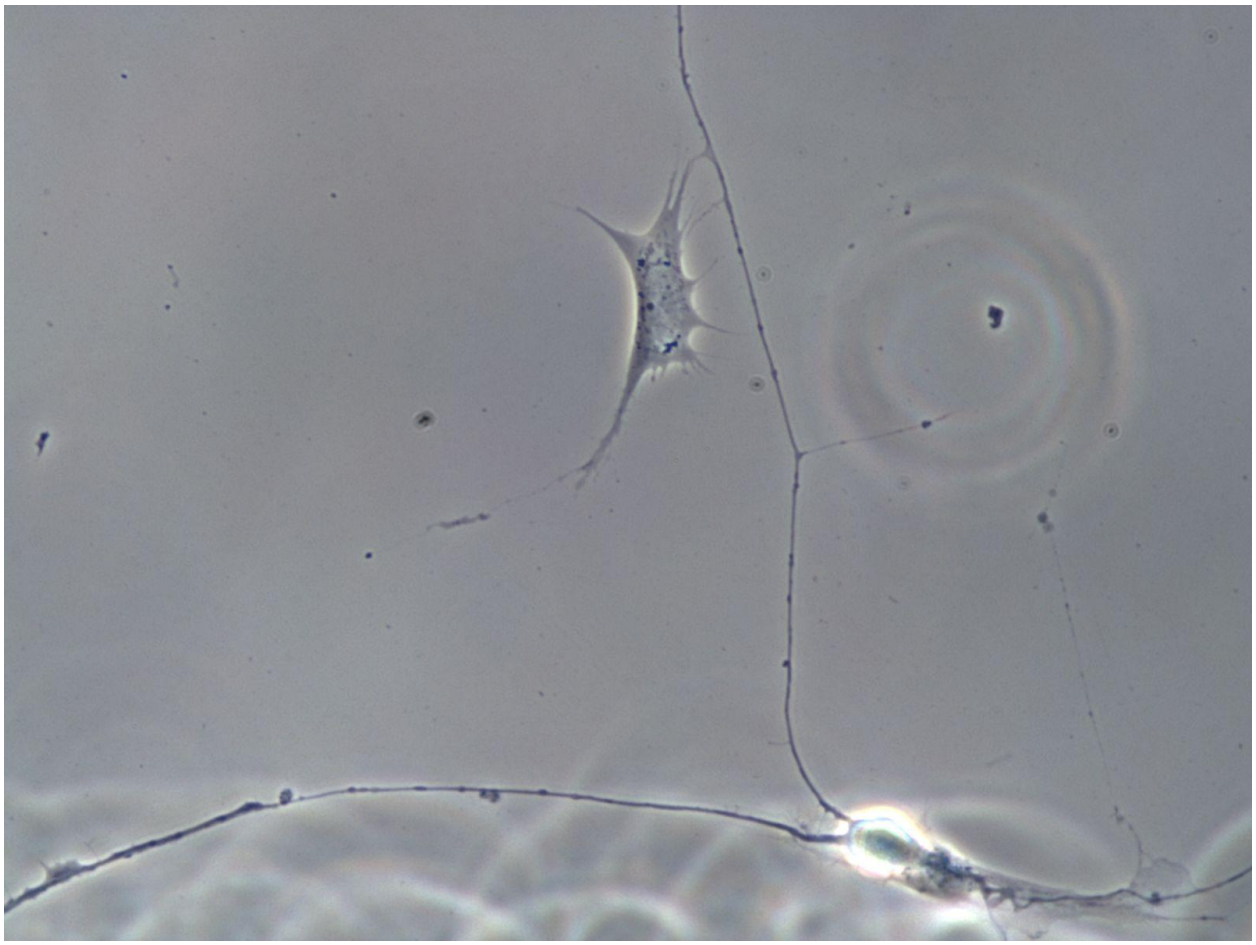

Figure 1. Glial cell interacting with a neuron under transmitted light treated with control solution. The filopodia of the glial cell have made contact with the axon of a nearby neuron. The periphery of the cell is clear and free of interference from other cells. Image was gathered in collaboration with Ellen Fossett.

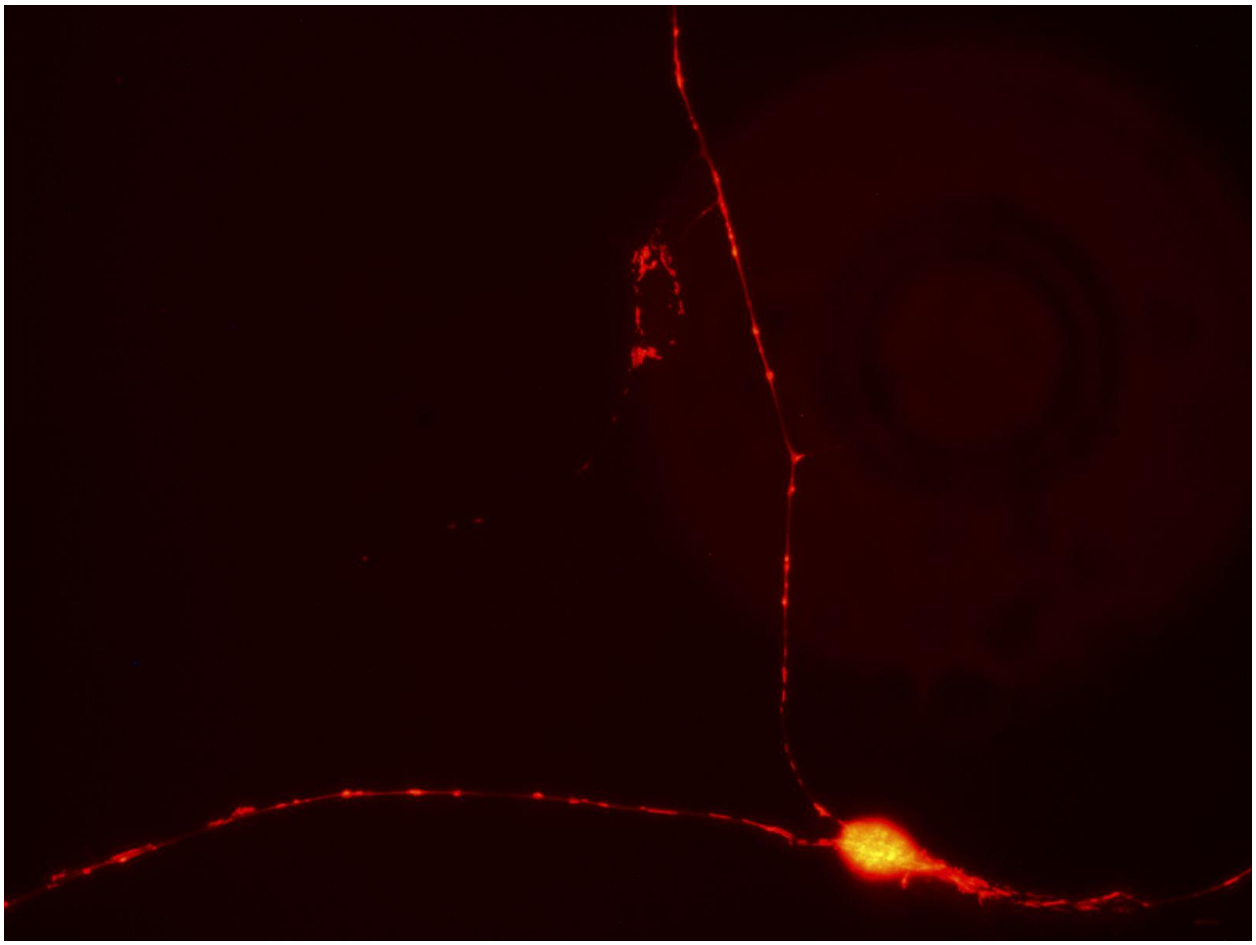

Figure 2. Glial cell interacting with neuron treated with control solution. This is the same subject as Figure 1 captured under 0.5s exposure to fluorescent light. This image shows the mitochondria of the cells labeled with MitoTracker® Orange as red cylindrical structures. Only the mitochondria of the glial cell have been labeled, the cytoplasm and other structures of the cell do not show on this image. The neuron at the bottom right of the figure is overexposed, as determined by the presence of yellow fluorescence however this cell is not being analyzed. Image was gathered in collaboration with Ellen Fossett.

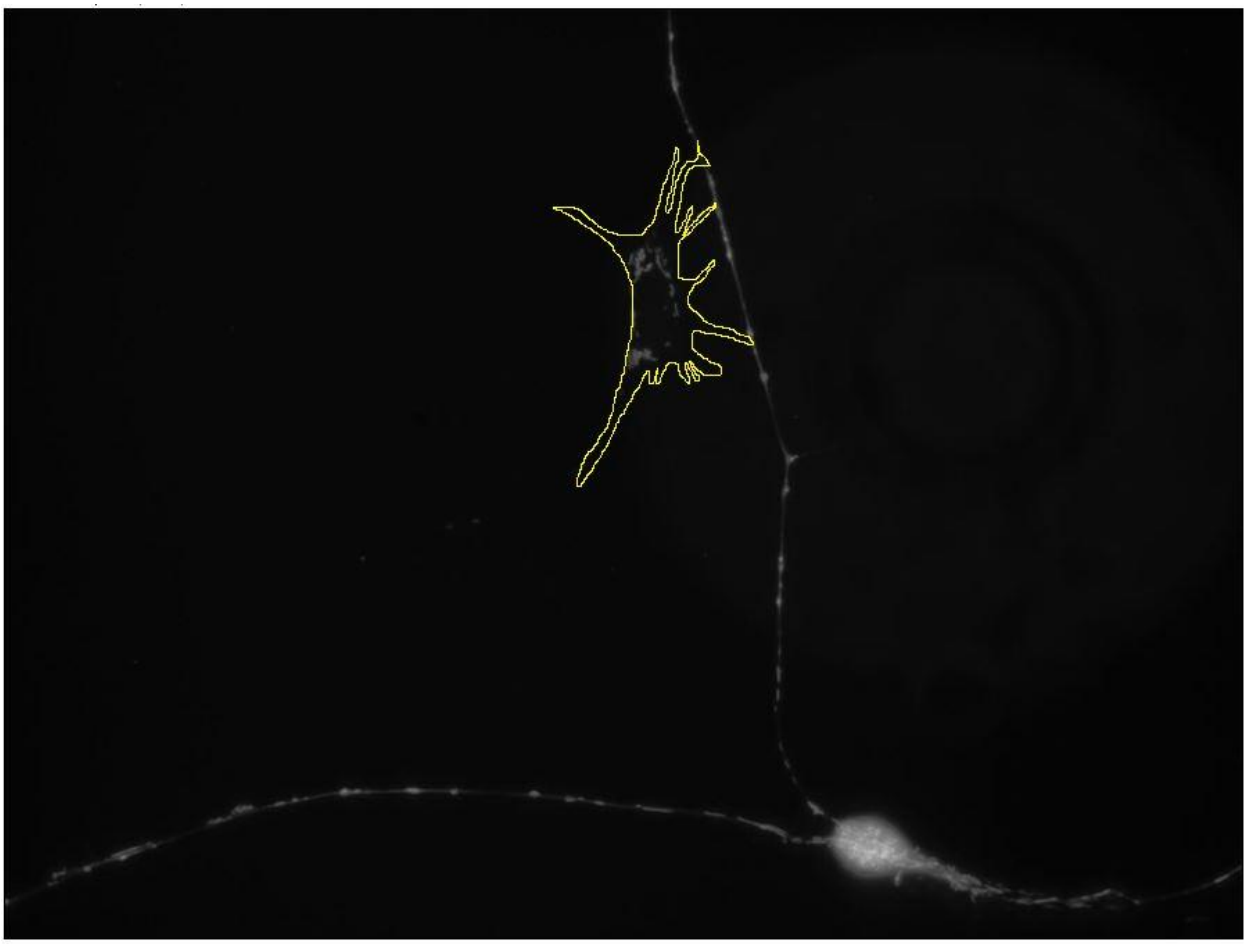

Figure 3. Glial cell interacting with neuron treated with control solution. This image was processed by ImageJ to convert Figure 2 into 8-bit grayscale. The red fluorescence is now represented by shades of gray determined by the variance in brightness. The periphery of the glial cell was traced using the free-form tool on the overlaid transmitted light image (Figure1). The size and shape of the cell are represented by this outline. Using the outline mitochondria can be seen within the cell body and the filopodia.

The following three figures show one of the four glia cells found in the experimental samples under transmitted light, excited under green fluorescent light with an exposure of 500 ms, and converted into 8-bit grayscale with the glial cell to be analyzed outlined. As with the control sample the isolation and juxtaposition of the glial cell show that the calculation of total cell fluorescence will not be skewed by the overlapping of another cell's mitochondria. The halo around the cells, the isotonicity, and the appearance of mitochondria under fluorescent light shows that the experimental concentration of Lithium (10 mM) is not fatally toxic at the cellular level.

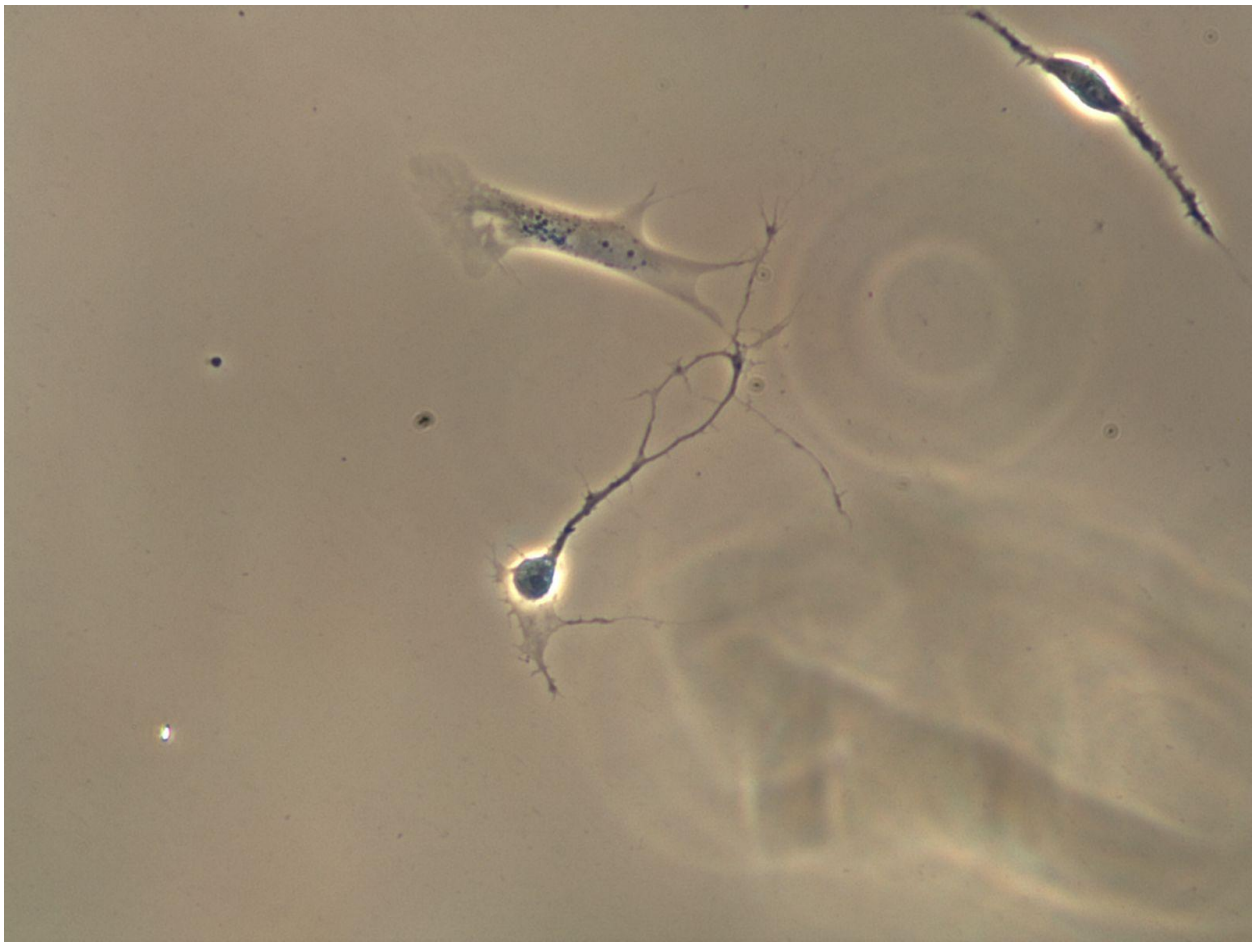

Figure 4. Single neuron and glial cell interacting under transmitted light perturbed with 10mM Li. Similarly to Figure 1 the periphery of the glial cell is clear and not obstructed by other cell structures. Filopodia of this cell can be seen interacting with the dendrites of a nearby neuron. Both cells appear healthy. Image was gathered in collaboration with Ellen Fossett.

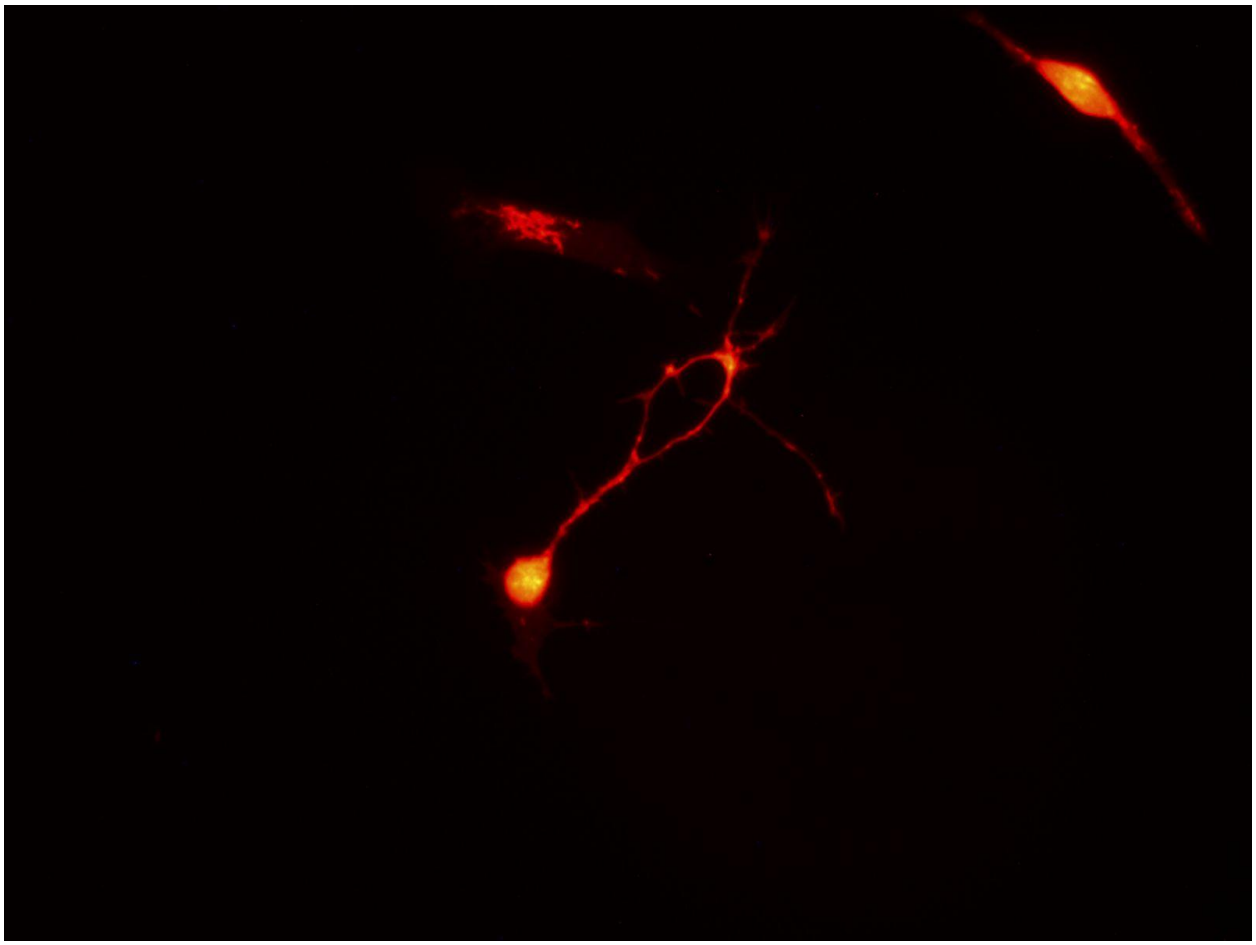

Figure 5. Single neuron and glial cell interacting perturbed with 10mM Li. This is the same subject as Figure 4 captured under 0.5s exposure to fluorescent light. This image shows the mitochondria of the cells labeled with MitoTracker® Orange as red cylindrical structures. Some of the cytoplasm of the cell appears to be labeled in addition to the mitochondria. The neuron in the middle of the figure is overexposed, as determined by the presence of yellow fluorescence however this cell is not being analyzed. Image was gathered in collaboration with Ellen Fossett.

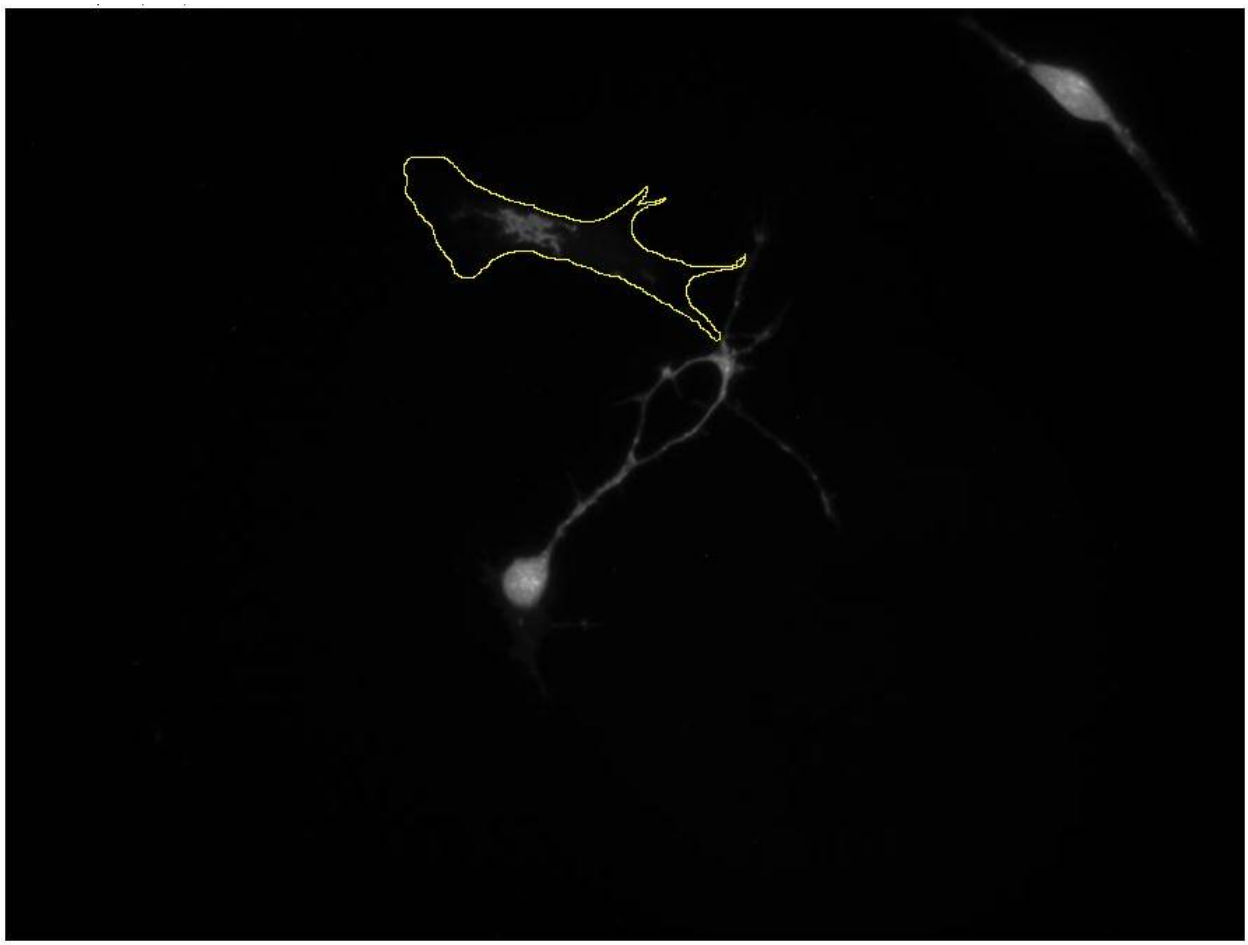

Figure 6. Single neuron and glial cell interacting perturbed with 10mM Li. This image was processed by ImageJ to convert Figure 5 into 8-bit grayscale. The red fluorescence is now represented by shades of gray determined by the variance in brightness. The periphery of the glial cell was traced using the free-form tool on the overlaid transmitted light image (Figure 4). The size and shape of the cell are represented by this outline. Using the outline mitochondria can be seen within the cell body and the filopodia. A lack of mitochondria is seen in the lamellipodia on the left side of the cell.

Figure 7 graphically represents the average corrected total cell fluorescence (CTCF) determined for both the experimental and control groups. The average CTCF for the control group was $3.43 \times 10^5 \pm 3.82 \times 10^5$ experimental group was $1.80 \times 10^5 \pm 9.51 \times 10^4$.

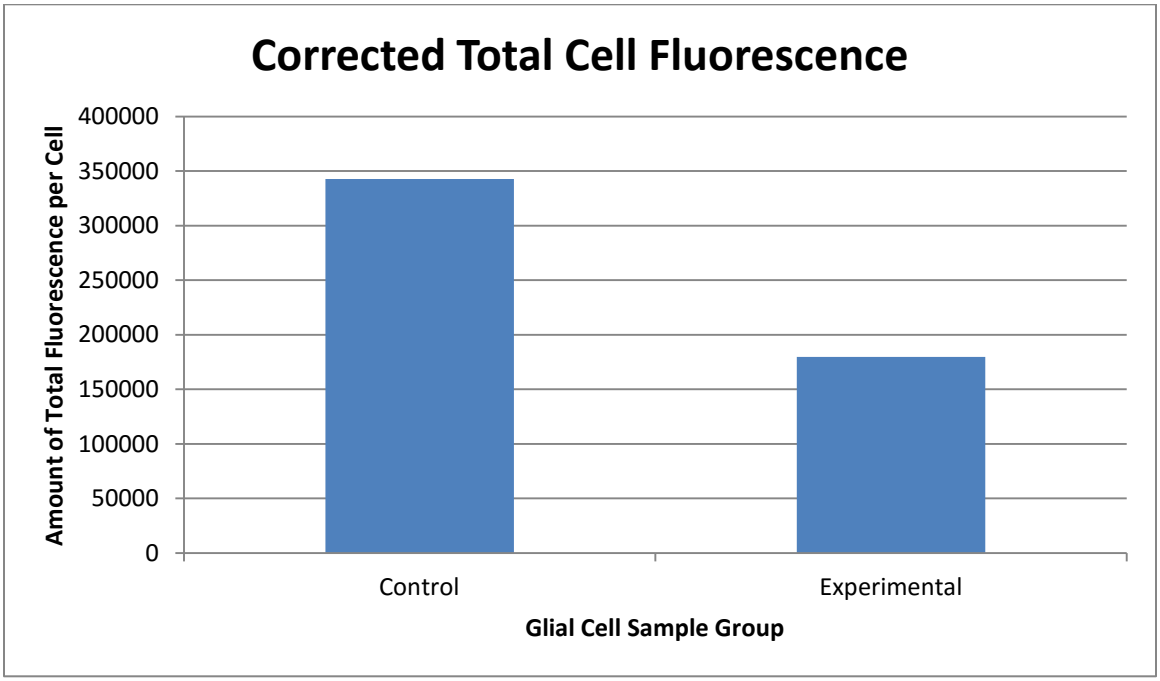

Figure 7. The average amount of corrected total cell fluorescence (CTCF) of the MitoTracker® Orange stained control and experimental samples. The CTCF was calculated for each cell in the sample population, n=3 for the control group and n=4 for the experimental group, and the averages were taken. The averages are on the same order of magnitude.

Discussion

The hypothesis that the perturbation of *Gallus gallus* embryonic sympathetic neurons with lithium will show an increase of the mitochondrial membrane potential of glial cells that are interacting with neurons was not supported by the result that the average amount of total fluorescence was on the same order of magnitude in the glial cells treated with 10mM lithium as the glial cells treated with the control solution. In fact the average CTCF value (arbitrary units) of the lithium experimental group falls within the standard deviation of the control group. From this it is not possible to distinguish the difference in total cell fluorescence between the two groups. Since the amount of fluorescent dye able to accumulate in the cell is dependent on the membrane potential, a greater amount of total cell fluorescence should correspond to an increase in mitochondrial membrane potential (MMP), however this was not supported by my results. The sample sizes of both the control and experimental groups were extremely small, which limits the significance of this study.

The hypothesis that lithium increases MMP was able to be supported by Bachmann et. al. (2009) in SH-SY5Y cells (a human neuroblastoma derived cell line) using the green and red fluorescence ratio in JC-1 stained cells. They found this by studying Methamphetamine-induced reduction of mitochondrial function and showing that lithium (and valproate) counteracts these effects. While some of the mechanisms for how Meth reduces mitochondrial function are known, the mechanisms for how lithium (and valproate) counteract these effects are not known, and they have only been assessed by MMP and mitochondrial oxidation (Bachmann et. al, 2009). It is also important to note that the Bachmann et. al. study involved chronic lithium exposure whereas the exposure in this study involved acute exposure.

Because the JC-1 stain was not available for ratiometric analysis, MitoTracker® Orange and corrected total cell fluorescence (CTCF) were used in the analysis. CTCF is useful in quantitative analysis because it allows for the reduction of background brightness (or background noise) which is a common issue when comparing fluorescent images (Pang et. al. 2011). Fluorescent images also cannot be compared if taken with different exposure times because there is a direct linear relationship between exposure time and brightness. Pang et. al. (2011) examine using dark pixel intensity to normalize different exposure time, however, following their methods was beyond the capabilities of this study. Instead I attempted to correct for this difference in an alternative way, adapted from Crespo and Cummings (2014), although this brings about a limitation in the significance of the results as this correction is not nearly as accurate as the methods of Pang et. al. (2011). If I were to repeat or further this experiment, given the appropriate materials, I would follow the methods set forth by Waters (2009) to achieve optimal fluorescent images for accurate and precise quantitative analysis, without having to correct for exposure time difference. There are additional techniques, controls, and complementary assays to employ when using fluorescent probes to monitor MMP that should be considered in order to ensure an accurate interpretation of the results which may benefit the validity of future research (Perry et. al., 2011).

If the hypothesis was able to be supported, it could be suggested that lithium has a stimulatory effect on MMP, which could be extrapolated to suggest a stimulatory effect on cell metabolism, assuming MMP function is a representative of or essential to cell metabolic functions (Perry et. al., 2011). One mechanism that may be related to this is lithium's ability to induce Bcl-2 expression and upregulation in cultured rat cerebellar granule cells. Bcl-2 is an anti-apoptotic protein inhibitor of cytochrome c release (an important protein in the electron transport

chain), which acts by regulating the permeability of the mitochondrial outer membrane (Chiu &Chuang, 2010). The significance of glial-neuronal interactions is that in the last ten years or so there has been substantial evidence to support the supposition that radial glial cells have the ability to guide newly formed neurons as well as to regenerate neurons and astrocytes (Manji, 2005). This relationship has been proposed for study in the treatment of bipolar disorder especially in relation to lithium's effects on oligodendrocytes and P2X signaling pathways within glial-neuronal networks. Although lithium is already used in the treatment of bipolar disorder, understanding lithium's neurobiological mechanisms will allow for targets to be identified for improved therapies.

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

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