Evidence that Mercury Exposure Decreases the Abundance of Mitochondria Located 30um Upstream of the Growth Cone of 7-10 Day Old Chick Embryo Peripheral Nerve Cells

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Introduction

Mitochondria are the organelles responsible for generating the cells ATP source and thus have been termed rightly the "powerhouse" of the cell. ATP, or adenosine triphosphate, stores energy within its chemical bonds and releases this energy to fuel cellular processes when needed. Although ATP, like other small molecules, is able to diffuse freely in the cytosol, most cells will position their mitochondria in areas with high ATP demand (Morris et al, 1993). Neurons are an example of these cells. Growth cones of neurons are located at the tips of developing processes and are responsible for extension, axonal pathfinding and target cell selection (Leong et al, 2001). Due to the asymmetric shape of neurons, there are different macromolecule and energy requirements in specific regions of the cell's cytoplasm (Morris et al, 1993). Evolution has provided a way for neurons to transport the needed substances within the axonal processes (Morris, 1993).

There have been many experiments performed as early as the 1920s that have deduced that large membranous organelles such as vesicles, mitochondria and elements of the smooth endoplasmic reticulum, are transported both to and from the cell body along the axon via fast axonal transport (Kandel et al, 2000). More recently using video-enhanced light microscopy, it has been revealed that these organelles are being transported along linear tracks aligned with the main axis of the axon (Kandel et al, 2000). These tracks are now known to be microtubules. This form of transport has evolved in response to the functional asymmetry of eukaryotic cells requiring different energy needs in different regions of the cell (Morris et al, 1993). However, our knowledge of this subject does not extend to knowing how the cell adapts to physiological changes (Morris et al, 1993).

Actin and tubulin comprise the bulk of growth cone cytoskeleton and are highly sensitive to various environmental signals (Leong et al, 2001). Microtubules are a chief protein of the cytoskeleton and are required for axoplasmic transport, membrane structure, and normal neurite outgrowth (Leong et al, 2001). It has been shown that mercury, either as a vapor or in the form of methylmercury (MeHg), is toxic to the central nervous system (Leong et al, 2001).

Inhalation exposure of rats to mercury vapor has inhibited the polymerization of tubulin molecules thus disrupting the brain's microtubule metabolism (Leong et al, 2001). A similar occurrence was found in the brains of 80% of Alzheimer disease patients but was not seen in a comparable sample of control patients (Leong et al, 2001).

Since it is known that most cells will position their mitochondria in regions of intense ATP consumption, it is not hard to imagine that the distal ends of growing axonal processes will have greater numbers of mitochondria due to their increased need for ATP (Morris et al, 1993). This was supported in the work of R.L. Morris and P.J. Hollenbeck. It is also recognized from C.C.W. Leong, N.I. Syed and F.L. Lorscheider's research that exposure to mercury ions notably disrupt the growth cones of neurons.

In this experiment, I will be working in collaboration with Elizabeth McKay (McKay, 2008) to determine the effects of mercury on mitochondrial quantity and activity in the peripheral nervous system of 7 – 10 day old *Gallus gallus* (i.e. the domestic chick). Elizabeth will be concentrating on mitochondrial activity, and I will be observing the number of mitochondria. Specifically, I am hypothesizing that mercury will decrease the number of mitochondria present in the 30um upstream from the growth cone of 7-10 day old chick embryo dorsal root ganglia. The distance of 30um was chosen because this appeared to be a distance of interested due to observable variability between neurons. Since mitochondria are the location of ATP synthesis, fewer mitochondria located in the growth cone may have cellular implications. A decrease in mitochondrial abundance could decrease the ATP concentration at the growth cone; this location should have the highest demand due axonal growth and retraction.

The fluorescent dye that will be used to label mitochondria is JC1. This is a cationic dye that indicates polarization by shifting its emissions from green to red (Molecular Probes, 2007). The color shift is based on concentration of J-aggregates (Molecular Probes, 2007). JC1 is more specific for mitochondria, has a consistent response to depolarization, and images easily (Molecular Probes, 2007). Some of the many uses of JC1 include apoptotic cell death detection, detecting changes in membrane potential associated with the mitochondrial permeability transition, and monitoring mitochondrial depolarization in response to excitotoxic glutamate stimulation of neurons (Molecular Probes, 2007). I have chosen JC1 as the fluorescent dye because it labels mitochondrial exceptionally well and will label them according to activity (Smiley, 1991). Highly active mitochondria will label red and less active mitochondria will label green; superimposing the images will produce a yellow color in regions were both red and green fluorescence coexisted (Smiley, 1991). This will help quantify the number of mitochondria present. Also, conclusions about mitochondrial activity can be made simultaneously.

I have chosen this topic because the specific movement of organelles within a cell is intriguing. It's fascinating that a cell can modulate its macromolecules and organelles to meet the needs of the different regions of its cytoplasm. There is strong evidence that mercury exposure causes a disruption in membrane integrity of neuronal growth cones which

may implicate mercury as a potential factor in neurodegeneration (Leong et al, 2001). There may also be a link between mercury exposure and Alzheimer disease (Leong et al, 2001). Alzheimer's is a terrible ailment that has affected many families across the nation. I have found this topic to be particularly interesting because of its clinical implications.

Materials and Methods

To set up the experiment and cells for observation:

The primary culture of *Gallus gallus* embryonic peripheral neurons were collected as described in my lab notebook pg 2/5/08 1 SMF – 2/5/08 14 SMF (Frasca, Suzanne, 2008). When appropriate, the materials utilized in the dissection were autoclaved and a sterile technique was followed. The dorsal root ganglia (DRG) that were collected were placed in a dish of warm Hanks Balanced Slat Solution (HBSS) to keep them alive. Cells will not grow in HBSS. The coverslips were cleaned with 200 proof ethanol. They were then treated with polylysine. To treat with polylysine, a 1mg/ml drop was put on the inside lid of a 110 mm Petri dish and one sterile, cleaned coverslip was then placed on top of the polylysine drop for 20-30 minutes. The coverslip was then rinsed with sterile water and allowed to dry. They were then coated with a solution of laminin in HBSS. It was important that the coverslips were kept wet until cell plating. This was done by leaving the coverslips in a dish of HBSS. When ready to use, the coverslips were rinsed with HBSS and then treated as above with laminin. The coverslips were kept wet to prevent denaturation by rinsing with HBSS (not water) and placed straight into a dish C medium. C medium contains Leibovitz L15 medium plus 0.5% methylcellulose, 10% fetal calf serum, 0.6% glucose, 2mM L-glutamine, 100 ug/ml streptomycin, 100 U/ml penicillin, 10-50 ng/ml NGF (Morris, 2008).

The suspension of dissociated cells was added dropwise to dishes already containing C-medium. They were set at a predetermined density of up to 2 DRGs or 2 sympathetic chains per 22mm coverslip or well. They began to extend axons within a few hours and were dissected in the 7-10 day old range (Morris, 2008).

To prepare slides for control data:

A 7-10 day old chick embryo DRG with a density of approximately 1 DRG in 3mL solvent per coverslip was obtained from 37°C incubation. The HBSS was aspirated. To make the JC1, 5mg JC1 powder was added to 2.5mL DMSO yielding 2mg/mL concentration which was then diluted 1:2 producing a 1mg/mL sample. This was then diluted 1:1000 in growth medium which gave us our final 1ug/mL concentration of JC1. Approximately 1mL of this 1ug/mL solution was added via a sterile Pasteur pipette. The JC1 was added steadily but gently to the outside edge of the dish which allowed the medium to flow over the wet coverslip and the cells. The lid was put back onto the dish and the coverslip

was then incubated for 5 minutes at 37°C. Due to the nature of JC1, it was vital that the slide containing neurons fluoresced with JC1 was kept in the dark when possible. Thus, tin foil was used to cover the slide whenever possible, such as during the incubation. After the incubation, the coverslip was washed gently three times with approximately 1 mL HBSS, letting the HBSS sit on the cells for 1 minute each before rinsing the next time. The coverslip was then washed once with enough growth medium just to keep the cells wet and keep a meniscus around the outer edge of the dish (Morris, 2008).

To seal the coverslips for observation, chip chambers were made. This was done by crushing an unused coverslip in a kimwipe into a fine glass powder. These shards were then placed in a square fashion slightly smaller in size then a coverslip itself (20mm x 20mm) on a clean, dry slide. A drop of growth medium was added to the center of the chip chamber to allow the cells to continue growth and movement. The coverslip was picked up out of the Petri dish using forceps with the cell side facing up. The back of the coverslip was wiped off with a Kimwipe and the coverslip was then dropped onto the slide with the cells facing downwards into the drop of growth medium. The edges of the coverslip were then painted with liquid VALAP to form a water impermeable seal. VALAP is a 1:1:1 ratio of Vaseline, laminin and paraffin with a melting point of 40°C (Morris, 2008).

The slide was then covered with tin foil during transportation to the microscope and then again once the slide was positioned on the stage of the microscope. The more the slide was covered, the less photo-bleaching occurred (Morris, 2008).

A space heater was set up 18 inches away from the stage of the microscope to maintain the temperature of approximately 37°C. This mimicked the temperature of the incubator which maintained the maximal growth and movement of the neurons (Morris, 2008).

JC1 enters the cell immediately, but there was a 60 minute waiting period before imaging was done. This was to allow the cell to respond to the mercury treatment during the experimental data. The control data needed to sit for the same amount of time as the mercury treated data to produce comparable results (Morris, 2008).

To prepare slides for experimental data:

To gather experimental data, the formerly mentioned control procedure was followed exactly the same, however, before the cells were stained with JC1, they were exposed to a 10nm dilution mercury treatment. The stock solution of $HgCl_2$ was 10nm in 0.5% HCl. The working $HgCl_2$was made in HBSS to yield a 10nm solution. After the HBSS was removed, 1-2mL of 10nm mercury was added. The lid was put back onto the dish and the coverslip was incubated at 37°C for 20 minutes. Following the incubation, the procedure for staining with JC1 was the same as was the rest of the

preparation used for the control data to prepare for imaging.

During each lab period in which data results were collected, both control and experimental data were collected at the same time. This helped alleviate some sources of error such as differences in reagents and made sure we were gathering comparable data in terms of exposure time (Morris, 2008).

Imaging:

All imaging was performed in the Imaging Center for Undergraduate Collaboration (ICUC) at Wheaton College, Norton, MA. The specific computer used was Capricorn with a 1.0x camera magnification, model #4.2. The images were taken with Spot, InSight QE programming. In terms of microscope magnification, the 40x lens was used with a 10x lens giving a total magnification of 400x. This magnification was used throughout all imaging.

To do the actual imaging, the phase of the lens was matched with the proper phase below the stage. A neuron was found under 400x magnification that had at least one lone visible growth cone that could be used analysis. The view was focused. In the Spot software, the top right pull down box was adjusted to “phase” and a live image was opened. Switching the image to the photo setting on the microscope produced the live image of the neuron on the computer scene. As long as the image was still in focus, a picture was taken. This produced a phase image of the neuron. The next step was to keep the same neuron in the view of the microscope and take fluorescent pictures. To do this, the bottom light source was covered and the knob above the stage was slide to position #4 (red). The shutter had to remain closed until absolutely ready to take the image or the neuron would bleach. On the computer, the top right pull down box must be changed to “JC1”. The “photo” preset was still set on the microscope and “live image” was running on the computer. The next step was done very quickly and in immediate succession. The shutter was flipped to “open” and the button was clicked to take the picture on the computer. The shutter was closed immediately after the 5 minute exposure picture was taken by the computer. This helped decrease overexposure. Our hands and elbows were removed form the desk to help alleviate as much vibration as possible. This should have helped take clearer images. This produced a red fluorescent image. The same procedure was repeated to take a green fluorescent image except that the knob above the stage was moved to position #3.

Since it was difficult to determine if each neuron would yield quantifiable results, we took as many pictures as possible before the slide began to bleach. As soon as the fluorescent images started to become faded and less clear, we ended our image collection. Overall, the experiment lasted 8 weeks. It took approximately three hours to successfully image both experimental and control images.

Quantification:

I wanted to look at and study the mitochondrial abundance in the area immediately upstream to the end of the growth cone. The growth cone was defined as beginning when then axon was no longer parallel. As soon as the two sides of the axon deviated from being parallel by increasing in size, I said that the growth cone had begun. Since I only wanted to observe activity in the axon, determining where the growth cone began and the axon ended was a very important first step. With the boundary set, I then began quantification.

I decided on a length of 30um to study. To determine how many pixels are in 30um, I used a stage micrometer. This is a ruler etched into a disc that was placed on the microscope stage so I could take a picture of the tick marks of the ruler (Morris, 2008). I then opened the image of the ruler in Image J, version 1.32j. Since I knew that the largest spaces measured 100um and the smaller spaces measured 10um (Morris, 2008), I used a segmented line and measured the number of pixels in 30um distance and transferred that over the neuron images.

The actual quantification was done in Image J. A stacked image was created of the phase image, and both the green and the red fluorescent images. To do this, the first picture was opened in Image J, then I clicked image – stacks – add slide. Another picture was opened in Image J. It was copied using the “copy” tool. I then went back to the first image and pasted the second image into the blank space created. This procedure was repeated to add the third picture into the same stacked image (Morris, 2008).

The phase image was used to determine the end of the growth cone because this was the image in which the growth cone was the most distinct. Therefore, the following steps were done using the phase image. The end of the growth cone was found based on previously stated prerequisites. A box then was created measuring 168 pixels of length beginning at the immediate point proximal to the end of the growth cone. This box represented the 30um distance from the growth cone that was of interest. The segmented line using the “polygon” selection of Image J was created following the outside border of the axon not including the glow of the outside of the cell. Once 168 pixels in length had been measured, the segmented line was continued perpendicular across the axon to the opposite side of the axon. The line was then continued distally until perpendicular to original starting point. A line was created across the axon to close the box. This gave a 30um distance of axon from the end of the growth cone. Since the images were stacked, the box that was created in the phase image was transferred over to both the fluorescent images.

To determine if I could quantify using only the red fluorescent image, I superimposed the red and green fluorescent images in the Photoshop software. Highly active mitochondria fluoresced red and those with a lower activity fluoresced green (Smiley et al, 1991). Superimposed, yellow fluorescence was observed in regions where both red and green fluorescence coexisted (Smiley et al, 1991). As long as the mitochondria fluoresced yellow in the region of interest, only the red images need be used since both images would yield the same quantification.

Although both the green and red fluorescent images were part of the stacked image, only the red fluorescent image was used in conjunction with the phase image. To determine the number of mitochondria present in 30um from the growth cone, the command: analyze – find edges was used. The computer then put predetermined edges around each mitochondrial organelle. The number of completely outlined organelles was counted, regardless of any size differentiation. In order to be considered in the final count, all of the organelle had to have been within the box. If a portion was not included in the 30um box, it was not included in my summation.

Once this quantification procedure was complete for three images of the control and the experimental data, the numbers of mitochondria were averaged to give an average number of mitochondria within the 30um distance from the growth cone both in the presence and absence of mercury. The control trials were both necessary and appropriate because they gave a set of data to which our experimental data could be compared. This helped me draw conclusions about the effect of mercury on mitochondrial activity in the 30um distance directly proximal from the growth cone. The experimental data were appropriate; however more data should be collected from both the experimental and control trials in order to draw more conclusive evidence. A histogram of the averages was created.

Results

The following figure is a representative phase image of a neuron from the control data (figure 1). The axon studied was the first short axon in from the right of the image.

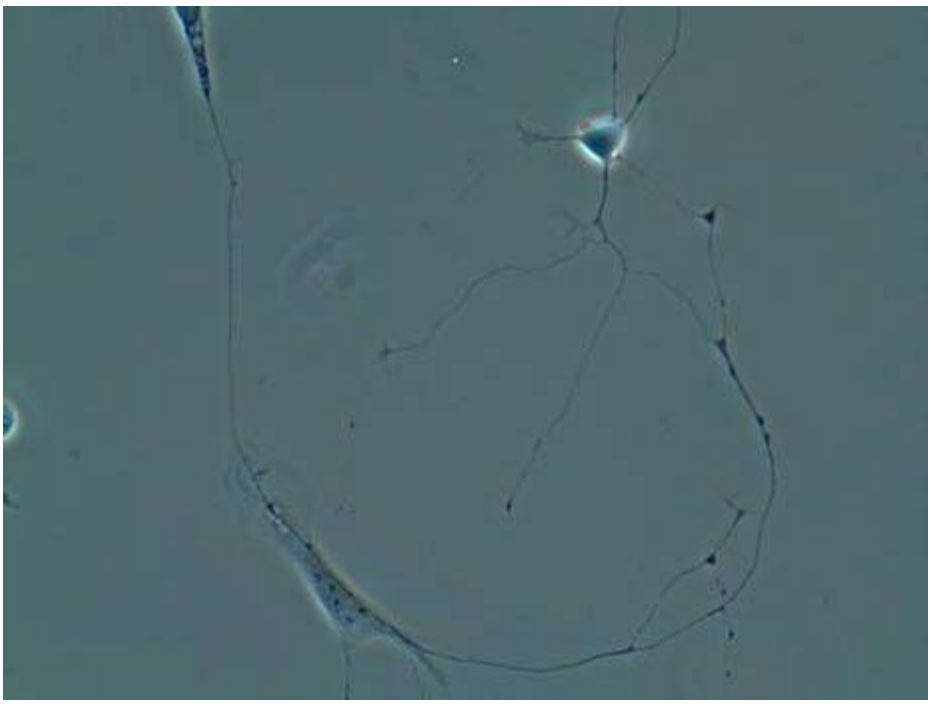

Figure 1: Phase image of a neuron from the control set of data; there was no mercury present. Notice cell body in the top right corner and the numerous axons projecting throughout the frame.

To determine if the mitochondria could be quantified using only the red fluorescent image, a superimposed green and red fluorescent image was created. The areas that glowed yellow indicate regions where red and green fluorescence overlapped. The following two images are representations of neurons from control and experimental experiments respectively.

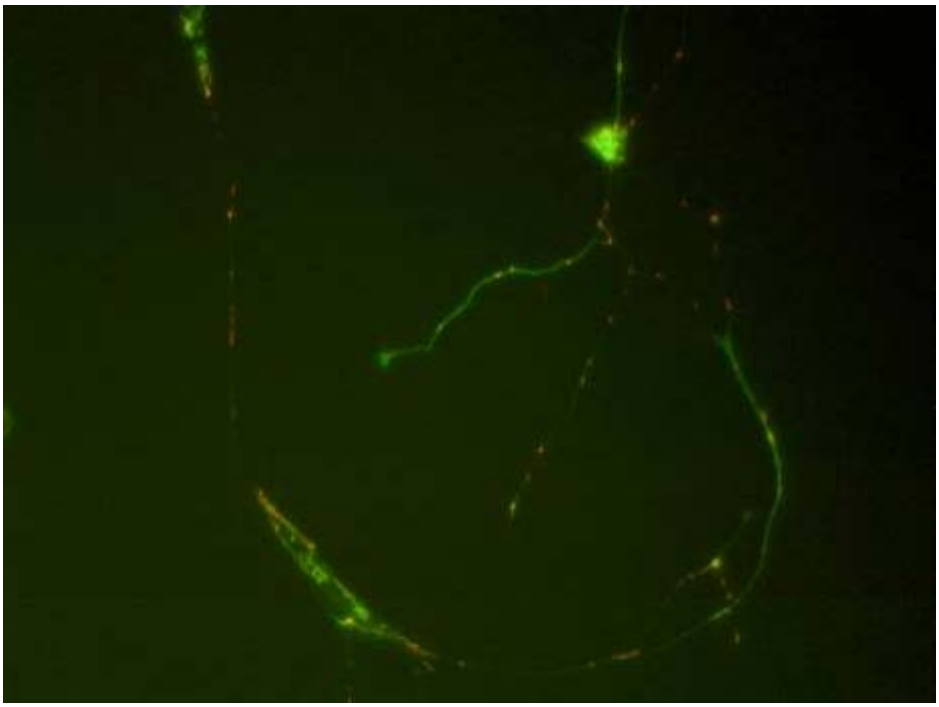

Figure 2: Superimposition of red and green fluorescent images of a neuron not treated with mercury. The first short axon from the right side of the image was the one that was observed. Notice that the mitochondria upstream from the growth cone contain yellow pigments indicating red and green mitochondria coexisting.

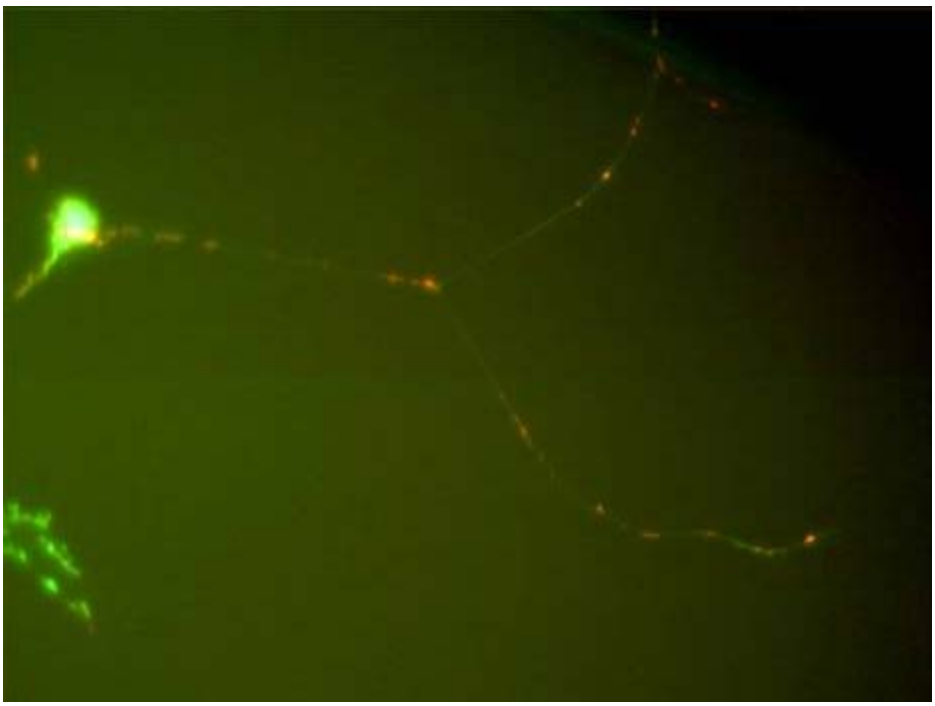

Figure 3: Superimposition of red and green fluorescent images of a neuron treated with mercury. The lower portion of the split of the axon was studied (i.e. the axon in the lower right corner of the image). Notice that the mitochondria upstream from the growth cone contain yellow pigments indicating red and green mitochondria coexisting.

The quantitative results show that there was an average of 3 mitochondria present in the 30um distance directly proximal to the growth cone with no mercury present (figure 4). In the neurons that had been affected by mercury, there was an average of only 2 mitochondria in the same 30um distance relative to the growth cone (figure 5). The following images are representations of the neurons observed in the control trials and experimental trials respectively.

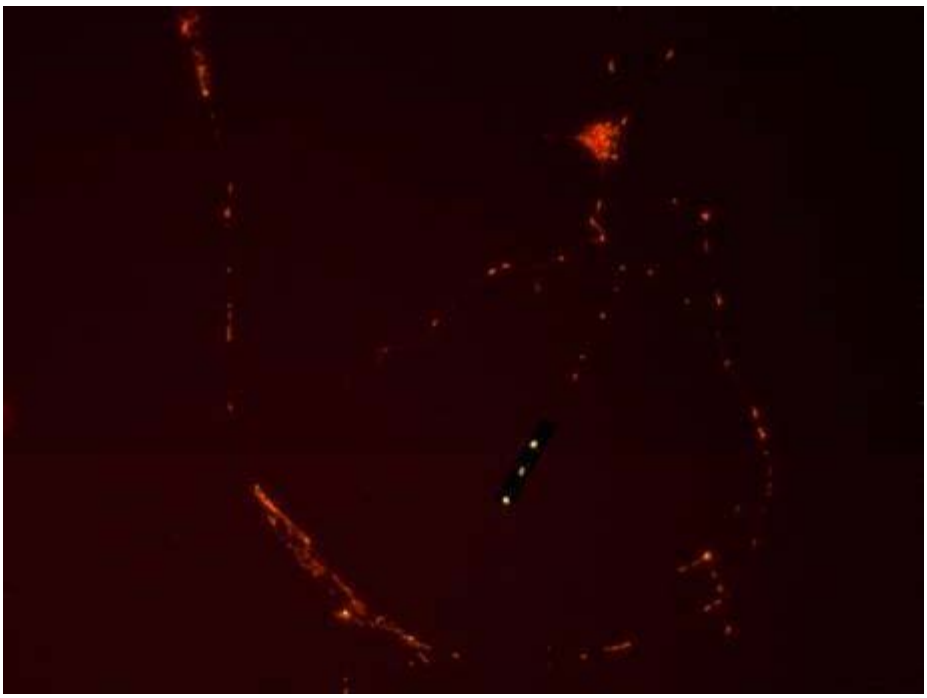

Figure 4: Representative image of control data. This neuron was not treated with mercury, just stained with JC1 fluorescent dye. The axon being observed is the one with the black box around it which represents 30um distance from the end of the growth cone. The distance from the growth cone was determined by using the phase image of the same neuron. The each round circle within the black box represents one mitochondrion.

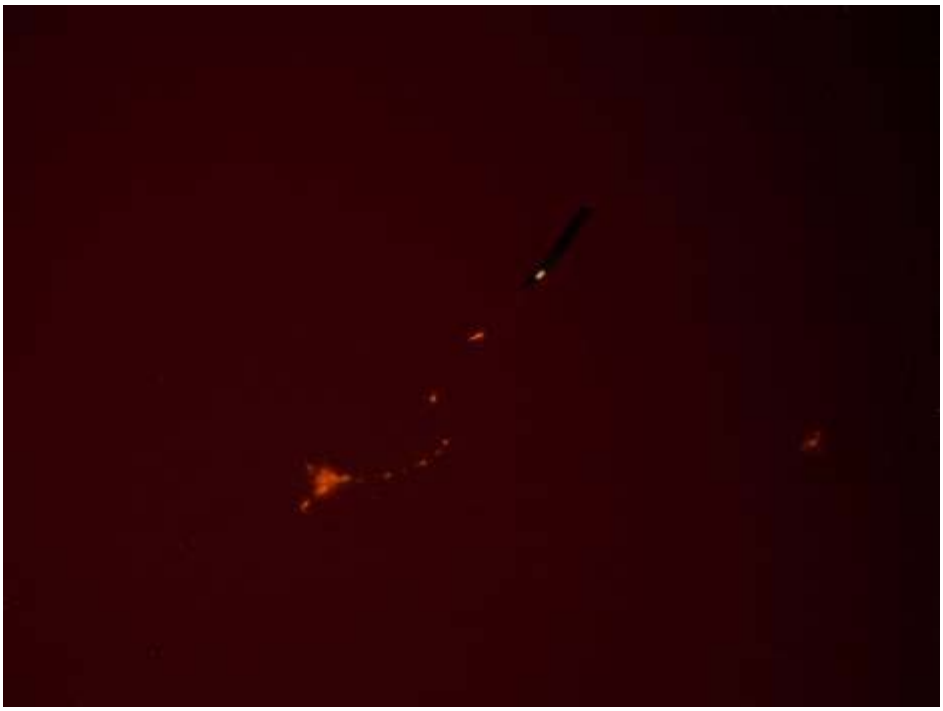

Figure 5: Representative image of experimental data. This neuron was treated first with mercury and then stained with JC1 fluorescent dye. The axon being observed is the one with the black box around it which represents 30um distance from the end of the growth cone. The distance from the growth cone was determined by using the phase image of the same neuron. The one round circle within the black box represents the only mitochondrion present in the studied area.

Each average consists of the number of mitochondria measured in 3 axons, each of an independent neuron, therefore, n=3. Notice that the number of mitochondria present in the 30um distance from the growth cone is less in the presence of mercury (figure 6).

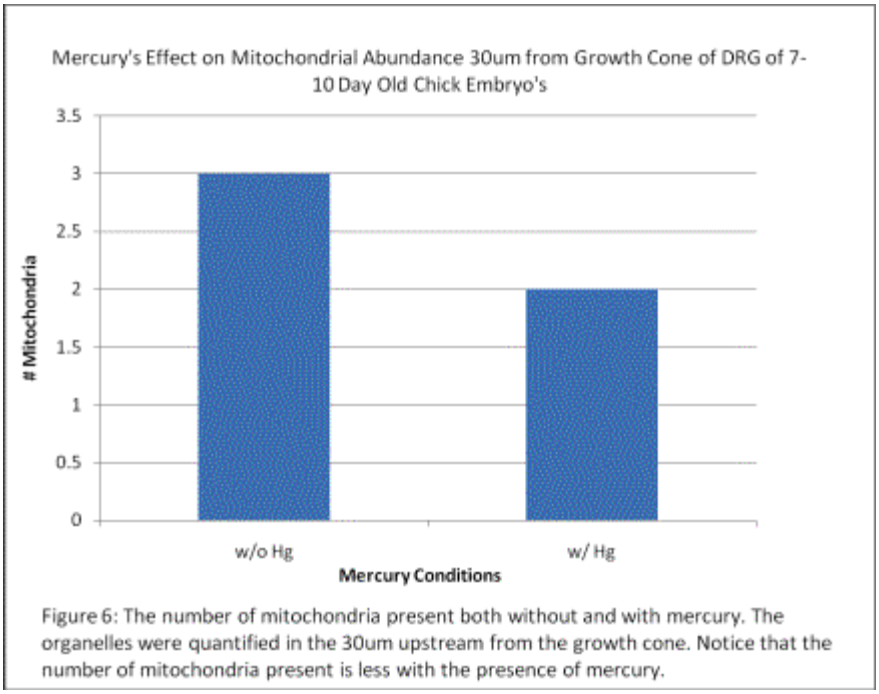

Figure 6: The number of mitochondria present both without and with mercury. The organelles were quantified in the 30um upstream from the growth cone. Notice that the number of mitochondria present is less with the presence of mercury.

There were many observations that could be made by taking the experimental and control data and looking at them collectively. When this was done, the axons of the neurons treated with mercury seemed to be about the same length as those without mercury. This is just an observation and was not measured. Other observations include that there appeared to be a difference in distribution along the axon and that the brightness of the mitochondria may have decreased.

Conclusion

The hypothesis of my experiment is that mercury will decrease the number of mitochondria in the 30um upstream of the growth cone. The results gathered support my hypothesis because there is a lower average number of mitochondria in the 30um region with the presence of mercury. There was an average of 3 mitochondria without mercury, and an average of 2 with the mercury. The findings of my collaborator, Elizabeth McKay, supported the idea that there is a decrease in activity of mitochondria due to the presence of mercury (McKay, 2008). Taking our conclusions together, we are able to support a broader hypothesis that mercury decreases abundance and activity of mitochondria in the 30um length upstream of the axon from the neuron's growth cone.

Although I was able to lend support to this idea, the results obtained from this particular lab do not touch on whether it is the mitochondria themselves that are being affected or the organelles involved with mitochondrial movement. Microtubules are involved with axoplasmic transport, membrane structure and normal neurite outgrowth (Leong et al, 2001). Perhaps exposing the neurons to mercury is causing the microtubules to retract the mitochondria back towards the cell body. If this was true, it would propose that it is not only the mitochondria that are being affected. Instead, it would suggest that the organelles involved with mitochondrial movement are affected by mercury exposure. This potentially still could cause a decrease in mitochondrial distribution near the growth cone.

There are a few ways I would like to build off of my experiment. I would like to see if mercury affects microtubules. If these organelles are adversely affected by mercury, then perhaps this is the reason for observed decrease in mitochondrial abundance in the distal end of the axon.

In subsequent experiments, more data would be necessary. If, in fact, I had collected enough data that showed a statistically significant difference between experimental and control data, we could draw more conclusive ideas. For example, I would be more confident in the fact that my hypothesis was supported which could then lead to more clinically applicable studies. For example, health specialists may be able to explain the results of mercury poisoning at a more molecular level. There is already some correlation between mercury exposure and Alzheimer disease (Leong et al, 2001). Or perhaps there are clinical implications to having fewer mitochondria at the growth cone since this may

decrease growth cone ATP concentrations.

One source of error came from the fact that we measured 168 pixels down the edge of one side of the axon. If the 30um length of axon was curved at all, then the distance observed would not have been exactly 30um. The next round of experimentation should take this into consideration and try to adjust for the error.

It is important to note that the mitochondria could potentially overlap within the axon. This could theoretically make quantification difficult. But since we have no reason to believe that mercury affects whether or not mitochondria are overlapped, we can assume that this factor will be present in both experimental and control neurons. Thus, we do not need to account for the overlap because it will be a common factor in both sets of data. Also, the slides may have bleached during imaging, but since this would have occurred with both the control and experimental slides, it can be deemed negligible.

I believe that this experiment showed great potential. As with any scientific experiment, there are always means of improvement. To refine this experiment, the most substantial aspect would be to collect more data. An n value of 3 for both the experimental and control groups is not high enough for substantial support. I would like to see more repetition. Quantitatively, I believe that I was on the right path but would like to see this process refined as well. Becoming more knowledgeable with Image J would be helpful because perhaps there is a more accurate way to count the organelles. I would try to keep all of these little aspects in the back of my mind when I was preparing for future experiments.

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