Number of Mitochondria Proximal to Growth Cones Decreases in Chick Sympathetic Neurons Following *In Vitro* Mercury Exposure.

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Introduction:

According to the Environmental Protection Agency (EPA) the average human is chronically exposed to mercury in their environment, as well as their diet, resulting in an average blood mercury content of 5.8 µg/L of whole blood (EPA). Low levels of methyl mercury have shown to result in severe nervous system damage in infants, while the consequences of chronic exposure for adults remains unknown (EPA). In 2001 Leong, Syed and Lorscheider demonstrated that *in vitro* mercury exposure of sympathetic ganglia resulted in cytoskeletal collapse of growth cones. Because synapse formation is vital for learning and nervous system development, the affect of substances which put synaptic formation in jeopardy is of upmost concern. This study aims at uncovering the mechanisms by which mercury degenerates nervous tissue by exposing sympathetic ganglion of embryonic chicks to acute levels of mercury. Specifically, this study will measure the effects of acute mercury exposure on amounts of mitochondria near the growth cones of growing neurons.

Growth cones of growing neurons are highly dynamic, making them an area of intense ATP consumption. In order to facilitate the cytoskeletal adjustments which make growth cones remarkably dynamic, a reliable source of ATP is needed. Because the growth cone is separated from the cell body by the axon, mitochondria are transported down the axon to locally supply ATP to the growth cone. In 2003 Chada and Hollenbeck supported that mitochondria move primarily anterograde to the growth cone during axonal elongation, but then become stationary while growth halts to later more equally distribute along the axon. Because the motility of mitochondria is influenced by the overall activity of neurite growth cones (Morris and Hollenbeck 1993) a shift in growth cone activity would be reflected by a change in mitochondrial transportation. In this study the hypothesis was tested there would be fewer mitochondria within a 400 pixel distance from the growth cone on average following exposure to mercury than in control groups. This hypothesis was tested using *in vitro* exposure to mercury solution with cultured neurons from the sympathetic nervous system of ten day old chicks (*Gallus gallus).*

Materials and Methods:

Materials List:

10 day old chick embryos

Hanks Balanced Salt Solution (HBSS)

Trypsin

50ng/ml Nerve Growth Factor

1mg/ml Polylysine

Laminin

10% Fetal bovine serum

.6% glucose

2mM L-glutamine

100.u./ml penicillin

100 µg/ml streptomycin

For complete list of materials for this dissection refer to the 28th of September, 2011 version of the "Procedure for Primary Culture of Chick Sympathetic Neurons: Bio 324 Neurobiology".

Rhodamine 123

Equipment List:

Dissection microscope

Incubator

BioRad MRC 600 confocal laser scanner (E200 Nikon Eclipse microscope with Sony DFW-X700 camera with C mount (1.0 magnification).

Image J software for measuring mitochondrial distribution.

Spot imaging software for image collection

Methods:

Chick embryos were collected and dissected in order to obtain ten day old nerve cells. The chicks were dissected using dissection microscopes while sitting in a Petri dish approximately half full of Hanks Balanced Salt Solution (HBSS). Sharp forceps were used to remove tissue and eventually separate sympathetic chains of neurons and ganglion cells from the spinal cord. These cells were then placed onto coverslips which were treated with poly-lysine and laminin in a small Petri dish of growth medium to foster attachment and growth. These cells were then incubated for at least 17 hours at 37 degrees Celsius until use. For full dissection procedure and coverslip treatments see Morris (2011a).

Control data was then collected using the first culture of chick embryonic cells. Cells were stained with Rhodamine 123 for fluorescence microscopy. The Rhodamine solution was 50 µg/ml concentration (for full procedure see Morris (20011c)). The coverslip with stained cells was then kept dark to keep from bleaching the stain. The coverslip was put onto a drop of growth medium in a chip chamber slide and the sides were sealed with VALAP to form a flow chamber. One set of control images were taken using transmitted light and fluorescence microscopy without flow through the chamber (fluorescent images were taken with a 2 second time of exposure). This set was composed of 6 fluorescent and 6 transmitted light photographs. Images were obtained and stored using the procedure found in Morris (2011b).

Following additional dissections, new sets of ten day old neurons were treated with Rhodamine solutions of the same concentration. These coverslips were prepared with the same procedure as the first control group. One control set of 6 fluorescent images and 6 transmitted light images were taken from cells which were then treated with a flow of buffer through the chamber and a 20 minute rest period. Following this rest period another post-flow set of 6 fluorescent and 6 transmitted light images were obtained. Then a separate experimental group of cells were treated with Rhodamine, imaged (6 fluorescent and 6 transmitted light), and received flow of mercury and twenty minutes of rest before mercury was exchanged with growth medium and 6 fluorescent and 6 transmitted light images were obtained. Mercury solution was taken from 19 milliliters of 100nm $HgCl_2$ in Hanks Balanced Salt Solution.

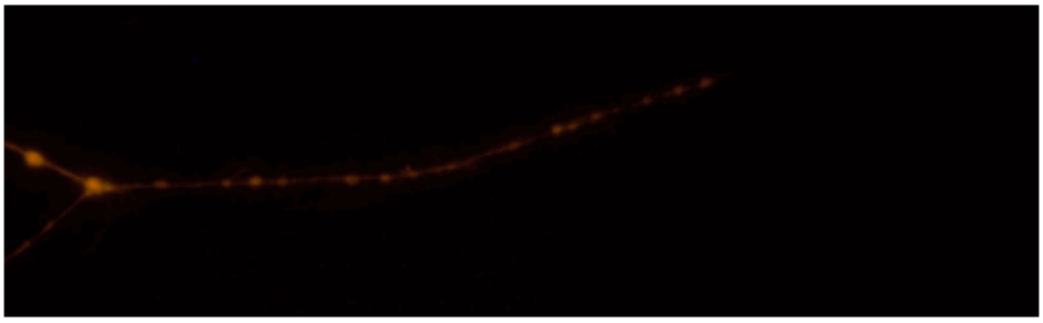
Images from both the flow control group, non-flow control group and the mercury exposed cells were immediately collected for quantification following completion of Rhodamine treatment and experimental treatment. The program Image J was used to display images and measure distances. Four hundred pixels down the axon was measured from the farthest point that the cell reaches in the direction of axonal growth (this measurement included the length of the growth cone which begins at the point where the axon width ceases to be consistent until the projection of cellular material terminates in any direction). The number of mitochondria found in this 400 pixel segment were summed and later

averaged between experimental and control groups. Mitochondria were defined as any bulge in the axon which glowed fluorescently. It should be noted that any fluorescent bulges larger than three times the width of the axon (directly preceding it) in any direction were not considered mitochondria (and were most likely a glial cells). The average number of mitochondria from each group were then displayed in a bar graph for further analysis.

Results:

This study found that mitochondria are less abundant in the last 400 pixels of axon following mercury exposure *in vitro* than in control groups. The average number of mitochondria found in the last 400 pixels near the growth cone was also less for the flow control group than the control group without the flow. Figure A1 below is an example of an axon from a set of control images without flow. Figure 1B shows the isolated fluorescent units which were calculated as mitochondria for the same control axon. These control images without flow, allowed the impact of flow to be quantified through their comparison with the control with flow group. The mitochondria in the last 400 pixels of axon in the non-flow group are noticeably more abundant than the quantity of mitochondria in comparison groups (demonstrated in figures 2 and 3). Figure 2 shows two typical axonal projections after flow of growth medium through the chip chamber. The mitochondria identified with arrows in figure 2A are less abundant near the tip of the axon and more densely packed retrograde down the axon. The axon on the right side of this image particularly demonstrates haw sparse the mitochondria were in comparison to those in figure 1. The group exposed to flow of mercury showed even fewer mitochondria in the region of interest. Figure 3 demonstrates the small number of mitochondria in the terminating 400 pixels of axon in an example axon following mercury exposure. Figure 3B also demonstrates that mitochondrial density increases within the 400 pixel length nearer to the cell body. The trends shown in these images were demonstrated by the average number of mitochondria for each group in the last 400 pixels of axon length. These relative averages are shown in figure 4 with the mercury exposed group having the smallest average number of mitochondria.

A.



B.

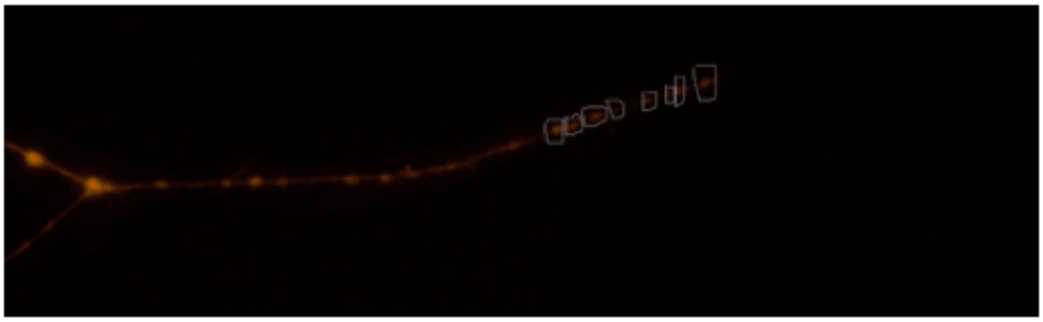
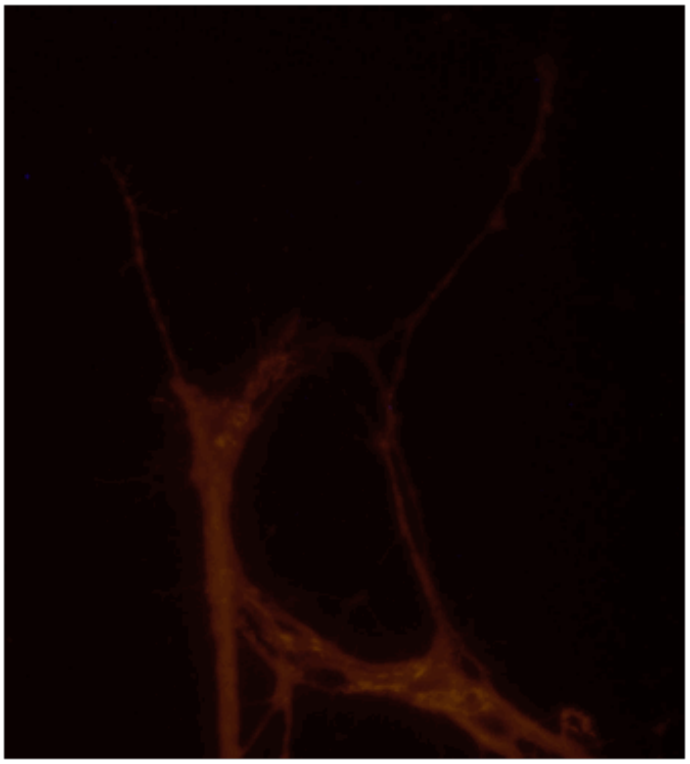


Figure 1: The above image is an axon from cell culture without experiencing flow or the presence of mercury (400x total magnification). Note the axon containing mitochondria and their abundance in the last 400 pixels of axon. Figure B is a duplicate of this image, however, units of fluorescence which were quantified as a single mitochondrion are circled in white (400x total magnification).

A.



B.

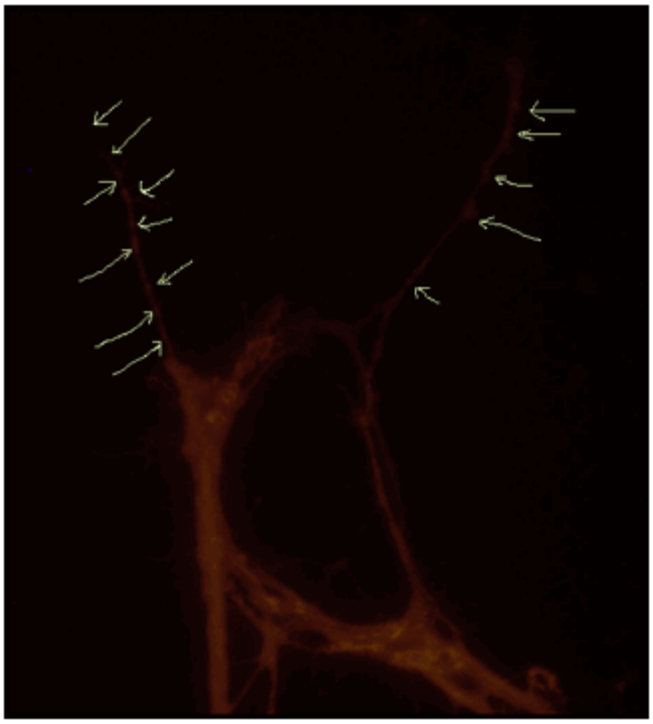


Figure 2: These images are of two axons which were observed after 20 minutes of flow of growth medium. Image 4 A is one of a data set which allowed the calculation of the impact of flow on the movement of mitochondria when compared to those in the data set of figure 2. Two axons project into the upper half of the image, these contain mitochondria (fluorescent bulges) which were quantified in the flow control group. Image 4B is the same photograph with the mitochondria pointed to with white arrows (400x total magnification). The axon in the right side of the image demonstrates the lack of mitochondria when compared to non-flow controls (figure 1).

A.

B.

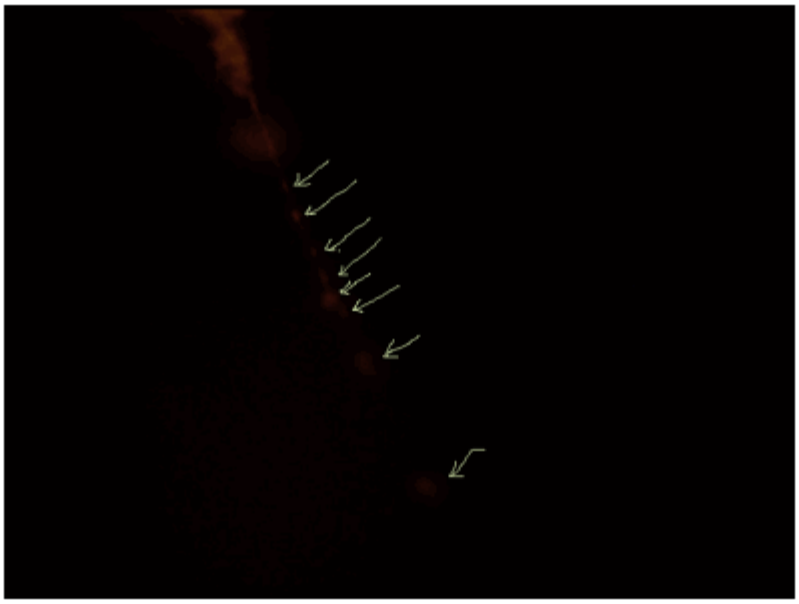


Figure 3: The above images are of an axon after mercury exposure for twenty minutes and an immediate exchange with growth medium. Part A shows the fluorescent axon, while part B includes white arrows pointing to the mitochondria which were quantified. This image demonstrates how the mitochondria are more abundant away from the end of the axon which is near the lower edge of the image (the end of the axonal projection was identified in the transmitted light image of the exact same area without adjustment of the slide or camera). Note the discrepancy between the single lowest mitochondrion, and the abundant group of mitochondria retrograde to it.

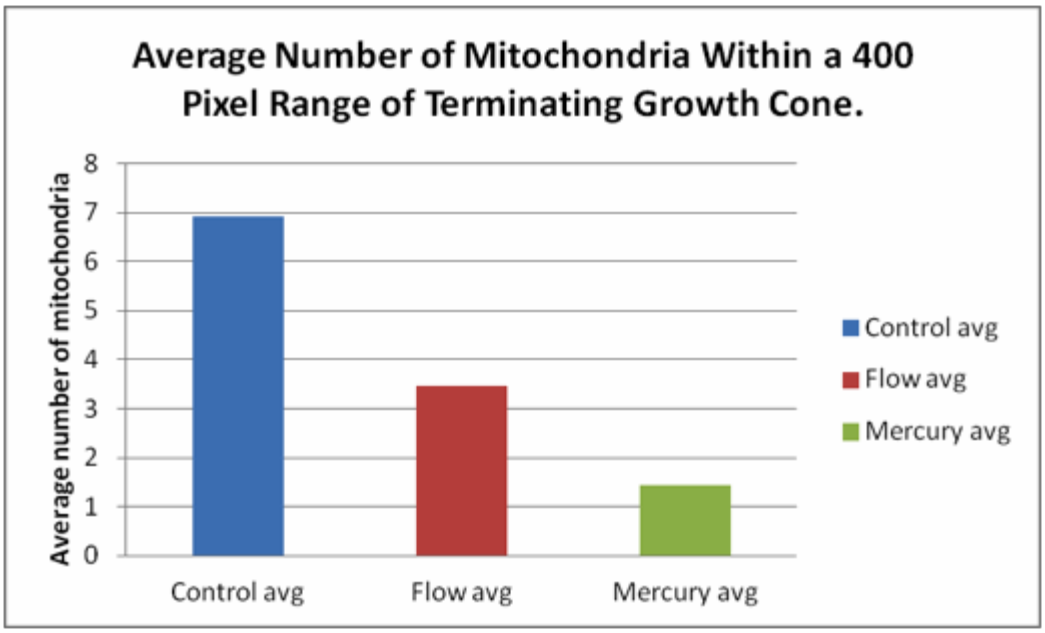


Figure 4: Average number of mitochondria following mercury exposure (shown in green) is less than the average number of mitochondria in both the flow and non-flow control groups (red and blue). A total of 23 400 pixel long axons were included in calculations from the control images without flow (n=23). 31 axons were included in quantification of mitochondria (n=31) in the flow control group and 30 axons in the exposed mercury experimental group (n=30).

Discussion and Conclusions:

In light of these results, the hypothesis was supported. A clear decrease was seen in the number of mitochondria present in the last 400 pixels of axon in groups of cells exposed to mercury when compared to control groups. The data support that flow itself has an impact on mitochondrial locations, decreasing their presence near the growth cone. However, the condition of exposure to mercury diminished the presence of mitochondria even further. Because the mercury exposed cells received an exchange of growth medium for mercury as well as an additional flow of growth medium following the exposure period it is possible that the results obtained are due to the impact of flow alone. In future experiments a control group which experiences flow twice would clarify the impact of mercury from that of flow. If this experiment should be replicated in this manner, with similar findings of decreased mitochondrial abundance near the growth cone it could be suggested that mercury has a negative effect on neurons possibly causing the mitochondria to be signaled to move retrograde back towards the cell body. This may imply that the cell is no longer requiring the ATP production of mitochondria and isn't able to extend and grow in an environment containing mercury. This could be shown by a depolymerization of microtubules in response to mercury exposure.

In future experiments measurements of total axonal length and number of axonal projections should be calculated in conjunction with location and number of mitochondria. Observations were made in this study which fell outside the parameters of our measurements. For example, in the mercury exposed cells fewer axons were available for measurement because the cells seemed to withdraw their axons. Therefore, it would be more accurate to have a calculation of total surviving axons so that the calculation of mitochondria remaining isn't a compilation of outlier data. Future experiments could also examine the signaling factors which guide the movement of mitochondria. Discovering the way these signaling factors operate in the cell might reveal how the extracellular environment is read by these signalers as toxic. Further investigation may also be more accurate if differences between mitochondria and other organelles and vesicles could be delineated for quantification. Overall, the impact of mercury on neurons should continue to be investigated as mercury is becoming a large concern in the environment and consequently public health.

References:

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