Mitochondrial Distribution in Axon Branch Points Affected by Mercury

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Introduction:

There are a few organelles in our cells that are known to be essential, without which the cell could not survive. One such organelle is the mitochondria. Mitochondria produce ATP for use by the rest of the cell, and are therefore commonly known as the "powerhouse" of eukaryotic cells. As discussed by Morris and Hollenbeck (1993), mitochondria in cells such as epithelia can position themselves around areas where high levels of ATP are needed. In fact, in neurons, mitochondria cluster around the terminal buttons of axons in order to supply ATP to various processes that occur there, including at the growth cone of elongating neurons (Frederick & Shaw, 2007). While transporting along the axon, the mitochondria reach branch points where the neuron must decide where to send the incoming mitochondria. These branch points serve as a relay station for the mitochondria, and the neuron will send the mitochondria down whichever growth cone has the greatest growth rate (Ruthel & Hollenbeck, 2003). During this stage of development, some substances have been shown to negatively affect the growth rate, and recent research has tried to isolate the various mechanisms by which brain damage from these exogenous chemicals can occur.

One such chemical under scrutiny is mercury. The EPA estimates that a typical level of mercury found in humans is below 5.8 micrograms per liter blood. From studies conducted by Leong et al. (2000) and Miura et al. (2000), it has been revealed that mercury can have an inhibitory effect on growing neurons. In order to expand our knowledge of how mercury affects neuronal growth, it is necessary to observe the effects of mercury on the distribution of mitochondria. By exploring the effects of physiological levels of mercury, we can better understand how these levels can affect human neural growth.

My experiment will investigate mitochondrial distribution using fluorescent vital dye (Rhodamine-123) to observe the effects of mercury on the axon branch points of chick (*Gallus gallus*) sympathetic neurons growing *in vitro*. Rhodamine-123 is used because it is attracted to the negative charges of a cell's organelles without damaging those structures. One highly negative organelle is the mitochondria, and therefore the concentration of Rhodamine-123 in these organelles is higher than for other organelles in the cell. In this study, I tested the hypothesis that the mitochondrial abundance would be less in the axon branch points after the application of mercury. We know from

Leong et al. (2000) that mercury causes axonal retraction, and that axonal outgrowth depends on mitochondria. Therefore, it is essential to observe what effect mercury has on the mitochondria that are necessary for axonal growth. By measuring the distribution before and after the application of mercury at the axonal branch points, we can better understand how this toxic substance harms our neurons.

Materials and Methods:

Materials:

For this experiment we used 10-day-old chick embryos dissected and stored in Leibovitz L-15 medium, with nutritional supplements added (2 mM L-glutamine, 0.6% glucose, 100 microgram/ml streptomycin, 100 i.u./ml penicillin, 10% Fetal Bovine Serum, and 50 ng/ml Nerve Growth Factor). Dissections were carried out in Hank's Balanced Salt Solution (HBSS, Sigma #9269). Individual sympathetic ganglion or dorsal root ganglions were then plated onto coverslips treated with 1 mg/ml poly-lysine, followed by laminin. The mercury chloride ($HgCl_2$) solution was made from a stock of 10ml of 10 μM $HgCl_2$ in 0.05% HCl. We used 0.19ml of this 10μM stock solution for our experiments to generate a working solution of 100 nM $HgCl_2$. Our cells were stained with 50μg/ml of Rhodamine-123 made from a 10mg/ml stock solution. The neuronal cells were viewed under 40x magnification. Pictures of neurons were taken with a Spot Insight 2 camera on an iMac OSX version 10.5.8. The camera is connected to a Nikon Eclipse E200 microscope with a 1.0x C-mount. Fluorescence was viewed via a Nikon Rhodamine filter set G-2E/C with an excitation of 528-553 and a dichroic of 565 and emission of 590-650. The software used to capture the images was Spot Advanced version 4.6.1.26. The data was analyzed using ImageJ version 1.45s. For a complete equipment list, see Morris (2011a), Morris (2011b) and Morris (2011c).

Methods:

Cell Culture:

The experiment started with cleaning the coverslips that would eventually be used to plate the chick ganglions using EtOH and kimwipes. The coverslips were prepared with poly-lysine and laminin (the coverslips sat in a drop of poly-lysine for 20 minutes followed by another 20 minutes in laminin). Cells were isolated from 10-day-chick embryos in HBSS and placed onto the prepared coverslips (there should be 2-3 dorsal root ganglion cells per 35mm coverslip). The neuronal cells were allowed to grow for at least 17 hours in an incubator set at 37°C. For a full procedure, see Morris, R. (2011a).

Staining and Observation:

These cells were then taken out of the incubator and stained with a Pasteur pipette full of Rhodamine-123 (concentration

of 50µg/ml). The cells bathed in the Rhodamine-123 in the incubator for 10 minutes after which they were rinsed three times with HBSS. The cells were kept in the dark while preparing the slides because Rhodamine-123 is light sensitive, and may be bleached from overexposure to light. The rinsed coverslip was placed on a chip chamber prepared slide and sealed with VALAP (wax mixture of Vaseline, lanolin and paraffin). The slides were then observed under a Nikon Eclipse E200 microscope at 40x magnification. Before any pictures were taken, the microscope was aligned for Koehler illumination to provide the greatest visual acuity in the photos. Four control pictures (2 transmitted light pictures and 2 fluorescent pictures of the same areas) were taken before flowing buffer and 0.5% HCl under the coverslip, and four control pictures were taken after the flow to account for any flow effects in our experiment. The experimental photos were taken with the same equipment as the control photos. Twelve photos were taken before exposing the neuronal cells to the 19ml of 10µM stock in HBSS methyl mercury solution (6 photos transmitted light, and 6 fluorescent photos of the same 6 regions). The methyl mercury solution was then flowed under the coverslip and let to sit for 20 minutes while exposing the neuronal cells to the mercury. During this time, the slide was covered with aluminum foil to protect the cells from photobleaching. After the 20 minutes, the slide was rinsed with HBSS to rid the cells of the mercury solution and observed under the same microscope at the same conditions as listed above. An additional 12 photos were taken (6 photos transmitted light, and 6 fluorescent photos in 6 different regions). Each of the fluorescent photos for both the control and experimental groups were taken with an exposure time of 2 seconds. All the photos were analyzed using ImageJ version 1.45s. Additional information about this procedure can be found in Morris (2011b).

Quantification:

The fluorescent photos were analyzed by selecting the regions of axon branch points and measuring the brightness (in pixels) of the selected area against the background of the area around the branch point. The background mean brightness for each of the selected branch points was subtracted from the corresponding branch point brightness to find the total brightness of the selected area. The mean values were then averaged together to find the total average brightness at each axon branch point for each of the treatment groups (control before flow, control after flow, experimental before mercury and experimental after mercury). Finally, the averages were converted into percentages to better compare the relative brightness of the control groups and the experimental groups. Additional information about this procedure can be found in Morris (2011c).

Results:

Data were analyzed by finding the percent maximum fluorescence of each individual axon branch point for each of the different groups (control before flow, control after flow, experimental before mercury exposure and experimental after

mercury exposure). From this analysis, Figure 3 was generated comparing the percent maximum fluorescence at the axon branch points. Figures 1, 2A and 2B are fluorescent photos of the neuronal cells under control conditions, before mercury exposure and after mercury exposure, respectively.

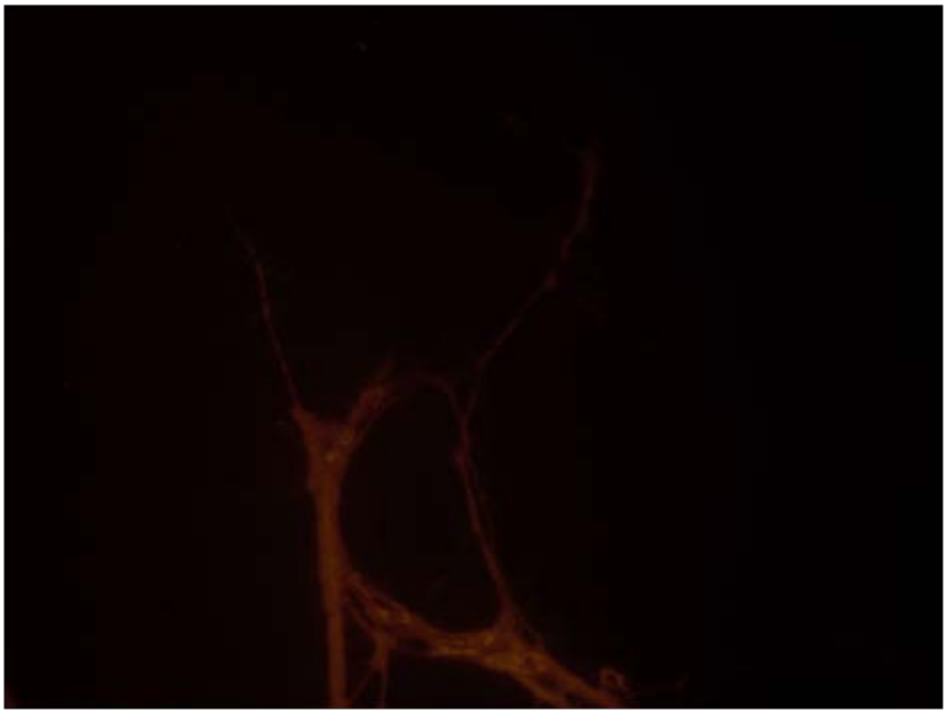

Fig. 1: Fluorescent neuronal cell control picture. This picture is of a fluorescent neuronal cell after the flow of buffer under the coverslip. Notice the bright points of fluorescence in the axons indicating a concentration of mitochondria.

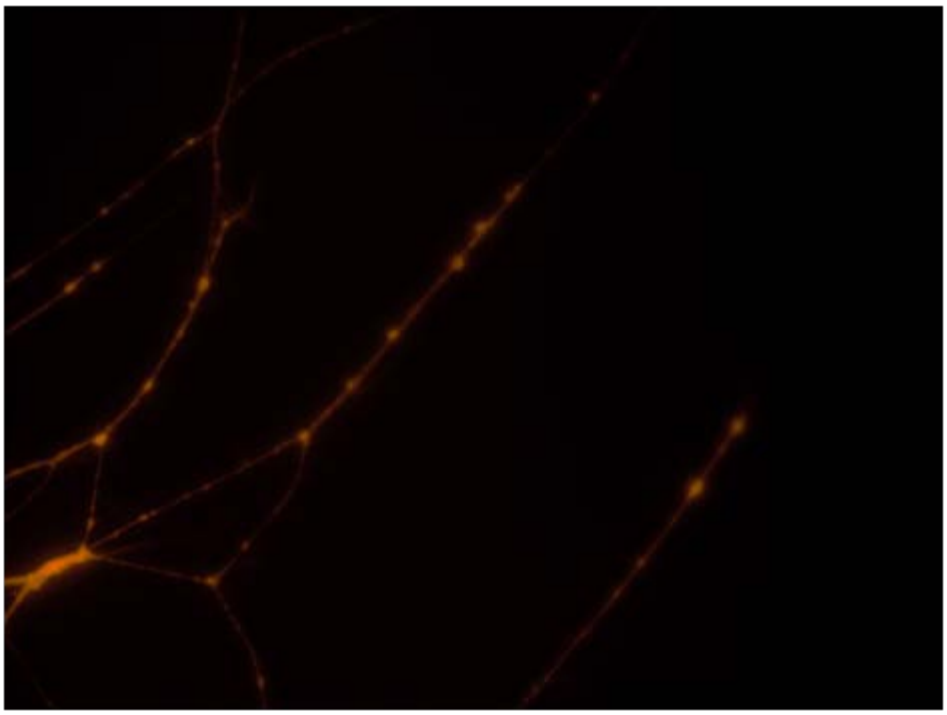

Fig. 2A: Experimental photo before exposure to mercury chloride in a neuronal cell. This photo shows axon branch points before the application of mercury. Notice the intense fluorescence of the tri-point branch points in the left lower quadrant where the mitochondria are concentrated.

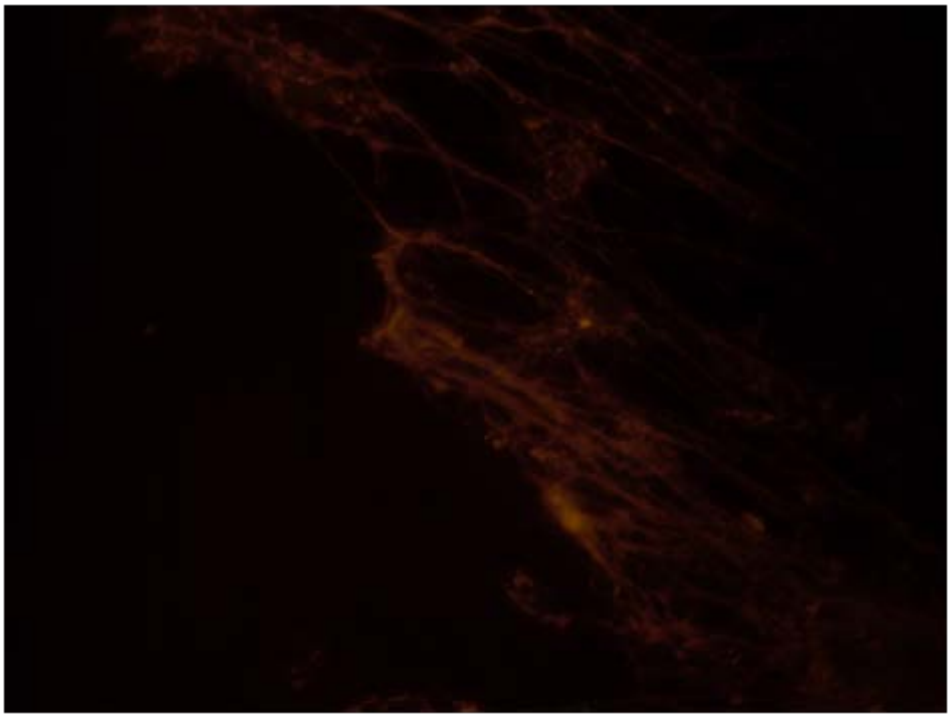

Fig. 2B: Neuronal cell after exposure to mercury chloride. This picture shows a neuronal cell after the application of mercury chloride for 20 minutes. Notice the dimmer quality to the axons, especially in the periphery where mitochondria are less abundant. The axons are in the upper portion of this photo extending from the middle of the top down to the lower right.

Comparing figures 2A and 2B, there is a marked decrease in the number of axonal outgrowths, and thus a decrease in the number of mitochondria in the periphery of these neurons and at the axonal branch points. In addition, there is a noticeable decrease in the intensity of the fluorescence before exposure to mercury (figure 2A) compared with after (figure 2B), especially in the axon branch points of this cell.

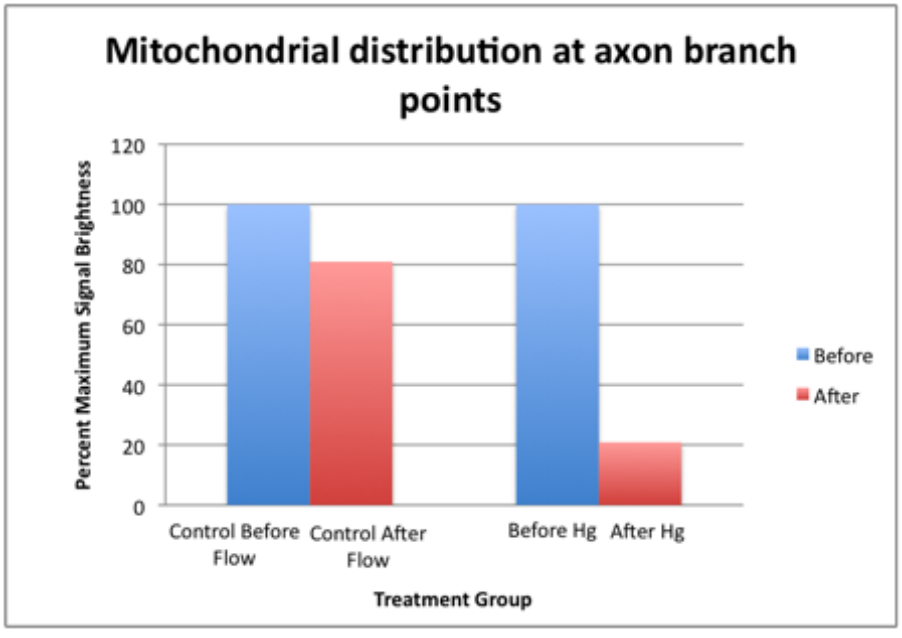

Fig. 3: Percent maximum brightness at axon branch points of neuronal cells before and after exposure to mercury chloride. This graph shows the relative fluorescence of axon branch points in a control setting (before flow n=2, after flow n=2) and in an experimental setting (before mercury exposure n=13, after mercury exposure n=13). Notice the large difference in the percent maximum fluorescence levels between the "before mercury" group and the "after mercury" group. The two groups shown on the left side of the graph show the control data (percent maximum signal brightness before and after the flow of buffer under the coverslip). The right side of the graph shows the percent maximum fluorescence of the axon branch points in neuronal cells before and after exposure to mercury chloride for 20 minutes.

This graph shows little difference in the relative levels of fluorescence between the control groups. There was, however, a large difference in the fluorescence before the application of mercury compared with after mercury exposure.

Discussion:

My hypothesis is supported by the data, which shows a dramatic difference in the relative maximum brightness of fluorescence between the axon branch points before mercury when compared to the axon branch points after exposure to mercury. This is most likely an indication that there is a smaller abundance of mitochondria at the axon branch points after mercury chloride exposure. These findings were expected because of the previous research that has been done with neuronal cells and mercury. Leong et al. (2001) found that growth cones retracted and degenerated in response to exposure to mercury. Since we also know that mitochondria are necessary components of the growth system in neurons (Morris & Hollenbeck, 1993), as well as every other living cell in our bodies, it is necessary to observe the effect that mercury has on the distribution of the mitochondria. This study attempts to provide some evidence toward this relationship.

From the findings in this study, we can conclude that the overall negativity of the branch points is greater before mercury exposure than after. Because Rhodamine-123 is attracted to negatively charged spaces, certain organelles such as mitochondria that are highly negatively charged attract more Rhodamine-123 than other structures. Therefore, for the branch points that have a higher percent maximum fluorescence, we can speculate that they are more negative than the dimmer branch points and therefore might contain more mitochondria in the brighter regions than the dimmer branch points. It is also possible that the mitochondria have a larger volume before mercury when compared to afterwards, which would also account for the dimming effect after mercury exposure.

If this experiment were conducted multiple times with the same results, and was found to be statistically significant, we would have stronger evidence that the overall abundance of mitochondria at the branch points are less after mercury is applied than before as seen in the relative fluorescence of each of the branch points. These results might be indicate that mitochondria are retracting and moving retrograde down axons back to the cell body away from the impinging toxic mercury. If the axon were no longer growing, and indeed were retracting, mitochondria would then have no purpose at the growth cone and would start to move back towards the soma, where they could be redistributed to actively growing

parts of the neuron.

It should be noted that in this experiment, the control groups were not allowed to sit in buffer for 20 minutes due to time constrictions of the experiment. If this experiment were repeated, a larger sample size would be useful to add power to the results in both the experimental groups and the control groups. In addition, future studies should determine what effect multiple exposure times to mercury and mercury concentrations might have on neuronal cells. Finding the lowest significant mercury concentration that affects neuronal cells might be useful for comparing to chronic and acute exposures to mercury for humans.

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