The Effect of Mercury on the Distribution of Neurite Area

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Neurobiology Short Report

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Introduction:

Mercury is known to have a variety of effects on humans, and most of them are nervous system related (Johansson et al. 2007). While mercury can cause symptoms in all ages of people, it is developing fetuses that are most at risk (EPA 2010). Mercury compounds such as methylmercury can cause neurobehavioral deficits at as little as 0.5μg/g, but neuropathological damage such as changes to neurite growth can take as much as 12μg/g methylmercury in humans (Johansson et al. 2007). Methylmercury is known to both inhibit microtubule polymerization as well as depolymerize microtubules (Vogel et al. 1985). The process through which this occurs involves the binding of methylmercury to sulfylhydryl groups on the microtubules (Vogel et al. 1985). Because microtubules are essential to axonal transport in it is reasonable to expect that mercury compounds would have an effect on neuronal growth neurons (Goldstein and Yang 2000). This study will focus on the compound mercuric chloride ($HgCl_2$) whose effect on neurons and microtubules is less well studied.

Neurite growth establishes the network of neuronal signaling in humans. The cells of dorsal root ganglia must span the distance between the spinal cord to the hands and feet (Kandel et al. 2000). I sought to investigate how the process of nerve growth, and thereby redistribution of area, is affected by the addition of mercuric chloride. My hypothesis was that the addition of $HgCl_2$ would induce the redistribution of neurite area from the growth cone toward the cell soma due to microtubule depolymerization. A prior experiment has found that mercuric chloride addition caused the neurites of the snail *Lymnaea stagnalis* to retract (Leong et al. 2000). I believe that retraction of the neurites will cause collapse of microtubules and thus the material at the front of the growth cone will be moved closer to the cell soma as was suspected in Leong et al. (2000). This is important because it can show that mercuric chloride causes neurite retraction. This could also provide a technique for quantifying the retraction activity of axons and growth cones in other mercuric chloride studies and ultimately contribute to describing the effects of mercuric chloride on the human nervous system. For this experiment we treated chick sympathetic neurons with mercury solutions and I measured the movements in area distribution in the neurons at different time points before and after mercury addition.

This study uses the model organism of chicken embryos. Chicken *Gallus gallus* embryos are easy to obtain, large enough to dissect for sympathetic neurons, and can be kept dormant (in arrested development) at low temperatures until needed. Chick embryos are used in many neuron studies where neuron cultures are required (Söderström and Ebendal 1995).

Materials and Methods:

Dissection

Chick sympathetic chains and dorsal root ganglions (DRGs) were dissected from 10-day-old chick embryos (Morris 2011a). The materials for this part of the experiment can be found in Morris (2011a). In addition to the listed materials the following were also used: Nikon SMz660 Dissection Microscope and Volpi Nifty 50 Light Source.

The control cultures were plated approximately 12 hours before the control observations and data collection. The mercury experimental cultures were plated approximately one week prior to the experimental observations and data collection. All cultures were incubated at 37°C. A collagen substratum was not used in this experiment. In the F-Medium the ingredients and their concentrations were: 100ml L-15, 2mM glutamine, 0.6% glucose, 100U,µg/ml penicillin/streptomycin, 10% Fetal Bovine Serum, and 50ng/ml Nerve Growth Factor.

Observation

Both the control and the mercury experimental cultures were observed using the protocol and materials listed in Morris (2011b). The imaging equipment that was used is as follows: Apple iMac computer with Mac OSX operating system version 10.5.8, Nikon E-200 Eclipse microscope with phase optics, DFW-X700 camera with 1.0X C-mount, BTV Imaging Software Version 6.01b, LASKO ceramic air heater, and a thermometer.

Flow Chamber and Observation

The methods and materials for making a flow chamber and observing it can be found in Morris (2011c). The imaging software and hardware was the same as used in the observation part of the experiment. The cells were located

on the cover slip and an axon with a growth cone was found. A picture was then taken at 40x magnification. All subsequent images were also collected at this magnification. The group waited five minutes before taking another picture in the same manner attempting not to shift from the frame captured in the previous picture. Immediately after the second picture flow was started through the flow chamber. For the control this flow was of 0.75ml of HBSS. For the experimental culture the flow was of 0.75ml of 100nM mercuric chloride. Both flows were done over a period of five minutes, but at the end of the five minutes of mercury flow the group washed out the mercury using an additional 0.75ml of HBSS that the control group did not recieve. Immediately after the flow steps another picture was taken. After five minutes another picture was taken. This results in a total of four images for the control neurons and four images for the mercury treated neurons. During the experiments the cells' temperatures were monitored at each time point where a picture was taken on BTV. These measured temperatures were never below 28.2°C and never above 32.8°C.

Quantitation of Data

The hardware used for the quantitation of the data was the same was the hardware used in the observation part of the experiment. The following software was used for quantitation: ImageJ 1.40g and Adobe Photoshop CS2 version 9.0.2. The images were transferred together into the ImageJ program for each experiment (the control flow and the mercury flow). Each image was set to a brightness minimum of 22 and a brightness maximum of 66. As mentioned before there was one trial with a total of eight images between the control and experimental groups. The first four images were for flow control. In each set of four images the first two images were a control for growth in that specific cell culture and that specific neuron. Stationary points were found on each image and lined up across images so that each matched for a stage of the experiment (control before flow, control after flow, experimental before flow, experimental after flow). With the images lined up according to stationary points, the (0,0) pixel coordinate on one image was calculated for the other. Using this system a pixel coordinate could be translated between the two images. A line was drawn to approximately move along a segment of the axon and the growth cone using the ImageJ "Straight Line Selections" function. The beginning and end points of this line were translated to the other image in the pair of photos.

The line was then divided into three segments of equal length. Perpendicular lines were drawn to extend 100 pixels from both sides of the line creating three boxes of equal total area (Figure 1). In each box the area of the neuron

was measured by using the “Polygon Selections” function of ImageJ. After outlining the neuron inside a section the “Analyze” and then “Measure” functions were used in ImageJ. The boxes were considered to be sections 1, 2, and 3. Section 1 was closest to the cell soma (the proximal section), section 2 was in the middle (the medial section), and section 3 contained the growth cone (the distal section). Photoshop was used to save steps of the experiment for reference when measuring the areas of each section. This procedure was done on each of the four pairs of images separated by five minute time points.

Figure 1

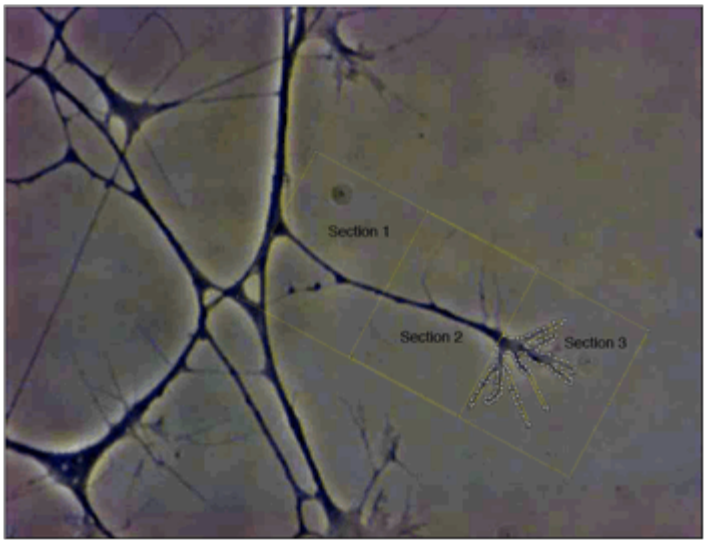


Figure 1 Legend: This figure shows the three sections (section 1, section 2, and section 3 representing the proximal, medial, and distal segments respectively) drawn using the ImageJ program. The area of the neuron is outlined with the “Polygon” function in the distal section only. The original line down the axon is not shown to limit confusion but it bisects all three sections. This particular image is an axon and growth cone right before introduction to mercuric chloride.

It is important to note that while filopodia were included in the area measurements, the lamellipodia were not due to their ability to drastically increase the area. Also, only the area for the one neuron was measured inside each section. The length of the starting line along the neuron was consistent throughout all pairs, and as such the boxes were of consistent area throughout the experiment. However the angle at which the initial line was drawn was not consistent between control images and experimental images, but it was consistent within each of those groups. For clarification on the setup for these methods refer to Figure 1.

A difference in area was calculated for images separated by five minute time intervals. This was done for changes in area between the first two images taken, and the final two images taken. For example the difference in the proximal section area of the control images from 5 minutes before flow to directly before flow was calculated.

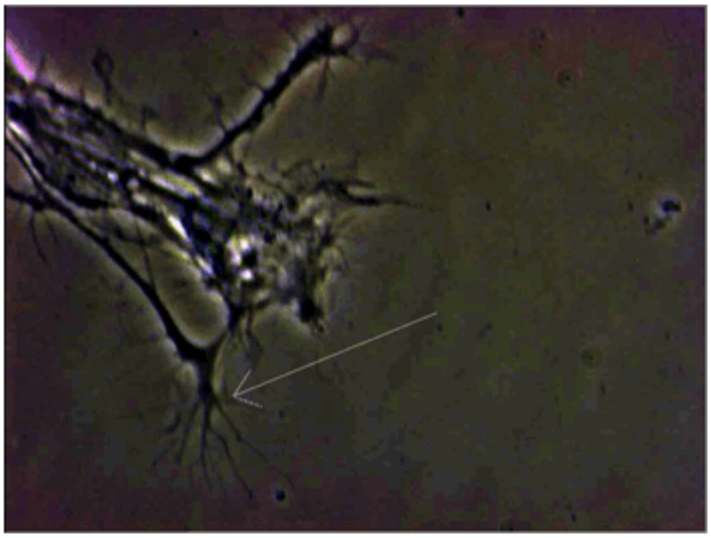
Differences were not calculated between images directly before and after flow. It should be noted that a positive difference corresponds to an increase in area after five minutes in a particular section whereas a negative difference corresponds to a decrease in area after five minutes in a particular section.

Results:

Both the control images and the experimental images showed growth before flow. DRGs cells were found in each culture and a single axon with its growth cone was chosen for quantitation in each image (Figure 2). Axons with growth cones were chosen that did not overlap very much with other cells.

Figure 2

A.



B.

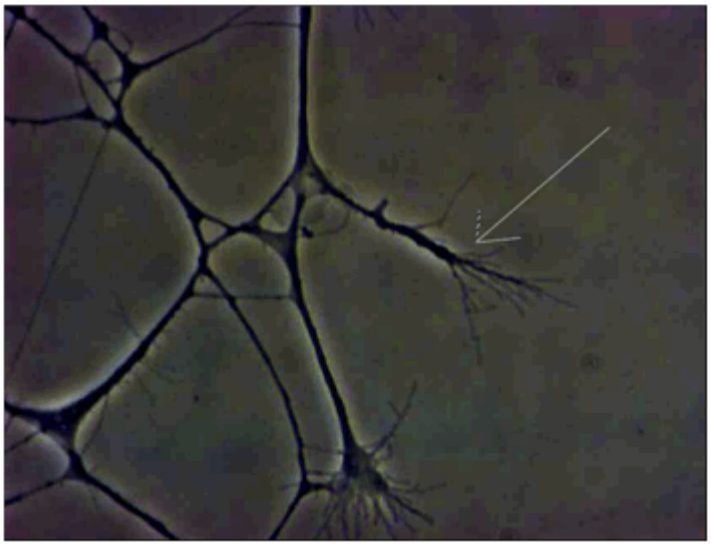


Figure 2 Legend: These images show examples of control and experimental axons used for data collection at 40x magnification in HBSS solution. Figure 2a shows a control image taken five minutes after HBSS flow. The growth cone furthest to the bottom was used for quantitation. Figure 2b shows an experimental image taken five minutes after mercury flow. The growth cone in the middle was used for quantitation.

The absolute values of the differences for each of the three sections were then averaged to find an average difference in area (Figure 3). These averages include both the control and mercuric chloride experimental results.

Figure 3

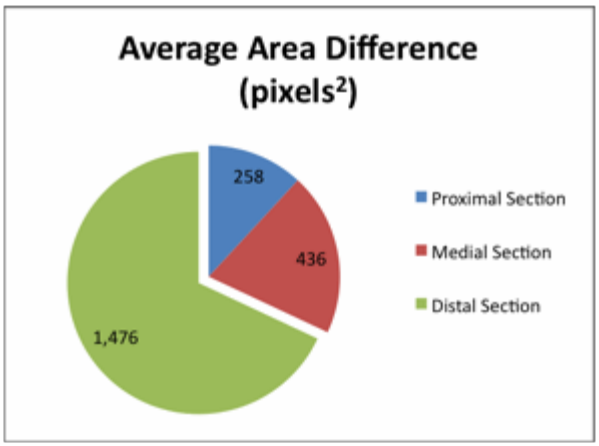
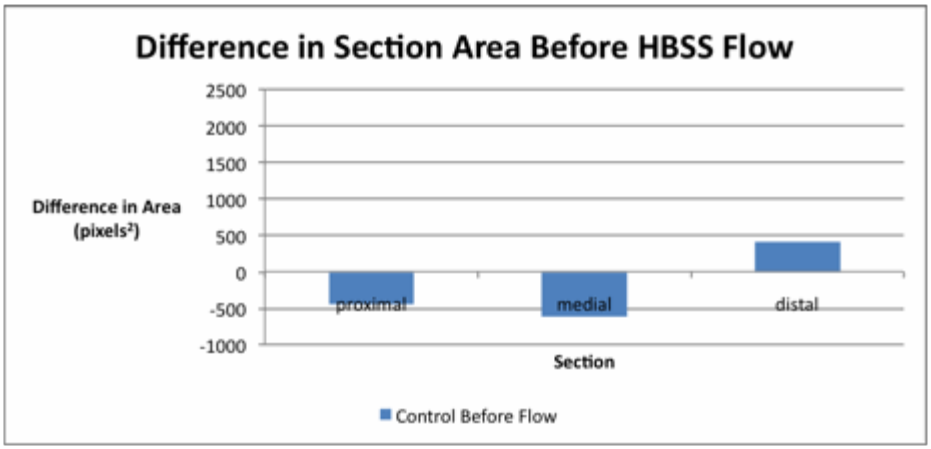


Figure 3 Legend: This pie graph depicts the average area difference in square pixels for each of the three sections described in the experiment. Section 1 is the section closest to the cell soma. Section 2 is the section in the middle. The distal section is the section that includes the growth cone. The n value for each section was 4.

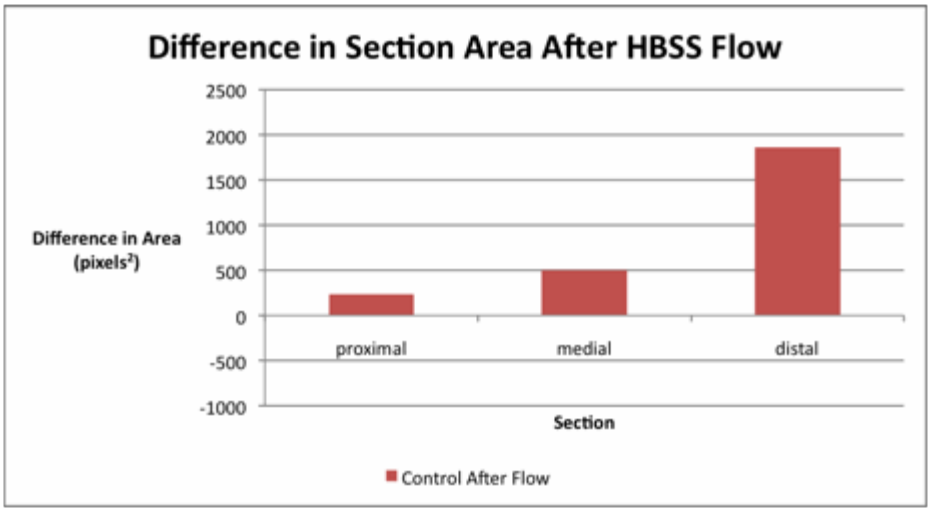
Figure 3 shows that the distal section has the largest average area difference between five minute intervals. It also constitutes an overall majority of average area difference for all three measured sections combined. The differences in sectional area were also compared across each time section of the experiment excluding across flow times (Figure 4).

Figure 4

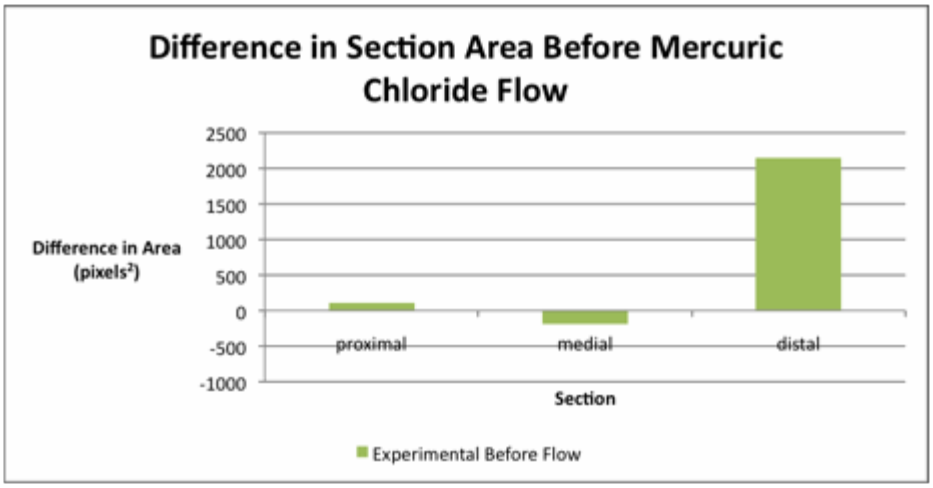
A.


Difference in Section Area Before HBSS Flow
2500
2000
1500
1000
500
0
-500
-1000
Difference in Area (pixels²)
proximal
medial
distal
Section
Control Before Flow

B.


Difference in Section Area After HBSS Flow
2500
2000
1500
1000
500
0
-500
-1000
Difference in Area (pixels²)
proximal
medial
distal
Section
Control After Flow

C.


Difference in Section Area Before Mercuric Chloride Flow
2500
2000
1500
1000
500
0
-500
-1000
Difference in Area (pixels²)
proximal
medial
distal
Section
Experimental Before Flow

D.

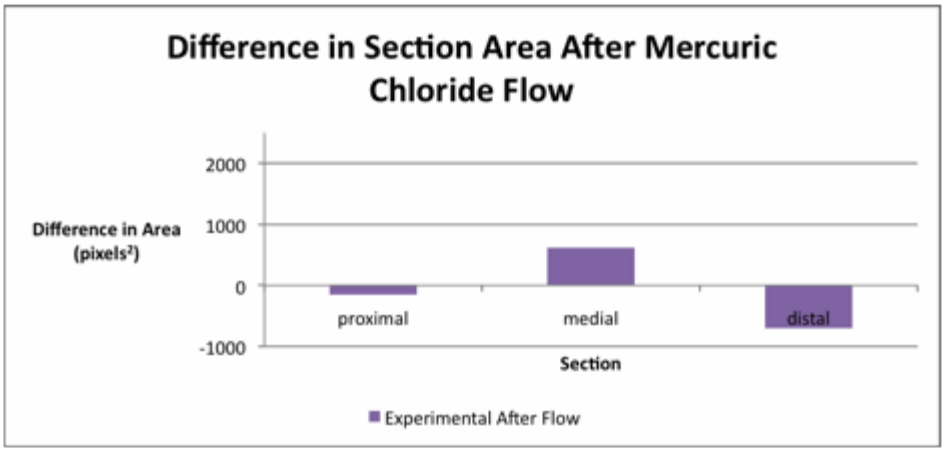


Figure 4 Legend: This graph shows the differences in area across five minutes intervals vs. the sections the areas were measured in. The four different five minute intervals were: before flow in the control (Figure 4A), after the flow in the control (Figure 4B), before flow in the experimental (Figure 4C), and after flow in the experimental (Figure 4C). The HBSS flow and mercuric chloride flows were applied over five minutes. There was an n value of one calculated area difference for each section across the two images of each five minute interval.

Figure 4 shows that difference in area is only negative in the distal section for the experimental group after flow. This indicates that the experimental group in lost area in section 3 after mercury flow and no other group had that quality. The two highest differences in area and the most negative difference in area were all calculated for section three.

Discussion and Conclusion:

These data support the hypothesis that the addition of mercuric chloride causes net retrograde area redistribution in neurites. Previous experiments (Vogel et al. 1985) suggest that this result could be caused by microtubule depolymerization in the axon. These data also support the hypothesis that this is a useful technique for quantifying retraction of axons and growth cones. There is a noticeable difference in growth in the distal section, which includes the growth cone, between the control after flow (Figure 4B) and the experimental after flow (Figure 4D). The experimental growth before the flow (Figure 4C) also appears to be very high in the distal section. This further supports that this loss of area was due to the mercuric chloride flow.

Figure 3 shows that the majority of the absolute area differences occur in section 3. This means that the proximal and medial sections are more likely to fluctuate between negative and positives changes in area because they don't have much overall area change. However, the area differences in the distal section are substantial. Therefore fluctuations in this section are more noteworthy.

If more axonal areas were gathered then it would indicate whether my data were significant. The experiment also used 0.75ml of flow for the controls and 1.5ml of flow for the experimental data because the mercury needed to be washed out. Next time a consistent flow volume should be used to ensure that the additional flow volume in the experimental group is not what caused net retrograde area redistribution in the axon. Even though the same brightness was used in each image the images were clearly different colors. Next time the images should have consistent light exposure to ensure that differences in image brightness do not increase or decrease the measured area values.

The cellular implications of these data are that the mercuric chloride may have caused the microtubules in the axon to depolymerize. It is unknown if this also works in the same was as methylmercury by binding to sulfylhydryl groups on the microtubules (Vogel et al. 1985). Leong et al. (2000) found that the collapsed part of the growth cone was “bulbous” in shape, but I found it to only increase the area of the axon in the medial axon section. Vogel et al. (1985) achieved a maximum axonal retraction rate of 1/3 of the axon length per minute by introducing 50μM methylmercury. It did not appear that retraction rates were that high for this experiment. Data was not collected over a long enough time to get a measured loss of directionality like Söderström and Ebendal (1995) or a lasting effect due to mercury like Grandjean et al. (1997). Future experiments could use this novel method of quantitation to investigate changes in axonal area distribution.

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Eastern Hsu assisted me in collecting data during the observation and flow chamber and observation segments of the experiment. Professor Robert Morris dissected the chick embryos used for this experiment and created the cultures for the DRGs afterward.

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

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