# A growth cone axon will undergo retraction *in vitro*, following exposure to mercuric chloride ($HgCl_2$).

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Neurobiology Short Report
Bio 324/Neurobiology
Wheaton College, Norton, Massachusetts, USA
November 29, 2011

## Introduction

Mercuric chloride ($HgCl_2$) is one of three forms that mercury can been exposed to humans. It is the least dangerous of the three, with methyl mercury ($CH_3Hg^+$) being the most dangerous, followed by elemental mercury (Hg) (Benoit, Wheaton College, personal communication, 9/21/11). Mercuric chloride used to be found in a variety of medicines and use in agriculture methods, but most of those uses have been banned in the United States (Thiessen, 1994). It is still used today in pesticides and for a various chemical synthesizes carried out in labs. According to the EPA assessment of mercuric chloride, up to 20,000 people in the U.S.A are exposed to the compound. (Thiessen, 1994). $HgCl_2$ has various effects on an assortment of human organs and cells. It can cause damage to the reproductive system of a male and female, mutations of the somatic cells can occur, and it can cause damage to the brain, peripheral nervous system (PNS) and central nervous system (CNS) (Mercuric Chloride, MSDS, 2005).

A group, from the Wheaton College, Bio class 324, focused on the effects of mercuric chloride on growth cones harvest from the sympathetic nerve chain and/or dorsal root ganglion of a domestic chicken embryo, *Gallus gallus*. Though the amount of past studies done on mercuric chloride's effects on nerve growth cones is small, the results are still relevant. A study

done by Leong, Syed and Lorscheider look at the effects of mercuric chloride on growth cones in 2000. They found that following exposure, neuron growth cones "ceased motility" and "exhibited robust collapse and retraction" (Leong, 2000). Recently, reports of exposure of Hg ions to microtubule tubulin monomers cause disruptions in the normal polymerization of the monomers into a microtubule polymer (Leong, 2000). The binding of GTP, which is necessary for tubulin polymerization, is reduced in the presence of Hg ions (Leong, 2000). Fluorescent images of stained microtubules and actin from the Leong study show a decrease in area from before treatment to after treatment of $HgCl_2$ (Fig.2. Leong, 2000). Another study done in 1996, show that inorganic mercury, such as mercuric chloride, can be taken up by motor neurons within the brain and spinal cord (Pamphlett and Waley, 1995). No conclusions were discussed on whether the inorganic mercury did damage to the motor neurons. The older study done by Imura, Miura, Inokawa and Nakada in 1980 showed that microtubules in mouse glioma cells injured microtubules by depressing tubulin polymerization (Imura, 1980).

As mentioned before, a group study looked at the effects of $HgCl_2$ on neuron growth cones. This study in particular, looked at the effects of $HgCl_2$ on the growth cone's axonal length. Growth cones from either a dorsal root ganglion or sympathetic nerve chain were harvest from the embryos of *Gallus gallus*. Growth cones are the terminal ends of a neurons' growing axon, which extend and explore the extracellular space of a neuron (Purves, 2001). Well-renown neurobiologists Ramon y Cajal said that "the growth cone was responsible for axonal pathfinding" (Kandel, 1991). Using chemical sensitivity, the growth cone will decide on the direction to go, and undergo dramatic structural changes to extend in the intended direction (Purves, 2001 and Kandel, 1991). Ultimately, the growth cone is looking for a synaptic target with which to associate with (Kandel, 1991). The microtubules in the axon play a crucial role in

the lengthening of axons and ultimate outward extension of a growth cone (Tanaka, Ho and Kirschner, 1995). Microtubules in the axon play a role in guiding the direction of the growth cone and the behavior of the microtubules determine the behavior of the growth cone (Tanaka, Ho and Kirschner, 1995).

I hypothesized that growth cone axons will undergo retraction after exposure to mercuric chloride ($HgCl_2$). This will be tested by exposing culture *Gallus gallus* neuron growth cone to a dilute solution of mercuric chloride via flow through a chip chamber. The measured distance of the axon between the growth cone and the connecting branch point will quantify outward growth. Axonal growth will stop because the mercuric chloride will disrupt the polymerization of microtubules and possible de-polymerization may occur. Since growth cones have not been exposed to mercuric chloride in the same way as the procedure used for this study, I expect different results then past studies.

# Methods and Materials

*Animals:* Chick embryos from Charles River Laboratories, Wilmington Massachusetts.

*Dissection and Cell Culture:* Dissection was carried out on ten-day-old chick embryos. Isolated one sympathetic chains and six dorsal root ganglions (DRG) (Morris 2011a). Two alterations occurred in the procedure for our experiments. The procedure labeled “Flame-constriction of Pasteur pipettes” was not used. The procedure labeled “Dissociation of ganglia part 1 – cells into trypsin” was also omitted. Instead the DRGs were culture in chunks.

*Control plating and imaging;* Control trial was performed on culture cells that had been incubated in Leibovitz L-15 medium. The procedure for creating the flow chamber can be found in part A of Morris (2011c). A Nikon E200 Eclipse Microscope with Phase Optics was used for viewing slide. The images were viewed on an iMac OS X desktop computer (version 10.5.8) using Sony DFW-X700, with C-mount 1.0x camera. The images were viewed in the application BTV (version 6.0bl) on the desktop and all images were taken in the Wheaton College ICUC room. Imaging procedure can be found in part B of Morris (2011c).

3 images were taken with three minutes between each image. Following the third image, the buffer solution was flown through the chip chamber. The solution was Hanks Balanced Salt Solution (HBSS) and a total of about 1mL was used to flow through the chamber. The procedure for conducting the flow chamber can be found in part C under Morris (2011c). Immediately following the flow chamber procedure, three more images were taken, each with three minutes in between. After the control trial a total of 6 images were collected.

*Experimental plating and mercuric chloride application:* Experimental trial was carried out on cells culture that had incubated in Leibovitz L-15 medium. The procedure for creating the flow chamber and imaging the cells are the same to the control trial and can be found in part A and B of Morris (2011c). A Nikon E200 Eclipse Microscope with Phase Optics was used for viewing slide. The images were viewed on an iMac OS X desktop computer (version 10.5.8) using Sony DFW-X700, with C-mount 1.0x camera. The images were viewed in the application BTV (version 6.0bl) on the desktop and all images were taken in the Wheaton College ICUC room. 3 images were taken with three minutes in between each image.

After the third image was taken a diluted solution of mercuric mercury was flown through the chip chamber. The concentration of the mercuric chloride solution was 10 µm in 0.05% HCl. A total of 190 µL was used to flow through the chip chamber. Once the diluted mercuric chloride had all flown through the solution was allowed to sit in the chamber for 20 minutes. After 20 minutes, 1 mL of HBSS was flown through the chip chamber to remove all traces of the mercuric chloride. Immediately following the buffer flow an image was taken. Two more consecutive images were taken with a three-minute space in between. A total of six images were taken during the experimental trial.

*Measurements:* Images from both control and experimental trial were imported into ImageJ. In each photo, the growth cone was located. A growth cone was defined as the terminal end of the neuron where the axon diverged from the parallel. The branch point was also identified in the photo as the point where the axon diverged from parallel to a mass that had multiple extensions. Once these two point were indentified, a line was drawn from the branch point where the axes of the axons were symmetric, to the center of mass of the growth cone. This measurement was recorded as part of the results.

# Results

Quantified data from the control trial showed growth in the axon before and after the flow of buffer, with an accidental coverslip jostling. An average length of 139.4 was calculated from each of the images. The increase in length of the axon from time 1 to time 6 was 28.2 pixels. The difference in visual length can be seen in Figure 1, which contains the first image

taken (1A) and the last image taken (1B). The axon's length did not grow steadily, but instead had variation in growth and retraction as seen in the graph in Figure 3. From time 1 to 3, which was before the HBSS exchange, there was a growth of 57.5 pixels and from time 4 to 6, after the HBSS exchange, there was a growth of 40.1 pixels. Time 1 was at 0 minutes, time 2 was 3 minutes into viewing the cell, time 3 was 6 minutes into viewing, time 4 was 29 minutes into viewing, time 5 was 32 minutes into viewing and time 6 was 35 minutes into viewing.

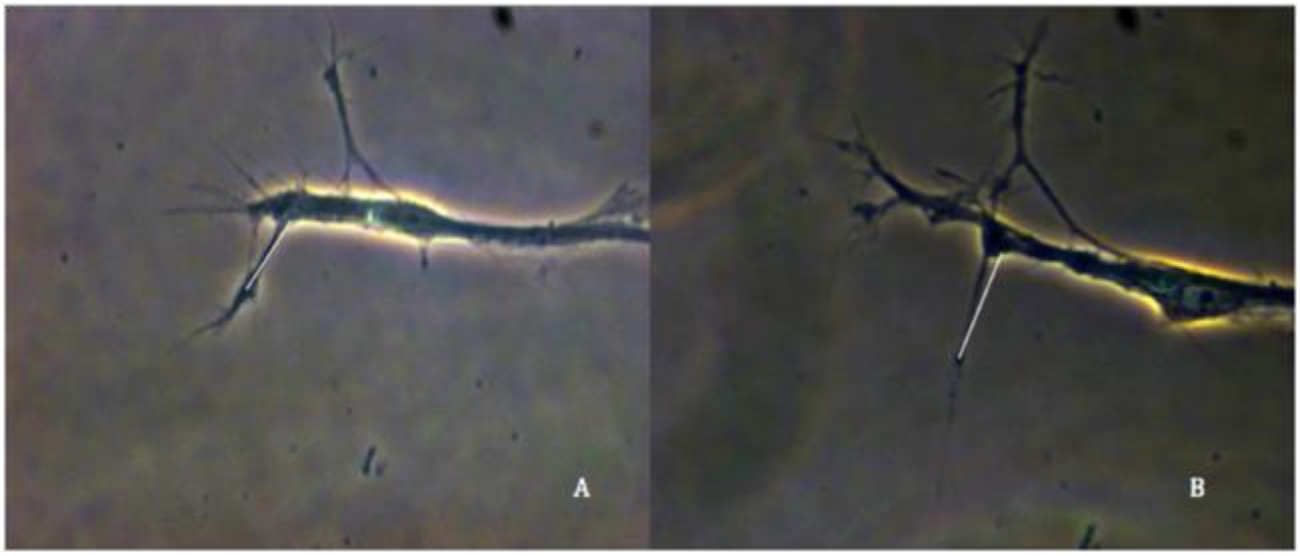


**Fig 1.** Difference in axonal length as seen in the images of the same growth cone at 40x from time 1 at 0 minutes (1A) to time 6 at 35 minutes (1B). The growth cone with the white line indicating the measured length from ImageJ was used to get the measured data. Image for time 1 (1A) occurred before the flow of 1ml of HBSS solution. Image for time 6 (1B) occurred 29 minutes after the start of the buffer exchange.

The quantifiable results from the experimental trial also showed growth of the axon before and after mercuric chloride was flown through, allowed to sit for 20 minutes and was then followed by HBSS flow. The average length of the axon from the six images was 142.6 pixels. From time 1 to time 6 there was a growth of 106.5 pixels. The difference in the lengths can be seen in

Figure 2. Visually, the growth cone center of mass is farther away from the branch point in the right image (2B) compared to the left (2A). The growth of the axon from time 1 to time 6 was not steady. Throughout the images there were variations of growth and retraction as seen in the continuous data graphed in Figure 3. From time 1 to time 3, which was before the exposure to mercuric chloride and HBSS, there was a growth of 24.6 pixels. From time 4 to time 6, which was after the 20-minute mercuric chloride exposure and buffer exchange, there was a growth of 2.7 pixels. Time 1 was at 0 minutes, time 2 was 3 minutes into viewing, time 3 was 6 minutes, time 4 was 35 minutes into viewing, time 5 was 38 minutes into viewing and time 6 was at 41 minutes. Figure 3 graphs a total of 12 images giving an n value of 11.

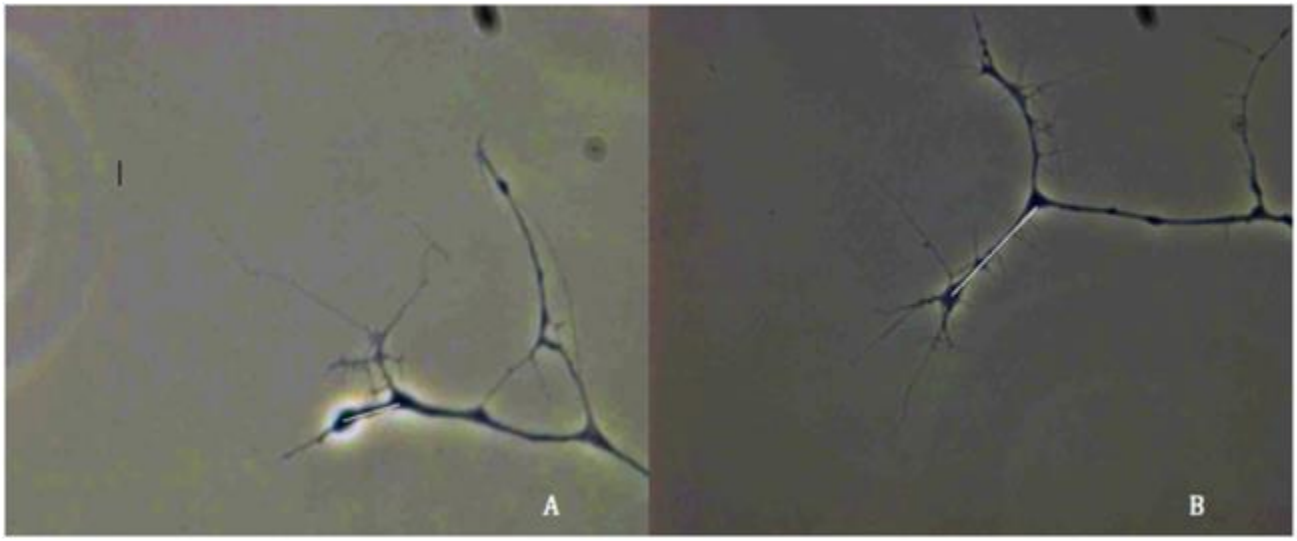


**Fig 2.** Difference in axonal length as seen in the images of the same growth cone at 40x from time 1 at 0 minutes (2A) to time 6 at 41 minutes (2B). The growth cone with the white line indicating the measured length from ImageJ was used to get the measured data. Image for time 1 (2A) occurred before the flow of 190 µL of mercuric chloride and 1 mL of HBSS solution. Image for time 6 (2B) occurred 35 minutes after the start of mercuric chloride flow.

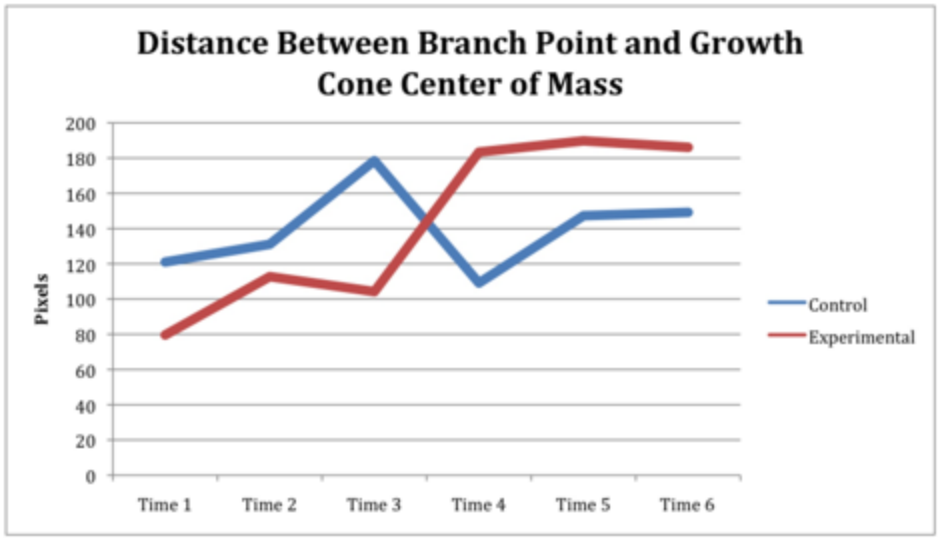


**Fig 3.** This graph shows the difference in lengths measured from six images in each trial. The times are different for each trial so the lines do not coincide in minutes. The first three points are before flow of any solution and the last three are after flow was finished. (n=11)

# Discussion

In this study the hypothesis that growth cone axons will undergo retraction after exposure to mercuric chloride ($HgCl_2$) was tested. The data collected did not support my initial hypothesis because the axon did not exhibit a decrease in length between the growth cone center of mass and the branch point. I was expecting to see a decrease in the lengths overall in times 4, 5, and 6 in the experimental trial. In conclusion, I would state that mercuric chloride does not cause de-polymerization of the alpha and beta tubulin of microtubules.

My collected data did supported conclusions drawn from previous studies, such as Pamphlett and Waley's study and Imura, Miura, Inokawa and Nakada's study. There was a 93.3% decrease in outward growth from the control trial after HBSS flow to the experimental trial of mercuric flow. The graph in Figure 3 shows the line representing the experimental data leveling out in time 4, 5, and 6, which were after the flow of mercuric chloride. There was still a

total growth of 106.5 pixels from time 1 to time 6, which skews the data. This could relate to the fact that while being exposed to the mercuric chloride and buffer, the growth cone had 20+ minutes to continue outward extension and should be taken into consideration. The papers mentioned before, state that following exposure to mercuric chloride, microtubules in the axons will cease polymerization, thus ceasing outward growth of the growth cone (Pamphlett and Waley, 1995) and (Imura, 1980). In conclusion, their data was further supported by my results and my hypothesis was not.

If the results were the same from an exact repeat of this study, but had a larger pool of data by either comparing in both the control and experimental trial, more than one axon per cell in a single culture or more than one axon from different cultures than I would be convinced that mercuric chloride does not cause retraction of the axon. And therefore, would be convinced that mercuric chloride does not cause de-polymerization of the microtubules' tubulin monomers. However, if this experiment was repeated with a larger pool of data and supported other results, such as retraction, than I would be convinced that mercuric chloride does indeed cause de-polymerization of the tubulin. This study only compares two different axons from two different cells, so a larger pool of data would give more support no matter the outcome.

Improvements for the experiment could also be used for future studies. Some suggestions would be to image the microtubules directly by immunofluorescence. This was unavailable to us, but might provide data on activities of microtubules after exposure to hard metals. Another suggestion for a future experiment would be to culture the cells in mercuric chloride instead of L-15 growth medium. This would be testing a different hypothesis since it no longer pertains to acute exposure but instead pro-longed exposure.

Some experimental errors occurred during this study. Mentioned before in the results section, the buffer overflowed in the control trial, touching the lens of the microscope. While cleaning the lens, the slide was jostled around and could have altered the data. There is some human error present in this study since the lengths taken could not be from a fixed point but instead were from my personal judgment. I decided to take this path even though a fixed point was available in each trial because the angle of the axon to the fixed point varied greatly and would have given unavailing data.

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