Axonal degeneration as a result of *in vitro* Mercury exposure

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Neurobiology Short Report

Bio 324 / Neurobiology

Wheaton College, Norton, Massachusetts, USA

11/30/11

Introduction:

Neural stem cells play an essential role in the development and maturation of the body's nervous system (Gage, 2000). However, the developing nervous system is very susceptible to damage from neurotoxins. Mercuric chloride has been found to be an extremely detrimental neurotoxin due to its interaction with tubulin which causes microtubule disassembly (Falconer et al., 1994; Tamm et al., 2006). At certain concentrations, mercury has also been shown to cause axonal and nerve fiber degeneration as well as the loss of synapses in the central nervous system (Capo et al., 1994). Mercury is also known to inhibit the production of neurotransmitters by disturbing calcium ion levels and thus calcium-dependent neurotransmitter release (Atchison and Hare, 1994). As detrimental as mercury is to neurons in the central nervous system, it is just as damaging to neurons of the peripheral nervous system. Mercury stays within neurons and causes a reduction in the size of myelinated motor axons (Pamphlett and Png, 1998). Many studies have looked at the detrimental effects mercury can have on neurons and cell growth, however, very few studies have looked at the rate of axonal degeneration after exposure to specific concentrations of mercury. In this study we used chick embryos (*Gallus gallus*) and treated the chick dorsal ganglion neurons with 100nM mercuric chloride. By using phase imaging techniques, it is the hope that this study can determine the rate of degeneration of nerve axons after being exposed to mercury and test the hypothesis that the velocity of axonal degeneration after exposure to mercury will be greater than the velocity of axonal growth or degeneration in the control nerve cell after the buffer exchange.

Materials & Methods:

Dissection:

10 day old chick embryos were used in this experiment. The methods used for this dissection can be found in Jenifer DeNormandie's notebook (9/28/11 9 JD). It was Professor Morris's procedure handout Primary Culture Of Chick Embryonic Peripheral Neurons 1: DISSECTION (Morris, 2011a). We followed all steps in the procedure to do a proper dissection of dorsal ganglion cells and sympathetic neuron chain (Thorne, 2011).

Observation and Data Collection:

Microscope used was a Nikon EFD-3 with sony DFW-x700 digital interface camera on a Mac OSX version 10.5.8. The imaging for the experiment was done using BTV software and then analyzed using imageJ version 1.40g.

While viewing the axons and growth cones under the microscope and taking pictures, the stage was kept warm using a LASKO ceramic Air Heater. The air around the stage was kept around 36-37°C and this was monitored using a digital thermometer.

Control:

This part of the procedure was taken from Professor Morris's procedure handout Primary Culture Of Chick Embryonic Peripheral Neurons 3: STAINING and OBSERVATION (Morris, 2011b). I followed all steps in part A, and part B through to step 10. Nerve axon and growth cone were imaged and using a Samsung cell phone as a stop watch, the nerve cells were imaged from time zero, then every 2 minutes for 6 minutes making a total of 4 images. We followed part C steps 26-34 of Morris's Primary Culture Of Chick Embryonic Peripheral Neurons 3: STAINING and OBSERVATION (Morris, 2011b). We then imaged the same cone every 2 minutes for 20 minutes for a total of 11 images including time zero (Thorne, 2011).

Observations: The buffer exchange did not harm the nerve axon or growth cones. Lamellipodia and filopodia were observed. Filopodia grew, became lamellipodia and then more filopodia grew off the lamellipodia (Thorne, 2011).

Experimental:

This part of the procedure was taken from Professor Morris's procedure handout Primary Culture Of Chick Embryonic Peripheral Neurons 3: STAINING and OBSERVATION (Morris, 2011b). I followed all steps in part A, and part B through to step 10. Nerve axon and growth cone were imaged and using a Samsung cell phone as a stopwatch, nerve cells were imaged from time zero, then every 2 minutes for 6 minutes making a total of 4 images. We followed part C steps 26-34 of Morris's Primary Culture Of Chick Embryonic Peripheral Neurons 3: STAINING and OBSERVATION, however instead of the buffer, 100 nM mercuric chloride was used (Morris, 2011b). The same nerve axon and growth cone was then imaged again starting at time zero after exchange then every 2 minutes for 20 minutes of exposure to mercuric chloride. We followed part C steps 26-34 of Morris's Primary Culture Of Chick Embryonic Peripheral Neurons 3: STAINING and OBSERVATION (Morris, 2011b). After exchanging the mercuric chloride for buffer, the same cone was then imaged at time zero then every 2 minutes for 20 minutes (Thorne, 2011).

For data collection and analysis, the program, ImageJ was used along with Microsoft excel. The images brightness minimum was set at 46 while the maximum was set at 196 for each image. The axon was measured from the point at which it grew out from the cell's soma or other given point of origin to the beginning of the growth cone. The beginning of the cell's soma was defined as the point where there was narrowing and increased contrast relative to the background of the cell. The given point of origin for the cell's axon in the experimental trial was the point of increased contrast between the cell and the grey background following a landmark filopodia on the cell's axon. The beginning of the growth cone was defined as being the beginning of the area where there the contrast of the cell relative to the background decreased or where there was a marked increase in the width of a the cell indicating the beginning of a lamellipodia. Filopodia and lamellipodia were not included when measuring the nerve axon length. Axon length was measured using the segmented line tool in image J. These lengths were then taken and compared to the original axon length at time 0 to determine the percent change in axon length over the 20 minutes. So for example to calculate the percent the length at time 2, the length at time 2 was divided by the original length at time 0 and then multiplied by 100. Though 11 images were taken during both the experimental trial after the mercury exposure and the control trial after the buffer exchange, only 4 images in from each trial used. Though it would have made sense to look at every 5 minutes since that would have made for 4 equidistant data points over the full 20 minutes, but since the cell was only imaged every 2 minutes, the 4 points ended up being 6 minutes apart. To include the last image at time 20 minutes, the initial image used had to be at time 2. So only the images taken at time 2, 8, 14, and 20 minutes were used and analyzed for change in axon length. Axon length was then compared using Excel and velocity of degeneration was calculated. This was done by looking at three time intervals and the length of the axon at the beginning and end of those time intervals. These intervals were 2 to 8 minutes, 8 to 14 minutes, and 14 to 20 minutes. Since velocity is change in distance divided by time, the axon length at the end of the time interval was subtracted from the axon length at the beginning of the time interval and then this change in axon length was divided by the time it took for that change and for each interval that time was 6 minutes.

Results:

In the control trial in which the neuron was not exposed to mercury, there is no significant change in the length of the axon. 8 minutes after the buffer exchange the length of the axon was at a high point at 584.241 pixels in length while at the end of the observation, after 20 minutes had passed since the buffer exchange, the nerve's axon was 563.263 pixels in length. The images of the nerve axon at these time points can be seen in Figure 1.

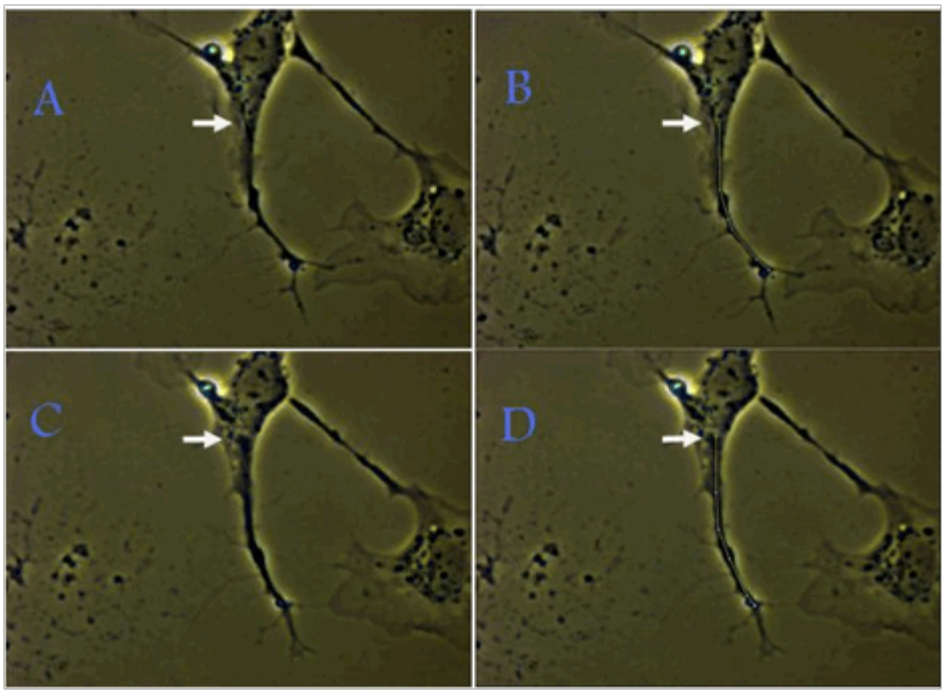

Figure 1: Control trial images of a nerve axon, the image at time 8 minutes and time 20 minutes. A and B show the nerve at time 8 minutes after the buffer exchange in the control trial. B also shows the line segment from image J measuring the length of the nerve axon. C and D show the nerve at time 20 minutes after the buffer exchange in the control trial. D also shows the line segment from image J measuring the length of the nerve axon. Note that the line segments shown in B and D are very similar in length.

In the experimental group, which was exposed to mercury, the axon length 8 minutes after the 20 minute exposure was 754.315 pixels and after 20 minutes after the 20 minute exposure the axon length dropped to 547.932 pixels. The images of the neuron at these time points can be seen in Figure 2.

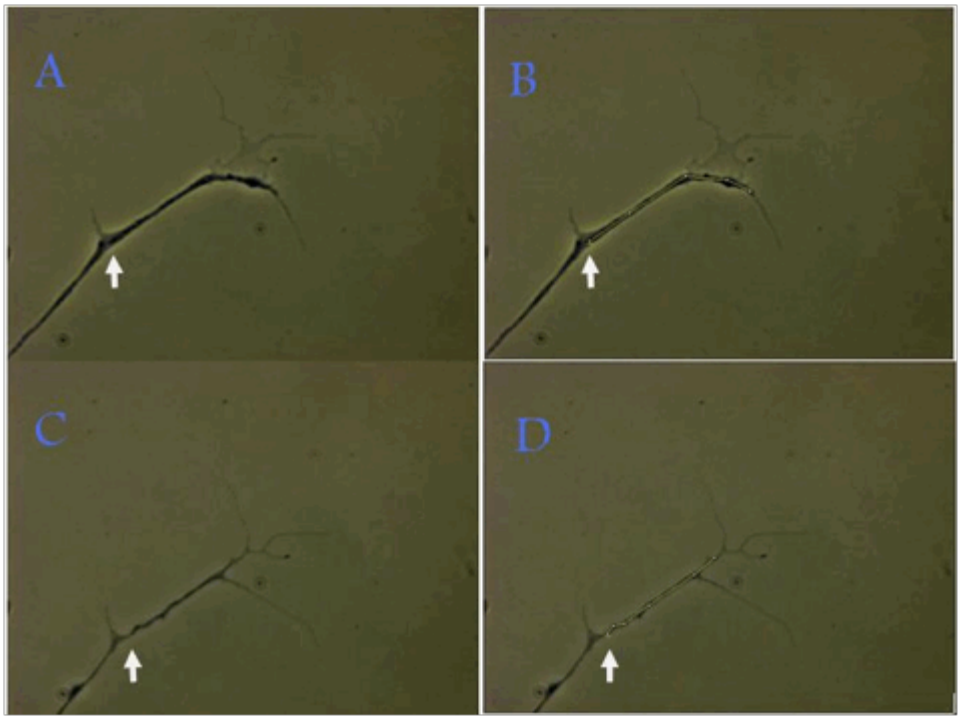

Figure 2: Experimental trial images of a nerve axon, the image at time 8 minutes and time 20 minutes. A and B show the nerve at time 8 minutes after the 20 minute mercury exposure and then the exchange of mercury for buffer in the experimental trial. B

also shows the line segment from image J measuring the length of the nerve axon. C and D show the nerve at time 20 minutes after the exposure and exchange of mercury for buffer in the experimental trial. D also shows the line segment from image J measuring the length of the nerve axon. Note that the line segment shown in D seems to be shorter than the line segment shown in B.

In order to determine whether mercury exposure would cause axonal degeneration and if the velocity of that axonal degeneration after exposure to mercury is greater than the velocity of axonal growth or degeneration in the control nerve cell after the buffer exchange, images were taken before and after the mercury exposure and buffer exchange. Axon length was measured in 8 images, 4 after the mercury exposure in the experimental trial and 4 after the buffer exchange in the control trial. These lengths and the changes in these lengths were quantified and converted to percentages using the procedure previously described. The results obtained are illustrated in Figure 3.

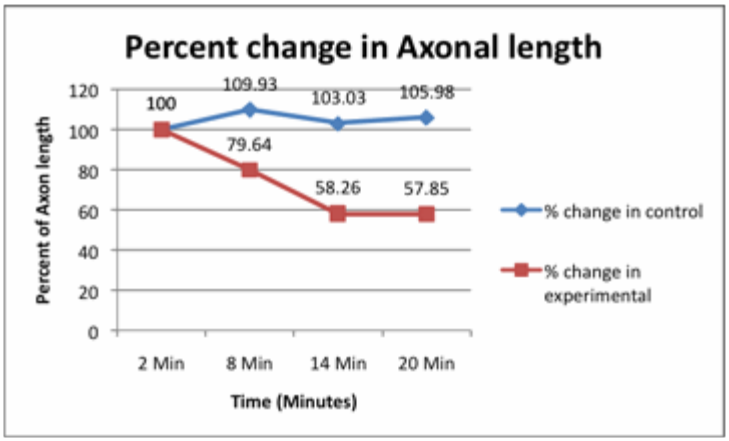

Figure 3: Graph of the percent axon length for the control and experimental trials. The graph demonstrates the change in the nerve axons length over time in both the experimental and control trials. These data that lead to these percentages were taken after the buffer exchange in the control trial and after the 20 minute mercury exposure and then buffer exchange (wash) in the experimental trial. So the times given in the graph pertain to how long after the buffer was exchanged in the control trial and how long after the mercury was replaced with buffer in the experimental trial. Note how the control percentages remain high, all greater than 100 percent. However there is some degeneration observed at 14 minutes, but the axon has some re-growth after this degeneration suggesting this may be due to pruning or simple changes in the shape and structure of the neuron.

The results from the two trials were further analyzed in that the lengths and the change in the lengths over time were quantified and displayed as velocity of axonal degeneration. Velocity was calculated using the procedure previously described. The results obtained are illustrated in Figure 4.

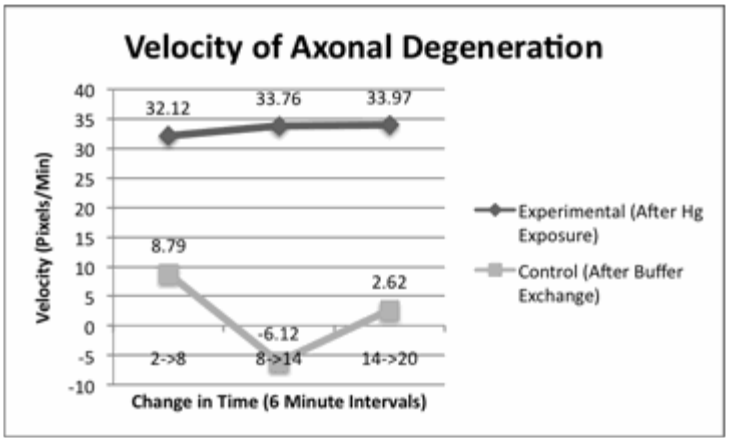

Figure 4: A comparison of the velocity of axonal degeneration in both the control and experimental trial over the three six minute time intervals. The times given in the graph pertain to how long after the buffer was exchanged in the control trial and how long after the mercury was replaced with buffer in the experimental trial. Note that the experimental trial has a greater velocity of degeneration than the control buffer exchange trial. The experimental trial's velocity of axonal degeneration remains consistent at an increasing rate of degeneration.

By looking at Figure 3 and Figure 4, it is evident that exposure to mercury had a significant effect on axon length and its rate of degeneration when compared to the control trial. During and immediately following mercury exposure, axonal degeneration was noticed as well as filopodia and lamellipodia degeneration.

Discussion:

The hypothesis of whether mercury exposure would cause axonal degeneration and if the velocity of that axonal degeneration after exposure to mercury would be greater than the velocity of axonal growth or degeneration in the control nerve cell after the buffer exchange was supported by the data which show that there was a dramatic decrease in axon length following mercury exposure where there was little change in the axon length of the control neuron which was not exposed to mercury. This indicates that at the concentration of 100nM, mercuric chloride can cause axonal degeneration that may be due to its interaction with tubulin causing microtubulin disassembly (Falconer et al., 1994; Tamm et al., 2006). These findings were expected because of the previous research done with mercury and neurons by Pamphlett and Png (1998). Though they only looked at myelinated motor axons in the peripheral nervous system, they found that mercury will stay within the neuron and cause the motor axons to reduce in size. Axons are a very important part of the neuron and the nervous system. If exposure to mercury causes a reduction in their length then this could cause problems with signal transduction since the spaces between neurons may become too great. Due possible neurological and physical implications as a result of axonal degeneration, it is necessary to study the relationship

between mercury and axonal degeneration and that is what this study attempted to do. The findings of this study suggest that there is a possibility that during the exposure to mercury, some mercury made it into the neuron and caused the constant and increasing velocity of axonal degeneration. If this study were repeated and enough of the same results were found to indicate significance, then there would be strong evidence suggesting that after exposure to mercury, axons will degenerate at an increasing rate which is much greater than that seen in axons not exposed to mercury.

It should be noted that due to the time constrictions of this experiment, the control neurons only had one buffer exchange and were not allowed to sit for 20 minutes in buffer that would have represented the 20 minute mercury exposure in the experimental trial. If this experiment were repeated with a larger sample size with different concentrations of mercury would be useful to address how much exposure to mercury is needed before major degeneration is seen. Future studies could also be interested in looking at what concentrations of mercury and length of exposure are necessary before neural cells are affected by this exposure. Such an experiment would be helpful for determining how much mercury humans can be exposed to before expecting any significant neurological problems.

References:

Atchison WD, Hare MF. Mechanisms of methylmercury-induced neurotoxicity. *FASEB J*. 1994;8(9):622-629.

Capo MA, Alonso CE, Sevil MB, Frejo MT. "In vitro" effects of methyl-mercury on the nervous system: A neurotoxicologic study. *J Environ Pathol Toxicol Oncol*. 1994;13(2):117-123.

Falconer MM, Vaillant A, Reuhl KR, Laferriere N, Brown DL. The molecular basis of microtubule stability in neurons. *Neurotoxicology*. 1994;15(1):109-122.

Gage FH. Mammalian neural stem cells. *Science*. 2000;287(5457):1433-1438.

Morris, R.L. (2011a) Neurobiology Bio324 Primary Culture Of Chick Embryonic Peripheral Neurons 1: DISSECTION. Available at http://icuc.wheatonma.edu/bio324/2011/LabProjProc/1.htm

Morris, R.L. (2011b) Neurobiology Bio324 Primary Culture Of Chick Embryonic Peripheral Neurons 3: STAINING and OBSERVATION. Available at http://icuc.wheatonma.edu/bio324/2011/LabProjProc/3.htm

Pamphlett R, Png FY. Shrinkage of motor axons following systemic exposure to inorganic mercury. *J Neuropathol Exp Neurol*. 1998;57(4):360-366.

Tamm C, Duckworth J, Hermanson O, Ceccatelli S. High susceptibility of neural stem cells to methylmercury toxicity: Effects on cell survival and neuronal differentiation. *J Neurochem*. 2006;97(1):69-78.

Thorne, B.L. 2011 Personal Communication.