Average Length of Axons Before, During, and After Incubation with DMEM and DMEM with Methylmercury

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I. Introduction:

The property of neurons studied was the growth and retraction of the axons, comparing the results of neurons incubated in DMEM and in DMEM supplemented with methylmercury for any significant discoveries of axonal behavior. The hypothesis tested in the experiment was if the embryonic peripheral neurons were exposed to a concentration of methylmercury, the axons will experience either a cease in growth or will retract (Maricle, C. 2014).

Studying of effects of methylmercury on axonal growth is important because many people suffer from methylmercury-based diseases that predominantly affect various parts of the brain (Grandjean, P., Weihe, P., et al., 1997) . Future neuronal development complications (Weston, H.I., Sobolewski, M.E., et al., 2014). are another concern to investigate, leading to the importance to study the effects of methylmercury on prenatal cells as much as mature cells (Benoit, J. 2014).

Primary cultured chick embryonic peripheral neurons of the species *Gallus gallus* were harvested and plated by Professor Bob Morris for the experiment. The background of methylmercury poisoning starts with the recognition that methylmercury is an example of a toxin made of inorganic material and is synthetic (Benoit, J. 2014). Human actions, including and not limited to coal burning or artisanal gold mining, have contributed a great deal of the atmospheric influx of mercury, which stays in the air for around a year and can travel great distances, capable of causing health concerns to other people across the globe (Benoit, J. 2014). Mercury can deposit into terrestrial and aquatic ecosystems (even by means of rain and snow) and any animal can be exposed to the mercury (Benoit, J. 2014). The collection of methylmercury brings another danger to organisms involving methylmercury bioaccumulation (while not the focus of the experiment) affects every organism in the food web (Benoit, J. 2014). Methylmercury can attack the mitochondria of cells directly, making the cell suffer degenerative effects as well as accumulating in reactive oxygen species (ROS's), where without antioxidants to keep ROS's in check, the mitochondria damage can ultimately lead to cell death, aging, and damaged or mutated mitochondrial DNA (Benoit, J. 2014). A lot is still unknown about the complete effects of

methylmercury on neuronal cells and generally in human health, especially on a cellular level, and little studies are focused on the side effects of methylmercury exposure with regards to axons. What would be the ultimate result if axons were to retract and not allow neurons to interconnect with one another?

For background on axonal growth, axons along with dendrites are ultimately instructed to form functional connections between neurons and target cells, which is important during the developmental stage and in later stages for any organism (Kandel, E.R., Schwartz, J.H., et al. 2013). Axonal growth cones, specialized tips at the very end of axons, are both sensory and motile in function (Kandel, E.R., Schwartz, J.H., et al. 2013). Filopodia, sandwiched with lamellipodia, receive signals from the environment of the neuron, which stimulates movement forward, backward, or to the side as the actin moves along the myosin to allow the neuron to outgrow farther (Kandel, E.R., Schwartz, J.H., et al. 2013). The binding of a ligand to the receptors of the axonal membrane is crucial to the advancement or retreat of axons of neurons, involving factors both inside and outside of the cell (Kandel, E.R., Schwartz, J.H., et al. 2013). What would happen if methylmercury were the ligand? Would the axons take the methylmercury to be a signal to retreat, advance, or stay stationary?

In the experiment, chick embryonic dorsal root ganglia sympathetic neurons were treated with a DMEM methylmercury solution and observed with a flow chamber and were later compared with sympathetic neurons treated with DMEM solution to compare the differences, if any exist, between axonal exposure to methylmercury and exposure to DMEM. The experiment used neurons from 9-10 day chick embryos, exposing the cells to DMEM as a control and to methylmercury as an experimental. Incubation was for twenty minutes, where pictures were taken before, during, and after incubation to measure the lengths of the axons in each picture and to determine the average of the lengths as well as determine if the axons were growing or retracting.

II. Materials and Methods:

Materials

All materials chosen from Professor Bob Morris's protocol for the chick embryo dissection (Morris, 2014b) and the protocol for the observation of unlabeled cells (Morris, 2014c) were used for the experiment. Primary neural cultures from 9-10 day old dissected chick embryos were used for the purposes of the experiment.

In the ICUC lab at Wheaton College, computers containing the software programs BTV 6.0b1 and ImageJ were used to capture images from compound light microscopes with a Sony DFW-X700 Camera and the Adapter C-mount 1.0x Magnification attached to the microscopes above the stage. A thermometer was also set on the stage of the microscope to maintain the temperature of the neurons for optimal activity. 40 nanomolar $HgCH_3$ (methylmercury) was

used in the experimental trials, which was diluted from a stock solution for the other researchers of 4.0 micromolar HgCH3.

Methods

Primary cultures were collected, plated, and incubated following the procedures written by Professor Bob Morris (Morris, 2014b). This procedure was altered to allow more axonal growth. The procedure was deviated a little by allowing the cells to settle for two days instead of one and treated the cover slips for three hours in poly-lysine and two hours in laminin. The amount of neural growth factor used was also increased to 200 ng/mL and the concentration of glutamine was to 4 micromolar in the growth medium.

All procedures involving flow chambers and observation of the cells were extrapolated from the protocol written by Professor Bob Morris (Morris, 2014c). For this experiment, flow chambers were the best choice to use to record data regarding the growth or retraction of axons for the target cells.

Before changing the buffer of the old growth medium with DMEM for the control samples, an anchored neuronal cell with visible axons was chosen and was brought into focus using Koehler Illumination (Morris, 2014a).

All slides were divided into two subgroups: experimental and control. All cells were observed with flow chamber in accordance with Professor Bob Morris's protocol on observing live unlabeled cells (Morris, 2014c).

The protocol for observing and recording data from the experimental methylmercury exposure and control DMEM exposure was as follows: Control and experimental cells were photographed directly after creating the flow chambers, marked down as pre treatment. DMEM was flown through the control cells, with accordance with the observation of unlabeled cells protocol (Morris, 2014c) and photographs were taken every five minutes after the flow. After the DMEM wash, a post wash picture was taken, followed by experimental cells having methylmercury flown through the chambers in accordance with Professor Bob Morris's protocol for the observation of unlabeled cells lab (Morris, 2014c). With replacing DMEM with DMEM methylmercury for the experimental trials, photographs were taken every five minutes after the flow of methylmercury through the flow chamber. After a DMEM wash to rinse off the remaining methylmercury, a post wash picture was taken.

The procedure for measuring cell lengths was as follows: A picture of a micrometer slide was taken under the 4x lens on a compound light microscope in the ICUC using BTV and imported into ImageJ (Pope, H. 2014). Once in ImageJ, the analyze button was clicked and the scale was set, the distance being measured to 0.00 (Pope, H. 2014). Using the ImageJ segmented line tool, a line was drawn from the edge of one millimeter marker to the opposite edge of the adjacent marker of the picture taken of a glass micrometer (used for scaling) placed on the stage of the microscope (Pope, H. 2014). Next, the cursor was positioned over each end of the line drawn and the x value was recorded (Pope,

H. 2014). The lower value was subtracted from the higher and the distance in pixels was measured as one millimeter for any object under the 4x objective lens (Pope, H. 2014). The pictures of the cells during the pre-wash, incubation, and post-wash of both the experimental and control trials were uploaded into ImageJ and the first few steps were repeated to set up the scale for the pictures (Pope, H. 2014). With the scaling measured, the distance in pixels was entered for one millimeter and the unite of length was changed to micrometers (Pope, H. 2014).

The segmented line tool was selected again and started to measure the axons from the base of the axon, attached to the cell body, to the most distinguished growth cone of one axon, returning the line to the beginning of the measurement to finalize the distance measured (Pope, H. 2014). The results were captured and were copied to a spreadsheet into an Excel document to make a graph (Pope, H. 2014). All procedures regarding the Methods and Materials section of this experiment were conducted and collaborated with Chris Maricle (Maricle, C. 2014).

III. Results:

During the experiment, the axons of the target cells underwent large amounts of activity prior, during, and after each treatment of both DMEM and methylmercury, captured as time elapsed through digital photographs. For the axons, there was no moment in time where the axonal activity was static. There was constant movement including both growth and retraction. All of the physical changes were sporadic and were not repetitive of one another prior, during, and after each time interval used to record data. While the most apparent or easiest axon to identify were used throughout each trial, there were several other axons responding to the surrounding buffer too, growing and retracting as well as changing directions rapidly.

From the three control samples, supported by the data shown in Figure 3 below, the axons did not deviate dramatically regarding growth or retraction. The data points collected showed the ‘dips’ in the growth of an axon being followed generally by more growth in the future (Maricle, C. 2014). From the two experimental samples (refer to Figure 2 for visual of post methylmercury wash), the point of interest to recognize on Figure 3 is the steep drop in the average length of axons between the second and fourth treatment of methylmercury to the cells. The drop is followed by growth like in the graph for the average growth of the axons in the control samples (refer to Figure 1 to view post DMEM wash), but the large change in growth is significant because it suggests methylmercury did have an impact in the growth of axons for prenatal neurons.

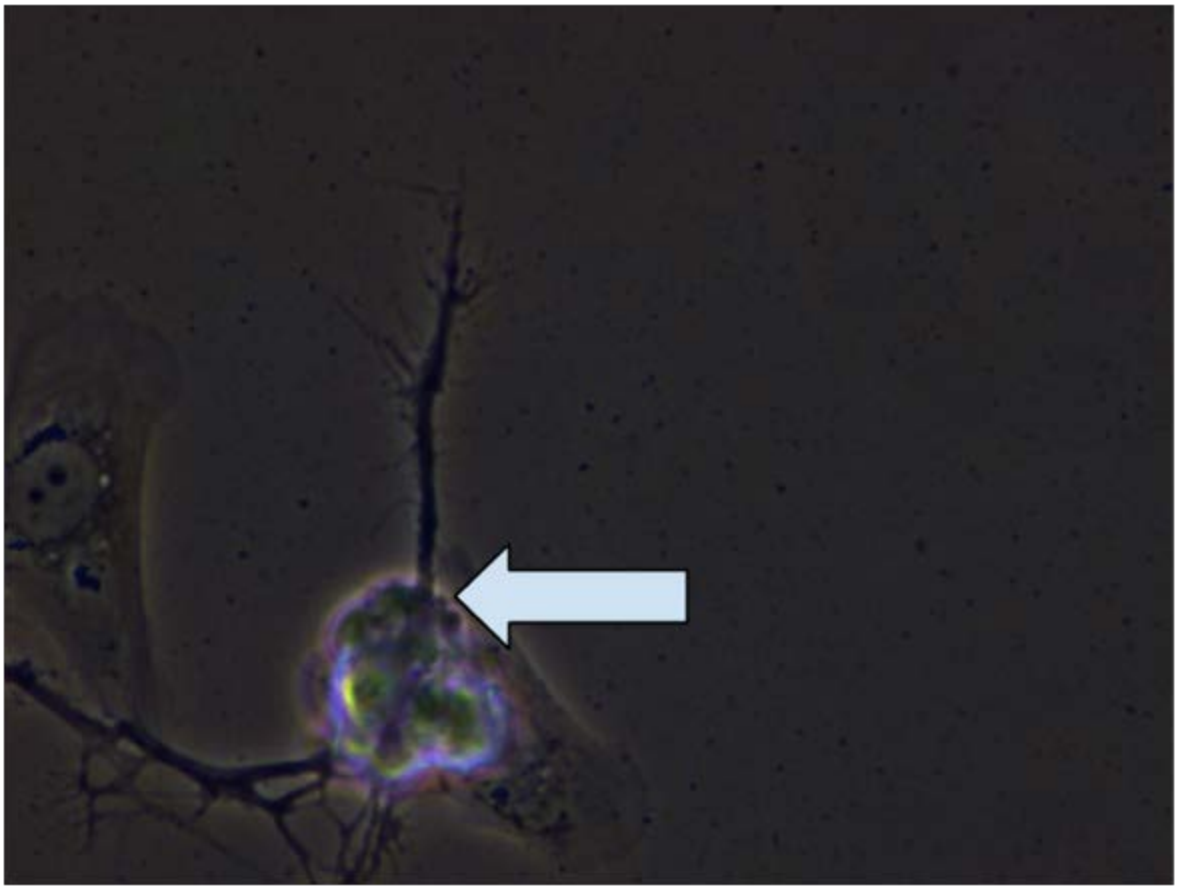

Figure 1. Post Wash DMEM Control Sample. This picture was taken after new DMEM was administered to the flow chamber once the twenty minute incubation period was complete. The arrow points to where the axon base is connected to the soma of the neuron and starts to extend out. All data was collected in collaboration with Chris Maricle (Maricle, C. 2014).

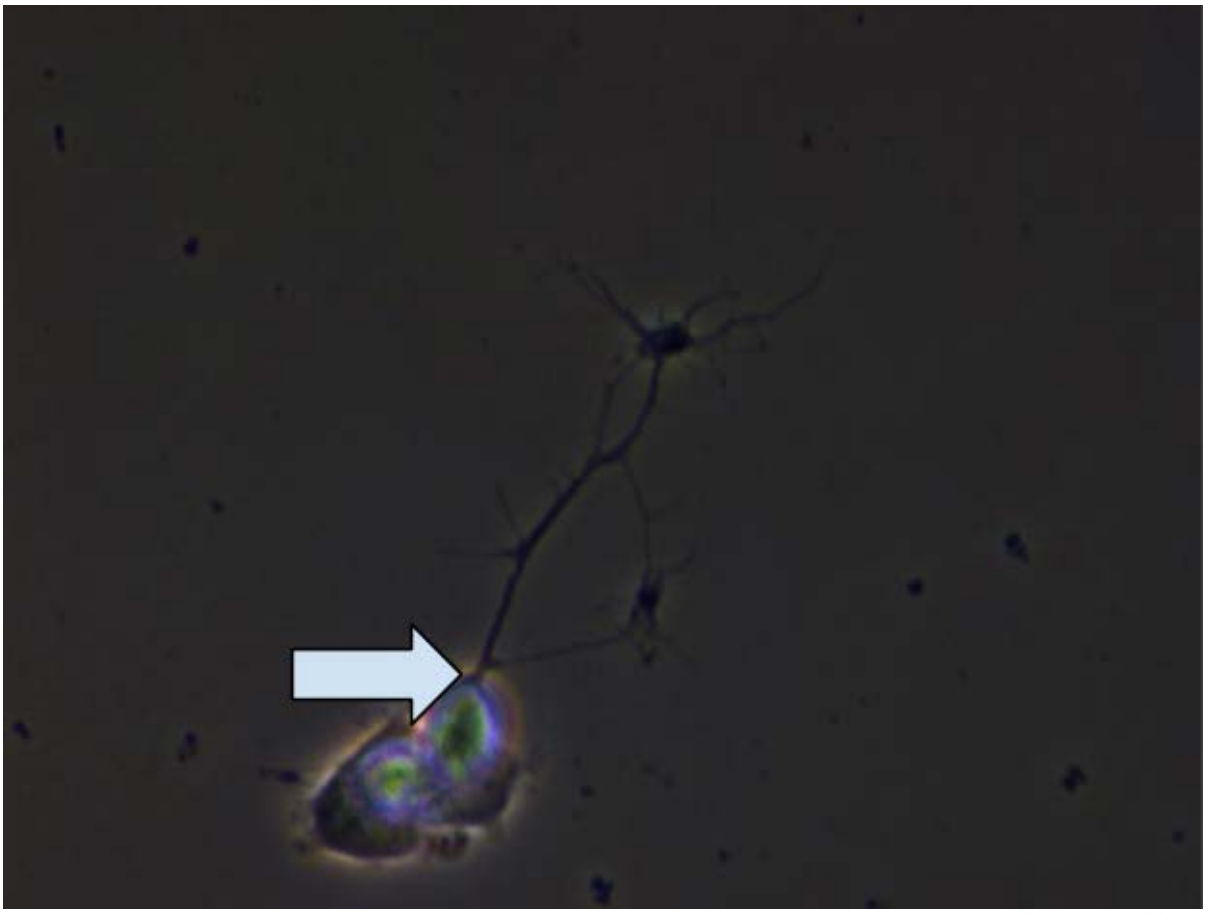

Figure 2. Post Wash Methylmercury Experimental Sample. This picture was taken after the buffer under the coverslip was changed with new DMEM after the twenty minute incubation period in methylmercury. The arrow points to where the axon begins from the soma of the neuron and starts extending out. All data was collected in collaboration with Chris Maricle (Maricle, C. 2014).

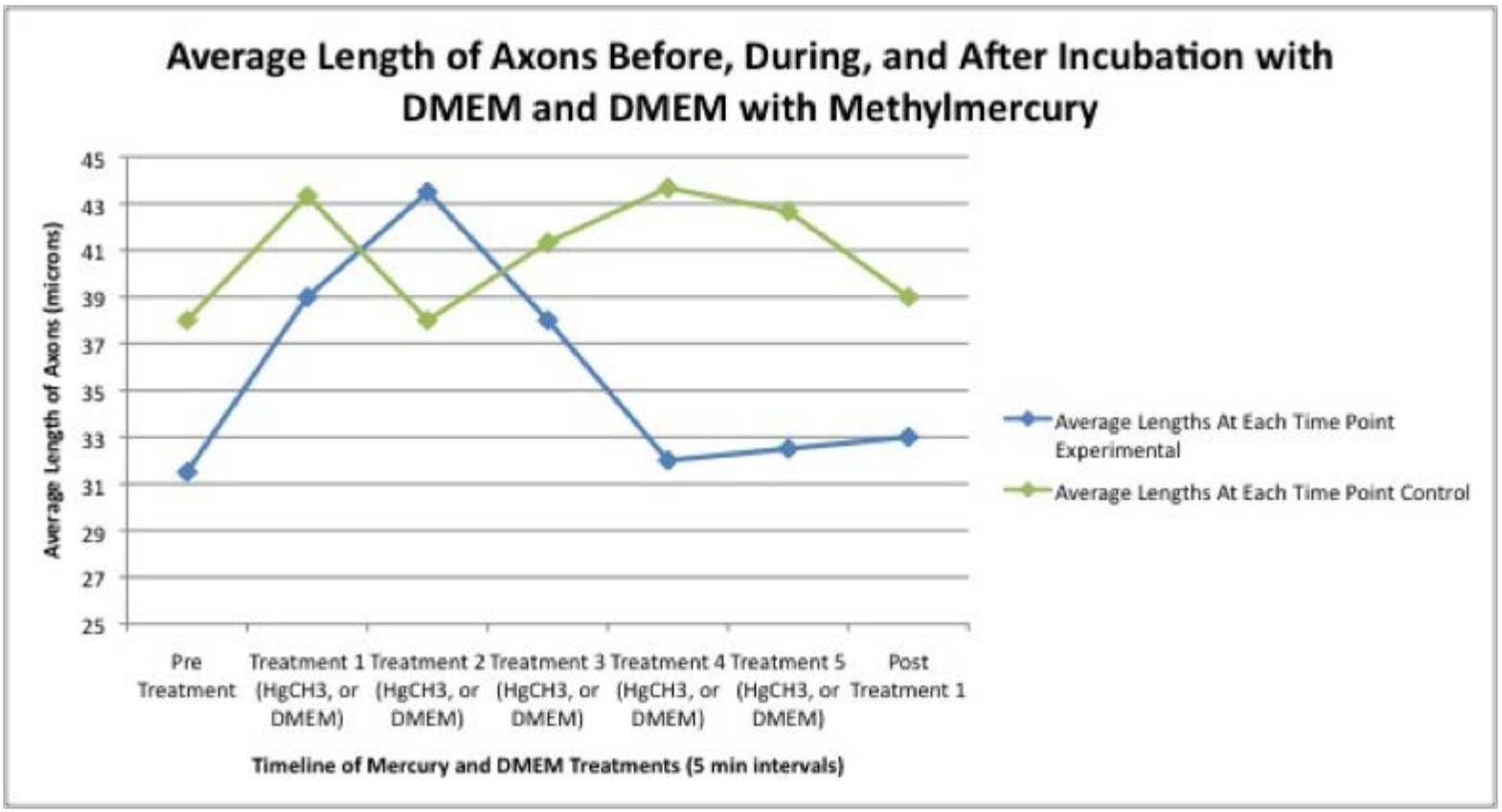

Figure 3. Average Length of Axons Before, During, and After Incubation with DMEM or Methylmercury. These two sets of results are the averages of measured axonal length from two experimental samples (n=2) and three control samples (n=3). Each sample was measured once prior to treatment of DMEM for controls or HgCH3 (methylmercury) for experimentals, five times during the treatment, and then once post treatment all in five minute intervals. From the data on the graph, treatment in methylmercury resulted in a large retraction in average axonal length when compared to the control. All data was collected in collaboration with Chris Maricle (Maricle, C. 2014).

IV. Discussions and Conclusions:

The data supports the hypothesis, where the introduction of the methylmercury did result in axons retracting from growing out unlike the control samples out of the two options of either showing retraction or showing a cease in growth. The second half involving a cease in growth was never demonstrated in the collected data and cannot be supported since there was no recorded data showing any stop in growth of the axons. Conclusions that can be drawn from the data are methylmercury had a negative effect on the length of axons in the experimental samples and the average length of the axons was far lower than the average length of axons in control samples. If the experiment was repeated a thousand times over, then it would be conclusive to say methylmercury, when exposed to prenatal cells, will have the axons retract during the incubation period and will have some growth post-incubation.

Regarding the biological system under experimental conditions, specific components of the microfilaments of the neuron that change the shape of the growth cones and extend axons may be more susceptible to being damaged by methylmercury than others (Berg, K., Puntervoll, P., et al., 2010). Due to the high affinity of methylmercury to proteins containing thiol groups, microtubules undergo depolymerization and ultimately result in a cascade of destructive events that would only start with the cease in growing of axons and result in retraction (Berg, K., Puntervoll, P., et al., 2010). From another chemical interaction perspective, neurons need directions to have the axons go in any direction and the expression of ligands and proteins of the neuron when treated with methylmercury were skewed and unbalanced, resulting in the retraction of axons again (Wilson, D.T., Polunas, M.A., et al., 2005). Ultimately, the continuous breakdown of the microtubule filaments structuring the axon as well as the rest of the cell can potentially lead to apoptosis, which seems to be linked to other nonneural cells following the same route (Miura, K., Koide, N., et al., 1999). The exposure of a neural cell to methylmercury carries a wave of destruction from the environment to the interior of the cell. All progression in growing stops and the cell breaks down from the inside out, leading to axons retracting back to the soma and potentially networks degrading down to minimal interweaving between axons.

The most prominent error was not being consistent with the number of washes between the controls and the experimentals. For the methylmercury samples, a second wash of DMEM had to be applied to the flow chamber to remove the methylmercury to observe the behavior of the cells after the incubation period. While the controls did not

have prenatal cells exposed to methylmercury, to remain consistent throughout the experiment, a second wash of DMEM to remove the incubating DMEM would have helped with the consistency of the data. Regardless of whether the cell's axons would have reacted differently with the second DMEM wash or not, the controls were tested differently compared to the experimental samples. Another error in the experiment involves the lack of considering observing multiple neurons and their networks. The networks of multiple neurons likely have very different behaviors compared to neurons by themselves. Would the same reaction of one neuron when exposed to methylmercury occur when there is a cluster of neurons instead? Since the option was never considered, the data collected from the experiment and documented in Figure 3 above cannot answer this question.

The next time the experiment would be conducted, the parts that would be refined include adding more samples for the control and experimental trials, given the limited time available for the researchers of the experiment and other researchers of various experiments to use the ICUC, equipment, and various reagents needed. Another modification would be to have a gradient of different concentrations of methylmercury to observe a potential range in the behaviors of the axons. This way, the different doses can be compared to one another to mark any trends or emerging behaviors at specific times along the concentration gradient. The final refinement would be to use peripheral neurons from differently aged eggs to see if the degenerative effects of methylmercury would be similar or different between younger and older chicks. Similar to the idea of a gradient of methylmercury concentrations, using peripheral neurons from differently aged developing chicks would provide another timeline for the rate of progression in degeneration in neurodevelopment in prenatal cells and possibly provide insight to how a mature chick would be functional with the exposure. As a future experiment, what should be done to extend the results in new directions is to examine networking neurons with interconnecting axons and what would happen to the networking net between the neurons when subjected to methylmercury. Would all of the axons retract? Would only some of them retract? Is retraction or elongation the only two behaviors that could happen if a cluster of neurons was exposed to methylmercury? Another future experiment could involve using a different organism's peripheral neurons, perhaps one organism with ancestry closer to human ties. The reasoning behind using a different experimental model is to not only replicate the experiment again to gather more data about the subject as a whole, but to also investigate if the behaviors could be different between species with the same concentration of methylmercury. Could the drastic neuronal developmental damage of a chick embryo be seen as a minor problem to the peripheral cells of a small rabbit, dog, or a chimpanzee?

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

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