Methylmercury's Effect on Axonal Growth

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Neurobiology Short Report
Bio324 / Neurobiology
Wheaton College, Norton, Massachusetts, USA
April 23, 2014

Introduction:

Mercury is a naturally occurring chemical on earth, which is distributed throughout and regulated within the environment. Human interaction and disruption of the environment has caused the amount of mercury in the air and water to rise (Benoit, 2014). This increases in the amount of free mercury causes the increase in methylation of mercury by anaerobic organisms living in the soil and under water (Benoit, 2014). Methylmercury is then taken up by organisms and bioaccumulates as well as biomagnifies (Benoit, 2014). In human adults, methylmercury has shown to cause ataxia, paresthesia, tremors, and mortal disorders (Benoit, 2014). Pre natal exposure to methylmercury can cause developmental problems, as well as language problems, and mental retardation later in life. (Benoit, 2014). The result of methylmercury poisoning on human fetuses is clear but the research as to how methylmercury has its impact on a cellular level has only begun to be studied. Axonal growth is an important part of neuronal development as shown by the complications that arise when abnormal growth occurs.

Microtubules have been shown to play an important part in the cytoskeletal support of growth cones (Sabry *et al.*, 1991, Yamada *et al.*, 1970); and in 1981, Nakada, S. and colleagues discovered that methylmercury caused the depolymerization of microtubules and slowed neurite outgrowth of chick embryonic sensory ganglia (Nakada *et al.* 1981). Miura, K. and colleagues have shown that methyl mercury impacts the microtubules within the axons of chick *Gallus gallus* dorsal root ganglia, causing the microtubules to depolymerize (Miura, K., et al, 2000). Research following this study showed that sub lethal *in vitro* incubation with methylmercury, on the scale of hours, would cause a reduced number of developing axons in chick (*Gallus gallus)* forebrain cells (Heidenmann *et al.*, 2001). Heidenmann and his colleagues also noted methylmercury's effect on microtubules of the axon, but the researchers were not able to show a lasting effect after a day of recovery (Heidenmann *et al.*, 2001). Based on the literature, we believe our experiment will lead to significant findings and the furthering of the scientific community. Although we did not have the opportunity to measure microtubule depolymerization we believe that methylmercury's effect on microtubules underlie the effects shown here. In the combined experiments of Melissa Darnley and Christopher Maricle, our hypothesis is as follows; If primary culture of chick sympathetic embryonic dorsal root ganglia cells are exposed to methylmercury in-vitro, then they will experience a decline in growth rate, or a complete reversal and start to retreating towards their cell bodies. This experiment will show the effect of methylmercury on the axons of single cells instead of axonal outgrowth from a dorsal root ganglia, as was shown by Nakada, S. and colleagues.

For our experiment we chose to use common domestic chicken embryos (*Gallus gallus)* as our model system. This choice was made based on the literature, where many researchers used domestic Chick neurons for their primary culture (Heidenmann et al., 2001, Miura, K., et al, 2000). The use of the domestic chick neurons as a model system comes from a need to easily procure primary neural cultures and at low cost and maintenance (Darnell D.K., & Schoenwolf, G.C., 2000). *Gallus gallus* neuron growth follows a similar process to laboratory rat neural growth (Heidenmann et al., 2001), but they do not require the same amount of care and oversight that a rat or mouse colony would need. We plan on testing our hypothesis by observing individual neurons over a period of twenty minutes of incubation in methylmercury. Neurons were defined by cellular protrusions with parallel sides extending away from the cell body.

Materials and Methods:

Materials

All materials chosen for our experiment were from Professor Bob Morris's protocol (Morris, 2014a) were used as well as all materials chosen for Professor Bob Morris's protocol (Morris, 2014b) We used primary neural cultures were collected from 9-12 day old chick embryos. Chicks embryos used were *Gallus gallus*.

We used the software programs BTV 6.0b1 and ImageJ with images captured from Nikon E-200 microscopes. We used Sony DFW-X700 Cameras with the Adapter C-mount 1.0x Magnification. A thermometer was also set on the stage of the microscope to maintain record of the temperature of the neurons for optimal activity. To expose the cells in the experimental trials with Methylmercury, we used 40 nanomolar HgCH3.

Methods

Primary cultures were collected, plated, and incubated using the procedures by Professor Morris (Morris, R.L., 2014a). We altered this procedure to allow for more axonal growth. We deviated from the procedure by allowing the cells to settle for two days instead of one. We treated the cover slips for three hours in poly-lysine as well as two hours in laminin. The amount of nerve growth factor used was also increased to 200 ng/mL. We increased the concentration of glutamine to 4 micromolar in our growth medium.

All procedures involving flow chambers and observation of our cells were extrapolated from the procedure of Professor Morris (Morris, R.L., 2014b). For this experiment, we chose to use flow chambers to better record data regarding the growth or retraction of axons for the target cells.

Prior to changing the buffer of the old growth medium with DMEM for the control samples, an anchored neuronal cell with visible axons was chosen and focused using Koehler Illumination.

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 (Morris, R.L. 2014b).

The protocol is as follows: Control and experimental cells were photographed directly after creating the flow chambers, marked down as pretreatment. Control cells then had DMEM flown through them, with accordance with Professor Morris's protocol (Morris, R.L., 2014b). Photographs were taken every five minutes after the flow of DMEM through the flow chamber. After the DMEM wash, a post wash picture was taken. Experimental cells had Methylmercury flown through their chambers in accordance with Professor Morris's protocol (Morris, R.L., 2014b); With the exception of replacing DMEM with Methylmercury. Photographs were taken every five minutes after the flow of Methylmercury through the flow chamber. After the DMEM wash, a post wash picture was taken.

Measuring of cell lengths procedure is as follows: Take a picture of a micrometer slide under the 4x lens on a microscope using BTV. Take that picture and import it to ImageJ. Once in ImageJ go to analyze, then set scale, make sure distance in pixels is 0.00 press ok. Use the ImageJ segmented line tool to draw a line from the edge of one-millimeter marker to the opposite edge of an adjacent marker. Position your cursor over each end of the line drawn and record the x value, which is displayed in the ImageJ toolbar. Subtract the lower value from the higher. This distance in pixels is one millimeter of any object under the 4x objective. Upload the pictures of the cells you want into ImageJ. Click on analyze, then click on set scale. Enter the distance in pixels for one millimeter. Change the unit of length to micrometers, and then press ok.

On the ImageJ toolbar, the line tool was selected and the segmented line option was selected. Measurements were started by clicking on the base of the axon where it meets the cell body. At each bend in the axon, the segmented line tool was utilized to change the direction of the line. The line ends once the growth cone of the axon is reached. The line was ended by double clicking. The results window was opened and the measurement was captured. Measurements were copied from the results window and transferred into an Excel document. These methods were used after communication with Hans pope (H. Pope, personal communication, April 8, 2014).

Results:

In our experiment we saw that there was a cellular retraction after incubation in methylmercury and a

fluctuation in the growth of our control cells. This retraction was quantitatively collected by measuring the length of axons every five minutes for twenty minutes in both experimental and control groups. These lengths were then averaged within control and experimental groups; and the averages were used to form figure two. Figure one shows the positions where we started and ended measurements of neurons prior to treatment of DMEM for controls, or methylmercury for experimentals. Measurements were taken once pretreatment, five times during the treatment, and once post treatment, all in five-minute intervals. As is visible from our graph, treatment in Methylmercury causes a large retraction in average axonal length. All data was collected in collaboration with Melissa Darnley (Darnley, 2014)

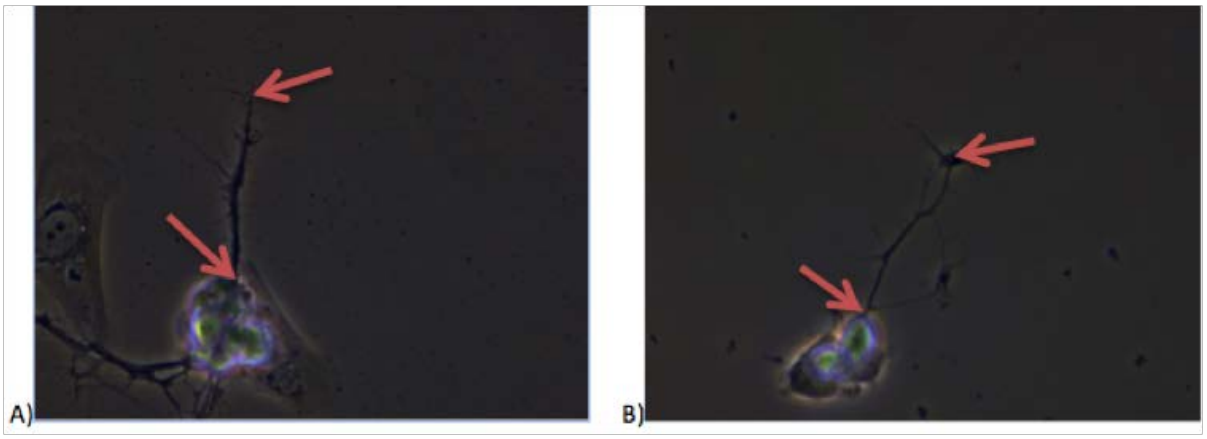

Figure1. A) A control neuron with its axon growing toward the top of the page. The red arrows show where our measurements started and ended. B) An experimental neuron post treatment and DMEM wash. The arrows show where measurements started and ended. All pictures were collected with Melissa Darnley (Darnley, 2014)

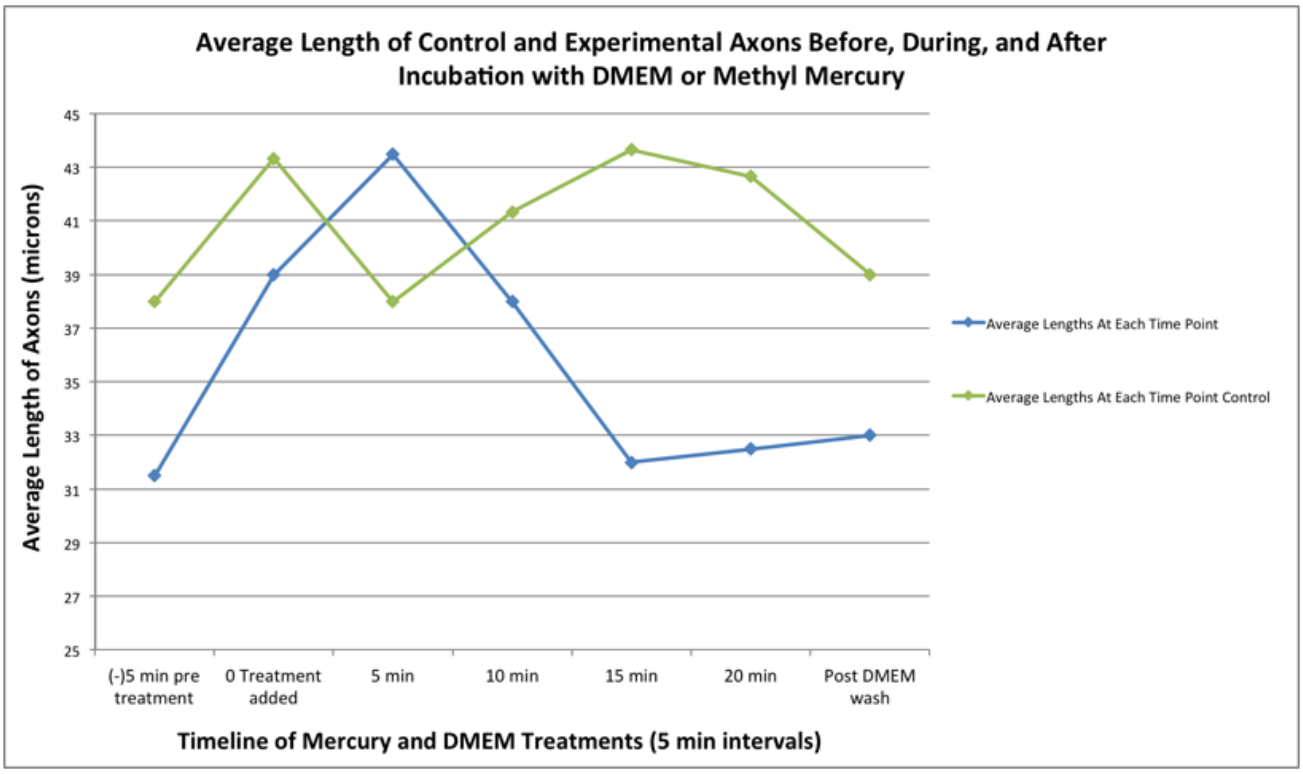

Figure 2. Average Length of Control and Experimental Axons Before, During, and After Incubation with DMEM or Methyl

Mercury. 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 at each time point. Treatment was added at time zero and pictures were taken for measurement at five-minute intervals after treatment was added. All data were analyzed with Melissa Darnley (Darnley, 2014).

Discussion:

Due to low n values our data is not statistically significant, but we do show trending results in congruence with our hypothesis. These data suggest there is a strong likelihood that even brief exposure to methylmercury can cause axonal retraction in developing neurons. If this experiment had been repeated a thousand times and we were still getting the same results, it would be strong evidence in favor of our hypothesis and would suggest that, in the presence of methylmercury single cells behave by retracting their axons. The next step in this hypothesis would be to track the polarization, and depolarization of microtubules in single cell's cytoskeleton, in order to replicate the results found in the literature. If similar results were found, we could start to look for more cytoskeletal or extracellular matrix components that may behave similarly to microtubules in the presence of mercury.

The number one source of error in our experiment was human error. An example of this is that our experimental cells lysed themselves during each experiment. If this was due to the mercury we would have expected to see this result in other people's experiments. It is more likely that our human error caused these cells to lyse. In future experiments, it would be useful to use a gradient of mercury to create a dose curve. A dose curve for methylmercury would be used to create a more standard dosage of methylmercury in *in vitro* experiments. It would also provide a base point for comparison between *invitro* and *invivo* experiments. This connection between *in vivo* and *in vitro* experiments is necessary for a strong understanding of the molecular effects of methylmercury and its impact on the organismal level. Currently there is no standard dose which represents common extracellular concentrations of methylmercury in subjects with methylmercury poisoning.

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I have abided by the Wheaton college honor code in this work.

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