In Vitro Methyl Mercury Decreases Axon Abundance

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Introduction

Proper axonal growth is extremely important to vertebrate survival. Neurons must form necessary connections between and within the central and peripheral nervous systems. Without these connections, an organism would unintentionally ignore much of the sensory input it could receive, putting it at a severe disadvantage.

Santiago Ramon y Cajal, who made many of the first observations of neurons and is considered to be the father of neurobiology, was the first to describe the axon. Since the Neuron Doctrine theory was introduced, it was discovered that axons are supported by a cytoskeleton of long microtubules that direct the flow of vesicles, and bundled actin filaments (Kandel, Schwartz, Jessel, Siegelbaum, Hudspeth, 2013). Axons are guided by Growth Cones to their various intended synapses throughout the organism, which (depending on the organism) can be over a meter away from the cell body. To reach destinations such lengths away, an axon must slowly interact with each cell it comes in contact with along the way, each pointing the growth cone in the right direction (Kandel et al, 2013). For an axon to be a stable entity, it must have continuous support from the cytoskeleton. The formation of the microtubules in this cytoskeleton is dependent on the binding of the GTP nucleotide to the beta-tubulin proteins (Yole, Wickstrom, Blakley, 2007).

Methyl mercury, also known as MeHg, is a potent neurotoxin. MeHg is also the most environmentally abundant form of mercury (Melo Reis, Herculano, Silva, Santos, Nascimento, 2007). MeHg has been shown to interfere with the binding of the GTP nucleotide to beta-tubulin (Yole et al, 2007). Inhibiting the polymerization of these subunits disrupts the structural integrity of neurite membrane structure. Without a strong supporting cytoskeleton, the axon will retract. This phenomenon seems to be specific for mercury and the disruption causes a similar molecular lesion to that shown in 80% of Alzheimer disease patients (Leong et al, 2000).

While nerve cells in the body grow a predictable number of axons, neurons grown in culture may grow many due to a lack of directional signals to the growth cones. This study used the sympathetic nerve chains and dorsal-root ganglia from ten-day-old *Gallus gallus* embryos. This experiment was designed to test the effects of methyl mercury on the abundance of axons. Due to the previous research on the influence of methyl mercury on the binding of beta-tubulin and GTP (Leong, Syed, Lorscheider, 2000) it was hypothesized that treatment and growth in methyl mercury would decrease the abundance of axons. This effect will be measurable in the average number of axons per neuron, as well as the average area of axonal matter (measured in two dimensions and therefore not a total representation of the axonal

volume).

Materials and Methods

Embryo Explanting

This experiment used sympathetic and dorsal-root-ganglion neurons from *Gallus gallus* embryos. These sympathetic neuron chains and DRGs were collected using the methods and materials described in Morris 2013a. The following changes were made: Treatment of the coverslips with Poly-K and Laminin were increased by two hours and one hour respectively, the nerve growth factor (NGF) concentration was increased four times, the Glutamine concentration was doubled, the number of sympathetic nerve chains and DRGs was increased by half. These steps were taken to increase the likelihood that neurons would adhere to the cover slip and grow axons and other processes.

Data were collected by creating a chip observation chamber using the methods and materials as described in Morris 2013b. The data were collected in the Imaging Center for Undergraduate Collaboration at Wheaton College in Norton, Massachusetts. The data collection took place using a C92 microscope, a Nikon Eclipse E200 with the 10x and 40x objective lenses with Koehler illumination through a phase optics condenser. The images were taken using a Sony DFW-x700 on a 1.0x camera mount connected to a Macintosh computer with OSX version 10.5.8. Images were collected using BTV software version 6.0b1.

The dosage of methyl mercury in the experimental condition used for incubation was 40nM in Dulbecco's Modified Eagles Medium. This concentration was calculated based on previous research by Leong et al. (2000). Cells were dissected and incubated with methyl mercury for 20 minutes, then plated out into culture dishes and grown. Data were collected 38 hours after dissection. Measurements were taken of live unstained cells.

Data Analysis

All measurements of images were made using Adobe Photoshop software. Each data point was the count of axons along with their respective length and width measured in mm. A neurite process was considered an axon if the length was greater than fifteen microns. From these data a two-dimensional area (length x width) was calculated for each axon, and from these areas an average area (in $mm^{2)}$ was calculated for the experimental and control conditions. The data pool for the control condition average included 17 nerve cells with 23 total axons. The data pool for the experimental condition consisted of 8 nerve cells with 10 total axons.

Results

The number of axons per neuron was recorded along with each axon's length and width. From those measurements an axonal area was calculated. Figures 1 and 2 are representative images of the control and experimental conditions (respectively) and were taken using a 10x objective lens.

Only neurons with defined axons fifteen microns or longer were included in the data set. The control condition provided many more neurons with defined axons than the methyl mercury condition. Further, the control condition produced an average of 1.35 axons per neuron, while the methyl mercury condition produced an average of 1.25 axons per neuron. The neurons that had been incubated post-methyl mercury treatment showed many more retracted processes than those that had not been treated with mercury. These cells were considered apoptotic. An example can be seen in *figure 3*, which was taken using a 40x objective lens.

As seen in *Figure 4*, axons in both conditions are approximately the same average width, however, the axons in the control condition are much greater in average length and therefore area.

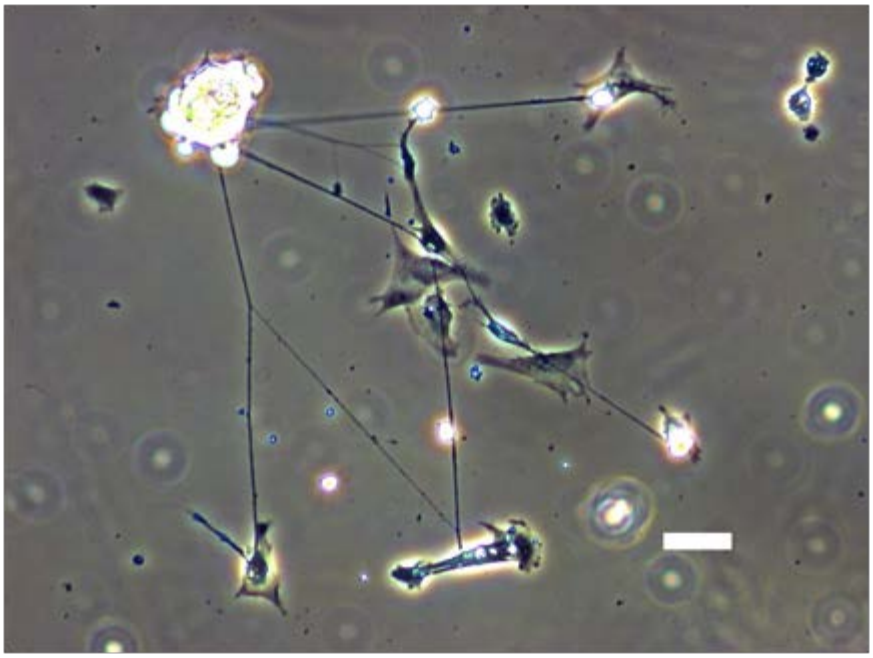

Figure 1. Control Representative Image. This is a representative image for the control (no mercury) condition. The white bar is a 50micron scale bar. Notice the abundance of long axons compared to the experimental condition.

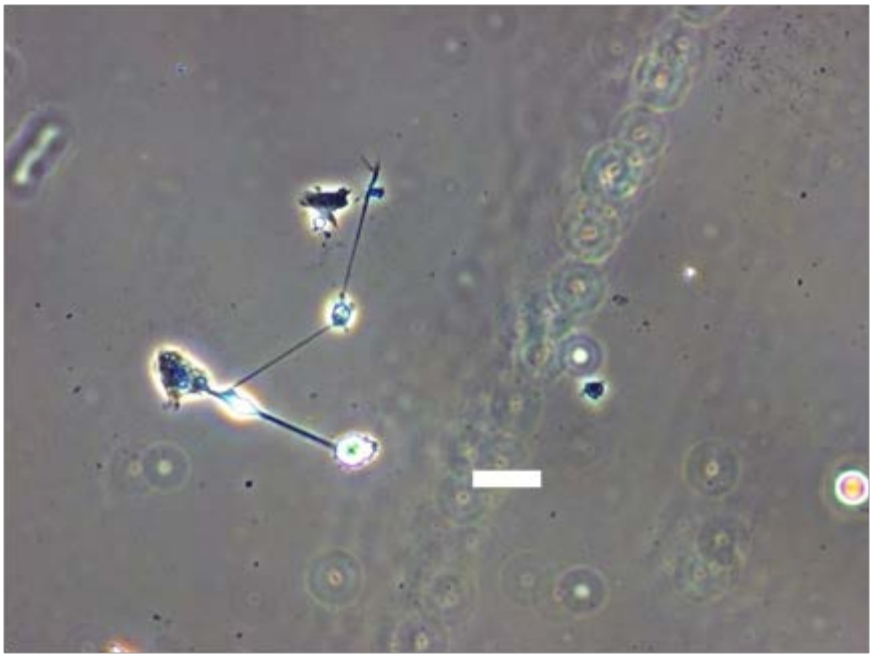

Figure 2. Experimental Representative Image. This is a representative image of the Experimental (Mercury) condition. Notice the short axons. The white bar is a 50micron scale bar

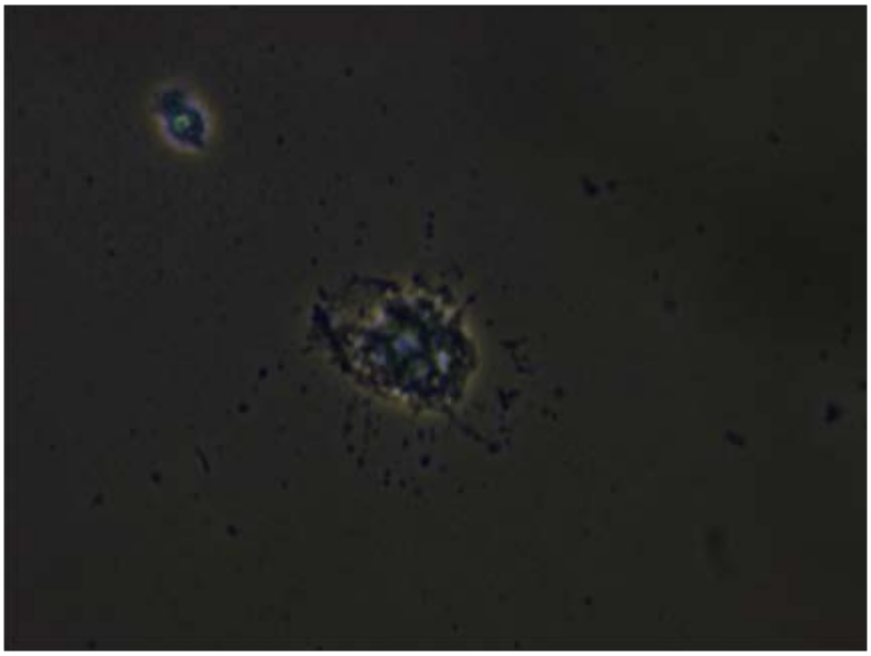

Figure 3. Apoptotic cell in the experimental condition. Notice the reduced brightness around the perimeter of the cell compared to the non-apoptotic neuron in the upper left corner of this image and the neurons in figures 1 and 2. Also notice the large quantity of cell matter outside the cell body.

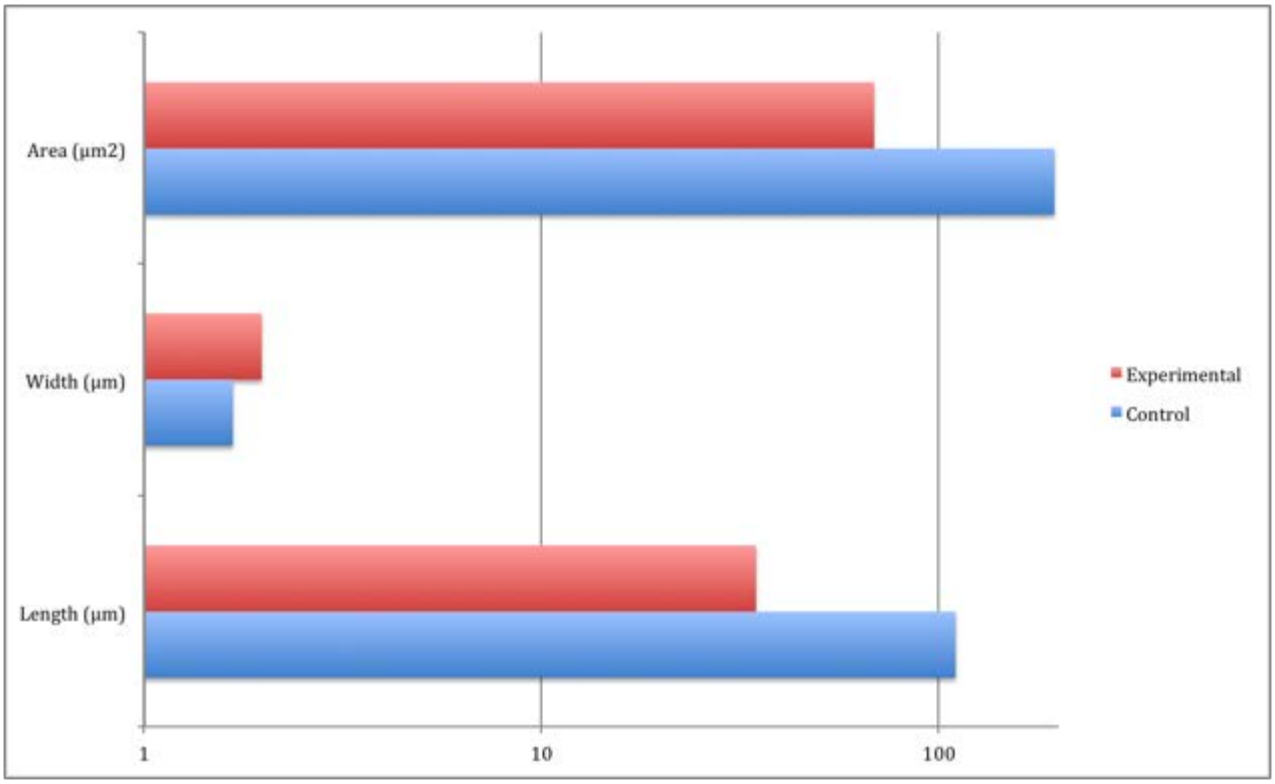

Figure 4. Measure of Axon Matter. This graph shows the average length, width and area for the experimental (mercury, n=10) condition, and the control (no mercury, n=23) condition. Average lengths for experimental and control are 34.6 microns and 109.8 microns (respectively). The average areas for experimental and control are 68.54 microns^2 and 195.0 microns^2 (respectively). Notice the average width is almost exactly the same, while the length, and therefore area, of the control is exceptionally higher than that of the experimental.

Discussion

Figure 4 demonstrates that the hypothesis was supported. The axon area was effected by the presence of methyl mercury in the growth medium. The neurons in the mercury condition averaged slightly less axons per neuron than the control, though it is unlikely that this difference affected the data. The average axon length for the control condition was over three times greater than the experimental, and due to the similar averages for width across the two conditions, the average area for the control axons is also over three times the average for the axons grown in 40nM methyl mercury. Due to the observation that width was seemingly unaffected by the conditions, this experiment lends support to the theory that methyl mercury inhibits beta-tubulin from binding to GDP (Leong et al, 2000; Yole et al, 2007). The increased frequency of apoptotic cells in the methyl mercury condition was seen throughout the culture dish (see figure 3). This could add support to the theory that mercury can not only prevent axons from forming but could also force axons to retract and cause cell death (Kuo, Lin-Shiau, 2004).

Had there been more time and resources and therefore more data to conclude from, we may be able to assert that

mercury does indeed disrupt all neural processes that are formed with microtubules. Unfortunately, only axons were studied in this experiment.

Unfortunately, the data collected were too limited to make the assertions stated above statically significant. This is due to several factors. The first is that the data set is relatively small. Only one culture dish was imaged for each condition, and only 17 neurons from the control and 8 neurons from the experimental were included in the data set.

When measuring axon area only length and width could be taken into account. Had the inclusion of a third dimension for measurement been feasible, the calculation of axonal volume would have been performed. Had axonal volume measurements been available, more descriptive conclusions could have been made. However, the data are such that the third dimension is not completely necessary for conclusions to be made.

To refine this experiment would require many more cultures for collecting exponentially more data. With 23 control axons and 10 experimental axons, it was shown that mercury likely effects axonal area. With more data, one may be able to show the effect with more certainty. If this experiment were replicated, the addition of a second experimental group with a higher mercury concentration would benefit the data. To show a more dramatic effect of methyl mercury on axon abundance, the data set should contain results from both low and high concentrations. Further, adding time as a variable would also benefit a future experiment.

While the data collected were not reliably significant, this experiment lends support to previous research that found methyl mercury to inhibit axon and growth cone growth (Yole et al, 2007). While this experiment cannot conclude that the disruption of binding of Beta-tubulin to GDP to form microtubules is the cause of differences in axon area between conditions, it does support previous research by way of the observed cellular response (Leong et al, 2000).

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