Active mitochondria in growth cones of neurons treated with methyl mercury

Lindsey Gillis
Neurobiology Short Report
Bio 324/Neurobiology
Wheaton College, Norton, Massachusetts, USA
April 23, 2014

Introduction

Methyl mercury is a naturally occurring contaminant. Human activity is drastically increasing the levels of methyl mercury in the environment and as such causes a threat to many organisms. When consumed by lower trophic level organisms, methyl mercury biomagnifies when it makes its way up the trophic levels as organisms are consumed by their predators. Humans are at the top of this hierarchy and experience the neurotoxic effects of methyl mercury through consumption, usually of fish (Castoldi et al. 2001; Sanfeliu et al. 2001). For adult humans, effects include paresthesia, ataxia, vision and hearing loss, speech impediment, trembling, abnormal eye movements, mental disorders and sometimes death (Castoldi et al. 2001). This is because methyl mercury is absorbed by the gastrointestinal tract and sent through the blood stream, allowing it to pass through the blood-brain barrier (Castoldi et al. 2001). Susceptibility to methyl mercury is increased during development. Symptoms and side effects include a wide range of neurological disorders and deficits (Castoldi et al. 2001; Sanfeliu et al. 2001).

Some studies find that the increased stress from oxidation is a potential cause of neural damage (Sarafian, 1999). Other studies have found that methyl mercury reduces axon growth in neurons due in part to the permeability of neuronal membranes to methyl mercury and its effects on microtubule formation (K. Miura et al. 2000). Microtubules are highly important in the transfer of mitochondria to the growth cone as a neuron develops (Hollenbeck, 1996; Verburg and Hollenbeck, 2008). Neurons have a high abundance of mitochondria within their growth cones when active (Verburg and Hollenbeck, 2008; Verburg et al. 2005; Hollenbeck, 1996). A study by Amiri and Hollenbeck in 2008 suggests that mitochondria also have biogenic capabilities within the axon. Reduced metabolic activity at the axon terminal is found in some neurodegenerative diseases (Collard et al. 1995). A study by Shenker et al. in 1999 proposes that mitochondria is the target organelle for methyl mercury based on significant changes in mitochondrial membrane potential in Human T-cells. The relationship between the role of mitochondria in metabolism and axon growth and its reaction to methyl mercury exposure could explain the biological mechanisms behind its neurotoxic effects.

This study was conducted in collaboration with researcher Sydney Damian-Loring. We use dorsal root ganglia and sympathetic nerve chains from embryos of Gallus gallus, the domestic chicken, to study the effects of methyl
mercury on developing nervous tissue. Chick embryos have a relatively fast rate of neural development and are similar to the human embryo at molecular, cellular and tissue levels (Vergara and Canto-Soler, 2012). Additionally, the nervous tissue of the developing chick embryo is relatively large and therefore easy to manipulate and visualize (Vergara and Canto-Soler, 2012) making it more practical to dissect and harvest. Fluorescence can be used to determine the presence of active mitochondria through use of stains such as MitoTracker (Lemasters and Ramshesh, 2007), which are reflective of the voltage of the mitochondrial membrane with brighter mitochondria being considered more active (Verberg and Hollenbeck, 2008). MitoTracker, the fluorescent probe used in this study uses a chloromethyl group that can permeate the membrane and form adducts between mitochondrial matrix proteins (Lemasters and Ramshesh, 2007). In this study, mitochondria will be considered active if they are above a set threshold brightness. In this study we test the hypothesis that there will be a decreased percentage of active mitochondrial coverage in the growth cone region of neurons exposed to methyl mercury.

**Methods**

**Dissection and Cell Culture**

Chick embryos were dissected after 10 days of incubation, removing dorsal root ganglia and sympathetic nerve chains following the protocol, “Primary Culture of Chick Embryonic Peripheral Neurons 1: Dissection” (Morris, 2014a) with modifications to the treatment of the coverslips and incubation periods. Coverslips were treated for 3 hours with poly lysine (this varies from the original Morris protocol in order to increase the probability of the attachment of axons) and for two hours with laminin (this time was increased in order to increase the probability of axon growth in the sample). The ganglia and sympathetic nerve chains harvested from the dissection were incubated with trypsin for 20 minutes and then dispersed and dissociated with trituration in a suspension of growth medium. The makeup of the growth medium was Leibovitz L1 5 medium plus 0.5% methylcellulose, 10% fetal calf serum, 0.6% glucose, 2mM L-glutamine, 100ug/ml streptomycin, 100u/ml penicillin, and 200nM NGF. These coverslips were then incubated in a vibration-insulated incubator for 38 hours to allow more time for cells to settle onto the coverslips.

**Staining and Imaging**

After incubation, the coverslips were stained following the protocol “Primary Culture of Chick Embryonic Peripheral Neurons 3: Staining and Observation of Live Cells” (Morris, 2014b) with modifications to the concentrations of the staining solutions and addition of methyl mercury to the experimental cells. Stain was applied to cells using 60nM MicroTracker in DMEM. Methyl mercury was applied to experimental cell stain solution during this step at
20nM MeHg in DMEM. To ensure the same concentrations of stain in both samples, DMEM was added to the control. Cells were incubated for 15 minutes based on times suggested in Morris (2014b) and Leong (2001) procedures. Dose of methyl mercury was modified for whole culture exposure from those used by Leong in 2001. After staining, coverslips were removed from solution and washed using Dulbecco’s Modified Eagles Medium (DMEM) and used with a small portion of growth medium to assemble an observation chamber on a microscope slide. Slides were viewed using a Nikon Eclipse E200 at 40X power illuminated by a tungsten bulb on a heated stage. One cell was imaged for each treatment and were chosen based on the visibility and presence of axons and growth cones attached to glial cells. Images were collected using an inSight camera on a Nikon 1.0x camera mount and SPOT software with both transmitted light and using fluorescence.

Analysis

Collected images were processed using ImageJ software. For each fluorescent image an average value was calculated for the background and a brightness value was set as a standard to determine the presence of mitochondria within the growth cone. There was one growth cone measured in each image. Using superimposed images of the transmitted light and fluorescent cells, growth cones were outlined using a selection tool in the ImageJ program. It was upon this selected area that a pixel count and histogram analysis was conducted. All pixels equal to or above the established mitochondrial brightness value within the selected growth cone region were counted. Size variation in the growth cone region indicated the need for a percent value in order to more accurately compare the mitochondrial coverage. The count of mitochondria described above was used to calculate a percent by dividing it by the total pixel count for the selected growth cone region.

Results

Images collected each depict one neuron attached to another cell (either neuron or glia). In the control sample the growth cone of the imaged cell (See Figure 1) was 64.2% covered by active mitochondria. The growth cone of the selected cell exposed to methyl mercury (See Figure 2) exhibited the highest percentage of active mitochondrial coverage at 92.5%. The percent of mitochondrial coverage in growth cones exhibits an unexpected pattern (See Figure 3). The N-value for each data set is 1. Upon examining the value histograms for the fluorescent images two peaks can be seen in each (See Figure 4). The first peak is indicative of the dark background value and the second peak illustrates where the bulk of the mitochondrial brightness values lie. These values were used to determine the background and mitochondrial brightness thresholds.
Figure 1. Fluorescent (left) and transmitted light (right) images of control neuron.
Axon can be seen extending from the neuron towards the top right and attaching to the neuron located towards the bottom corner. The growth cone region (marked with the green arrow) can be seen at the top right of the lower cell. These images were captured using a Nikon Eclipse E200 Microscope at 40x using a Nikon 1.0 C-mount and InSight camera. The round nature of both of these neurons indicates healthy growth.

Figure 2. Fluorescent (left) and transmitted light (right) images of neuron exposed 20mM of methyl mercury.
Axon can be seen connecting the neuron located above the glial cell to the top right to the glial cell on the lower left of the image. The growth cone region (indicated by the green arrow) can be seen clearly as a triangle of dense bright mitochondria on the bottommost glial cell. These images were captured using a Nikon Eclipse E200 Microscope at 40x using a Nikon 1.0 C-mount and InSight camera.
Figure 3. Percent of mitochondrial coverage of growth cone region for control and methyl mercury dose.
Data was collected with an n-value of 1. The high dose of methyl mercury showed a higher level of mitochondrial coverage compared to that of the control. There is a difference of 28.3 percent between the two samples.

Figure 4. Value histograms for growth cone regions from fluorescent images.
The y-axis in each chart indicates the number of pixels at a given brightness value, 0 being the darkest and 255 being the lightest. The cluster indicated by the blue arrow in each histograms marks the range for brightness values in the
background. The second cluster indicated by the orange arrow in the histograms for the control and high MeHg dose illustrates the range for brightness values in the regions of the growth cones covered by mitochondria. The pixel count (indicated by the “Count:” header in the histograms above) for the growth cones illustrates the difference in growth cones size.

**Discussion**

The hypothesis was not supported by the results provided in this experiment. Cells exposed to methyl mercury showed a higher percentage of active mitochondrial coverage than the control. The control growth cone is attached to a neuron while the methyl mercury exposed growth cone was attached to a glial cell. This could have attributed to differences in mitochondrial abundance and would require investigation in future studies. Based on the results from Leong’s 2001 study, microtubule disintegration resulted in the collapse of growth cones and could explain the difficulty in this experiment in finding axons and growth cones in cells after extended exposure to methyl mercury. Time between the staining incubation of the coverslips and the time cells were observed and imaged could have induced the neuronal degradation that made larger data sets unachievable in our study. Neuronal degradation occurred within 15 minutes of methyl mercury exposure in Leong’s 2001 indicating that this should be considered in future studies. A study by Drieem et al. in 2005 asserts that mitochondrial activity and membrane potential are both reduced in neurons treated with methyl mercury, which contradicts the results of this experiment as such it would be pertinent to continue our investigation of mitochondria as it may have implications on other research. In research conducted by Bellairs it was observed that the proportion of mitochondria in neurons decreases between 4-10 days of development and between 3-4 days the axonal growth is decreased (Bellairs, 1959). Based on this information, it might be beneficial to measure the mitochondria at different times over the span of axonal development to determine if there is any difference in the susceptibility of neurons and growth cones to methyl mercury at varying points of development which could explain the contradictions to existing literature. We would need more data in order to apply these results to a larger population. Further studies with a larger sample size would need to be conducted to determine the regularity of the pattern observed.

**References Cited**

Research and data collection was completed with the assistance of Sydney Damian-Loring


