The Effects of Mercury on Primary Process Growth, Branching Patterns, and Length in Chick Sympathetic Neurons

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I. Introduction

Mercury is a heavy, soft metal that is naturally occurring in the environment. However, human activity has released so much of the metal from the earth's crust that it is present in high enough concentrations to be deemed an environmental contaminant. The industrial revolution and its coal plants, waste incinerators, and metal smelting have mobilized Hg in the environment. Although these processes generally release inorganic and elemental forms of mercury, once they enter the biogeochemical cycle they are easily transformed to the organic forms, such as methyl mercury (MeHg) which is causative of most health effects associated with mercury (Fitzgerald and Clarkson, 1991).

The most common source of exposure to this methylated form of mercury is through fish consumption. As elemental and inorganic mercury cycles through the environment, sulfate reducing bacteria have been shown to be the primary methylators in anoxic sediments where they utilize sulfate ions as terminal electron acceptors during carbon metabolism (Morgan, 2004).

The neurotoxic effects of mercury have been known since the 19th century when the metal was observed to exert behavioral effects on workers in the felting industry. Many studies have been conducted to demonstrate the effects of mercury exposure in utero as well as at various developmental time points and ages, with both intellectual and behavioral effects observed in their results (Pressinger, 1997).

Few studies have been published to demonstrate the mechanism for neurite damage due to mercury exposure that leads to the observed changes in behavior. The Neurobiology class of Wheaton College in the Spring of 2006 will attempt to characterize the effects of mercury on primary culture chick sympathetic neurons by closely examining neuronal cell interaction, growth cone activity, and axonal outgrowth. Leong et al observed disruption of the microtubule metabolism as one mechanism for neurite degeneration under Hg medium, which may in turn induce behavioral changes in vivo (2001). In our assays we will look at the previously mentioned cellular attributes and I will specifically investigate the effects of mercury on neuronal branching in culture.

Bray et al demonstrated that sensory neurons cultured from chick embryos display different patterns in overall growth and morphology at various ages and under variable conditions of substratum (1987). Knowing that embryonic development has sensitive periods where teratogens may exert greater visible toxicity, I believe that the methodology in the afore mentioned research will be useful in observing the effects of mercury on the sensory neurons of the dorsal root ganglion.

As Leong et al observed a disruption in microtubule metabolism under mercury medium (2001), I would also hypothesize that the neurotoxin will reduce the overall growth of axons, and in turn the later maturation of frequent branches. Therefore, I propose to investigate the frequency and length of primary neurite growth and branching under control solution of 5% HCl in Hanks Balanced Salt Solution and with the addition of mercury standard to the control solution. Using microimaging and counting, and length calculating software as outlined in the methodology, I will attempt to characterize any adverse effects of mercury on primary neurite growth.

II. Materials

Nikon Eclipse TS100 Inverted Microscope
Macintosh G4 with OS X 10.2
ImageJ Image analysis software
Transfer pipettes
6 well dishes
37 degrees Celsius incubator
Corning glass slides
Lamenin/poly-lysine treated, ethanol washed and baked cover slips.
Sympathetic chick neurons from 9 day old chick at a 1x density
Hanks Balanced Saline Solution (HBSS)
F+ growth medium

1:1000 0.5%HCl: HBSS
1:1000 10mM $HgCl_2$: HBSS for a 10nM $HgCl_2$
Sorvall tabletop centrifuge

III. Methods

Chick Embryos

Chick eggs were obtained from Charles River Laboratories and placed in an incubator at 37ºC for 9 days. Eggs were then observed with candle lighting techniques for viability. For all experiments, embryos were dissected for culture of dorsal root ganglia and sympathetic chains.

Primary Culture of Chick Sympathetic Neurons (techniques prescribed by Peter Hollenbeck and modified by Robert Morris)

Viable eggs were oriented with blunt end up and sterilized with EtOH spray and allowed to dry before dissection. A 110mm Petri dish was placed on the microscope stand and a sterile flame drawn pipette was used to dispense 5mls of Hanks Balanced Salt Solution (HBSS) to maintain adequate conditions during culture. The blunt end of forceps was used to tap through the blunt shell end and then to snip a circle around the shell to remove the top centimeter. The embryo was then lifted with forceps from the shell and its head gently lifted away, placing the trunk in HBSS on the stage and the head in dish for waste. The remaining trunk was oriented dorsal side up in the dish and the limbs and viscera were segmented using the forceps and discarded with the waste of the egg and head. Additional tissue was removed to the sides of the embryo to expose the spinal cord in order to gently tease away the sympathetic chains and DRG. The embryo was rinsed with fresh HBSS throughout dissection and collected ganglia were placed in a separate 25mm Petri dish with HBSS.

Dissociation of ganglia (techniques prescribed by Peter Hollenbeck and modified by Robert Morris)

Collected ganglia were rinsed twice in HBSS before removal of the salt solution with trypsin solution (Ca/Mg-free HBSS containing 0.25% trypsin) using a sterile flame drawn pipette. The ganglia were then incubated in the trypsin for 15-20 minutes at 37ºC to allow for dissociation. After incubation trypsin was carefully removed and the ganglia resuspended in HBSS, titrating gently to dissociate ganglia into single cells.

Preparation of laminin substratum

Glass coverslips were cleaned by EtOH rinsing, wiping, and further by baking in an oven to sterilize. On the inside cover of a Petri dish one drop of polylysine (1mg/ml) per coverslip was placed, keeping them isolated from one another. One sterile, clean CS was placed on each drop and allowed to set for 20 minutes. Coverslips were then floated off the dish using sterile water and then held with forceps while rinsing with the sterile water. Coverslips were allowed to dry before repeating the droplet coating with laminin in HBSS and incubating again for 20 minutes. Coverslips were rinsed with HBSS right before adding cells.

Plating Cells

To obtain desired density of 1x, F+ growth medium was added in accordance with how many ganglions were initially dissociated. One ganglion per ml was dispensed on top of a coverslip in two of the six 35mm dishes with 8mls of medium per dish. The six well dish was then centrifuged at 700 RPM for 15 minutes (adapted from experiments done by Rosie Weld in P.J. Hollenbeck lab 5/7/92) to rapidly settle cells with a 6 well plate with an equivalent mass of water in the wells for counterbalance and were incubated at 37ºC for one hour.

Mercury Application

Ganglia plated at 1x on one coverslip per well were removed from the incubator and the following solutions added: Control – 1ml of 1:1000 0.5%HCl: HBSS, Exposed – 1ml of 1:1000 10mM HgCl2: HBSS for a 10nM HgCl2

exposure and incubated for 20 minutes at 37ºC. The solutions were removed after the incubation with a sterile pipette and discarded in appropriate disposal bottles before replacing removed solutions with 3ml of F+ medium per well and incubated for 15 hrs.

Imaging and Quantification

Control and mercury exposed coverslips were divided into nine quadrants each and imaged at 10x with a Nikon Eclipse TS100 inverted microscope. Total cell count was conducted along with total number of neurons having primary processes. Neuronal cells were defined as those with approximately the same diameter as a cell with obvious growth of processes, and a spherical somal shape. Also, cells were counted on the same plane and therefore also had characteristic halos as shown in Figure 1. A primary process was any extension off the cell body longer than the diameter of the cell body itself. The data from the nine quadrants were combined and the total percentage of cells having processes was calculated for both variables.

Of the cells that were observed at 10x to have primary processes, images were taken at 20x and 40x to decipher the presence of branching. From the total number of neurons with processes, the frequency of branching was calculated for the control and mercury exposed cells. A branch was defined as an extension off of the cell body longer than the diameter of the cell body, as shown in Figure 1.

Using Image J software, the total length of processes was determined in pixels for each image at 20x. The lengths of processes for each condition were summed together and divided among the number of cells that had processes to determine the average length of processes per cell.

For all results, standard deviation of the data range was calculated to determine statistical significance.

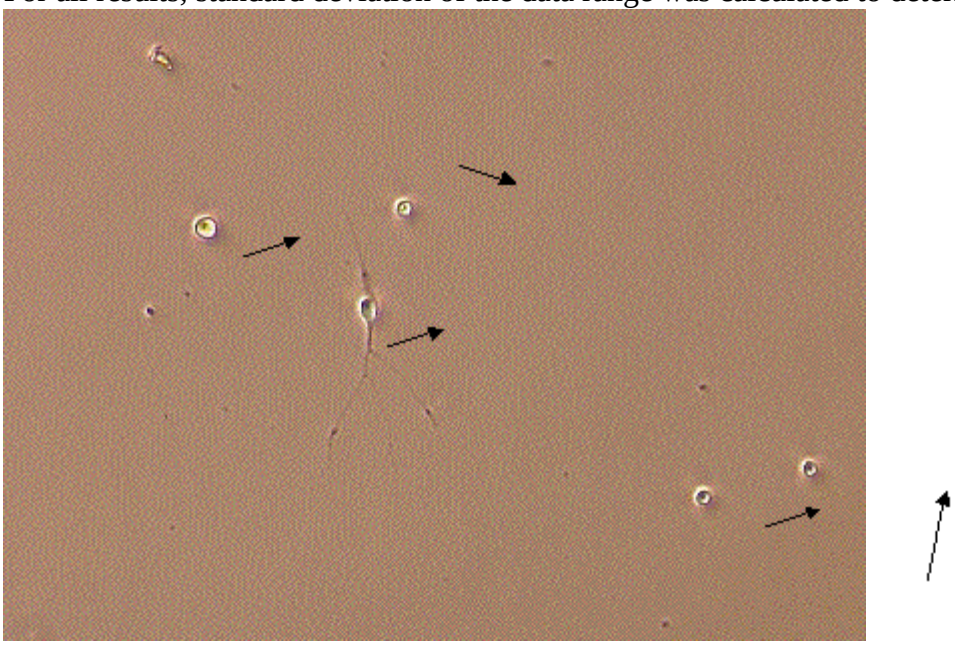

Figure 1. Defining neuronals, processes, and branching events. Those cells with arrows are classified as neuronals due to their diameter, shape, and hallow signifying an early stage of development. The neuron with obvious process growth also demonstrates branching.

IV. Results

Analysis of control and experimental images focused on the frequency of primary processes, the frequency of branching in these processes, and the overall length of processes to demonstrate the effects of mercury on axon morphology. General observations of these variables deviated greatly. As shown in Figure 2, no significant difference was observed in the frequency of primary processes or axons.

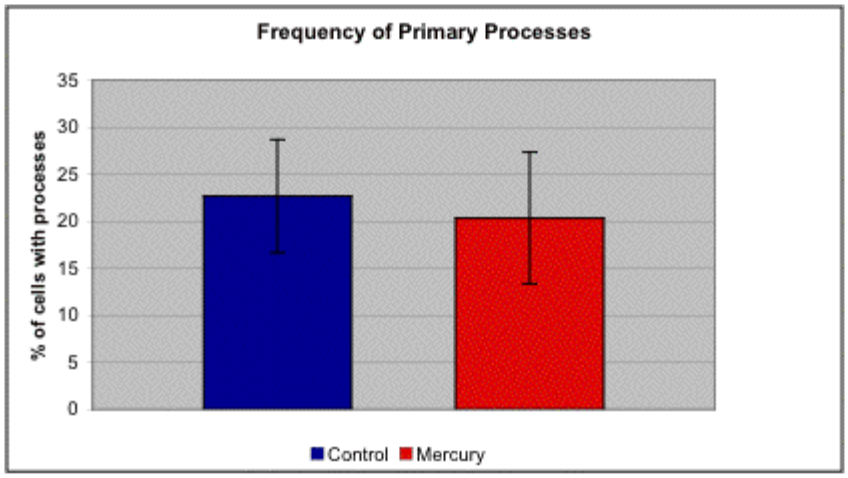

Figure 2. Percentage of observed cells at 10x with primary processes. n=20 cells.

Although the occurrence of primary processes generally showed no significant difference between control and experimental conditions, the prevalence of branching in these processes was significantly greater in control cells. As demonstrated by Figure 3, 70.6% of control processes branched while branching occurred in only 33.3% of processes grown after exposure to mercury.

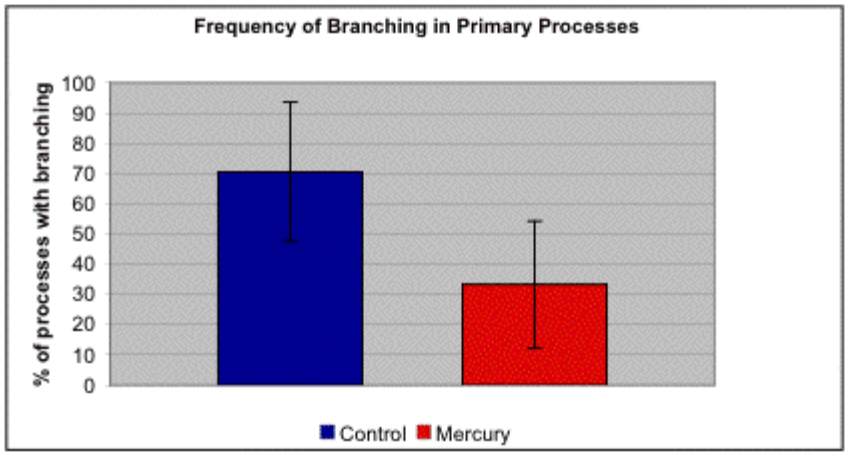

Figure 3. Percentage of primary processes demonstrating branching events when observed at 20x and 40x. n=20 cells.

Lastly, axons initially appeared to be longer in mercury treated neurons than in control. However, analysis showed that this difference was statistically insignificant. This is likely due to the observation of a few exposed neurons with exceptionally long neurons that is counterbalanced by the deviation among cells as demonstrated in Figure 4.

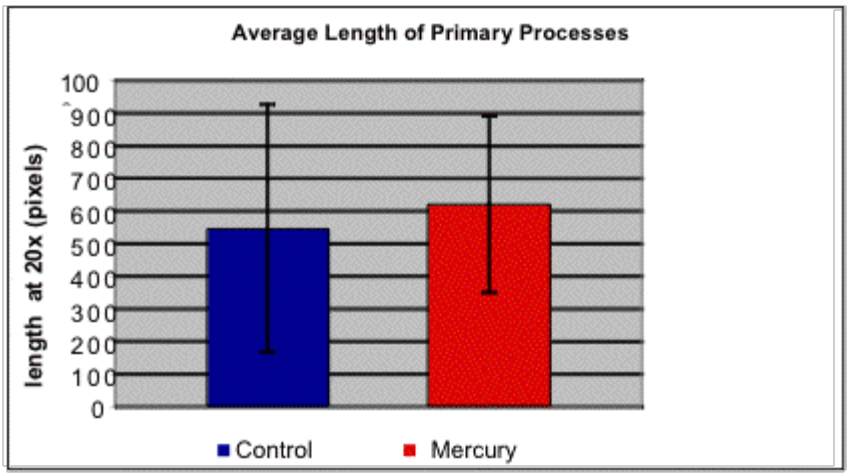

Figure 4. Average length of primary processes in pixels at 20x determined using Image J software. n= 18 cells.

V. Discussion and Conclusions

Results for mercury treated neurons suggest that this experimental condition reduces the rate of axon branch formation. However, variations of axon length analysis and primary process frequencies among control and experimental conditions were insignificant in their averages and greatly deviated. These results suggest that mercury exposure during early embryonic development has variable effects on the morphology and growth of axons. The conclusion that aspects of axon development are variably effected also support my hypothesis that there may be time points sensitive for certain aspects of axon development, such as branching, that are significant for normal growth. Exposure to mercury or other neurotoxins during these sensitive periods potentially disrupts the mechanisms for normal growth, such as the disruption of the microtubule metabolism suggested by Leong et al (2001).

Fellow researchers in parallel experiments suggest that axonal transport of organelles, mitochondrial metabolism, and actin cytoskeletal dynamics in particular are adversely affected by exposure to mercury (Bhatia et al, 2006). These findings suggest multiple disruptions of molecular mechanisms under exposure to mercury.

In replications of this experiment, it would be beneficial to fix cells to their coverslips and to seal them to glass slides for observation to produce clearer images. Difficulties arose in maintaining growth conditions such as temperature during observation while in the 6 well plates. However, by observing cells for growth at multiple time points, it should be possible to determine a consistent time at which to fix cells following adequate growth and therefore image and quantify cells at a uniform period of growth and development.

Furthermore, to expand upon these results, it would be useful to examine morphology at multiple points of growth after experimentation. From this a relationship of rates could be calculated for the variables of morphology examined. Additionally, initial mercury exposure could be applied at variable times following culture and at different concentrations to fully explore the effects of mercury as a neurotoxin.

VI. Bibliography

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