Mercury disrupts the arrangement of axonal microtubules in primary culture chick sympathetic neurons

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I. Introduction

Microtubules are cytoskeleton components that both determine the shape of the cell and function in a variety of movements, including some cell locomotion and intracellular transport of organelles (Cooper et al, 2004). It is due to this function that microtubule integrity is such a crucial part of the neuron, or any other cell for that matter. If the microtubules become corrupted, proper cellular transport cannot take place.

Microtubules are made up of tubulin dimers, consisting of two polypeptides, a-tubulin and b-tubulin. Tubulin dimers can polymerize to form microtubules and depolymerize to break down. b-tubulin binds to GTP in order to polymerize and form microtubules. When the GTP is hydrolyzed into GDP, the microtubules depolymerize. The

presence of Hg interferes with the GTP binding site similar to the hydrolysis of GDP causing the tubulin to depolymerize (Leong et al, 2000).

Since mercury is thought to cause microtubule depolymerization, it would be accurate to deduce that neurons treated with mercury are going to have less microtubules or shorter microtubules. In either case, this would effect the brightness value of the axon, making a mercury-treated axon less bright than a control neuron that has not been treated with mercury.

Chicken embryos were used in this experiment due to the relative ease of which they can dissected and neurons can be located. In addition, they are warm-blooded animals and vertebrates, so the information gained from chick embryonic neurons is likely to be more applicable than that gained from an invertebrate, for example a snail such as *Lymnaea stagnalis*, as Leong et al did in their research with the affects of mercury on membrane structure integrity (2000).

This experiment tests the hypothesis that the addition of mercury has an effect on the arrangement of microtubules in the axons and growth cones of primary culture chick embryonic sympathetic neurons. Due to the developmental nature of the cells, this experiment could propose potential physical events during fetal mercury exposure. This is an extension of the Leong et al paper, *Retrograde degeneration of neurite membrane structural integrity of nerve growth cones following* in vitro *exposure to mercury* (2000). Some changes, such as the concentration of mercury used was altered to reflect the Environmental Protection Agency's guidelines as to maximal recommended intake, as was the method of introduction of mercury. Leong et al used a microinjector to deliver a steady stream of mercury to cells (2000), whereas the cells in this experiment were treated with a solution of mercury in Hanks' Balanced Saline Solution.

Collaborators

Amy Silviri collaborated by observing the effect of mercury on another cytoskeletal element, actin. By acquiring data on both cytoskeletal structures, we can gain a better understanding of how Mercury is affecting the cell. We can hypothesis where it interacts by understanding what is happening to the microtubules versus what happens to the actin. Brittney Chick is also collaborating by observing growth cone morphology after exposure to mercury. It has been hypothesized that the axons are not dissociating, but simply altering their shape. Brittney's research will assist in this determination.

Jasmine Bhatia collaborated by observing the amount of axonal transport in mercury-treated axons as opposed to non-mercury treated axons. This extends the information gathered from this experiment to explain what the effects of mercury-treated axonal microtubules are on organelle transport. (Bhatia, 2006)

II. Materials and Methods

Materials

The materials used in this experiment include F+ Medium, Hank's Balanced Saline Solution (HBSS), HCl HBSS, HgHCl HBSS, forceps, flame-drawn pipette, Pasteur pipette bulb, small Petri dishes to plate cells, clean coverslips, eggs, 90%EtOH spray bottle, trypsin, Petri dishes for dissections, incubuator, poly-L-lysine, water, 2 sparsely plated coverslips with primary culture chick embryonic neurons, 4 medium density coverslips, 2 densely plated coverslips, 30 mL fix/permeabilization buffer (30 mL L-15, 1.62 mL 37% Formaldehyde, 0.12 mL 25% Gluteraldehyde, 1.2 g sucrose, 0.6 mL 25% TX100, 0.3 mL and 0.2 M EGTA), Fix alone (30mLs L-15, 1.62 mL 37% Formaldehyde, 0.12 mL 25% gluteraldehyde, 1.2 g sucrose, and 0.3 mL 0.2 M EGTA),
PBS/Triton x-100 (500 mL PBS and 2.5 mL 100% Tx-100), Block Buffer (50 mL PBS and 1.5 g BSA), 1¼ Ab (1 mL Block Buffer and 2 µL DM1-A), 10 slides, kimwipes, nail polish, 2 humidity chambers, 10 pedestals, 37¼ C Incubator, Nikon Eclipse 80-I microscope, 60x oil immersion lens, ImageJ software, Spot Advanced Imaging software, Adobe Photoshop 7.0 software, and Microsoft Excel software.

Methods

Dissection of Primary Culture Chick Embryonic Neurons:

Eleven day old eggs were sprayed with 90% EtOH to sterilize them. A Petri dish with warm HBSS was then set up. Sterile, dull forceps were used to tap through the shell about 1cm down from apex and the top of the shell was removed. Using forceps, the embryo was lifted out of the shell and placed in the Petri dish containing HBSS. The head was then separated from the rest of the body, along with the wings and legs. The internal organs were then removed. The embryo was then placed with the ventral side up as the tissue was carefully removed, revealing the spinal chord. Ganglia were dissected by carefully removing tissue on the sides of the spinal chord, Sympathetic nerve chains and dorsal root ganglia were then carefully removed and placed in HBSS. (Morris, Hollenbeck)

To Dissociate the Ganglia

The ganglia were washed twice with HBSS. The HBSS was then replaced with a trypsin solution (Ca/Mg-free HBSS containing 0.25% trypsin) and were allowed to incubate for 20 minutes at 37¼C. After the incubation, the trypsin was

removed gently and a small amount of HBSS was used to resuspend the ganglia. A flame-drawn Pasteur pipette was used to tituate the cells until they were dissociated into single ganglia.

Preparation of Laminin Substrata

Coverslips were treated with 1mg/ml poly-L-lysine by placing drops of poly-L-lysine onto the inside lid of a Petri dish, and a clean coverslip was placed on top of this drop. This was allowed to sit for 30 minutes before being rinsed with water and allowed to dry. The coverslips were then treated with a solution of laminin in HBSS for 30 minutes and kept wet with either laminin or HBSS until the cells are set.

Plating the Cells

Drops of the suspension of dissociated ganglia and sympathetic nerve chains were placed in dishes coverslips and growth medium. The cells were plated at different densities to allow for the best results. Low densities included ½ DRG or sympathetic nerve chain. Medium densities included 1 DRG or sympathetic nerve chain, and high densities included 2 DRGs or sympathetic nerve chains.

To Prepare Mercury-treated Immunofluorescence:

Growth medium was removed from pre-plated cells. 10 Hg HBSS was added and allowed to incubate for 20 minutes. After 20 minutes the Hg HBSS was removed and F+ medium was added for a 10-minute recovery period. After this period the F+ was removed and the cells were washed with warm HBSS from the incubator. The HBSS was then removed and 2 ml fix/perm was gently added to each dish. This was allowed to sit for 15 minutes. The fix/perm was removed after this time and fresh fix only was added and allowed to sit for 15 minutes. This was removed from the cultures after the 15 minutes and they were then washed with PBS/Tx-100. 3% BSA in PBS was used to block the cells after that. They were allowed to incubate in 37¼ C for 20 minutes before each cover slip was transferred to a pedestal in a humidity chamber. They were then labeled with 1¼ Ab by dropping 100 μL of the stain onto the coverslips using a pipetman and allowed to incubate for one hour. These were then washed three times with PBS - Tx-100 before being mounted on slides and painted with nail polish to seal the coverslips onto the slides.

To Prepare Negative Control for Hg-treated slides:

Growth medium was removed from previously plated cells. 10 Hg HBSS was added to the cells and left to incubate for 20 minutes. Hg HBSS was removed and F+ medium was added for a 10 minute recovery period. F+ medium was removed and the cells were washed with warm HBSS from the incubator. The HBSS was then removed and 2 ml

fix/perm was gently added to each dish and incubated for 15 minutes. After 15 minutes, the fix/perm was removed and fresh fix only was added and allowed to sit for 15 minutes. The fix was the removed from the cultures and PBS/Triton-X 100was used to wash the cells. 3% BSA in PBS was added in order to block.. This was allowed to incubate in 37¼ C for 20 minutes. Each cover slip was carefully transferred to a pedestal in a humidity chamber and left to incubate for one hour. After the hour, each coverslip was washed three times with PBS - Tx-100. After washing the coverslips were mounted on slide and paint with nail polish to seal.

To Prepare Control Slides

Growth medium was removed from cells. HCl HBSS was added and allowed to incubate for 20 minutes. HBSS was removed and F+ medium was added and allowed to incubate for 10 minutes. F+ medium was removed and the cells were washed with warm HBSS from the incubator. HBSS was removed and 2 ml fix/perm was gently added to each dish and allowed to incubate for 15 minutes. The fix/perm was removed after fifteen minutes and fresh fix only was added to the cultures and allowed to sit for 15 minutes. The fix only was removed and the cells were washed with PBS/Tx-100. After the wash, the cells were blocked with 3% BSA in PBS at 37¼ C for 20 minutes. After blocking, each coverslip was transferred to a pedestal in a humidity chamber and labeled with 1¼ Ab by dropping 100 µL onto the tops of the coverslips. These were allowed to incubate for one hour. After labeling, the cells were washed three times with PBS - Tx-100, mounted on a slide and painted with nail polish to seal them.

To Prepare Negative Control for Control Slides

Growth medium was from pre-plated cells. HCl HBSS was added and allowed to incubate for 20 minutes. After 20 minutes, the Hg HBSS was removed and replaced by F+ medium for a 10 minute recovery period. F+ medium was removed and the cells were washed with warm HBSS from the incubator. The HBSS was remove and gently add 2 ml fix/perm to each dish. Allow this to site for 15 minutes. Remove fix/perm and add fresh fix only for 15 minutes. Remove and wash with PBS/Tx-100. Block with 3% BSA in PBS. Incubate in 37¼ C for 20 minutes. Wash three times with PBS - Tx-100. Mount on slide and paint with nail polish to seal.

Data Collection

A Nikon Eclipse 80-I 60 x oil immersion lenses was used to detect cells. To locate appropriate specimen, the filter was switched to position 3 for microtubules. The transmitted light was shut off by placing a piece of aluminum foil over the

light source. The shutter was opened to search for cells. Spot imaging software was used to obtain images.

To quantify images, photos were saved as 16-bit tif images and opened in ImageJ software. The region of interest was selected, in this case the axons. Histogram, under the analyze menu was selected. These values were then copied and pasted into Microsoft Excel. In addition, a dark region where no axons are located was selected and these values were also copied into Excel. Any axonal brightness values less than the dark region values were deleted. These values were averaged by multiplying the number of pixels by the brightness and adding these values to get total brightness, then dividing by the sum of the number of pixels. This is the average brightness value for the axon. The average brightness of the cell body was also taken using this same method. To find the percent brightness, the average brightness for the axons, an average of all of the axons of the cell, was divided by the average brightness of the cell body. This average was found in order to account for any discrepancies from adjusting the camera brightness, etc.

III. Results

The mercury-treated axons exhibited a lower brightness percentage in comparison to the cell body than did the axons that were not treated with mercury. Figure 1 shows an example of a neuron and glial cell stained with both Rhodamine Phalloidin, to detect the actin, and DM1-A to detect the microtubules.

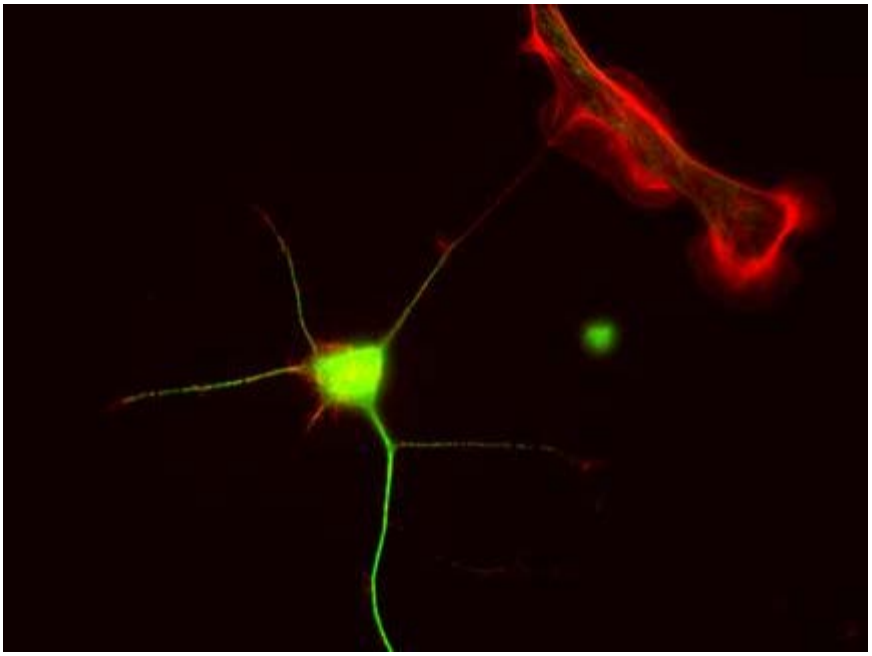


Figure 1. A neuron and glial cell stained with Rhodamine Phalloidin (red) and DM1-A (green). The green fluorescence is the microtubules and the red is the actin. The bright spherical region is the cell body. The growth cones are visible, along with neuronal-glial interaction. The image was taken on a Nikon Eclipse 80-I microscope with a 60x oil immersion lens, by a Spot insight camera.

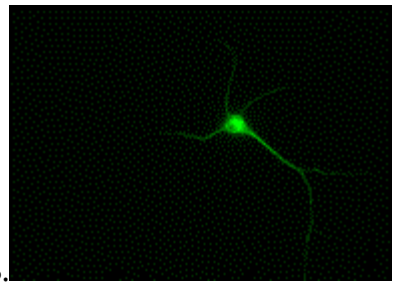
A.  **B.** 

Figure 2. **A**. Mercury-treated primary culture chick embryonic neuron. Axons were selected and measured for their brightness value using ImageJ software. The values of all experimental (mercury-treated) axons were averaged and compared to the average brightness of the cell body. **B**. Control primary culture chick embryonic neuron. The brightness of these axons was assessed equally to the mercury treated neurons.

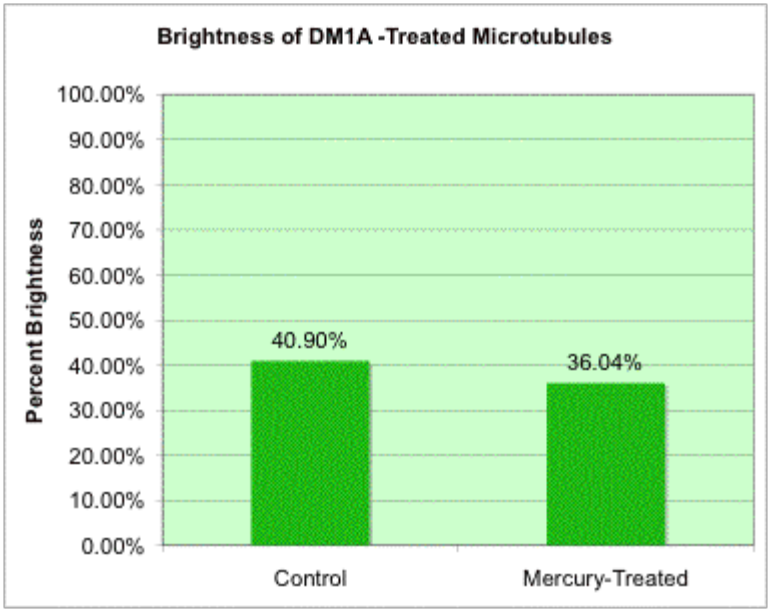


figure 3. This plot exhibits the percent brightness of DM1-A stained axonal microtubules in comparison to the cell body for both control cultures and mercury-treated cultures. Brightness is greater for Control cultures. n=14 axons

As shown by figure 3, mercury-treated axons had a lower percent brightness than those that were not treated with the metal. The percent brightness of the control axons was 40.90% of the cell body. The percent brightness for the mercury-treated axons was 36.04%. The axons that were not treated with mercury had an average brightness that was 3.86% brighter than those that were treated with mercury.

IV. Discussion and Conclusions

The average percent brightness of the Control cells was found to be 40.90%. The average percent brightness for the mercury-treated cells is 36.04%. This suggests a discrepancy between the treatments and supports the research form the University of Calgary by Leong et al. Though these results are not as dramatic as those reported by Leong and his

team, it is likely that Leong used a greater magnitude of mercury to treat their cells. Also, the method of introduction is different between the two studies.

Lowered brightness due to mercury treatment could suggest a lower concentration of microtubules in mercury treated cells. Lowered brightness values would then support the original hypothesis that mercury affects the integrity of microtubule structure. These findings also support those by J. Bhatia.

In her research concerning the amount of axonal transport in neurons exposed to mercury, Bhatia found less transport in mercury treated axons than in axons not treated with mercury (Bhatia, 2006). This suggests the possibility of lower percent brightness being due to lower levels of microtubule concentration, therefore allowing for less transport in mercury treated neurons.

Research conducted by Silveri showed a greater percent brightness average for mercury-treated axons than for the control axons (Silveri, 2006). This information may seem counter-intuitive, but it is explained fairly easily by the research completed by Chick. Chick found that though there appeared to be additional growth cones in the mercury-treated group, they exhibited less activity than the growth cones of neurons not treated with mercury (Chick, 2006).

There could very well be multiple sources of error, simply due to the complexity of the experiment and the number of different solutions that had to be made up fresh each trial. In addition, it is possible that error arose in locating cells, and that the cells that are being used for sampling are not typical of other control or experimental cells. Lastly. It is likely that sources of error could have arose during quantification of data due to sampling errors and human error, either within the region of interest or due to calculation and mathematical errors.

If this experiment were to be repeated in the future, more time should be given to preparation, imaging, and quantifying data. A larger population of axons should have been used in order to make speculations about how mercury is affecting the brightness of the microtubules. Further experimentation should be done to determine what the effect of mercury is on mature neurons, instead of embryonic, developing neurons. This should be done so that the information gathered can be applied to more than just fetal development and an understanding of the impact of mercury ingestion in adults can be gained.

V. Bibliography

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