

The Effects of Mercury on Endocytosis in *Gallus gallus* Sympathetic Neurons

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Introduction

Metals, such as mercury have been known to have neurodegenerative effects on the brain and its function. There is presently scientific literature that has investigated mercury and its ability to inhibit brain function (Yokel, 2006; Leong et al., 2001). Some research supports the hypothesis that mercury has negative effects on the brain. For example, occupational exposure to mercury appears to be a risk factor in the development of Parkinson's disease (Yokel, 2006).

My hypothesis for the following study is that a level of 100 nM HgCl₂ concentration applied to a neuron will result in lower mean brightness of endosomes within that neuron. The significance of this is that a lower brightness implies mercury is inhibiting the process of endocytosis within the neuron. This is a particularly interesting aspect to study since the amount of scientific literature investigating endocytosis in neurons is small. However, there are studies that have tested other features of the neuron, such as degeneration of nerve cones. (Leong, et al., 2001). A study by Leong et al. found that after mercury exposure, mercury ions disrupted membrane structure and growth rates, as well as microtubule structure disintegration.

Neurons from the embryos of the domestic *Gallus gallus* will be used to test the hypothesis. Accessing neurons via humans is not viable in my situation, so the best alternative is dissecting a vertebrate with a nervous system similar to a human. It also is much easier and safer than obtaining cells from a human.

Endocytosis is an important process for the proper functioning of a neuron (Schwartz et al., 2000). After exocytosis of neurotransmitter vesicles, used vesicle membranes are returned to the cell body for reuse or degradation. The process has other important functions as well (Schwartz et al., 2000). For example, it alters the function of regulatory molecules such as receptors and adhesion molecules (Schwartz et al., 2000). A large proportion of endocytic traffic is carried by clathrin-coated vesicles, which process is referred to as receptor-mediated endocytosis, since components of the clathrin coat specifically identify transmembrane receptor proteins in the extracellular space (Schwartz et al., 2000). Endocytosis occurs most often at nerve terminals when dealing with the recycling of vesicles

(Schwartz et al., 2000).

Two main experiments will be performed with varying parameters. A control concentration of 0nM Hg will be applied to dissociated neuronal cells, as well an experimental concentration of 100nM Hg to another set of dissociated cells. Fluorescein-dextran dye will be used to label the endosomes. It is normally used for studies involving endocytosis because it is internalized inside the vesicle when it passes through the membrane, and thus the dextran will fluoresce under a fluorescent-imaging microscope and appear green. Any bright green area will be labeled endosomes.

(Gustafson, 2003).

In this study, I treated chick sympathetic neurons with 0nM Hg solution and 100uM neurons, fluorescently labeled them with fluorescein-dextran, took images of both and collected data based off those images. To supplement my study, Catherine Forant and Mark Halbach will be performing similar experiments. Ms. Forant will be investigating the effects of mercury on growth cone morphology and retraction using various concentrations. Mr. Halbach will be examining organelle transport and the effects of various mercury concentrations have on this particular process.

Materials

Materials needed to carry out the experiment were forceps, sterile Pasteur pipettes, small Petri dishes (110 mm/25mm), coverslips, 8-10 day chicken eggs, Ethanol (EtOH), Hanks Balanced Salt Solution, C-medium, Trypsin, kimwipes, incubator, dissection scope, polylysine, water, laminin, Ei80 microscope (equipped with camera), Xcite 120 EXFO Fluorescence Illumination System, Uniblitz shutter control box, model VMM-D1 shutter driver slides, VALAP and aluminum foil.

Several solutions were created. The solutions were 0 nM HgCl₂ in 0.5% HCl, 100 nM HgCl₂ in 0.5% HCl and Fluorescein-dextran dye (1 ml of 50uM in methanol used at 1:100 in Growth medium),

Method

The following methods were all obtained from *Primary Culture of Chick Embryonic Peripheral Neurons* by Peter J. Hollenbeck and Robert L. Morris.

Chick embryo dissection

To obtain the neuronal cells, Professor Morris performed dissections on chick (*Gallus gallus*) embryos that were 8-10 days old. We all had practical experience with dissection prior to our experiments, however; in the interest of time, Professor Morris conducted all the dissections. A beheaded chick embryo was placed in a 110mm Petri dish. Dorsal root ganglia and sympathetic nerve chains were accessed by using forceps to cut along the rib cage. Tissue and organs

were carefully removed exposing the spinal cord. Once the dorsal root ganglia or sympathetic nerve chains were separated from the cord, they were placed in 25mm Petri dishes in HBSS.

Dissociation of ganglia

The ganglia were cleaned and washed at least 2 times with HBSS. The HBSS was replaced with trypsin and the ganglia were incubated for 15-20 minutes at 37 C. The trypsin was removed gently and the ganglia were resuspended in a small volume of HBSS. They were triturated with a Pasteur pipette until dissociated into single cells.

Preparation of laminin substrata

1 mg/ml polylysine was applied to the surface of a coverslip for 20 to 30 minutes, then it was rinsed with sterile water and allowed to dry. The slip was then covered with laminin in HBSS for 20-30 minutes. Coverslips were kept wet until cell plating.

Plating out mercury/fluorescent-treated coverslips

I obtained 2 coverslips with dissociated cells from the incubator. I removed the growth medium from both 25mm Petri dishes using a Pasteur pipette. I placed 1 ml of 0 nM Hg HBSS on one coverslip and labeled it. This served as my control. I also placed 1 ml of 100nM Hg HBSS onto a coverslip and labeled it. I returned both to the incubator at 37 C for 30 minutes. After, I used a pipette to remove the mercury solution to a waste beaker. Then, I rinsed both slips three times with HBSS, with 2ml per wash. Then I added 1ml fluorescein-dextran dye to my coverslips. I wrapped both Petri dishes with aluminum foil and incubated for 10 minutes. Following the pulse of dye, I returned the fluorescein-dextran to its original test tube for later use. Both coverslips were then washed a total of 5 times. Each time, I washed 2 times with HBSS and incubated the coverslips for 5 minutes at 37 C. At the end of the wash period, I created two chip chambers on two individual slides and put one drop of growth medium in the middle of each chamber. I placed the coverslips on top of these drops and made sure the opposite side was dry with kimwipes. To seal the coverslips, I applied VALAP and cleaned the top of the coverslip with water.

Imaging

I used a Nikon Eclipse 80i microscope equipped with the necessary tools (Uniblitz shutter control box, model VMM-D1 shutter driver to image fluorescence; Xcite 120 EXFO Fluorescence Illumination System. All images were viewed at 400x magnification. Exposure time was set to 10 seconds and gain was set to 4. All images were captured using SPOT software.

Data collection

Four trials were performed for the control cells, yielding four control slides. Two trials were performed for the

experimental cells, yielding two slides. Images were formatted in ImageJ to enhance brightness. I adjusted brightness as well as contrast. I made the same adjustments for control and experimental images. They were: brightness (-1) and contrast (+61). After having collected two suitable images, one control and one experimental, I chose three areas along each neuron containing fluorescein-labeled endosomes. I calculated mean brightness of those areas and mean brightness of the background directly adjacent to those areas. This was done using a histogram with the program ImageJ. I took the difference between mean brightness of the endosomes and mean brightness of the background and averaged the values, respectively. These were then used to create bar graph with Excel.

Results

Based upon the collected data, it appears that the experimental images have a higher mean brightness value than the control images. The average mean brightness value for the control was 3.6. The average mean brightness value for the experimental was 8.8. See page 51 in Alexander Meyer's lab notebook for data tables.

Phase images were taken in addition to the fluorescent images. These were used to identify the correct regions of neurons where endosomes were located. Figure 5 provides a bar graph showing a comparison of brightness values between control versus experimental endosomes.



Figure 1. This is a neuron stained with fluorescein-dextran, treated with 0nM Hg concentration.

The brighter, visible green areas are labeled endosomes. The image was taken with a Nikon 80i microscope at 400X.



Figure 2. This is an image of the cells that were stained seen in phase treated with 0nM Hg concentration (Fig.1).



Figure 3. This is a neuron stained with fluorescein-dextran, treated with 100nM Hg concentration. The brighter, visible green areas are labeled endosomes. The image was taken with a Nikon 80i microscope at 400X.



Figure 4. This is an image of the cells that were stained seen in phase treated with 100nM Hg concentration.

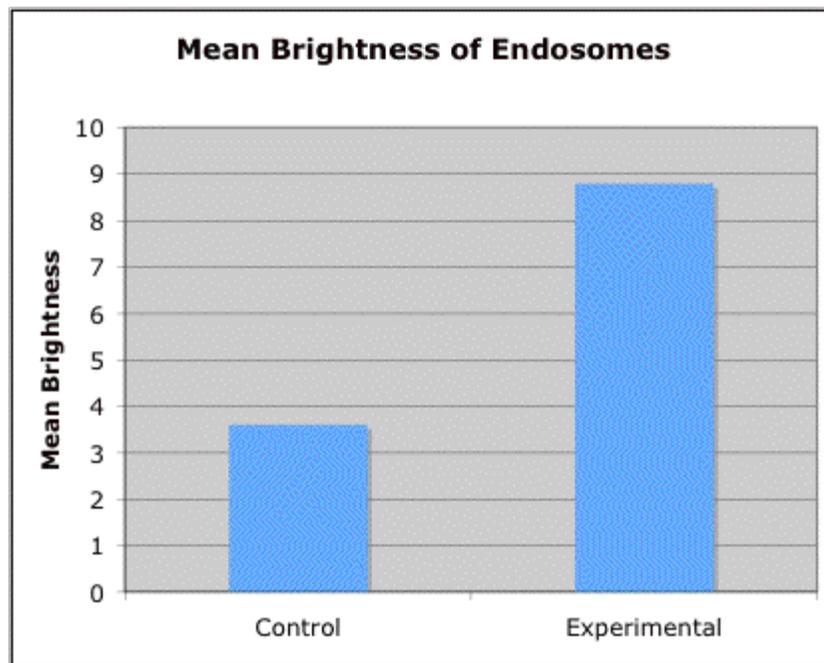


Figure 5. This graph represents the mean brightness of both control (0uM Hg) and experimental (100nM Hg) labeled neurons (n =6).

Discussion

My hypothesis that stated a 100 nM mercury concentration would have a negative effect on endocytosis was not supported by the data I collected. Therefore, I refute my hypothesis. From the data we can make several conclusions.

The difference between mean brightness values of experimental and control is small. This would lead us to believe that mercury does not have a significant effect on endocytosis within a neuron. Such a conclusion would not be consistent with other projects that support the hypothesis that mercury can have negative or harmful effects on neurons and their processes (Leong, Syed, Lorscheider, 2000). If my hypothesis were to be supported by my data, I would have to see a significantly higher mean brightness value associated with my control compared to my experimental. This would signify that less endosomes would be forming under the influence of a higher concentration of mercury.

At the cellular level, a 100 nM Hg concentration did not have an effect on endocytic traffic within the axon and cell body because the control brightness and mean brightness are similar in value. Retrograde axonal transport of endocytic vesicles therefore, is unaffected.

I believe that during the experiment, I had various sources of error. I'm particularly concerned with my control image. It is harder to identify neuron cellular structure compared to the experimental image. I had to perform the experiment several times before I had any discernible image. This probably was a result of either too much fluorescein-dextran dye or not enough washes. Washing out the dextran is very important, especially after the pulse (Moris, 2008). It is necessary to completely remove the excess dextran so that when imaging, only the endosomes appear and not the entire background (Morris, 2008). Another source of error could be my imaging technique. The problem stemmed from changed settings on the computer, specifically exposure time and gain. Also, the time from VALAP application to imaging might have taken too long or that I might have bleached the neurons so they could no longer fluoresce. My final trial yielded the best images (Fig. 1,3). Previous trials failed at producing images because of altered computer settings. I was able to get an image only after help from ICUC staff and others (Rossetti; Morris, 2008). I changed exposure time from 1 second to 10 seconds and increased the gain, or sensitivity, to 4.

If I were to do this experiment again, I would do several things. I would have a more accurate protocol, so I wouldn't have to alter steps or concentrations. I would also give myself plenty of time to image each concentration. I often felt pressed for time during my data collection period. I would also like to work in closer conjunction with a fellow student. Although I performed similar experiments with other students, I was the only student using fluorescein-dextran. I would benefit from another researcher who was using dextran because we could compare protocols and adjust accordingly.

In the future, I would continue to study endocytosis and would continue to use dosages of mercury. This independent project did not yield the results I had hoped for, so I'd like to continue with endocytosis within neurons. I would want to use various concentrations but try dosing cells with 200 nM Hg instead of 100 nM to see if extreme concentrations have any effect.

References

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