The Effect of Mercury on the Ratio Between Polymerized and De-polymerized Actin in Glial Cells

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I. Introduction

Brain function and development is largely dependent on glial cells which are known to encourage the survival and differentiation of neurons as well as stimulate the migration of the neuronal somata and axons (Pfriegaer). Glial cells provide the brain with structure and are appropriately known as the “glue” of the nervous system as they surround the cell bodies, axons, and dendrites of neurons (Kandel, 20). Glial cells are also known to perform tasks which include, removing debris following injury or neuron death and absorbing the neurotransmitters released by neurons during synaptic transmission (Kandel, 20). In addition to this, certain types of glial cells direct migrating neurons and guide the outward growth of axons throughout the brains development (Kandel, 20).

There are two different types of glial cells, oligodendrocytes and Schwann cells. These two types of glial cells are required for proper functioning of the central nervous system as they both produce myelin which serves as insulation on the nerve cell axons (Kandel, 20). Previous studies have determined that Schwann cells advance the growth of axons during regeneration (Bixby). Alternatively, Oligodenrocytes are responsible for elevating the amount central myelin, which typically ensheathes many axonal processes (Kandel, 85).

The occurrence of specific diseases can be attributed to the disruption of the specialized properties of neurons (Kandel, 86). For this reason the following experiment was designed to evaluate the effect of mercury on actin dynamics. In doing so, this study tested the hypothesis that mercury disrupts actin dynamics and affects the ratio of polymerized vs. de-polymerized actin. Since mercury exposure often has a negative impact on the brain, investigating the effect of mercury on actin dynamics will provide insight for studying neurological abnormalities. In addition, this experiment may help to identify the specific portions of the brain that are most affected by mercury.

The cells required for completing this study were obtained from domestic chicken, or *Gallus gallus*, embryos. This specific organism was selected because chick embryos produce a number of viable neurons that be conveniently dissected. It is also beneficial that these organisms are warm-blooded vertebrates for data gathered from them is more applicable to the study of neurobiology in humans. In this study chick sympathetic ganglia were treated with solutions of Hank’s Balanced Salt Solution that contained varying concentrations of mercury. A method of fluorescent double

labeling was employed to label the F-actin and G-actin, and brightfield and fluorescence microscopy was used to evaluate the results.

This project was completed in collaboration with Blair Rossetti, Michael Grimaldi and Michael Ophir. Blair designed an experiment that allowed him to study the ratio of polymerized actin versus de-polymerized actin in neurons. Michael Grimaldi organized an experiment that allowed him to measure the amount of polymerized actin in neurons, and Michael Ophir measured the amount of actin in Glia. Since these projects were so closely related, we were able to obtain a large amount of quantifiable data.

II. Materials and Methods

i. Materials

The materials utilized in this experiment included Hanks Balanced Salt Solution, growth medium, sucrose, 37% formaldehyde, 25% gluteraldehyde, 25% Triton X-100, 0.2M EGTA, Sodium Hydroxide, 3% BSA, Deoxyribonuclease I (Alexa Fluor® 488 conjugate), Alexa Fluor® 546 phalloidin, G-actin/F-actin Fluorescent Buffer, 1°Ab, Mercury, VALAP, Fix/Permeabilization Buffer, Nikon B-2E/C (Medium Band Blue Excitation) Filter Block, Nikon G-2E/C (Narrow Band Green Excitation) Filter Block, SPOT RT Color camera (model 2.2.0), SPOT Advanced Software, Chip Chambers, Coverslips, Incubator, Humidity Chamber, forceps, 90% EtOH solution, glass pipettes, Pasteur pipette bulbs, small Petri dishes, large Petri dishes, nail polish, kimwipes, and plated coverslips with primary culture chick embryonic neurons.

ii. Dissection of Primary Culture Chick Embryonic Neurons

Eleven-day-old chick eggs were sterilized with 90% ethanol solution. Once the eggs were sterilized, and a sterile pair of forceps were used to break through the shell leaving a tiny hole approximately 1 cm in diameter. The chick embryo was removed through the hole using the sterilized forceps, and placed into a Petri dish containing Hank's Balanced Salt Solution (HBSS). The embryo's head was removed and separated from the remainder of the body along with the legs and wings using the forceps. The remainder of the embryo was dissected in a manner that allowed the spinal chord to be come exposed. Once the spinal chord was visible, ganglia and sympathetic nerve chains were carefully removed and placed in HBSS. (Morris, Hollenbeck)

iii. Buffer Preparation

The HgHBSS buffer was prepared by placing 10M of mercury chloride ($HgCl_2$) in 0.5% hydrochloric acid (HCl). We then diluted the mercury solution with HBSS to a 1:1000 ratio to achieve a 10nM HgHBSS stock solution. Preparation of the Fixation/Permeablization Buffer (FPB) was completed by mixing: 30mL L15 growth medium, 1.2 g

sucrose, 1.62mL of 37% formaldehyde, 0.12mL 25% gluteraldehyde, 0.6mL of 25% Triton X-100 (TX-100) and 0.3mL of 0.2M EGTA. The buffer was then neutralized to a pH 7 by adding drops of sodium hydroxide. In order to prepare the Fixation Buffer (FB) 30mL of L15 growth medium was mixed with 1.2 g sucrose, 1.62mL of 37% formaldehyde, 0.12mL 25% gluteraldehyde, and 0.3mL of 0.2M EGTA. This buffer was also neutralized to a pH of 7 by adding drops of sodium hydroxide. Preparation of PBS/Triton X-100 was achieved by vigorously mixing 500mL PBS and 2.5mL 100% T X-100. Block Buffer was prepared by dissolving 1.5g BSA (3% BSA final) in 50mL PBS. The 1°Ab was prepared when 1:500 DM1A was added to 2µL DM1A in 1mL Block Buffer and finally the G-actin/F-actin Fluorescent Buffer were prepared by mixing 200 µL of a 9 µg/mL (0.3 µM) solution of Deoxyribonuclease I (Alexa Fluor® 488 conjugate), 200 µL of and 0.165 µM solution of Alexa Fluor® 546 phalloidin. This information was provided in Fluorescent Deoxyribonuclease I Conjugates Product Information (Revised: 25-October-2005). **NOTE:** We were careful not too shake this buffer!

iii. Preparation of Neurons and Ganglia

The neurons and ganglia gathered during dissection were washed twice with HBSS. The HBSS was then removed and the cells were placed in trypsin solution (Ca/MG- free HBSS containing 0.25% trypsin). The neurons and Ganglia were then left to incubate in the trypsin solution for a period of 20 minutes at room temperature or 37°C. Once the incubation period passed, the trypsin was removed and the cells were re-suspended in HBSS.

iv. Preparation of Lamanin Substrata

The coverslips utilized in this experiment were treated with lamanin to induce the growth of neurons. In order to accomplish this we coated the surface of the cells with 1 mg/ml pf ply-L-lysine and put drops of 1 mg/ml polylysine on the inside lid of a 110mm Petri dish. Immediately following this, on sterile coverslip was laced on the drop and left there for 20 minutes. When the 20-minute period was over, the coverslip was rinsed with distilled water and left to dry.

v. Plating Cells

The suspensions of dissociated cells were added to the dishes containing coverslips and growth medium in a dropwise manner. The cells were set at varying densities to ensure the best results.

vi. Mercury Treatment

Mercury treatment was accomplished by placing the cultured experimental cells on coverslips and treating them with a mercury concentration of 10nM (HgHBSS) for 20 minutes. No mercury treatment was received by the cultured control cells. Growth medium along with F+ was added to the coverslips for 10 minutes. Immediately following this, the coverslips were washed with HBSS.

vii. Fluorescent Double-Labeling of F-Actin and G-Actin

The fixed cultured cells became adhered to the coverslips coverslips when 2mL of FPB was added to the cells for 15 minutes. The FPB was then withdrawn and fixation was continued by adding 2mL of FB to the cells and allowing then to remain at room temperature for 5-10 minutes. The coverslips were then washed with one portion of PBS/Triton X-100 and incubated for 20 min at 37°C in Block Buffer. During the incubation period, humidity chambers were assembled. Once the incubation period was complete, the coverslips were transferred to the humidity chambers and left to incubate in 100μL of 1°Ab for 1 hour. The coverslips were then washed with three portions of PBS/Triton X-10 and two portions of PBS. Next the G-actin was labeled with Deoxyribonuclease I, Alexa Fluor® 488 conjugate and the F-actin was labeled with Alexa Fluor® 546 phalloidin. The coverslips were allowed to incubate in the G-actin/F-actin Fluorescent Buffer for 15-20 minutes then they were allowed to air dry.

viii. Brightfield and Fluorescence Microscopy

The fixed cells were imaged using a Nikon 80*i* Eclipse equipped with a SPOT RT Color CCD camera by Diagnostic Instruments, Inc. In order to view the cells the EXFO X-Cite 120 Fluorescence Illumination System was turned on and set to highest setting. The SPOT RT Color CCD camera was then turned on followed by the microscope power supply. Next the Uniblitz Model VMM-D1 Shutter Driver by Vincent Associates was turned on long with the Mac G4 computer. Then the PHOTO/BINO slider was set to BINO. The Nomarski Analyzer was removed from the light path and the filter turret was set to open position. While the fluorescent shutter was closed the objective turret was rotated to Nikon Plan Fluor 40x/0.75 Ph2 DLL inf/0.17 WD 0.72 objective. The Turret was then rotated to Ph2. The condenser aperture was then completely opened along with the field stop. Any remaining filters were removed from the light path and the specimen was placed on the stage. The specimen was located by using a scanning slide technique in raster motion. We then Aligned for Kohler Illumination and opened the SPOT program, selecting phase mode. The images were acquired using Automatic Exposure. Then the microscope lamp was covered to block all light. The filter turret was then rotated to Nikon B-2E/C Filter Cube. We acquired a standard AF 488 image with and Exposure of 4.5 seconds and a gain of 4. 24 Bits per Pixel (RGB). We acquired the optimal AF 488 image with a gain 4. 24 Bits per Pixel (RGB). We then rotated the filter turret to the Nikon G-2E/C Filter Cube and acquired a standard AF 568 image. Exposure lasted 0.4 seconds. Gain was 4. 24 Bits per Pixel (RGB). Next an optimal AF 568 image was acquired for which exposure varied and gain was 4. 24 Bits per Pixel (RGB). All gathered images were saved as TIFF.

iix. Quantification

In order to quantify the data gathered in this experiment, all AF 568 images were opened in ImageJ (1.32j) and the background was subtracted by utilizing the Subtract Background function with a Rolling Ball Radius of 50 and White Background selected. The Images were then converted to black and white mask by selecting the threshold function under the Binary menu. The AF 568 or AF 488 image of interest was opened and the Image Calculator was used to add the mask to the image of interest. The radius of interest was roughly selected and a histogram was obtained. The histogram was copied and pasted into excel so that a mean GV for all pixels except 255 could be calculated (Mean = Sum(GV*count)/Sum(count)). These steps were repeated for all images of interest.

The standardization of exposures of fluorescently labeled F-actin and G-actin in Neurons was completed according to the protocol written by Michael Grimaldi and Blair Rossetti. These procedures can be viewed in each of their research papers if further reference is necessary.

Please note that these procedures were written in collaboration with Blair Rossetti, Michael Ophir, and Michael Grimaldi. Procedures found in this section are based on existing labeling procedures that were provided in the following scientific papers

Akisaka, T., Yoshida, H., Inoue, S., Shimizu, K. (2001). Organization of cytoskeletal F-actin, G-actin, and gelsolin in the adhesion structures in cultured osteoclast. *J. of Bone and Mineral Research* 16, 1248-1255.

Paglini, G., Kunda, P., Quiroga, S., Kosik, K., Cáceres, A. (1998) Suppression of radixin and moesin alters growth cone morphology, motility, and process formation in primary cultured neurons. *J. of Cell Biol.* 143, 443-455.

Pichon, S., Bryckaert, M., Berrou, E. (2004). Control of actin dynamics by p38 MAP kinase-Hsp27 distribution in the lamellipodium of smooth muscle cells. *J. of Cell Science* 117, 2569-2577.

Silverio, A. (2006). Rhodamine phalloidin stain indicates greater amount of actin in mercury-treated primary chick sympathetic neurons. *ICUC Server*.

III. Results

The results of the experiment determined that the mercury treated glial cells have the same ratio of polymerized and de-polymerized actin as the mercury treated cells. In order to evaluate the ratio of G-actin vs. p-actin we took a number of exposures at the control level and at the mercury treated level.

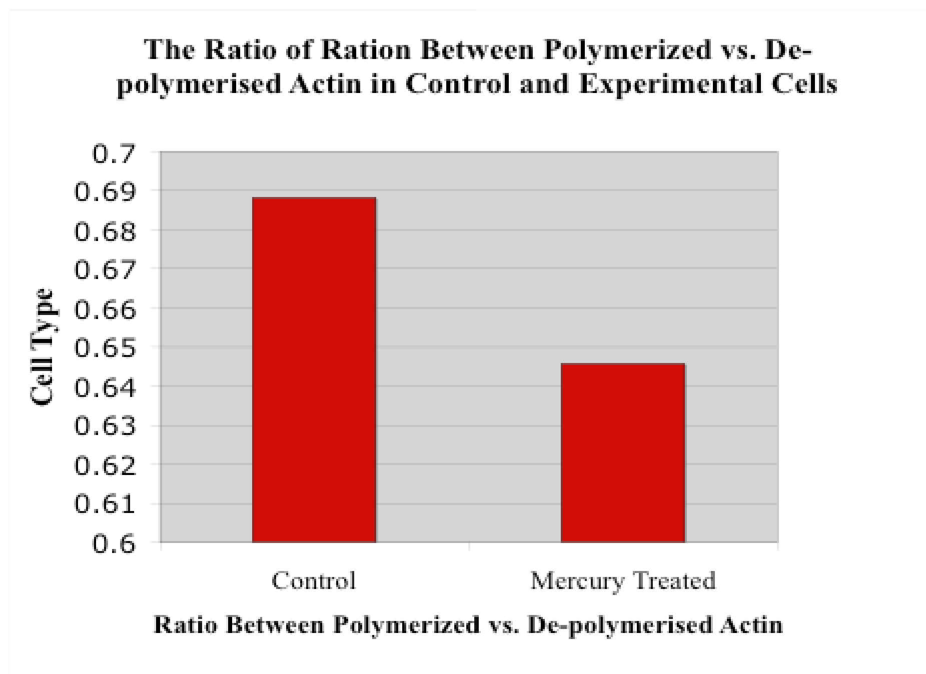

Figure 3. This graph shows the ratio between polymerized vs. depolymerized actin in control and mercury treated cells.

The results of the graph clearly indicate that the ratio between the polymerized and de-polymerized actin does not vary in response to mercury exposure. The overall ratios of mercury treated cells vs. control cells was .646 to .688. The .042 difference between the two groups is not at all significant. Images of glial cells were included to demonstrate the physical characteristics of these unique cells. For the most part, the cells took on the shape displayed in each of the figures. In collecting our data, we calculated the mean gray scale value for each of the glial cells. The details of this procedure are include in the methods section of this report. In gathering data this way, we calculated the mean gray scale value for 16 control slides and 15 mercury treated slides.

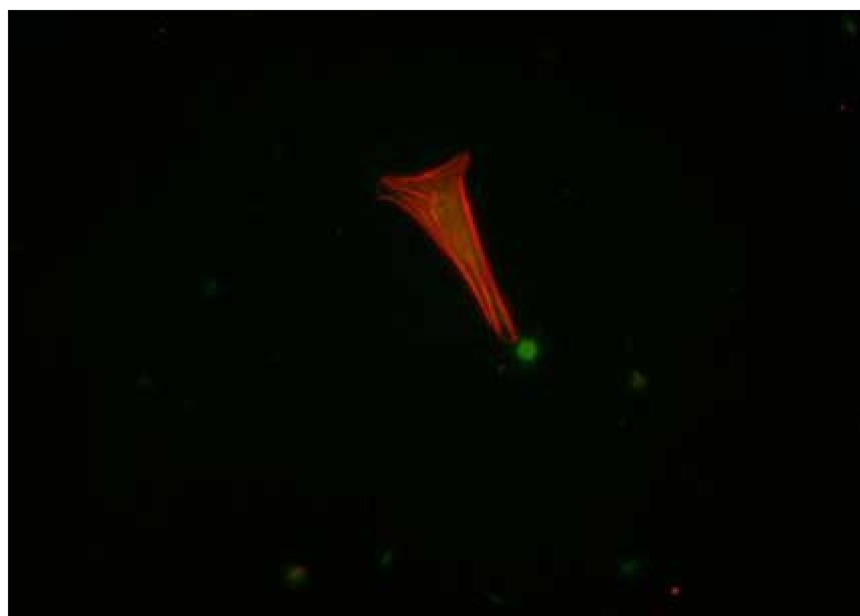

Figure 1. The image above displays a glial cell stained with Deoxyribonuclease and phalloidin. This particular photo shows one of the glial cells that was obtained from the control group. This image was taken using a Nikon 80*i* Eclipse microscope equipped with a Spot insight camera.

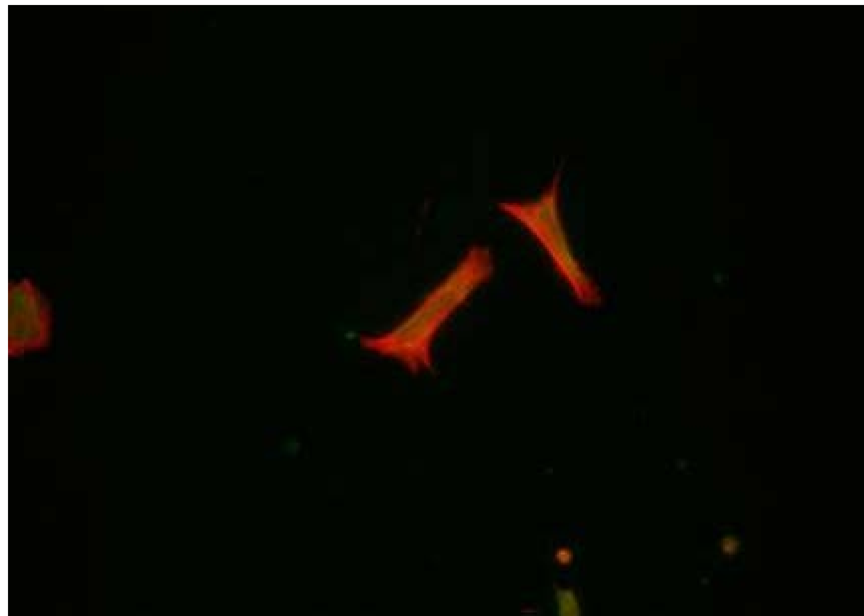

Figure 2. The image displayed as figure 2. Shows two glial cells that have been stained with Deoxyribonuclease and phalloidin. Both of the cells include in the image were also treated with mercury that had a concentration of 10nM.

V. Discussion and Conclusions

The results obtained from this experiment failed to support our hypothesis that the presence of mercury disrupts the actin dynamics of glial cells. This result suggests that mercury does not affect the actin within neurons. This outcome suggests that neurological conditions that arise after mercury expose are not a result of dysfunctional glial cells.

These findings are consistent with those found Blair Rossetti's a study which investigated the ratio between G-actin and F-actin in neurons. He too found that the ratios remained fairly consistent between the control and experimental groups.

Some sources of error were discovered in completing this experiment. The glial cells selected for imaging were chosen at random, and we imaged as many glial cells as were available on each slide for the control group and mercury treated groups. This is a feature of the experiment that probably could not have been prevented however, since we did not have direct control over the number of glial cells that were successfully fixed. Additionally these results were also in agreement with those of Michael Grimaldi who discovered that mercury does not have an affect on F-actin in neurons. Other sources of error may be attributed to the complexity of the experiment. Many of the techniques employed in this investigation were used for the first time. For this reason there may be some minor inaccuracies in locating cells, or standardizing data.

If this experiment were to be repeated, additional time to image more cells would be beneficial. Increasing the concentration of mercury may also lead to different results. The effects of a test like this may prove beneficial in further neurological studies.

VI. Acknowledgements

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I have abided by the Wheaton College Honor code in this work.