The Effects of Methyl Mercury on the Rate of Endosomal Movement within Chick Embryonic Glial Cells

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Introduction:

Neuroglia cells, or glial cells, are non-neuronal cells that play a significant role in the proper functioning of neurons and the rest of the nervous system. In Greek, the term *glia* actually means "glue." This meaning is representative of the cell's general role of supporting and maintaining the nervous system balanced. Some of the roles of glial cells include maintaining an ionic environment for neurons, regulating the rate of nerve signal transmission, regulating synaptic actions by controlling the reuptake of neurotransmitters, providing a framework for some aspects of neural development, and aiding in recovery from neural injury (Purves et al., 2001). Glial cells have been differentiated into three types: astrocytes, oligodendrocytes, and microglial. While astrocytes, oligodendrocytes, and microglial cells are restricted to the central nervous system, another type of glial cell known as Schwann cells are restricted to the peripheral nervous system. These cells form myelin sheaths around the axons of motor and sensory neurons and therefore have effects on the speed of action potentials. The cells dissected from the primary chick culture all belonged to the peripheral nervous system.

According to Smythe and Ayscough (2006), endocytosis is defined as the process through which the plasma membrane invaginates into the cell and forms a vesicle that fuses with endosomes and eventually enters the endo-lysosomal membrane system. The process can be carried out via two different pathways—clathrin independent and clathrin-dependent endocytosis—and has major roles involving plasma membrane lipid recycling, protein trafficking, and the uptake or down regulation of cell-surface receptors (Smythe & Ayscough, 2006). Two topics of interest regarding endocytosis within the nervous system include endocytic recycling of myelin proteins and actin regulation in endocytosis. Endocytic sorting and recycling of myelin proteins PLP, MAG, and MOG aid in the remodeling of the plasma membrane during morphogenesis of myelin subdomains (Winterstein et al., 2008).

Significance of this aspect of endocytosis in terms of glial cell exposure to methyl mercury conditions lies in the importance of endocytic recycling for cell morphogenesis once the cell is exposed to MeHg. Given that methyl mercury is a lethal toxin, exposure to MeHg may result in mechanisms involving endocytic recycling and the next topic of discussion, actin regulation. In their analysis of actin regulation in endocytosis, Smythe and Ayscough relate the manner in which actin structures in the cytoskeleton are organized to the remodeling of the cell surface to allow inward movement of vesicles. Kinetic aspects of endocytosis linking the recruitment of actin to endocytosis were presented in 2003. According to Smythe and Ayscough, endocytic vesicles coat proteins are recruited to sites on the plasma membrane and deform the membrane, causing invagination (Smythe & Ayscough, 2006). Actin is then recruited to assist in vesicle formation and movement of the vesicle away from the membrane and towards the lysosome.

The following study tested the hypothesis: as doses of methyl mercury increase, the rate of endosomal movement within a glial cell will also increase. It analyzed the rate of endocytosis of glial cells in the peripheral nervous system of chick embryos after exposure to methyl mercury. By using glial cells from embryonic *Gallus gallus* as a model system, the study effectively modeled growing glial cells in humans. Because chickens are vertebrates, their development process is similar to that of humans. Studying *Gallus gallus* has provided much understanding into the human nervous system specifically, how cells migrate and differentiate and much understanding into the molecular basis of limb development (AnimalResearch). Once inorganic mercury is converted into organic mercury (MeHg), called methyl mercury, in water, the metal is known to be in its most prevalent and toxic form. Many symptoms of methyl mercury poisoning lie within the spectrum of neurological disorders and irregular limb development making chick embryos optimum model systems for the following experiment. Some of these symptoms include: mental retardation, primitive reflexes, coordination disturbance, dysarthria, limb deformation, growth disorder, chorea-athetose, and hypersalivation (Ni, 2011).

To test the effects of methyl mercury on the rate of endosomal movement within glial cells, I treated chick glial cells with a methyl mercury solution, labeled the endosomes using Fluorescein Dextran in growth medium, and measured the velocity of control and experimental “particles.” Once absorbed by the body through the lungs, skin or GI tract, methyl mercury accumulates in red blood cells and is slowly re-distributed to the other organ systems including the nervous system (Ni, 2011). Dysfunction of glial cells is associated to methyl mercury induced brain damage. Reasons to hypothesize that methyl mercury exposure would increase rates of endocytosis and endosomal movement toward the soma can be attributed to electron microscopy of human autopsy described in Ni’s dissertation. Results of the microscopy showed that rough endoplasmic reticula were lost, neurons were lost in

basal ganglia, ribosome aggregation, and segmental demyelination of dorsal fibers as a result of methyl mercury exposure. In addition, nerve cells were shrunken and the tubulin structure of growth cones was disintegrated after methyl mercury exposure (Ni, 2011). These forms of neuronal degradation are all associated with the process of endocytosis and endosomal movement within a cell. Though the results in Ni's dissertation describe those for nerve cells exposed to methyl mercury, inferences regarding demyelination, shortening of cells and disintegration of growth cones in nerve cells can be related to those in glial cells. Therefore, similar mechanisms may play a role in glial cell degeneration under the influence of methyl mercury.

Materials and Methods:

Cell Culture

The dissection protocol of *Gallus gallus* peripheral nerve cells can be found on the Wheaton College ICUC database (Morris, 2014a) A few modifications to the procedure include the utilization of DMEM, Dulbecco's Modified Eagle's Medium, instead of HBSS, Hanks Balanced Salt Solution, as the buffer solution used throughout the experiment and the elimination of the "flame-constriction of Pasteur pipettes" procedure mentioned as one of the preparation day steps. After experiencing sub-par axonal growth after the first week of experimentation, a couple additional steps were taken to amplify cell growth. To help the cells latch onto the dish faster and stronger, the environment of the cells was made stickier by increasing the poly-K treatment of the coverslips to 3 hours. The laminin treatment of the coverslips was increased to 2 hours to increase signaling of the cells. The concentrations of both the nerve growth factor and of glutamine were increased. The concentration of NGF was increased to 200ng/ml and the concentration of glutamine, Q, was increased to 4mM. In addition to increasing concentrations and treatment times, the shelf inside the incubator was vibrationally isolated, 60 ganglia instead of 40 ganglia were plated, and the cells were incubated a total of 38 instead of 24 hours in the hope of expedited cell growth.

Control Dish

A petri dish containing live cells on a cover slip and growth medium was removed from the incubator after 38 hours. All growth medium was removed and DMEM (Dulbecco's Modified Eagle's Medium) was added to the dish. The dish was then incubated for twenty minutes. Once the incubation time was complete, DMEM was withdrawn. A wash with DMEM followed.

Experimental Dish

A second petri dish containing live cells on a cover slip and growth medium was recovered from the incubator after 38 hours. All growth medium was removed and a DMEM plus methyl mercury solution was added to the dish. This solution had a concentration of 40 nM of methyl mercury in DMEM (Leong, 2001).This was considered a high dose of MeHg. The dish was then incubated for twenty minutes. The DMEM and methyl mercury solution was removed and a new DMEM plus MeHg solution was added to the dish and used to perform a wash.

Endosome Labeling

Endosome labeling occurred in both the control and the experimental dishes. After the washing steps, DMEM was removed from the control dish and DMEM plus MeHg was removed from the experimental dish. Once this removal was complete, the subsequent steps for endosome labeling were applied to both the control and experimental dishes. 2 mL of 10 μM Fluorescein Dextran in growth medium was divided between and applied to both petri dishes (Bi & Morris, 1997). The two dishes were incubated for thirty minutes. After the incubation, the Fluorescein Dextran solution was removed and DMEM was used to wash the cultures. A color assay was performed a total of four times until the color of DMEM matched the color of the growth medium instead of the yellow color attributed to Fluorescein Dextran in solution.

Observation Chamber

The observation chamber protocol can be found on the Wheaton College ICUC database (Morris, 2014b). Because an observation chamber was made instead of a flow chamber, the coverslip was sealed on all four sides with valap.

Fluorescent Microscopy

When a cell was located under transmitted light, the pin of the microscope was pulled so that the image would be sent to the computer screen. The restart button was clicked and the image was focused on the computer using the microscope's fine focus lens. Once the picture was optimized on the computer, a fluorescent picture was taken. The transmitted light was cut off and the fluorescence slider was set to position three. Settings on SPOT microscopy software were set so that the slides experienced a 4 sec manual fluorescence exposure time. The shutter was then opened and the image was taken and saved. All images were taken under 40X lens of a Nikon Eclipse E200 microscope, with the Insight Firewire- 2megasample SPOT camera and using SPOT, the fluorescent microscopy software. The images were then opened with Image J software in the ICUC and the velocities of control and experimental endosomal "spots" were recorded. Velocity is expressed by dividing displacement by time. The

displacements calculated using imagJ were divided by 10 seconds. Viewing individual endosomes within the glial cell was not possible due to visual limitations. Therefore, an endosome or small group of endosomes was represented as a “particle” and was defined as a small, circular, isolated particle within a glial cell region. The particles utilized are close representations of the fewest number of endosomes per “spot.” In fluorescent images, glial cell boundaries cannot be seen. Instead, one can see green blobs, which represent many endosomes in that one area of the glial cell. We identify this glial cell region in fluorescent images using glial cell boundaries visible in transmitted light images.

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Results:

As time progressed from 10 seconds to 20 seconds, the distance from one particle to the center of the glial cell decreased. The distance from one control particle exposed to the fluorescent light for 10 seconds to the edge of the glial cell region with the highest endosome concentration was 4.909 μm. After 20 seconds of exposure, the distance from the same control particle to the edge of the region with high endosome concentrations was 3.273 μm. The velocity of one control particle exposed to methyl mercury was 1.636 μm/10sec (Fig.1-b).

Both experimental velocities were averaged and resulted in an average experimental velocity of 0.157 μm/10sec (Figure 3).

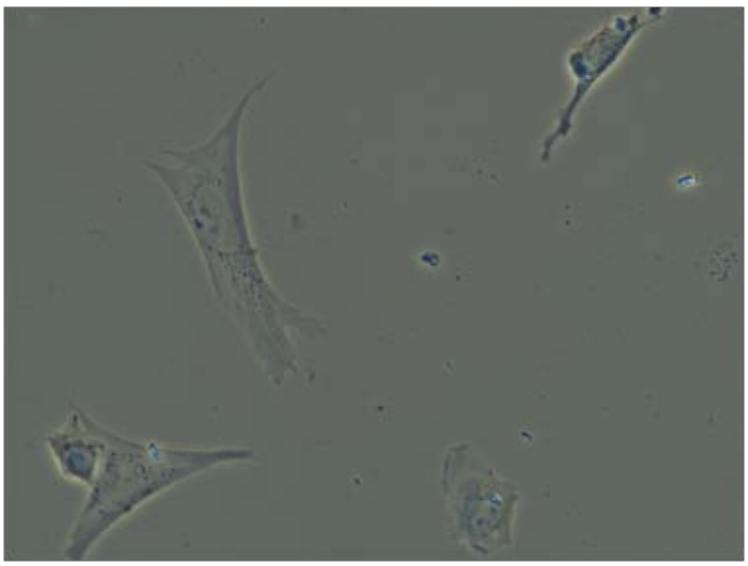

Figure 1-a. Control glial cells under transmitted light microscopy.

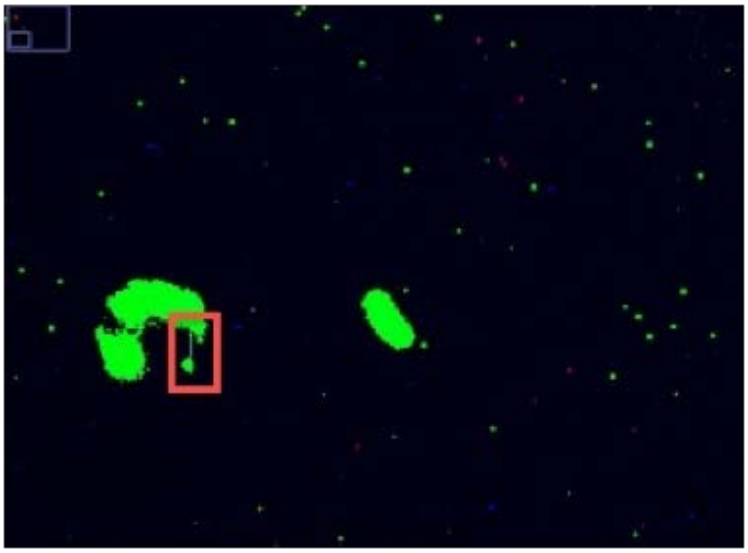

Figure 1-b. Fluorescent light microscopy of a control particle. The blue line in the red box represents the distance travelled in 20 seconds by the control particle. The line/distance travelled in this image is 1.636 μm shorter than the line travelled in the first image captured after 10 seconds of fluorescent light exposure.

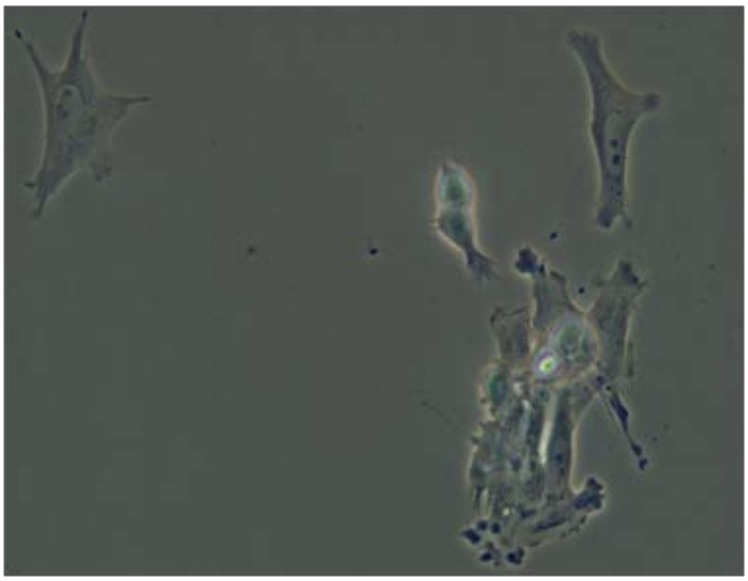

Figure 2-a. Methyl Mercury treated glial cells under transmitted light microscopy. Image collaboration with Megan Donahue.

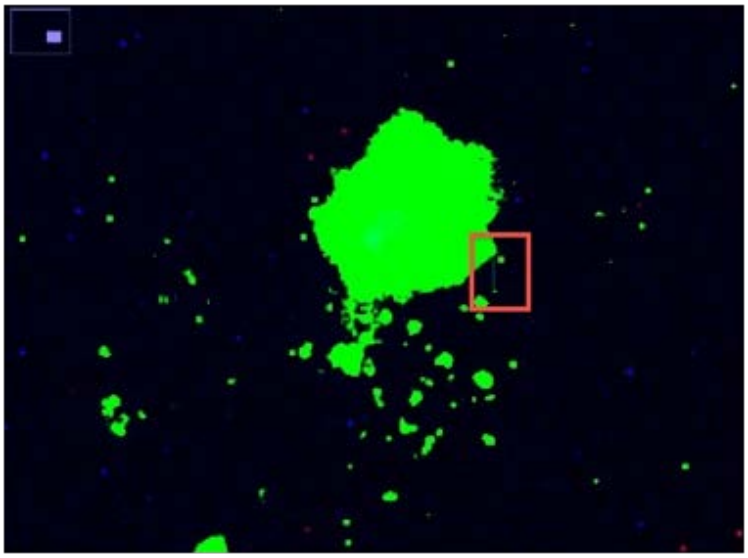

Figure 2-b. Fluorescent Microscopy of first experimental particle after 10 seconds of travelling. The blue line inside the red box represents the distance travelled in the first 10 seconds from first experimental particle to the edge of the glial cell region with the highest endosome concentration. All measurements were taken as if the particle was travelling in a straight-line forward. Image collaboration with Megan Donahue.

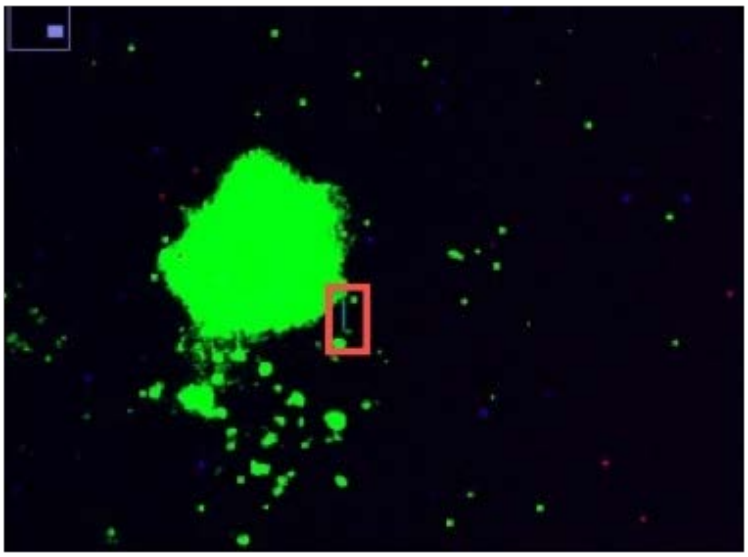

Figure 2-c. Fluorescent Microscopy of first experimental particle after 20 seconds of travelling. The blue line inside the red box illustrates the distance travelled in 20 seconds from the first experimental particle to the edge of the glial cell region with the highest concentration of endosomes. Image collaboration with Megan Donahue.

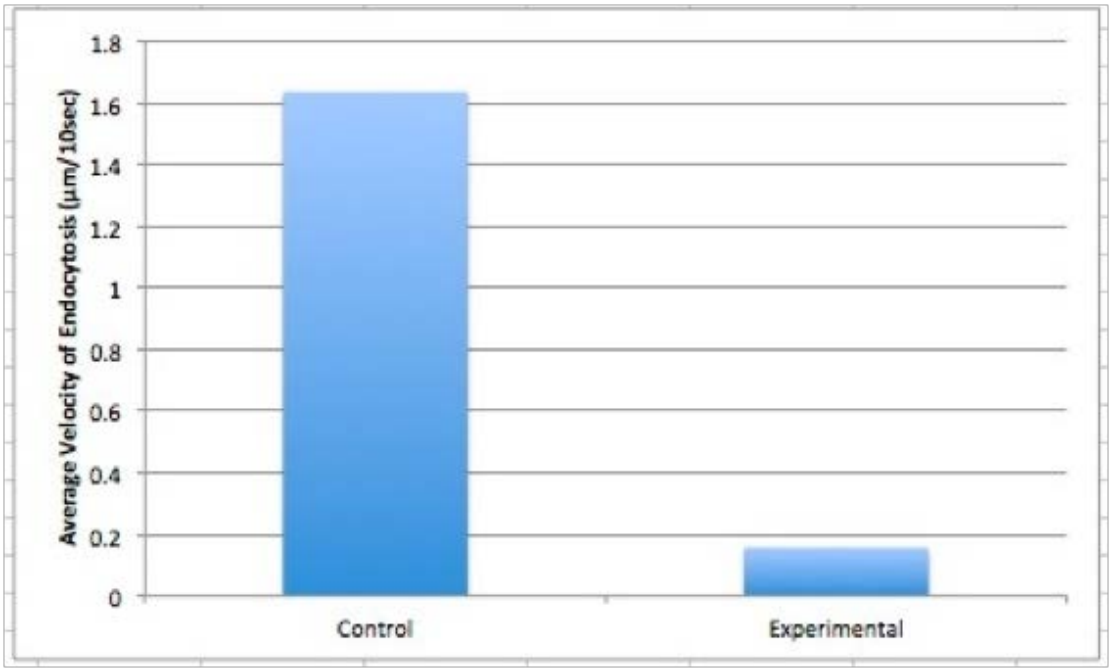

Figure3. Average velocity of control particles and average velocity of experimental particles. The control particles, which were not administered methyl mercury, experienced a much greater velocity than the experimental particles, which were administered methyl mercury experienced. The velocity of the control particle was about 12.5 times greater than the average velocity of the two experimental particles.

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Discussion:

Based on velocity results, the hypothesis stating that the rate of endosomal movement within a glial cell will increase as doses of methyl mercury increase was disproved. Refuting this hypothesis gives insight into the effects of methyl mercury on the rate of endosomal movement within a glial cell. Though methyl mercury was expected to expedite the rate of endosomal movement, the rate was significantly reduced. If the study had been repeated a thousand times and gotten the same result such that all experimental data was statistically significant, it could be concluded that methyl mercury exposure actually reduces the rate of endosomal activity within these glial cells. There are a couple possible explanations for such a result. One possible explanation can be attributed to actin structures in the cytoskeleton and their regulation in endocytosis. In their article, Smythe and Ayscough located actin filaments at sites of endocytosis at the synapse and described the effects of certain drugs on endocytosis in terms of the actin cytoskeleton. After studying certain drugs that inhibit endocytosis it has been confirmed that the exposure reduces the rate of internalization instead of blocking it completely (Smythe & Ayscough, 2006). Though the actin does not have a mandatory role in endocytosis it can be inferred that actin filaments in the cytoskeleton are sensitive to foreign substances such as drugs and possibly organic metals like methyl mercury. Results in figure

3 also coincide with the reduction of endocytosis rate rather than the blockage of endocytosis because plasma membrane invagination occurred throughout the experiment without subsiding completely. Another possible explanation for a decreased rate of endocytosis was described in a journal article about the effects of endocytic recycling of myelin proteins on membrane remodeling. In the article, authors relate variation in endocytosis rates to the relative composition of different maturation stages. When they tested oligodendrocytes cultured for more than five days, internalization of myelin proteins was virtually absent. It was then concluded that mature cells would undergo endocytosis and that terminally differentiated cells would exhibit reduced endocytic activity (Winterstein et al., 2008). Based on the results of my study and the results of Winterstein, Trotter, and Kramer-Albers, it can be confirmed that a developing nervous system is much more susceptible to methyl mercury than a mature nervous system is and could be one of the most viable reasons for my unpredicted results.

There were many aspects of this experiment from which sources of error could have derived. Though no sources of error became overbearingly apparent resulting in termination of the experiment, there was potential for error during cell culturing, incubation, and data collection and analysis. Images of the control data were taken before images of the experimental data were taken. A potential reason for low velocity values of experimental "particles" could be that the initial, fast occurring moments of endosomal movement occurred before I captured the experimental images. Following the endosomes until they reach the lysosomes and qualitatively recording their interaction could be an appropriate extension to this experiment. In addition, studying the effects, if any, of myosin regulation in endocytosis would also be a great contribution to the study. Because actin and myosin are utilized in association with each other during muscle contractions, it could be interesting to study the relationship between myosin and endocytic reuptake and then evaluate the endocytic process utilizing analysis from both the extended experiment and the experiment performed in Smythe and Ayscough's journal article.

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Images in collaboration with Dana MacDonald and Megan Donahue