Evidence That the Addition of Mercury Decreases Area and Slows Overall Rate of Growth of Lamellar Region in Developing Chick Embryonic Glial Cells

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Introduction

The growth of glial cells, the supporting cells of the central nervous system, is primarily guided by actin extensions of the cell known as filopodia and a corresponding actin gel matrix called the lamellipodia. These structures are present on the leading edge of what is termed the lamellar region of a glial cell, which is analogous to a neuron's growth cone. The growth process that developing glia (and neurons) undergo involves the extension of filopodia to read and transduce signals from the cell's surroundings. These structures are made of tightly bound actin bundles, which are an integral part of a cell's cytoskeleton. The filopodia are highly sensitive due to receptors located within the membrane; as a result, the filopodia react to any slight chemical changes in a cell's external environment and direct the cell's movement accordingly (Kandel et al., 2000). The lamellipodia, a highly motile structure, fills in between the filopodia, allowing the cell's microtubules to advance forward and create a new extension of the cell (Kandel, 2000). The maintenance and constant activity of microtubules and actin fibers is the mechanism by which glia move and continue to grow.

In this experiment, we will be studying the ways in which the structure and activity of the glial lamellar region is affected by the addition of mercury chloride. Mercury is an element that exists in a variety of forms that can be toxic with acute exposure to an organism. The element can be found in water due to deposits from the air. Once in the water, many microorganisms convert the element into methylmercury, which is an extremely toxic form of the substance that accumulates in aquatic animals (EPA, 2007). Humans receive most of their mercury exposure by consuming these water animals such as fish and crustaceans.

This can put a person at risk for mercury poisoning, particularly young children and infants, causing brutally impaired neurological development. On a molecular level, this includes disturbances in axonal transport and cytoskeletal growth (EPA, 2007). Leong et al. performed a study with cultured central ring ganglia of the snail *Lymnaea stagnalis*, exposing the cells to various metal ions including mercury. Their results showed significant disruption in the structure of the neurons as well as slowed growth rates of the neurites when mercury was added as opposed to other metal ion compounds (Leong et al., 2001). Similarly, Brittany Chick conducted an experiment in which she exposed peripheral embryonic chick neurons to a mercury chloride solution and observed any effects on

growth cone activity. Her data supports Leong's in that mercury-treated neurons displayed significantly slower extension and retraction rates and less overall filopodial activity than nonmercury-treated cells (Chick, 2006). These data suggest that mercury has a detrimental effect on the structure and morphology of neurons.

In this experiment, I tested whether or not similar results could be found by exposing glial cells to mercury instead of neuronal cells. Specifically, I decided to analyze and quantitate lamellipodia of glia, observing any change in area and rate of change due to the supplement of mercury chloride. I tested this phenomenon on primary culture chick sympathetic glia. I hypothesized that the addition of mercury chloride to glia would decrease size and slow overall growth rates of lamellar area in the cell.

My collaborators on this experiment were Sarah Karevicius and Chelsea Nardone. Chelsea's experiment directly relates to mine; she will be studying filopodial morphology of neurons with added mercury chloride. Sarah's experiment correlates with both of ours and bridges the gap between them; she will be observing and analyzing the effects of mercury exposure on neuron-glia interactions.

Methods and Materials

Primary Cell Culture (protocol by Hollenbeck et al., 1998)

To begin the experiment, we created a primary culture of chick embryonic peripheral neurons by dissection of 9-11 day old chick embryos. We used sterile technique by spraying hands, dull forceps and the apex of the egg with ethyl alcohol. A Petri dish was prepared with approximately 5mls of room temperature HBSS (Hanks Balanced Salt Solution). Using the dull forceps, we gently tapped through the eggshell about 1-2cms down from the apex and, using the forceps as scissors, snipped off and discarded the top of the shell. The embryo was gently removed from the egg by the neck, the head was pinched off and discarded, and the body was placed into the prepared Petri dish. Under the dissecting microscope, the body was placed ventral side up as we pinched off the legs and wings, and carefully removed the viscera, ensuring the spinal cord remained intact. The sympathetic nervous chain was removed by gently teasing the forceps along the length of the spine. In addition, the dorsal root ganglia (DRGs) were removed by gently scraping away their attachment to the spinal cord. HBSS was administered occasionally throughout this procedure using a pasteur pipette to clear away any accumulated debris. The collected DRGs and sympathetic nervous chains were placed into a 25mm Petri dish with HBSS.

We dissociated the ganglia by first rinsing them with HBSS. We then replaced HBSS with trypsin solution (CA/Mg-free HBSS containing 0.25% trypsin) and incubated the ganglia for 15-20 minutes at 37ºC. The trypsin solution was carefully removed and the ganglia were placed back in a small amount of HBSS (approximately 1

drop/ganglion). We triturated these cells using a pasteur pipette until they were completely dissociated into single cells.

We then prepared the laminin substrata on which the cells would grow. To do this, we coated the surface of a coverslip with 1mg/ml poly-K-lysine by placing one drop of the solution on the bottom of a 110mm Petri dish, then positioning the coverslip overtop of the drop and letting it sit for 1-24 hours. The coverslip was rinsed once with sterile water and allowed to fully dry again. The same technique was used to coat the coverslip a second time with laminin in HBSS. This was allowed the sit for 1-2 hours. Making sure to keep the coverslip wet at all times, we rinsed the laminin coverslip with HBSS and placed the entire coverslip, poly-K/laminin side up, into a 25mm Petri dish containing growth medium.

To plate out the cells, one drop of the dissociated cells solution was placed on the poly-K/laminin side of the coverslip. The cells were left to grow in the incubator at 37ºC for 19-25 hours.

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Imaging

To prepare the cultured cells to be viewed under the microscope, we made chip chamber slides. This was done by shattering a dry coverslip wrapped in a Kimwipe, and placing a number of the pieces on a clean, dry slide. The pieces were arranged in the outline of an intact coverslip square, so as to support the laminin-coated coverslip. Using a pasteur pipette, one drop of the growth medium from the cultured cells was placed in the center of the chip chambers. Then, using sharp forceps, we carefully removed the coverslip from the 25mm Petri dish, keeping track of which side the cells were on. A Kimwipe was used to dry the non-laminin side of the coverslip, and the cell side was slowly lowered down onto the chip chamber so as to reduce the formation of any air bubbles. The edges of the coverslip were sealed with VALAP, and the top of the slide was rinsed with water to remove any formed salt or debris. The slide was then ready for viewing. This procedure deviated slightly when creating a control or experimental slide. After obtaining the cultured cells, we removed the growth medium and replaced it with approximately 10ml of 0.5% HCl in HBSS or 10ml of 10nM $HgCl_2$ in HBSS, respectively. This was left to sit for 20 minutes at 37ºC. The mercury solution was removed from the coverslip, which was rinsed with HBSS and placed on the chip chamber as described above.

Using the Nikon Eclipse E200 microscope with an attached Spot Insight QE camera, we imaged the cells using phase microscopy and the Spot program on the Gemini computer in the ICUC. The chip chamber slide containing either the control or experimental solution was placed under the microscope. A glia cell was located and the image was optimized at 40X magnification. We then increased the magnification to the 100x oil objective. A heater was placed approximately 2 feet from the microscope stage, which maintained a heat of 37-42ºC on the cells during imaging. Three

image sequence trials were performed: one trial analyzed a selected glial cell from the control coverslip and two trials analyzed glial cells from the experimental coverslip. A total of 3 cells were imaged. Each imaging sequence was taken for one hour, with a photo being snapped every 15 seconds. This allowed for a total of 240 images for each of the three trials.

Quantification of Lamellar Area

To quantitate the collected data, every 20^{th} image, beginning with Image 20, was extracted from the control image sequence and one experimental image sequence for one hour. This provided a total of 24 images to be analyzed. Each image was formatted into an 8-bit image using the Image J program. Contrast and sharpness were also increased to improve measurement accuracy. The lamellar area was then measured using the polygon tool in Image J.

The lamellar area was defined as the area on the leading edge of a glia cell that is devoid of any organelles and excludes filopodia. The filopodia were defined as long, thin extensions ending when the sides of the base of the shaft are no longer parallel to one another, approximately doubling the diameter of the extended filopodium shaft. On the outer edge of the cell, the lamellar boundary was defined as often jagged edges that displayed an area of sudden and high contrast between the lamellipodium and the gray background of the coverslip. The boundary between the organelles and the lamellipodium was defined at the vertices of stress fibers, which were characterized by dark, straight lines extending from the center of the cell just outside the nuclear envelope. Also, mitochondria and cell nuclei were often beneficial markers of the intercellular boundary.

Since the lamellar area was calculated in pixels, we had to convert that measurement to square-micrometers. This was done by taking a photo of a stage micrometer with Spot (at 100x magnification) and transferring the picture to Image J. Using the length tool, we measured the segment in pixels, knowing its length to be 10 micrometers across. We measured this stage micrometer length 3 times, and calculated the average length to get as accurate a conversion as possible, which was 109 pixels in 10 micrometers. This equaled 0.09 micrometers/pixel, a length which had to be squared to find the area per pixel. The final conversion came out to be 0.0081 square-micrometers/pixel. Once we had this conversion, we could multiply it by the total number of pixels as calculated by Image J in each lamellar region measured.

We also calculated the rate of change of lamellar area over time in the control and experimental cells. To do this, we implemented the formula $|T_i-T_f|/300$, where T_f is the final time from one interval and T_i is the initial time from the previous interval. This represents the absolute value of the difference in area at some initial time to the area at the following final time, divided by the 300 seconds, which separates those two measurements in the imaging sequence.

Results

In collecting data for both the control and experimental trials, we observed that the experimental glial cells displayed very scalloped, jagged leading edges. The average lamellar area of the control glia was 517 square-micrometers, while the average lamellar area of the experimental glia was 333 square-micrometers. Total lamellar area, as measured in square-micrometers, is shown in Figure 1.

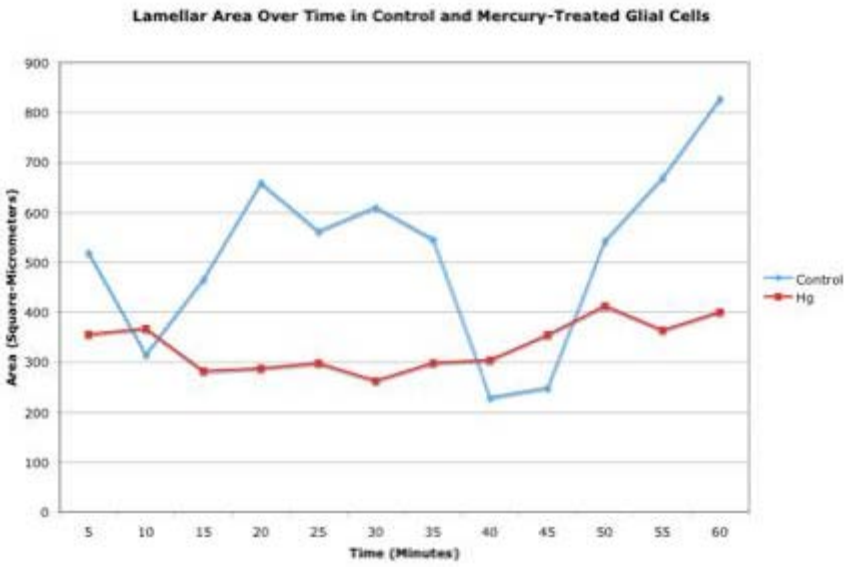


Figure 1. Lamellar Area Over Time in Control and Mercury-Treated Glial Cells. Note the difference in minimum and maximum values between control and experimental groups, as well as consistency in values of experimental group vs. control.

We also observed the presence of more distinguishable stress fibers in the experimental groups versus the control group. These stress fibers are vague in the “edge-detected” formatted images (Figures 2 and 4), but are apparent in the original photos before they were optimized for analysis (Figures 3 and 5).

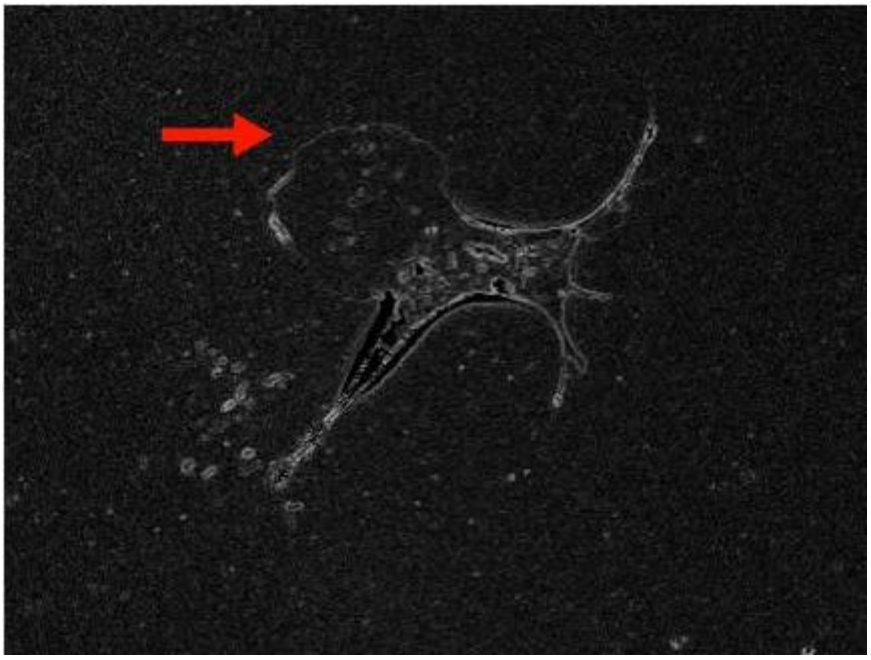
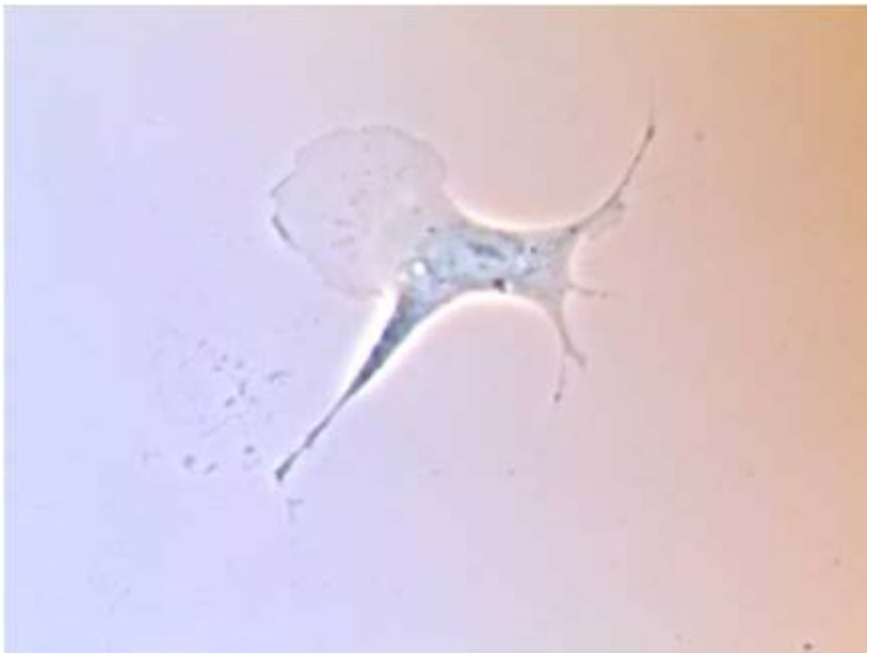


Figure 2. (above) Edge-Detected Control Glial Cell at 20 Minutes. Notice the smooth, uninterrupted edges of the lamellar region's leading edge

Figure 3. (below) Original Control Glial Cell at 20 Minutes. Notice the lack of distinguishable stress fibers in the lamellar area.



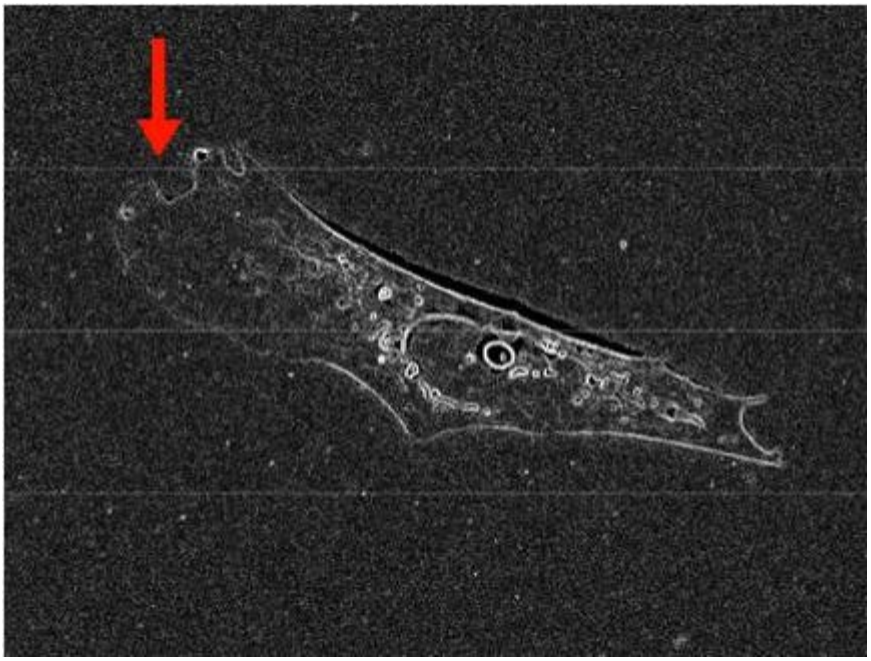


Figure 4. (above) Edge-Detected Mercury-Treated Glial Cell at 15 Minutes. Notice the jagged, scalloped edges of the lamellar region's leading edge.
Figure 5. (below) Original Mercury-Treated Glial Cell at 15 Minutes. Notice the distinguishable stress fibers in the lamellar region.

From the data in Figure 1, we can create a second figure displaying the rate of change of lamellar area in the two groups. This data is shown in Figure 6.

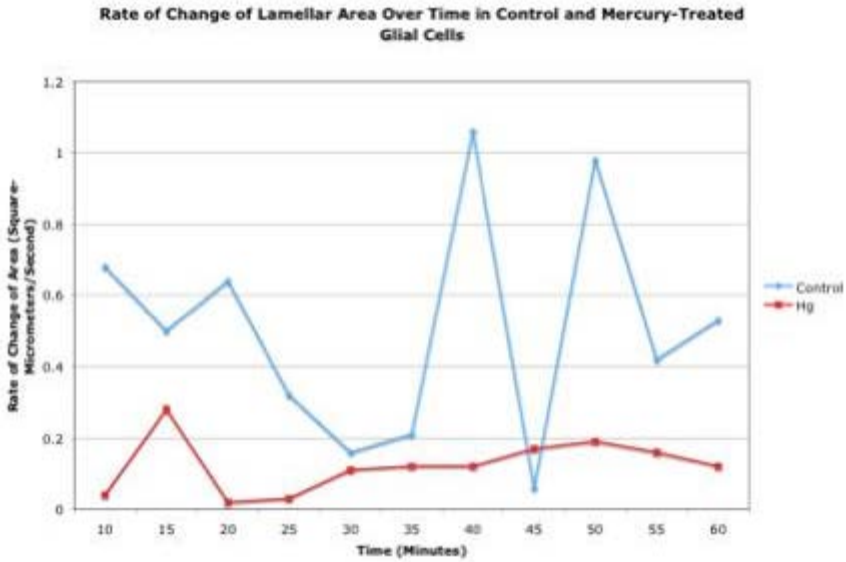


Figure 6. Rate of Change of Lamellar Area Over Time in Control and Mercury-Treated Glial Cells. Note the overall higher and larger variability of values in the control group versus the experimental group

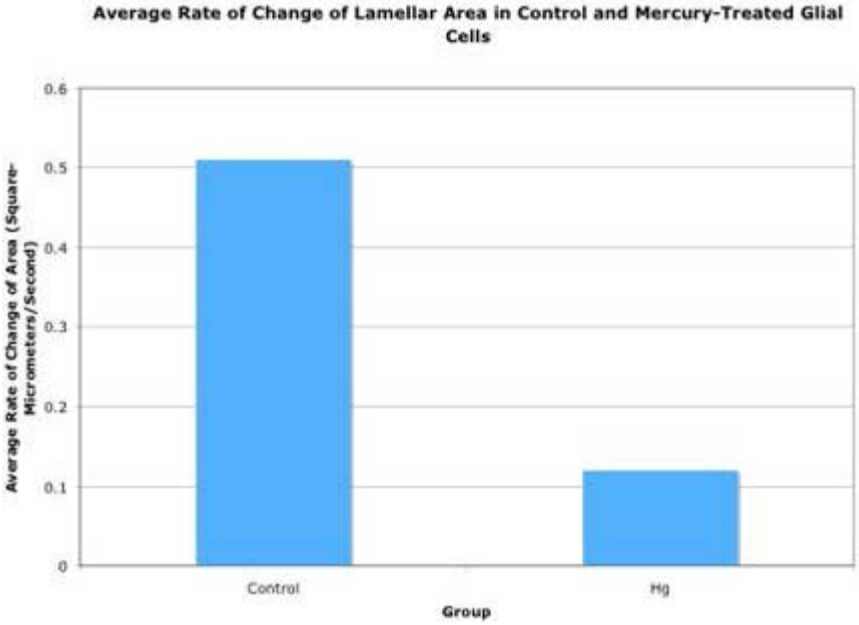


Figure 7. Average Rate of Change of Lamellar Area in Control and Mercury-Treated Glial Cells. Note that the rate of the control group is more than three times the rate of the experimental group.

Additionally, if we were to plot a line-of-best-fit for each of the graphs in Figure 6, we would see that the average rate of change for the control group is significantly higher than the average rate of change for the experimental

group. These data are illustrated in the bar graph of Figure 7.

Discussion and Conclusions

Collection and analysis of this preliminary data supports our hypothesis that the addition of mercury to developing glial cells in the nervous system decreases size and overall rate of growth of the lamellar area of chick sympathetic glial cells. Observed scalloped edges and changed membrane dynamics along the leading edge of the lamellar region in the mercury-treated glial cells suggest disrupted morphology and a disturbed actin gel matrix, creating growth patterns that are inconsistent from the control cells to the mercury-treated cells.

The conclusion that mercury decreases lamellar area is also supported by our data in Table 1. The average lamellar area of the control group is greater than the average lamellar area among the experimental group. The data suggest that the addition of mercury interfered with nutrient absorption by the cell, which would normally promote healthy growth and extension of the lamellipodia (Kovacs, 2006).

In addition, the difference in lamellar region area, as illustrated in Figure 1, was much more varied in the control group than in the experimental group, suggesting that the mercury may impede the busy extension and retraction activity that characterizes a typically functioning glial cell. In Figure 6, the higher maximums and lower minimums of the control group over the same time period as the experimental group suggest a much higher rate of activity and movement without the presence of mercury; the larger variability of values in the control group versus the experimental group also supports this idea. These data demonstrate that the lamellar region may be disrupted by the addition of the mercury, affecting both structure and movement.

Had this experiment been carried out across many more trials, with the same end results, we could run a statistical analysis to determine if deviations in the collected data were significant, providing even stronger evidence that mercury has a negative effect on glial lamellar region function. Biologically, this would further support the idea that the nutrients required to fuel healthy extension, retraction and formation of the lamellipodia are not reaching the cell due to the concentration of mercury present. Or, as Leong et al. suggest, microtubule polymerization is being hindered due to the inhibition of GTP binding caused by the presence of mercury (Leong et al, 2001)

Several sources of error were present in my experiment. In making the cell culture for the experimental trial, a longer than usual laminin treatment was used on the coverslips (25 hours as opposed to ~19). A longer exposure to laminin may cause the cells to grow multiple and longer filopodia and more expansive lamellipodia once placed on the coverslip (Morris, 2008). Had the laminin treatment been the usual 19 hours, we may have observed even less lamellar area among the experimental group, translating into an even greater negative effect caused by the mercury.

Another potential cause of error concerns my control solution. In order to create a completely accurate control trial, the cells should have been placed in the chip chamber with one drop of 10nM HCl in HBSS, since the experimental group used 10nM $HgCl_2$ in HBSS. However, in my trial, the control cells were placed on the coverslip with one drop of growth medium. This difference could have allowed the control cells to grow more rapidly with the provided nutrient-rich solution. (Although data was collected using the correct control solution, the trial was not as valuable for accurate quantification and so was discarded.)

In performing this experiment again, I would correct both the above-mentioned errors by better controlling the length of the laminin treatment as well as using the correct control solution for precise comparison of variables. Furthermore, I would perform more trials, as many as 10 each for control and experimental, to assure that my data is consistent. This would ensure that my results were not caused by errors in the experiment. Further experimentation might test varying concentrations of mercury on the area and activity of glia lamellipodia. By increasing the added dosage, we could see the effects, if any, of minor versus major mercury exposure, or if it is simply an all-or-nothing threshold effect. As a result, we might gain a better understanding of the varying degrees of mercury poisoning and what that means for cellular health.

References

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[Discussion with Sarah Karevicius and Chelsea Nardone, collaborators, 2008.]

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