Observing the effects of toxin mercury on lamellipodia and filopodia in relation to neuron-glia interactions

Sarah Karevicius
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I. Introduction

Neuronal plasticity, or neuroplasticity, is the ability of neurons within the central nervous system to modify the way in which they react to stimuli, which can thereby create functional changes to an organism (Kandle, Schwarts, and Jessell, 2000). In other terms, the plastic behaviors of neurons within the brain and spinal cord are what allow humans to learn, adapt and grow, as well as repair itself after injury. (Pluchino& Martino, 2007).

Basic physiological processes of neurons that allow them to be considered plastic involve interactions between neurons at their synapses (Will et al, 2007). However neurons are not the only cells involved with synaptic transmission. Glia cells that are immediately associated with neurons at their synapses are able to regulate synaptic connections (Burnstock& De Ryck, 2008) which results in playing a major role in brain function as neuronal signaling is the basis.

In growing and developing neurons, axons extend outward with protuberances called growth cones. The growth cones consist of three regions including a central region, primarily composed of mitochondria and microtubules. Extending from this region are slight extensions of cytoskeletal actin fibers called filopodia while another type of actin filled region called lamellipodia exists between filopodia augmentations, forming a web like appearance (Kandel, Schwarts, and Jessell, 2000). As the filopodia reach outward through the extracellular environment, the matrix consisting of lamellipodia soon fills in after it, creating a cyclic phenomenon which allows the axon of the neuron to grow and reach its target destination (R Morris, public presentation 2.5.08). Therefore, growth of neurons and glia cells includes both extension and retraction. Specifically, the membranes of the filopodia hold receptors which react to certain extra cellular cues, thus guiding the axon to grow by navigation (Kandel, Schwarts and Jessell, 200).

Likewise growing glia cells act in the same manner and have growth cones consisting of the same actin fibers filopodia and lamellipodia that function in the same manner as they do in neurons; retracting and extending outwards while navigating towards a target by means of membrane receptors. As the glia's main function is to assist neurons in signaling as well as to provide them with protection and nourishment, the target for glia cells are neurons (Kandle,

Schwarts, and Jessell, 2000). The connections and networks made by neurons with their associating glia cells are the basic units which provide the central nervous system its ability to control both conscious and subconscious functions (Kandel, Schwarts, and Jessell, 2000).

Current and past research observing interactions between glia and neurons specifically have noted that illnesses such as epilepsy, schizophrenia and other infections as well central nervous system dysfunctions can result when glia aren't capable or permitted to carry out their function (Burnstock& De Ryck, 2008). Furthermore, current research in the fields of neuroscience and psychology indicate that on top of possible genetic and other environmental factors, mercury exposure—in amounts unknown—may lead to autism spectrum disorders specifically in developing children (Williams et al, 2007).

Mercury (Hg) is a toxic substance that can cause such complications such as coughing, chest pains, further impairment of pulmonary functions, dyspnea or shortness of breath, hemoptysis or coughing up blood, and has even shown possible interstitial pneumonitis when exposed to an individual in acute amounts (ADSTR, 2007). However, what is not easily known or understood is how long term mercury exposure affects individual cells with in the human body, specifically the filopodia and lamellipodia of neuron and glia. Since these two types of actin fibers are the primary reasons that neuron and glia cells are able to grow, branch and develop synapses, thereby creating networks which provide the basis for the most basic forms of behavior (Kandel, Schwarts, and Jessell, 2000) it could be beneficial to observe how they function in the presence of mercury.

In this study, filopida and lamellipodia of interacting neuron and glia cells were observed in environments both with and without mercury to test the hypothesis that mercury negatively affects their growth rates. My collaborators for this experiment were Becky Halmo who studied the effects of mercury strictly on the lamellipodia of glial cells, while Chelsea Nardone studied the effects of mercury on neuronal filopodia.

II. Materials and Methods

All procedures for this experiment followed that of handouts and lectures provided by Dr. Robert L. Morris, Wheaton College. Mercury solutions were provided by Dr. Janni Bennoit, Wheaton College.

Culture of Chick Embryonic Peripheral Neurons

Nine to ten day old chick embryos were dissected to culture peripheral neurons as well as dorsal root ganglia. To ensure that any collected ganglia or peripheral neurons survived all materials were autoclaved and/or sterilized. The chick embryos were removed from their shells and placed in 110mm Petri dishes along with approximately 5ml of warm Hanks Balanced Salt Solution (HBSS). Under a dissection microscope the head, wings, lower limbs, viscera,

sternum, ribs and any other redundant tissues were removed from the body using blunt and sharp forceps to expose the developing spinal cord. Using sharp forceps, dorsal root ganglia and/or peripheral neurons were carefully separated from the body and placed in separate 25mm Petri dishes with HBSS.

Dissociation of Cultured Ganglia

Collected ganglia were washed with HBSS. Following the second wash, the ganglia were placed in a trypsin solution (Ca/Mg free HBSS containing 0.25% trypsin) and incubated for 15-20 minutes at relatively 37^{o}C. The trypsin solution was removed carefully from ganglia subsequent to incubation, and placed in HBSS with a volume of approximately 1 drop per ganglion. Using a flame drawn pipette, the ganglia were then triturated cautiously until they were dissociated into single cells.

Preparation of Substrata and Plating Cells

Cover slips were first treated with a 1mg/ml poly-L-lisine solution by placing them on top of small poly-L-lisine drops on the inside lid of a Petri dish, for 20 minutes. The cover slips were then rinsed with sterile water and allowed to dry for approximately 20 minutes. After the cover slips had dried, they were coated with a solution of laminin and HBSS for about 20-30 minutes before one final rinsing with HBSS. These treated cover slips were positioned laminin side up on the bottom of a 110mm Petri dish containing a growth medium (Leibovitz L15 medium, l0% fetal calf serum, 0.6% glucose, 2mM L-glutamine, 100ug/ml streptomycin, 100U/ml penicillin, and 10-50ng/ml nerve growth factor (NGF)). Following dissociation, cells were placed in the growth medium and permitted to settle and grow onto the treated cover slips for at least one day in an incubator set to maintain temperature at relatively 37^{o}C.

Preparing Cover Slips for Observation

To prepare for observations of controlled cell growth, cover slips were removed from the growth medium and placed into a Petri dish containing a solution of HBSS and 0.04mL of HCl solution for 20 minutes. They were then rinsed with HBSS before being placed cell-side down onto a chip chamber with a drop of HBSS and sealed with VALAP that had been heated to about 40^{o}C. The same procedure was used for experimental observations, only cover slips were placed in a Petri dish containing a mercury solution of HBSS and .04mL HgCl for 20 minutes following their removal from the C medium, before being placed on a chip chamber and sealed with VALAP.

Collecting Data for Observation

Prepared slides were placed under microscopes at 40x initially to locate interactions between neuron and glia cells. Interactions were defined as neuron(s) and glia(l) cells whose lamellipodia and/or filopodia appeared to be in contact or shared with both types of cells or in a shared area. Once interactions were located, magnification was increased to 100x. Control images were collected simultaneously with 15 seconds between each exposure for a period of 10 minutes with a program called SPOT. Due to laboratory constraints, mercury treated slide images were captured using a program called BTV Pro in which time lapse movies were taken. However, frames were extracted from these movies with 15 seconds between each exposure, also for a 10 minute period.

Analyzing Data Collected

First, images that represented data in one minute increments were edited to allow for easier quantification. Using a program called Image J, these selected images were converted to 8-bit images and then enhanced in contrast so that the edges and area of the lamellipodia and filopodia could be clearly identified.

Interactions between neuron and glia cells for both controlled and experimental data were quantified by analyzing both the lamellipodia and filopodia. Lamellar area was defined as the area of a glia cell or neuron that is devoid of any organelles and filopodia were defined as the long, thin extensions where the sides of the shaft are parallel to one another as (Halmo, 2008 & Nardone, 2008) as shown in Figures 1 & 2.

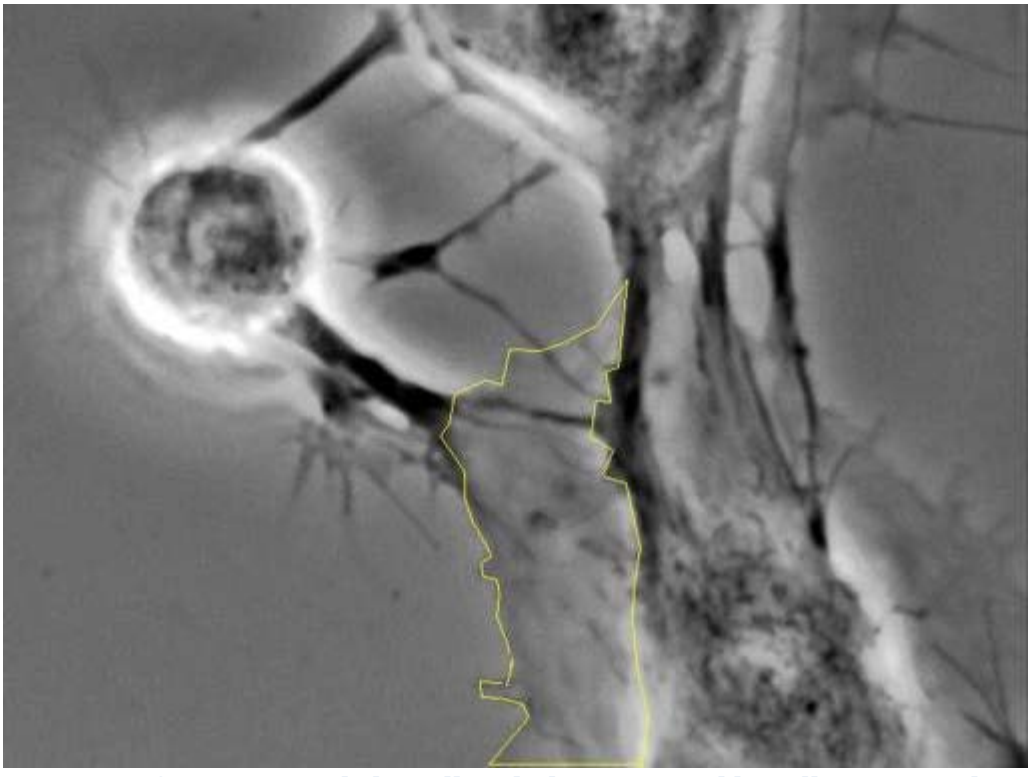

Figure 1 picture of a neuron and glia cell with the measured lamellar area outlined in yellow

A selection tool was used to measure the lamellar area that was in contact with both the neuron and glia cells within each image. All data was originally recorded as pixel measurements and then converted to micrometers using a stage micrometer as a means of conversion. Separately, the observed number of filopodia associated with the measured lamellar regions were quantified in number within each image as well, for both control and experimental data.

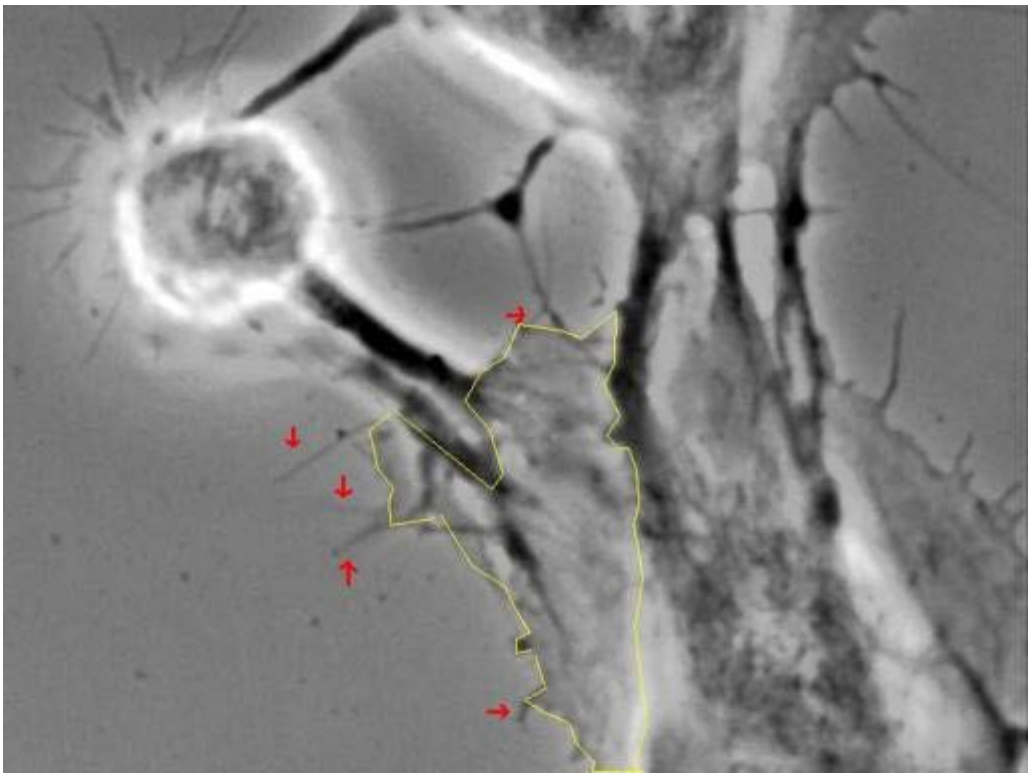

Figure 2- picuture of a neuron and glial cell with the quantified filopodia indicated with red arrows

III. Results

All results observed are that of interactions between one neuron and one glia cell. If images included multiple glia or multiple neurons, only one interaction was monitored and measured for data analysis.

General observations on the effects of Mercury on cell appearance

Initial observations made from images taken of neuron-glia interactions treated with HBSS and 0.04mL HCl solution showed that growth was steady and constant, with a calculated area oscillating within 100-200um^2 between each image measured. Due to the relatively small amounts of change between each exposure, images collected of this data remained relatively unchanged as Figure 3. Also the number of associated filopodia remained constant between images but were dynamic in nature; changing in length and position throughout the collected images but never in

quantity.

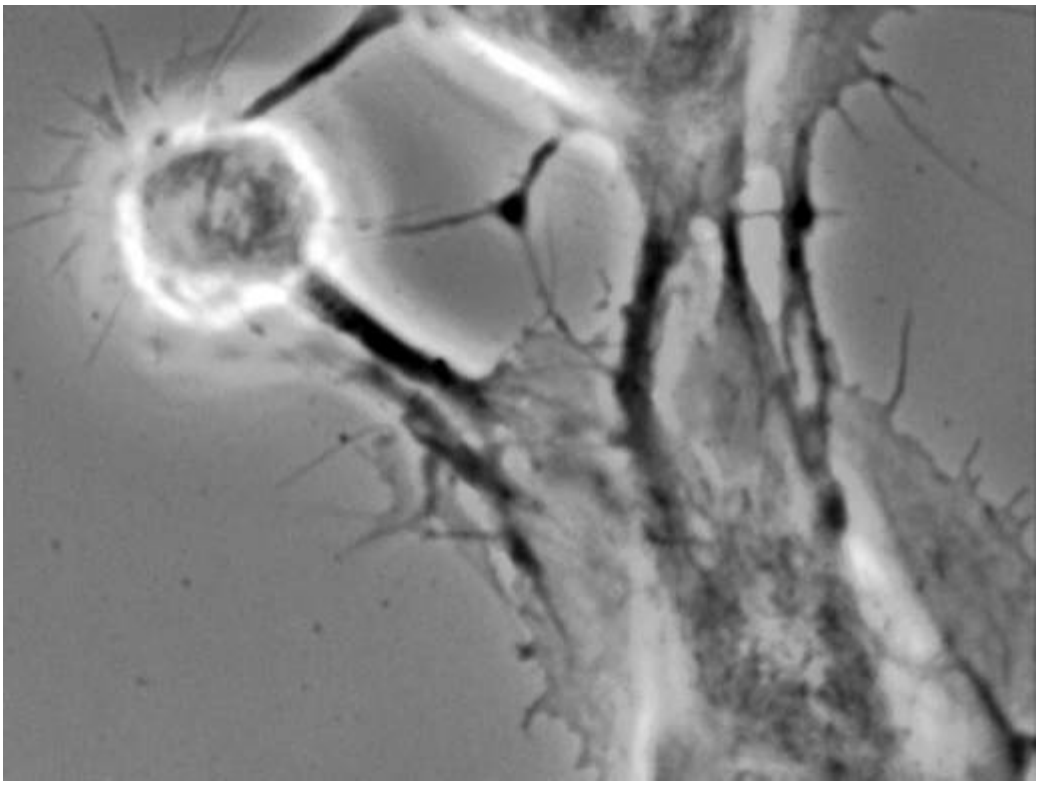

Figure 3 neuron-glia interation in the absence of mercury

Observations between images of neuron-glia interactions subjected to the HBSS and 0.04mL HgCl mercury solution showed steady decrease of lamellar area and ultimately interaction with a calculated initial lamellar area of 354.4um^2 and a final measurement of 176.6um^2 as seen between the images in Figure 4, representing the initial and final images. The presence of filopodia in these images is also relatively minimal, as none can be observed in either of the images of Figure 4.

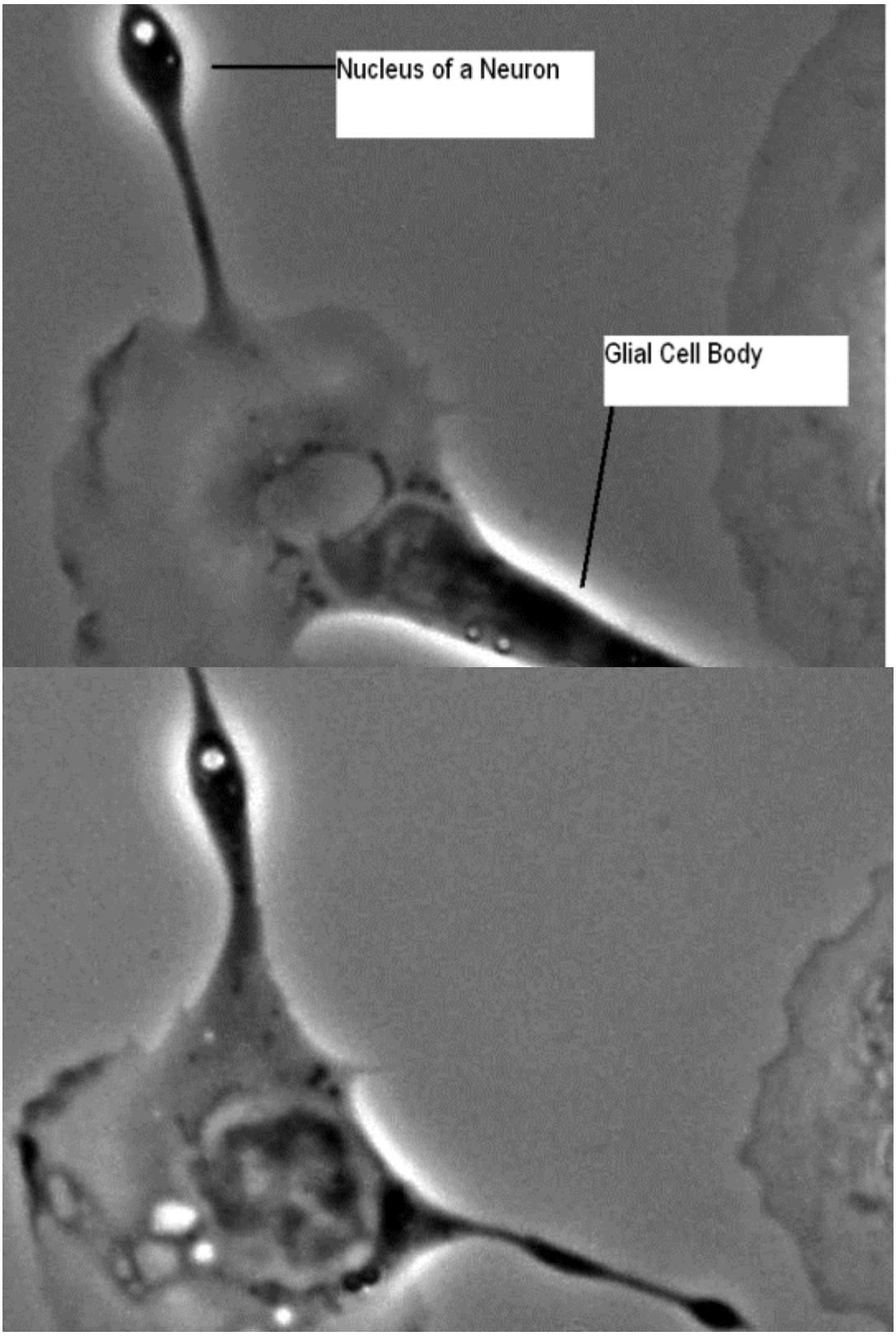

Figure 4 images of neuron-glia interaction in presence of mercury

Data showing effects of mercury on growth rates

Measurements taken on the images collected of neuron-glia interactions subjected to the 0.04mL HCl solution, in the absence of mercury, showed consistent fluctuations of connecting lamellipodia area with measurements mostly

staying in the range of 300-600um^2 as shown in Figure 3.

Data also showed overall trends towards positive growth in absence of mercury, as 1.99um^2 per minute on average as well as exhibiting overall larger area with an average of 500.9um$^{2.}$ The number of filapodia in interacting lamellar areas in the absence of mercury were also consistent, existing in an average ration of .013 filopodia per um^2 of lamellar area as seen in Figure 6.

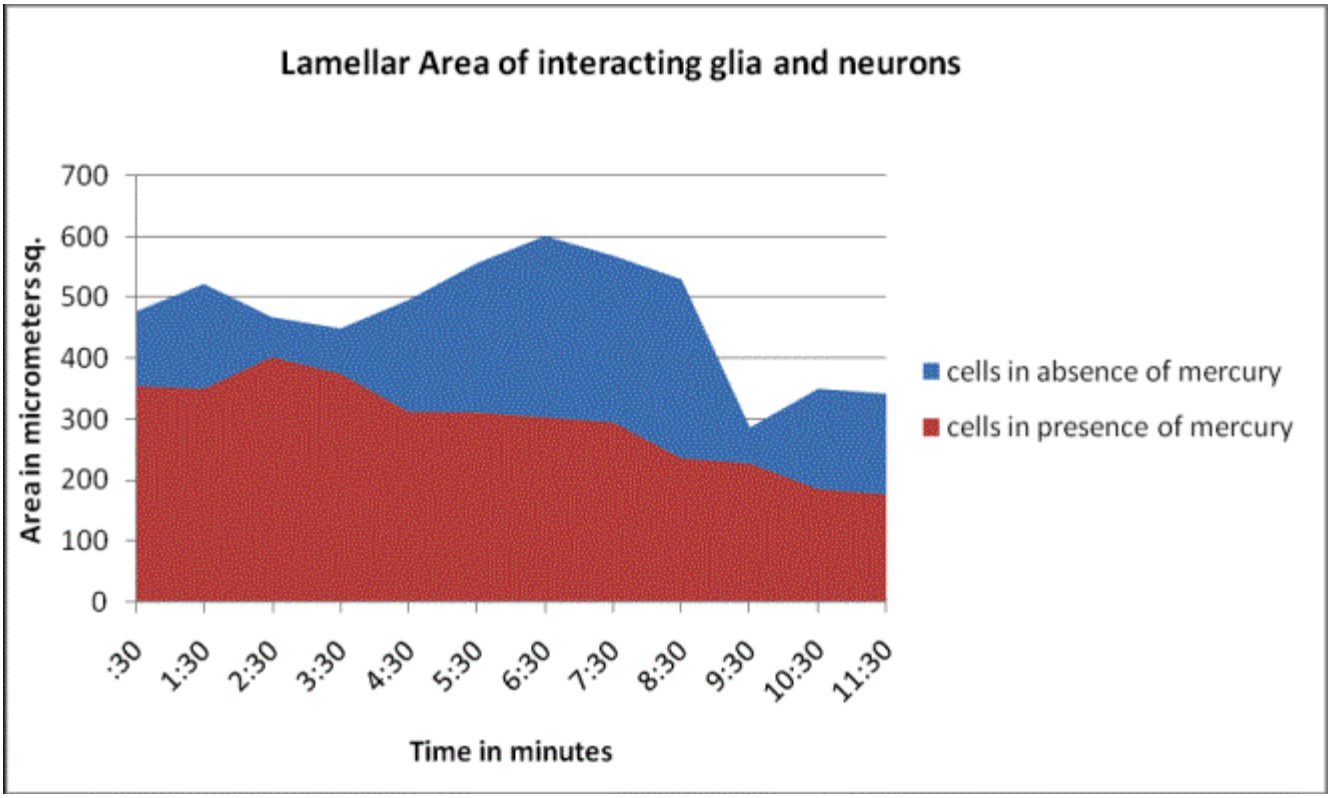

Figure 5 Graph showing data collected on lamellar area of interactions between 1 neuron and 1 glia in absence and presence of mercury

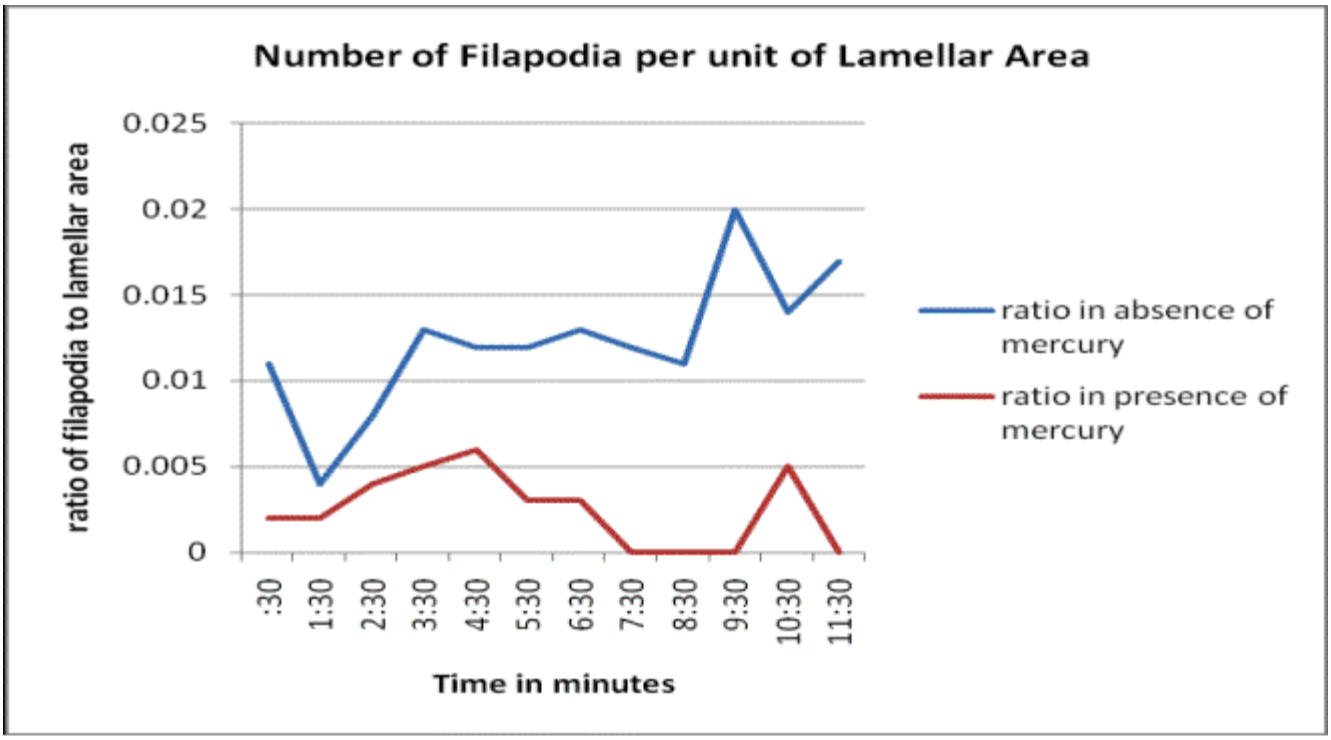

Figure 6 graph showing data collected on filopodia counts of neuron-glia interactions in the absence and presence of mercury

Data collected on neuron-glia interactions exposed to the 0.04mL HgCl mercury solution showed steady decrease in lamellar area from highest measurement of 401.4um^2 at 2:30 minutes, to the lowest measurement of 176.6um^2 (Fig. 3). Growth rate of these cells also was much slower, exhibiting changes of only 0.85um^2 on average between each image as well as totaling a smaller average area of 302.1um^2. Interactions of neurons and glia under the influence of mercury also showed little to no presence of filapodia projections, with an average ratio of .002 filopodia per um^2 of associated lamellar area (Fig 4).

IV. Discussions & Conclusions

Based on the results of the experiments, it appears that mercury does disrupt the mechanisms necessary to produce interactions between neuron and glia cells from occurring as hypothesized. Comparisons between controlled and experimental data suggest that cell growth, accounting for both extension and retraction of lamellipodial and filopodial actin between cells, is more normal with cells in the absence of mercury. As figure 5 shows, the lamellar area connecting a single neuron and glia cell fluctuated suggesting both retraction and extension were taking place. Similarly, the number of filopodia seemed to fluctuate in relation to the fluctuations of lamellar area measured, as shown in figure 6. These data suggest that the individual actions of the lamellipodial and filopodial actin fibers were

typical, and capable of functioning at a normal level.

However in the presence of mercury the lamellipodial and filopodial actin fibers did not behave typically. As figure 5 shows, the interconnecting lamellar area between a single neuron and glia decreases, suggesting that retraction is the only capable action of the lamellipodial actin fibers or that perhaps this region is destroyed. Relatively, there was little to no presence of filopodia in these regions as ratios show in figure 6. These data can suggest two things. Firstly, filopodial actin can neither exist nor function properly if lamellipodia cannot do the same. It could also suggest that even if the two types actin fibers are affected by mercury separately, mercury still greatly inhibits their activity and function which is an important aspect for all developing neurons and glia (R Morris, public presentation 2.5.08), in particular, their ability to form associations with neighboring cells.

The implications of these results may enhance the findings of other research going on in the field of neuroscience. Several researchers have explained the importance of glia and their interactions with associated neurons, specifically how they help to regulate synaptic transmission (Burnstock& De Ryck, 2008). In a series of complicated mechanisms, glia cells are capable of regulating levels of various neurotransmitters in the synaptic cleft, such as glutamate, GABA, norepinephrine, dopamine, serotonin, and acetylcholine (De Keyser et al, 2007). As lamellipodia and filopodia fibers are partly responsible for growth of these glia and neurons, which would ultimately create connections and associations between the two, it can be suggested that their inhibition by mercury is problematic and could result in the formation of brain dysfunctions in vision, sensory impairment, hearing loss and mental deterioration (De Keyser et al, 2007).

Had these results been the only of its kind after multiple repetitions of the same experiment, it could be suggested that perhaps mercury's effect on neuron-glia interactions is minimal and ultimately indifferent. However, had these experiments been repeated multiple times, all yielding the same results as described above, it could be inferred that mercury does in fact greatly and negatively affects the growth of lamellipodia and filopodia of interacting neuron and glia cells. Results of this sort would continue to suggest that the toxin mercury could be a reason for many neurological and psychological illnesses and disorders, specifically with the way glia cells maintain neuronal synapses (Yin et al, 2006).

Of course it is also possible that non-related factors could have influenced the observed results. In order for neurons and glia to grow properly, they need to be in a suitable environment including a temperature surrounding 37C. If at any moment during the dissection, incubation or observation the temperature varied, growth rates would have been easily affected. To avoid this, heating fans were used to maintain constant temperature once cells were placed on slides but it is possible that such efforts were ineffective.

If given the opportunity to test the same hypothesis a second time, it would be ideal have more time in order to collect more data. As this experiment was carried out in a matter of a few weeks, there was a relatively minimal amount of specimen to observe and analyze. Although another suggestion to use the exact same cells in comparisons between normal and mercury induced environments may seem beneficial, it could be suggested that the results would not be affected by such steps as having multiple specimen would carry the same effect. I would also suggest to be much more attentive to the temperature the cells were left in as well as the cleanliness of the tools used (R Morris, public presentation 2.5.08).

Furthermore, future experiments analyzing neuron-glia interactions may want to consider toxins other than mercury such as cadmium, arsenic or perhaps even fluoride to study. Cadmium for example, which a substance found mostly in workplaces where cadmium products are made or even more commonly in cigarette smoke (ADSTR, 2007), has research to suggest that exposure particularly in developmental stages may create problems with perceptual abilities (Hen Chow et al, 2007). Arsenic, another common substance to be in contact with, is normally found in combinations with other substances such as oxygen, sulfur and chlorine, and naturally comes from volcanic eruption. It is found in such things as herbicides, insecticides, leaded gasoline, wood preservatives and antifouling paints. The affects of arsenic on the nervous system result in peripheral neuropathy or degradation of peripheral neurons (ADSTR, 2007). Specifically it is possible that arsenic causes apoptosis of neurons (Namgung & Xia, 2001) as well as creating problems with axonal transport (DeFuria, 2007).

Ideally, the purpose of the blood-brain barrier which exists in the human brain is to restrict the ability of any toxins to exit the capillaries and enter space occupied by glia and neurons (De Keyser et al, 2007). However if at any point this function was to be disrupted; whether from injury or some other factor, functions of individual neurons and glia could be compromised, resulting in impaired abilities for interactions which form the basis of basic nervous system function. It would be imperative to study these substances and possible associated dysfunctions on a cellular level in relation to neuron and glia interactions, in order to further our understanding of neurological and psychological illnesses and disorders in hopes to discover ways to correct and prevent them.

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