Mercury Shows Varied Influence on Distribution Between Actin in Developing Glial Cells and Neurons

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Introduction

Currently Alzheimer's disease (AD) and Autism have mainly been identified and studies put forth to address the symptoms, though recently there has been more of a focus towards the causes or triggers for these diseases. Recently theories been proposed linking mercury exposure and with the symptoms of AD and Autism. As a result, extensive research is underway that aims to explain in depth how these health issues are related to genetics, our environment, and increased mercury exposure. Mercury exposure has increased with use of specific dental fillings, fish dependent diets, medications and elsewhere in our environment that may be linked with the increase in both AD and Autism in the world (Mutter 2005). In 1992 roughly 70% of mercury ion exposure was claimed to result from amalgam (Aposhian et al, 1992). These types of dental fillings use mercury to keep the filling pliable during the treatment, and then it will evaporate and leave behind a hardened material that will protect the tooth (Benoit 2008). Mutter *et al.* state the current stance on autism very clearly in that the actual causes are unknown but that genetic and environmental risk factors are somehow involved (Mutter 2005). They propose this theory on the basis that mercury levels have risen along with autism and children with autism have shown to have a decreased detoxification for mercury (Mutter 2005). In recent years mercury has been released into the environment by power plants, chemical manufacturers, and other sources where it settles into the waterways and builds up in the fish we eat from these sources. Once mercury enters a water source, bacteria in the water convert the mercury into methyl mercury which is easily absorbed and extremely toxic for humans (CDC 2005). For the purpose of this study we are using ionic mercury which has a recommended maximum concentration of 0.0002 mg/m^3 (Benoit 2008). In 2005, the Center for Disease Control concluded that one in 17 women of childbearing age have a concentration of mercury in their blood above 5.8 micrograms (CDC 2005). Further, in recent studies it has been theorized that mercury actually concentrates in the umbilical cord, which would make levels as low as 3.4 micrograms a serious consideration for fetal development (CDC 2005).

Neuron's cytoskeletons contain three main components; microtubules, neurofilaments and actin microfilaments (Kandel *et al.,* 2000). They work together mainly as the cytoskeleton and undergo rapid cycles of polymerization and depolymerization during development (Kandel *et al.,* 2000). In addition to this, they function in part of some cell movement and intracellular transport (Kandel *et al.,* 2000). Previous research has shown mercury to negatively influence tubulin polymerization but have yet to show any variation for F-actin (Leong et al, 2001). There is also much debate about the actual concentration that becomes effective within the body since minute amounts of toxins may not show any significant effect. Therefore, we will be testing one high concentration of mercury on chick neurons to determine both the effect on tubulin and F-actin. Although only tubulin has been shown to be affected by Hg, the use of a high dose will also help determine if there is a higher threshold for Hg to affect actin than previously tested (Leong et al, 2001). Chick embryos were used because of the relative size for dissection and imaging as well as the availability for consistent dissection. The information for supported research should also be more available for chicks as it is a very commonly studied species.

If the microtubules in a neuron are impaired in any way, then proper cell movement and function cannot take place. Therefore, under the assumption based on previous research, the following experiment has been adapted from a previous study conducted by Amy Silviri, Brianne Jeffery, and Jasmine Bhatia in 2006 to determine the effects of mercury exposure on neuron development and function. More specifically I will be focusing on the effects on actin and then collaborating with Ashley Furr who will be focused on tubulin. This experiment tests the hypothesis that exposure to high concentrations of mercury will negatively affect the concentration and arrangement of of F-actin.

Materials/Methods

*The following materials and methods were adapted from Amy Silviri and Brianne Jeffrey's experiment in 2006 (Jeffrey 2006).

Materials

** The following materials are adequate to start the experiment though additional solutions were made to allow additional samples. Adjustments can be made according to available ingredients.

Materials needed to complete this experiment include 10 day Chick embryos, Rhodamine Phalloidin, DM1A, a 37˚ C incubator, L15 Growth Medium, 90% EtOH solution, Hank's Balanced Saline Solution (HBSS), HgHBSS (0nm and 100nm), sucrose, 37% Formaldehyde, 25% Gluteraldehyde, 25% Triton-x 100, 0.2M EGTA, trypsin (1ml for each dish of dissociated tissue), Sodium hydroxide, blunt and sharp forceps, flame-drawn Pasteur Pipettes and bulbs, small petri dishes to plate cells (one for each coverslip), trypsin, poly-L Lysine, slides, all Buffers, kimwipes, nail polish,

humidity chambers, Nikon Eclipse 80-I microscope, Image J and Spot imaging software, and Microsoft Excel.

Several solutions were also prepared. These included 30ml fix/permeablization buffer (30ml L-15, 1.62 ml 37% Formaldehdye, 0.12 ml 25% gluteraldehyde, 1.2g sucrose, 0.6 ml 25% TX100, and 0.3 ml 0.2 M EGTA), fix alone (30ml L-15, 1.62 ml 37% Formaldehdye, 0.12 ml 25% gluteraldehyde, 1.2g sucrose, and 0.3 ml 0.2 M EGTA), PBS/Triton x-100 (500ml PBS and 2.5 ml 100% Tx-100), Block Buffer (50ml PBS and 1.5g BSA), and 1:1000 DM1A and 1:500 Rhodamine Phalloidin fluorescence (2ml per cover slip).

Solution Preparation

Fix/Permeabilization Buffer

First add to 30ml L15 growth medium the following ingredients; 1.2g sucrose, 1.62ml of 37% formaldehyde, 0.12ml 25% gluteraldehyde, 0.6ml of 25% Triton x 100 (Tx 100) and 0.3 ml of 0.2M EGTA. Then combine solutions and shake vigorously. Set at pH of 7.0 by adding sodium hydroxide.

Fix Buffer

First add to 30ml L15 growth medium the following ingredients; 1.2g sucrose, 1.62ml of 37% formaldehyde, 0.12ml 25% gluteraldehyde, 0.6ml of 25% Triton x 100 (Tx 100) and 0.3 ml of 0.2M EGTA. Combine solutions and shake vigorously. Set at pH of 7.0 by adding sodium hydroxide Add to 30ml L15 growth medium

PBS/Triton x 100

Combine 500ml PBS and 2.5ml 100% T x 100 and shake vigorously.

Block Buffer

Combine 1.5g BSA (3% BSA final) with 50ml PBS and shake vigorously.

**Note: The following reagents are to be prepared as close to their use as possible to avoid photo bleach.

DM1A

To create 1:1000 DM1A in block buffer, add 3µl DM1A to 3ml block buffer.

Rhodamine Phalloidin

Add 6µl Rhodamine Phalloidin to 3µl block buffer to create 1;500.

Methods

* The following methods were adjusted from Morris and Hollenbeck's experiment in 2006 (Morris and Hollenbeck, 2006).

Dissection of Primary Culture Chick Embryos

Ten day old eggs were sprayed with 90% EtOH to sterilize. Several petri dishes with 5mls of warm HBSS were prepared while eggs dried. Use sterile, dull forceps to tap through shell about 1 cm down from apex and shell debris was removed. The embryo was gently removed from the shell and placed in petri dish containing HBSS. The head, wings, and legs were then removed from the body. The internal organs were then removed by proceeding through the sternum (chest plate) of the chick until the spinal cord was removed. Tissue on either side of the spinal cord was then removed so that the ganglia and sympathetic nerve chains could be removed and placed in HBSS. This process is very delicate, so gentle teasing of the tissue seems to be the most effective way to remove undamaged cells (Morris and Hollenbeck).

Dissociation of the ganglia

The dissected cells were washed two times with HBSS. The HBSS was then removed and replaced with trypsin (Ca/Mg-free HBSS containing 0.25% trypsin) and incubated for 20 minutes at 37˚ C. The trypsin was gently removed in order to not disturb the chains too much as they are very delicate during this stage. The trypsin was replaced by HBSS to resuspend the ganglia. A flame-drawn Pasteur pipette was then used to tituate the cells until they dissociated into single ganglia.

Preparation of Laminin Substrata and plating cells

Although neurons can grow on plain glass, they are typically plated onto one of two substrata for optimal results. Depending on the experiment, one may use laminin to grow cells faster, or collagen to create better adherence to the cover slip. For the purpose of this experiment we decided to use laminin. In order to do this, we first coated the surface with 1mg/ml poly-L-lysine by placing a single drop on a petri dish and then placing the cover slip on top of the drop. This was allowed to sit for 1-24 hours before it was rinsed with sterile water and allowed to dry. Coat the cover slip with laminin solution in HBSS for at least 1-16 hours. The cells were then submerged in growth medium until they are ready to plate. Rinse with HBSS prior to plating. Drops of suspension containing dissociated ganglia and sympathetic nerve chains were placed on laminin side of cover slips and growth medium. Growth should begin within a few hours after plating.

Slide preparation

Slide preparation is almost identical for all slides in this experiment so a detailed description will be provided for the experimental and then a brief explanation of how the controls differ by removing Hg or Fluorescence will follow.

Growth medium was removed from pre-plated cells. Since the purpose of this experiment was to determine the varied affects of different Hg concentrations, four solutions of HgHBSS were prepared by Professor Morris though able to duplicate on our own (0nM, 10nM, 25nM, and 100nM). For all cells receiving Hg, we added 1-2ml of designated solution to cells (consistently 1ml was used for our purpose) and left to incubate for 20 minutes. HgHBSS was then removed and growth medium was added for the duration of 10 minutes for recovery of cells. The growth medium was removed and cells were washed with warm HBSS from incubator. HBSS was removed and 2ml of fix/perm solution was added around the perimeter of the petri dish so as to not to disturb the cells. The cells were then allowed to sit for 20 minutes. It is important at this stage that you do NOT place these cells back in the incubator as the formaldehyde evaporation from the fix/perm solution could potentially kill or damage living cells from previous stages of the experiment. The fix/perm was then removed and fresh fix solution was added and left to sit for an additional 15min. Only 5 minutes is considered necessary for this stage, though the longer you are able to expose the cells to the fix buffer, the more stable the cells will potentially be. After this period, the fix buffer was removed and cells were rinsed gently with PBS/Tx-100. 3% BSA in PBS was used to block cells and were allowed to sit for at least one hour. If the cells needed to be left in block for longer than 24 hours, we added a sodium azide solution to prevent any unwanted growth in the samples (Morris 2008). Concluding the blocking step was often a good time to take a break.

To begin the fluorescence labeling stage, the cover slips were transferred to a pedestal in a humidity chamber with the cells remaining on the top of the cover slip. The cells were labeled with 100µl of Ab solution and allowed to sit for 1 hour. We made sure the cells are not exposed to excessive light during the transfer and incubation process so the Ab does not photo bleach too much. We kept our cells covered with tin foil or in a drawer to achieve this. The cells were then washed with PBS/Tx-100 and then mounted on slides on top of a single drop of PBS/Tx-100 to keep cells hydrated for imaging. Cover slips were placed cell side down and then sealed with nail polish on all four edges.

As explained above, not all cover slips received the same protocol for mounting. Since we used 4 complete cover slips, we opted to have two controls of 0nM and two 100nM. One of the controls received no fluorescence as well as no Hg to make sure unaltered cells did would not fluoresce. One of the 100nM cover slips also did not receive fluorescence to make sure the Hg treatment did not create fluorescence on its own. It was not necessary to do this for each concentration of Hg because if the Hg will fluoresce, we expected it will do so at the highest concentration. The method we used to divide our cover slips into the necessary categories is represented below.

	Mercury exposed(Hg)	Fluoresced?
0nM	No	No
0nM	No	Yes
100nM	Yes	Yes
100nM	Yes	No

Data Collection

For the entirety of the experiment a Nikon Eclipse 80-I microscope was used. Location of the specimen was done using a 40x objective and Kholer Illumination. Once a specimen was located on the microscope the image was refocused on the computer using Spot software since the camera is located at a different distance from the slide. Once proper focus was achieved, one photograph was taken of the phase picture, and then through trial and error for proper exposure of the picture was taken using the appropriate filter while the transmitted light was blocked by covering it with tin foil.

Quantifying the data could be done in several different ways and many options could potentially work as long as they are consistent and you account for possible error that may occur as a result. There were several attempts to equate exposure times between slides and standardize our data to make the applications more universal. The main one was devised by colleague Blair Rossetti which used ImageJ software. This process utilized 5 pictures for each cell (1 phase, 1 optimally exposed red fluorescence, 1 optimally exposed green fluorescence, 1 standard red and then 1 standard green). They first opened the optimal image, subtracted the background, then selected ‘binary’ and then ‘threshold’ under the ‘process’ tab in the program. This created an image with significant contrast as the background was now white. This provided an image that was easier to determine its edges for proper analysis. Then they opened the standard image and selected ‘image calculator’under‘ processes. The standard image was selected for the first box, ‘add’ was selected for the second, and then the optimal placed in the third. We then generated a histogram and it was then analyzed and data copied to excel for further analysis. This process was ideal for looking at fluorescence that was too dim by utilizing a brighter fluorescence to define cell boundaries. Since this experiment is primarily looking at actin alone, a simplified version was used to quantify.

To quantify the image, the photos were first converted to 8-bit tif images in ImageJ. The threshold was then adjusted under the ‘image’ tab and the minimum was set to 1. The wand tool provided by ImageJ that selects well-defined regions of an image. This tool was used be more consistent than manually selecting a region. A histogram was then generated and the mean was recorded. Since the majority of the images for rhodamine phalloidin were exposed for 3 seconds, varied exposure did not have to be accounted for. The means and stand deviation was then copied to excel

for further analysis. This process was repeated for three successful trials though only the final set resulted in consistent data for analysis.

Time

Planned timing is essential in this experiment in order to work around other students' schedules and keep each sample consistent with others. We discovered that creating the solutions took about 1 hour, and additional 1.5 hours was generously allowed to get to the blocking step. At this point we found it to be a good to take a break. After this there was roughly 1 hour to fluoresce cover slips and 15 minutes to mount on slides. It is best to image as close to time of fluorescence as possible, though if need be slides can be kept in a closed container in the refrigerator. We had a lot of difficulty with the initial rounds of imaging, but once it became routine we allowed about 2 hours per slide to get adequate results.

Results

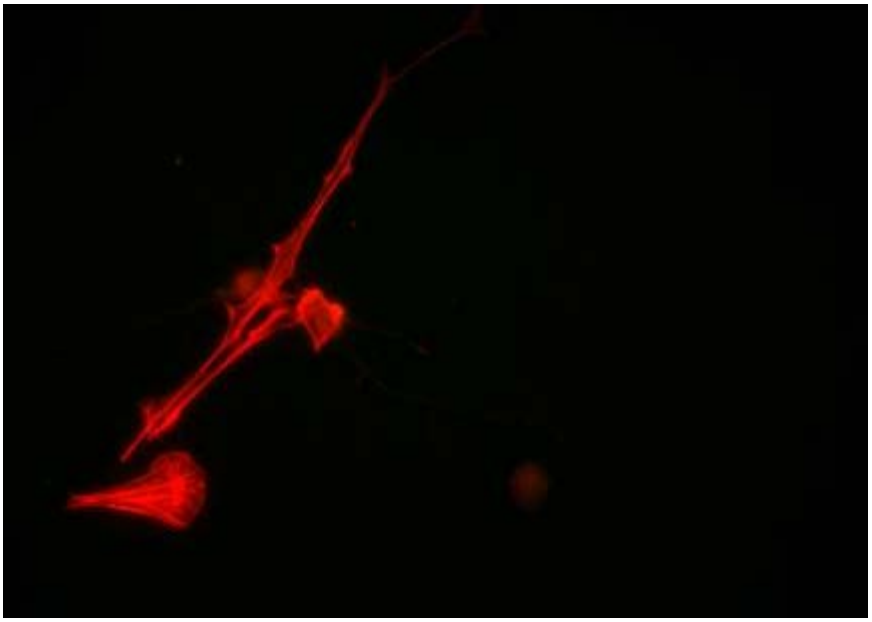

Figure 1: This image represents double fluorescence of DM1A (green = tubulin) and red = Rhodamine Phalloidin (F-actin) in the control (no Mercury) neurons and glial cells. The DM1A is mostly hidden by the Rhodamine Phalloidin as a result of insufficient brightness.

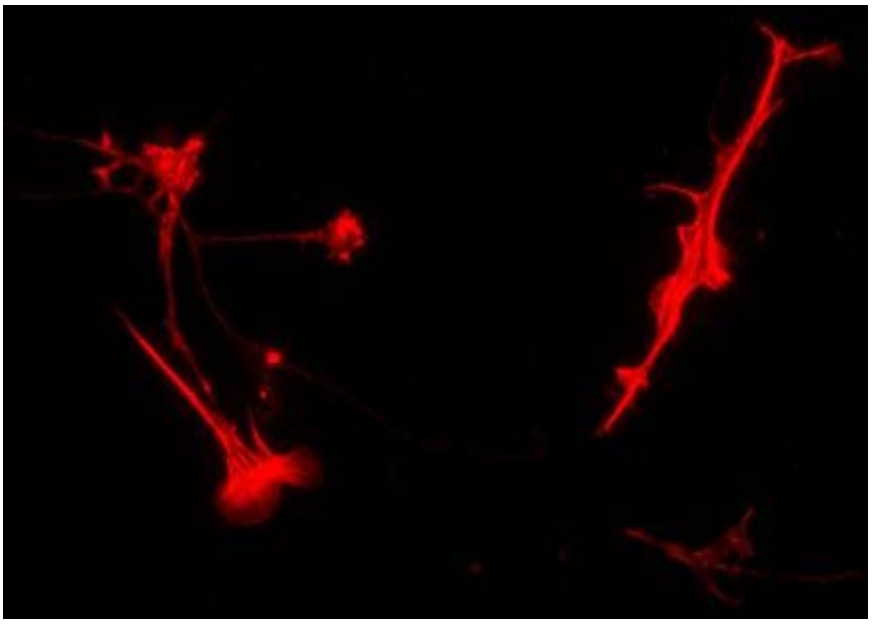

Figure 2: This image represents single fluorescence of Rhodamine Phalloidine (actin) in the experimental group that received 100ml of mercury. Clear axon growth is shown to be slightly dimmer than fluorescence in the glial cells emitting the highest brightness. The actin produces very striated accents in the image and appears to be well distributed.

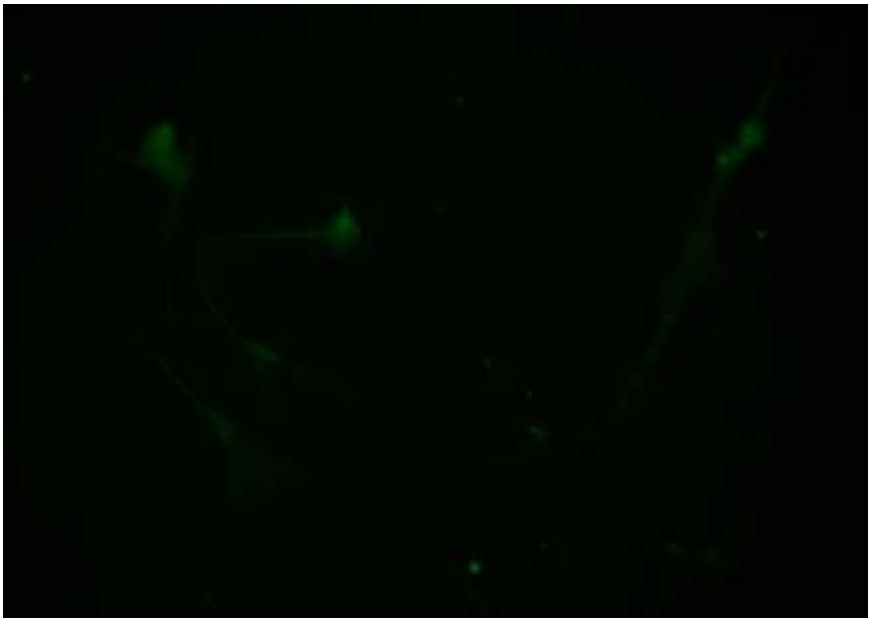

Figure 3: This image represents the single fluorescence of DM1A in the experimental group that received 100ml of mercury. The DM1A represents tubulin and is noticeablydimmer than the Rhodamine Phalloidine in Figure 2. The tubulin appears to be less striated than the Rhodamine Phalloidine and concentrates in the cell body.

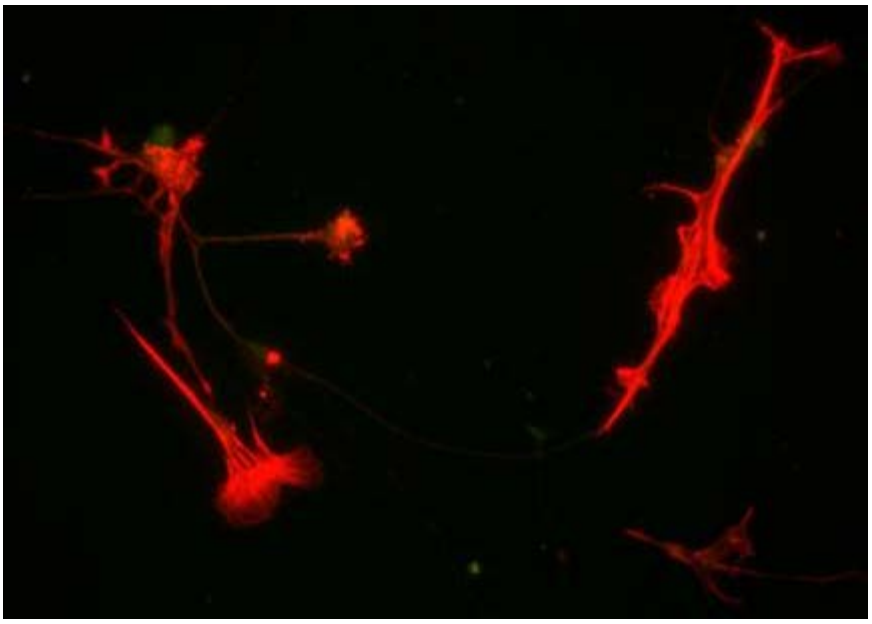

Figure 4: This image is a representation of how Figures 2 and 3 would be stacked on top of each other to create a representation of the ration between tubulin and actin. Slight differences can be seen in the cell bodieis and axons though the image is difficult to analyze due to the insufficient brightness of DM1A.

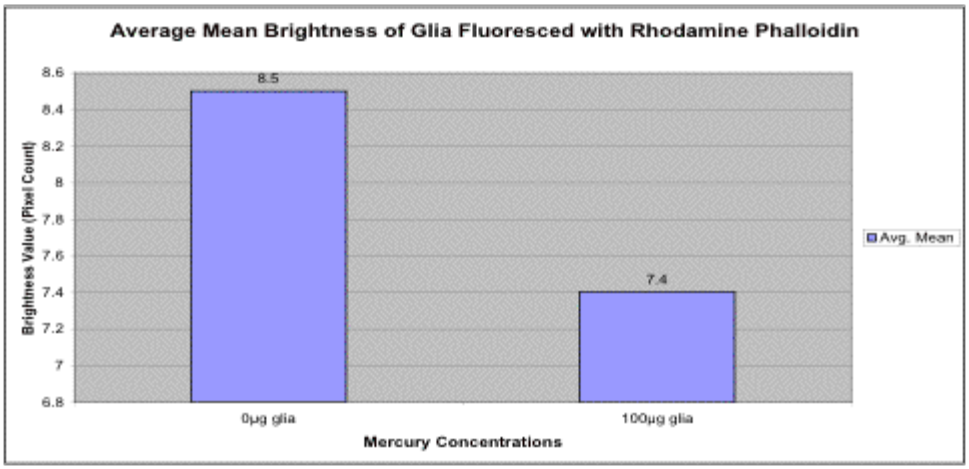

Figure 5: This graph is a depiction of the Average Mean of Brightness Values for glia. The graph is segregated based on the Mercury (Hg) concentration it was exposed to during growth. Notice the negative affects on the Brightness Values for the mean as the glia are exposed to Hg.

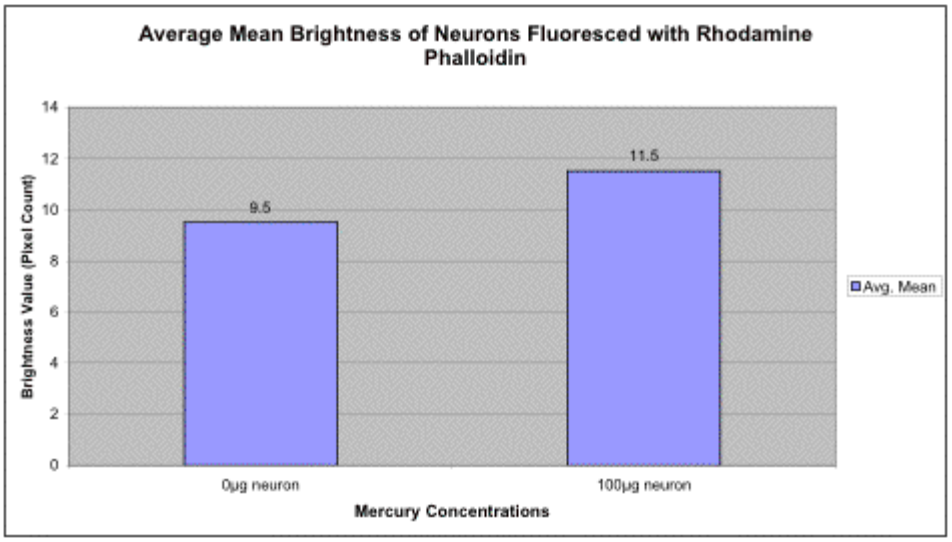

Figure 6: This graph is a depiction of the Average Mean of Brightness Values for neurons. The graph is segregated based on the Mercury (Hg) concentration it was exposed to during growth. Notice the positive affects on the Brightness Values for the mean as the neurons are exposed to Hg.

As is depicted in Figure 5, mercury showed to have a negative effect on brightness values of glia.Both the mercury treated glia resulted in a lower brightness value for the average mean of the cells. The mercury treated glia showed a decreased average mean by 1.1. The opposite effect occurred with neurons as mercury had a positive effect on the brightness value of neurons. The mercury treated neurons showed an increase of 2 for average mean. There was no visual difference in brightness between the control and experiment, though decreased levels of F-actin were noticed in long axons of the experimental, yet tubulin seemed to remain consistent

Discussion

To test the effects of Hg on neuron and glial cell development the concentration of Hg was altered and compared against a control. To provide a visual representation of the effect we used a double fluorescence of Rhodamine Phalloidin and DM1A to provide a possible ratio of F-actin and tubulin within the cells. As seen in Figure 5, there was a slight drop in brightness value for F-actin for the average mean for glial cells. This effect of increased brightness for F-actin is similar to those proposed by Leong (Leong et al 2001). Interestingly, however, the neurons in this experiment exposed to the same process of Hg exposure and double fluorescence as the glia actually showed the opposite effect with decreased brightness values for F-actin. These results seem to negate the effect on glial cells, but this may provide further research onto which types of cells may be affected and why.

Leong reported that the strongest support for his findings was on how Hg effected the development of neurons and degeneration of growth cones upon exposure to Hg (Leong et al, 2001). Leong's results also showed no effect of

exposure to other heavy metals on neurons and determined that the most likely influence in his experiment was actually on GTP nucleotide binding to β–tubulin (Leong et al, 2001). This step of nucleotide binding is necessary for tubulin polymerization and therefore depicts strong similarities between Hg exposure and neuronal disease such as AD or Autism.. Unfortunately, the images of DM1A staining tubulin in this experiment were inconsistent with each other and often too dim to provide adequate analysis. This leaves little ability to support or reject Leong's results.

Leong's results on Hg exposure on tubulin, however, were mainly attributed to disruption of microtubule-tubulin metabolism and evidence put forth for having no effect on actin (Leong et al, 2001). These results support the depiction in Figure 4 as there is no negative effect of Hg exposure on brightness value of actin, but in fact a slightly positive one as the average brightness value increased by almost three. The presence of Hg actually resulted in a slight increase in brightness value for the average mean. However, in light of our recognized sources of error, we believe that these results are not distinguished enough to be conclusive about the effects of Hg. Instead, we are only able to gain support for the fact that there was minimal effect of Hg on the brightness value of actin. Further experiments would be necessary to provide enough information to determine the exact method of Hg inhibition on tubulin.

During the first two trials we noticed a difficulty with DM1A staining and attempted to remedy this for the last experiment by increasing the concentration of DM1A to 1:250 instead of 1:1000. Despite our attempts to brighten the results for quantification, this change showed little or no effect on imaging. Therefore, for the purpose of this experiment DM1A seemed to be ineffective in measuring the effects of Hg on tubulin as we could not efficiently stain the cells. This does not allow us to make any conclusive statements about tubulin, though through qualitative observation we noticed a possible influence based on the green fluorescence we were able to acquire. There appeared to be a general trend of consistent tubulin in visible axons often where actin was absent. These observations may lead to other research that would focus on the ratio between tubulin and actin in solely axon growth.

There are several sources of error that may attribute to our inconclusive results. One may be our difficulty with DM1A fluorescence as despite attempts to boost the concentration of the antibody to 1:250 from the original 1:1000 we were unable to achieve adequate images. Another source would be the multiple rounds of buffers that were made for each trial as different collaborators may make different solutions depending on the stage we were at in the experiment. A third source of error may have been the inconsistency of locating and fluorescence cells properly. We had a significant learning curve with regards to imaging efficiently as well as preparing slides in a routine manner. Therefore, earlier samples may have been in a more aggressive environment with prolonged transfer periods and intense rinsing. We also noticed as we began quantifying that our actin was not fully exposed in every picture. Although they were consistent with a three second exposure, an optimal exposure to determine the true cell boundary would have been

useful and also provided more consistent data for analysis. This also would have allowed us to use Rossetti's theory for analysis described in the methods.

The potential application for these studies possibly would be further understanding of Alzeihmers, Autism, or those with amalgram dental fillings containing Hg. Hg has been proposed to have either causal or activational effects for these diseases though have not shown any conclusive results to determine the mechanism. If we had seen similar results in our experiment as Leong (2001) or Jeffrey (2006), we would be able to support the theory that Hg may disrupt microtubule polymerization by inhibiting GTP nucleotide binding to β–tubulin (Leong et al, 2001). However, we were unable to acquire results that supported conclusions either way and therefore are unable to support our own hypothesis that mercury would disrupt tubulin and actin structure.

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