Rhodamine Phalloidin Stain Indicates Greater Amount of Actin in Mercury-Treated Primary Chick Sympathetic Neurons

Introduction

The cytoskeleton is the major intrinsic determinant of the shape of a neuron (Kandel *et al.*, 2000). It contains three main filamentous structures: microtubules, neurofilaments and actin microfilamets (Kandel *et al.*, 2000). Microfilaments, 3-5 nm in diameter, are the thinnest of the three main types of fibers that make up the cytoskeleton. They are polar polymers of globular actin monomers wound into a double-stranded helix (Kandel *et al*, 2000). Actin filaments form short polymers and are concentrated at the cell's periphery in the cortical cytoplasm, lying just underneath the plasmalemma, where, together with a very large number of actin-binding proteins, they form a dense network (Kandel *et al.*, 2000). This matrix plays a key role in the dynamic function of the cell's periphery, such as the motility of growth cones during development (Kandel *et al*., 2000). Microfilaments are in a dynamic state and undergo cycles of polymerization and depolymerization, which is the same with microtubules (Kandel *et al*., 2000). The state of actin within the cell is controlled by binding proteins (Kandel *et al.*, 2000). These proteins facilitate assembly and block changes in polymer length by capping the rapidly growing end of the filament or by severing it (Kandel *et al*., 2000). The dynamic state of microtutubles and microfilaments permit the mature neuron to retract old processes and extend new ones (Kandel *et al*., 2000).

In addition to serving as cytoskeleton, microtubules and actin filaments act as tracks along which other organelles and proteins are driven by molecular motors (Kandel *et al.*, 2000). Since these filamentous polymers are polar, each motor drives its organelle cargo in one direction only (Kandel *et al.*, 2000). Actin motors, called myosins, mediate the extension of the cell's processes (Kandel *et al*., 2000). Myosin is also thought to translocate membraneous organelles within the cortical cytoplasm (Kandel *et al.*, 2000).

Elongating axons terminate in a protuberance called the growth cones (Kandel *et al.*, 2000). The growth cone is both a sensory structure that receives directional cues from the environment and a motor structure whose activity leads to axon elongation (Kandel *et al.*, 2000). It has been asserted that the growth cone guides the axon by transducing positive and negative cues into the signals that regulate the cytoskeleton and thereby determine the course and rate of axon outgrowth (Kandel *et al*., 2000).

Growth cones have three main regions. The central core is rich in microtubules, mitochondria and a variety of other organelles (Kandel *et al.*, 2000). Projecting from the body are long slender extensions called filopodia (Kandel *et al.*, 2000). Between the filopodia are lamellipodia, which are motile and give the growth cone its characteristic ruffled

appearance (Kandel *et al.*, 2000). The sensory capabibility of the growth cone depends in large part on its filopodia (Kandel *et al.*, 2000). These rod-like, actin-rich, membrane-limited structures are highly motile (Kandel *et al.*, 2000). When receptors on the filopodia encounter signals in the environment, the growth cone is stimulated to advance, retract, or turn (Kandel *et al.*, 2000). Several motors involving actin, myosin and membrane components power these reactions, and the contribution of each molecular motor to advance of the growth cone likely varies from one situation to another (Kandel *et al,* 2000). The final step involves the flow of microtubules from the central core into the newly extended protrusion, thus moving the growth cone ahead and leaving behind a new stretch of axon (Kandel *et al.*, 2000).

Studies have shown that mercury ions effect growth cone morphology and tubulin polymerization of microtubules, and Leong *et al.* set out to see whether mercury-induced degeneration of growth cone structure involved actin/tubulin architecture of newly assembled cytoskeletal elements. What they saw after exposing the growth cones of the snail *Lymnaea stagnalis* to mercury ions and fixing and processing the collapsed cells for actin/tubulin immunofluorescence was that the mercury ion treated growth cones exhibited a high degree of disintegration of the tubulin/microtubule structure (Leong *et al.*, 2001). This result led to the supposition that mercury-induced degeneration of growth cone structure probably involves microtubular disassembly (Leong *et al.*, 2001).

A question that arose from the Leong *et al.* paper was whether the mercury-induced degeneration of the neuron membrane was solely due to the disruption in microtubule metabolism. According to Duhr *et al.*, mercury was shown to have no effect on GTP binding to actin as it had on binding to β-tubulin. Leong *et al.* concluded that structural disassembly of the neurite membrane was a direct effect of Hg on tubulin rather than actin.

By following a very similar procedure to Leong *et al.*, this study investigated the effects of mercury on the cytoskeletal assembly and dynamics of actin on primary chick sympathetic neurons. The goal of this experiment was to compare the amount of actin filaments found in neurons that have not been exposed to mercury to the amount of actin filaments found in neurons after exposure to mercury.

Mercury is a highly toxic environmental contaminant which forms a serious hazard to the public heath and a threat to most life forms (Braeckman *et al.*, 1997). It is of utmost interest to see how exactly mercury can effect the developing nervous system. Knowing that the cytoskeleton plays an extremely pivotal role in maintaining the cell's integrity, it is of utmost interest to determine the negative effects that mercury has on cytoskeletal structures. Whereas Leong *et al.* used a very high concentration of mercury of 200mM, this experiment used a concentration of 20mM, which is the concentration that is normally found in the environment (Morel and Hering, 1993). Based on the conclusions drawn by Leong *et al.*, it was hypothesized that there would not be an effect on the synthesis of new actin

filaments after the neuron's exposure to mercury.

I collaborated with Brianne Jeffrey on this experiment. While I was analyzing the effects of mercury on actin filaments, she was analyzing the effects of mercury on microtubules. Both of us were interested in analyzing the effects of mercury on the cytoskeleton of the chick embryo neurons. We used the same cells, but different fluorescent antibodies in order to make our own observations for our different areas of study. In addition, I collaborated with Brittany Chick and Sara Tower, as they were also analyzing the effects that mercury had on actin filaments, particularly the filopodia. Chick was studying filopodial activity and Tower was studying neuron-neuron interactions and filopodial branching.

Materials and Methods

Materials:

The cells used in this experiment were sympathetic chain ganglia that were dissected from 9-11 day chick embryos. All the reagents that were used were obtained from Sigma Chemical Co. Rhodamine Phalloidin was obtained from Molecular Probes Inc., and DM1A anti-tubulin was obtained from Boehringer-Mannheim Biochemicals. A 37°C incubator was used to culture the cells. In order to collect fluorescence data a Nikon Eclipse 80-1 microscope was used, using a 60x oil immersion lens and a SPOT insight camera. To analyze the brightness of the fluorescence of the cells, Image J software was employed. An EXCEL spreadsheet was used in order to carry out all the necessary calculations to determine the relative brightness of the actin filaments in the neuron.

Methods:

The variables in this experiment were the concentrations of the neuronal cells being analyzed and whether or not the cells are exposed to mercury. Six medium density, two high density and two low density poly-lysine coverslips were analyzed. These cells were allowed to grow overnight in F^+ medium in the 37°C incubator. The concentration of 10nM of mercury (HgHBSS) was delivered to the experimental cells, and this concentration was not varied. The HgHBSS was prepared by Professor Morris by keeping the mercury in solution by placing it in acid (10μM $HgCl_2$ in 0.5% HCl) and then diluting it to 1:1000 in HBSS to get a 10nM $HgCl_2$ in HBSS stock solution. For every experimental coverslip, there was a control coverslip, which was not treated with mercury. It was believed that better results from the medium density would be obtained, since there would not be as many interactions as is seen in the high density cultures, and there would be more cells to observe than in the low density culture. As it turns out, cells were seen in all of the cultures and were not too crowded. Therefore, it may not be completely necessary to vary the

concentrations, and one concentration could be chosen. However, it is still apparent that the more cells that there are, the more likely it is that the treatment for fluorescence will work properly. By changing the concentrations, we were trying to optimize our results by not restricting the amount of cells that we attempt to treat and analyze under the fluorescence microscope.

The reagents that had to be made before the experiment was performed included the fix/permeabilization buffer, the fix buffer, the PBS/Triton x 100, and the block buffer.

Fix/Permeabilization Buffer

An amount of 3mL of this buffer was to be delivered to each coverslip that we had. Since we were analyzing 10 coverslips in total, we wanted a total of 30mL of this buffer. To 30mL L15 growth medium, 1.2 g sucrose, 1.62mL of 37% formaldehyde, 0.12mL 25% gluteraldehyde, 0.6mL of 25% Triton x 100 (Tx100) and 0.3mL of 0.2M EGTA were added. The formaldehyde, gluteraldehyde and triton x 100 were delivered by a calibrated plastic pipette under the hood. The EGTA solution was prepared by Professor Morris and delivered to the mixture under the hood. The solution was well shaken.

This buffer was brought up to a pH of about 7.0 by adding drops of sodium hydroxide.

Fix Buffer

To 30mL L15 growth medium, 1.2 g sucrose, 1.62mL of 37% formaldehyde, 0.12mL 25% gluteraldehyde and 0.3mL of 0.2M EGTA were added. The formaldehyde, gluteraldehyde and triton x 100 were delivered by a calibrated plastic pipette under the hood. The EGTA solution was prepared by Professor Morris and was delivered to the mixture under the hood. The solution was well shaken. This buffer was brought up to a pH of about 7.0 by adding drops of sodium hydroxide.

PBS/Triton x 100

An amount of 500mL PBS and 2.5mL 100% T x 100 were added together and shaken vigorously.

Block Buffer

1.5g BSA (3% BSA final) was added to 50mL PBS and shaken vigorously so that the BSA dissolved completely.

The following reagents were to be prepared right before they were to be used because they are apt to photobleach. They

contain DM1A and Rhodamine Phalloidin, which are fluorescent antibodies.

1º Ab

In order to make up 1:500 DM1A in block buffer, 2µL DM1A was added to 1mL block buffer.

Rhodamine Phalloidin

A volume of 50µL Rhodamine Phalloidin was added to 950µL block buffer.

Out of the 10 coverslips that were analyzed in this experiment, the following is the breakdown between the controls and the experimental cells.

Table 1: Summary of the number of coverslips needed and the densities of the cells needed on each one, for both the control and experimental cells.

Control	***Experimental***
1 low density	1 low density
2 medium density	2 medium density
1 high density	1 high density
Negative Control	***Negative Control***
No Fluorescence	No Fluorescence
Medium Density	Medium Density

Two 6-welled plates were used to carry out steps 3-10. One plate was for the controls and the other plate was for the experimental cells, which were to be exposed to HgHBSS. The wells were appropriately labeled with the different coverslip densities that each were going to contain.

The growth medium, F+, was removed from the cells that have been incubating overnight. The coverslips were gently removed from the Petri dishes and placed into the labeled six-welled plates. HgHBSS was added to the experimental coverslips and control HBSS to the control coverslips for 20 min. HgHBSS was then removed from the experimental coverslips and HBSS was removed from the control coverslips. F+ was added to each batch of coverslips for 10 min. All coverslips were washed with warm HBSS once. A volume of 2mL Fix/Perm was added to each dish, and the coverslips were allowed to incubate in this buffer for 15 min. The Fix/Perm was withdrawn, and fresh fix buffer (without Tx100) was added for 5-10 min. The coverslips were washed with PBS/TX100 one time. Block buffer with 3% BSA in PBS was then added to each coverslip and allowed to incubate for 20 min at 37°C. During this time, a humidity chamber was constructed. After the incubation period, the coverslips were transferred to pedestals in the

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humidity chamber. An amount of 100μL 1 Ab was added to the coverslips and they were allowed to incubate with this antibody for 1 hr. The coverslips were washed with PBS/Tx100 3 times and then washed with PBS alone 2 times. Rhodamine Phalloidin was then added and the coverslips were allowed to incubate with it for 15 min. They were then washed with PBS-Triton x 100 3 times. Chip chambers were made and the coverslips were mounted on slides. The coverslips were painted onto the slides with nail polish to seal. The cells were observed under the fluorescence microscope, the Nikon80-1, using a 60x oil immersion lens. Pictures were taken using SPOT software on the computer.

In total, it took about 30 minutes to make all the solutions up and then it took approximately 55 minutes to get up to the blocking step. This was a good break point. We had run only two trials using this procedure, and in each one, we had stopped at the blocking step, meaning that after the block buffer had been added to the cells, they were placed in the 37^{o}C incubator, but for a longer time frame (approximately two hours instead of the 20 minutes), and then placed in the fridge. The reason for the longer incubation period in the 37^{o}C incubator was due to the fact that the very first time that we carried out this experiment, we were not able to locate the rhodamine phalloidin or the DM1A stains. We therefore could not proceed with the procedure, but were not sure what to do with the cells. It was only after 2 hours that we decided to put them in the fridge, and for this reason, this same step (of incubating the cells for approximately two hours in the 37^{o}C incubator) was performed for both trials, in order to obtain reproducible results. After the incubation with the 1^{o}Ab (DM1A) and the rhomadine phalloidin, it took about 35 minutes to prepare all the slides for viewing. Taking pictures takes a long time, in that the cells were usually hard to locate, and then once they were located, the pictures had to be taken immediately to avoid photobleaching. Locating the cells always proved to be the most challenging aspect.

In order to analyze the results that are obtained from this experiment, the brightness of the actin of the control and experimental cells was examined. All images from SPOT were saved as 16-bit tif images and opened with the Image J software. A region of interest, meaning either the axons or cell body, was selected using the mouse. The Histogram feature was selected from the drop down menu under Analyze on the main menu bar. The List option was selected and all the values were copied and pasted into an EXCEL Worksheet. It is important to note that two columns of values were listed in this list, and the first one included the brightness value and the second included the number of pixels that corresponded to that brightness value. A dark region where no cell existed (a region where the color looked like the background of where the axon or cell body of interest lay) was then selected with the mouse. Again, the Histogram feature was selected from the drop down menu under Analyze on the main menu bar. The List option was selected, and the highest value from the region of “non interest” (the dark region) was found. Any of the values from

the region of “interest” (the axons or cell body) below this value were deleted from the EXCEL spreadsheet. With the remaining values in the EXCEL spreadsheet, the brightness value was multiplied by the number of pixels. This means that the values that were selected from the list for the region of “interest” were multiplied by the number of pixels. A sum of the number of pixels and a sum of these multiplied values were found. The multiplied values were divided by the number of pixels. This value gave the brightness of the area of “interest”.

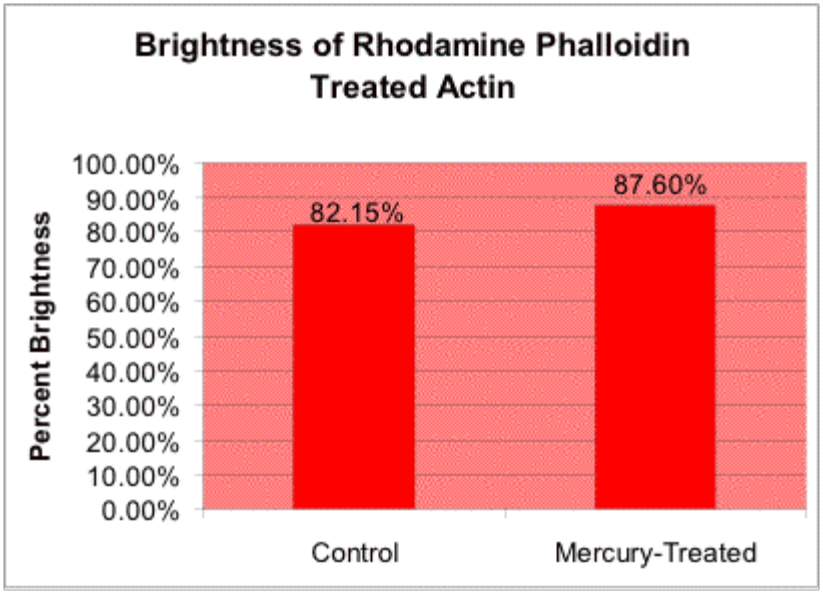
The brightness of the axons and of the cell bodies in each of the photographs was compared to that of the background using this procedure. On average, there were usually two to three axons in a specific region that were analyzed together and compared to the same background. Then there were a lot of branched axons coming off of the cell body, which were analyzed separately from one another. There were approximately three regions on each photograph that contained different axons stemming from the cell body, and therefore, three different brightness values were obtained for the axons of the same photograph. The brightness of axons was then compared to the brightness of the cell body. There was no average brightness taken for the cell body, as this was the only one that was present in the photograph.

Results

When analyzing the neurons under the microscope, it was interesting to observe that the red Rhodamine Phalloidin dye was very bright. For our first trial, there was an overwhelming amount of red background. After careful analysis of our procedure, it was observed that we had skipped the step at which the Rhodamine Phalloidin was to be washed off of the coverslips using PBS-Triton x 100. For the second trial, when the Rhodamine Phalloidin had been appropriately washed off of the coverslips, the background was darker, meaning that there was not an overwhelming amount of the Rhodamine Phalloidin in the background. In order to get a relative brightness value for the axons, and to ensure that the brightness being recorded was only from the neurons and not from the background, these two brightness levels had to be compared. In addition, the brightness value that was obtained from comparing the axonal brightness to that of the background was then compared with the brightness value of the cell body when compared to the background. What resulted was an analysis of the percent brightness of the axons in relation to that of the cell body for the control and experimental cells. On average, the cell body was significantly brighter than that of the average axon brightness that was determined from the neurons being analyzed, both for the control and for the experimental cells.

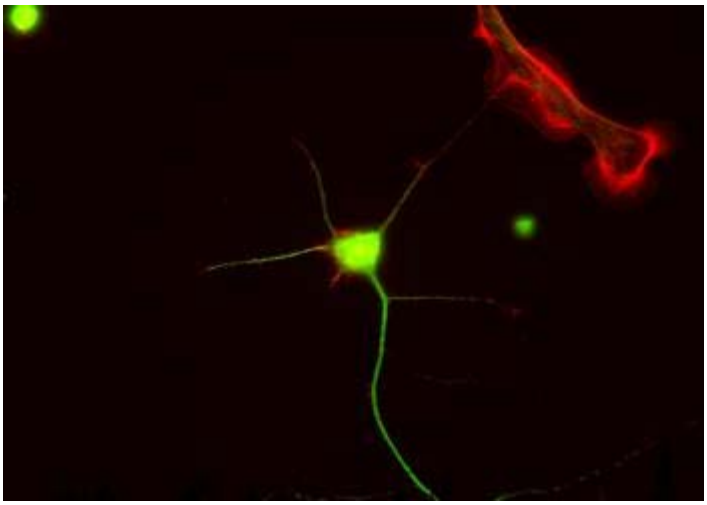
An average of the control values and an average of the experimental values, which included an average of the axon brightness and an average of the cell body brightness for each, were determined. The average axon brightness for the control was divided by the average cell body brightness for the control and multiplied by 100 in order to obtain a percent brightness value of axon to cell body for the controls. The same was done with the experimental cells. From the data that was collected when measuring the brightness of the actin in comparison to that of the background and cell body, the following graph was generated (Figure 1).

Figure 1: Brightness of Rhodamine Phalloidin stained actin is greater in axons exposed to mercury. The y-axis represents the percent brightness of the region's actin relative to that of cell body. The small difference between control and mercury-treated neurons is likely attributed to sampling errors, and not due to the effects of mercury. N=14.



Using a combination of Jeffrey's results and my results, we were able to get a superimposed image of both fluorescence staining. This resulting image is shown in Figure 2.

Figure 2: A neuron and glial cell stained with Rhodamine Phalloidin (red) and DM1-A (green). The bright spherical region is the cell body. The growth cones are visible along with neuronal-glial interaction. The image was taken on a Nikon Eclipse 80-I microscope with a 60x oil immersion lens, by a Spot Insight camera.



Discussion and Conclusions

As can be seen from the results depicted in Figure 1, the Rhomadine Phalloidin staining was slightly brighter on neurons exposed to mercury than on untreated neurons. If this result is supported by further analysis, it suggests that mercury may affect the actin cytoskeleton. Although the reason for this result is not immediately clear, it corresponds to the results found by Chick, 2006 and Tower, 2006. Chick, 2006 found that filopodial activity remained constant when the neurons were exposed to mercury. This means that there were more extensions and retractions of filopodia in the untreated controls than what was seen with the mercury-treated neurons. The actin filaments were assembling and disassembling more frequently when they were not exposed to mercury. At any given time, the filopodia (actin) present in the mercury treated cells would be greater than that of the untreated control, because the number of filopodia did not fluctuate. Tower, 2006 found that neuron-neuron interactions were decreased with exposure to mercury and the frequency of triple points resulting from filopodial branching was higher in neuronal cell cultures exposed to mercury. The work from these other scientists seems to relate very closely to my results.

When the cells were fixed for my experiment, it was not known at what process in the development stage they were at. It is very possible that I fixed the cells exposed to mercury when the filopodia activity remained constant. This would cause it to seem as though there were more actin filaments present in the cells than what would be expected if the actin was assembling or disassembling instead. It is also possible, based on Tower, 2006 results, that there was more actin present due to a higher degree of filopodial branching. However, if Chick's results are accurate, and there was a constant amount of filopodia in the mercury-treated cells, then it could be assumed that the number of branches that occurred did not necessarily increase, but they never were able to retract and extend as should be the case with the untreated control. In other words, in the presence of mercury, the filopodia could stop their outgrowth, so that the number of filopodia in the growth cone remains constant, and what Tower was observing was the filopodial branching

in the cell that occurred prior to when the cell was exposed to mercury. The cell might just not be able to extend and retract as efficiently after exposure to mercury. In this case, the number of neuron-neuron interactions in the controls decreased because the growth cones are not continuing to extend and retract. Whatever the case may be for the greater amount of actin in the mercury exposed neurons, it is a result that was continuously being found in this investigation, and should therefore be studied more in depth in future experiments.

The main source of error in this lab has to lie in the way that the results were analyzed and interpreted. By using the software, Image J, the brightness of the actin could be determined. However, the way that the brightness was analyzed from one axon, or cell body, to another was never the same. It is hard to master a specific technique for this analysis, due in part to the fact that it is based more on how a particular person characterizes what part of the picture should be determined the background, and how much of the axon or cell body should be analyzed at any given point. It is believed that the method used for this experiment was consistent throughout, meaning that what was considered background in one photo was considered the background in all subsequent photos, but there is still some uncertainty that there was complete consistency. It would be beneficial to find another means of quantifying the results, but as it stands, it can be concluded that even though this may have been a source of error, it is not believed to be extremely significant.

It would be interesting to carry out all these experiments again in order to determine if we can reproduce all these findings. In addition, it would be intriguing to see if different concentrations of mercury would give the same results as what was found for this experiment. Of utmost interest would be to find out if actin filaments are actually brighter after exposure to mercury, indicating whether there really is an increase in the actin filaments after the cells are exposed to mercury. Although the results found in this experiment are preliminary, they show that the addition of mercury does have some effect on actin filaments. Leong *et al.* had stated that structural disassembly of the neurite membrane was a direct effect of Hg on tubulin rather than actin. Jeffrey, 2006 had found that there was a greater amount of microtubules in the mercury-treated neurons than in the control neurons. It is very possible that the cells that we were analyzing were not able to effectively fluctuate between assembly and disassembly phases, and that we just happened to be analyzing neurons that had their growth cones halted by the presence of mercury. If this were the case, it would not necessarily go against the claim made by Leong *et al.* because they had analyzed the disassembly of these structures. Nevertheless, since it was apparent that these cytoskeletal structures were able to assemble even after mercury exposure goes to show that a neuron's exposure to a concentration of 2mM does not exactly have the potency to completely disassemble cytoskeletal structures, microtubules in particular, as had been seen in the Leong *et al*. paper when they had used a much higher concentration of 200mM of mercury. Data seems to support Leong *et al.*'s

speculation that actin integrity may not be compromised after mercury exposure, but further experimentation should be performed for these results to be conclusive. It seems as if the structure is not harmed, but it seems as if the function of actin, and in particular, filopodia, may be affected with the introduction of mercury.

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