100nM HgCl Does not have an Immediate Affect on F-actin in 10-day Chick Embryo Fixed Sympathetic Neurons

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INTRODUCTION

Mercury (Hg) is a rare metal found in the environment, and which accumulates through the food chain (Augusto de Melo Reis *et al.* 2007). It exists in three forms: elemental mercury (Hg^0), inorganic mercury (I-Hg) and organic mercury known as methyl mercury (MeHg) as reviewed in Counter *et al.* (2004). Although it is rare to find on the earths crust, Hg exposure is becoming more common, concerning many health professionals (Kaur *et al*. 2006). Researchers have placed mercury on a list with other metals including lead (Pb) and cadium (Cd) as harmful elements that may impair health (Counter *et al*. 2004). Research suggests Hg specifically affects the central nervous system (C.N.S.) and is more harmful for children than adults (Counter *et al*. 2004). Current studies have examined methyl mercury through *in vitro* models such as chick embryos ranging from 7 to 12 days old (Heidemann *et al*. 2001; Hoffman-Kim *et al*. 2002; and Augusto de Melo Reis *et al.* 2007) and mice (Akisaka *et al*. 2001 and Kaur *et al*. 2006).

Methyl mercury poses the largest threat for human exposure and is the most common source of mercury found in the environment (Augusto de Melo Reis *et al.* 2007). MeHg has been found in increasing numbers in species of fish around the world. Most ingested MeHg is absorbed through the gastrointestinal tract, where it easily enters the blood stream and can bind with sulfhyldryl groups (Counter *et al*. 2004). MeHg then crosses the blood-brain barrier via proteins, which eventually accumulate in the brain (Counter *et al*. 2004 and Augusto de Melo Reis *et al.* 2007). The effect of methyl mercury on peripheral neurons is not currently understood in its entirety. Mercuric chloride (HgCl) is a derivative of methyl mercury that essentially has the same effect as methyl mercury (Professor Jani Benoit, lecture, 04/09/08). There are two different types of HgCl; $HgCl_2$ and $HgCl^2_4$ (Huang *et al*. 1996). The type used for this experiment ($HgCl_2$) was found to be less dangerous and hazardous according to Huang *et al*. (1996). In addition to $HgCl_2$ affects on neurons as one cell, there is evidence that suggests that $HgCl_2$ has also been found to affect Potassium (K^+), Calcium (Ca^{++}) channels and other receptors in sympathetic neurons (Huang *et al*. 1996).

Filamentous actin, also known as F-actin consists of small globular subunits called G-actin (Akisaka *et al*. 2001).

F-actin is important to all eukaryotic cells because it is involved in many important cell functions such as, cell shape and cell signaling (Akisaka *et al.* 2001). F-actin polymerizes by using an ATP molecule to bind to the G-actin, which form oligomers and eventually thin filaments. The current experiment will explore the immediate effect of $HgCl_2$ exposure on F-actin in10-day chick sympathetic neurons.

Chick *Gallus gallus* embryos were chosen for this experiment because they are effective *in vitro* models to study in the laboratory. Chick embryos have been used in numerous experiments on studies of caffeine exposure (Bruyere *et al*. 2006) and fetal alcohol syndrome (F.A.S.) (Cartwright *et al.* 1995). Specific physiological structures in the chick models such as blood vessels, optic nerves, and various major organs such as the heart have also been studied in this model because of its easy accessibility for viewing and experimentation (Armstrong *et al*. 1994 and Professor Robert Morris, lecture, 10/09/07). The central nervous system, a highly sophisticated network of cells has also been studied in chick embryos, these studies include neurons and are considered to be especially effective models because of their similarity to human neurons (Hoffman-Kim *et al.* 2002 and Heidemann *et al.* 2001). The current experiment is significant because $HgCl_2$ has been found to have degenerative effects on the C.N.S. according to (Counter *et al*. 2004 and Augusto de Melo Reis *et al.* 2007) and with studying a specific area or single structure of the neuron we can then determine if $HgCl_2$ has specific effects on a single structure. From these findings we can conduct further and more specific experiments targeting areas of interest such as other organelles or intracellular structures.

In collaboration with B. Rossetti, A. Rawson, and M. Ophir, I will be testing $HgCl_2$ effect on F-actin in 9-11 day old chick embryo sympathetic neurons. Similar experiments have studied effects of $HgCl_2$ in mice and found MeHg decreases an important antioxidant in our bodies (glutathione), which in turn increases MeHg concentrations in neurons (Kaur *et al*. 2006). Another similar study Heidemann *et al*. in (2001) found that MeHg did not kill 7-8 day chick embryo forebrain neurons *in vitro* but inhibited axonal morphogenesis. Based on results from other experiments I aimed my hypothesis at addressing that $HgCl_2$ disrupted F-actin polymerization in axons in 9-11 day chick embryos sympathetic neurons. Each member of this team conducted the same experiment measuring different cells and different actin filaments. The different cells that were studied were G-actin and F-actin in glial cells and G-actin and F-actin in neurons. A. Rawson studied the ratio between the G-actin in glial cells and neurons, B. Rossetti studied the ratio of F-actin in glial cells in neurons, M. Ophir studied F-actin in glial cells and I studied F-actin in neurons. This method provided an adequate sample size for collecting data and provided an appropriate *in vitro* method for experimentation, which allow us to explore our interests with Hg and its effect on neuronal development.

MATERIALS AND METHODS

Materials

All materials for tissue culture were obtained from Sigma Chemical Co. (St. Louis, MO). Alexa Flour 568 phalloidin and deoxyribonuclease I Alexa Flour 488 conjugate were obtained from Invitrogen Molecular Probes Inc. (Eugene, OR). Chicken embryos were obtained from Charles River SPAFAS (Franklin, CT). The mercury chloride was provided by Dr. Jani Benoit, Wheaton College Department of Chemistry. Chick sympathetic neurons were dissected by Dr. Robert Morris, Wheaton College Department of Biology.

Primary Tissue Culture

Sympathetic chains and dorsal root ganglia were dissected from 9-10 day chick embryos and were dissociated and grown as single cells. Cells were plated on coverslips placed in small Petri dishes that were treated with poly-L-lysine for 1 -24 hours and laminin for 1-16 hours. The plated cells were covered with L-15 growth medium that consisted of (100mL L15, 2mM glutamine, 0.6% glucose, 100µg/mL pen/strep, 10% fetal bovine serum FBS, and 50ng/mL rat nerve growth factor) and kept in the incubator at 37°C for 24 hours as described in (Morris and Hollenbeck 1993).

Buffer Preparation

1L PBS buffer consisted of 40g of NaCl, 1g of KCl, 13.4g of $NaHPO_4$, 1.2g of K_2PO_4 in 400ml of H_2O. pH was adjusted to 7.4 by adding HCl and was tested by using a Diagger 5500 pH meter. Fixation/Permeabilized buffer was composed of, 1.2g sucrose, 1.62mL of 37% formaldehyde 0.12mL of 25% gluteraldehyde, 0.6mL of Triton X-100 and 0.3mL of 0.2M EGTA in 30mL of L-15 growth medium. Fixation/permeabilized was neutralized to pH of 7 by adding drops of sodium hydroxide. Fixation buffer consisted of the same amounts of sucrose, 37% formaldehyde, 25% gluteraldehyde as used for fixation/permeablization and 0.3mL of 0.2M of EGTA in 30mL of L-15 growth medium. Fixation buffer was neutralized to pH 7 by adding drops of sodium hydroxide and was tested with litmus paper. PBS/Triton X-100 buffer consisted of 2.5mL of 100% Triton X-100 in 500mL of PBS and was mixed vigorously. Block buffer consisted of 1.5g BSA in 50mL of PBS. Buffers were modified from (Jeffrey 2006), (Silverio, 2006).

Mercury Treatment

Mercury solutions were made on the day of experimentation and were kept in 15mL poly ethylene vials. For experimental (100nM $HgCl_2$ in 8mL HBSS) and control (100nM HCl in 8mL HBSS), coverslips were provided by Dr. Robert Morris.

Preparation of Neurons and Glial cells

9 already plated coverslips with live neurons and glial cells were obtained from the incubator on day of experiment. L-15 growth medium was removed from all 9 coverslips immediately after incubation. Upon removal of coverslips the 5 experimental coverslips each received 2mL of 100nM $HgCl_2$ and the 4 control coverslips each received 2mL of 100nM HCl using the P20 pipette. All coverslips were placed back into the incubator for 20 minutes. Coverslips were removed from incubator followed by mercury extraction, washed twice with HBSS, which replaced the mercury. After second wash, 2mL of fixation/permeablization was added and allowed to sit for 15 minutes. Fixation/ permeablization buffer was then removed and the coverslip was washed with PBS/Triton-X 100 twice. PBS/Triton-X100 was removed and the fixation buffer was then added to the coverslips for 15 minutes, after 15 minutes coverslips were washed with PBS. (All procedures with formaldehyde and gluteraldehyde were done under the hood in the biochemistry laboratory at Wheaton College Norton, MA). Block buffer was added to the coverslips and stayed in the buffer overnight following washes with PBS. Procedure was modified from (Jeffrey 2006) and (Silverio, 2006).

Fluorescent Double-Staining for G-actin and F-actin

Prior to fluorescent staining, 9 coverslip pedestals were constructed using epindorf tube caps that were freely attached to parafilm. The parafilm lined the bottom of 2 large Petri dishes. The Petri dish covers were covered in tinfoil to keep coverslip pedestals out of the light.

Coverslips with block buffer were washed twice with PBS and replaced by fluorescent solution which consisted of G-actin/F-actin fluorescent buffer consisted of 5.4µl of 5mg/mL of deoxyribonuclease I Alexa Flour 488 conjugate diluted to 3mL for final solution for 9 coverslips each coverslip contained 0.34µl of Alexa Flour 488 and 5µl of Alexa Flour 568 phalloidin. For 7 coverslip solutions, 5.4µl of Alexa Flour 488 and 75µl of Alexa Flour 568 were added to glass vial with 3mL PBS and were kept in the dark). 7 experimental coverslips were exposed to 200µl of fluorescent solution along with 1negative control, the negative experimental coverslip 1 exposed to PBS. Humidity chambers were constructed with water and Kimwipes and were located at opposite ends of Petri dishes. The coverslips were extracted from small Petri dishes with forceps and gently placed on pedestals. Cover slips were then exposed to the fluorescent and PBS solutions for 20 minutes; solutions were added by pipetting 2mL of each solution using the P1000 onto the coverslip that was placed onto the pedestal. Petri dish covers were immediately placed on dishes after. After 20 minutes fluorescent and PBS solutions were removed from the coverslips and placed back into small Petri dishes and washed with PBS.

After the coverslips were placed back into small Petri dishes each coverslip was placed on a microscope slide

and sealed with nail polish. Performed one at a time, each coverslip was taken out of PBS, extracted from small Petri dish, and placed on microscope slide with one drop of PBS solution that was placed in the middle of the slide. The coverslip was then carefully placed on microscope slide upside down. The coverslip was positioned on the microscope slide the edges dried (leave enough PBS under the slide or cells will dry out). The coverslip was centered and sealed with 2 coats of nail polish. All microscope slides were placed in desk drawer and given enough time for nail polish to completely dry. The slides were then placed in microslide box and stored in refrigerator at -20°C.

Fluorescence Microscopy

Fluorescent and phase images were captured on the Nikon 80*i* Eclipse microscope equipped with a SPOT RT Color CCD camera with a .76x mount by Diagnostic Instruments, Inc. in the Imaging Center for Undergraduate Collaboration (ICUC) at Wheaton College Norton, MA. Each image was capture using the Nikon Plan Flour 40x objective. SPOT software Mac: version 4.1 was the program used to adjust settings for imaging. For capturing images for phase mode set SPOT software to phase auto option and capture the image. For capturing images for G-actin (deoxyribonuclease I Alexa Flour 488 conjugate) the exposure time was set to 4.5 seconds, the gain was set at 4, and 24 Bits per pixel (RGB). For capturing images for F-actin (Alexa Flour 568 phalloidin) the exposure time was set at 0.4 seconds, the gain was set to 4, and 24 Bits per pixel (RGB). Each image was saved as a TIFF file.

Quantification of Fluorescent Images

Each image was collected in collaboration with M. Ophir, B. Rossetti, and A. Rawson. Images were opened in ImageJ version 1.32j software. For each image the background was subtracted by using the subtract background option with Rolling Ball radius of 50 with a white background selection. We converted each image to black and white mask by selecting the threshold option under binary menu. The same image was opened again, unchanged, in ImageJ where the image calculator option was selected and the two images were then superimposed. A region of interest was selected around the neuron and a histogram of the region of interest was calculated. The histogram was then pasted into an Excel document that calculated the brightness value (GV) of all the pixels except for 255 and the mean= Sum (GV*count)/Sum (count). This was repeated for each image.

Standardizing Images with CCD Camera

Nail polish was painted on a microscope slide and placed on the Nikon 80*i* Eclipse microscope. 34 pictures of the same image were taken using different exposures for the deoxyribonuclease I Alexa Flour 488 conjugate, the image was moved to a different area using the same exposure times. This procedure was performed 3 times for the deoxyribonuclease I Alexa Flour 488 conjugate and for the Alexa Flour 568 phalloidin but with different exposure times. Histograms were calculated in ImageJ and the mean was copied and pasted into an excel document where the

means were graphed. This produced a standard curve of brightness vs. exposure time that was used for normalizing our data.

RESULTS

Analysis of Standardizing Exposures in Fluorescently Labeled F-actin in Neurons

To determine if we can use the same exposure time to compare relative brightness we calculated an exposure curve for the green channel fluorescence (Alexa Flour 568 phalloidin). The same procedure was performed for the deoxyribonuclease I Alexa Flour 488 conjugate but in studying F-actin the curve did not serve any significance for the current because I did not study G-actin. We placed nail polish on a microscope slide and imaged the nail polish through the green channel where the image appeared red Figure 1. The nail polish was not labeled but did fluoresce green.

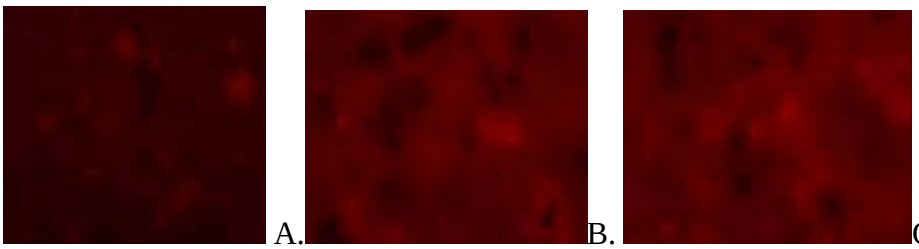


Figure 1. Different images of the same nail polish microscope slide captured at the same exposure time under the green fluorescent channel. Image A was the fist image captured at 0.3 seconds and is considerably darker than images B and C. Image B was the second image captured at 0.3 seconds is brighter than image but darker than image C and image C is most bright. This can be seen by looking at the dark specs in the nail polish there are less dark specs in image C than in image B and the both background in image B and C are brighter than image A.

In all 29 images of each picture were imaged at different exposures, Figure 1 represents only one exposure time for each trial. The exposures ranged from 0.05 to 0.3 seconds and can be seen in Figure 2. Figure 2 represents all 29 images of for each trial at different exposure times, which were calculated by taking the mean GV of the histogram and plotting it along the y-axis and plotting the exposure time in milliseconds.

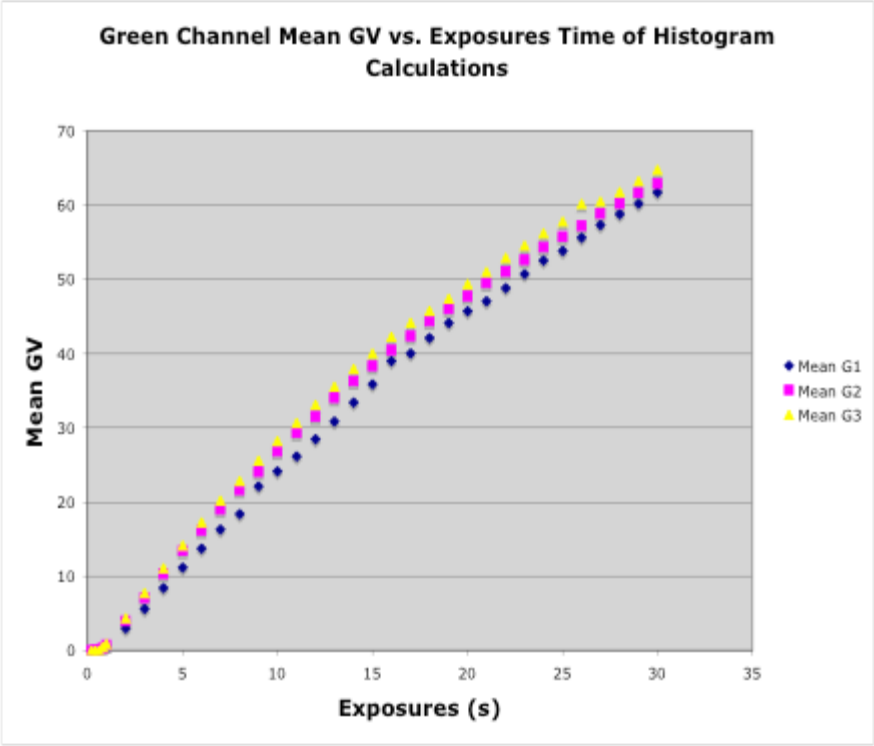
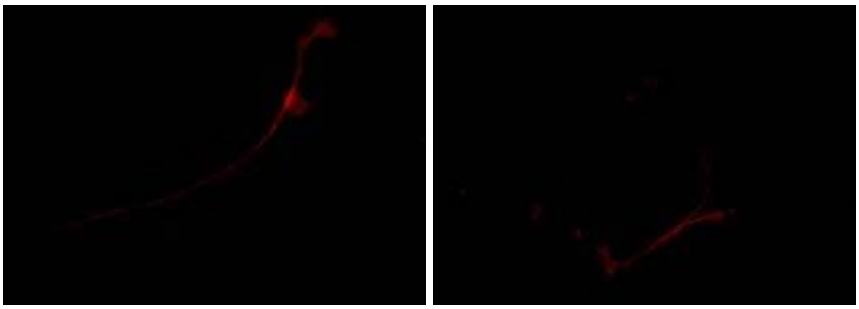


Figure 2. This is a graph of histogram calculations of green channel that were performed in ImageJ and the mean GV were pasted into an excel document. We see a logistic curve rather than a linear relationship between the means. The longer the exposure time the further the curves deviate from each other. Mean 1, the blue curve was the first trial, the pink curve was the second and the yellow curve was the third, each time the curves were brighter than the last. The curves did follow a similar path and stayed relative to each other despite separations. This means that each image taken even if at different exposure times could be compared to another image taken at a different exposure time.

Analyzing Fluorescently Labeled F-actin in Neurons

Both images the axons have more brightness than the cell body; some axons however are difficult to see because they are long and narrow. The cell bodies are easy to recognize, round in shape and are usually less bright but have the same consistency of brightness. The branching patterns are similar, we found 2 to 3 very long prominent axons projection from a cell body. Both images look very similar when considering brightness.



Figures 3 and 4. Control (left) and $HgCl_2$ treated (Right) 10-day chick sympathetic neurons. Both images have long axons with a prominent cell body and 2 or 3 axons projecting away from the cell body. Both images appear to have the same brightness.

Two coverslips were the negative controls, which were completely black, three control coverslips accounting for 27 images with one or more neurons in the image, and four experimental coverslips accounting for 40 images with one or more neurons in the image. These images were all calculated the same and graphed the same, which can be seen in

Figure 5. There is no significant difference between the average mean GV for the control and for the experimental as shown by the standard deviation.

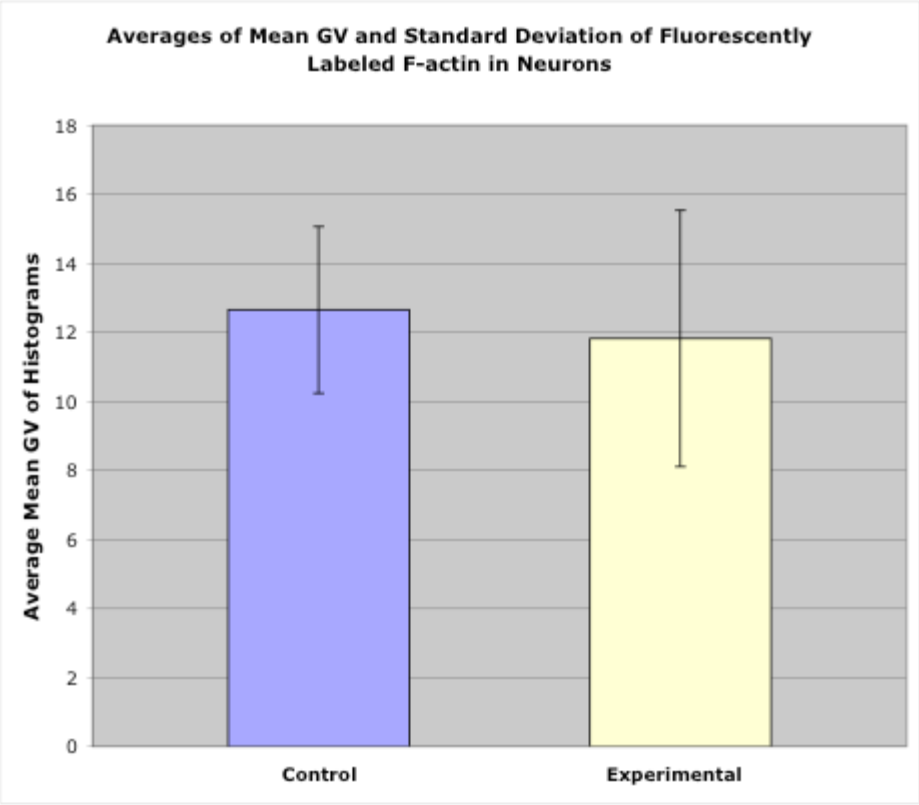


Figure 5. The graph represents the averages of the mean GV of the fluorescently labeled F-actin in 10-day chick embryo sympathetic neurons. Both data sets are similar as shown by the standard deviation lines.

DISCUSSION

Analysis of 100nM HgCl on Brightness of Fluorescent F-actin in 10-day Chick Embryo Sympathetic Neurons

After analyzing all the data we refute our hypothesis that treatment of 100nM $HgCl_2$ has an immediate effect on F-actin in 10-day chick embryo sympathetic neurons and rather the data suggests the 100nM $HgCl_2$ has no effect on brightness of F-actin in 10-day chick embryo sympathetic neurons. The evidence for refuting our hypothesis can be seen in figure 5 where the control and experimental averages of the mean GV were similar differing by 1.1%. The mean GV only calculated the number pixels at a specific brightness on a scale from 0 to 254 and the mean GV was the average of the number of pixels that appeared in the images. The standard deviation reinforced the idea that 100nM $HgCl_2$ does not affect the F-actin, because the deviations overlapped each average mean GV suggesting again the data were not different.

The experimental data are not statistically significant because there was only one trial, if we were to conduct this experiment again and find the same results then we would call these data statistically significant. Furthermore, if the experimental data were statistically significant then 100nM $HgCl_2$ is not a high enough dose to illicit any effect on brightness of F-actin in 10-day chick embryo sympathetic neurons. 100nM $HgCl_2$ is also a safe amount of mercury that a 10-day chick embryo can be exposed to without any effect on brightness of F-actin in sympathetic neurons.

As explained in (Huang *et al* 1996) $HgCl_2$ is membrane permeable to living cells. Our understanding is that in fixed cells the membrane has been removed allowing full exposure of $HgCl_2$ to intracellular structures, which resulted in a 1.1% difference in mean GV of brightness (Figure 5). Therefore our assumptions for any effect $HgCl_2$ has on membrane permeability can be refuted. Outside the cells $HgCl_2$ binds to hydroxyl groups in high extracellular pH conditions (Huang *et al*.1996). In the current experiment pH was tested for Fix, Fix/perm and PBS buffers both were basic 7.0 and 7.4 respectively. The buffers were used to wash and fix neurons and from this data we assume that the extracellular pH was also basic, in which $HgCl_2$ in a basic environment would illicit similar results as the $HgCl_2$ with hydroxyl groups (Huang *et al*.1996). Extracellular pH is also known to affect specific ligand-gated ion channels such as K^+, Na^+, and Cl^- (Huang *et al*. 1996). Ebner *et al*. (2005) found that Cl^- may have an effect of F-actin in cell volume changes. This is important because increased amounts of Cl^- may affect the shape of the cell and therefore the structure of F-actin in the presence of $HgCl_2$. However, the results suggested that there was no difference in brightness found between the control and experimental groups. For a dimmer image we would assume that $HgCl_2$ denatures the both G/F-actin and thus being less sites for the fluorescent dye to bind.

For sources of error, 255 was deleted from mean GV so that any pixels with a brightness value of 255 were not calculated in the histogram and only 0 to 254 were calculated in the histogram. The value of 255 is the brightest pixel in the image. The reason for deleting this was to be able to use the 255 value as a template to accurately measure the F-actin in the image. Washing the coverslips more carefully may have allowed more cells to stay attached to the coverslips altering the number of cells viewed on each slide. Longer exposure to the fluorescent light photo-bleaches the image thus dimming the fluorescent labels. The selection of neurons were control by the experimenters and not randomly, with a random selection method our data cannot be bias and more accurate.

In refining this experiment allowing more time for quantitation and analysis would be beneficial. Future experiments could measure F-actin with higher concentrations of $HgCl_2$ and other kinds of Hg in 10-day chick embryo sympathetic neurons.

Analysis of Exposure Standard Curve of Green Channel Fluorescence

An exposure standard curve was created to determine if the same exposure time could be compared to relative brightness. This curve represents that at different exposure times each image stays at relatively the same brightness and can be quantified as a group. If we did not construct this standard curve we could not state the data were subjected to the same experimentation and there not pool our data.

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