The abundance of mitochondria in growth cones of chick embryos when exposed to different levels of methylmercury

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Introduction

Methylmercury is a neurotoxin that has infiltrated many levels of the food chain causing it to be prominent in the diets of humans. The bioaccumulation of methylmercury is especially dangerous because it has the ability to pass through the blood brain barrier, when present in humans, and disrupts important neurological mechanisms. The effect of the disrupted neurological processes include negative impacts on cognitive thinking, memory, attention, language, and fine motor skills (Hasset-Sipple, 1997). Exposure to methylmercury in developing neurons have an even more disastrous effect and can lead to impaired vision, hearing, walking, and an increased risk of fetal brain damage. However, the listed symptoms are present when there is a great amount of methylmercury exposure to developing brains of humans (Castoldi, 2001). Today there is still disagreement over the effects of exposure to lesser amounts of methylmercury but there is little debate over the fact that understanding the mechanism behind the debilitating effects of methylmercury is of great importance.

An important process of neuronal development is the extension of axons from the cell body that leads to synaptic connections between other neurons and cells. The extension of axons is lead by the growth cone, and the elongation of microtubules and actin. Not only is the support structure for the cell assisting in creating neuron pathways but also helping other organelles, such as mitochondria and endoplasmic reticulum, traverse the axon and reach the growth cone in order to provide further support to the growth cone (Miura 2000). In fact, a large amount of mitochondria are dispersed throughout active growth cones but if the growth cone becomes inactive the presence of mitochondria moves to the axon. (Hollenbeck, 1996). There is strong evidence that when methylmercury is introduced to the dorsal root ganglia of chick dorsal root ganglia growth is inhibited due to the sensitivity of microtubules to methylmercury (Miura, 2000).

Unfortunately many studies do not focus on the influence of methylmercury on mitochondria brought to the

growth cone by microtubules. Mitochondria are very important in providing energy for axon growth and extension; therefore it is very likely that the mitochondria are heavily influenced by exposure to methylmercury, just as microtubules are. The research done by Miura, Himeno, Koide, and Imura, N.in 2000 also includes impacts of inorganic mercury, which has a strong effect on the plasma membrane barrier of the cell. A membrane similar to the plasma membrane of the cell also protects mitochondria; therefore the effect of mehtylmercury on the membrane of mitochondria might be similar to what is described in the study (Miura, 2000). With the information available it is very likely that the abundance mitochondria in growth cones of developing nerve cells that are exposed to methyl will decrease over time, and will decrease as the dose of methylmercury increases.

The following experiment takes advantage of chick embryos as a model organism for the human nervous system due to the availability and relative ease of using chick embryos, or *Gullus gullus*. Chicken eggs are always available throughout the year, are relatively inexpensive and can be incubated to any stage of interest, which allows experimenters to have more freedom within their schedules and allows more flexibility in the timing of the experiment. The development of the nervous system of chickens also develops quickly (within 2 to 3 days), and after removing the chick embryo from the egg it is fairly easy to see through the skin into the internal tissues of the chick (Darnell, 2000).

The research outlined below tested the hypothesis that the abundance of mitochondria in growth cones of developing neuron cells, harvested from *Gullus gullus,* would decrease as the amount of methylmercury is increased. There will be two different conditions in the experiment where cells will be grown on coverslips, one coverslip with developing neurons will be exposed to no methylmercury, and the second coverslip with developing neurons will be exposed to a high dose of methylmercury. The cells will then be exposed to a fluorescent dye that stains the mitochondria specifically, the results will be examined and captured under a microscope and the brightness of the mitochondria will be analyzed to determine the abundance of these organelles within the growth cones.

Materials and Methods

Culture Preparation

The ganglia cells and sympathetic nerve chains used in the lab were dissected from chick embryos and placed on previously cleaned coverslips following the procedure outlined by Morris under the subheading **Preparation Day steps** with some slight modifications. The coverslips had been previously treated with polylysine for three hours and then treated with laminin for two hours. The medium used was C medium that includes Leibovitz Ll 5 medium plus 0.5% methylcellulose, 10% fetal calf serum, 0.6% glucose, 2 mM L-glutamine, 100 ug/ml streptomycin, 100 U/ml penicillin,

and 10-50 ng/ml nerve growth factor (Morris, Preparation Day steps, 2014). Approximately 40 dorsal root ganglia cells and 4 sympathetic nervous chains were harvested from chicken embryos. The coverslips also underwent incubation during chemical treatment (Morris, 2014, Dissection part 1).

Control Culture and Staining

There will be two different cultures: a control and a high dose. The coverslip and culture for the control is assembled normally following parts one through four of the coverslip treatment procedure within the Dissection procedure (Morris, 2014, part 1-4). When beginning to make an observational chamber the growth medium is taken out and pipettes into large petri dish. Afterward 1 mL of Dulbecco's Modified Eagle Medium (DMEM) is added along with 1 mL of green MitoTracker as a fluorescent dye in order for the mitochondria to be visible (Vital-stain Fluorescence Microscopy, Morris, 2014, part A). After adding the green MitoTracker to the petri dish the coverslip was incubated for 15 minutes. After incubating the DMEM and green MitoTracker solution was removed and the coverslip was washed three times with DMEM. An observational chamber was then created using cover slip chips and vasoline laminin parafilm (VALAP) was painted onto the edges of the coverslip, similar to the steps given by the Staining and Observation of Live Cells procedure, however all four sides were covered with VALAP to make an observational chamber rather than just two.

Experimental Culture and Staining

The same procedure for the control culture was used to create the experimental culture with the high dose of methylmercury except 1 mL of MeHg of 10 nM MeHg was added with 1 mL of green MitoTracker. For the low dose 0.5 mL of MeHg was added with 1 mL of green MitoTracker (Morris, 2014).

Data Analysis

In order to collect data a bifocal Nikon Eclipse E200 microscope was be used to take an image of the brightness of the mitochondria presumably flowing from the axon to the growth cone. The exposure will take on a range of 64.46 to 525.5 in order for there to be optimal imaging quality and for clarity of the image. A bar graph comparing the brightness of pixels between each culture measured using ImageJ for each dose was used in order to clearly show the differences in mitochondrial abundance between each culture. In order for the data to be comparable an average for the background values was taken using the values given in the program being used to analyze the pictures (ImageJ 1.48u4). An average of the brightness of pixels that were considered bright enough to be counted as mitochondria was then calculated and termed the brightness threshold.

A histogram plot was created next by looking at the options under the "Analyze" menu and choosing "Histogram." There should be a button under the histogram plot labeled "List." After clicking this button a list of pixel

values should be given in the left column and of the number of pixels associated with the pixel values should be given in the right column. The brightness threshold value could be found in the right column, all of the values listed in the right column that correspond to the values listed under the brightness threshold in the left column were added together. A percentage was then found using the added values of the right column and dividing by the total number of pixels and multiplying by 100. The program ImageJ version 1.48u4 was used to make the histogram by outlining a specific area of the picture, looking at the options under the "Analyze" menu and choosing "Histogram."

Results

Table 1

The following table describes the difference in the abundance of mitochondria based on the brightness of the mitochondria in images. The table shows a great difference in percentage of bright pixels within the growth cone region between the control and high dose group. The data was gathered using the values from the histogram plot created through the program ImageJ.

	Control	High Dose
Average Background Pixel Value	3	4
Mitochondria Threshold	31	32
Pixel Count Above Threshold	866	272
Percentage	99.8	42.3

**Figure 1- Fluorescent (bottom) and Bright-Field (top) Image of Control Cells**

The above image shows a growth cone extending from a neural cell (top right) to a glial cell (bottom left) after exposure to a high dose of methylmercury. The yellow arrow is highlighting the area used to find the average background pixel value, the pink arrow is highlighting the area used to set the threshold for what would be counted as bright and the yellow amorphous circle is where the growth cone was found based on the transmitted light image. The image was captured by a Nikon 1.0 C-mount Eclipse E200 at a magnification of 40X and InSight camera, captured by Sydney Damian-Loring and Lindsey Gillis
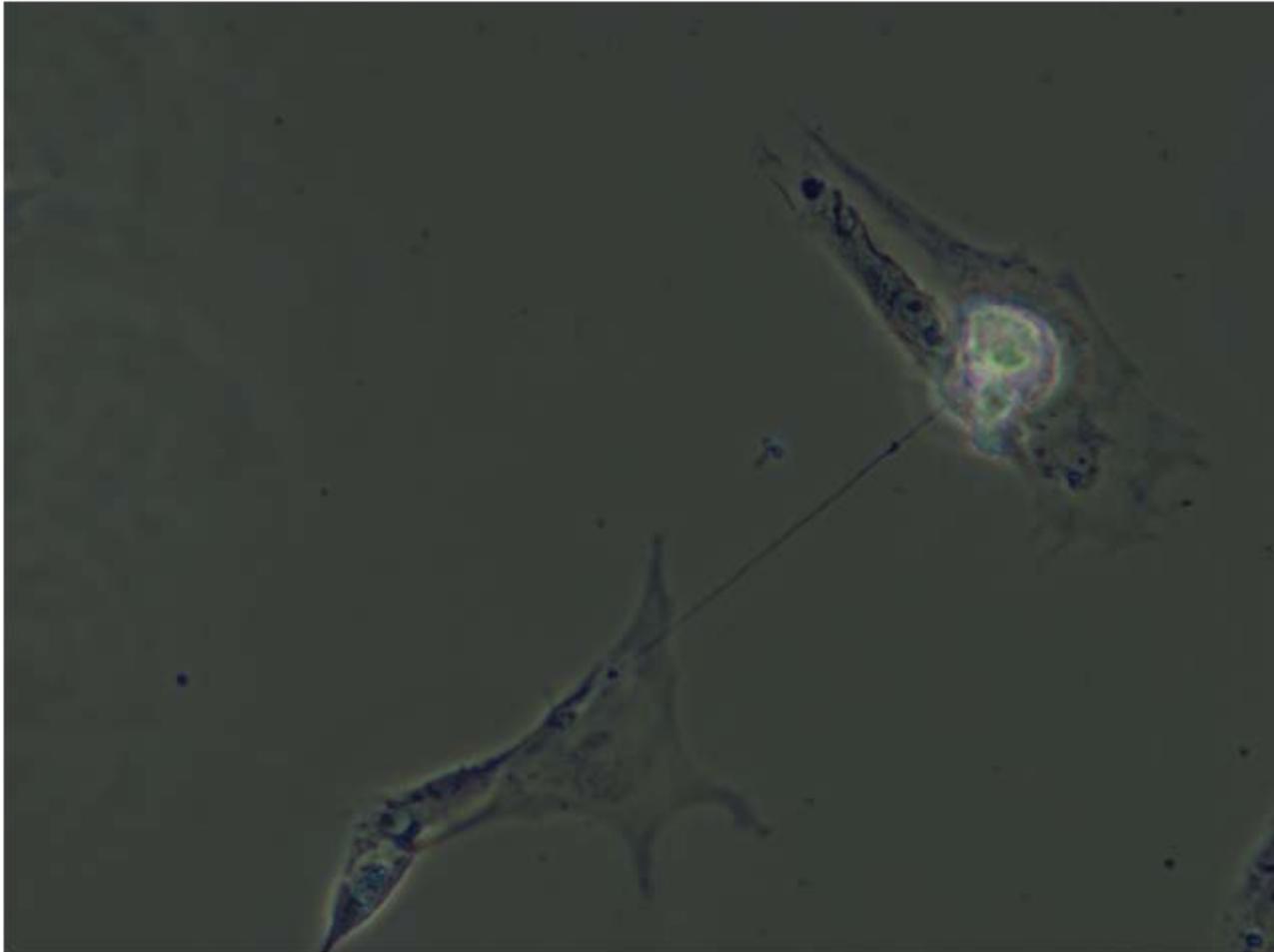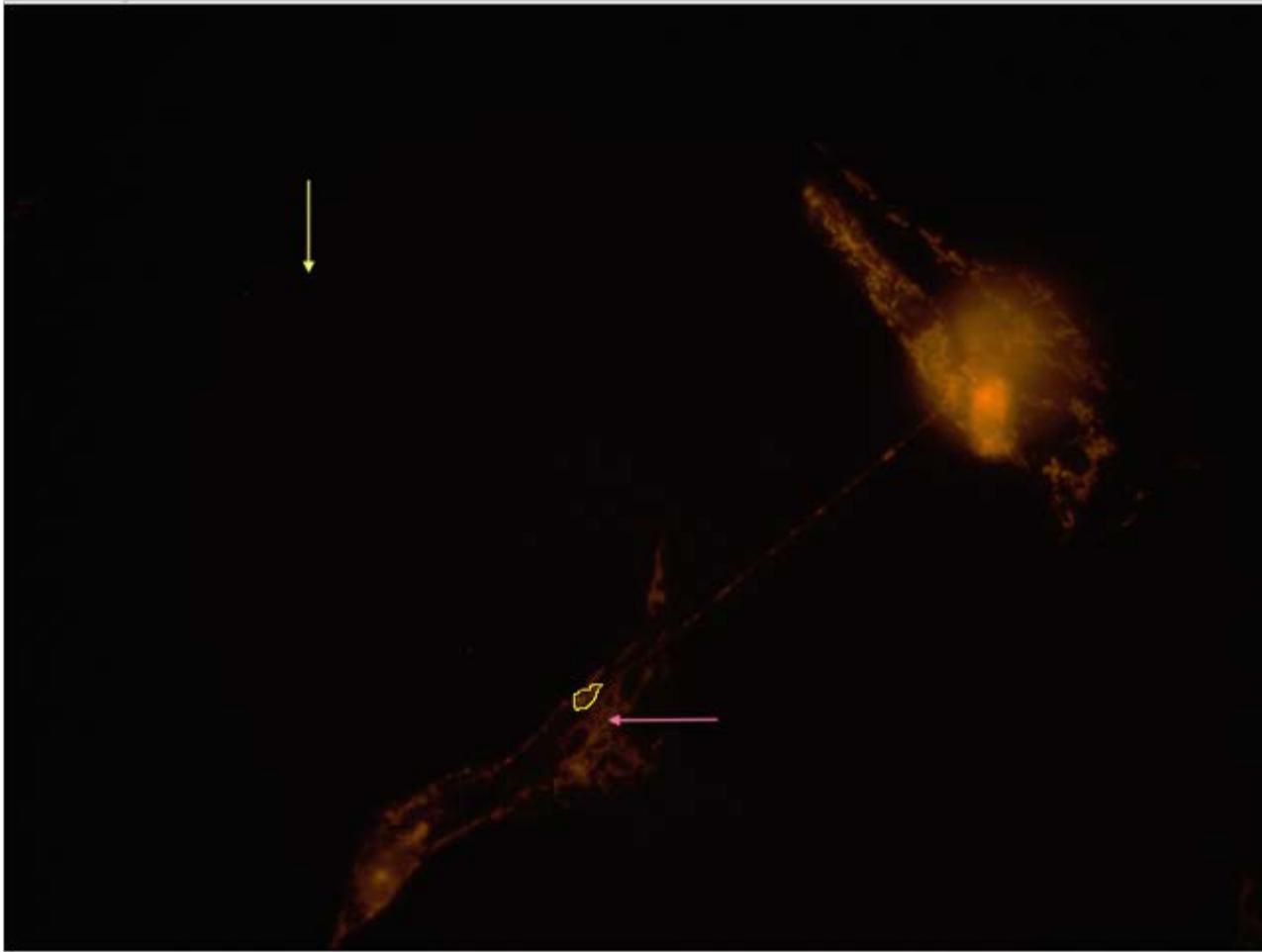
Figure 2-Fluorescent (bottom) and Bright-Field (top) Image of High Dose Cells

The above image shows a growth cone extending from a neural cell (top right) to a glial cell (bottom left) after exposure to a high dose of methylmercury. The yellow arrow is highlighting the area used to find the average background pixel value, the pink arrow is highlighting the area used to set the threshold for what would be counted as bright and the yellow amorphous circle is where the growth cone was found based on the transmitted light image. The image was captured by a Nikon 1.0 C-mount Eclipse E200 at a magnification of 40X and InSight camera, captured by Sydney Damian-Loring and Lindsey Gillis.
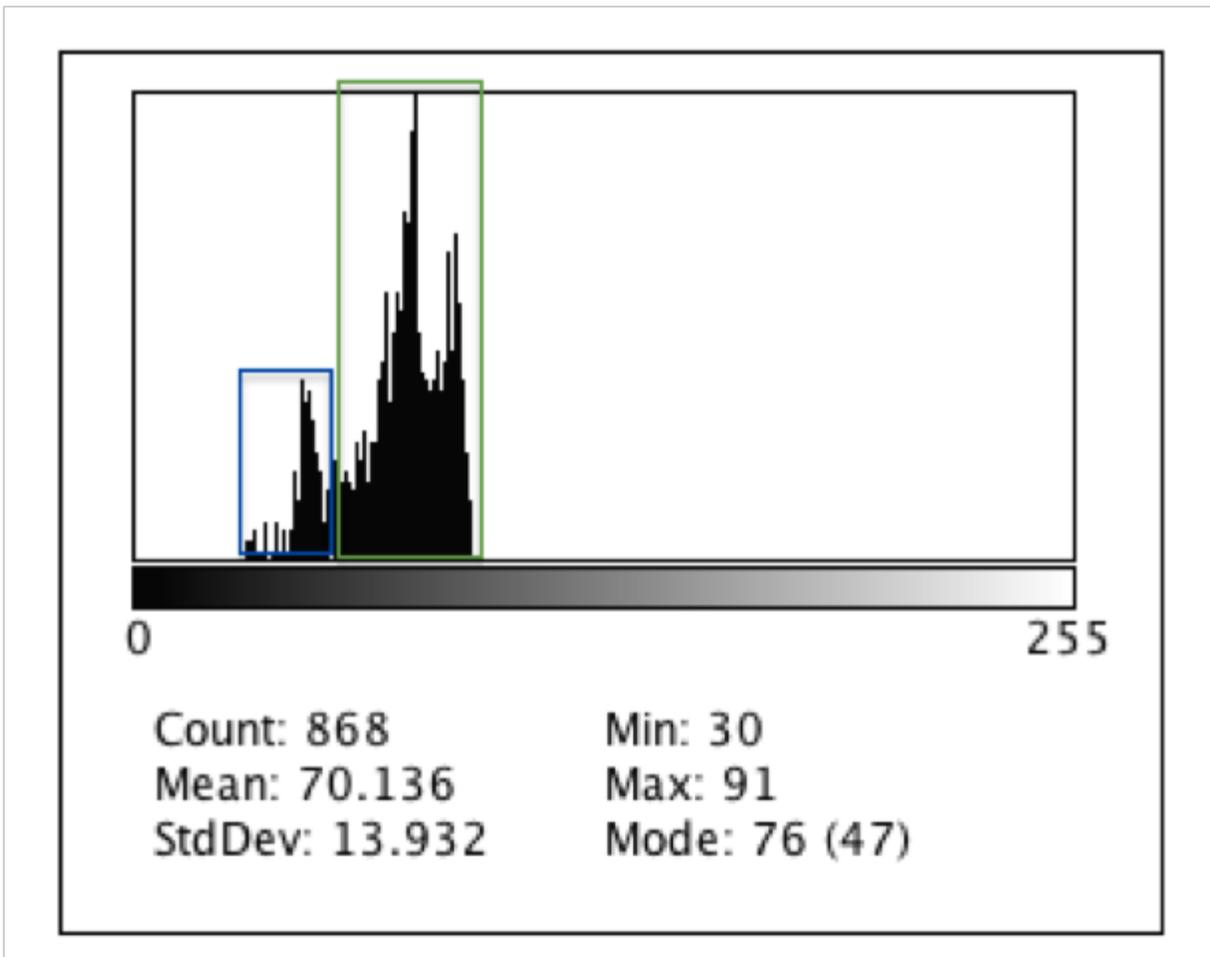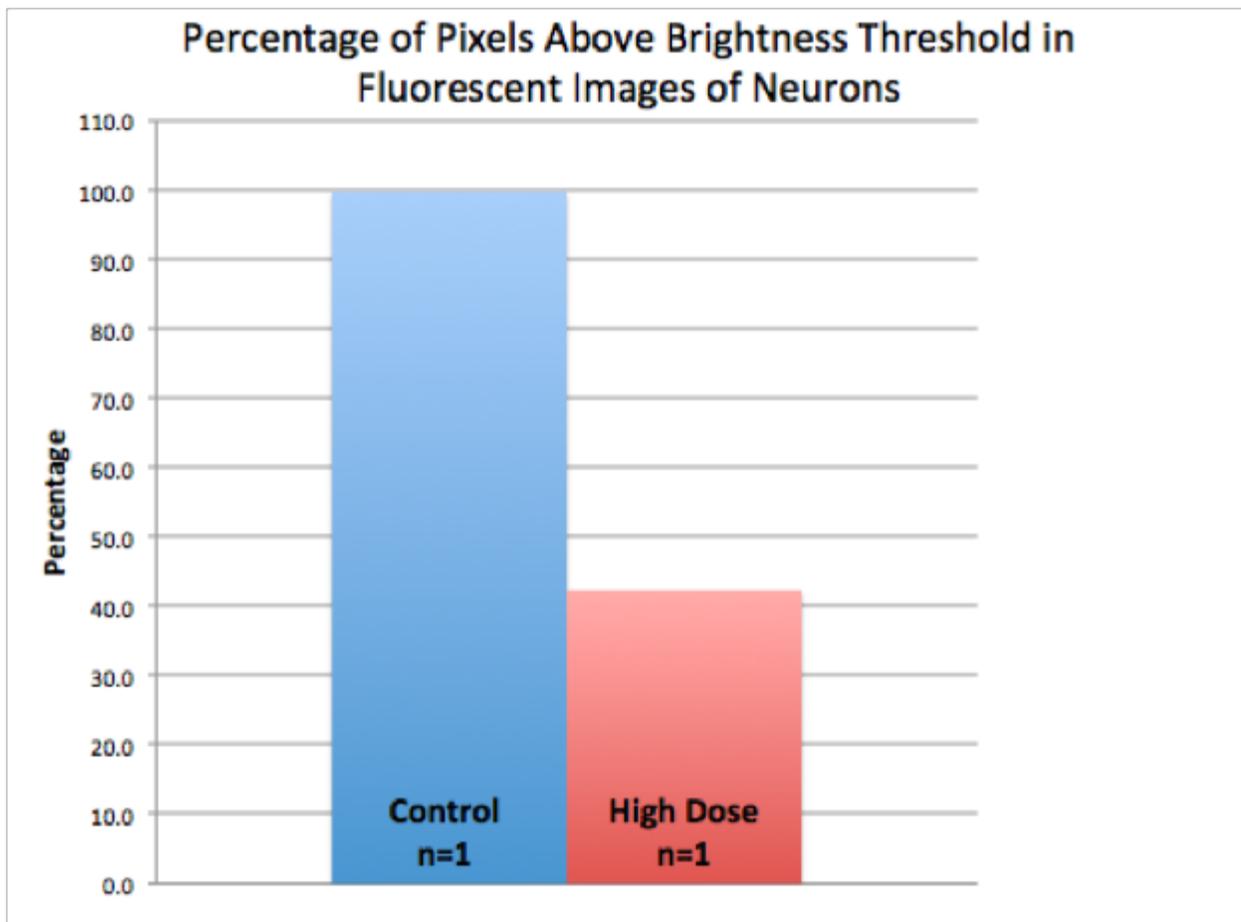
Figure 3

The histogram above shows the count (868) used to calculate the percentage of bright pixels of the control image. The groups of peaks at the beginning (encased in a blue box) indicate the brightness of pixels in the background of the image (pointed out by the yellow arrow in Figures 1 and 2) and the second grouping of peaks (encased by the green box) illustrates the brightness of the pixels of the neuron. The program ImageJ was used to make the histogram by outlining a specific area of the picture, looking at the options under the "Analyze" menu and choosing "Histogram."
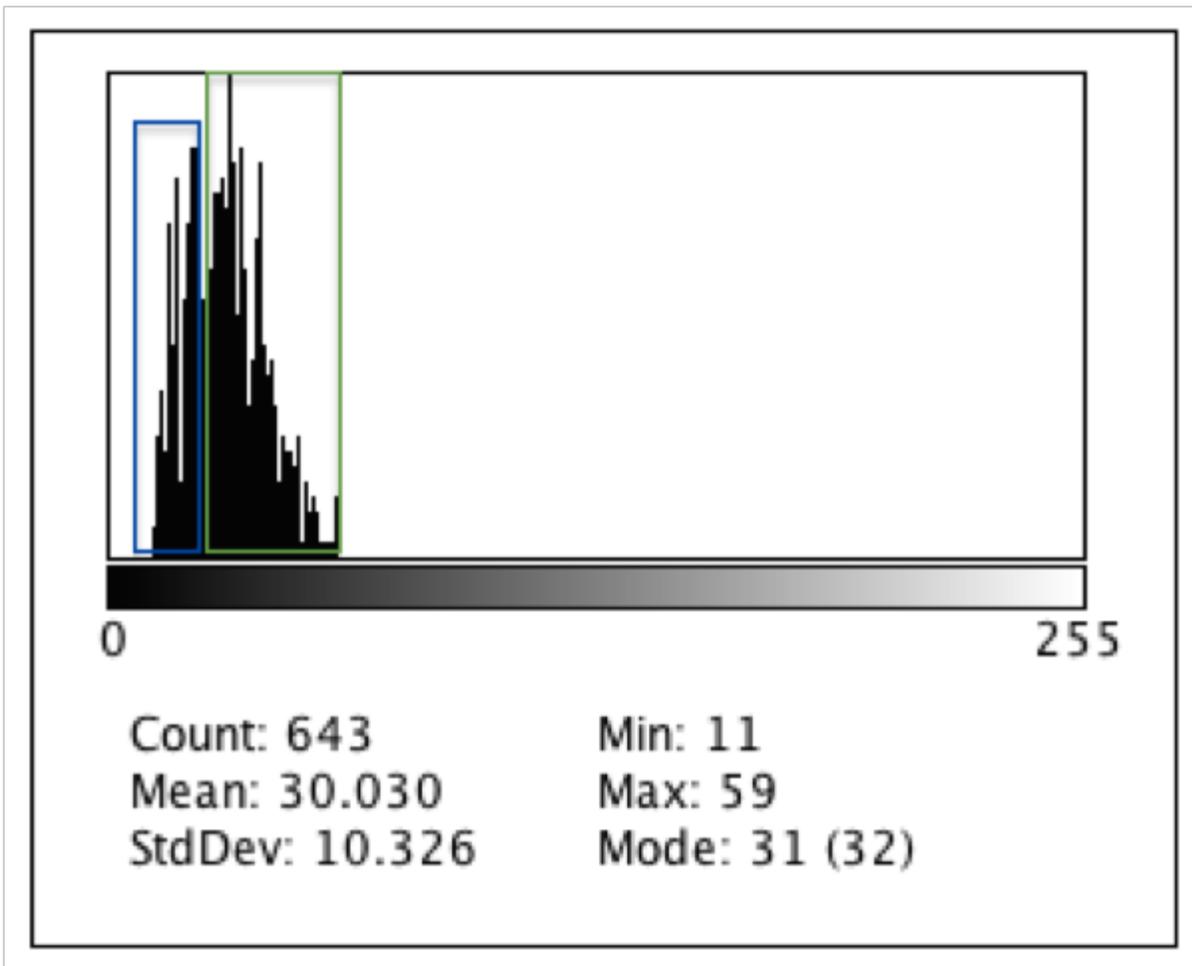
Figure 4

The histogram above shows the count (868) used to calculate the percentage of bright pixels of the high dose image. The groups of peaks at the beginning (encased by the blue box) indicate the brightness of pixels in the background of the image (pointed out by the yellow arrow in Figures 1 and 2) and the second grouping of peaks (encased by the green box) illustrates the brightness of the pixels of the neuron. The program ImageJ was used to make the histogram by outlining a specific area of the picture, looking at the options under the "Analyze" menu and choosing "Histogram."

Percentage of Pixels Above Brightness Threshold in Fluorescent Images of Neurons

Figure 5

For each sample the value of n=1 because only one cell was present within the sample and only one growth cone of one cell was measured in each case. There is a great difference between the control group and the high dose group concerning the total value for brightness. Overall brightness percentage decreased when the cells were exposed to a high dose of methylmercury.

Discussion and Conclusions

The data analyzed fully supported the hypothesis, which was that the abundance mitochondria in the growth cones of developing nerve cells that are exposed to methylmercury will decrease as the dose of methylmercury increases. The data showed that the control group had a higher percentage of bright pixels when looking at the growth cone specifically, especially when compared to the high dose group. The higher percentage of bright pixels implies that there is a greater abundance of fluorescence and a greater number of mitochondria. An issue with the data analysis is that the brightness may indicate the number of mitochondria present but it is also an indication of mitochondrial metabolic activity. The data could be a representative of a few bright mitochondria rather than a lot of mitochondria with a similar

brightness all grouped together. To combat this issue future experiments should use different microscope settings when focusing on the cells.

The mechanism that causes such a disproportion is not entirely known however it seems that mitochondria may undergo ultrastructural changes in other areas of developing neurons that are exposed to methylmercury. It is very likely that similar changes occur in the growth cones observed above, however the study by O'Kusky only focused on the axons, dendrites, and presynaptic terminals, and O'Kusky had the ability to use electron microscopy to examine the developing cells. Once exposed to mercury the mitochondria increase in abundance in axons (O'Kusky, 1983). If there were similar equipment available during the present study it may have been possible to study the abundance and behavior of the mitochondria. It would also make sense to examine the number of mitochondria in the axons too.

In the future the experiments that include exposure of neurons to methylmercury should include a time component in order to analyze the difference that different developmental stages have on mitochondrial abundance in growth cones. Future studies could also take into account the amount of time that the cells were exposed to methylmercury and look at the development of growth cones and presence of mitochondria over days rather than hours. Many studies also involve the use of neural cells from rats, it would be interesting to see if there are any derivations in data based on what animal the cell came from (Yee, 1996). It would also be helpful to know the effect of methylmerury on the abundance of mitochondria in growth cones over days at a time rather than hours at a time. If this study were to be done again the researchers should manipulate the period of exposure along with the concentration of methylmercury the cells are being exposed to.

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The data was gathered above with Lindsey Gillis