Evidence that mercury has no immediate effect on the velocity of mitochondrial movement along the axon of chick peripheral neurons

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Introduction

Neurons are unlike other cells in many ways, and one of their most defining characteristics is their unique shape. Nerve cells consist of a nerve body with arm-like extensions protruding away from this body. These extensions are called axons. Most of the cytoplasm of a neuron exists within the axon, yet these axons a lack synthetic capacity and are supported by the bidirectional traffic of organelles between the cell body and the terminal region (Morris et al, 1993).

Mitochondria are organelles that supply energy, in the form of ATP, to cells. They are abundant within neurons, and are large enough to be easily identified with adequate magnification. Mitochondria travel in a linear path within the constrained length of the axon (Morris et al, 1993). Mitochondria move in retrograde and antrograde directions. Retrograde movement refers to the movement of the organelle towards the cell body of the neuron, while antrograde movement refers to the movement of the organelle towards to nerve terminal (Karpow, 2008).

Mercury (Hg) is a non-radioactive toxic element and comes in a variety of forms. The three main forms of mercury are Elemental Mercury, Ionic Mercury, and Methyl Mercury (Benoit, 2008). Elemental Mercury (Hg(l) or Hg(g)) is used as a catalyst, and also has been used in thermometers, fluorescent lights, and dental amalgams. Dental amalgams were a widespread way to fill tooth cavities, and then the risk of ingesting mercury vapors became a known threat to the human brain. Due to the fact that Elemental Mercury can lead to brain damage, its use in dental settings has been banned (Benoit, 2008). Ionic mercury ($HgCl_2$) is a rapidly processed form of the element that is processed in the body and excreted through the kidney. Ionic mercury is most noted for its contribution to kidney damage because it is processed there. Although Ionic Mercury doesn't bioaccumulate (meaning accumulate in the body over time with more exposure), it also produces neurological affects because it can cross the blood-brain barrier (Benoit, 2008). The third form of mercury is Methyl Mercury (CH_3Hg^+) which is known best for its concentration in predatory fish such as tuna, shark, and swordfish. Methyl Mercury finds its way into oceans and lakes through the burning of fossil fuels,

specifically coal (Benoit, 2008). In a ten acre lake, only a half a gram of mercury would warrant the establishment of a fish advisory (Connealy, 2006). Methyl Mercury is a neurotoxin and targets the brain because it readily crosses the blood brain barrier, especially the fetal brain (Benoit, 2008). Maternal exposure to Methyl Mercury can lead to deficits in motor function of the child and also in memory and other forms of development (Benoit, 2008). The Environmental Protection Agency refers to cases where a mother has been exposed to a high dose of mercury while pregnant, then gave birth to children with nervous system malfunction (EPA, 2007).

Acute exposure to mercury refers to one exposure of the toxin at a high dose in a short period of time. I was interested in testing to find if an acute exposure of Ionic Mercury had any effect on the way a neuron functioned, particularly in the way that it transported its mitochondria. Mitochondria were the chosen organelle for study not only for the fact that they are easily identified due to their size relative to the diameter of the axon along which they are traveling, but also because of their ability to produce ATP (Morris et al, 1993). Neurons position mitochondria in parts of the axon where there is a great need for ATP (energy). In the growing axons of growing peripheral neurons, the active growth cone requires intense ATP consumption. The growth cone is so distant from the cell body, that it requires ATP through the positioning of mitochondria, which are regulated through axonal transport (Morris et al, 1993). Mitochondria are needed to fuel the growth cone, and I hypothesized that an acute dose of Ionic Mercury would immediately affect chick peripheral neurons by slowing down mitochondrial movement (velocity) along the axon of the neuron. This retard in the mitochondrial velocity would be due the affect of Ionic Mercury on the nervous system, more specifically Ionic Mercury's ability to easily cross the blood brain barrier (Benoit, 2008). By using chick peripheral neurons in the experiment, I would be removing the blood brain barrier all together, hopefully showing the effect that Mercury would have on the function of neurons in *Gallus gallus* (much like how it would affect a human fetus). My research team also consisted of Danica Peterson and Celeste Karpow.

Materials and Methods

Primary Culture of 10 Day Chick Embryo Peripheral Neurons

I made sure that the wider end of a 10 day chicken egg was pointed face up. This created an air space on the top section of the egg. I squirted the entire egg with 70% ethyl alcohol (EtOH) for sterilization purposes. I cracked the wide end of the egg with a pair of sterilized sharp forceps. As soon as there was a slight crack or hole in the egg, I used the forceps to lift away the small cracked parts so that the top part of the shell could be lifted away, and the embryo exposed. The head of the chick was separated from the body by snipping it off with the sharp forceps. The body was

lifted with the forceps and placed in a Petri dish filled with “Hank’s Buffered Saline Solution (HBSS).” Using a sterile pasteur pipette, the body of the embryo was rinsed several times with HBSS. With sharp forceps, I removed the limbs, and I set the body of the embryo ventral side up and removed all tissue that was covering the spinal cord. This was a process was done carefully, as to not pull away the sympathetic nerve chain or dorsal root ganglia. The sympathetic chains are hard to see and easily ripped, so they should be teased away from each side of the spinal cord. Dorsal root ganglia can be ripped off by their tails and are much easier to see because they resemble tiny balls. The sympathetic nerve chains and the dorsal root ganglia were placed in a separate 25mm Petri dish filled with HBSS. The embryo can be rinsed with HBSS using a sterile pasteur pipette at any time during the dissection (Hollenbeck and Morris).

The ganglia, which consisted of sympathetic nerve chains and dorsal root ganglia were rinsed thoroughly with HBSS. I filled the Petri dish that they were set in with HBSS, and then removed the ganglia with another sterile pasteur pipette. The HBSS was replaced with a trypsin solution that was Ca/Mg free HBSS containing 0.25% trypsin. The ganglia can then be left in a 37 degree Celsius incubator for 1 to 24 hours. Carefully the trypsin was taken from the 25mm Petri dish and HBSS was added again (about 1 drop per ganglion) and titrated gently with a drawn pasteur pipette until the ganglion were dissociated into separate cells (Hollenbeck and Morris).

The neurons will grow out best if they are set on a cover slip that is first coated in laminin. Before use, cover slips were rinsed with pure ethanol and then wiped back and forth with a kimwipe. They were baked at a temperature of 200 degrees Celsius for two hours. The surface of a sterile and clean 22mm cover slip was first coated with 1 mg/ml poly-L-lysine and set aside for 20-30 minutes. It was then rinsed with distilled water and allowed adequate time to dry after being placed poly-L-lysine side up on the edge of a 25mm Petri dish. I then lifted the cover slip and washed it with HBSS, because water would have denatured the cells. I set the cover slip in a dish of growth medium, which was called L-15 (contained amino acids, glucose, nerve growth factor, and fetal bovine serum). The surface of the cover slip was then coated with a solution of laminin in HBSS for at least 30 minutes. The cover slips were left in a dish of HBSS (Hollenbeck and Morris).

Making up the Rhodamine 123 Solution for Florescence

I obtained a solution with 1mg/ml Rhodamine 123 in DMSO. This solution was diluted 1:1000 Rhodamine 123 in growth medium. In a test tube I combined 5µL of Rhodamine 123 with 5ml of growth medium (final concentration). I covered the test tube with tin foil to prevent bleaching and set aside for later use (Bhatia, 2006).

Creating Control and Experimental Slides

I obtained Petri dishes with dissociated neurons on cover slips that were immersed in growth medium. With a pasteur pipette, I removed the growth medium from all Petri dishes. Two solutions were made up. One was a *control* solution, which contained a solution of HBSS in 1.5% HCL. The other was an *experimental* solution, which contained 10nm HgCl2 + HBSS in 0.5% HCL. In control Petri dishes, I added the control solution and was sure to label the Petri dishes because the control and experimental solutions looked the same. In the experimental Petri dishes, I added the experimental Hg solution and labeled these dishes as well. The control and experimental dishes were then incubated in a 37 degree incubator for 20 minutes.

After the 20 minutes was up, the control and experimental Petri dishes were removed from the incubator and the contents of these dishes were pipetted out. I was sure to dispose of the Hg solution properly, as it is toxic. I added the working solution of Rhodamine 123 to all Petri dishes and closed the lids once again. All the Petri dishes were wrapped in tinfoil in the same way that the test tube containing the Rhodamine 123 solution was covered. I incubated the dishes again, this time for 10 minutes in the incubator set at 37 degrees Celsius.

While these cells were incubating, a chip chamber was prepared. You crack a cover slip into tiny pieces in the middle of a kimwipe, as to be sure to not allow glass to protrude from the kimwipe and cut your hand. 5-10 of these small chips of glass should be taken and the placed in the center of a clean slide. Spread them into a 20mm by 20mm square.

After the 10 minutes of incubation time was up, I removeed the Petri dishes from the incubator and removed the Rhodamine 123 solutions from each dish using a pasteur pipette. I rinsed by adding HBSS to the Petri dishes as soon as the Rhodamine 123 solution was pipetted out. I removed all liquid from the Petri dishes with a pasteur pipette. Using a pasteur pipette, I added one drop of HBSS to the center of my chip chamber slide while the cells were incubating. Using sharp forceps, the cover slips were lifted from the bottom of the Petri dishes, (being sure to be pay close attention to what each was labeled as (control or experimental)). I gently placed the cover slip face DOWN onto the chip chamber. The edges of the cover slip were sealed with VALAP.

Taking Fluorescent Images

The stage heater was turned on so that the base of the microscope was being kept warm at about 37 degrees Celsius, and the cells were mobile throughout the image process. I opened up “Spot Advance,” which was done on a Microsoft computer. The camera I used was an RT Spot color from Diagnostic Instruments Inc. All images were viewed at 40x magnification.

Before taking a picture, I pulled the photo pin from the microscope, because this allowed me to see on the computer what the camera is seeing. From here I pressed the “Live” setting on the “Spot Advance” Program, so that I could properly focus my image. The camera was switched to “Rhodamine 123 mode” so that when I looked at the image on the computer screen, the color was mostly green. I placed a sheet of tinfoil on top of the transmitted light on the microscope and moved the slider on the microscope to position 3. Immediately I snapped a picture and as soon as the image was captured on that computer, the slider was moved back to position 1. After waiting two minutes and once again moving the slider to position 3, I took another picture, and moved the slider back to position 1. I did this until the neuron was bleached and I could no longer make out mitochondria in my photographs.

Quantifying Data

In order to quantify the length with which the mitochondria moved I found a 0.1mm/0.01mm ruler. I took a picture of the image of the ruler at 40x magnification on the same camera that the images of the neurons were taken.

Once all the images were taken, I opened them up in a program called “Image J” which was generated for a Mac. The program allows for quantification of the brightness of pixels. All images are 8 Bit brightness (256 levels of brightness where 0 is maximal white and 255 is maximal black). Each image is shown in a 1520x1080 pixel frame. I opened the picture of the ruler, and using the “straight line” selection tool. I selected from one of the larger bars to the next larger bar. I made sure that the angle reading read 0.00 degrees. A reading on the image showed how many pixels I captured in a .1mm length. I found that there are 408.00 pixels in .1mm.

I opened images in the order that I took them for one time lapse. In the top menu, I selected “Image”, “Stacks,” “Convert Image to Stacks.” Each mitochondrion was identified by brightness level. In my experiment, where there seemed to be a mitochondrion along the length of an axon, the location of the mitochondrion was measured by the brightest spot in that area.

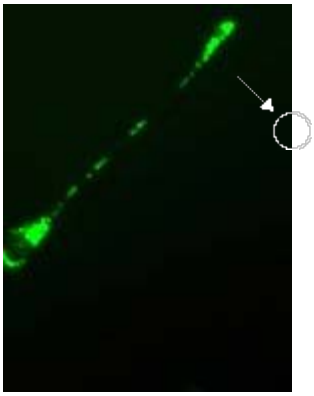
In order to identify mitochondria more clearly in each image in the time lapse, in top menu I selected “Image,” “Adjust”, “Brightness/Contrast.” I moved the “minimum” bar to the right until the line on the histogram displayed any pixel below a brightness level of 55 as a color close to total black. This made the mitochondria stand out more so that they were easier to view. Using the “magnifying glass” icon, I zoomed into the image 400%. Using the “segmented line” tool, I clicked on the far end of one speified mitochondrion. I used the right arrow key to move to the next step of the time lapse. I double clicked on the same part of the same mitochondrion that I did in the first frame. I then selected “Analyze” in the top menu, and “Measure.” A box popped up on the screen displaying the length measured in pixels. I

converted to mm. This was repeated until I had measured the distance the mitochondrion moved in each frame.

Once all data was collected, I converted the velocity that each mitochondrion moved by dividing the distance the mitochondrion traveled (in mm) by the time (in seconds).

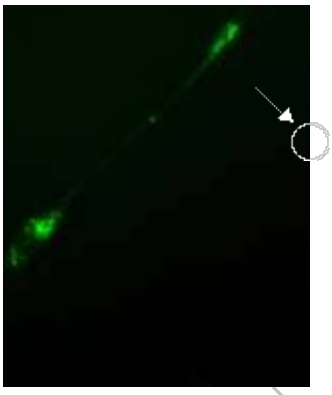
Results

After carefully viewing the time lapses taken of all the control and experimental neurons, I looked for even the slightest movement in any of the mitochondria. Once I identified a mitochondrion that had visibly moved along the axon at some point in the six or eight minutes that the photographs were taken, specific mitochondrion were identified and labeled as either A, B, C, D, E, F, G, H, or I. I measured the distance in pixels that each mitochondrion moved in the time that it took for the Rhodamine123 dye to bleach in the organelle. This distance was then converted from pixels to mm. With the distance traveled by the identified mitochondrion, and the time that it took that mitochondrion to move this distance, the velocity with which the organelle was traveling was calculated.



0 minutes

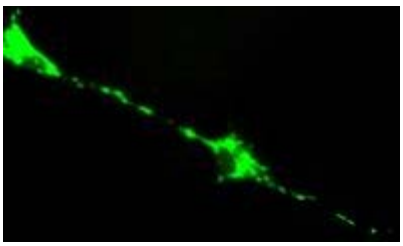
2 minutes



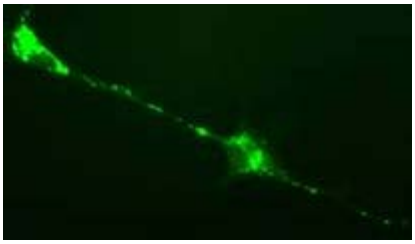
4 minutes

6 minutes

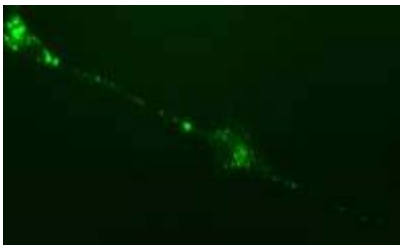
Figure 1: Doing a manual time lapse included taking pictures of the same image every two minutes until the mitochondria that were dyed with Rhodamine 123 completely bleached, and were therefore no longer visible. This took up to eight minutes. In this time lapse of a Control neuron, a mitochondrion was identified so that its velocity could be measured by calculating the distance it traveled in mm divided by the time it took (in seconds). The photos were taken every two minutes for a total of six minutes with an RT Spot Color camera from Diagnostic Instruments, etc.



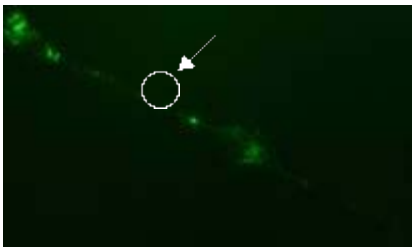
0 minutes



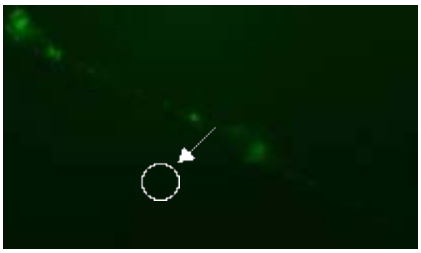
2 minutes



4 minutes



6 minutes



8 minutes

Figure 2: The time lapse done for the Experimental slide shows a specific mitochondrion being identified and tracked for an eight minute period. A picture was taken every two minutes using an RT Spot Color camera from Diagnostic Instruments. Using Image J software, I was able to calculate the velocity with which the mitochondrion moved this period of time.

Figure 3 shows that the average velocity with which the mitochondria moved along the axon. The values for the control group that was not exposed to the dose of Ionic Mercury came from a sample size n=3. The values for the experimental group that was exposed to an acute dose of Ionic Mercury came from a sample size n=6. The average value of the data sets are within only .06 *10E-5 mm/sec of each other. Control mitochondria moved at an average velocity of 1.91 *10E-5 mm/sec and the experimental mitochondria moved at an average velocity of 1.97 *10E-5 mm/sec.

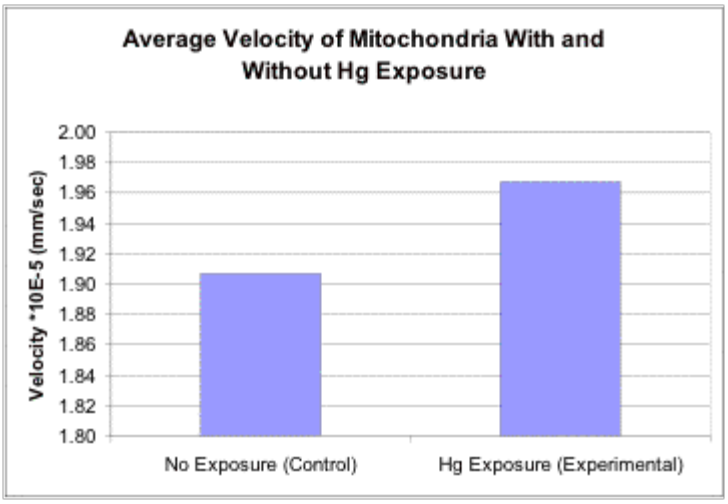


Figure 3: The average velocity with which the mitochondria moved is displayed in mm/second. Control mitochondria moved at an average velocity of 1.91 *10E-5 mm/sec and the experimental mitochondria moved at an average velocity of 1.97 *10E-5 mm/sec.

Discussion

Due to these preliminary findings, there is not sufficient evidence to show that an acute dose of Ionic Mercury on 10-day chick peripheral neurons has an immediate effect on the velocity of mitochondrial movement. My hypothesis was that an acute dose of mercury would affect the primary culture of a chick peripheral neuron by slowing down mitochondrial movement (velocity) along the axon of the cell, and my results did not yield sufficient evidence to support this claim. The results of my experiment did not show a significant difference in mitochondrial velocity in mercury treated neurons versus neurons that were not treated with mercury. The average velocities of the control data and the experimental data only varied .06 *10E-5 mm/se. From a cellular standpoint, when a neuron from a chick fetus is exposed to mercury, it may or may not slow down or speed up the movement of mitochondria along the axon of the nerve cell, therefore affecting the amount of ATP that the growth cone receives.

A probable explanation for the lack of evidence supporting my hypothesis could first and foremost be explained in the lack of data that was collected. Due to the short time frame that was given to complete the experiment, my group members and I only had time to image a few neurons and organize them into time lapses. We did not have sample sizes in our control or experimental groups that were large enough to make any conclusions because n=3 for control neurons and n=6 for experimental neurons. The results that I drew throughout the experiment were based on the movement patterns of only nine specified mitochondria, some of which existed in the same neurons. This sample sizes is too small

for a comparison of average velocity in the two groups to yield any definite conclusions. I also did not use the same amount of mitochondria in my control and experimental quantifications. This may have statistically altered the results that I obtained.

The apparent lack of effect that Ionic Mercury exposure had on the neurons in my experiment was not expected, and might also be due to that fact that an acute dose of mercury may not immediately slow down mitochondrial movement in the axon of a chick peripheral neuron. Only a small amount of mercury accumulates in the body at a time, and most of the time it is excreted in the natural form by urine, stool, and expired gas (Kwok-Keung et al, 2007). If the mercury was going to affect the velocity of the mitochondria in the axon at all, maybe an acute dose of Ionic Mercury just wasn't enough to show that.

Another possibility for why my results may not be conclusive, is because I used my own judgment to assume that the specified mitochondria that I was watching in each time lapse, was in fact that same one after every two minute interval. I may have labeled one mitochondrion in one frame, a different mitochondrion in the next frame because the mitochondria could not be marked. Mitochondria in close proximity to each other and on the same axon seemed to share similar movement and displacement patterns (Karpow, 2008).

Future experiments might use a higher concentration of mercury solution in order to yield more conclusive data. Also increasing the amount of time between time-lapse images from two minutes to a longer time span, may show a longer movement pattern in specific mitochondrion, and therefore make for quantifying velocity of the movement of mitochondria in the axon of a chick peripheral neuron easier and more obvious.

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