Evidence that Invitro Mercury Exposure Decreases Strength of Charge of Individual Mitochondria Located in Axons of Embryonic Chick Peripheral Neurons

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Introduction

I studied broadly the process of generating axons from in-vitro dorsal root ganglia of embryonic chick cells and more specifically the effects of the neurotoxin Mercury (Hg) on mitochondrial charges in these axons. The hypothesis was that individual mitochondria in Hg exposed axons will have a lower charge than individual mitochondria in control, unexposed axons.

Mitochondria play a crucial role in living cells by producing ATP which is the basis of cellular energy and a key factor in cellular communication and differentiation as well as other cellular functions. (Ekizian, 2007) The inner membrane and external membrane of mitochondria have specific charges that allow the production of ATP to occur. If these charges are altered there can be negative results on cellular health. (Treadwell, 2005) A rising concern is the effect of neurotoxins and their increasing appearance in our environment today. Methyl mercury is a toxin that is most commonly found in certain foods we eat, particularly fish. It has been proven that low level exposure of Hg can exacerbate neuro – degenerative diseases and effect cell mechanisms and structure. (Benoit, 2011) It has also been proven that Mercuric chloride can contribute to neurodegeneration (Leong, Syed & Lorscheider, 2000). It is clear today that neurons are sensitive to the toxicity of Hg. (Benoit, 2011) Hg exposure to neuron's mitochondria will be explored in this experiment.

Studies on Neurogenesis have shown that the growth of new neurons in the brain can be effected by mitochondria (Volobоueva, 2011). Some recent studies have been done on mitochondrial involvement in neurogenesis, specifically on inflammation and how it can inhibit mitochondrial function (Volobоueva, 2011) and on toxins such as Hg being another predator able to target mitochondria and alter their functions. Embryonic chick (*Gallus gallus*) peripheral neurons were used to obtain neurons for culture. Dissection and culture of dorsal root ganglia allowed for the direct observation of individual mitochondria in the axons of these ganglia. Experimental Mitochondria were pre-exposed with ionic Hg (which was dissolved from mercuric chloride) and R123 was used to observe each individual mitochondrion through fluorescence microscopy.

Materials and Methods

*I collaborated with Natasha Colon-Ortiz on this project. See references for her report information.

Materials: All materials used were the same as those listed in "Morris (2011a,b,c)."

Camera and Software Specifics:

* ICUC at the Wheaton College Mars Center for Science and Technology building

- Spot Insight FireWire 2 mega sample camera, model number 18.2 color mosaic, Nikon phase contrast 0.90 DRY Japan, 40x objective, Microscope Nikon Eclipse E400

- Mac iMac running OS X version 10.5.8, Processor 2 GHz Intel Core 2 Duo computer

- Spot software version 4.6 Copyright 2009 Diagnostic Instruments Inc.

- ImageJ 1.40g Wayne Rasband Java 1.5.0_30

- Preview Version 4.2 (469.5) copyright 2007 Apple Inc.

Methods:

Dissection and Observation: The first steps in the experiment were the dissection and primary culture of chick embryonic peripheral neurons found in “Morris (2011a)." Cells were then observed after incubation following the steps found in "Morris (2011b)." Photos obtained after these procedures were followed were used solely to observe the normal activity of mitochondria. For the sake of time, the actual neuron cultures used for the control and experiment were dissected and grown by Professor Morris following the same procedure as “Morris (2011a)".

Staining: Neurons, grown by Professor Morris, were plated and stained. Video-enhanced phase microscopy and vital-stain fluorescence microscopy were used to observe and quantify. These steps can be found in “Morris (2011c).” The concentration of R123 used was 5 ug/ml. A 20 minute buffer exchange took place to obtain a control photo.

Mercury Exposure: To obtain a mercury exposed experimental photo the same steps as the procedure in “Morris (2011c)” were followed however there was a 20 minute mercury exposure instead of a 20 minute buffer exchange. This took place by bathing the coverslip in mercury after staining of R123 and before it was mounted onto a slide. The mercury concentration used was 100 nM $HgCl_2$.

*The camera, microscope, computer and software information used to capture the mitochondria are listed in the materials section.

*heaters are mentioned in “Morris (2011b)” but where not used in this experiment.

Quantification: The control consisted of a fluorescence microscopy image focused primarily on axons after changing the buffer with growth medium for 20 minutes. This photo was used as the control in quantification. For the experimental photo axons were exposed to mercury for 20 minutes and 3 fluorescence microscopy photos

were taken. Three photos were taken instead of two so a clearer picture could be obtained. This was a change to the procedure in mentioned in "Morris, 2011c". The control photo and the best photo of the 3 mercury exposed photos were opened up with ImageJ software. Each individual mitochondrion's brightness was calculated by the computer using the polygon selections tool. This was done for both the control and the experimental photo using axons in each picture that showed clear enough mitochondria to depict from other organelles. A bar graph was then created by plotting the averaged brightness of the individual control mitochondria and likewise for the experimental mitochondria. Nine mitochondria were used for the control and four were used for the experimental. This is due to the fact that after Hg exposure, many of the mitochondria were too dim to distinguish from other organelles.

Important Definitions: Mitochondria were defined in this experiment as round, small objects within fibrous axons. Mitochondria must be distinguished from the round swellings known as glial cells that also appeared in the photos. Change in charge was defined as the brighter the mitochondrion the higher the charge, the dimmer the mitochondrion the lower the charge. The control axons are different looking than the experimental ones because it wasn't important to our experiment that we have the exact same axons before and after mercury exposure. It was only important that we had axons to compare our Hg exposed axons to because we were not exploring topics such as movement or regeneration of axons.

Organism Used: *Gallus gallus* was used because it is a prime organism for primary tissue culture due to the fact that the eggs are readily available and their incubation time is short allowing for quick development of the nervous system (Price , Jarman, Mason & Kind , 2011).

Results

It is important to re-state that the guidelines for identifying a change in charge for the sake of this experiment are that the mitochondrion with a higher average brightness is

more highly charged than a mitochondrion with a lower average brightness. Overall, average brightness of control mitochondria was higher than the average brightness of Hg exposed mitochondria as seen in Figure 3. Averaged together, the total averaged brightness of all the control mitochondria was almost 2 times higher than the total average of all experimental mitochondria. There were less Mitochondria seen in the Hg exposed axons permitting the number of individual mitochondria able to be measured to 4 as opposed to the 9 measured in the control axons. Figure 1 shows the control photo taken and works as a comparison for Figure 2 demonstrating the lack of mitochondria in the axons after being exposed to Hg.

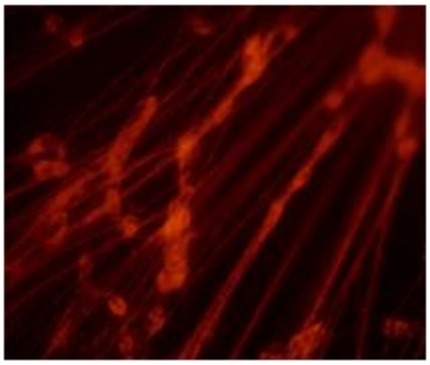

Figure 1. Control axons as linear fibers. Round swellings are glial cells; pay no attention to these. R123 used.

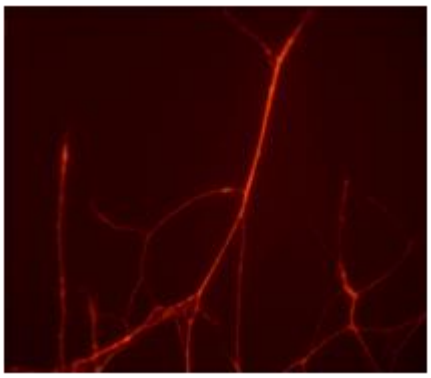

Figure 2. Experimental fibrous axons after being exposed to Hg for 20 minutes. R123 used.

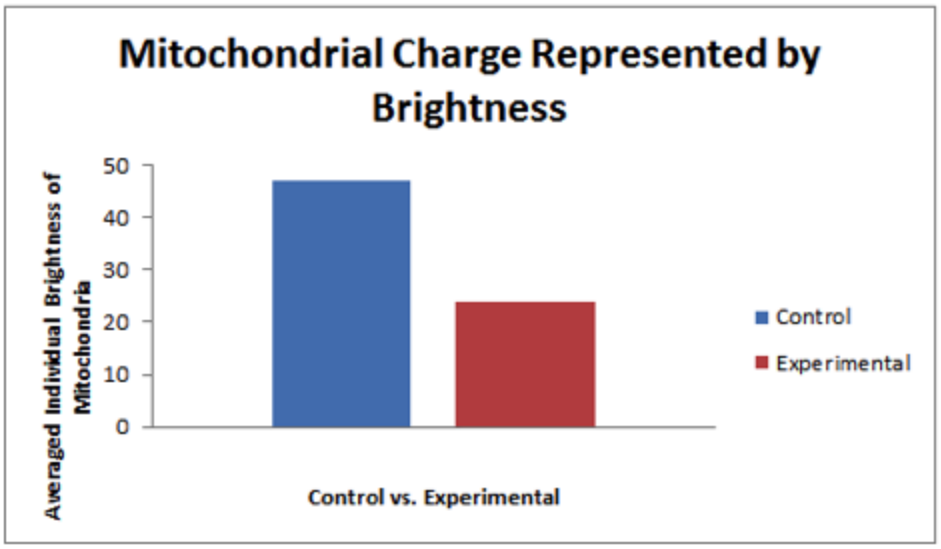

Figure 3. The averaged brightness of the control mitochondria compared against the averaged brightness of the mercury exposed mitochondria. Before mercury (n = 46.901), after mercury (n = 23.821).

Discussion and Conclusions

Individual mitochondria in control axons had a higher charge than individual mitochondria in Mercury exposed axons. This supported my hypothesis and allowed me to conclude that Hg was toxic to the mitochondria in this experiment making their charges lower after a 20 minute exposure. The control mitochondria as a whole were about 2 times brighter than the experimental mitochondria demonstrating that the controls had a higher charge. A 2 times higher charge may not seem like a big number but when we're talking about mitochondria and whether or not they will be able to function properly with a weaker charge, this number is very significant.

If I was able to repeat this experiment several more times to increase the amount of times I can prove my hypothesis I would be able to conclude that Hg is toxic to mitochondria thus making their charge weaker when exposed for at least 20 minutes. The results I obtained are likely a hint that Hg is causing something to go wrong with

the normal functions of mitochondria. Other studies have proven that mercury and other toxins change the membrane of mitochondria thus causing influxes of ions and negatively affecting the mitochondria's normal functions. (Esquivel, Zazueta, Chontal, Resendiz, Pavon, Chavez, 2011) In this case it may be that the mercury exposed to my neuronal axons caused reactive oxygen species buildup in the mitochondria. In mitochondria GSSG (glutathione disulfide – oxidized form of glutathione) has to depend on other factors to reduce it to GSH (glutathione). This makes it more susceptible to oxidative stress. GSH is what will protect the mitochondria from oxidative stress. Oxidative stress occurs when reactive oxygen species build up in the mitochondria. Therefore, If GSSG was not able to be reduced in my mitochondria the result could be mitochondrial dysfunction. (Garrecht, Austin, 2011) The weaker charged mitochondria (dimmer in brightness) could be exhibiting this dysfunction due to their mercury exposure.

To refine the experiment further I would attempt to get more focused axons in my pictures. This would allow for better quantitation of the brightness of individual mitochondrion. The pictures I came up with would be my source of error for this experiment. They were not as sharp as I had hoped and didn't allow me to quantify as many individual mitochondria as I had hoped for. It's vital in proving an experiment that enough numbers are quantified, therefore better pictures would solve this problem for me. With that said, this experiment, although successful, would be much more convincing if more mitochondria were quantified.

In future experiments I might explore what specific parts of the mitochondria are being affected by the mercury and causing a weaker charge. This would take much more knowledge at the cellular level and different lab instruments. This information would be important because knowing what Hg specifically targets may allow scientists to create a defense mechanism against Hg. This information could also be used to delve more into the details of how Hg actually changes mitochondrial charge and maybe lead to explanations of mitochondrial failure which could then lead to a

cure, because specific details could be found. I would also be interested in testing other toxins on mitochondria to see if the same results occur. It is important to find out which toxins are harmful to mitochondria not only for safety reasons but also to aid in the research of these toxins that are becoming more prevalent in the environment and are capable of altering these energy producing organelles. For example, one toxin may affect mitochondria differently than another, or it may not have any affect at all. These answers are gateways to treatments and progressive studying of mitochondria.

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