Analyzing Motility Behavior and Anchoring Action of Mitochondria Exposed to Mercury during Transport within Chick Sympathetic Neurons

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ABSTRACT

Following evidence of mitochondrial motility through molecular motors, several pieces of research have further indicated the apparent “anchoring” or membranous organelles such as mitochondria to microtubules or neurofilaments as a means to stabilize their distribution (Hollenbeck, 1996). Due to the limited research addressing the effect of mitochondrial motility properties in coordination with altered axonal physiology, my experiment was specifically designed to examine the regulation of motility in mitochondria in chick sympathetic neurons when exposed to mercury. Using the fluorescence imaging technique, I quantified my data by counting the number of mitochondria that were moving per each experimental slide (mercury treated neurons) in comparison to the control slides (non-mercury treated neurons). Converting each measurement into a percentage of movement over non-movement and then averaging this for both the experimental and control groups, I was able to demonstrate that a fewer amount of mitochondria in axons exposed to mercury exhibited movement when compared to mitochondria under normal conditions.

INTRODUCTION

Located along the axons of neurons, mitochondria are best known for their primary function of axonal transport in which they undergo the essential role of aerobically producing ATP and regulating intracellular calcium levels within a cell (Hollenbeck,1996). Mitochondria are often transported to regions in which the demand for energy is likely to be high and dispersed only when the local energy needs of the neuron changes. Their unique motor activities involve movement in both anterograde and retrograde directions, and include either regulated, saltatory bidirectional movement in which the organelles make frequent stops and starts, often changing direction after halting, or prolonged periods of time in a fluctuated, stationary phase (Hollenbeck, 1996).

Of the three major systems of cytoskeletal filaments in the axon consisting of microtubules, microfilaments, and neurofilaments, mitochondria primarily move along microtubules which serve as stationary, linear tracks aligned with the main axis of the axon (Kandel, Schwartz, & Jessell, 2002). Although a large body of evidence has suggested that microtubules play the only role for axonal transport across the membrane, a recent study in 1995 by R. L. Morris and P. J. Hollenbeck discovered that axonal transport is able to occur along microtubules as well as microfilaments in vivo, but with different velocities and net transport properties (Morris & Hollenbeck, 1995). The movement of these specific organelles is powered by molecular motors, which are specific to the direction of the organelles motility. Anterograde axonal organelle transport is thought to be supported by members of the kinesin family of molecular motors, while retrograde movement seems likely to be driven by members of the cytoplasmic dyenin family (Hollenbeck, 1996). Although mitochondria contain both anterograde and retrograde motor activities, the motility behaviors necessary to

achieve these redistributions seems to rely largely upon regulation of the anterograde motor activity alone (Hollenbeck, 1996).

A further element in the regulation of mitochondrial motility and distribution throughout the axon is the evident "anchoring" of mitochondria to microtubules or neurofilaments. This process has been found to stabilize their distribution once regulated motility has moved the mitochondria to appropriate sites of energy need (Hollenbeck, 1996). While little is known about the mechanism for recruitment of mitochondria between stationary and motile states, numerous studies have revealed distinct cross-bridges between axonal mitochondria and these particular cytoskeletal elements. (Hirokawa, 1982). Furthermore, these cross-bridges have been most often observed between mitochondria and microtubules, but also with neurofilaments in which rapid fixation and freezing techniques have revealed interactions between motor proteins and mitochondria. Furthermore, the transport properties of the two cytoskeletal filament systems are assumed to be balanced with each other so that they produce a coordinated net transport of organelles, which functions to meet both the cell's regional and physiological needs (Morris & Hollenbeck, 1995). In the study conducted by R. L. Morris and P. J. Hollenbeck. (1993), the researchers supported this specific anchoring phenomenon through their observations as to the specific balance between motile and stationary mitochondria and its dependence upon the growth state of the axon in suggesting that this specific "docking" phenomenon plays an important role in maintaining a mitochondrial distribution that is appropriate to the physiological state of the axon (Hollenbeck, 1996).

Because so much recent attention has been paid to the transport differences of organelles, there has been limited research conducted on addressing the effects of physiological changes on the axon's response of transporting these specific organelles to their required regions. Based off of the evidence that indicates that the motility of mitochondria is profoundly regulated by the physiological state of the neuron, I have adapted the techniques of Hollenbeck and Morris (2003) to look at how the impact of the introduction of a chemical agent such as mercury has on the anchoring function of mitochondria during transport in *Gallus gallus* chick embryo sympathetic neurons. As a result of this altered physiological state, I have hypothesized that we will see a fewer number of moving mitochondria in the mercury treated neurons in comparison to the non-mercury treated neurons.

MATERIALS AND METHODS

Materials

Forceps, pasture pipettes, 25mm and 110 mm Petri dishes, coverslips, coverslip fragments, glass slides, 8 - 15 day chicken eggs, 18-26 hour grown sympathetic chick neurons, 37 degrees Celsius incubator, 100% EtOH sterilizing

solution, Hanks Balanced Saline Solution (HBSS), Trypsin solution (Ca/Mg-free HBSS containing 0.25% trypsin) C medium: Leibovitz L-15 medium plus 0.5% methylcellulose, 10% fetal bovine serum, 0.6% glucose, 2mM L-glutamine, 100ug/ml streptomycin, 100U/ml penicillin, 10-50ng/ml nerve growth factor, poly-lysine/laminin treated coverslips, 1 mg/ml Rhodamine 123 in growth medium, Nikon Eclipse 80i Microscope with fluorescence capabilities, RT Color-Diagnostic Instruments Spot Insight Camera, Spot Advance 4.1 software, Macintosh G4, portable heater, ImageJ image analysis software, Microsoft Excel.

Methods

Neuronal Cell Culture/Plated Cells

Professor Morris provided us with all plated neurons. “Using 8-15 day old chick embryo’s, Morris dissected out the sympathetic chain ganglia which was then cultured as either whole ganglia or dissociated ganglia and grown as single cells on cover slips. Cells were grown for 18-26 hours in a 37 degrees C incubator in Liebovitz L-15 medium supplemented with 0.5% methylcellulose, 10% fetal bovine serum, 0.6% glucose, 2mM L-glutamine, 100ug/ml streptomycin, 100U/ml penicillin, 10-50 ng/ml nerve growth factor. Coverslips were treated with 1 mg/ml polylysine followed by a laminin-enriched fraction of condititioned medium for 20-60 minutes before plating out cells.” (Dissections performed according to *Primary Culture of Chick Embryo’s*, a protocol by Peter J. Hollenbeck with slight modifications by Robert L. Morris).

Ganglia Staining/Coverslip Preparation

Once obtaining the dissociated cells in growth medium, we had assigned both a control and experimental cell to each coverslip. The experimental cells were treated with 1 mL of 10 nM HgCl2 in HBSS, and treated the control with 1 mL of 0.5% HCl in HBSS, and incubated for 20 minutes at 37 degrees C. After incubation, the solution was removed using a sterile pipette and then 1 mg/ml Rhodamine 123 in growth medium was added. The cells were wrapped in tin foil, and then incubated again at 37 degrees C for approximately 10 minutes. After the 10 minute incubation, the ganglia coverslips were removed from the incubator and the Rhodamine 123 was pipetted out. The coverslips were then washed several times with HBSS to remove any dye that may still exist. Chip chambers were then prepared chip using coverslip fragments. Using forceps, our ganglia coverslip was removed and placed up-side-down on top of the coverslip chamber, excluding any air. The edges of the coverslip were sealed with heated valap. The top of the coverslip was washed off with deionized water to get rid of any salt. Four trials of ganglia staining and coverslip preparation were performed in subsequent weeks. For the first trial, 1 experimental and 1 control slide were created but not included in our results. The second trial produced 2 controls and 1 experimental slide. The third trial produced 2 controls and 2

experimental slides. The forth trial produced 2 controls and 2 experimental slides.

Identifying the Target Regions/Imaging the Cells

Positions of individual mitochondria were revealed by fluorescent imaging, using the Rhodamine 123 fluorescence dye. Still kept at 37 degrees C, the neurons were observed under a Nikon Eclipse 80i Microscope and located by phase microscopy on the basis that a cell body, axon, and synapse were visible. Images were then transferred to the Spot Advanced 4.1 software program on a Macintosh G4 computer system which was first used to create a live image in order to maximize the focus for the neuron of interest. Images were taken using blue-emission, green-emitting fluorescence, under 40x magnification. Imaging settings for Spot program were set at 6 sec exposure time using green fluorescence only. Around 4-5 images were manually taken, 2 minutes apart from each other. Three trials using the fluorescence technique were performed. For the first trial, 2 control and 2 experimental images were taken which took 3 hours total. For the second trial, 2 control and 2 experimental images were taken which took 6 hours total. For the third trial, 3 control and 3 experimental images were taken which took 3 hours total.

Quantifying the Data

Altogether 5 experimental and 5 control images were used in our quantification. Mitochondrial images were transferred to the Image J program and arranged in stacks subsequent to their 2 minute exposure differences, and labeled according to what treatment was used, slide number, and day they were taken. Once in stacks, the images were all individually enhanced using the brightness and contrast tool in order to get the best pixel to background contrast in order to better identify the mitochondria. Using the zoom option from the image J tool bar, I had zoomed in on each axon of interest to get the best possible view of the mitochondria altogether. The first slide of each stack was temporarily set to 8 bit option in order to take away the color, and then inverted so that the mitochondria were black and the background white. This image was then printed off and used manually in circling and numbering the visible mitochondria, and thus served as a guide in labeling the mitochondria throughout the rest of the stack. Targeting each individual mitochondrion at a time, I scrolled through all of the images in the same stack with focusing on my mitochondria of interest in order to determine whether or not the mitochondria had moved positions along the axon. I recorded this for each visible mitochondrion, separating my data by the treatment and slide number. This was repeated for all 5 control and 5 experimental slides.

For each of the treatment slides, I added up the stationary vs. movement patterns of mitochondria. Because the number of mitochondria observed was different for both the experimental and control treatments, I calculated the percentage of motile mitochondria per each neuron by taking the total number of mitochondria observed within the selected neuron, and then dividing the number of motile mitochondria by this total. This was done for all 5 control and

5 experimental slides. I then averaged these percentages for both the control and experimental group, rounding each number to three decimal places.

RESULTS

My own personal observations were used in quantifying the movement of the mitochondria in either supporting or discarding the hypothesis of decreased movement found in the experimental slides when compared to the control slides. Regions of interest were easily identified under the Nikon Eclipse 80i Microscope as indicated by the visualization of individual images of mitochondria, also demonstrating that the fluorescence staining technique had been successful. However, when my collaborators and I took images using the Spot program, the fluorescence of the mitochondria started to become blurry after 2 to 3 image captures, making it harder to observe the motility behaviors of mitochondria for longer periods of time.

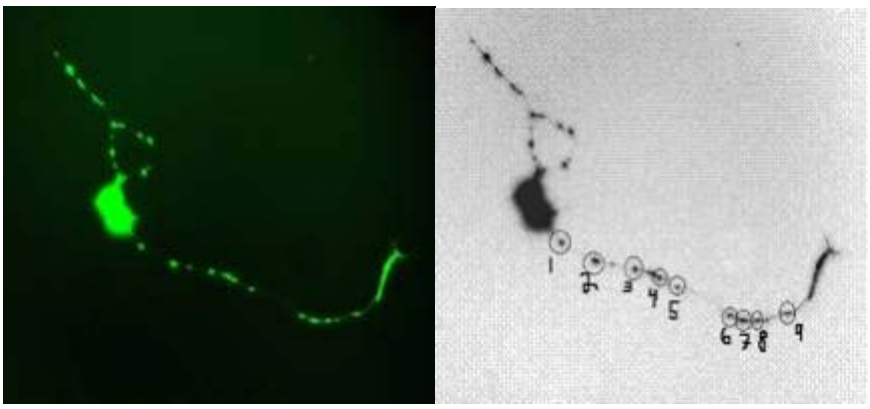
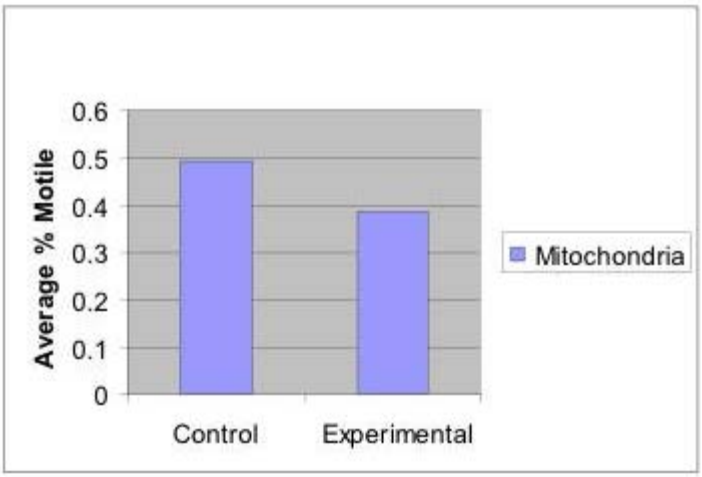


FIGURE 1 – Side-by-side figures of a control image taken by the 80i microscope at 40x. Image on the left shows the original fluorescence image in which the mitochondrial regions are clearly identified. The image on the right shows the quantification technique of numerically labeling the visibly identifiable mitochondria along a single axon.



GRAPH 1 – Motility behavior analyzed from mitochondrial measurements for control vs. experimental groups. Percentages of motile mitochondria per neuron were averaged for both the control (n=66) and experimental group (n=52). Note that the control group shows a higher average percentage of mitochondria exhibiting movement in comparison to the experimental group.

DISCUSSION

Looking at the evidence that indicates that the motility of mitochondria is profoundly regulated by the physiological state of the neuron, I was able to successfully conduct my experiment using the compound mercury in which to further examine this. According to my results, I was able to support my hypothesis in that a fewer amount mitochondria in axons exposed to mercury exhibited movement when compared to mitochondria in axons not exposed to mercury. This may indicate that when a neuron exhibits an altered physiological state such as the addition of mercury, this will have no effect on the anchoring action of the mitochondria to certain cytoplasmic elements, but rather produce a halt on their motility behavior.

A relevant study by Nobataka Hirokawa in 1982 explored the cross-connections that membranous organelles such as mitochondria had with cytoskeletal elements such as microtubules and neurofilaments, particularly when the neuron's physiological state was altered. Using the quick-freeze, deep-etch method, Hirokawa compared experimental neurons that had either been chemically extracted with 0.1% saponin or physically ruptured by gentle homogenization before freezing, to untreated control neurons. Through his own visual analysis, Hirokawa had found no detectable differences between the cross-bridges between both the membranous organelles and the neurofilaments of both the fresh and manipulated axons. The only noticeable impact of the physical rupture on the neurons consisted of a slight difference in the morphology of the microtubule cross-bridge in which the surface substructure had been obscured. This study relates to mine in that Hirokawa was able to indicate cross-connections between mitochondria and microtubules as well as mitochondria and neurofilaments, in both untreated and manipulated samples. Thus, through Hirokawa's indication that mitochondria remained immobile in both samples, my results seem to further suggest that when mitochondria were exposed to mercury, they still exhibited motile behavior, but at a less amount compared to the un-manipulated axons. (Hirokawa, 1982).

If I had repeated my experiment a thousand times and gotten the same results so that all experimental data diverged from my controls was statistically significant, I would be able to support that mercury definitely inhibits the motility functioning within mitochondria. However, although I would like to assume that this halt in mitochondrial motility when exposed to mercury supports an increase in the anchoring function to these certain cytoskeletal structures, there is simply not enough evidence to exclude other explanations. If I were to refine this experiment, I would have used the quick-freeze, deep-etch method described in Hirokawa's experiment in order to analyze the effect of mercury specifically on the cross-bridge formation.

Although there were no sources of error in my experiment that I was aware of, some possibilities may include the fact that I used my own visual analysis in recording whether or not mitochondria exhibited movement. Due to the

fact that some of the fluorescent images were more blurry than others, I may have incorrectly labeled a mitochondrion as moving when it could have just been the blur in the image. Also, my groupmates and I did not use the same amount of mitochondria for both the control and experimental quantifications, so that may have statistically altered our results. If I had a very large amount of data in such that I was convinced that all the trends and differences between the experimentals and controls were real, from a cellular standpoint I would explain this in that because mercury ruptures the physiological structure of the cytoskeleton, the hault in movement reflects the halt in either the distribution of energy needs within the cell, or an alteration of the molecular motors thus rendering them stationary. Future experiments using the quick-freeze technique should be done to analyze the effect of mercury specifically on the cross-bridge formation, in which researchers could further explore how physical alteration affects motility and cross-bridge formation, especially it's effects on the energy needs of the cell or the molecular motors of mitochondria.

REFERENCES

Hirokawa, N. (1982, July). Cross-linker system between neurofilaments, microtubules, and membranous organelles in frog axons revealed by the quick freeze, deep-etching method. *The Journal of Cell Biology.* 94: 129-142.

Hollenbeck, P. J. (1996). The pattern and mechanism of mitochondrial transport in axons. *Dept. of Neurobiology, Harvard Medical School, 220 Longwood Avenue, Boston, MA 02115, USA.* Date Retrieved 2/9/08. http://www.bioscience.org/1996/v1/d/hollenb1/htmls/91-102.htm

Kandel, E and Schwartz, J. and Jessell, T. M. (2002). *Principles of Neural Science, 4th edition.* McGraw-Hill Company, NY, pp 99-103.

Karpow, Celeste (2008) [personal communication, Wheaton College, Norton, MA]

Langevin, Amy (2008) [personal communication, Wheaton College, Norton, MA]

Morris, R. L and P. J. Hollenbeck. (1993). The regulation of bi-directional mitochondria is coordinated with axonal outgrowth. *Journal of Cell Science.*. 104: 917-927.

Morris, R. L. and P. J. Hollenbeck. (1995, December). Axonal transport of mitochondria along microtubules and F-action in living vertebrate neurons. *Journal of Cell Biology.* 131(5): 1315-1326.