The Effects of Mercury on Microtubule Polymerization in Axons of *Gallus gallus* neurons

Adam Askew
Neurobiology Short Report
Bio324/ Neurobiology
Wheaton College, Norton, Massachusetts, USA
April 24, 2013

Introduction:

Microtubules are cytoskeletal filaments that are responsible for vesicular trafficking in neurons (Alberts, 2012). Microtubules are polymers of α- tubulin and β-tubulin (Alberts, 2012). The motor proteins kinesin and dynein are responsible for the unidirectional transport that occurs in all cells (Alberts, 2012). However, in neurons, this transport system is slightly more nuanced. Kinesins are responsible for anterograde axonal transport, meaning that they transport vesicles from the cell body to the synapse. Dyneins are responsible for the opposite, retrograde motion, carrying materials from the synapse to the cell body (Goldstein, 2000). Specialized cells also have special arrays and organizations of microtubules (Goldstein, 2000). Microtubules are dynamic, polymerizing and depolymerizing to suit the transport needs of a cell. This dynamic nature is important in processes such as axonal branching, where an axon splits into two or more axons, innervating many targets (Dent, 2001).

Tubulin depolymerization is cause for concern in the study of disease. It is one of the key features of Alzheimer's disease, the most widespread cause of dementia. The polymerization process can be interfered with by other molecules (Santa- Maria, 2005). Mercury vapor has been shown to inhibit the binding of GTP to tubulin in rat brains, inhibiting the polymerization of tubulin into microtubules (Leong, 2001). Evidence shows that mercury ions are also neurodegenerative, destroying neurons in several ways (Leong, 2001). There have been parallels between autism spectrum disorders and mercury intoxication, making mercury affected neurons prime targets to study in regards to autism spectrum disorders (Kern, 2012). Amyotrophic lateral sclerosis (ALS) is another neurodegenerative disorder that leads to the deterioration of muscle strength. ALS patients and those who inhale low doses of mercury over a long period of time show similar inhibition of motor function when autoradiograms of their spine are taken (Roos, 2012).

Mercury exposure is inevitable, however. Mercury is added to the biosphere constantly by human activity. Unfortunately, this has had negative consequences for biological rhythms, such as disturbances in hair growth. The inhalation of mercury vapors causes autonomic nervous system (ANS) degradation without showing other signs of mercury intoxication, causing a more difficult diagnosis of mercury intoxication(Lombardi, 2012).

In the current study, microtubule densities in the axons of neurons were examined in conditions with mercuric chloride treatment and without. In this study, two sets of neurons of 10-day old *Gallus gallus* embryos were cultured, treated with mercury chloride, labeled with immunofluorescent antibodies, then imaged and the density of microtubules was quantified by image brightness. It is hypothesized that cells under the mercury chloride condition will show less polymerized microtubules, resulting in quantifiably dimmer images.

This hypothesis is significant to investigate as mercury degeneration could be used as a model for other forms of degeneration. Depolymerizing microtubules with mercury can leave researchers with model specimens for study. These specimens could be used to find a process to reverse neurodegeneration, starting with microtubules. Furthermore, these specimens could be used to study molecular neurodegenerative mechanisms. In effect, this field of research could lead to a better understanding of several neurodegenerative diseases such as Alzheimer's, leading to a higher quality of life and a higher survival rate for those with the diseases.

Materials:

Materials in this experiment included neurons from 10-day-old chick embryos, coverslips, EtOH, 110 mm petri dishes, kimwipes, forceps, regular and constricted pasteur pipettes with bulbs, 35 mm petri dishes, DMEM, growth medium, trypsin, poly-lysine, laminin, and rubber gloves during the dissection process. The cells were treated with 100 nM $HgCl_2$. Cells were fixed using a recipe of 2% Formaldehyde, .01% glutaldehyde, 0.12 M sucrose, .05% TX-100, 2

mM EGTA in two 6 well-plates and washed with DMEM and Tyrode's solution. The cells were stained with 100nM Mitotracker (prepared by adding 50 micrograms of the Mitotracker to 117 microliters of DMSO and diluting it to 1:2000 solution to get 500nM. This was then added to 6 ml of DMSO, resulting in a 100nM solution) , 1:50 solution of DM1A, and 1:10,000 Hoechst stain. Slides were sealed with Revlon Cherries in the Snow nail enamel. All images were captured in the Imaging Center for Undergraduate Collaboration at Wheaton College, Norton Massachusetts on Nikon Eclipse E801 epifluorescence scope with the Spot RT3 camera with .76x C- mount and 1x eyepiece on Spot software version 4.6 on a Macintosh iMac (operating system OSX) in red fluorescent channel. All analysis was done on ImageJ software version 1.46r 32-bit. All images were captured with a .5 second exposure time.

Methods:

Six *Gallus gallus* samples were prepared using the protocol described in Morris (2013). The three experimental groups were exposed to mercury chloride solution in Tyrode's solution for 15 minutes in the incubator. The three control samples were incubated for 15 minutes in Tyrode's solution. The cells were fixed and stained using the protocol from Morris (2012) using the concentrations and solutions listed in the materials section and using the neuron slides instead of HeLa cells.

Imaging:

All cells were imaged 2 days later with the 40x objective lens at 0.5 second exposure time under the red fluorescence channel to standardize results. Images were saved and quantified using the ImageJ program.

Quantification:

Axonal brightness was determined by the ImageJ software using the multi-point selection tool to minimize background darkness or glow. Multiple points were selected along each axon and measured for mean brightness. Background brightness was not taken into account, but was averaged into all results.

Certain standards were set to keep quantification standardized and precise. Axons near ganglia were avoided in order to decrease the chance of plotting data points on more than one axon. Branching axons were taken into account, however, no points were plotted at the point of branching to account for the excessive axonal size, which would increase brightness. Points on the axon were plotted at random to maintain a large and accurate sample size that included dark and bright axonal regions. Mean brightness was taken from the points as a representative brightness.

Results:

The data shows that there was an overall difference between the control and experimental groups, with the control group having the higher brightness value. Although the brightness value is measured in arbitrary units computed by the ImageJ software, they still show a difference in brightness.

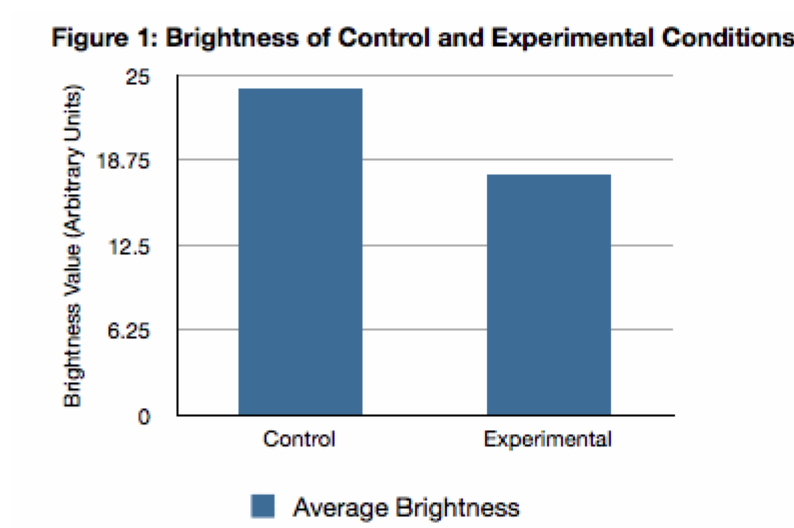

Figure 1: Bar chart that shows the average brightness values for experimental and control groups. Notice how the

control group is brighter than the experimental group. The control condition used 34 axons from 34 different cells to have n= 341. The experimental group used 29 axons from 29 different cells to have n= 435

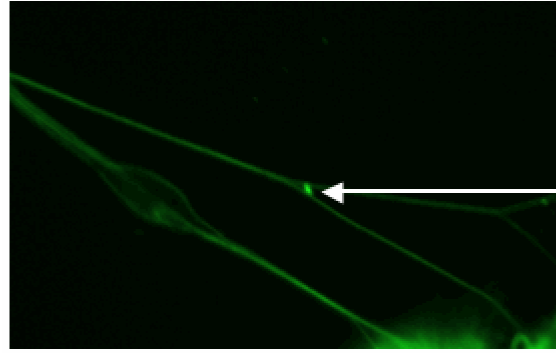

Figure 2: Single axons in a control slide. The control slide looks far brighter in certain regions than most points in the experimental slide. Furthermore, the arrow denotes a point of axonal branching in which excess neuron tissue increases the mean brightness.

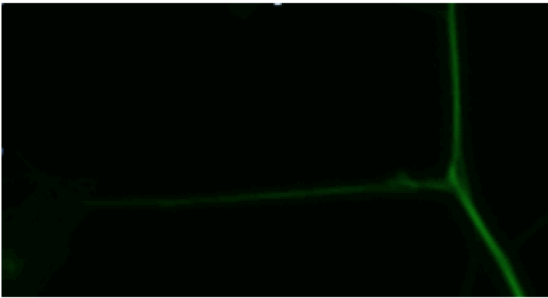

Figure 3: Single axons in an experimental slide. These axons in particular show the general fading that occurs in mercuric chloride treated axons.

Discussion and Conclusions

The evidence collected supports the hypothesis that mercury chloride interferes with tubulin binding, causing less microtubules to polymerize shown in the decreased brightness value of the experimental condition. Therefore, we can conclude that mercuric chloride is likely to be neurodegenerative as it adversely affects sub-cellular elements of neurons that play a key role in intracellular transport. Bringing these results to a bigger spectrum, mercury exposure may be the cause of neurodevelopmental and neurogenerative disorders, such as autism (Mutter, 2005). In further research, mercury may be used as a controlled substance that causes neurodegeneration and the inhibition of the polymerization of cytoskeletal filaments like microtubules. Mercury substances could be used to study how neurons function under neurodegenerative conditions and how those conditions may be reversed.

As stated in Leong, 2001, mercury ions inhibit tubulin polymerization by binding to tubulin. This would account for the fewer amount of microtubules polymerized in the experimental group.

Incubation times were exact, however, better results may have been achieved through longer incubations or higher concentrations of solutions. One of the experimental slides did spill during one of the staining procedures, causing a potential source of error in which not enough fluorescent marker was able to penetrate the cell, causing a dimmer cell. Although cells were kept dim until photographing, photobleaching may have adversely affected the slides differently. Certain slides had fewer neurons and were harder to image. Therefore, they were exposed to the fluorescent light and the natural, dim sunlight that leeched into the lab. Furthermore, non-representative cells of the sample may not have been imaged, leading to biased results.

In order to improve the experiment, less stains should be used in order to have less procedural time, resulting in less photobleaching. More stains also introduce more accidents that can occur in the lab. Using one stain would focus the results of the experiment to just tubulin staining. Different doses of mercury should be used. Higher doses of

mercury should, in theory, result in less polymerization. This further research can illuminate what levels of mercury are safe for neuronal tissue, if any, and what occurs in neurons at different levels incubated for different times. Dissociating the cells more would also result in better, single axons that could be measured more accurately.

Further experiments may look at mercury's affect on other cytoskeletal filaments, like actin. By looking at movement or other cellular activities that involve actin, further research could be conducted with mercury. Performing a time lapse of cells under a mercury condition could also aid in furthering the knowledge of mercury's affects on neuronal tissue. Time lapse video of cells can show blebbing and other cellular activities that can not be shown in static photos. These sort of videos show more short term affects, which may illuminate mechanisms of long term affects

References:

Alberts, B., Bray, Dennis, B., Hopkin, K., Johnson, A., Lewis, J., Raff, M., Roberts, K., Walter, P. (2010) Essential Cell Biology (3rd ed.). New York, New York: Garland Science.

Dent, E.W., Kalil, K. (2001) Axon Branching Requires Interactions between Dynamic Microtubules and Actin Filaments. The Journal of Neuroscience. 21(24). 9757-9769

Goldstein, L.S.B, Yang, Z. (2000) Microtubule-based transport systems in Neurons: The Roles of Kinesins and Dyneins. Annual Review of Neuroscience. 23. 39-71.

Kern, J.K., Geier, D.A., Audhya, T., King. P.G., Sykes, L.K., Geier, M.R. (2012). Evidence of parallels between mercury intoxication and the brain pathology in autism. Acta Neurobiology Experimentius. 72. 113-153.

Leong, C. C. W., Syed, N. I., Lorcheider, F. L. (2001). Retrograde degeneration of neurite membrane structural integrity of nerve growth cones following *in vitro* exposure to mercury. *Neuroreport*, *12*(4), 733-737.

Lombardi, G., Lanzirotti, A., Qualls, C., Socola, F., Ali, A. Appenzeller, O. (2012) Five Hundred Years of Mercury Exposure and Adaptation. *Journal of Biomedicine and Biotechnology.* *2012*. 1-10

Morris, R.L. (2012). Immunofluor staining of HeLa Cells- MeOH fixation.

Morris, R.L. (2013). Primary Culture of Chick Embryonic Peripheral Neurons: Dissection. Available at http://icuc.wheatoncollege.edu/bio324/2013/morris_robert/BIO324_Lab_Proc_1_Dissection.htm

Mutter, J., Naumann, J., Schneider, R., Walach, H., Haley, B. (2005). Mercury and autism: Accelerating Evidence?. *Neuroendocrinology Letters. 26*(5). 439-446

Roos, P.M., Dencker, L. (2012) Mercury in the Spinal Cord After Inhalation of Mercury. *Basic Clinical Pharmacology & Toxicology. 111(*2). 126-132.

Santa-María, I. Smith, M.A., Perry. G., Hernández, F. Avila, Moreno, F.J. (2005) Effect of quinones on microtubule polymerization: a link between oxidative stress and cytoskeletal alterations in Alzheimer's disease, *Biochimica et Biophysica Acta (BBA) Molecular Basis of Disease*. *1740*(3). 472-480.