The Effects of Mercury on Chick Embryonic Neuron Growth Cones

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Introduction:

Chicken Embryo's are frequently used in Neuron Research due to their accessibility of primitive Peripheral neurons. Neuron studies are imperative in such medical areas as Neurogenerative Disorders, Chemical toxicicity, and neuronal injuries. Neurons are cells that upon electrical excitation can process and transmit information (Kandel et. al 2000). One of the most concentrated areas of study with neuron cells is the brain and the effects that Methyl Mercury has on the tissue and neurons. The primary tissue affected by Methyl Mercury in humans is the brain. Adults who are exposed to MM and express paresthesias of the circumoral area, visual field constriction, and ataxia after Neuropathological examination all revealed localized destruction of neurons in the visual cortex and cerebellar granule cells (Clarkson et al. 2003). Even more susceptible to mercury-induced damage than a human brain is a fetal brain. Not only does Methyl mercury disrupts the cytoarchitecture of the young brain, but also inhibits the division and migration of neuronal cells (Blocks et al. 2004). The wiring of the nervous system depends on the correct navigation of nerve growth cones along stereotyped pathways during development (Mallavarapu, 1999). Methyl mercury has been a known neurotoxin for years, and is the most susceptible form of mercury that bioaccumulates in organisms (ATSDR, 1999). The compound consists of a mercury atom with a methyl group bonded to it. Methyl Mercury is most popular for its large impact on fishing industries, medical effects on pregnancies and brain cells.

In this Neurobiology experiment, we will be studying the effects of Methyl mercury on cultured neurons from chicken embryos. More specifically, analyzing and studying the effects that MM has on growth cone morphology and retraction rate. Our experiment hypothesizes that after exposure to certain concentrations of MM, our cultured neurons will shows morphological signs of retraction and disconnection. The sub stratum and certain points anchored by the neuron will begin to morphologically degenerate. Growth cones and their corresponding filopodia continually change shape as they extend toward the filopodium tip. The extension and retraction of typical growth cones and filopodia, are compromised of a bundle of 15-20 actin filaments oriented with their fast growing ends toward the tip (Mallavarapu,1999). Our data will be quantified by comparing rates of growth cone morphology of neurons exposed to Methyl mercury to neuron growth cone rates of our control neurons, which will not be exposed to mercury. These growth rates will be studied over a certain time period quantitatively, but also this experiment will allow us to qualitatively analyze our growth rates by visual identification and comparison of retracted neurons.

Other neuron experiments performed at Wheaton College study rates of endocytosis, neuronal transport, and neuronal interactions after exposure to MM. These will provide sequential information about nerve cell characteristics and behavior. Such information as cell signaling, degeneration and changes to cellular membrane such as asymmetry attachment or fragmentation, allow similar neuronal research areas to collaborate information and benefit from others research. Other similar experiments using fluorescent compound labeling with neurons that have been exposed to mercury, allow scientist to study cell and organelle movement, and vesicle endocytosis.

Studying the effects that Methyl mercury has on neurons helps scientist to better understand the impact and implications that this toxin has. Particularly nerve cell interactions, processing and transport of information.

Materials and Methods

Materials

Hanks Balanced Salt Solution (HBSS) along with C medium, Polylysine, Laminin, Trypsin and Mercury Chloride were previously obtained from the Wheaton College science department and used to carry out these experiments.

Methods

Chicken Embryo Dissection and Dissociation: Nine-day-old chicken embryos were dissected using an aseptic technique and utensils were washed with 92% ethanol solution (ETOH). First the chicken's head was removed and body eviscerated leaving only spinal chord region in cavity. The remaining body cavity was placed ventral side up, carefully removing tissue with forceps revealing the spinal chord. Along each edge of the spinal column are the sympathetic nerve chains. These were carefully tweezed away under a dissecting microscope. There are three obvious dorsal root ganglia at the bottom of the column, which can be plucked away and pulled out. The dissected ganglia are placed in a 25mm Petri dish containing HBSS. The ganglia is washed twice with this salt solution, removed and placed in Trypsin (Ca/Mg free HBSS containing 0.25% trypsin). This solution is then incubated for 15-20 minutes at 37 degrees Celsius. Placing careful attention, the trypsin and removed and the ganglia are suspended in one drop of HBSS per ganglia. The ganglia are then titrated until they become completely dissociated into single cells.

Preparation of Substrata: The substratum was constructed by coating the surface of the coverslip with 1mg/ml poly-L-lysine. The drops of polylysine are placed on the inside lid of a 110mm Petri dish labeled number one. After cleaning a coverslip with 22ml absolute ethanol, the CS is dried and baked for 2hrs. at 200 degrees Celsius. The coverslip was then placed on the drop for 20-30 seconds, rinsed with sterile water, and left on the edge of the Petri dish polylysine side up, and allowed to dry. Then coat with solution of Laminin to keep wet, lift up, rinse with HBSS, and add to Petri dish number two.

Preparation of Media and Plating: Culture neurons in C medium, which contains the following:

Leibobovitz L-15 medium + 0.5% methylcellulose + 10% fetal calf serum + 0.6% glucose+ 2mM L-glutamine + 100ug/ml streptomycin + 10 OU/ml penicillin + 10-5- nm/ml Nerve Growth Factor.

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To plate the neurons, the test tube that contains the dissociated cells was added to dish number 2 containing the L-15 medium. To insure good quality growth cones, isolated cells are obtained ½- 2/3 dorsal root ganglia or sympathetic nerve chain per coverslip. The DRG's are then placed in an incubator at 37 degrees Celsius for 24hrs. After incubation using a pipette, 1ml of sterile water is added to the slide and several glass fragments are placed in a circular manner on the slide. The coverslip is then positioned onto the slide, covering the glass fragments. The edges of the CS are sealed with hot VALAP in a continuous and fast motion.

Preparation and Methods of Mercury Concentrations:

Three experimental concentrations of mercury were utilized and tested in this experiment. The control used for the three different mercury concentrations was 10ml of HBSS + 0.5% HCl. The three different concentrations of mercury tested were as follows: 10 milliliters of 0nm, 10nm, and 100nm mercury concentrations were prepared in a 1:100 ratio. After the neurons are incubated for 24 hrs, the growth medium is removed and the coverslip washed in HBSS. One milliliter of the desired mercury concentration was added. The coverslip was submerged in the control, 0nm, 10nm, and 100nm mercury concentrations for 20 minutes. The mercury was then removed, growth medium was replaced, and the CS was positioned on a chip chamber slide for microscope analysis.

Imaging

Viewing the neurons exposed to the three different mercury concentrations was done in the ICUC at Wheaton College. Using a Nikon Eclipse E200 under phase 2 (x40) my neurons were imaged and analyzed. While imaging, the neurons were kept at 37 degrees Celsius, using an electric space heater. After locating a neuron and focusing in, time lapsed images were recorded. Using the programs BTV Pro I was able to capture the neuronal growth cones at t0, t15, t30, and t45, for the control and experimental concentrations.

Measuring and Quantifying:

To analyze and quantify this data, the program Image J was used. In Image J, the desired file could be opened under open file. Under process, enhance contrast, and equalize equilibrium was selected to distinctly outline the edges of the growth cone. In this experiment to measure the growth cone the definition that the axonal shaft angle increases about 30 degrees is the base/start of the growth cone to be measured, was utilized. The free hand selection tool was used to outline the entire growth cone at the start of the bulbous enlargement. To compute this measurement, under the analyze menu, hit measure. The area of the growth cone previously measured is displayed. To convert these 1024 ´ 768 pixel images to quantifiable data, a picture was taken using the same Nikon eclipse camera, x40, and Image J program, of a stage micrometer. Since the number of pixels in a given growth cone area were computed in Image J, the pixels can then be converted to micrometers using the stage micrometer. The total area of a neuronal growth cones was found using the 1024 x 768 image, area measurements, and stage micrometer picture to convert to micrometers. The area of each growth cones for the control and each experimental at t0, t15, t30, and t45 are then plotted to display any possible retraction or growth trends.

RESULTS

To test the effects of Methyl Mercury on neuronal growth cone retraction, neurons were exposed to different concentrations of MM and growth cone areas were measured over a forty-five minute period at fifteen-minute intervals. The overall areas for the experimental growth cones (10nm, 100nm) demonstrated a regressive trend during the forty-five minute time period, while the control demonstrated a progressive trend, and the 0nm concentration fluctuated between outgrowth and retraction.

Control Neuron absent from exposure to mercury

Fig.1

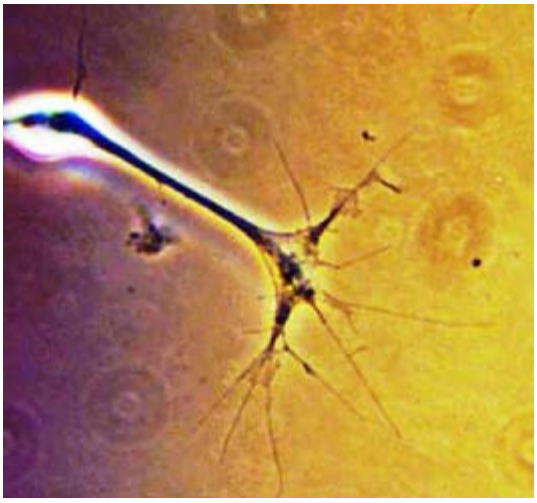

Figure 1 displays a typical neuronal growth cone and filapodia. This control neuron was imaged at 37 degrees Celsius,

t40, and using the program BTV Pro. The picture was edited and quantified using the Image J program.

Experimental Neurons Exposed to Mercury

Fig.2

Figure 2 was exposed to 100nm mercury concentration for duration of 20 minutes. The image was captured using BTV Pro at t45. The overall growth cone area displays growth cone retraction and degradation.

Figure. 3

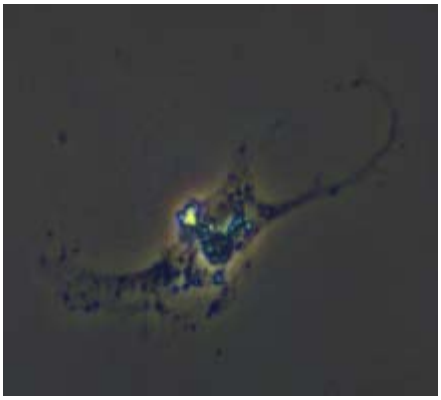

Figure 3 represents a neuron, which has been exposed to 10nm mercury concentration for 20 minutes. Using BTV Pro the neuron was imaged at t45. The overall area of growth cone demonstrates retraction and morphological changes.

Neuron Growth Cone Areas vs. Time

Fig. 4

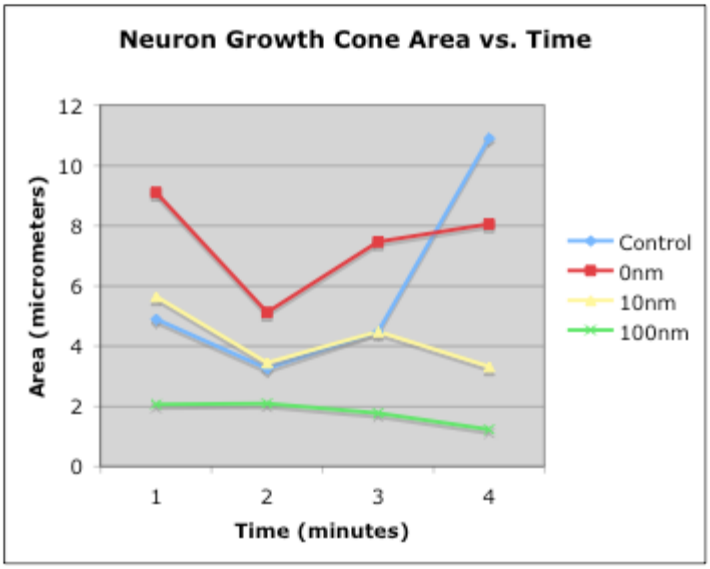

Figure 4 displays neuron growth cone area versus time. Control and experimental growth cone areas were recorded at time zero, time fifteen, time thirty, and time forty-five minutes. The control and 0nm concentration display an increase in growth cone area, versus the 10nm and 100nm concentrations, which display regression. n= the area of growth cones. For the control n= 9.7, for the experimental 10nm concentration n=4.5.

Linear Tredline Equations for Figure 4

Fig. 4.1

Concentrations	Linear Line Equation and R-Squared Value
Control	Y= 1.923x + 1.075 R_2 = 0.5277
0 nm	y = -0.084x + 7.64 R_2 = 0.0042
10nm	y = -0.593x + 5.705 R_2 = 0.5042
100nm	y = -0.277x + 2.475 R_2 = 0.8242

Figure 4.1 displays the tredline equation and R-squared values for the control and experimental data. The tredline for the control displays a positive growth rate, while the tredline for the experimental data shows an overall negative rate.

Discussion and Conclusions

The Effects of Mercury on Neuron Growth Cones

The three experimental mercury concentrations, which were administered to the neurons, appeared to cause morphological changes and growth cone retraction. The data collected from this experiment suggest the more concentrated the mercury and neuron exposure time, the greater the implications on neuronal structure and function. There are several environmental factors, which are extremely specific, to a nerve cell's function and survival. Variables

such as temperature, incubation time, exposure to solutions, mercury concentrations, and moisture content, weigh heavily on normal neuronal growth cone function. On the molecular level, the growth cone degeneration seen in the experimental solutions could be attributed to the mercury chloride ions interacting with voltage-gated and ligand-gated ions channels. This would have resulted in growth cone retraction and disrupted cell function. However, the data collected from the experimental concentrations demonstrated a variation in retraction and outgrowth. The experimental results overall did demonstrate growth cone retraction, but did not irrefutability support the hypothesis that the mercury would cause an indefinite and immediate response to growth cone function and morphology. Growth cones the corresponding filopodia are constantly changing shape and form, but in normal cells are continuously progressing as seen in the control. The experimental data suggest there was still growth along with retraction. However, the observed experimental mercury concentrations did overall show a more significant growth cone retraction and morphological change than the control.

It was observed from the experimental data, that the longer the neurons were exposed to mercury, the greater the effects to the neuron. To perform this experiment again or future experiments, testing mercury concentration on neuronal function, scientist should consider the long-term effects of this toxin on cell maintenance and function. In society the exposure to methyl mercury, typically persists over a longer period of time. To study and observe a greater and significant effect on growth cone retraction and degradation the same concentrations of mercury should be administered to the neurons, however the time of observation should be extended to days, weeks, or months. Increasing the data size would also elicit more accurate and comparable results. Measuring the areas of hundreds of growth cones exposed to mercury over a longer period of time, then averaged together would produce a more accurate experimental data set to draw conclusions from. In future experiments, the mercury chloride ions should gradually be administered to the neuron, to decrease the damage to the cell. The sudden and immense impact of the influx of ions could damage the cell, and interfere with accurate data collection and observation. Measuring and analyzing neurons that are relatively the same size will provide more accurate data to compare and draw conclusions from. I measured all different proportions of nerve cells, and would do it differently in future experiments. Finding similar nerve cells to measure would produce more accurate results.

In conclusion, the experimental data and control demonstrated the effects that mercury has on nerve growth cone function and morphology. The hypothesis was not completely supported, and therefore would require additional experiments with the suggest of longer exposure time, longer imaging period, analyzing and measuring similar cells,

and the slow addition of mercury to nerve cells. Overall, the experimental data did represent the detrimental effects mercury can inflict on growth cone function, progression, and morphology.

Bibliography:

Aneil Mallavarapu and Tim Mitchison Regulated Actin Cytoskeleton Assembly at Filopodium Tips Controls Their Extension and Retraction J. Cell Biol., Sep 1999; 146: 1097 - 1106

Block, Lawrence S., Patterson, Bill, Ryan, James, Dickey, Jefferson H., Wendroff, Arnold P., Ross, David A., Koral, Stephan M., Clarkson, Tom, Magos, Laszlo, Myers, Gary The Toxicology of Mercury N Engl J Med 2004 350: 945-947

Clarkson, Thomas W., Magos, Laszlo, Myers, Gary J.The Toxicology of Mercury -- Current Exposures and Clinical Manifestations N Engl J Med 2003 349: 1731-1737

E.R. Krandel, J.H. Schwartz, and T.M. Jessel. Principles of Neural Science. McGraw Hill/ Appleton and Lange, Norwalk, Connecticut. 2000.

Esther, Kovacs. Mercury-treated Neurons Show Slower Growth Cone Retraction Rate Than Non-Mercury Treated Neurons on Laminin Subtratum. Wheaton College, 2006. http:/icuc.wheatonma.edu

V Rehder and SB Kater Regulation of neuronal growth cone filopodia by intracellular calcium J. Neurosci., Aug 1992; 12: 3175 - 3186.