Objective:

The Effect of Mercury on Neuron-Neuron Interactions of Nine Day Chick Embryo Dorsal Root Ganglia and Sympathetic Chain Neurons

By Sara Tower

Introduction

Mercury is a neurotoxic (Leong et al., 2000) metal which, when exposed to the human body in large quantities, can cause damage to the brain, nervous system and the kidneys (Facts: Mercury in Drinking Water, 1998) and effect neuronal cell ultra structure (Braeckman et al., 1997). Through increased burning of coal and oil, and incineration of materials such as batteries, (Facts: Mercury in Drinking Water, 1998) mercury is becoming more prevalent in the environment. For this reason, it has become important to investigate the effects that mercury has on the human body in an attempt to understand potential hazards to which humans are increasingly exposed on a daily basis.

Previous investigations into the effects of mercury on neuronal cell cultures have outlined mercury's detrimental effect on normal neuronal growth and activity. Leong et al.'s article on the *Retrograde degeneration of neurite membrane structural integrity of nerve growth cones following* in vitro *exposure to mercury* (Leong et al. 2001) most readily pertained to this investigation and found that mercury ions disrupted membrane structure and linear growth rates of neurites in 77% of all nerve growth cones (Leong et al. 2001). In an effort to complement Leong et al.'s findings, this study will investigate the effects of mercury on neuron-neuron interactions of nine day old Dorsal Root Ganglia (DRGs) and Sympathetic chain neurons obtained from the chick embryo.

Although neuron-neuron interactions in normal neuronal cell cultures are not completely understood, it is hypothesized, based on previous investigations into the effects of mercury on other aspects of neuronal activity, that neuron-neuron interactions will be decreased in dense neuronal cell cultures exposed to mercury. Based on preliminary observations, it is hypothesized that this decrease will be seen as distinct decreases in axonal interaction. It is hoped that this research will be complemented by several other ongoing investigations with collaborators including B. Chick's investigation into the effects of mercury on growth cone activity, A. Silverio's investigation into the presence of actin with and without mercury exposure and J. Fess's research into the effects of mercury on neuron-glial interactions. Through this analysis, and in conjunction with these other researchers, it is hoped that a better overall understanding of the effects of mercury on neuronal cell cultures can be obtained, and that a clear picture of neuron-neuron interaction and mercury's subsequent effect on that interaction can be understood.

Materials and Methods

Materials for Neuron Dissection (As adapted from Morris) 2/14/06 7. S.A.T.

Objective:

3 x 35 mm Petri dishes (one for dissection collection, two for plating coverslips), 2 x 110 mm Petri dishes (one for dissection, one for CS treatments), Poly K, Laminin, 1 x 50 mls Hanks Buffered Saline Solution (HBSS), Sterile forceps bag, Pasteur pipette container, Bulb for Pasteur pipettes, Plastic tray, Dissection scope, Disposable gloves, 2 x coverslips, Waste Bowl, 9- Day Chicken Eggs, 70% Ethanol, Kimwipes

Materials for Independent Investigation

F+ medium, 10nM Hg/HBSS solution, 10nM HCl/HBSS solution, Distilled Water, Pasteur Pipette, Slides, Coverslips, Kimwipes, Forceps, VALAP wax, Hot Plate heater

Paint Brush, Standard Microscope fitted with Camera and SPOT imaging technology,

Space Heater

Procedure for Neuron Dissection (As adapted from Morris) 2/14/10 3. S.A.T

Before preparing eggs, one 110 mm Petri dish was set up and in it, approximately 5mls HBSS were added.

The blunt egg of an 8-12 day incubated chick egg was sterilized with ETOH. Then, using the blunt end of sterile forceps the shell was broken and the top of the shell was lifted away and discarded. Approximately 3mls of the egg yolk was then removed. Using the sterile forceps, the embryo was lifted out of the egg and placed into the 110 mm Petri dish containing HBSS. The head was then removed by gently pinching the neck area, and was then placed back into the egg. Again with sterile forceps, the visceral organs (wings and legs) were removed by gently snipping the soft tissue connecting them to the body with sharp forceps. The embryo was then positioned ventral side up and the tissue surrounding the spinal cord was gently pushed away with the forceps, washing with HBSS when necessary. This exposed the spinal cord and the Dorsal Root ganglia (DRGs) were observed. Using the forceps, the round ball-like ends of the DRGs were gently pulled away from the spinal cord on each side and set into a small 25mm Petri dish (previously set up with 1ml HBSS). Once all visible DRGs were removed from the spinal column, the sympathetic chain was gently teased up and away from the top portion of the spinal column. The sympathetic chain was also placed in the 25 mm Petri dish along with the previously collected DRGs.

Dr. Bob Morris then collected the DRGs and sympathetic chains and treated them with a solution of 0.25% trypsin and allowed them to incubate for 15-20 minutes at 37 degrees Celsius. He did this by removing the HBSS and adding the trypsin solution. After the incubation period, the trypsin solution was removed and HBSS was again added to the dish and the neurons were triturated gently with a sterile pipette.

The neurons were then ready to be plated. Coverslips were treated with two types of substrata, both laminin and collagen. To make laminin substratum on the coverslip, the surface of a cleaned coverslip was coated with 1mg/ml

poly-L-lysine by placing the coverslip into a 110mm Petri dish containing the poly-L-lysine. The coverslips were then rinsed with sterilized distilled water and allowed to dry. After drying, the coverslips were coated with a laminin/HBSS solution for 20-30 minutes at which time they were coated with a dilute solution of neutralized collage (0.1 mg/ml) again for 20-30 minutes.

The neurons were then ready to be plated on the treated coverslips. There were three cell densities which were made; sparse (.5 DRG/coverslip with 24 hours growth), medium density (1 DRG/coverslip with 24 hours growth) and high density (3 DRG/coverslip with 24 hours growth).

The coverslips were then incubated between 24-48 hours at 37 degrees Celsius upon which time investigations into Neuron-Neuron interactions were began.

The previous procedures were conducted in entirety by Dr. Bob Morris 1-2 days before Independent Investigations were conducted by individual students and the procedures were repeated as needed (as often as independent investigations needed neuronal cell cultures).

Procedure for Independent Investigation (as adapted from Tower) 3/21/06 ***4*** *S.A.T*

Before treatment, two solutions were made by Dr. Bob Morris. The control solution was a 10nM HCl/HBSS solution. The experimental solution was a 10nM Hg/HBSS solution.

Two 24-48 hour long incubated dense neuron cultures were removed from the incubator. In each dish, the F+ medium was removed using a non-sterile Pasteur pipette and 1ml of the control or experimental solution was added to each respectively labeled dish. Both dishes were then incubated at 37 degrees Celsius for a 20 minute period. After the incubation time, the Hg/HBSS or HCL/HBSS solutions were removed from the dishes and 1mL of F+ medium was added to both dishes and the dishes were allowed to recover at room temperature for 10 minutes.

After this 10 minute recovery period, chip chambers were made for both the control and experimental coverslip. To make a chip chamber, a slide was cleaned using a Kimwipe. Four glass shards (made from a crushed coverslip) were placed equidistant on the slide in the shape of a square (size similar to a coverslip). All of the F+ medium was then removed from each Petri dish using a pipette. One drop of medium was placed in the middle of the square made by the shards on the slide. Carefully yet quickly to avoid drying, using forceps, the coverslip was lifted out of the dry Petri dish and placed inverted (so neurons were faced down) onto the four glass shards. A kimwipe was used to wick away extra F+ medium from the sides of the coverslip and the edges were sealed using VALAP. To do this, VALAP was melted at 48 degrees. When liquid, a brush was used to paint the sides of the coverslip with VALAP wax. When dry, the top of the slide was gently washed using distilled water and dried using a kimwipe, being sure to wipe away from the center of the coverslip.

The slide with the coverslip was then viewed using a standard microscope at 10X. Pictures and time-lapse videos were taken of the neurons using SPOT in the Imaging Center for Undergraduate Collaboration (ICUC) at Wheaton College. During imaging time, the cells needed to be kept warm (at approximately 37 degrees Celsius) to continue their normal activity. This temperature was maintained by using space heaters that were placed next to the microscope stage while cells were being imaged.

To quantify data, time lapse videos of mercury exposed and normal neuronal cell clusters which were representative of neuronal interactions throughout the culture, were taken for a period of 12 minutes each. After the 12 minute video was obtained and saved to a suitable computer file, images corresponding to time 0 minutes and time 12 minutes were extracted from the videos for both normal and mercury exposed neuronal cell cultures. These images were printed using inkjet printers available in the Wheaton College ICUC and then labeled clearly normal or mercury and 0 or 12 minute.

Images were analyzed for four different types of occurrences. Triple points from two different axons were traced in green ink, triple points resulting from branching were traced in red ink, triple points occurring from unknown sources were traced in blue ink and all filopodia were traced in purple ink. For the purpose of this investigation, triple points were designated as a point from which a Y type formation was seen when two branches either come together to form one branch, or one branch splits into two branches. Also for the purpose of this investigation, filopodia were designated as all branches of less than 5 mm in length.

Once all branching events (triple point and filopodial contributing) were accounted for in each image, the total number of triple points from two different axons, triple points from branching events and triple points occurring from two unknown sources were manually counted and totaled in both control and mercury exposed cell cultures. The rate of triple point formation from these so called “long branching events” was calculated for each. The number of filopodia contributing to triple point presence was also manually counted in each image and the percent of filopodia contributing to triple points at time 0 and time 12 for both control and mercury cultures was calculated.

Results

To determine whether mercury exposed cultures effected neuron-neuron interactions in dense cell cultures, the overall rate of triple point formation as well as rates of triple point formation resulting from long branching and filopodial events were calculated based on representative images obtained from both control and mercury exposed cultures. Image 1 and 2 below are the actual representation images that were used. Image 1 represents the control neuronal culture and Image 2 the mercury exposed culture.

Objective:

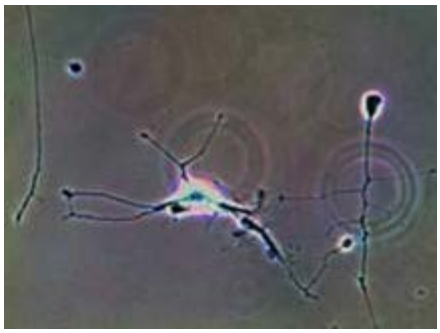

Image 1: Representative image of neuronal cluster in control dense neuronal culture.

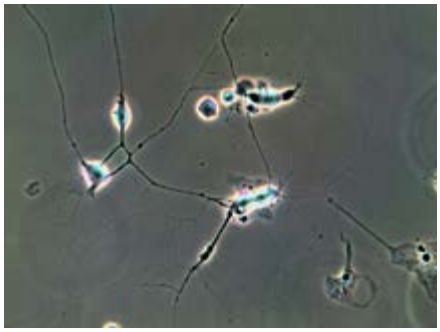

Image 2: Representative image of neuronal cluster in mercury exposed dense cell culture

Figure 1 below is a graphical representation of the overall rate of triple point formation resulting from long and short branching events in both mercury exposed and control neuronal cell cultures. Because the data was obtained from only one representative photo from each culture (control and mercury exposed), the n value for this experiment is one still image or an estimated 7 neuron network in the mercury image and a 5 neuron network in the control image

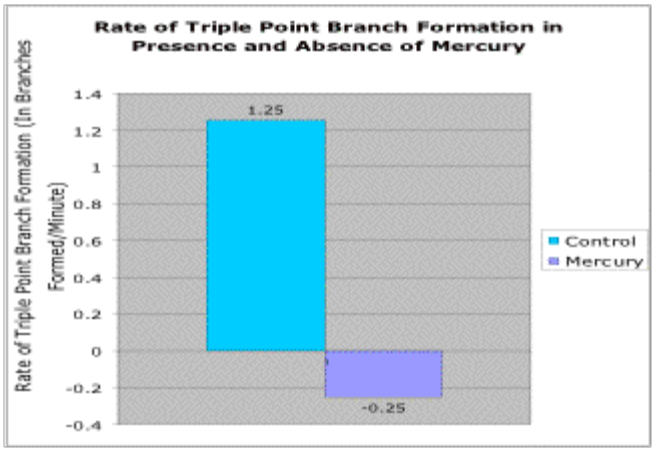

Figure 1: Rate of triple point formation resulting from long or short branching events in dense neuronal cultures exposed to mercury was significantly less than in dense control neuronal cultures. This preliminary data indicates mercury may decrease neuron-neuron interactions by limiting triple point formation.

In the control images, the number of triple point occurrences resulting from any type of long or short branching

axonal interactions continually increased over the 12 minute observation time and a rate of 1.25 (triple points formed/minute) was observed. Dense cell cultures exposed to mercury, however, resulted in fewer triple point occurrences over the 12 minute observation period and a negative rate of -0.25 (triple points formed/minute) was calculated.

Filopodial branching events, those branches of less than 5 mm in length, for the purpose of this study, did not represent a true triple point or neuron-neuron interaction because they were a clear result of branching near the axon terminal. For this reason, the frequency of triple points resulting from filopodial branching events was calculated and represented in Figure 2 below. Because the data was obtained from only one representative photo from each culture (control and mercury exposed), the n value for this experiment is one still image or an estimated 7-neuron network in the mercury image and a 5-neuron network in the control image

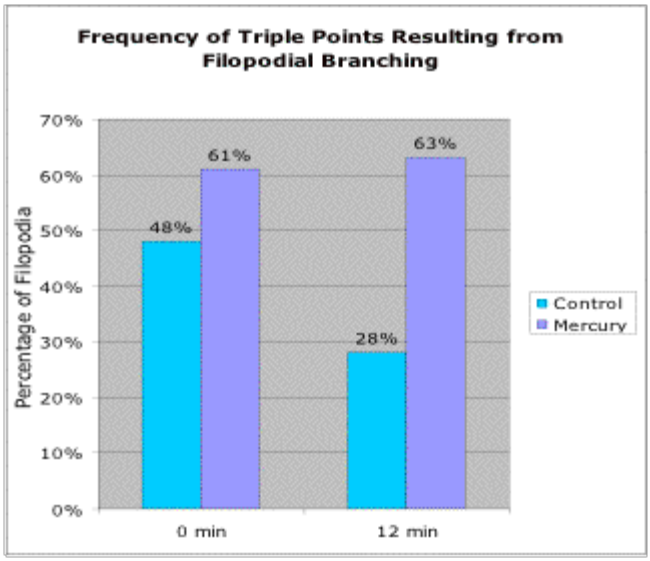

Figure 2: The percentage of triple points in representative images at time 0 and 12 minute, resulting from filopodial branching events. Mercury exposed neuronal cultures maintained a high frequency of triple points resulting from filopodial branching while filopodial branching was decreased in control neurons after the 12 minute incubation period.

Conversely, triple points resulting from long branching events increased over the 12 minute observation period in the representative cluster from the control neuronal culture while long branching events ceased to increase over the 12 minute observation period in the representative clusters of the mercury cultures. This data can be seen in Figure 3 below.

Objective:

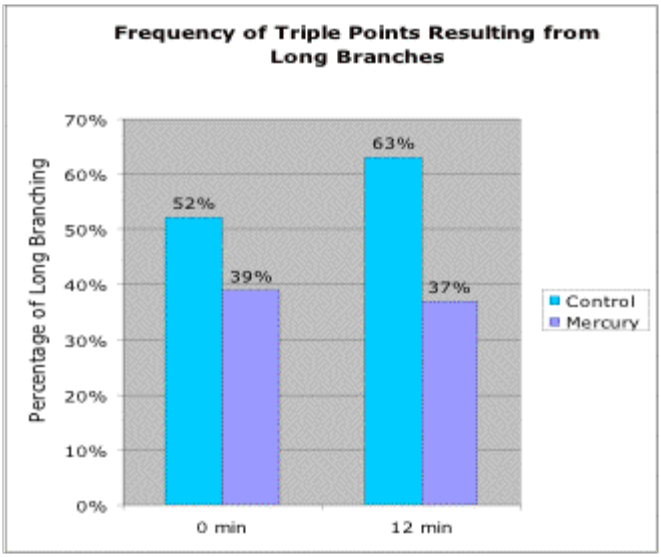

Figure 3: Frequency of triple point occurrences resulting from branching of long axonal outgrowths was greater fro the representative control clusters after both 0 and 12 minute observation periods. If long branching triple point formation accurately models neuron-neuron interaction, this data may indicate that mercury decreases neuron-neuron interaction.

Again, because the data was obtained from only one representative photo from each culture (control and mercury exposed), the n value for this experiment is one still image or an estimated 7 neuron network in the mercury image and a 5 neuron network in the control image.

Overall, no significant change in the position or interaction between neuronal cell bodies was observed. They seemed to remain stationary.

Discussion

This study has shown that neuronal exposure to mercury decreases triple point formation rate and triple points resulting from long branching events. It has also demonstrated that triple points resulting from filopodial branching occur at a higher frequency in neurons exposed to mercury than in control neurons. It was shown that the overall rate of triple point formation occurring from all contributing factors was higher in control than in mercury exposed neuronal cultures. All contributing factors included triple points resulting from all long branching events (triple points from two different contributing axons, triple points from branching events and triple points resulting from unknown neuronal sources) as well as filopodial branching events. This total number of branching events did not discriminate as to the source of the event, only to its presence. This lack of discrimination was necessary to eliminate error that may have

resulted from the objectiveness of the quantification process if researchers needed to individually determine which type of event led to the triple point itself. Determination of filopodial events was easier to subjectively quantify however, and they were quantified as all outgrowths of 5mm in length or less.

If triple point formation resulting from long branching events is indeed an accurate representation of neuron-neuron interactions, than this preliminary data indicates that mercury may decrease neuron-neuron interaction by limiting triple point formation. Although the frequency of triple points resulting from long branching (Figure 3) are not significantly decreased after exposure to mercury and a subsequent 12 minute observation period, their presence remained relatively constant which suggests that mercury may not break down interactions which were present before mercury exposure, but rather limit future triple point formations and in turn, limit future neuron-neuron interactions. This is supported by the control data in Figure 3 which demonstrates that triple points from long branching events not only occur at a higher frequency initially, but new triple points continue to form over time.

Because a detailed understanding of the mechanisms of normal neuron-neuron interactions has not yet been developed, researchers in this project are forced to speculate as to what causes neuron-neuron interactions, as shown by triple point formation, to decrease in the presence of mercury. It is speculated that mercury may degrade adhesion proteins located in the plasma membrane of neuronal cells and axons which aid in the formation of en passant synapses (Morris et al., 2005) and contribute to neuron-neuron interactions. This type of plasma membrane degradation was also seen in Leong et al.'s 2000 study. We were also forced to speculate as to why filopodial activity seems to be increased in neuronal cell cultures exposed to mercury. It is thought that mercury may not damage the integrity of the actin which constitutes filopodia.

Collaborators in this experiment who investigated the effects of mercury on other aspects of normal neuronal activity obtained results which correlated with the findings of this study. Chick, B. (2006) found that filopodia activity increased after exposure to mercury and interestingly, Silverio, A. (2006) observed that fluorescence for actin was brighter in the neurons treated with mercury. This data supports this studies speculation that actin integrity may not be compromised after mercury exposure. Fess, J. (2006), who studied the effects of mercury exposure on neuron-glial interactions, found that neuron-glial interactions were also decreased after mercury exposure in dense cell cultures. This finding is particularly pertinent to this papers stipulation that neuron-neuron or perhaps simply cellular interactions may be decreased after cell exposure to mercury.

To further the advancement of this investigation, it may be necessary to obtain a better understanding of normal neuronal activity, and specifically normal neuron-neuron interactions. If a mechanism can be derived which would outline normal neuron-neuron interaction, than reasonably, it would be more feasible to design experiments which

could explain the mechanism by which neurons interact. Similarly, one of the weakest portions of this experiment was our inability to define "neuron-neuron" interaction. Perhaps because the mechanism by which neurons interact is not understood, a generally guideline which describe neuronal interaction has also been neglected. For the purpose of this experiment neuron-neuron interactions were labeled as "triple points" however a more universal term or definition may be desirable if future investigations into this topic are pursued. Another adjustment in methods which would help to increase the accuracy and precision of this investigation would be to quantify the data using several representative images from each of the neuronal cultures. This would increase the n value (which in this experiment was only one image or one mercury exposed neuron cluster consisting of approximately 7 neurons and one control neuron cluster consisting of approximately 5 neurons. A higher n value would increase the validity of future experiments.

Sources Cited

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