Substantial retraction of growth cones resulting immediately after exposure to mercury

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Introduction:

Many forms of mercury such as ionic mercury and methylmercury are known neurotoxins (Zahir et al., 2005). The effects of methylmercury have been extensively researched at the cellular and organismal level. The symptoms of mercury exposure include memory loss, attention deficit, and autism (Zahir et al., 2005). David Brown et al. studied the effects of methylmercury treatment on the microtubules of mouse lymphocytes and found that microtubule disassembly occurred after exposing the lymphocytes to 1-10 μM of methylmercury (Brown et al., 2004).

The effects of ionic mercury at the cellular and organismal level are relatively unknown. Mercury ions are suspect for hindering tubulin polymerization. Leong et. al fixed neurites that were exposed to mercury chloride for actin/tubulin immunofluorescence (Leong et al., 2001). They reported that compared to a control, the mercury treated growth cones had less tubulin/microtubule structure (Leong et al., 2001). This data from immunofluorescence suggest that exposure to ionic mercury alters microtubule growth (Leong et al., 2001).

The current study was conducted to better understand the effects of ionic mercury on neurons at the cellular level. Specifically growth cones of neurons in *Gallus gallus,* domestic chicken embryos, were studied. The chick embryo was chosen as a model system because it is easy to obtain and maintain and has many similarities to mammalian development. (Rashidi et al., 2009).

In this experiment chick neurons were treated with 100nM $HgCl_2$ to test the hypothesis that axons retract after mercury exposure as measured by an increase in axon thickness and a simultaneous decrease in growth cone area.

It is expected to see evidence in support of this hypothesis because researchers have discovered that exposure to mercury results in the retraction of growth cones (Leong et al., 2001). If growth cones retract, it is plausible that the axon of the neuron becomes thicker to take in the retracting growth cone.

Materials and Methods:

Primary Culture of Chick Embryonic Peripheral Neurons:

10 day old chicken embryos were dissected according to Morris (2011a). A Nikon SMZ-660 dissection microscope was used to recover dorsal root ganglia (DRGs) from the spinal cord. It was discovered that the neurons grew well in the L15 growth medium containing 50ng/ml of Nerve Growth Factor and 100Uμg/ml pen strep. This growth medium was used for the duration of the experiments. The DRGs were incubated at 37°C overnight before observation.

Observation, Creating Control and Mercury Exposure for Chick Embryonic Peripheral Neurons:

16-hour-old neurons were observed according to Morris (2011b) and Morris (2011c). The observations for the control neurons and the mercury exposed neurons were made in the ICUC at Wheaton College in Norton, Massachusetts. A Nikon Eclipse E200 with phase optics was imaged through a SONY DFW-X700 with a 1.0x C-mount. The imaging software used was BTV version 6.0b1. The computer used was the iMac at station "Leo" which had operating system Mac OS X version 10.5.8. The images were taken at 40X objective on the Phase 2 setting on the aperture. The heater used was a ceramic air heater.

The protocol followed in both the control experiment and the mercury exposure experiment followed the basic procedure outlined in Morris (2011c). In the control experiment, after finding the neurons under the microscope the heater was turned on, and the neurons were warmed for one minute. Then an image was captured and called time 0. The temperature was recorded at the time when each picture was captured. The heater was left on for the remainder of the experiment (with the temperature remaining between 31-34°C), and pictures were captured in two minute intervals until 8 minutes was reached (time 0, time 2, time 4, time 6, time 8). Then the buffer was changed which allowed a control for flow. The buffer change included adding 1ml of growth medium to the flow chamber on the microscope slide. After change of buffer, the slide was refocused. After one minute, the first image was captured and labeled as time 0. Then images were captured at 2 minutes, 4 minutes, 6 minutes and 8 minutes.

For the mercury experiment, a 100nM solution of $HgCl_2$ in Hank's Balanced Salt Solution (HBSS) was made from 10ml of 10μM $HgCl_2$ in 0.05% HCl. The experiment with mercury was performed in the same manner as the control experiment as described above, except that the change of buffer involved adding 1ml of the 100nM solution of $HgCl_2$ in HBSS to the flow chamber. The $HgCl_2$ solution was left in the chamber for 10 minutes with the heater left on (with the temperature remaining between 31-34°C). At the conclusion of the 10 minutes, a second buffer change occurred which included replacing the $HgCl_2$ with 1ml of growth medium as described above. Then pictures were captured as described above with one at time 0, time 2, time 4, time 6, and time 8.

Analyzing Data:

The data was analyzed with Elyse Doherty (Doherty, 2011). Because the hypothesis of this experiment was

testing the behavior of neurons after exposure to mercury, the control images from after buffer change (t-0,2,4,6, and 8) and the images from post-mercury exposure (t-0,2,4,6, and 8) were analyzed. To analyze the data ImageJ version 1.40g was used. First the brightness scale for each set of pictures was altered. For the control pictures and the experimental pictures the brightness scale was altered such that the minimum input was 2 and the maximum input was 71.

ImageJ was used to measure axon thickness and growth cone area in each of the captured pictures. The axon thickness was measured in pixels using the straight line selection tool as the point where the growth cone met the axon where the sides of the axon became parallel. The growth cone area was measured in pixels using the freehand selection tool. With the freehand selection tool, the entire perimeter of the growth cone was traced starting at the point where the axon thickness was measured and continuing around the growth cone. All filopodia were included. All unobstructed neurons were measured. This resulted in 1 neuron being measured in the control and 3 neurons being measured in the experimental.

Once measurements were collected for axon thickness and growth cone area for each neuron, the ratio of growth cone area to axon thickness was calculated for each neuron at each time point. These ratios were averaged in the experimental pictures where there were three neurons per picture. These ratios were plotted in a line graph to show the growth and or retraction of the growth cones in the experimental and control as seen in Figure 3.

Results:

Four additional images, like the one in Figure 1 were captured for the control at time 0, time 4, time 6, and time 8. Four additional images, like the one in Figure 2 were captured for experimental at time 0, time 6, and time 8. The frame in each of the 5 control images and the frame in the 5 experimental images remained constant such that the same growth cone present in Figure 1 was measured in all 5 control images and the same 3 growth cones present in Figure 2 were measured in all 5 experimental pictures.

The ratio of growth cone area to axon thickness in the control figures were compared to the same ratio in the experimental figures at the same time point after either buffer change or mercury exposure. Therefore this ratio in Figure 1 (control at 2 min) was compared to the ratio of growth cone area to axon thickness in Figure 2 (experimental at 2 min).

These ratios were plotted as seen in Figure 3 to show whether the ratio increased or decreased from one time point to another. An increase in this ratio was seen as a line with a positive slope in Figure 3 such as the blue line (control group) between 4-6 minutes. A decrease in this ratio was seen as a line with a negative slope in Figure 3 such as the red line (experimental group) between 0-2 minutes. The ratio of growth cone area to axon thickness in Figure 1 is

shown as the second blue point in Figure 3 (control at 2 min). The average of the ratios of the three growth cones present in Figure 2 is shown as the second red point in Figure 3 (mercury exposed time 2). As seen in Figure 3, at every time point excluding 0 min, the ratio of growth cone area to axon thickness was higher in the control group than in the experimental group.

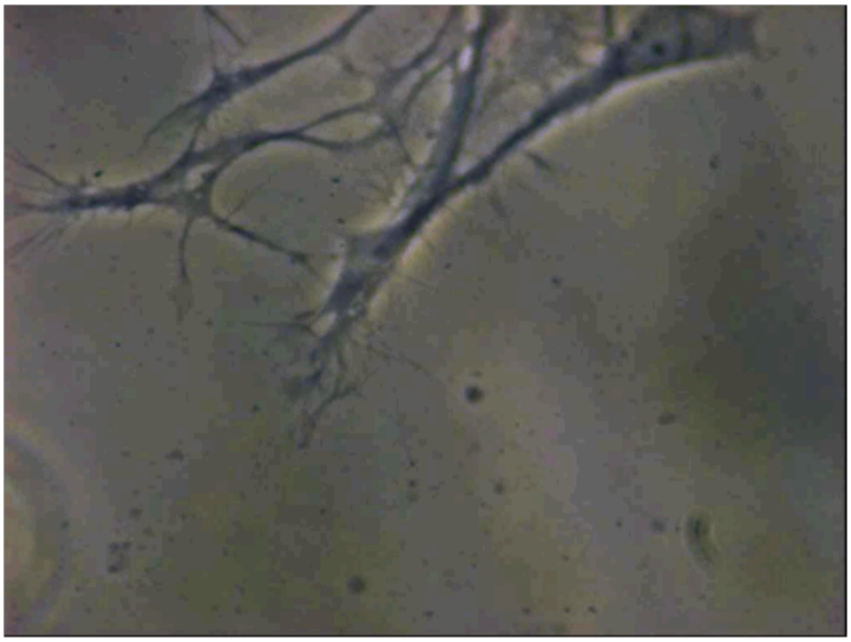

Fig. 1: Control image captured from growth cones of cultured *Gallus gallus* neurons at 40x magnification. This image shows a growth cone 2 minutes after buffer change. Note that there is one distinct growth cone and axon at the top of the image just left of the center. Also note that the majority of the cells present are glial cells.

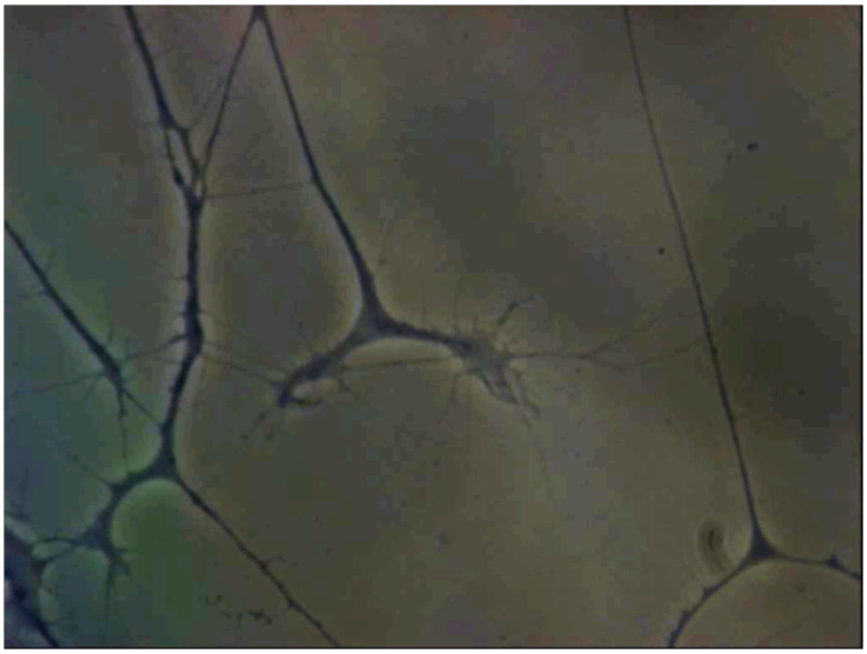

Fig. 2: Experimental image captured from growth cones of cultured *Gallus gallus* neurons at 40x magnification. This image shows growth cones 2 minutes after mercury was removed from the neurons. Note the presence of three distinct growth cones and axons (two in the center of the image and one in the left edge of the image).

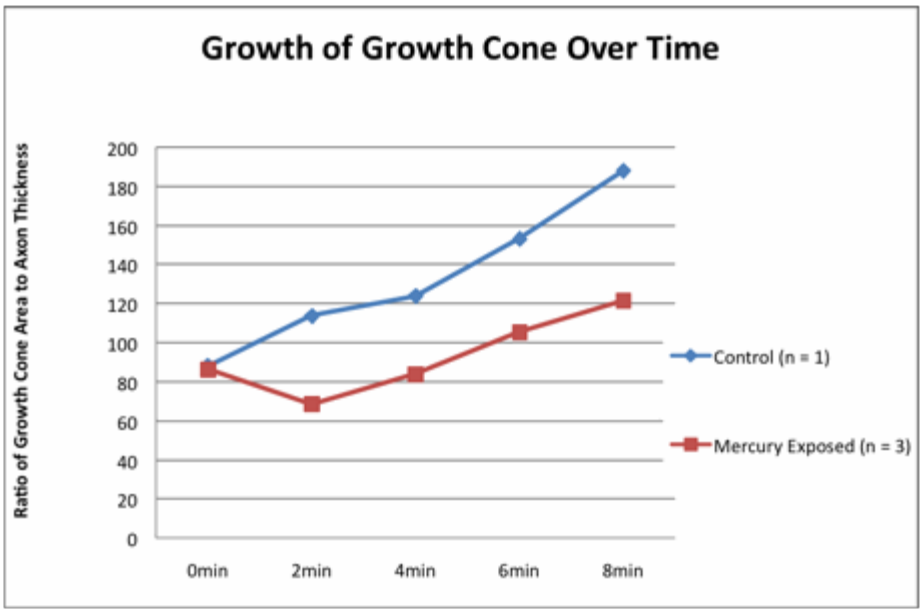

Fig. 3: The ratio of growth cone area to axon thickness at the time point when images were captured indicates whether the growth cone was growing or retracting. Notice the consistent increase in growth in the control compared to the decrease in growth from 0-2min after mercury exposure and the subsequent increase in growth from 2min- 8min after mercury exposure in the experimental.

Discussion:

My hypothesis was supported from 0 minutes to 2 minutes after mercury was removed from the growth cones: in this time interval the thickness of the axon increased and the area of the growth cone decreased indicating that the growth cone retracted. This retraction likely resulted from depolymerization and simultaneous inhibition of polymerization of microtubules in the growth cone. Although it is not known how mercuric chloride affects microtubules at a cellular level, there is evidence to suggest that methylmercury has its effects on microtubules because it binds to free sulfhydyl groups on the ends and on the surface of microtubules inhibiting their ability to polymerize (Vogel et al., 1985). It may be likely that mercuric chloride acts in a manner similar to methylmercury.

Interestingly, from 2 minutes after mercury was removed from the neurons through 8 minutes after mercury was removed, the ratio between growth cone area and axon thickness increased indicating that the growth cone grew. This suggests that the growth cones were able to recover from the effects of mercury exposure within 4 minutes after exposure, and continue growing. Irreversible damage can be seen in cells after 15 minutes of exposure to methylmercury (Toimela et al., 2004). It would be suspected that the recovery from mercuric chloride would be similar to the recovery from methylmercury. Because the neurons were only exposed to the mercury for 10 minutes, the total mercury concentration in the neuron may not have been significant enough to permanently alter the cell physiology. In fact cells treated with mercury may be able to recover depending on the total mercury concentration measured in their cells (Sager et al., 1984). In the control, the ratio between growth cone area and axon thickness increased from 0 minutes after buffer change to 8 minutes after buffer change indicating that the growth cone continuously grew.

To refine this research the same experiment would be conducted with more trials to increase the population size. If the population size was increased and enough supporting data was gathered to yield statistical significance, the claim that mercury inhibits growth immediately after exposure if exposure lasts for less than 15 minutes could be made with more certainty. In the future the ability of cells to recover from mercury exposure would be explored. This could be done by increasing exposure time to mercury to 15 minutes or longer to see if growth persists.

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