Neuronal exposure to mercury II chloride decreases the number of endosomes present in the growth cone of a degenerating neuron

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Introduction

Evidence has shown a strong link between functional actin patches and successful endocytosis (Huckaba et al., 2004, Samaj et al., 2004 and Smythe et al., 2006). Many of the proteins necessary for endocytosis localize themselves on actin patches (Huckaba et al., 2006 and Shurety et al., 1998). Additionally, previous studies have proven that mercury chloride exposure can cause degeneration of actin filaments (Leong et al., 2000). Mercury chloride can be chemically transformed into mercury II chloride. Therefore, the hypothesis tested states that neuronal exposure to 100nM HgCl₂ for 20 minutes will cause a decrease in the number of endosomes present in the growth cone. The potentiality of endosome formation depends on the presence of an effective actin cortex along the pre-synaptic membrane.

Sympathetic neurons isolated from a domestic chicken, Gallus gallus, embryo are used because the Gallus gallus has a nervous system comparable to that of a human.

This study is important in understanding the effects of mercury on neuronal activity because neurons must take up, often though endocytosis, various growth factors and nutrients from the extracellular space in order to grow (Samaj et al., 2004). Specifically, nerve growth factor, a small protein integral in the growth, maintenance, and survival of nerve cells, is brought into the cell via endocytosis (Samaj et al., 2004). The Environmental Protection Agency has noted an increase in the number of mercury poisonings and has stated that it can result in a non-functioning nervous system (“Mercury”, 2010). This paper aims to identify one component of a functioning neuron that becomes damaged as a result of mercury poisoning. In this study, a chick sympathetic neuron is exposed to mercury and then the numbers of endosomes present in the growth cone pre-Hg and post-Hg exposure are compared.

Materials and Methods

Fertilized domestic chicken (Gallus gallus) eggs were purchased and incubated at 37°C for ten days. After ten days, the fertilized eggs were removed from the incubator and inspected for the presence of an embryo. The protocol for the dissection as described in “Primary Culture Of Chick Embryonic Peripheral Neurons 1: DISSECTION” was followed.
Two coverslips, on which the sympathetic nerve chains and DRGs were eventually placed, were then prepared. The protocol used for the preparation of the coverslips is also described in “Primary Culture Of Chick Embryonic Peripheral Neurons 2: DISSECTION” (Morris, 2011a).

The sympathetic chains were placed in the center of one prepared coverslip and two DRGs (sliced in half) were placed in the center of the remaining prepared coverslip. These coverslips were placed in the incubator at 37°C and allowed to incubate and grow overnight. The next day, the two small petri dishes were removed from the incubator the dish containing the DRGs was looked at under a compound light microscope to observe that growth had occurred. The protocol that was followed to observe growth is called “Primary Culture Of Chick Embryonic Peripheral Neurons 3: OBSERVATION” (Morris, 2011b).

A flow cell was created to allow for a buffer exchange system that was used to test exposure to mercury. The protocol for making a flow chamber is detailed in “Primary Culture Of Chick Embryonic Peripheral Neurons 3: STAINING and OBSERVATION” (Morris, 2011c). The slide was taken to the ICUC at Wheaton College and viewed, using the 40x objective, under a Nikon eclipse E200 with phase optics. An initial image of a growth cone was taken through the microscope using a Sony DFW-x700 1.0x C-mount and BTV version 6.0b1 imaging software. The computer used to view the images was an iMac with Mac OSX version 10.5.8 operating system. Maintaining the initial image on the screen, the growth medium under the coverslip was exchanged with 1mL of HBSS buffer to act as the control for the rest of the experiment. A pipette was used to inject HBSS under the coverslip from one open end of the coverslip. At the other open end, a Kimwipe was used to collect the growth medium that was being removed from under the coverslip. This control was necessary to demonstrate that the exchange of the buffer alone does not induce a decrease in endocytosis. The HBSS was left under the coverslip for five minutes and then was exchanged again for growth medium. An image was taken after the coverslip was once again in growth medium. These two images together served as the control.

To perform the experimental aspect of this study, a new slide was prepared according to the protocol described above. The coverslip containing DRGs was plated on growth medium. Video-enhanced phase microscopy, using the equipment detailed above, was used to view the cells. A heater was placed approximately 1.5 feet away from the microscope and the environment surrounding the cells was maintained at a temperature of 37-39°C. After aligning the microscope for Koehler illumination, the all of the growth cones were examined. A growth cone was defined in this experiment as beginning where the walls of the axon diverge from being parallel. Adding to this definition, a growth cone is located at the distal end of a neurite. Four images were taken of growth cones that were representative, with
respect to the number of endosomes present, of all of the growth cones on the slide. For the purpose of this study, an endosome was defined as a phase-light, round object of at least 3.50 pixels in length.

The next step was to introduce the mercury (100nM HgCl₂ in HBSS). Using the flow chamber, 1.0mL of the working mercury solution was added under the coverslip in exchange for removal of the growth medium. Once the cells were submerged in mercury, the period of exposure lasted for 20 minutes. At 20 minutes post-introduction of mercury, the mercury solution was exchanged with 1mL of fresh growth medium. Immediately after the mercury was removed, the growth cones throughout the slide were once again examined. Four more images were taken of growth cones that were representative, with respect to the number of endosomes present, of the entire population of growth cones present on the slide. It was important to do this step relatively quickly so as not to allow for possible regeneration and rehabilitation of the growth cone region. One “trial” was conducted in which four pre-Hg and four post-Hg images were taken. The four pre-Hg images represent the process of endocytosis as it occurs in the absence of mercury and the four post-Hg images taken represent the process of endocytosis as it is affected by exposure to mercury.

Data was collected by taking images of the growth cones before and after they were exposed to mercury. The data was quantified by counting the number of endosomes present and visible in the primary growth cone of each image. ImageJ was used to adjust the contrast of the image in order to focus on the phase-light endosomes. The collected data was pooled and the number of endosomes was averaged. In order to analyze the data, the average number of endosomes present per growth cone before exposed to mercury was compared to the average number of endosomes present per growth cone after 20 minutes of exposure to 100nM HgCl₂.

Results

The data collected from this experiment show that the average number of endosomes per growth cone is greater before the neuron is exposed to mercury as compared to after a 20 minute exposure to 100nM HgCl₂. Before the mercury solution was introduced to the cells, an extremely high percentage of growth cones contained endosomes. In comparison, it was highly unlikely to see a growth cone containing any endosomes after the cells were allowed to sit in the mercury solution for 20 minutes. Each of the growth cones imaged pre-Hg exposure contained endosomes whereas none of the growth cones imaged post-Hg exposure contained a single endosome. These images were representative of the entire field of growth cones on the slide. Figure 1 below shows the control before and after buffer exchange.
Figure 1 – This figure represents the control for this experiment. Two growth cones extend down from the top of the image toward the bottom left of the image. The image on the left was taken when the cells were sitting in growth medium, before the buffer was exchanged. The image on the right was taken after the buffer was exchanged (HBSS replaced the growth medium). The reader should notice that the growth cone regions do not degenerate after buffer exchange.

Figure 1 is significant as it demonstrates that the act of exchanging the buffer did not cause a change in the growth cone integrity. This allowed the endosome counts to be directly related to the presence of the mercury.

Figure 2 below depicts the effects that mercury has on the number of endosomes present in a neuronal cell growth cone.
Figure 2 – This figure represents the effects of mercury on the number of endosomes present in the growth cone. In this study, the growth cone begins where the walls of the axon diverge from parallel. The image on top shows a growth cone, slightly up from the center of the image, before it was exposed to mercury. The phase-light, round objects seen within the growth cone are endosomes. The figure on the bottom depicts a growth cone after it was exposed to mercury. It contains no endosomes.

As seen above in Figure 2, the pre-Hg exposure images demonstrate that endosomes are present in growth cones of a fully-functioning neuron. Four images were taken that were representative of all of the growth cones before toxic exposure.

Each pre-Hg growth cone imaged contained endosomes and the total number of endosomes in all four growth cones was 24. When averaged, each growth cone contained six endosomes. On the contrary, four images were taken that were representative of the entire field of growth cones after the cells were exposed to mercury for 20 minutes. None of the growth cones imaged contained endosomes because in the entire field of growth cones after exposure, only one growth cone was noted to contain a single endosome. Therefore, on average, each growth cone post-Hg exposure contained zero endosomes.
Figure 3 below shows a graphical comparison of the total number and average number of endosomes present in the growth cones before and after a 20 minute mercury exposure. Before exposure, there are 24 endosomes distributed among four growth cones, with each growth cone containing endosomes. After exposure, there are no endosomes present in any of the four growth cones. Thus, red bars are not visible in Figure 3.

![Endosomes in the growth cone, pre and post mercury exposure](image)

Figure 3 – This figure represents the pooled and averaged data from the experiment. The total number of endosomes in four growth cones pre-Hg exposure was 24 whereas post-Hg exposure was 0. The average number of endosomes, then, per growth cone before the addition of mercury was 6. In contrast, the average number of endosomes after mercury exposure was 0. Both n-values for post-Hg exposure are 0, which is why no red bars are visible on the chart.

It is important to note that although the growth cones studied expressed no endosomes, there were some endosomes present throughout the axons. However, these endosomes not in the growth cone were not the focus of this experiment and thus were not included in any data.

**Discussion**

The data analysis indicates that the hypothesis is supported. After exposure to HgCl₂, the growth cones showed an absence of endosomes, which may suggest that endosome formation in the growth cone region was possibly be halted in response to mercury exposure. This could perhaps suggest that the cells were no longer able to carry out endocytosis; however, there are alternative explanations as to why endosomes may not have been observed in the post-Hg growth cones. Mercury may potentially disrupt a cell’s ability to form endosomes, thus causing an absence of visible
endosomes in the growth cone region. Alternative explanations to this absence of endosomes are various. For example, it has been demonstrated that endosomes fuse with lysosomes, so it is possible that mercury exposure speeds up the fusion, making the endosomes essentially invisible to the eye (Luzio et al., 2000). It is also possible that the presence of mercury causes the endosomes to be transported very quickly to the axon closer to the cell body or even the cell body itself. Research has shown that mercury causes a retrograde degeneration of neurites (Leong et al., 2000), so it is possible that the contents of the endosomes are transported very quickly to where the cell needs the nutrients the most.

Having minimal and limited data makes it impossible to ensure that the data from this experiment is significant and accurate. If enough data was compiled, as to allow for statistical significance, one could conclude that an explanation for these data is that mercury does have an effect on the number of endosomes visible in the growth cone.

Researchers previously demonstrated that mercury does have a degenerating effect on the membrane of the neuron in addition to the actin within neurites (Leong et al., 2000). Significantly, many proteins necessary for endocytosis localize themselves on actin patches associated with the membranes (Huckaba et al., 2006 and Shurety et al., 1998). Thus, it is possible that mercury-induced deterioration of the growth cones’ actin filaments eliminated vital actin patches that hold proteins essential to endocytosis.

A source of error in this experiment involves the control images. The “control” growth cone did not contain any endosomes. Therefore it is not definite that the buffer exchange alone did not cause a reduction of endosomes in the growth cone. An appropriate control would have included many growth cones that would have held many endosomes. Additionally, only one trial was conducted, using just one slide. Additional trials are necessary to support or refute the hypothesis. To improve this experiment, three trials would be conducted and more images would be taken. Also, mercury could be left in the chamber while imaging to ensure that the growth cones have no time to regenerate.

To extend this work, an experiment could be designed to look directly at possible formation of endosomes after mercury has been removed. If endosomes are eventually able to reappear in the growth cone after a period of time post-exposure, that may indicate how long the effects of mercury last for a specific exposure time under a certain mercury concentration.

**References Cited**


