The effect of methyl mercury on the distribution of mitochondria in glial cells

Anya Sokolova
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Introduction

Methyl Mercury (MeHg) is a neurotoxin that affects the CNS in both developing brains and mature brains. The damage caused by MeHg cannot be undone (Aschner et al. 2006). Methyl mercury is present in the food chain and it poses a problem of toxicity (Atchison & Hare, 1994). At high doses, MeHg can cause the loss of neurons in humans and animals. At low doses, when there is no evidence of loss of neurons, MeHG can deplete the number of microglial cells (Eskes et al. 2002). Methyl mercury toxicity can cause many different neurodegenerative disorders (Aschner et al. 2006) including, but not limited to, Parkinson’s disease, Alzheimer’s disease, and epilepsy. The mechanism by which mercury induces neuronal degeneration is not very well understood (Chen et al. 2012). There has been evidence of the negative effects of methyl mercury on neurons, but there is not much known about the effects of methyl mercury on glial cells. The focus of this research is the effect of methyl mercury on glial cells because the glial cell shows the earliest signs of neural toxicity (Eskes et al., 2002). This experiment specifically looks at how methyl mercury could affect the length of distribution of mitochondria clusters away from the nucleus.

Mitochondria are organelles of cells that live in the cytoplasm (Lovas & Wang, 2013). They are necessary for the survival of neurons (Amiri & Hollenbeck 2008) because of their responsibility of making ATP by means of aerobic respiration, and for creating a homeostasis of Ca^{2+} ions. The quality of interactions between glial cells and neurons depends on the proper location of mitochondria in the glial cell (Lovas & Wang, 2013). Although mitochondria spend a large amount of time in the cell inactive (Chada & Hollenbeck, 2003), mitochondria trafficking occurs in response to provide ATP to regions where is a high demand of energy, or to help maintain the homeostasis of Ca^{2+} (Honick et. al. 2005). Glial cells communicate through signaling techniques that activate Ca^{2+} signaling. Proper homeostasis of distribution Ca^{2+} in mitochondria is
needed to effectively maintain a healthy glial cell (Lovas & Wang, 2013).

The purpose of this study is to test the effect of methyl mercury on the maximum mitochondria distribution from the nucleus of the glial cell. The experiment is tested on dorsal root ganglia and sympathetic nervous chain from ten-day-old chick embryos. In this experiment we hypothesize that methyl mercury would cause a negative effect on the maximum length of distribution of mitochondria from the nucleus of the glial cell. The higher the dosage of methyl mercury, the shorter the length of mitochondria distribution would be. Since methyl mercury is a neurotoxin that is detrimental to the proper development of neurons, it is important to look at the interactions within glial cells to show evidence for the harmful effects of methyl mercury on mitochondria, and on the central nervous system. This experiment was conducted in collaboration with Lauren McClelland and with the Neurobiology Class 324 of 2014 at Wheaton College MA.

Materials and Methods

Dissection

Chick embryos were dissected by the Neurobiology Bio324 Primary Culture of Chick Embryonic Peripheral Neurons 1: DISSECTION (Hollenbeck & Morris, 2014). After dissection, the isolated cells from the dorsal root ganglia and sympathetic nerve chain were incubated for at least 24 hours.

Labeling and Treatment

For our control and experimental treatments the procedure was almost the same other than the incubation of MeHg treatments. The control was incubated in DMEM and the experimental conditions were incubated in a low (20nM) and high(40nM) concentration of MeHg. Three petri dishes containing cells were taken out of the incubator. Each petri dish was labeled with permanent marker corresponding to the condition: control, low, and high. The growth medium was removed from the petri dish and set aside in a different petri dish. The control and both experimental treatments received a treatment of 10uL of 236 uM of Mitotracker diluted in 500uL of DMEM. We used this concentration of Mitotracker because we have 50mg of Mitotracker from our stock solution, and we wanted approximately 100nM final concentration on our cells.

The control and both experimental treatments were incubated for 10 minutes with the Mitotracker. The
lighting in the laboratory must be minimal to keep the Mitotracker in the dark. After incubation, the Mitotracker was washed for control and experimental treatments three times. This means that Mitotracker was taken out by means of unsterilized Pasteur pipettes, and fresh DMEM was added to the control and experimental treatment petri dishes with a fresh unsterilized Pasteur pipettes. Each new wash was kept in the petri dish for 1 minute. The petri dishes were kept under a long piece of aluminum foil for that minute. After the third wash of DMEM, the control petri dish got a fresh amount of DMEM. The experimental treatments got different dosages of MeHg. The high condition got 40nM of MeHg in DMEM. This concentration of MeHg was used based on the study done by Leong (2000), but the concentration was modified for our whole culture incubation conditions because Leong and colleagues applied their MeHg as an application through a pipette needle. The amount of MeHg was lowered because in this experiment the MeHg was placed directly on cells. For the experimental treatment of the low condition, the concentration of MeHg was cut in half to be 20nM of MeHg. This was done by adding eight drops of the high concentration (40nM of MeHg in DMEM) and eight drops of DMEM. The control and both experimental conditions were placed back into the incubator for another 10 minutes of incubation. During that time, three observation slides were labeled corresponding to condition: control, low, and high. The slides were then set up based on part B of the Neurobiology Bio324 Primary Culture of Chick Embryonic Peripheral Neurons 2: OBSERVATION of LIVE UNLABELED CELLS (Morris, 2014).

*Image taking*

Pictures of all three slides were taken with an immunofluorescence Nikon eclipse E200 microscope. The transmitted light images were taken using a Nikon camera at exposure 250 and gain 1 and the SPOT program. To take a picture of the same glial cell under immunofluorescence the exposure was changed to 600 and the gain at 2. These picture-taking steps were applied to both experimental conditions as well.

*Analysis*

Once all of the photos were collected, the pictures of the fluorescent glial cells were opened in Image J. The length of each glial cell, or the distance along the longest axis of the cell, was measured by using the straight-line measurement tool in Image J on the transmitted light image. The length included the filopodia and the lamellipodia. To measure the length of the cell the straight-line button was clicked, a line was drawn from one end to the other. To see the length, the tab ‘analyze’ was clicked, and then the button ‘measure’ was
clicked. The length was given in pixels. The length of each glial cell was recorded in Microsoft Excel. The length of the glial cell was measured and documented. The average length of dispersal of mitochondria from the nucleus is relevant in proportion to the size of the cell. The nucleus and nucleus center were first located using an image of the glial cell that was taken with transmitted light microscopy, and then located on the fluorescent image. For each glial cell, six measurements to the center the farthest cluster of mitochondria from the center of the nucleus were measured. A cluster of mitochondria in this experiment was defined as an area that emits Mitotracker dye. The farthest cluster of mitochondria that emitted any Mitotracker dye was measured using the same straight-line tool, and the same sequence of steps, that were used to measure the length of the glial cell. The average maximum length of the mitochondria dispersion was calculated by adding up all of the lengths and dividing the total number by 6. The average length was then (in pixels) was converted to micrometers. 5.4 pixels was equivalent to 1um. This conversion was determined in a previous experiment done in Neurobiology 324 by photographing a ruler and measuring the length of a millimeter on image J. The length in pixels per mm was converted to pixels per um. Once all of the averages were converted into um, the average information for each condition was graphed in one bar graph. Next, the ratio of the average distance the mitochondria traveled from the nucleus and the average size of glial cell were calculated for each condition. This information was used to create a column graph.

Results

To assess the effects of methyl mercury on dispersion of mitochondria in glial cells pictures were taken by fluorescence imaging and transmitted light imaging. These images can be seen in Figures 1 and 2. A glial cell that did not receive methyl mercury treatment can be seen in figures 1A through 1E. A glial cell that received methyl mercury treatment can be seen in Figure 2. To be able to see the potential changes of mitochondria distribution, glial cells were stained with a red fluorescent Mitotracker. The Mitotracker dye can be seen in figures 1A, D, E, and figure 2. Transmitted light images of each of the glial cells were also taken in order to measure the length of a glial cell and to find the nucleus of a glial cell. The transmitted light images can be seen in figures 1B and C. Figure 1B shows how the length of the glial cell was measured, and figure 1C shows how the nucleus of the glial cell was found. The complexity of Figure 1 is designed to
explain exactly how the length of each glial cell was measured, and how the length of the mitochondria
distribution was measured. The average length of the glial cells for each condition was graphed along with
the length of average maximum distribution of mitochondria from the center of the nucleus for each
condition. This can be seen in figure 3. In figure 3 it seems that the mitochondria clusters in the low
conditions are the farthest away from the center of the nucleus. However, the length of the average maximum
mitochondria cluster from the center of the nucleus for each condition need to be seen in proportion to length
of glial cell to accurately deduce if there was a change in length of mitochondrial distribution between each
condition. This proportion of length of mitochondrial distribution to length of glial cell can be seen in figure
4. The mitochondria in each condition were all 25% of the total length of the glial cell away from the center
of the nucleus. If a quarter of the length of the glial cell was measured, that would be equal to the average
maximum distance that the mitochondria were from the center of the nucleus. We found that there was no
obvious difference in the distribution of mitochondria clusters between the control and the experimental
conditions, which can also be seen in figure 4.

In the control condition and the experimental conditions there were qualitative differences between
mitochondria clusters and glial cell qualities of the difference conditions. The clusters of mitochondria in
figure 2 look like they are being distributed farther from the nucleus than the mitochondria clusters in figure
1A. The mitochondria clusters in Figure 2 look longer and more spread apart than the mitochondria clusters
in figure 1. The lamellipodia and filopodia of the glial cell that was not given any methyl mercury (figure 1B)
seem to be longer than the ones of the glial cell that was given methyl mercury (figure 2A). Although the
mitochondria clusters may look like they are distributed farther from the center of the nucleus under the
treatment of methyl mercury, we found that there was no obvious difference in the distribution of
mitochondria between the control and the experimental conditions. This is shown in figure 4.
Figure 1. Fluorescent images (Figures 1A, 1D, and 1E) of a cultured glial cell stained with Mitotracker (red), and transmitted light images (Figures 1B and 1C) of the same cultured glial cell before methyl mercury exposure. Pictures all taken with 40x objective. Figure 1A shows a fluorescent picture of chick embryo glial cell. N=1. The mitochondria clusters can be seen close together in Figure 1A and there is not much dark space seen between the clusters. Figure 1B shows how the glial cell length was measured. N=1. The length of the glial cell includes the filopodia and the lamellipodia, which are the hairy like extensions of the glial cell. Figure 1C and figure 1D shows how the nucleus of the glial cell was located on a transmitted light image and then on a fluorescent image. N=1. Figure 1E shows the manner in which 6 data points were collected. N=6.
B. Figure 2. Transmitted light image (A) and fluorescent image of same cultured glial cells stained with Mitotracker (red)(B) after 40nM of methyl mercury exposure in DMEM. N=1. Pictures both taken with a 40x objective . The lamellipodia and filopodia can be seen extending from the bottom of the glial cell in Figure 2A. The mitochondria in Figure 2 can be seen around the nucleus with dark space in between each cluster. The mitochondria clusters have a ‘stringy’ appearance in Figure 2A.

Figure 3. Average length of glial cell (in blue) next to average length of maximum dispersal of mitochondria clusters (in red). The average length of a glial cell that was given the high dose of methyl mercury was about 80 micrometers. The average length of a glial cell that was given the low dose of methyl mercury was approximately 110 micrometers. The average length of a glial cell that was not given a dose of methyl mercury was about 70 micrometers. The mitochondria clusters for the high condition traveled, on average, 20 micrometers from the nucleus of the glial cell. The mitochondria clusters for the low condition traveled, on
average, 30 micrometers from the center of the nucleus of the glial cell. The mitochondria clusters for the control conditions traveled, on average, less than 20 micrometers from the nucleus of the glial cell.

Figure 4. The calculated ratio, in percentage, of the average maximum length of dispersal of mitochondria clusters from the nucleus center, to the average length of the glial cell. The average length of the glial cell is the length of the longest axis. The mitochondria in the glial cell that was treated with a high dose of methyl mercury show an average maximum dispersal of 25% of the length of the glial cell away from the center of the nucleus. The mitochondria in the glial cell that was treated with a low dose of methyl mercury showed an average maximum dispersal of 25% of the length of the glial cell away from the center of the nucleus. The mitochondria in the glial cell that was not treated with a dose of methyl mercury show an average maximum dispersal of 25% of the length of the glial cell away from the center of the nucleus.

**Discussion**

This experiment does not support the hypothesis that exposure to methyl mercury causes a change in the length of the distribution of mitochondria from the nucleus. Once the glial cells were exposed to the methyl mercury, there was no change in the ratio of distance mitochondria traveled from the nucleus to the size of the glial cell. This can be seen in Figure 4. In Figure 4 the mitochondria in the control and both experimental conditions traveled a quarter of the length of the glial cell away from the center of the nucleus. Figure 4 shows that the exposure to methyl mercury does not cause a change in the distribution of mitochondria from the nucleus.

As our hypothesis states, Lauren and I were expecting that the methyl mercury would hinder the distribution of mitochondria from the nucleus. We expected that glial cells that were treated with methyl
mercury would show clusters of mitochondria that do not disperse away from the nucleus. Lauren and I expected these results because mitochondria move depending on local calcium levels. The blocking of Ca$^{2+}$ uptake (Atchison & Hare, 1994) by methyl mercury could cause a negative effect in the distribution of mitochondria from the nucleus of the glial cell. We also expected to see an overall decrease in mitochondrial, but we did not look at, or measure, the overall brightness of the mitochondria in this experiment. As was just stated, our results did not support our hypothesis, and there was no evident difference of the maximum length of distribution of mitochondria from the nucleus between the control and the experimental conditions.

An explanation for the lack of difference in mitochondrial distribution could be that a change in calcium levels can occur without affecting mitochondrial movements (Baker et al. 2013). In addition, if the uptake of Ca$^{2+}$ was blocked by methyl mercury, then there could be an abnormal increase of calcium levels which could cause the production of ROS (reactive oxygen species) and the possibility of apoptosis, or cell death could occur (Cosentino & García-Sáez, 2014). The unhealthy mitochondria could have suffered the fate of apoptosis, and the mitochondria that were present in our methyl mercury treated glial cells were healthy. This would support why the mitochondria in the control and the experimental glial cells all distributed the same length from the nucleus in proportion to the length of the glial cell. Although the sample size was relatively small, if the sample size was larger and this experiment was redone with the same results, it could further confirm that there may not be a strong relationship between methyl mercury, calcium levels, and mitochondrial movement or methyl mercury could trigger mitochondrial apoptosis, leaving only healthy mitochondria in the glial cell.

There could be other explanations for the results that were attained. In order for a damaged mitochondrion to be properly repaired it may be cleared from the cell, or repaired by fusion of said mitochondrion with a different, healthy, mitochondrion. A damaged mitochondrion could be sent back to the cell body (Lovas & Wang, 2013). There could have been mitochondria movement retrograde, meaning going back to the cell body, and not anterograde, meaning going away from the cell body. We did not distinguish between mitochondria that were being trafficked anterograde and retrograde. This could be researched in a future experiment because it could help the study by accurately assessing the length of mitochondrial
dispersion that is traveling away from the center of the nucleus. This type of experiment could be problematic because clusters of mitochondria could be comprised of both mitochondrion that are traveling away from the cell body, and mitochondrion that are traveling back to the cell body.

A different future experiment could be conducted with a longer incubation time with the same dosage of methyl mercury, or the same incubation time and a larger dose of methyl mercury. A longer incubation time, say 15 minutes instead of 10, may yield different results because of the amount of time methyl mercury is affecting the glial cells and the mitochondria. This experiment may also not show any differences in results. Increasing the dose of methyl mercury, say to 100nm as a high dose and 50nm as a low dose, but keeping the incubation time the same (10 minutes) may show a difference in the maximum length of distribution of mitochondria between the control and the experimental conditions. An issue with increasing the dosage by two times is that it would create a significant amount of methyl mercury waste. Another future experiment could be done with time lapse video to see the distance mitochondrion, or clusters of mitochondria travel between a control and experimental conditions. It would be interesting to see the distance a mitochondrion travels over time because then, if there is no effect of methyl mercury on the distribution of mitochondria, there may be an effect of methyl mercury on the rate of travel of mitochondria. The problem of this type of experiment is that one would have to identify one mitochondrion, or cluster of mitochondrion, and be able to see it, or them, move. If the mitochondria are packed closely together like they are in Figure 1, it may be difficult to see specific clusters of mitochondria move. There are many different experiments that could be conducted, but these are the few that I think could improve and build upon this experiment.

A few errors may have occurred during this experiment. There could be an error in the accuracy in lengths of the glial cells because the lamellipodia and filopodia may not be entirely visible in the transmitted light images. Another error may be that there was no definite way to define the nucleus and the center of the nucleus. The nucleus is a spherical 3 dimensional area and the images are 2 dimensional. There may have been an error in accurately finding the entire nucleus for every glial cell. There could also have been an error in accurately finding the center of the farthest cluster of mitochondria. This experiment could be improved by gathering data from glial cells of the same shape because some glial cells were triangular, and some were rectangular. The shape could have also contributed to an inaccurate measurement of glial cell length for the glial cells that had a triangular shape, as seen in Figure 2. The length, or the longest axis of the glial cell, may
have not represented the glial cell in the best manner in the case of a glial cell like the one in Figure 2. A larger sample size would have benefitted this experiment greatly. These errors may have contributed and affected the results, and it is imperative that this study be re-done in the exact same manner to see if the results are the same.

References


Hollenbeck, P. J., Verburg J. (2008). Mitochondrial membrane potential in axons increases with local NGF or...


