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" Effects of common perturbing agents on neurons and glial cells in culture"

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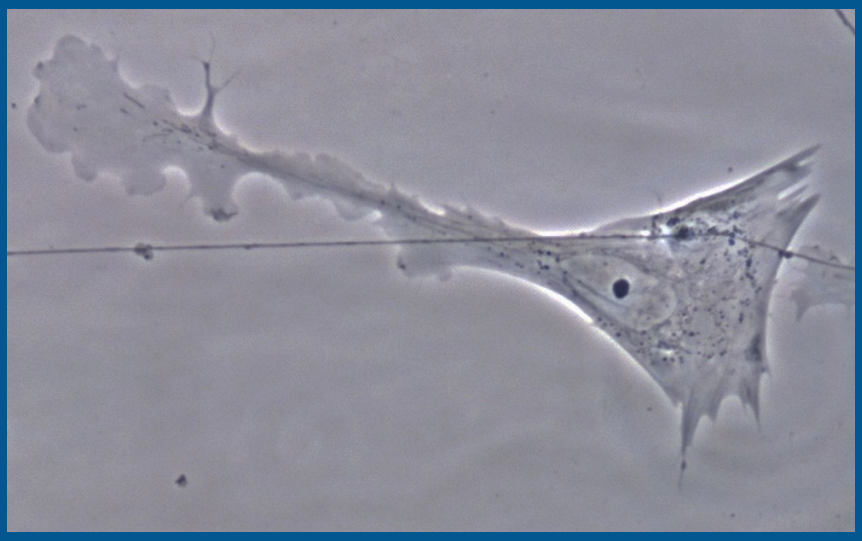

Evidence of chronic and acute exposure time effects of lipopolysaccharide on glial cell motility

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Introduction

Neurodegenerative diseases are distinguished as the slow progressive loss of neurons in the central nervous system that can strip away all of a person's abilities, often leaving them mentally and/or physically debilitated (Gao & Hong, 2008). These diseases often don't manifest until later in life and are an immense physical, mental, emotional and financial burden that affect 50 million Americans a year, yet so much is still not known about the causes and cures (Brown, Lockwood, & Sonawane, 2005; Kaiser, 2018).

Inflammation is known to be a component for multiple neurodegenerative diseases such as Alzheimer's disease, Parkinson's disease, Huntington's disease and multiple sclerosis (Chang et al., 2001; Gao & Hong, 2008). Evidence shows that when inflammation occurs due to injury glial cells are activated and respond; microglia, astrocytes in the CNS, and Schwann cells all have vital roles to play in their microenvironments (Chang et al., 2001). Glia cell then may go on to cause an adaptive immune response, which has been linked to be an accelerator of the onset of neurodegenerative diseases; it creates the ideal condition for neurodegeneration to occur (Nguyen, Julien, & Rivest, 2002; Whitney, Eidem, Peng, Huang, & Zheng, 2009). The inflammatory response occurs as a reaction to stress, injury or infection to protect and defend the central nervous system, (Whitney et al., 2009). Microglia are specifically immune cell however, they are similar to the glial cells seen in this experiment because all glial cells are known to play a housekeeping role to aid neurons in functioning properly (Barres, 2008). Microglial cells are active on sites of injury and inflammation, they are triggered to release cytokines such as tumor necrosis factor- α (TNF- α) and nitric oxide that can be toxic to neurons (Drew & Chavis, 2000).

The endotoxin, lipopolysaccharide (LPS) is commonly used to induce a strong inflammatory response and stimulate the production of TNF- α (Qin et al., 2007). However it has also been demonstrated in an *in vitro* study that with chronic exposure of 72 hours to LPS, microglia show signs to have a neuroprotective effects (Cacci, Ajmone-Cat, Anelli, Biagioni, & Minghetti, 2008). Findings from this study show that during chronic stimulation, microglia showed a reduction in pro-inflammatory cytokines and more similarities to unstimulated microglia. This suggest that there is a relationship between exposure time and glial cell response (Cacci et al., 2008).

In this experiment, LPS was used to treat cultures of *Gallus gallus* sympathetic cells from 10-day old chick embryos at different exposure lengths to see how prolonged exposure time changes how glia responds. It has been seen in previous studies that motility rates of glia decrease with higher levels of TNF- α produced by LPS, suggests its toxicity (Nimmerjahn, Kirchhoff, & Helmchen, 2004). Higher motility rates were seen with lower levels of TNF- α,

representing protective effects that occur (Alden, 2017; Whitney et al., 2009). In this paper, it is predicted that glial cells will have lessened motility rates with acute exposure to LPS and glial cells with chronic exposure to LPS will show greater motility rates, similar to rates of unexposed glial cells. If LPS is applied to primary tissue culture for 72 hours, less cytokines and TNF- α will be released so glial cells will become more motile due to the less toxic environment. Therefore, it is hypothesized in this experiment that glial cell motility will increase with chronic exposure and decrease with acute exposure to LPS.

Materials and Methods

Primary Culture

In this experiment, first coverslips were cleaned thoroughly and pasture pipettes were contracted with a flame in preparation by following *Preparation Day Steps in the Primary Culture of Chick Embryonic Peripheral Neurons 1: Dissection* protocol (Morris, 2015a). Dissection of Sympathetic nerve chains and dorsal root ganglia from 10-day old chick embryos were conducted as instructed by the guidelines from *Preparation Day Steps in the Primary Culture of Chick Embryonic Peripheral Neurons 1: Dissection* protocol (Morris, 2015a). Coverslips were treated with polylysine and laminin during dissections. Hanks Balanced Salt Solution (HBSS) was used as the dissection medium.

Lipopolysaccharides Concentrations

For this experiment, one stock solution of lipopolysaccharide was prepared. LPS was reconstituted into a 1mg/mL solution by adding 13.3mg of powdered LPS to 13ml of Hanks Balanced Salt Solution as a buffer. The mixture was stirred gently until the LPS powder was completely dissolved. Subsequent dilutions were made for the does used in this experiment based off of a previous experiment performed which showed that the optimum does of LPS is 1μg/ml (Shi et al., 2016). Using the LPS stock solution created of 1mg/ml, the does was prepped by adding 2μl of stock to 2ml of F+ medium right before the application of the solution to the desired petri dish. The LPS stock solution was kept in chilled storage at 2-8° C. Reagents were shared with collaborators Joey Batson, Amanda Swanson and Harmony Ring.

Experimental and Control

Five primary tissue cultures at low cell density were attained for experiment for a control, 24, 48, 72- and 96-hour exposure periods. All cultures were plated on the same day, thus were the same age throughout the experiment and were stored in an incubator at 37° C. This experiment was carried out over the course of a five-day period. For the acute exposure conditions, the determined dose of 1μg/ml LPS recommended by Shi et al., (2016) was applied with a sterile pipette after 24 hours on a 2-day old primary tissue culture and after 48 hours on 3-day old primary tissue culture. For the chronic exposure conditions, the same 1μg/ml dose of LPS was applied after 72 hours on 4-day old primary tissue culture and after 96 hours on 5-day old primary tissue culture. Over the 5-day experiment, every 24-hours, all of the cultures received fresh 2ml of F+ medium with LPS to already-treated cultures or just F+ medium alone to cultures yet to be treated with LPS. The control was left without any LPS, only receiving fresh F+ medium over the course of the five-days. This was done by first removing the existing medium in the culture dish and replacing it with fresh medium with a sterile pipette. Two trials of this experiment were performed following this protocol.

Preparing Slides

Five chip chambers were created using the protocol of *Primary Culture of Chick Embryonic Peripheral Neurons 2: Observation of live unlabeled cells* (Morris, 2015b). Growth medium was removed from cultures, allowing for the removal of the coverslips. Coverslips were then placed on slides and sealed with VALAP (1:1:1 mix of Vaseline, Lanolin, Paraffin). Each slide was labeled as control or LPS exposure time.

Microscopy and Imaging

A Nikon Eclipse E200 microscope with a SPOT Insight camera was used to take Video-enhanced phase microscopy images on. The SPOT 5.3 imaging program version 5.3.5 was used to capture still frames. The chip chambers were kept at 37 °C with an electrical space heater placed adjacent to the microscope and monitored with a digital thermometer taped to the stage of the microscope.

All slides were first scanned at a 10x objective for lone glial cells not being overlapped or interacting with other glial cells or neurons. Glial cells were identified by their ruffled edges, and oblong triangular shape. When a one or more glial cell not being touched or overlapped by other glia or neurons were found in the frame, it was imaged at 40x objective with phase 2 light. The SPOT software was used for time-lapse microscopy, capturing still frames every ten minutes for 30 minutes, focusing on the glial cells in frame.

After imaging of all five slides were completed, each still frame was uploaded to ImageJ. Images were taken in the ICUC lab at Wheaton College, Ma.

Image Analysis

For the purpose of quantification, cells that were not completely in each frame and cells with unclear boundaries were excluded from quantification. Three glial cells area and perimeter were averaged for each of the conditions. Using the freehand selection tool, the identified glial cell's edges were outlined in each frame. The length scale was set on SPOT using a stage micrometer to determine the pixels/micron. The area and perimeter of the cell were measured and recorded for the starting glial cell position and tracked in each frame. The difference of pixels/micron in area and perimeter were calculated from picture to picture in order to obtain average absolute values of change in area and perimeter. Area and perimeter measured the changing shape and growth occurring. This process was repeated for all frames. The areas and perimeters from each cell were averaged per experimental group and complied into two categories: average absolute values of change in area and average absolute values of change in perimeter.

Results

The motility rates can be clearly observed and compared between control group and the 24-hour condition in figure 1. Common trends can be seen in the condition groups, with 24 hours of LPS exposure glial cell area and perimeter changes continually increasing in figures 2 and 3. The 72-hour LPS exposure condition group show similar spikes to the control group, however is also inconsistent with increasing and decreasing rates in figures 2 and 3. With the 96-hour condition group a similar trend is noticed, and the average change of perimeter spiked higher than the control in figure 3.

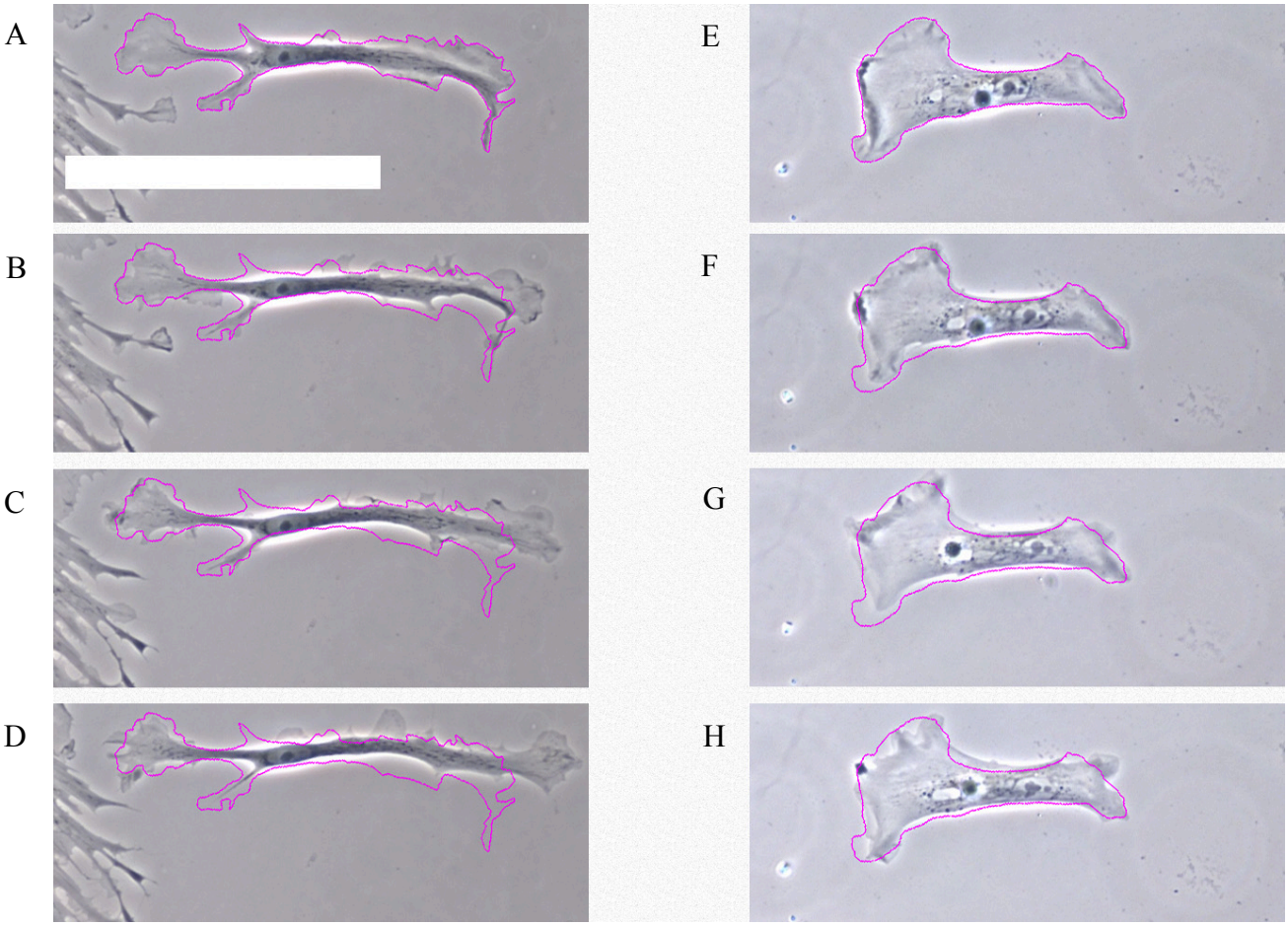

Figure 1. Motility of control glial cell compared to glial cell exposed to LPS for 24 hours
Control cell images (A, B, C, D) and 24-hour condition cell images (E, F, G, H), were viewed at 40x under transmitted light microscope. In images A, B, C, D show motility of control glial cell recorded over 30 minutes. Images E, F, G, H show motility of glial cell exposed to LPS in the 24-hour LPS exposure condition. Notice motility in the images and how the control images show more motility than the images from the 24-hour LPS exposure condition. Scale bar represents 50 microns.

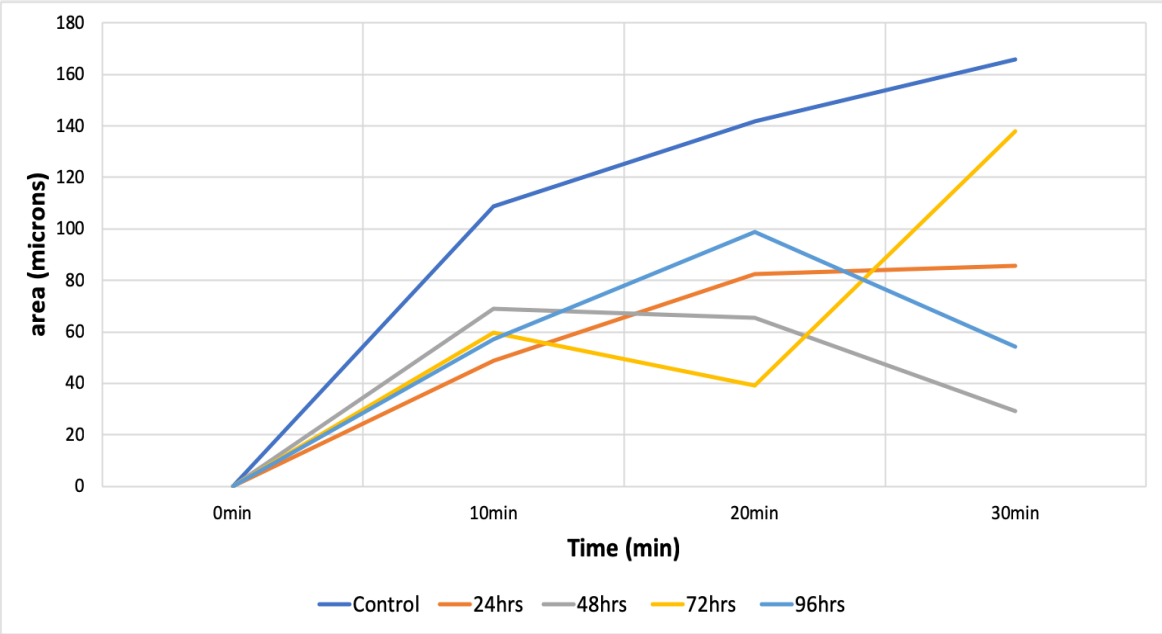

Figure 2. Average of absolute values of change in area seen every 10 minutes
Absolute values of area measurements were taken from three glial cells from each experimental group and were averaged. Averages were recorded in each group every 10 minutes for 30 minutes. All experimental group averages and the control group average showed in increase in area change at 10minutes. At 30 minutes the control group and the 72-hour condition group show similar spikes in motility while the three other conditions motility rates decrease.

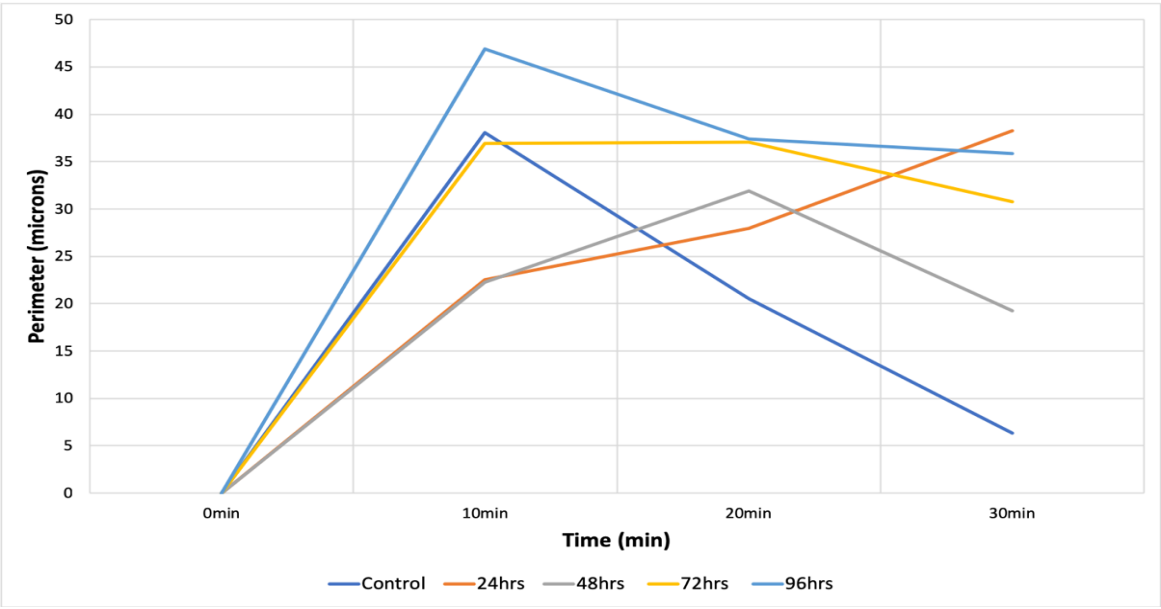

Figure 3. Average of absolute values of change in perimeter seen every 10 minutes
Absolute values of perimeter measurements were taken from three glial cells from each experimental group and were averaged. Averages were recorded in each group every 10 minutes for 30 minutes. Over the 30-minute time period the 72-hour and the 96-hour condition show the most consistent changes in perimeter over the 30-minute period. After 10 minutes for the 24, 28- and 96-hour condition group show a decline in perimeter change activity

Discussion & Conclusion

These preliminary results support the proposed hypothesis that a chronic exposure time of LPS for 72-hours increase glial cell motility, which as is suggested by prior research is an indicator for neuroprotective effects occurring in the environment (Nimmerjahn et al., 2004). The changes in area and perimeter for the 72-hour and 96-hour condition seen in the results section is the most similar to the unstimulated control group, as was hypothesized. The 24, 48- and 96-hour condition groups all showed similar decreased rates of change in area and perimeter, which may be a sign of a toxic environment. The increased movement at 72-hours and 96-hours may be a result of the reduction of pro-inflammatory cytokines released as seen in microglia (Drew & Chavis, 2000). Although microglia and the glial cells observed in this study differ, these types of glial cells are also thought to release neurotropic signals to promote neuron survival (Barres, 2008). Activated glial cells have been thought to show neuroprotective as well as neurotoxic functions on neurons, when the environment is chronically altered (Cacci et al., 2008; Nimmerjahn et al., 2004). The data presented supports Nimmerjhan's 2004 study, as the it shows how during acute exposure glial cells may respond with the over-activation release of cytokines. This may cause the environment to become more toxic and motility rates to decrease. While after 72-hours of chronic exposure, motility rates increase due to the less toxic environment. These changes seen between chronic and acute exposure in properties displayed by glial cells are consistent with research from Cacci et al., (2008) which suggests that distinct glial phenotypes can be affected with acute or chronic stimulation.

However, it is important to acknowledge that not all studies agree with this hypothesis. In other studies, evidence has been found that suggests that different doses of LPS can produce the changes in effect of glial seen in this study. In one *in vitro* study glial cells were exposed to LPS at 2.5µg/ml, 5 µg/ml and 10 µg/ml. The author suggests that a LPS dose of 2.5µg/ml causes a neuroprotective effect due to small amounts of TNF- α produced, whereas the overexposure of TNF- α triggered by the higher doses of LPS causes decreased motility, a sign of toxicity and cell death (Alden, 2017). This study done by Alden, (2017) adds a dose component that was not considered for this experiment. Using different doses of LPS as an addition to study to determine the combination of dose and exposure time effects on neuroinflammation would be a beneficial follow up to this experiment.

It would be beneficial in future studies if levels of TNF- α were to be measured. This would allow more concrete conclusions to be made about what is occurring and why in these cultures. This experiment could also be improved by having a larger data set with a longer imaging time-lapse period. This would give more evidence and could show stronger, significant correlations between exposure times and glial cell motility. Different doses of LPS should also be investigated to further results found by Alden (2017). Possible sources of error in this experiment could come from inconsistent selection of glial cells for quantification. To ensure the most reliable and valid data, identifying more similar glial cells between cultures to compare would be beneficial.

The inflammatory response is an essential regulation function of homeostasis when trauma or injury occurs. However, when this response becomes uncontrolled and excessive many larger, damaging effects can occur (Gao & Hong, 2008). Research shows that inflammation contributes to the neuronal loss in neurodegenerative diseases. However so much is still unknown about the role that inflammation plays in the progression of these diseases (Gao & Hong, 2008). Utilizing the findings from this study to determine the optimal exposure time of LPS could help scientists to identify the ideal levels and dangerous levels of proinflammatory

cytokines. This information could be a big step in identifying and eventually preventing the main factors of neurotoxicity and inflammation such as, the role glial cells play.

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