

The Effect of Chlorpyrifos Exposure on NFH Concentration in Dopamine-Producing Cells

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Introduction

Neurofilaments (NF), which are a crucial building block of the cytoskeleton of neurons, are composed of three subunits, one of which is neurofilament heavy (NFH). The phosphorylation of NFH has been tied to axonal growth, in that it allows for axonal transport to occur properly. Axonal transport is crucial for a neuron so that proteins and other molecules that are necessary for survival may be exchanged between the cell body and the axon terminal of the neuron. Without proper axonal transport, neurotransmitters cannot travel to the terminus of the axon and be released by the neuron. Previous studies have seen that axons lacking NFs show a decrease in cross-sectional area, meaning that they are smaller than axons with higher NF concentrations (Boquet *et al.* 2009). Previous studies suggest that NFH acts as a biomarker for nervous system distress. High concentrations of NFH have been observed in patients with neurodegeneration, such as spinal cord injuries, cognitive impairment from chemotherapy, and delirium (Sumitani, *et al.* 2016; Mietani, *et al.* 2019; Perrot, *et al.* 2008).

Chlorpyrifos (CPF) is a commonly-used organophosphate insecticide that leads to neurodegeneration through the inhibiting of AChE, the enzyme responsible for breaking down acetylcholinesterase (Williamson *et al.* 2013). AChE inhibition disrupts the communication between neurons, preventing vital information from being conveyed between cells. Chlorpyrifos can cross the blood-brain barrier (BBB), entering the brain and affecting normal gene expression of cells composing the BBB. This permanently renders the BBB from functioning properly, as the cells responsible for keeping out large, charged, and lipophobic molecules are no longer able to do so (Wu, *et al.* 2017).

Chlorpyrifos crossing the BBB is a concentration-dependent response, with concentrations above 10 μM CPF leading to decreased neurite length and number of neurons in cell cultures. At concentrations of 30 μM CPF, cell death is observed (Wu, *et al.* 2017). Other research has found that CPF induces apoptosis in dopamine-producing cells at 25 μM CPF (Raszewski, *et al.* 2015). Other studies have seen that, in the cells that survive CPF exposure, neurite outgrowth is significantly inhibited at 50 μM CPF (Wu, *et al.* 2017). The

hypothesis of this study was that if dopamine-producing cells are exposed to CPF, then cells that are exposed to 25 μM CPF will have the highest concentration of NFH, as compared to cells exposed to 0 μM , 5 μM , or 20 μM CPF.

Methods

Cultures of SH-SY5Y cells were maintained in HAMs F-12 media with 10% FBS. Cultures were maintained at 37°C in a humidified incubator. To count cells, media was removed from the flask, cells were washed with 1X DPBS, and 0.25% trypsin was added to lift the cells from the flask. The flask was incubated for two minutes at 37°C, and media was added to neutralize the trypsin. This solution was centrifuged at 1200 RPM for 7 minutes to concentrate the cells at the bottom of the tube. The cells were then resuspended in 1X DPBS. Trypan Blue was added to 25 μl of the cell suspension in a 1:1 ratio. Cells were counted to determine the needed volume of media that would provide 5 x 10⁴ cells per ml. Cells were plated at 5 x 10⁴ cells/ml to each of twelve wells in a cell imaging plate (Eppendorf).

The cells were treated the following day with 5 μM , 20 μM , or 25 μM of CPF or media only (0.01% ethanol). Chlorpyrifos was diluted in 100% ethanol. Final concentrations were made in media with three wells per treatment. The plate was incubated for 24 hours at 37°C.

The media was removed, and the cells in each well were pre-fixed with 2% PFA for 10 minutes and then with 4% PFA for 15 minutes. Cells were washed three times with 1X DPBS and treated with 0.1% Triton X for 10 minutes to allow for permeation of cell membranes. The cells were washed three times with 0.1% PBST and blocked with BSA for one hour at room temperature. The PBST was again used to wash the cells three times. Anti-NFH antibody (1:1000 dilution in 1% BSA in 0.1% PBST) was added to each well. Cells were incubated in the anti-NFH antibody at room temperature for three hours. The antibody was removed, and the cells were washed three times with 0.1% PBST. The secondary antibody (1:1000 dilution in 1% BSA in 1X PBS) was added to each well, and cells were incubated at room temperature for 45 minutes. The cells were washed three times with 0.1% PBST

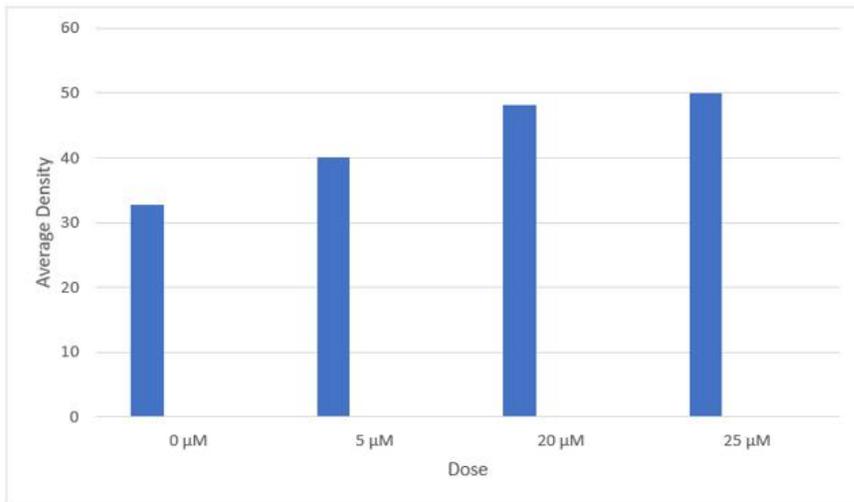


Figure 1: Average density of NFH in neurons exposed to various concentrations of chlorpyrifos. Cells exposed to CPF had significantly more NFH than control cells. Cells exposed to 20 μM had significantly more NFH than cells exposed to 5 μM of chlorpyrifos.

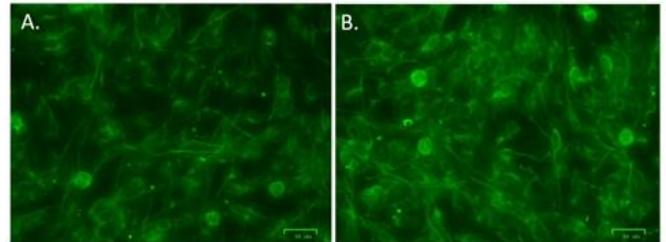


Figure 2. NFH protein in neurons exposed to 0 μM CPF (A) and 25 μM CPF (B). There was significantly more NFH protein in cells exposed to 25 μM CPF, compared to 0 μM CPF.

and stored at 4°C until they were imaged the following day using a ZOE Cell Imager (BioRad).

Results

The average density of NFH in the control was 32.71 (+/- 11.48). In neurons exposed to 5 μM CPF, the average density of NFH was 40.04 (+/- 11.93). Neurons exposed to 20 μM CPF had an average density of NFH of 48.12 (+/- 14.36), and neurons exposed to 25 μM CPF had an average NFH density of 50.27 (+/- 13.31) (Figure 1).

There was significantly more NFH in neurons exposed to 5 μM, 20 μM, or 25 μM CPF, compared to the control ($p < 0.001$) (Figure 2). There was significantly less NFH present in neurons exposed to 5 μM CPF, compared to neurons exposed to 20 μM CPF or 25 μM CPF ($p < 0.001$). There was no significant difference in NFH density between neurons exposed to 20 μM CPF and 25 μM CPF ($p = 0.66$).

Discussion

The hypothesis stated that dopaminergic cells exposed to 25 μM CPF would have a higher concentration of NFH, compared to cells exposed to 0 μM, 5 μM, or 20 μM CPF. There was significantly more NFH in cells exposed to 20 μM CPF and 25 μM CPF, compared to cells exposed to 5 μM CPF, and in all CPF concentrations compared to the control. This suggests that neurons exposed to 20 μM CPF produce significantly more NFH than neurons exposed to 5 μM CPF. Neurons exposed to 20 μM and 5 μM CPF produced significantly more NFH than neurons that were not exposed to any CPF. There was no significant difference between the NFH density of neurons exposed to 20 μM CPF, compared to 25 μM CPF.

This finding is consistent with studies on NFH concentrations in patients with traumatic brain or spinal cord

injuries. Studies suggest that higher levels of NFH were present in patients with severe spinal cord injuries and encephalitis, compared to control participants (Sing, *et al.* 2017; Li, *et al.* 2019).

Neurofilament heavy protein (NFH) is important for myelination of neurons, as well as for ensuring that the cytoskeleton of the cell is stable. Without NFH, a cell would not have the necessary conduction or stability for transporting signals throughout the neuron. Increased NFH production is the cell's defense to nervous system distress and its effort to increase axonal growth and, thus, transport. When damage is inflicted to the nervous system, a proteolytic cascade occurs. This leads to neurons releasing more NFH than they would if unaffected (Petzold and Plant 2012).

Cells exposed to 25 μM CPF had the highest average NFH density of all the lower CPF concentrations. This is consistent with expectations that neurons who are exposed to higher CPF concentrations will produce more NFH. The fact that there was no significant difference in NFH density between cells exposed to 20 μM CPF, compared to 25 μM CPF, may be due to the fact that 25 μM is nearing the concentration of CPF at which cells die (30 μM CPF). It is possible the rate of NFH production slows as CPF concentration increases beyond 20 μM CPF and the cells begin to undergo extreme distress or death.

Studying the effects of CPF in neurons is crucial to fully understanding how CPF affects the nervous system. By using NFH as a biomarker for nervous distress in cell cultures, researchers may be able to determine which concentrations of CPF are detrimental to human health and the extent to which they cause harm. This could have effects on legislation to further ban use of CPF or to give benefits to people exposed to CPF.

References

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