Application Note

Spatially restricted exposure of toxins to neurons and its impact on axonal degeneration using AXIS®

Kevin Long, Ferogh Ahmadi, Mike Kunis, and Wayne Speckmann
Merck Millipore Corporation

Abstract

Neuronal cells are structurally complex with large somas, highly branched dendritic arbors, and axonal projections that often extend great distances from the cell body. Due to their size, shape, and function, neurons and their distal axons are continually exposed to differing factors which can mediate neurotoxic effects. Neurotoxicity can impair normal cellular activity and can even lead to the death of neurons. Examples of external sources of neurotoxicity include radiation, heavy metals, pesticides, alcohol, food additives, drugs, cosmetics, biological toxins, etc. For many of these neurotoxins the underlying mechanism of damage and the site of cellular interaction are unknown. Investigations into these effects have been hampered by the lack of an *in vitro* test system capable of separating axons from their cell bodies. Utilizing a novel microfluidic AXonal Isolation System (AXIS®) we were able to spatially isolate axons so they could be directly and specifically exposed to neurotoxins. Our goal was to determine if the neurotoxic effects observed were due to direct axonal exposure or mediated by the neuronal cell body. Three neurotoxins, MPP+, acrylamide, and rotenone, were analyzed to determine if the site of cellular exposure mediated the neurotoxic effect. Acrylamide and MPP+ both caused neurotoxicity to cells when exposure was limited to axonal exposure. In contrast, rotenone exposure to axons caused no detectable change whereas its exposure to cell bodies caused cell death and neurite retraction. Thus, our analysis has determined that site-specific exposure of cells to toxin can result in different cytotoxic outcomes.

Introduction

Neurotoxins are molecules that kill, damage, or impair the function of neuronal cells. Although the intracellular mechanism of action of numerous neurotoxins has been identified, the entry route for many of these molecules is unknown. This discrepancy is concerning, given that neuronal cells often have long cellular projections that extend great distances from the cell body, with proximal or distal toxin exposure possibly inducing dramatically different outcomes. Yet, most neurotoxin investigations are conducted by completely immersing cells cultured *in vitro* in experimental substances. Although effective for determining cellular toxicity, this approach does not provide spatial resolution regarding site of entry. With this in mind, we utilized AXIS® devices to determine what effect three known neurotoxins would cause when exposure was confined to only the axonal processes. In addition, nocodazole, a well known microtubule polymerization inhibitor[1], was also analyzed as a positive control reagent as it is known to induce axonal retraction.

The three neurotoxins analyzed in this study were MPP+, acrylamide, and rotenone. MPP+ (1-methyl-4-phenylpyridinium) is a positively charged molecule that is often used as a herbicide. In neurons, it can interfere with oxidative phosphorylation in mitochondria and block ATP production which can result in cell death[2]. In addition, it has been found to reduce dopamine levels in the brain and inactivate the enzyme tyrosine hydroxylase[3]. It triggers parkinsonism in primates by killing dopaminergic neurons in the substantia nigra

area of the brain[2]. These neurons are dead or are greatly reduced in human patients with Parkinson's disease.

Acrylamide is a monomer of polyacrylamide, a reagent commonly used in industrial and laboratory processes. While polymeric forms of acrylamide are relatively benign, the monomeric form is highly toxic. Chronic treatment of laboratory animals has been shown to produce tumors in tissues from the mammary gland, thyroid, and nervous system[4]. Despite a growing body of research on acrylamide's toxicity, the mechanism of induction is still unresolved. Although it has been shown to bind directly to DNA, it is unclear if this effect is responsible for the acrylamide-mediated toxicity.

Rotenone is an odorless chemical naturally found in the roots and stems of several different species of plants. It has been widely used as a broad-spectrum insecticide and less commonly as a piscicide (fish killer)[5]. Rotenone can be applied as either a powder or a liquid. Its mode of action involves blocking the electron transport chain in mitochondria, leading to a block of ATP production. Low doses of rotenone in lab animals cause the death of dopaminergic neurons in the substantia nigra (similar to MPP+) and lead to Parkinson's-like symptoms[5]. Both rotenone and MPP+ are lipophilic and therefore can cross the blood brain barrier.

Illustration of the Plasma-bonded AXIS® Device

AX50005PB

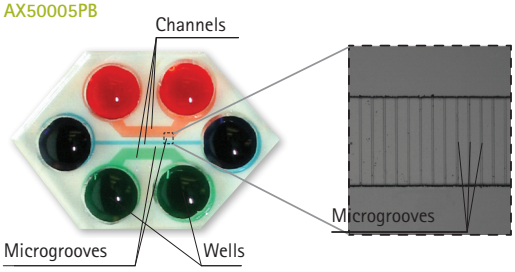

Figure 1. This device contains three chambers that are highlighted in red, blue, and green. Each chamber is composed of two wells that are interconnected by a channel. Two sets of 500 µm-wide microgrooves connect the central blue chamber with each of the outer chambers. The diameter of the microgroove opening is too small to permit neuronal cell bodies from passing through, but axons can grow into and through them. In these studies, E18 rat cortex cells were loaded in the central (blue) chamber.

Materials and Methods

Cell Culture

Plasma-bonded AXIS® devices (Merck Millipore, Cat. No. AX50005PB) with 500 µm microchannels were sterilized with ethanol then coated with polyethyleneimine as per the standard protocol. E18 rat cortex primary neurons were subsequently loaded into the central channel of the device (Figures 1 and 2). The cells were then cultured at 37°C with 5% CO_2 for seven days in standard neuronal medium. On day 4 post plating, the medium in both outside chambers was removed and replaced with fresh medium. On one side of the device, the fresh medium contained neurotoxin (300 µM MPP+, 5 mM acrylamide, 500 nM rotenone, or 5 µg/ml nocodazole), while on the other side, the medium was toxin-free (Figure 2). In addition, unequal amounts of medium were added to different wells of the device to fluidically isolate the neurotoxin so that it was confined to one chamber as described previously[6]. To accomplish this, the wells connected to the central channel were each filled with 250 µL of medium, while the other four wells of the device were only filled with 100 µL of medium.

Immunocytochemistry

All test reactions were fixed seven days after the start of the experiment with 4% paraformaldehyde then stained with Milli-Mark™ FluoroPan neuronal marker, a fluorescently labeled monoclonal antibody blend (Merck Millipore Cat. No. MAB2300X), and DAPI (a nuclear stain). Fluorescent images were captured using a Leica® DMI6000 inverted fluorescence microscope. Measurements of axonal length were captured using the Leica LAS AF software. The number of axons extending from all of the microgrooves within each experiment (control or toxin-treated) was counted manually.

Reaction Setup in AXIS® Devices

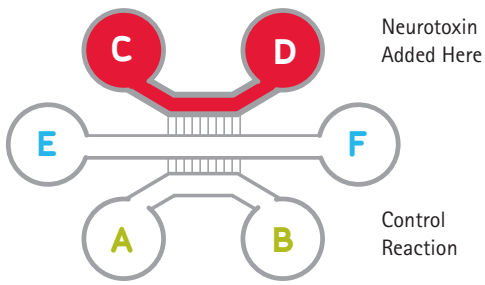

Figure 2. E18 rat cortex cells were loaded in the central channel between wells E and F and cultured to allow axonal growth and differentiation. At day 4 after cell loading, the medium in the outer chambers was replaced. The fresh medium added to chamber side C-D contained neurotoxin, while the medium added to the A-B chamber did not.

Results

All three neurotoxins (MPP+, acrylamide, and rotenone) were mildly to highly toxic to cells when applied directly to cell bodies in the central channel (data not shown).

MPP+ exposure that was microfluidically localized to only axons had dramatic effects on their physical appearance (Figures 3 and 4). The toxin caused a decrease in the number of axonal projections as well as a decrease in the length of the projections. In addition, the axons that remained appeared unhealthy; showing an unusual, punctate staining.

Acrylamide, much like MPP+, caused dramatic effects when exposed to axons. The toxin caused a decrease in the number of axonal projections as well as a decrease in the length of the projections. In addition, the axons that remained showed an unusual morphology with both punctate staining and swelled or puffed axons (Figure 4).

Effect of MPP+ Exposure on Axonal Extension

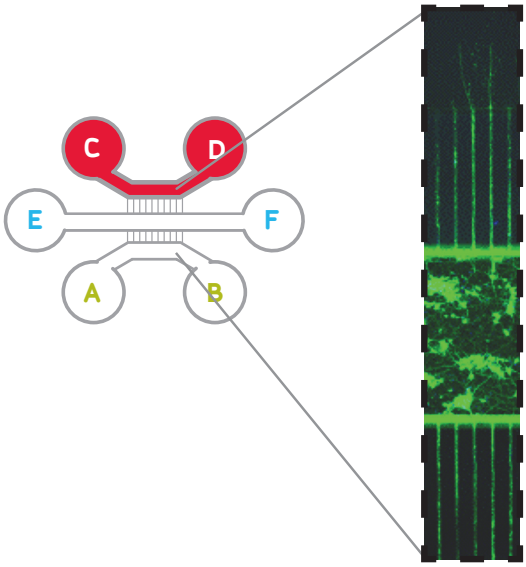

Figure 3. Shown above, right is the result from an experiment where the neurotoxin MPP+ was site-specifically added to one side of the device at day four. A representative series of images was captured and subsequently stitched together to allow complete visualization of axonal extension from both the control and treatment side of the AXIS® device. Reductions in the number of axons as well as their extension lengths are readily apparent from the treatment of the axons by toxin.

Effect of Three Neurotoxins on Axon Growth Visualized by Fluorescence Microscopy

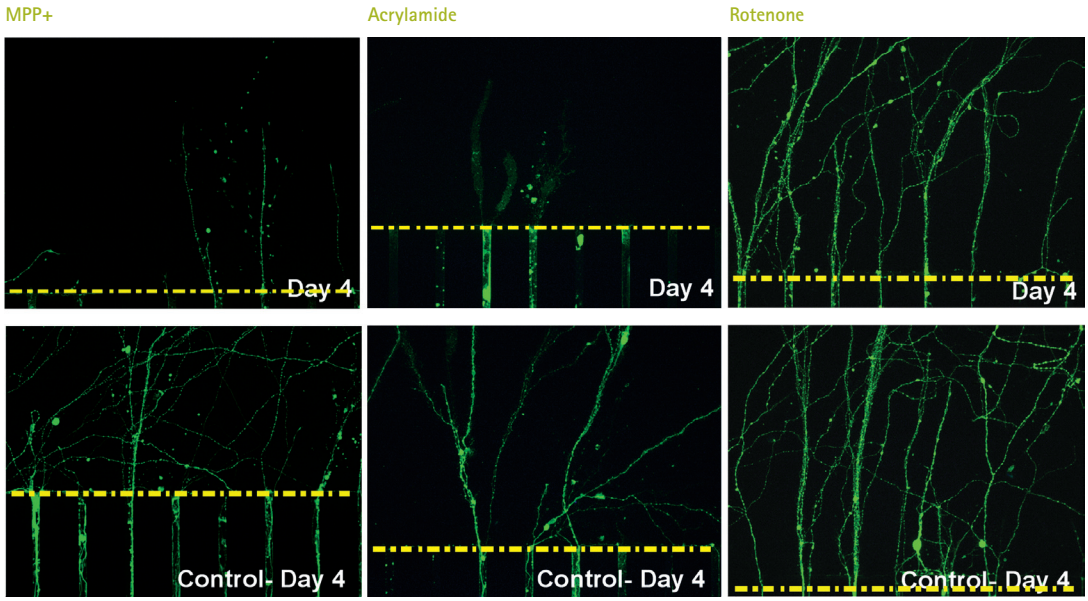

Figure 4. Axons were exposed to 300 µM MPP+, 5 mM acrylamide, and 500 nM rotenone starting at day 4 post plating, exposing the axons to toxins for 3 days. The top row shows axonal extensions that were exposed to toxin, while the bottom row shows the corresponding control axons. The dotted yellow line indicates the end of the microgrooves.

In contrast to both MPP+ and acrylamide, rotenone exposure to axons had little, if any, effect on axonal morphology (Figure 4). Axonal segments treated with toxin showed similar numbers of extensions and similar lengths of projections as compared to untreated axons. In addition, there were no readily apparent differences in staining characteristics of treated and untreated axons.

Quantitative microscopy showed that acrylamide and MPP+ caused a ~60% decrease in the number of axons when toxin exposure was limited to axons. In contrast, rotenone exposure to axons had essentially no effect on axon number (Figure 5). Axon length was measured as shown in Figure 6. Three days of exposure to acrylamide and MPP+ both caused dramatic reductions (~50%) in axon length.

Effect of Toxins on the Number of Axon Projections

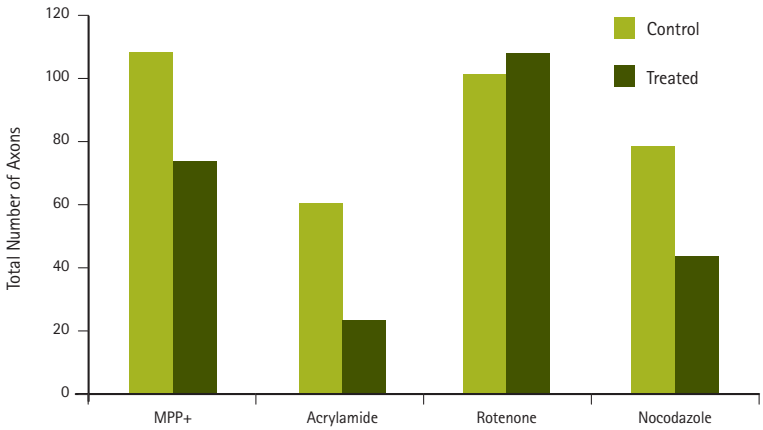

Figure 5. The axons extending from all of the microgrooves within each experiment (control or toxin-treated) were counted and the results are summarized above. MPP+ and acrylamide caused dramatic decreases (~60%) in the number of axons when toxin exposure was limited to axonal projections. In contrast, rotenone exposure had essentially no effect, as the number of axons for both control and treatment were very similar. Nocodazole produced dramatic decreases in axon number, as expected.

Measurement of Axon Length

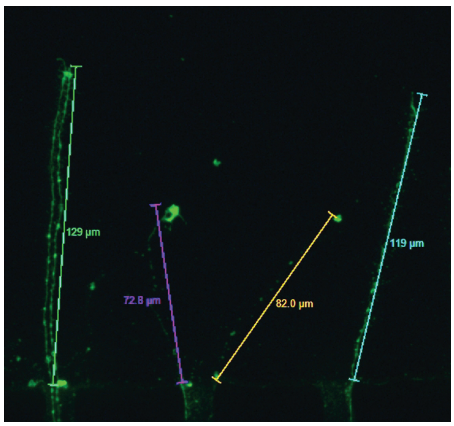

Figure 6. The distance that axons extended beyond the microgrooves was measured using the Leica LAS AF software. An example of how the measurements were made is provided above. The dotted yellow line indicates the end of the microgrooves.

Rotenone exposure had virtually no impact on the lengths of the axons (Figure 7). As expected, the positive control reagent, nocodazole, caused dramatic decreases in the number of axons as well as decreases in axon lengths (Figures 5 and 7). This drug was highly toxic to the cells, as many appeared to be dead or dying (data not shown).

Conclusions

Using the novel AXIS® platform, we were able to direct neurite outgrowth restricted to microgrooves, fluidically isolate toxins and medium components, and quantify the effect of toxins on neurons with unprecedented, high spatial resolution. By exploiting these capabilities, we were able to show that spatially localized exposure of neurons to neurotoxins induced dramatically different outcomes. Specifically, axonally restricted exposure of acrylamide and MPP+ appeared to induce degeneration of axons, while rotenone did not.

Neurotoxin Impact on Axon Length

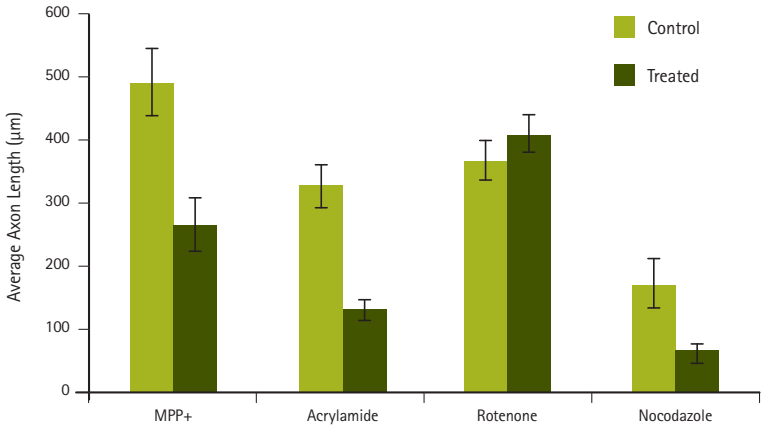

Figure 7. The length of the axons extending from the microgrooves was measured to determine the effect of each of the neurotoxins. To reduce user bias, the first 20 axons (starting on the extreme left and counting to the right) were analyzed for each condition. Acrylamide and MPP+ treatments both caused dramatic reductions (~50%) in axon length. Rotenone exposure had virtually no impact on the lengths of the axons. Nocodazole exposure reduced axon lengths by 40 – 50%. Error bars represent standard error of the mean (SEM).

References

1. Bereiter-Hahn, J., et al. *Structural implications of mitochondrial dynamics.* 2008; 3(6):765-80.
2. Schmidt, N. and Ferger, B. *Neurochemical findings in the MPTP model of Parkinson's disease.* J. Neural Transm. 2001; 108(11): 1263-82.
3. Ghosh, A., et al. *Neuroprotection by a mitochondria-targeted drug in a Parkinson's disease model.* Free Radic. Biol. Med. 2010; Epub Sep. 7.
4. Klaunig, J. Acrylamide *carcinogenicity.* J. Agric. Food Chem. 2008; 56(15):5984-8.
5. Cicchetti, F., et al. *Environmental toxins and Parkinson's disease: what have we learned from pesticide-induced animal models?* Trends Pharmacol. Sci. 2009; 30(9):475-83.
6. Park, J., et al. *Microfluidic culture platform for neuroscience research.* Nat Protocols 2006; 1: 2128-2136.

To Place an Order or Receive Technical Assistance

In Europe, please call Customer Service:

France: 0825 045 645
Germany: 01805 045 645
Italy: 848 845 645
Spain: 901 516 645 Option 1
Switzerland: 0848 645 645
United Kingdom: 0870 900 4645

For other countries across Europe, please call: +44 (0) 115 943 0840

Or visit: **www.merckmillipore.com/offices**

For Technical Service visit: **www.merckmillipore.com/techservice**

Get Connected!

Join Merck Millipore Bioscience on your favorite social media outlet for the latest updates, news, products, innovations, and contests!

facebook.com/MerckMilliporeBioscience

twitter.com/Merck4Bio

MERCK MILLIPORE

www.merckmillipore.com/offices

FSC logo

Lit No. AN4463ENEU LS SBU-12-06546 5/2011 Printed in the USA.