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Morphometric Assessment of Toxicant Induced Neuronal Degeneration in Full and Restricted Contact Co-cultures of Embryonic Cortical Rat Neurons and Astrocytes: Using *m*-Dinitrobenzene as a Model Neurotoxicant

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Abstract

With *m*-Dinitrobenzene (*m*-DNB) as a selected model neurotoxicant, we demonstrate how to assess neurotoxicity, using morphology based measurement of neurite degeneration, in a conventional “full-contact” and a modern “restricted-contact” co-culture of rat cortical neurons and astrocytes. In the “full-contact” co-culture, neurons and astrocytes in complete physical contact are “globally” exposed to *m*-DNB. A newly emergent “restricted-contact” co-culture is attained with a microfluidic device that polarizes neuron somas and neurites into separate compartments, and the neurite compartment is “selectively” exposed to *m*-DNB. Morphometric analysis of the neuronal area revealed that *m*-DNB exposure produced no significant change in mean neuronal cell area in “full-contact” co-cultures, whereas a significant decrease was observed for neuron monocultures. Neurite elaboration into a neurite exclusive compartment in a compartmentalized microfluidic device, for both monocultures (no astrocytes) and “restricted” co-cultures (astrocytes touching neurites), decreased with exposure to increasing concentrations of *m*-DNB, but the average neurite area was higher in co-cultures. By using co-culture systems that more closely approach biological and architectural complexities, and the *directionality* of exposure found in the brain, this study provides a methodological foundation for unraveling the role of physical contact between astrocytes and neurons in mitigating the toxic effects of chemicals such as *m*-DNB.

Keywords

m-Dinitrobenzene (*m*-DNB); cortical neurons; cortical astrocytes; co-culture; microfluidic; morphology

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Introduction

Interactions between neurons and astrocytes contribute to the maintenance of homeostasis in the central nervous system (CNS).^{1–4} Unraveling the complexities of intercellular relationships that mediate responses to toxic chemicals is necessary to gain a better understanding of the progression of neurotoxic states in the CNS. Astrocyte processes make physical contact with postsynaptic and presynaptic neuron terminals at the “tripartite synapse,” where an astrocyte can receive synaptic signals and in turn modulate neuronal activity of the central nervous system.^{2, 5} This intimate interaction governs a host of physiological and pathophysiological events associated with energy metabolism, neurotransmitter release, and signal transmission.^{1–3, 6, 7}

To simulate the interplay among different cells of the CNS, including astrocyte-neuron dialogue, co-cultures are often exploited to reconstruct cell-cell interfaces found in the CNS. Biological events within the CNS are complex, and elucidation of molecular mechanisms of toxicity between multiple cellular players can benefit from an arsenal of *in vitro* co-culture methods that are used to make inferences about *in vivo* occurrences. Neuron-astrocyte co-cultures are customarily characterized as being either contact or non-contact in form, based on the level of physical interaction between the different cell types contained in the cultures.⁸ In contact co-cultures, one cell type is in physical contact with another cell type. One such co-culture involves the placement of neurons on top of a confluent bed of astrocytes.^{9, 10} On the other hand, in non-contact co-cultures, different cell regions can be positioned in close proximity to one another, but physical contact is hindered. Non-contact co-cultures can be established by various systems, such as those comprised of two discrete cellular regions that are divided by a porous physical barrier (transwell),¹¹ sandwiched between coverslips (each coverslip containing a different cell type, where coverslips are separated by small spacers),^{12, 13} or confined in a shared cell culture vessel.^{14, 15} In these non-contact co-culture set-ups, mechanisms of cell-cell communication are limited to diffusion of soluble factors.¹⁶

A newly emergent form of neuron-glia co-culture is a “restricted-contact” co-culture, in which only the neurites extending from neurons, as opposed the entire neuronal cell, touch a non-neuronal cell. In the “restricted-contact” co-culture, minimal contact exists between unique cell populations. This type of restricted co-culture can be rendered from a compartmentalized microfluidic device that was initially developed to facilitate culture of CNS neurons in a Campenot-like construction. In this microfluidic Campenot-like device, two chambers are separated by a series of microgrooves. Neurons are cultured in one chamber, while their neurite processes, through size exclusion, can enter the microgrooves and emerge into the second chamber. Also, by adjusting the medium levels in the devices, secreted or exogenous factors can be fluidically isolated to a preferred compartment.¹⁷

Increasing utilization of this class of compartmentalized devices for elaborate cell cultures, which mimic innate complex cellular hierarchy, facilitates the investigation of physiological^{18–21} and pathophysiology^{22–26} events in the nervous system. Device-based monocultures are useful in evaluating the neurite outgrowth characteristics of neurons

following their exposure to molecular factors at various developmental stages.^{18, 20, 21} In one such study, IL-1 β , a pro-inflammatory cytokine, inhibited axonal growth of developing neurons, suggesting that inflammation may be important in the regulation of neuronal development.²⁰ Models of neuropathological disorders developed with the aid of compartmentalized devices, include Alzheimer's disease,²² Parkinson's disease (PD),²³ ischemia,²⁴ glutamate excitotoxicity,²⁵ and peripheral neuropathy.²⁶ These devices can be used to clearly visualize and quantify trafficking of molecules or the occurrence of cellular processes, such as mitography,²³ within neurites of afflicted neurons. In many of these device-based studies, levels of degeneration were primarily assessed from a measurement of neurite length^{24, 26} or other characteristics of neurite morphology, such as beading and fragmentation.²⁵

The utility of these devices can be extended to include co-cultures of neurons and a second cell type, enabling the exploration of the intricacies of cell-cell communication between neurons and glia.²⁷ Device-based co-cultures were used to demonstrate that microglia have a tendency to dock to areas containing damaged neurons more often than regions with healthy neurons, and axon-specific signals may have promoted this microglia migratory behavior.²⁸ A separate device-based co-culture revealed that astrocytes touching neurites in a neurite-astrocyte compartment express higher levels of GLT1 glutamate receptor than other astrocytes in the same compartment that are not in physical contact with neurites. This observation suggests that GLT1 receptor expression is influenced by synaptic activity between astrocyte and neuron processes.²⁹ As a variation to the aforementioned models, we present a compartmentalized system as a means to decipher the relevance, if any, of minimal physical contact between neurons and astrocytes in lessening neurotoxicity.

Neurite degeneration can be used as a measure of neurotoxicity in the CNS;^{30–33} colchicine, acrylamide and ethylene oxide³⁰ are a few of the neurotoxicants that cause structural aberrations, such as focal swellings configured as beads, in neuronal processes.^{30, 32} Neurite beading is associated with the increased concentration of organelles in the varicosities that occur regularly along the length of neurites,³⁰ and is a hallmark of neurotoxicity in numerous neuropathological conditions, including Alzheimer's disease^{31, 32} and ischemia.³² Here, we propose to use both a modern "restricted-contact" co-culture technique and basic neuron morphology assessments, including the aforementioned structural abnormalities in neurites, as a dual approach to gauge levels of neurotoxicity in a neuron-astrocyte environment.

In this study, we made use of two co-culture systems, one involving "full-contact" and the other "restricted-contact" between neurons and astrocytes, which are illustrated in Fig. 1. To achieve full-contact of heterogeneous cells in *in vitro* co-cultures, neurons were plated atop a bed of astrocytes, and all cells were "globally" exposed to a flood of *neurotoxicant* solution. In restricted-contact co-cultures, microfluidic devices were used to confine neurons and astrocytes in separate compartments, allowing neurites to touch astrocytes via interconnecting microgrooves, after which astrocytes were "selectively" exposed to a neurotoxicant to gauge their neuroprotective capabilities. A selective exposure co-culture system may be beneficial for determining the effects of neurotoxicants or drug therapies on particular portions of a neuron. A device-based study backing this supposition demonstrated

that axons are more susceptible to toxicity induced by Paclitaxel, a common chemotherapeutic drug, and human erythropoietin was found to supply neuroprotection regardless of being administered to either somata or axons.²⁶ Axonal degeneration, as opposed to somatic degeneration, has been proposed as a key mechanism of CNS disorders,³⁴ and these neuronal region-specific mechanisms can be evaluated in compartmentalized devices.

Here we employ *m*-Dinitrobenzene, a widely used chemical intermediate in the manufacture of explosives, plastics and dyes,^{35, 36} as a model neurotoxicant. The toxic effects of *m*-DNB on the CNS shares similarities with acute energy deprivation syndromes (AEDs).^{37, 38} Characteristic of AEDs, histopathological profiling of brains from rats dosed with *m*-DNB reveal gliovascular lesions in selective regions of the auditory and vestibular complexes of the brainstem, where damage to neurons occurs only after the morphological integrity of the macroglia has been compromised.³⁶

A host of *in vitro* monocultures have been used to help decipher the respective roles of neurons and astrocytes in AEDS, particularly emphasizing the role of astrocytes in neuroprotection. These collections of monocultures demonstrate cell-specific sensitivity to *m*-DNB toxicity, not only among different types of nervous system cells or cell models, but also between the same types of specialized cells isolated from different brain regions within the CNS.^{39, 40} Co-cultures can uncover key interactions between astrocytes and neurons involved in astrocytes' protection of neurons from *m*-DNB induced toxicity.

Using conventional and modern co-culture systems, we aimed to determine if exposure of neurons to *m*-DNB leads to degeneration of neurons if they were in full contact with astrocytes, and if so, whether or not minimum contact of astrocytes with neuronal processes was sufficient to provide protective effects to attenuate the level of degeneration of neuronal processes post toxicant exposure. Using a thresholding tool from NIH Image J software, we quantified the area of neuronal somata, their processes, or the entire neuron following exposure to *m*-DNB, in either monocultures lacking astrocytes or in co-cultures containing astrocytes.

Materials and Methods

Tissue Preservation and Cell Culture Reagents

Neurobasal, antibiotic-antimycin, glutamate, 0.25% trypsin, and horse serum, were all purchased from Invitrogen. Embryonic day 18 (E18) rat cortices and the complementary tissue preservation medium, Hibernate E, devoid of Calcium (HE-Ca) were obtained from BrainBits, LLC (Springfield, IL).

Immunocytochemistry

Paraformaldehyde (PFA), Aqueous Solution (16%) fixative agent and Fluoromount G mounting agent were obtained from Electron Microscopy Sciences. Image-iT® FX Signal Enhancer and normal goat serum staining reagent was from Invitrogen. The following fluorophore-conjugated monoclonal antibodies were supplied by Millipore: Anti-Tubulin β -III, clone TU-20, Alexa Fluor555 Conjugate (CBL412A5), Anti-Tubulin β -III, clone TU-20,

Alexa Fluor488 Conjugate (CBL412X), Anti-Glial Fibrillary Acidic Protein Antibody, Cy3 Conjugate (MAB3402C3) and Anti-Glial Fibrillary Acidic Protein Antibody, clone GA5, Alexa Fluor488 Conjugate (MAB3402X). Triton-X100 was acquired from Invitrogen. Rectangular (40mm × 24mm, 12-544-12) and round cover glasses (18mm dia., 12-545-100) were purchase from Fisher Scientific.

Chemicals

Cytosine β -D-arabinofuranoside (C1768), Poly-D-lysine (P0899) and 1,3 DNB (*m*-DNB) were purchased from Sigma. Lyophilized papain (LS003119), Dimethylsulfoxide, DMSO (D128-500), sterile distilled water (15230-089), and Ethanol (2701) were acquired respectively from Worthington Biochemical Corporation, Fisher Scientific, Invitrogen, and Decon Laboratories, respectively.

Cell culture substrates and devices

Microfluidic devices (SND 450), cover glasses, and glass bottom 6-well (diameter = 20 mm) plates were ordered from Xona Microfluidics, LLC (Temecula, CA), Fisher Scientific, and MatTek Corporation (Ashland, MA), respectively.

Substrate and Instrument Preparation

Rectangular cover glasses were sonicated in warm deionized water for 1 hr to remove surface debris. They were then transferred to a sterile cell culture hood and successively rinsed once with ethanol for sterilization and twice with sterile distilled water to remove residual ethanol. Cover glasses were allowed to dry overnight prior to storage in a poly-d-lysine solution (PDL, 0.5 mg/mL in Borate Buffer, pH~8.5) for a minimum of two days. The PDL solution was transferred to a container to be stored for later reuse. The cover glasses were rinsed with copious amounts of sterile distilled water to remove unbound PDL and any traces of boric buffer. The glass regions were allowed to fully dry in the biosafety cabinet prior to use. Cover glasses were removed from the PDL solution and rinsed with copious amounts of sterile distilled water.

PDL solution was added to the glass depressions in the glass bottom well plate, and allowed to adhere for at least one day. The PDL solution was removed and the glass regions were rinsed thoroughly with sterile distilled water.

Devices were soaked in ethanol for 12–24 hr to leach out free silicone elastomer that can be detrimental to neuron health.⁴¹ In a sterile cell culture hood, the devices were removed from ethanol and left overnight to dry completely.

Isolation of Cortical Cells from Rat Tissue

Cortical neurons and astrocytes were isolated, promptly upon tissue delivery, from E18 Rat Cortices. One pair of rat cortices was enzymatically digested in 2 mL papain solution (0.5 mg/mL in HE-Ca, dissolved 10 min at 30°C) for 10 min at 30°C, agitating tissue at 5 min. To inhibit further enzymatic digestion, the papain solution was replaced with 1 mL fetal bovine serum (FBS)⁴² and incubated for 5 min (agitating FBS solution every min). Note: For the experiments detailed here, neutralization of papain contributed to prolonged neuron

viability inside microchannels. The tissue was rinsed 5 times with 1 mL neuron culture medium. Fresh neuron culture medium or conditioned tissue support medium (containing Hibernate, B27 and Glutamax, reserved from the original shipment vial of BrainBits tissue) was added to the tissue. The cortices were then mechanically digested roughly 10 times with successively narrow fire-polished Pasteur pipettes containing a 1 mm and 0.5 mm diameter opening. Tissue debris was allowed to settle for one minute, and the supernatant was centrifuged for 1 min at 1100 rpm ($200 \times g$). A small volume ($\sim 50 \mu\text{L}$) of medium was used to dissociate the cell pellet into a single cells suspension, and, the volume was increased to 1 mL. Cells were counted with a Countess® automated cell counter (Invitrogen), and viable cells were identified by their exclusion of Trypan blue (0.4% solution). Cortical cells were then diluted in medium, and color coded according to desired cell type: neuron medium, devoid of phenol red, or astrocyte medium, containing phenol red.

Dissociated cortical cells were plated in either neuron or astrocyte specific medium. Neuron maintenance medium contained Neurobasal, 2% B27, 1% antibiotic-antimycotic (100x), and 0.25 mM GlutaMAX. In addition to being cultured in a serum-free environment, neurons were subsequently treated with 0.25 μM Ara-C, to inhibit growth of glial cells. Astrocyte maintenance medium was composed of Neurobasal, 10% horse serum, 1% antibiotic-antimycotic (100x), and 3 mM GlutaMAX. Astrocytes were expanded to 100% confluence and not used past 2 passages.

Cell Cultures on Glass Bottom Wells

To create monocultures of neurons, a 0.5 mL cortical cell suspension (6.28×10^5 cells/mL, 1×10^5 cells/cm²) was placed in the recessed glass base region in each well of a 6-well glass bottom plate. After 1 hr (time necessary for cells to fully settle to the bottom of the well and adhere), the medium volume in each well was increased to 2.5 mL. The following day, medium was replaced with maintenance medium supplemented with 0.25 μM Ara-C.

To create co-cultures, astrocytes (4×10^4 cells/cm², 2.5 mL medium/well) were first grown on the glass regions of the 6-well glass bottom plates, and then expanded for six days to create astrocyte beds. A day prior to cell isolation, half of the astrocyte medium was replaced with neuron medium. Isolated cortical cells were added to the well using the same procedure for generating neuron monocultures, with the inclusion that cortical cells were plated on a confluent bed of astrocytes. Neuron maintenance medium was added to the wells containing both neurons and astrocytes. The following day, half of the medium was replaced with neuronal medium, supplemented with 0.25 μM Ara-C to curb proliferation of microglia in confluent astrocyte beds.

Cell Cultures in Compartmentalized Devices

Devices were assembled by using ethanol-sterilized tweezers to press microfluidic chambers, center aligned, against PDL coated cover glasses, arranged in a non-treated square Petri-dish. All wells were filled with neuron medium to condition the substrate for cell seeding.

Cortical cells ($10 \mu\text{L}$, 5×10^6 cells/mL) were seeded in the main channel of one compartment through the top well of the device and allowed to adhere for 5 min. Cells were added in a

same manner to the bottom well of the same compartment. All compartments were filled with 150 μ L of medium. On isolation days, fresh medium was introduced at the top wells, to wash away residual Papain. The cells were left undisturbed in the incubator for 3 hr to allow cells to fully adhere, after which an additional 150 μ L of medium was added to each well.

In co-cultures, one day following neuronal seeding, astrocytes were added to the second compartment of the device (5 μ L, 5×10^6 cells/mL) and allowed to adhere for 10 min. The neuron and astrocyte compartments were filled with medium corresponding to the cell types they contained, and the neuron medium was supplemented with 0.25 μ M Ara-C.

Exposure of Cultured Cells to Toxicants

DMSO was used to dissolve *m*-DNB to prepare stock solutions at a concentration of 10 mM, 100 mM, or 250 mM. Since *m*-DNB may interact with serum proteins, stock solutions were diluted in neuron medium. Working *m*-DNB solutions were prepared by dissolving stock solutions in neuronal medium so that the final concentration of DMSO was 0.1%.

For global exposure co-cultures, where all cells were subjected to *m*-DNB, 1.5 mL of the medium was removed from each well and replaced with 2 mL working solution (extra 0.5 mL to compensate for evaporation) so that final concentrations of 10 μ M or 100 μ M were achieved. The cells were exposed on day 4 post-neuron plating, and experiments were terminated 12 hr or 24 hr later.

To achieve selective exposure of *m*-DNB in microfluidic devices through fluidic isolation, 350 μ L (175 μ L in both the top and bottom wells) of medium was added to the neuron compartment, while 300 μ L (150 μ L in both the top and bottom wells) of the *m*-DNB solution was added to the neurite/astrocyte compartment. In the neurite/astrocyte compartment, 25 μ L of *m*-DNB solution was removed from the bottom well and 25 μ L was added to the top well. The 25 μ L was allowed 5 min to completely flow over the cells and allow the medium volumes in the wells of the compartment to re-equilibrate. This treatment was repeated 2 more times so that a total volume of 75 μ L of the *m*-DNB solution flowed over the neurites/astrocytes. This triplicate exposure to *m*-DNB solution was used to achieve toxicant exposure that was equivalent, based on toxicant concentration per unit area of substrate covered by cells, to that received by cells cultured in the glass-bottom wells.

In the device-based experiments, neuron monocultures were maintained for a total period of five or seven days. In co-cultures, neurons and astrocytes, respectively, were maintained for a total period of seven and six days. An *m*-DNB solution was added to the chamber, containing either neurites alone or both neurites and astrocytes, on the last two days of the culture period. Cells were fixed on the last day of the culture period.

The timeline for adding and treating cells in the well-based and device-based co-cultures is shown in Supplementary Fig. 1.

Immunocytochemistry and Fluorescent Microscopy

Glass Bottom Wells—Unless otherwise specified, all the following procedures were conducted at room temperature. A 4% PFA solution (in PBS, diluted from 16% stock) was

added to each well and incubated for 15–20 min. The fixative was removed with 3 rinses of cold PBS. Cells were permeabilized with 0.25% Triton-x for 10 min, and washed with PBS (3x, 5 min each). Image Signal Enhancer was used to minimize background fluorescence and nonspecific staining. The solution was added at a volume sufficient to cover the entire glass portion of the recessed region of the well, and rinsed away with PBS (3x, 5 min each) after 30 min. Cells were blocked with 10% normal goat serum (NGS, in PBS) blocking buffer, for 1 hr, and efficient blocking was facilitated by placing the plate on a titer plate for at least 15 min of the 1 hr incubation. The blocking buffer was replaced with an antibody-containing solution comprised of anti- β tubulin III antibody conjugated to Alexa 555 (1:200) and anti-GFAP conjugated to Alexa 488 (1:1000) in blocking buffer, where the solution volume was confined to the well recession. The plates were stored at 4 °C. The following day, the plates were placed on a microtiter plate for 30 min. Residual unbound antibody solution was washed away with PBS (3x, 5 min each on microtiter plate). Cellular nuclei were stained with 4',6-Diamidino-2-Phenylindole, DAPI (0.5 μ g/mL) for 2.5–4 min and thoroughly rinsed (3x, 5 min each on microtiter plate). The plates were filled with PBS and stored at 4 °C until cells were imaged. For long term storage at 4 °C, the PBS was removed and Fluoromount G was added to the well recession that was subsequently covered with an 18 mm-diameter round coverslip.

Microfluidic Devices—The cells in the devices were stained with a procedure similar to that used for the glass bottom well plates. All media in the microfluidic devices was aspirated, except in the microchannel region. Incubations, except for those of overnight duration, were conducted at room temperature on a rocking platform shaker (VWR). During rocking, plates were rotated by 90 degrees, approximately every 15 or 30 min, to permit flow in channels and grooves that are perpendicular to one another. Additionally, the volume of solution in the soma compartment was kept 50 μ L higher than that in the neurite compartment to encourage flow through microgrooves. Cells were permeabilized for 30 min with 0.2% Triton, treated 30 min with Image Signal Enhancer (1–2 drops/well), and blocked 1 hr with blocking buffer (10% NGS). A neuron-staining solution, containing anti- β tubulin III conjugated to Alexa 488 (1:500 in blocking buffer), was added to the top wells and stored overnight at 4 °C. The following day, the devices were rocked to distribute the antibody solution through the channels. Following rinsing (3x, 5 min each), cells were blocked for 1 hr with blocking buffer. An astrocyte staining solution, anti-GFAP conjugated to Cy3 (1:500 in blocking buffer), was added to the devices and incubated overnight. The next day, the nuclei were stained with DAPI (0.5 μ g/mL). The wells were filled with PBS until imaging. For long-term storage at 4 °C, 40 μ L of Fluoromount G was added to the top wells.

Images of stained substrates were captured in the widefield fluorescence mode with SlideBook™ 5.0 software that was interfaced with an Olympus Disk Scanning Confocal Unit (DSU), outfitted with a Hamamatsu camera.

Quantification of Neurite Outgrowth and Axon Degeneration

Fiji software (NIH) was used to process images. Raw fluorescent images of neurons were thresholded to create a contour of the area of the cell. In some instances, the Enhance Local Contrast (CLAHE) plug-in was used to allow the cell morphology to be more precisely

outlined. Thresholding was used to convert the fluorescent images into binary black and white images. A threshold tool was applied to assign background regions a pixel intensity value of 0 (black), and fluorescent regions outlining cells a pixel intensity value of 255 (white). The area measurements were confined to the white regions. The Count Particles plug-in was used to identify neurite fragmentation, and based on visual observations of several images of neurites, particles below 2.5 μm were considered to be either debris or background artifacts. Fluorescent images were color corrected according to the fluorophore used for cell type specific or nuclear staining. The CLAHE was applied to some groups of images, as needed, to improve image clarity. Two stitching plug-ins, 2D or Mosaic, were used to respectively fuse or manually stitch images of neurites in the macrochannel together vertically. ImageJ can also be used, but doesn't contain CLACHE and stitching plug-ins.

Statistical Analysis

The one way analysis of variance (ANOVA) method, followed by a Tukey's post hoc test, was used to make comparisons between control and treated groups. Means of populations are reported as mean $\pm$ SD, with a *p*-value of 0.05 or less considered statistically significant. Statistical analysis was performed using the Minitab statistical package.

Results

To determine the effects of *m*-DNB toxicity on neuronal or neurite morphology, two separate co-cultures were used: 1) global exposure of *m*-DNB on neurons atop an astrocyte bed and 2) selective exposure of neurites elaborated onto an adjacent, but physically-isolated astrocyte bed.

Monoculture and "Full Contact" Co-Culture in Glass Bottom Wells

During the duration of "full contact" co-culture, the astrocytes in the underlying bed shifted from being a population of cells displaying a homogenous polygonal shape towards a heterogeneous cell mixture that exhibited both polygonal (type 1), and stellate (type 2), phenotypes.^{43, 44} This morphological transformation by the astrocytes is attributed to the use of serum-free medium, which has been reported to induce stellation.^{45, 46} More extensive neurite arbors formed in co-cultures of neurons and astrocytes than in monocultures of neurons, an occurrence that is expected since astrocytes can secrete factors that encourage neuron survival and neuritogenesis.^{47, 48}

In the co-cultures, extensive elaboration of neuronal processes made it difficult to fully quantify distinct features of neurite morphology that are characteristic of toxicity, such as fragmentation. Instead, the toxicity assessment was based on quantification of the total area of the entire neuron population (somata and neurites) in one field, obtained by applying a threshold to fluorescent images to contour the cells (Fig. 2A). Fuji software was used to calculate the neuron area in the field restricted to the thresholded region, and this total cellular area was considered a metric of degeneration. It cannot be definitively stated that the neurodegeneration resulted exclusively from decreased neurite elaboration since, in some conditions, a proportional concentration dependent decrease in cellular area and cell number was observed, which suggests the occurrence of cellular death. The total cell area in the

experimental groups was expressed as a percent of the total cell area from the control (vehicle) group.

Neuron monocultures and “full contact” neuron-astrocyte co-cultures received exposures of 0 μM (vehicle alone), 10 μM or 100 μM *m*-DNB for 12 hr or 24 hr. Fluorescent images of total neuron area analyzed for both cultures at 12 hr and 24 hr are shown in Fig. 3. At the 10 μM concentration, both neurons and astrocytes had similar total neuron areas that were equal to ~100% of the vehicle control for 12 hr and ~90% for 24 hr (Fig. 3C, F). Thus, 10 μM of *m*-DNB appears to have only a mild neurotoxic effect, based on neuron area, after 24 hr. However, neurons in monoculture, exposed to 100 μM DNB had a significantly lower mean total neuron area than those exposed to the 10 μM DNB, with areas equal to roughly 70% and 25% of the control at 12 hr and 24 hr respectively (Fig. 3C, F). In co-culture, no significant difference in mean total neuron area was found between the 10 μM DNB and 100 μM DNB concentrations for 12 hr and 24 hr (Fig. 3C, F), suggesting that the astrocytes provide some neuroprotective support in an environment subjected to an *m*-DNB insult.

Monoculture and “Restricted Contact” Co-Culture in Microfluidic Devices

With the capability afforded by compartmentalized devices to polarize somas and neurites into separate compartments, the effects of *m*-DNB on neurite area could be better visualized and characterized. With the use of the compartmentalized device, localization of chemicals to a single compartment can be achieved through fluidic isolation.¹⁷

In one set of device-based experiments, we determined the effects of *m*-DNB on neurite fragmentation. We chose to analyze neurite fragmentation in 3-day-old cultures, because cells cultured for longer periods of time (~1 week) displayed a large number of tiny neurite residues that were difficult to quantify reliably with our morphometric assessment technique. Three days following seeding, prior to the neurites fully extending into the opposing compartment, the medium in the neurite compartment was exchanged with either 0 μM (vehicle), 100 μM or 250 μM *m*-DNB solution for a two day period. Cells were fixed and stained (Fig. 4A) for subsequent Fiji analysis (Fig. 2B) on day 5. In addition to quantifying total neurite area (Fig. 4B), we used Fiji to identify fragments within the neurite arbors (Fig. 4C). We observed that both the 100 μM and 250 μM concentrations yielded significantly lower mean neurite areas, with respect to the control, that were in both cases around one-fourth of the control. Similarly, the average fragment area was lowest for concentrations of 100 μM and 250 μM , respectively 320 μm^2 and 150 μm^2 , compared to 1500 μm^2 for the control (Fig. 4B). Also, there was a linear increase in the total number of fragments with increasing *m*-DNB concentration (Fig. 4C).

A second set of device-based culture experiments were used to compare monoculture to co-cultures. In these experiments, the neurons were in culture for a total of 6 days prior to dosing, to allow astrocytes sufficient time to establish a confluent astrocyte bed. In device-based co-cultures, neurons were seeded into one chamber of the microfluidic device, and astrocytes were seeded into the second chamber the following day. Astrocytes formed confluent monolayers after four days. Five days and four days post-seeding of the neurons and astrocytes, respectively, the medium in the neurite compartment was displaced with either 0 μM (vehicle), 100 μM or 250 μM *m*-DNB solution. Exposure was repeated for a

second day, and cells were fixed for subsequent morphometric analysis on day 7. In both device-based monocultures and co-cultures, maintained for 7 days, we observed a concentration dependent decrease in mean total area of the neurites (Fig. 5C). In the monocultures, the mean neurite area was approximately 50% of the control value for the 100 μ M *m*-DNB, and 16% for 250 μ M *m*-DNB (Fig. 5C). In the co-cultures, mean neurite area was approximately 70% of the control value for the 100 μ M *m*-DNB, and 36% for 250 μ M *m*-DNB (Fig. 5C).

It was easier to quantify neurite fragmentation in the neurite compartment of the devices than in the wells. As compared to neurites in the wells, the neurite networks isolated in one chamber of the device were less intricate, and showed some linear orientation with respect to the long axis of the microgrooves.

Determining *m*-DNB effects on the morphology of astrocytes presented challenges, since the number of visible cells in separate planes of a fully confluent astrocyte bed varied, and morphological comparisons might be misleading. The same morphological assessment used for neurons was applied to the characterization of astrocyte area in the “full contact” co-culture. Even though a reduction (~15%) in average cell area values for astrocytes was observed in co-cultures exposed to 100 μ M *m*-DNB, no significant difference in total astrocyte area at 24 hr was observed amongst all concentrations of *m*-DNB (data not shown).

Discussion

Existing *m*-DNB CNS neurotoxicity studies could benefit from unique, facile, and advanced *in vitro* device-based co-culture approaches that more closely recapitulate the complex cellular architecture of the native CNS environment. Through the image analysis of neuron morphology in “full” contact and “restricted” contact co-cultures, comprised of astrocytes and neurons, a first attempt has been made to quantify effects of *m*-DNB toxicity on the morphology of primary neurons physically associated with primary astrocytes *in vitro*. Fluorescence image analysis was used to identify a reduction in neuronal cell area, reduction in neuron elaboration, or structural aberrations of neurites consequent to exposure of mono- or co-cultures to *m*-DNB.

The results demonstrated that *m*-DNB, regardless of *m*-DNB concentration, induced no significant change in mean neuronal cell area when the neurons were cultured on top of an astrocyte bed, whereas there was a significant decrease in mean cell area for neurons cultured alone. More extensive neurite elaboration on the neurosupportive astrocyte beds, when compared to bare culture surfaces, was also observed.

In the device-based experiments, neurons were cultured in one chamber of the microfluidic device. Neurites, arising from the neuron chamber, traversed a set of microgrooves and emerged onto either the bare floor of a chamber or an astrocyte bed covering the floor a chamber. Neurites or both neurites and astrocytes in the “neurite” chamber were selectively exposed to *m*-DNB.

In one set of microfluidic device neuron monoculture experiments, neurites were exposed to *m*-DNB to quantify both neurite area and fragmentation. The mean neurite area was one-

tenth of the control for both the 100 μm and 250 μm concentrations of *m*-DNB. Also, the average fragment area was indirectly proportional to the total number of fragments, with the total number of fragments increasing with increasing concentrations of *m*-DNB.

In a second set of microfluidic device culture experiments, neuron-astrocyte co-cultures were compared to neuron monocultures. In the co-cultures, astrocytes were seeded into the neurite compartment three days following the seeding of neurons. The mono- and co-cultures in these second set of experiments were maintained for five days before being exposed to *m*-DNB. Elaboration of neurites onto either an astrocyte bed or a bare channel floor decreased with an increasing concentration of *m*-DNB, but the area of neurite arbors was always higher for neurites touching astrocytes.

In the “restricted” contact co-cultures, the astrocytes maintained a proteoplasmic phenotype when touching neurites, but some astrocytes in “full” contact co-cultures adapted a stellate phenotype. This could indicate that cell somas or whole neurons, rather than neurites alone, express or release factors that influence the shape of the astrocytes that they physically contact. In future investigations, we will collect medium from the neurite/astrocyte compartment of the device, and test the medium for the presence of soluble factors that *m*-DNB induces astrocytes to secrete.

Neurite degeneration occurs to various degrees in an assortment of neurodegenerative disorders and diseases.^{31, 32} In the present experiments, we evaluated the loss of neurite structural integrity as a sign of neurodegeneration,^{30–33} and we stained neurons for the expression of the neuronal tubulin, a type of cytoskeletal protein. The fragmented staining of neuronal tubulin that demarcated varicosities along the length of neurites, indicates a degradation of cytoskeletal structure. Calpain is a protease whose common targets are cytoskeletal proteins.⁴⁹ In other experiments, protease inhibitors can be applied to the neurite compartment to determine if *m*-DNB modulates activity of proteases, such as Calpain, and any resulting neurite fragmentation can be readily quantified.

Morphometric analysis of neurites has been used as a sensitive neurotoxicity assessment tool,³⁰ and there is an ongoing refinement of imaging techniques that permits simple and precise quantification of morphological alterations in complex arrangements of neuronal processes.^{50–55} These techniques can be used to acquire simple morphometric measures, such as average neurite length, which were not evaluated in this study due to the complex neurite network formed by the dense neuronal cultures. However, our technique can be used to approximate the density of complex neuronal networks based on the percent area that the neurites cover in a specific region of interest. In the neurite compartment of the microfluidic device, the contours of whole neurites or fragmented neurites can be readily identified. Despite the simplicity of our current analysis technique, it still suffers from some of the same drawbacks as the other aforementioned imaging techniques.^{50, 51} First, it is difficult to outline cells with complex neuritic arbors, and as shown in our experiments, the level of neurite arbor complexity increases with the presence of astrocytes. Second, the extent of neurite elaboration increases with longevity of culture time, requiring the creation of composite images to analyze long neurites that can span several image fields. Third, not only can distinct cells be positioned on different focal planes, such as astrocytes residing

under neurons in our co-culture systems, but the neurites themselves can extend through different focal depths. Thus, heterogeneous cell culture systems with increasing complexity demand more advanced imaging and analysis techniques, such as one that is coupled with confocal microscopy for multiple plane analysis, to unveil features of cell morphology in all dimensions.

A similar device-based morphometric analysis was employed to quantify the level of neurite degeneration resulting from excitotoxicity. Fluorescent neuronal images, obtained from the neurite compartment following selective exposure of hydrogen peroxide to either a soma or neurite compartment, were overlaid with a square grid. Neurites in each grid were sorted into bins representative of neurite physical appearance: beaded, fragmented, or whole, and the level of degeneration was interpreted as the percentage of the sum of axons fragments scored as beaded or fragmented. In our device-based studies, we analyzed all axons in the neurite compartment, instead of individual fragments, and interpreted degeneration as a measure of total neurite area.²⁵ Our method provides the added value that it may be able to determine early stages of onset degeneration based on a reduced area of neurite outgrowth prior to the physical manifestation of beading or fragmentation in individual neurites.

This study can be expanded to evaluate the roles of other major cellular players, in addition to neurons and astrocytes, in mitigating the effects *m*-DNB toxicity. It may be beneficial to learn the role of microglia in culture with astrocyte and neurons, since microglia have been reported to perform both agonistic and antagonistic roles as they interact with neurons.³² Conveniently, one design variation of the existing commercialized device includes a third compartment that could house microglia.

Device-based queries could clarify a relationship, if any, between the events of neurodegeneration and mitochondria trafficking that are associated with *m*-DNB toxicity. Research indicates that mitochondrial movement is significantly impaired following neuronal injuries, such as glutamate excitotoxicity and zinc neurotoxicity, which led to some effects observed in ischemia.⁵⁶ The microgroove component of the microfluidic device could permit facile quantification of retrograde and anterograde trafficking of mitochondria along neuron processes,^{57, 58} making the device an ideal system to explore relationships between axonal degeneration and mitochondrial dysfunction in neurons cultured alone or in the presence of other cells.

Conclusion

This article offers a simple neurite morphology assessment coupled to a unique comparative co-culture system as a means to study cell-cell interactions' effects in neurotoxic environments. The present neurotoxicity analysis of *m*-DNB-induced effects, based on the extent of neurons' physical contact with astrocytes is in its infancy. Yet, it was discovered that astrocytes in physical contact with neurites offer some form of neuroprotective support against the effects of *m*-DNB exposure, and may even regulate neurite elaboration. Using a "restricted-contact" co-culture, we quantified levels of neurite degeneration resulting from the targeting of *m*-DNB to the neurites only. Neurite degeneration resulting from selective *m*-DNB exposure to the somas will be the focus of upcoming experiments. Even though we

limited the morphological indicator of neurodegeneration to observations of transformations to external neuronal architecture, a more powerful indicator of the degeneration status of neurons can be acquired from the inclusion of nuclear morphology in our assessment.⁵⁹ This unique “restricted” co-culture approach can be extended to investigate the mode of action for an assortment of other neurotoxicants, such as ammonia, that like *m*-DNB, provokes a neuroprotective action from astrocytes.⁶⁰

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

- Astrocytes and neurons physically contact each other in two distinct co-cultures.
- In the “full” contact co-culture, neurons lay atop an astrocyte bed.
- In the “restricted” contact co-culture, isolated neurites elaborate onto an astrocyte bed.
- Based on the co-culture type, cells are selectively or globally exposed to *m*-DNB.
- Physical contact between astrocytes and neurons mitigate toxic effects of *m*-DNB.

Figure 1.

Design Overview of “Full” and “Restricted” Co-Culture Systems. A) In the “full” contact co-culture model, neurons are plated atop a confluent astrocyte bed, where maximum cell contact is permitted. Also both astrocytes and neurons are bathed in medium containing a neurotoxicant. B) In the “restricted” contact co-culture model, the somas are restricted to one compartment while neurites migrate through a series of microgrooves to fill an adjacent compartment, which contains astrocytes. Neurotoxicant enriched solutions can be restricted to either compartment through fluidic isolation. Fluorescent images of C) “full” and D) “restricted” co-cultures. Nuclei are stained with DAPI. Anti- β tubulin III antigens in neurons were recognized with antibodies conjugated to Alexa 555 (C) or Alexa 488 (D). Astrocytes were visualized with antibodies, conjugated to Alexa 488 (C) or Cy3 (D), specific for against glial fibrillary acidic protein, GFAP. Scale bar = 100 μ m.

Figure 2. Technique for Measuring Morphological Area of Cells. The threshold tool in Fiji was used to transform raw grayscale images of a) neurons, in glass bottom wells, or b) neurites in the neurite region of compartmentalized microfluidic devices (b) into binary black and white images. Background was always assigned the color black, and cell associated regions the color white.

Figure 3.

Effect of DNB on Morphology of Neurons in “Full” Contact Neuron-Astrocyte Co-Cultures Compared to Neuron Monocultures. Representative fluorescent images of a monoculture (□) of neurons (stained with anti-β tubulin III-Alexa 555 conjugate) at 12 hr (a) and 24 hr (d) and co-culture (■) of neurons and astrocytes t 12 hr (b) and 24 hr (e) post exposure to 0 μM, 10 μM or 100 μM *m*-DNB. Neurons and astrocytes were respectively stained with anti-β tubulin III conjugated to Alexa 555 and anti-GFAP conjugated to Alexa 488. Scale bar = 200 μm. Comparison of cellular area between monoculture and co-cultures at 12 hr (c) and 24 hr (f). Cell area measurements were averaged from 5 image fields taken from each well. Data shown for each group was obtained from three biologically independent experiments with two replicates per experiment, and the mean cell area measurement for each group is expressed as a percentage of the control (* denotes $p < 0.05$).

Figure 4.

Effect of DNB on Neurite Elaboration and Degeneration in Compartmentalized Microfluidic Devices. A. Fluorescent images of neurites that were allowed to extend for three days into and through microgrooves into an environment *m*-DNB devoid (0 μM , vehicle control) or enriched (100 μM or 250 μM) environment. Measurements were collected from 10 centermost fields along the neurite containing microchannels of four devices for each concentration condition. Images shown are 2 image fields stitched together with Fiji. Scale bar = 200 μm . B. The area of the neurites was measured with Fiji software, which showed that the neurite area decreased with increasing DNB concentrations. C. Fiji was used to recognize fragments in neurite arbors, and the graph displays an inverse relationship between the average fragment area (○) and the total number of fragments (■). The mean neurite area measurements were obtained from 4 devices (two biologically independent experiments with two replicates per experiment) for each concentration (* denotes $p < 0.05$).

Figure 5. Effect of DNB on Morphology of Neurons in Compartmentalized “Minimal” Contact Neuron-Astrocyte Co-Cultures Compared to Compartmentalized Neuron Monocultures. Neurites traverse microgrooves and elaborate either on a bare surface (a) or an astrocyte bed (b), where the neurites or neurites and astrocytes are exposed to 0 μM, 100 μM or 250 μM DNB. Neurites are stained with anti-β tubulin III-Alexa 488 conjugate. Astrocytes are stained with anti-GFAP-Alexa Cy3 conjugate. Scale bar = 100 μm. (c) Comparison of total neurite area in the presence of DNB with or without a supportive underlying astrocyte layer. The mean neurite area measurements for monocultures (□) were obtained from 3 devices, for the 0 μM and 100 μM concentrations, and 1 device (due to experimental constraints), for the 250 μM concentration (*denotes p<0.05; † denotes group was excluded from statistical analysis). The mean neurite area measurements for co-cultures (■) were obtained from 5 devices, for the 0 μM and 100 μM concentrations, and 4 devices, for the 250 μM concentration.