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Single-Neuron Axonal Pathfinding under Geometric Guidance: Low-Dose-Methylmercury Developmental Neurotoxicity Test

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Abstract

Because the nervous system is most vulnerable to toxicants during development, there is a crucial need for a highly sensitive developmental-neurotoxicity-test model to detect potential toxicants at low doses. We developed a lab-on-chip wherein single-neuron axonal pathfinding under geometric guidance was created using soft lithography and laser cell-micropatterning techniques. After coating the surface with L1, an axon-specific member of the Ig family of cell adhesion molecules (CAMs), and optimizing microunit geometric parameters, we introduced low-dose methylmercury, a well-known, environmentally significant neurotoxicant, in the shared medium. Its developmental neurotoxicity was evaluated using a novel axonal pathfinding assay including axonal turning and branching rates at turning points in this model. Compared to the conventional neurite-outgrowth assay, this model's detection threshold for low-dose methylmercury was 10-fold more sensitive at comparable exposure durations. These preliminary results support study of developmental effects of known and potential neurotoxicants on axon pathfinding. This novel assay model would be useful to study neuronal disease mechanisms at the single-cell level. To our knowledge, the potential of methylmercury chloride to cause acute in vitro developmental neurotoxicity (DNT) at such a low dosage has not been reported. This is the first DNT test model with high reproducibility to use single-neuron axonal pathfinding under precise geometric guidance.

Introduction

Exposure of the nervous system to toxicants during its development can cause long-lasting and possibly heritable effects.¹⁻³ Recent work has focused on developing models to determine developmental neurotoxicity (DNT), especially at the cellular and molecular level.⁴

Exposure to methylmercury (MeHg) inhibits neuronal development in humans,^{5, 6} and both acute and chronic exposure to mercury cause a variety of neurological and psychiatric disorders.⁷⁻⁹ The risks associated with low-dose MeHg are an area of urgent concern.^{1, 10} It is critical to public health to determine the threshold of risk associated with MeHg exposure to determine whether subclinical effects may vary with the toxic load and any benefits that may be expected with remediation. To detect DNT secondary to acute- and low-dose exposure and investigate cellular and molecular mechanisms of toxic action require development of DNT-test models with higher sensitivity than the currently available assays. Because MeHg decreases neurite outgrowth at concentrations that do not affect neuronal viability,^{10, 11} assays based on neurite outgrowth provide greater screening sensitivity than do cell-viability assays. Thus, neurite-outgrowth models are the most widely used methods for study of *in vitro* DNT.

Because conventional neurite-outgrowth models use monolayer cell-culture methods, in which neighboring cells provide trophic support that can mask toxic perturbations, they lack the sensitivity to elucidate molecular mechanisms. For *in vivo* cell viability, support from neighboring neurons may be physiologically relevant. However, *in vivo* single-cell events, such as pioneer-neuron axonal pathfinding,^{12, 13} exist during neuronal development. These developing neurons are more sensitive to toxic exposure than those in monolayer cell cultures because pioneer neurons lack neighboring neurons to compensate for neurite inhibition or pathfinding errors. Moreover, in conventional neurite outgrowth assays only parameters like neurite length and outgrowth rate are tested. Thus the success rate of actual axonal pathfinding to the intended target under physiological guidance cues, a key step in correct neuronal-circuit formation that is distinct from neurite-outgrowth rate, can only be inferred. To address these issues, we developed a microfabricated biochip for single neurons under geometric guidance to detect a developmental process that can be perturbed at concentrations less than those required to inhibit neurite outgrowth. These perturbations may harm isolated neurons that cannot communicate with others and thus are more vulnerable and sensitive to toxicants.

Here we report development of the biochip and its application for DNT testing of acute, low-dose MeHg. The biochip provides multiple identical microunits for data acquisition for statistical analysis. Each microunit is composed of three cell-culture microwells connected by microchannels in either a “T” or “Y” pattern. The laser cell-micropatterning technique is used to place a single cell into one microwell in each microunit. The channel connecting the microwells provides geometric guidance, allowing investigation of defined single-neuron axonal pathfinding. The channel, which is open, provides each microunit with uniform cell-survival cues from the shared medium. Therefore, pathfinding by each single cell is exposed to an identical microenvironment.

Before introducing MeHg into the model, we tested low-dose MeHg DNT in a neurite outgrowth assay based on conventional random cell culture to identify the concentration ranges for testing. Then, MeHg was introduced into the culture medium to test the model presented in this work: After incubation in shared medium for 4 hr and recovery for 48 hr, axonal branching rates under geometric guidance of all groups (5 nM, 10 nM, and 20 nM) were significantly inhibited. Compared with the neurite outgrowth assay, the DNT model established in this study was more sensitive than the MeHg DNT based on conventional random cell culture, and its detection threshold was 10-fold lower. Acute MeHg DNT at such a low dose has not been previously reported; moreover, this is the first DNT-test model to apply axonal pathfinding of single-neurons under geometric guidance.

Materials and Methods

Biochip design and fabrication

An on-chip DNT-test model based on axonal pathfinding of single neurons was constructed with multiple identically microfabricated geometric-guidance microunits consisting of microwells and microchannels in a “T” or “Y” pattern. The microwells were designed to confine the cell body, and the microchannels were designed to guide axonal growth along a defined path. Microwells and microchannels were the same depth.

The biochip was microfabricated in polydimethylsiloxane (PDMS) using standard photolithography and soft lithography techniques.¹⁴ The microscale features were designed using AutoCAD and printed on a film as masks (CAD/Art Services Inc.). The replica molds were made of SU-8 2050, 2025, or 2005 negative photoresists (MicroChem Inc.), which was spun on silicon wafers at different speeds for 45 s and then UV-exposed under masks using a Karl-Suss MJB aligner to achieve pillars with a height of 80 μm , 40 μm , 20 μm , or 5 μm . After hard baking at 137 °C for 2 hr, the silicon molds were silanized with trimethylchlorosilane to aid removal of elastomeric membranes. For example, to obtain PDMS membranes with a thickness of approximately 80 μm , a 10:1 mixture of the base agent and the curing agent Sylgard™ 184 (World Precision Instruments Inc.) was spun on the mold at 1000 RPM for 1 min. The PDMS membranes were cured at 95 °C for maximum crosslinking and immersed consecutively in three solvents (triethylamine, ethylacetate, and acetone), each for 2 hr, to extract short chain oligomers. Then, the membranes were baked in a vacuum oven at 137 °C overnight to remove the solvents. Before surface modification, each PDMS membrane was bonded to a clean coverglass (22×22 mm) and heated at 137°C for 2 hr to create a permanent bond.

Surface modification

The surface of a newly fabricated PDMS biochip is hydrophobic and thus not suitable for cell attachment and growth. Therefore, the biochip was treated for 10 min with oxygen plasma, which replaces the -CH₃ groups with -OH groups, converts the surface from hydrophobic to hydrophilic, and adds some -O⁻ groups, which give the surface a negative charge. The cell membrane is also negatively charged. To reverse the biochip's charge and thereby improve neuronal attachment and growth, it was immersed in positively charged 0.01 mg/ml poly-L-lysine (PLL, Sigma) diluted in Milli-Q filtered water in a 35 mm dish at

room temperature overnight, which provided enough time to ensure that the microunit could physically absorb PLL molecules. The biochip was rinsed three times in 48 hr in Milli-Q water. This procedure was repeated to produce enough biochips to perform the experiments.

Following PLL-coating, the biochips, except for those to be used as control groups, were immersed in 25 µg/ml recombinant human NCAM-L1/Fc chimera (L1, R&D systems) diluted in Milli-Q water for 4 hr at 37°C and rinsed once in M199 medium before neuronal culture.

Cell culture and laser cell-micropatterning

Chick forebrain neurons (CFNs) were used for their stereotypical morphology as a model for cortical pyramidal neuronal projections. Neurons from the forebrain (telencephalon) of 6- or 7-day-old White Leghorn chick embryos were harvested as described by Heidemann.¹⁵ Individual neurons were deposited in the microwells and geometric guidance channels using the laser cell-micropatterning technique.

The technique utilizes optical force, which can confine a single cell in the axis of a laser beam and guide it downward to a substrate.¹⁶ By moving the substrate relative to the laser beam, selected cells can be moved transversely while they are pushed downward to create a specific cell pattern. The details of our system are described in our two previous publications.^{17, 18} Our real-time laser cell-micropatterning video is provided in Movie Supplement 1.

Prior to laser cell-micropatterning, 1 ml neuron-culture medium was added to a 35 mm petri dish. The culture medium consisted of medium 199 (without phenol red) supplemented with 1% antibiotic/antimycotic (10,000 units/ml penicillin G sodium, 10,000 µg/ml streptomycin sulfate), 50 µg/ml gentamicin, 2.5 µg/ml amphotericin, 10% fetal bovine serum, 2% B27, and 100 ng/ml NGF 7s. All supplements were purchased from Invitrogen Life Technologies. Immediately after neuronal patterning, the biochip was removed from the cell-deposition chamber, placed at the bottom of the 35 mm petri dish, and incubated (37 °C and 5% CO₂).

MeHg exposure

CFNs were placed in biochips coated with PLL and L1. After approximately 16 hr in culture, they attached to the culture surface. Axons grew rapidly in the following several hours.

In both the neurite outgrowth assay and the single-cell, on-chip DNT-evaluation model studies, CFNs were exposed to methylmercury chloride (Sigma) after 16 hr in culture. Because MeHg can bind to free sulfhydryl groups and to cysteine in particular,^{19, 20} MeHg was diluted in M199 medium without serum or growth supplements at concentrations of 5, 10, 20, or 50 nM. CFNs were exposed to the MeHg medium for 4 hr. The control group was exposed to unsupplemented M199 medium without addition of MeHg for the same period. After 4 hr incubation, medium in all groups was replaced with full culture medium (M199 with FBS, B27, and NGF) for recovery. Data were collected after 24 hr and 48 hr.

Immunocytochemistry and microscopy

Live-cell phase microscopy was performed on a Zeiss microscope equipped with an onstage incubator maintained at 37 °C and 5% CO₂, and the immunostained biochips were observed under the same microscope equipped with fluorescence-imaging ability.

After 3 days, the neurons on biochips (inside or outside microwells) were fixed with 4% paraformaldehyde/glucose in PBS and stained with neuron-specific antibody β 3-tubulin (abcam). Alex Fluor 488 goat anti-mouse (Invitrogen) was used as the secondary antibody. Finally, slides were prepared with ProLong® gold antifade with DAPI reagent as the mounting medium (Invitrogen).

Statistics

There were three possible outcomes for axonal pathfinding along microchannels by single-neurons in microwells: turning to either side of the microchannel, branching to both sides, or neither turning nor branching. Each condition was studied under different doses of MeHg exposure. We performed a statistical analysis using the chi-square (χ^2) test, which is typically used for comparing experimental frequency distributions to an expected distribution. The chi-square test was also used to compare correct polarity rates of CFNs in wells of different depths, axonal branching rates along with axonal morphogenesis rates of CFNs on PLL-only- or PLL+L1-coated surfaces, and axonal branching rates of CFNs exposed to different doses MeHg in the neurite-outgrowth assay.

One-way ANOVA was used to compare 1) axonal length of CFNs on PLL-only- or PLL+L1-coated surfaces and 2) axonal length of CFNs exposed to different doses MeHg in the neurite-outgrowth assay.

Results

Surface modification

For conventional *in vitro* neuronal cultures, which are typically high density, coating the substrate with PLL is sufficient for cell attachment and growth. In our microwell model, cell-to-cell signalling is so limited that maintaining viable single neurons is difficult; achieving successful axonal pathfinding under geometric guidance is even more challenging. To improve the likelihood of successful pathfinding, the biochip was first coated with PLL and then coated with L1, an axon-specific member of the Ig family of cell adhesion molecules (CAMs) and a potent promoter of neuronal attachment and neurite outgrowth.²¹ After 3 days on flat PDMS membranes, neurons cultured on surfaces coated with PLL+L1 had larger somas and longer neurites with branches than those on surfaces coated with PLL-only (Fig. 1).

The quantitative differences between neurons cultured on PDMS membranes coated with PLL-only and PLL+L1 are shown in Fig. 2. In this research, the longest neurite is designated as the axon.²²

At 16 hr *in vitro* culture, there was no significant difference in axonal morphogenesis (measured as the percentage of the number of neurons that generate axons out of the total

number of neurons) in the PLL+L1 group and the PLL-only (Fig. 2A). However, at 48 hr and 72 hr, the promotion of axon branching by L1 was significant (Fig. 2B). Furthermore, CFN axonal length when cultured on the PLL+L1 surface was significantly longer than on the PLL-only surface at time points of 16 hr, 48 hr, and 72 hr (Fig. 2C).

Optimization of geometric guidance parameters

Geometric parameters are critical for successful DNT testing *in vitro* using single-neuron axonal pathfinding. In a previous study, researchers found that axonal guidance could be regulated by varying the turning angle in open microchannels.²³ In the study reported here, we chose to investigate the effect of turning angles on DNT evaluation using a model with two different turning angles for axonal pathfinding, a T-pattern (90°) and a Y-pattern (60°). First, we conducted experiments to determine the optimal size for the geometric-guidance unit.

A microwell's main purpose is to confine cell bodies; a microchannel's main purpose is to guide axonal outgrowth. The average diameter of dissected neurons is 8-10 μm ; after adhesion, they may be approximately 20 μm . Accordingly, we designed microwells in two diameters (25 μm and 50 μm) and microchannels in two widths (15 μm and 20 μm). Each microunit had equally deep microwells and microchannels.

To investigate the effect of depth on neuronal viability, we studied microwells of a number of different depths: 80 μm , 40 μm , 20 μm , and 5 μm . For both the untreated and the treated group, neurons from the same suspension were placed in biochips that varied in microwell diameter, channel width, and microwell depth.

Our experimental data demonstrated that relatively shallow microwells promoted neuronal growth, but microwells that were too shallow did not effectively guide axonal extension, which was shown in Fig 3. The 5 μm deep microwells had this result (Fig. 3A), which was defined as “incorrect polarity.” The majority of cells in 20 μm wells extended neurites along the microchannels (Fig. 3B), which was defined as “correct polarity.” There was a significant difference in polarity between 5 μm and 20 μm wells (Fig. 4). A study by another group found that when channel depth was higher than 22 μm , axons had difficulty extending outside channels.²⁴

For the microwell diameter, although 25 μm microwells are sufficiently large for the soma, the dendrites, the signal receptors, need more space. Our result demonstrated that in 50 μm diameter microwells, the viability of neurons in microwells is higher than that in 25 μm (data not shown).

Microchannel width appeared to be important for neurite extension in microwells of the selected diameter and depth (50 μm and 20 μm). For example, a greater number of neurites extended in 20 μm wide microchannels than in 15 μm wide microchannels (data not shown). One study by others found that turning frequency increased as the microchannel width did, but no statistically significant difference between 20 and 50 μm widths was found.²³ In another study, the guidance effect of microchannels was strongest for neurons in channels 20 μm wide.²⁵

Of the geometric parameters tested, a single-neuron axonal pathfinding model with a microwell diameter of 50 μm and a microchannel depth and width of 20 μm was optimal for neuron viability and polarity. After 2-3 days in culture in this geometry, more than 60% of cells were polarized by the geometric guidance patterns, as shown in Fig. 5. In both T and Y patterns, axons extended along the microchannels and turned to one side or branched to both sides. The immunostaining results are shown in Fig. 6.

Low-dose MeHg DNT evaluation

Exposure of randomly cultured CFNs to MeHg—To determine the concentration range of MeHg for investigation in our on-chip model, CFNs were placed on flat PDMS membranes at low density (50 cells/ mm^2). The two most widely used parameters of the conventional neurite outgrowth assay, axonal length and primary branching ratio, were used to evaluate the DNT of MeHg at several concentrations: Cells were incubated with MeHg in concentrations of 5 nM, 10 nM, 20 nM, or 50 nM for 4 hr and allowed to recover for 24 or 48 hr (Fig. 7).

In the 50 nM group, both axon length and primary branching ratio were significantly lower than those of the control group (without MeHg addition). No significant difference was found between the control group and the 5 nM, 10 nM, or 20 nM groups. Therefore, 5 nM, 10 nM, and 20 nM MeHg were applied in our single-neuron axonal pathfinding DNT-test model to explore its sensitivity.

Exposure of single neurons on chips to MeHg—To be successful under geometric guidance by microchannels, axons of neurons patterned in microwells must extend along the channel and pass the T or Y node to turn to either side or branch to both sides (Fig. 5 and Movie Supplement 2). The essential concept of applying this geometric-guidance phenomenon to DNT testing is dependent on the hypothesis that with the addition of toxicants, the ability of axons to pass the node will be completely or partially inhibited.

Successful single-neuron axonal pathfinding in each microunit is defined as either turning to one side (turning) or branching to both sides (branching). The successful pathfinding rate is thus the summation of the turning rate and the branching rate. The successful pathfinding rate, turning rate, and branching rate were compared between groups with different MeHg doses and the control group to study low-dose MeHg toxicity. With this, the neuronal morphological indicator (turning or branching), the microchannel pattern (T- or Y-pattern), and the time-point (24 hr or 48 hr recovery time) that were most sensitive to MeHg exposure were quantified.

When turning and branching were not distinguished, and the successful axonal pathfinding rate alone was considered, the rate of only the T-pattern model 20 nM group with recovery for 24 hr after 4 hr MeHg incubation was significantly lower than that of the control group. When the three MeHg groups were recovered for 48 hr, none showed a significant difference compared with the control group (Fig. 8A). In the Y-pattern model at 24 hr and 48 hr-recovery time points, no significant difference was found between any test group and the control group (Fig. 8B).

When branching and turning rates were each compared with control, results indicated a greater level of sensitivity than when they were not distinguished. Fig. 9A and B show results at the 24 hr recovery-time point. In the T-pattern model, the branching rates of both the 10 nM and 20 nM groups were significantly lower than that of the control group; the turning rate of the 20 nM group was significantly lower than that of the control group (Fig. 9A). In the Y-pattern model, the branching rate of the 20 nM group was significantly lower than that of the control group; turning rates of the three MeHg dosage groups were not significantly different than the control group (Fig. 9B).

At the 48 hr recovery-time point, 1) in the T-pattern model, the branching rates of all three dosage groups were significantly lower than those of the control group, and the turning rates between any of the three dosage groups and the control group were not significantly different (Fig. 9C); 2) in the Y-pattern model, none of the three MeHg dosage groups was significantly different in branching rate or turning rate than the control group (Fig. 9D).

Discussion

L1 CAM for surface modification

L1 is primarily expressed in the developing nervous system. Its functional importance in axon extension and guidance is emphasized by neurological syndromes (e.g., MASA syndrome and X-linked hydrocephalus) that result from mutations of the single gene that encodes L1 in humans.²⁶ Our data demonstrate that L1 in our single-neuron-pathfinding assay promoted neurite outgrowth and axon turning and branching. However, although L1 coating can promote neurite outgrowth from the start of culture, it cannot promote axonal morphogenesis, and its role in branching is not significant until later. It may be that neurons possess a degree of intrinsic programming that remains unmodified by this extracellular clue.

Geometry selection

Our results show that 50 μm microwells were better at promoting cell viability and functionality (e.g., neurite outgrowth rate) than 25 μm microwells. One possible reason for this result is that although 25 μm microwells were spacious enough for the soma, they lacked space for dendritic (signal receptor) growth. It may be that the greater space in 50 μm microwells increases neuronal viability. The results that led to the selection of a 20 μm channel-depth may be related to receiving nutrition and exchanging metabolic wastes, which are more difficult in 80 μm and 40 μm deep channels than in 20 μm and 5 μm deep channels. Additionally, unbound PLL molecules, which are toxic to neurons, are more difficult to remove by cleaning in deep microchannels. Thus, shallow microchannels allow more neurite growth than deep channels.

Axonal pathfinding under geometric guidance and branching inhibition

Our data show that regardless of recovery time, CFNs in the T-pattern model were more sensitive to MeHg than those in the Y-pattern model. It may be that because the turning angle of the Y channel was smaller, passing the Y microchannel node was easier than passing the T node.²³ If T-pattern turns are more challenging to neurons, they may become

more sensitive to lower doses of MeHg. In general the time-point most sensitive to MeHg was the 24 hr recovery point (acute effect); however, for branching in the T-pattern model, the 48 hr recovery point was the most sensitive. How the geometry and the MeHg exposure might be related to this is unknown. It may be that if branching an axon into a tight angle is inherently more difficult, it is likewise more difficult to remediate during recovery, and thus the defect becomes a permanent morphological condition (branching occurs mainly during a culture stage corresponding to the 48 hr recovery time point). Or, MeHg may inhibit neuritogenesis in a general manner, making such a branch difficult to establish or maintain. Or, perhaps MeHg may interfere with a specific mechanism of axon branching that is not fully elucidated under these study conditions. Further investigation of small-angle branching including physiological relevance (*in vivo* branching) is needed to determine the nature of high axonal sensitivity to MeHg.

It is well established that a neuron, particularly in the mammalian central nervous system, establishes correct connections not only by extending an axon, but also by branching the primary axon to the appropriate targets.²⁷ To our knowledge, this study is the first to differentiate the response of axonal branching to geometric guidance from that of axonal extension and growth by use of a simple geometric-guidance circuit for single-neuron axonal pathfinding. We found that 1) rate of axonal branching under geometric guidance was more vulnerable to low-dose MeHg than length of axonal extension and 2) the underlying mechanisms of axonal branching versus simple turning at geometric corners in the pathfinding process were distinct with respect to exposure to MeHg. MeHg is known to inhibit Rac activity.²⁸ In studies by other groups, axonal-branching defects occurred with partial loss of Rac activity, whereas guidance and growth deficits occurred with more severe loss of Rac activity.^{29, 30}

Acute and low-dose DNT of MeHg

The developing nervous system is particularly vulnerable to toxic insults. Many, if not most, developmental neurotoxicants have been identified as such after incidents of human exposure caused clinical injuries. To detect potential developmental toxicity before human exposure is critical to public health.³¹ Among 80,000 known chemicals, only 201 have been effectively tested for neurotoxicity to humans.¹¹ The cost, ethical issues, and time associated with the current use of animals make *in vitro* toxicity studies for rapid chemical screening a critical need. Additionally, current test methods may not take into consideration level of sensitivity and differential effects of exposure on humans and developing fetuses.

In our DNT tests with low-dose MeHg, after single and acute 4 hr exposure and recovery for 48 hr, the branching rate was inhibited in even the 5 nM group. This study demonstrated the efficacy of our geometric guidance model on a neuronal biochip: It delivered precise chemical evaluation at much lower doses than other techniques. Additional work will determine if the morphological effect observed represents an appreciable risk to humans.

A chemical's DNT potential can vary depending on the stage of neuronal development during exposure and the processes (e.g., axonal elongation, turning, or branching) that individual neurons are undergoing at the time of exposure. In this study, cells were cultured in regular medium 16 hr and then incubated 4 hr with MeHg; this period is relevant to the

embryo in an early stage (critical for axonal morphogenesis). At a comparable exposure duration, Heidemann found that 250 nM MeHg significantly inhibited axonal morphogenesis, but did not cause cell death.¹⁰ Accordingly, the group assumed that inhibition of axonal morphogenesis by acute, sublethal concentrations of MeHg might contribute to developmental syndromes (Parkinson's syndrome, autism, and epilepsy) caused by environmental exposure to MeHg. Most studies evaluate basic neurite morphology and neurite extension. This research, however, focused on axonal branching and turning rates in a defined choice point as distinct and important neurodevelopmental processes. In the current study, axon branching in the T was the most sensitive to MeHg at 5nM, causing significant deterioration. Compared to the corresponding neurite outgrowth assay (Fig. 7), the microunit-based DNT model exhibited a 10-fold sensitivity increase to MeHg. It is also notable that regarding the sensitivity in the random cell-culture, the axonal branching assay (Fig. 7B) showed no significant difference in comparison with that of the neurite extension assay (Fig. 7A). However, axonal branching in our microunit model, which was induced by defined geometric guidance at the single cell level, showed a distinguishably high sensitivity (Fig. 9) when compared with the random culture.

Using a 24-fold increased exposure duration, Radio exposed NS-1 (PC-12) cells to chemicals 2 hr after plating and then incubated them for 96 hr, a much lengthier period than was used here.¹¹ When unconfined neurite extension was measured, the lowest dose of significant MeHg DNT was 1 nM, indicating that other neuronal processes with extreme sensitivity to MeHg may remain unknown. This suggests that future research should study longer exposure times and lower MeHg concentrations to determine a minimum threshold dose and exposure duration. Additional research using geometry to investigate different neurodevelopmental processes at various dose and exposure-duration ranges will determine how this model relates to stages of *in vivo* development and human risk-assessment.

Consumption of fish containing mercury during pregnancy has been implicated in development of autism, but there is no direct evidence of a causal relationship. Research employing MRI has shown that to some degree, autism involves connection deficits in the axonal tracts in the brain.^{32, 33} In this study, we modeled a geometrically well-defined *in vitro* axon pathfinding choice-point and found that MeHg inhibited neuronal ability to branch during neuronal-network formation. Such a process *in vivo* may be correlated with fewer connections with target neurons. Therefore, in addition to precise elucidation of axon pathfinding mechanisms, this model may be useful for investigation of neuronal disease mechanisms at the single-cell level.

Conclusion

This on-chip model of axonal pathfinding of single neurons under geometric guidance is a highly sensitive test for DNT of acute and low-dose MeHg. This model's low-dose MeHg detection threshold is 10-fold lower than the conventional neurite outgrowth assay. The high-sensitivity and control of neurotoxicant exposure and differentiation of axonal pathfinding responses (elongation, branching, or turning) at the single-neuron scale in this on-chip model present a new avenue for developmental neurotoxicity research.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Fig. 1. CFN (3 days in culture) morphological differences on PDMS with different coatings. (A) PLL-only; (B) PLL+L1. Neurons were stained with β -tubulin III (green) and DAPI (blue). The scale bar for both images equals 20 μ m.

Fig. 2.

Quantitative results of CFN neurite outgrowth on different coatings. (A) Axonal morphogenesis, chi-square test, $n=100$; (B) Axonal branching rate, $*p < 0.05$, chi-square test, $n=100$; (C) Axonal length, $*p < 0.05$, one-way ANOVA, $n=100$.

Fig. 3. CFNs (3 days in culture) in wells of the same diameter (50 μm), but different depth (H). (A) H=5 μm; (B) H=20 μm. The scale bar is for both images and equals to 20 μm.

Fig. 4. Comparison of polarity in wells of different depth. * $p < 0.05$, chi-square test, $n = 50$.

Fig. 5. Typical successful axonal pathfinding of CFNs (3 days in culture) in microwells with optimal geometry. (A) Branching in T-pattern; (B) Turning to one side in T-pattern; (C) Branching in Y-pattern; (D) Turning to one side in Y-pattern. The scale bar, for all images, equals 20 μm .

Fig. 6. Axonal branching of CFNs (3 days in culture) in microwells with different microchannels were stained with neuron-specific β -tubulin III antibody (green) and DAPI (blue). (A) T-pattern; (B) Y-pattern. The scale bar, for both images, equals 20 μm .

Fig. 7. Neurite outgrowth results of MeHg neurotoxicity based on different indices. (A) Axonal length, * $p < 0.05$, one-way ANOVA, $n=150$; (B) Axonal branching rate, * $p < 0.05$, chi-square test, $n=150$.

Fig. 8.

Effects of MeHg neurotoxicity on axonal pathfinding rate of single-neuron on-chip DNT test model after MeHg incubation for 4 hr and recovery for 24 hr and 48 hr. (A) T- pattern; (B) Y-pattern. $*p < 0.05$, chi-square test, $n=40$.

Fig. 9.

Branching and turning rates in the single-neuron on-chip DNT test model after MeHg incubation for 4 hr. (A) T-pattern and recovery for 24 hr; (B) Y-pattern and recovery for 24 hr; (C) T-pattern and recovery for 48 hr; (D) Y-pattern and recovery for 48 hr. * $p < 0.05$, chi-square test, $n=40$.