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Quantitative Analysis of CNS Axon Regeneration Using a Microfluidic Neuron Culture Device

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Abstract

CNS axonal regeneration has been widely studied in order to develop strategies for overcoming Myelin mediated inhibition. However, there are no suitable in vitro methods to distinguish and assess regeneration of severed CNS axons. In this paper we describe the use of a new microfluidic neuron culture device that can be applied quantitatively to investigate the effect of myelin inhibitors on the regeneration of injured CNS axons. The device has two isolated compartments separated by a physical barrier with embedded microgrooves. The device has been successfully used for long-term culture of primary CNS neurons while isolating the somata in one chamber and directing growth of axons to the other chamber. We cultured cortical neurons in the devices and subjected them to reproducible axotomy by vacuum aspiration. After 24 hours, the lengths of regenerated axons were measured for quantitative analysis. NOGO-66 concentrations above 10 nM consistently resulted in ~20% reduction in length of regenerated axons. MAG protein also inhibited axonal regeneration. The length of regenerated axons decreased with addition of myelin inhibitory proteins after injury. These results suggest that the device can serve as an in vitro model for axonal injury and regeneration by simulating the microenvironment around the injury site.

Keywords: Cortical neurons, Nogo, MAG, Microfluidics

Introduction

Trauma to the adult central nervous system (CNS) is often associated with devastating functional deficits owing primarily to incapability of mature axons to regenerate after injury. According to previous studies[1-3], regenerative failure of the adult mammalian CNS axons has been postulated to be due to the presence of inhibitory proteins associated with damaged myelin. Efforts that promote axons to overcome the growth inhibitory nature of myelin and its associated inhibitors can therefore represent new therapeutic strategies in recovery after CNS injury.

In pursuit of such strategies, a variety of in vitro assay systems with controlled experimental conditions have been traditionally employed, where direct effects of the neuron outgrowth inhibitors can be investigated at the cellular and molecular level. Progress in regards to the myelin inhibitors, for instance, has been supported to a large degree by two in vitro assay systems. The first is the neurite outgrowth assay, which is performed in cell culture plates coated with myelin or myelin inhibitor proteins. The second assay is the growth cone collapse assay, which is based on ability of the myelin or myelin associated outgrowth inhibitors to cause the acute collapse of the growth cone[4]. Through the use of these two assays, three major cell-surface molecules with axon outgrowth inhibitory activity were identified in CNS myelin. The three proteins are Nogo, myelin-associated glycoprotein (MAG), and oligodendrocyte myelin glycoprotein (OMgp)[5-10]. While these assays have their own advantages and have proved useful for addressing questions related to myelin associated growth inhibitor molecules in neuronal development and elongation, they suffer from two major limitations. First is the lack of an appropriate method to probe the axons independently from the cell bodies. The second limitation inherent in the assay principle itself is the lack of an appropriate method to induce axonal injury.

To overcome the latter limitation, a variety of different tools have been employed to mimic CNS axonal injury, including sharp metal blades[11], pulled glass electrodes[12], and rubber impactors controlled by electrically driven shafts[13]. These procedures, however, must be conducted through the use of sophisticated computer-controlled micromanipulators, and

can cut only a few axons at a time precluding high throughput experimentation.

A notable exception is the Campenot chamber, which first allows for isolation of axons from the cell bodies in different compartments and second; allows for severing the isolated axons by directing a water jet towards one of the side compartments[14]. This method has proven successful in generating a uniform front of regenerating Peripheral Nervous System (PNS) axons, and has facilitated quantification of both the pattern and the timing of regeneration. CNS neurons involved in the pathology of most neurodegenerative diseases and injuries, however, have not been successfully cultured in Campenot chambers. These neurons are generally more difficult to culture and do not have the same dependency on neurotrophic factors for axonal growth as their peripheral counterparts. In addition to restricted use for PNS neurons, the Campenot chamber and its variations suffer from number of other limitations. For example, these chambers are extremely challenging to fabricate and assemble, have a tendency to leak, and have demonstrated restricted adaptability for sophisticated microscopy techniques.

To overcome the limitations described above, a novel microfluidic neuron culture device was developed[15]. The device, which truly exemplifies the evolution of the Campenot chambers through microfabrication technology, has two isolated compartments separated by a physical barrier with embedded microgrooves, permitting the passage of axons but not the cell bodies. We have successfully demonstrated long-term culture of CNS neurons without the use of neurotrophins and other targeting molecules[15,16]. In addition to compatibility with live-cell imaging techniques, these chambers have several other advantages including the ability to (a) isolate CNS axons without somas or dendrites and (b) apply localized physical and chemical treatments to CNS axons. Utilizing this technology, we have quantitatively investigated the effects of myelin inhibitors on regeneration of injured CNS axons.

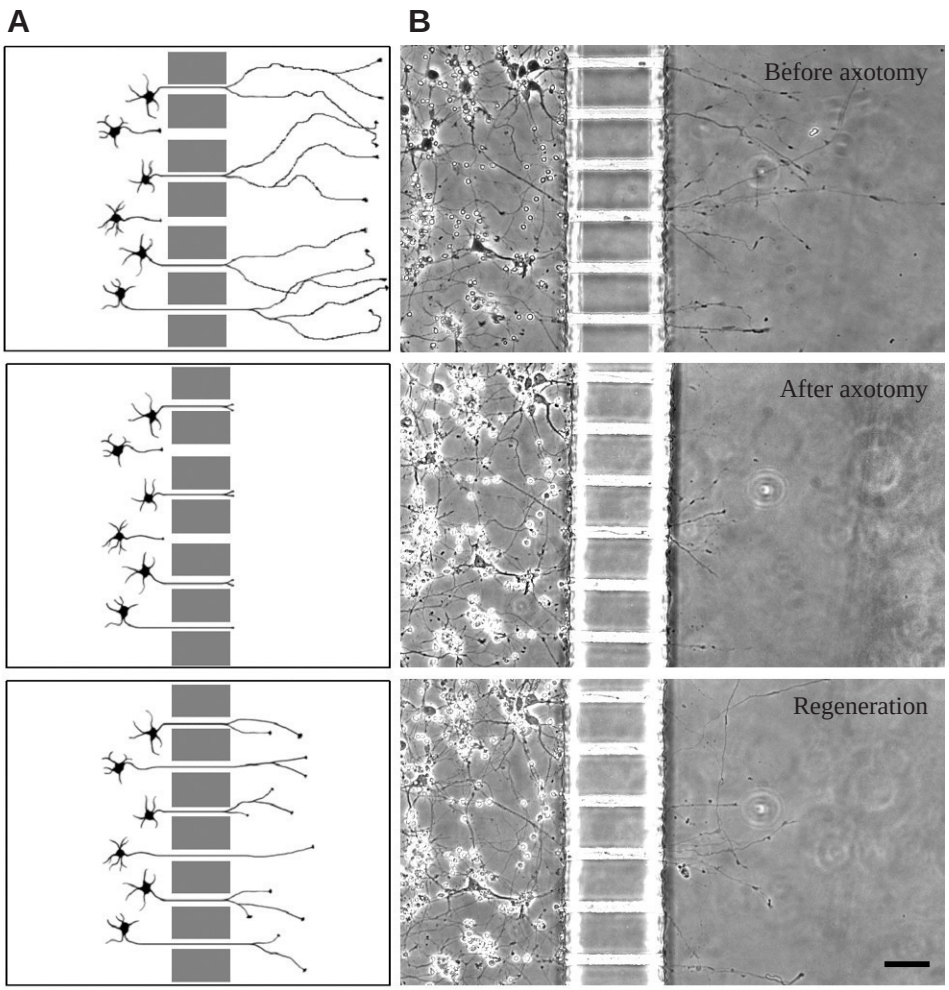

Figure 1. Schematic of the procedure for inducing axonal injury and monitoring regeneration. The neuron culture chamber has two compartments connected by microgrooves. Rat cortical neurons are added to the left-side of the reservoir and are drawn in the channel by capillary action. Axotomy by aspiration is performed from the right-side. (A) Schematic outline of the procedure. Neurons are cultured in the microfluidic devices (top). Isolated axons are cut by aspiration (middle). Injured axons are then incubated for 20-24 hr neurobasal medium (bottom). (B) Phase-contrast micrographs of the schematic outlined in (A). The scale bar is 100 μm.

NOGO-66 and MAG-Fc proteins were employed as model myelin inhibitors along with rat E18 cortical neurons to represent CNS. Neurons were cultured in the microfluidic devices for 7 days to ensure the passage of axons through the microgrooves and into the opposite compartment. To induce in vitro axonal injury, axons were removed from axonal compartments by vacuum aspiration. Owing to high fluidic resistance of the microgrooves, the aspiration did not disturb the cell bodies in the somal compartment. This was followed by treatment with NOGO-66 or MAG proteins. Both myelin inhibitory proteins reduced the length of regenerated axons by about 80% as compared to controls. Our results suggest that microfluidic devices for neuron culture can be used as an in vitro model for axonal injury and regeneration by simulating the microenvironment around the injury site, and can be used to statistically quantify the inhibitory effect of Nogo and other myelin inhibitors on axonal regeneration.

Results and Discussion

In vitro Model of CNS Axonal Injury and Regeneration

We have previously reported the development and characterization of a microfluidic-based neuron culture device that could be utilized as an in vitro model for CNS axonal injury and regeneration[16]. Figure 1 outlines the procedure, where rat E18 cortical neurons were seeded into the somal (left) compartment and cultured for 7 days. The isolation and the growth pattern of the axons through the microgrooves and into the axonal compartment (right) is further demonstrated by the phase-contrast micrograph (Figure 1B, top). Isolated axons were cut by vacuum aspiration from the axonal compartment (Figure 1, middle & Figure 2). Due to high fluidic resistance of the microgrooves, however, the cell bodies in the somal compartment were not disturbed. After injury, neurobasal medium was added to both compartments and the samples were incubated for 24 hr. Figure 1B (bottom) shows the regeneration of the injured axons into the axonal compartment after 1 day incubation.

Regeneration of Injured Axons

To monitor the regeneration of injured axons, time-lapse images were taken for 16 hrs post axotomy (Supplement movie 1). Figure 3 shows the re-growth patterns of the injured axons in the axonal compartment. In this configuration the truly re-generating axons (white arrows) can be distinguished from their un-injured cohorts (black arrows).

NOGO-66 and MAG Show Inhibitory Effects on Axonal Regeneration of Rat E18 Cortical Neurons

Before the effect of myelin inhibitors on axonal regeneration was investigated, the expression level of Nogo receptor in the cultivated rat E18 cortical neurons was examined. Cell lysates were prepared from cultured primary neurons after 4, 7, 11, and 14 days. After 7 days, expression of Nogo receptor was detected by Western analysis (Figure 4C).

To demonstrate the potential utility of the method and to investigate the inhibitory effect of myelin proteins on injured axons, soluble NOGO-66 and MAG-Fc proteins were used. It has been previously shown

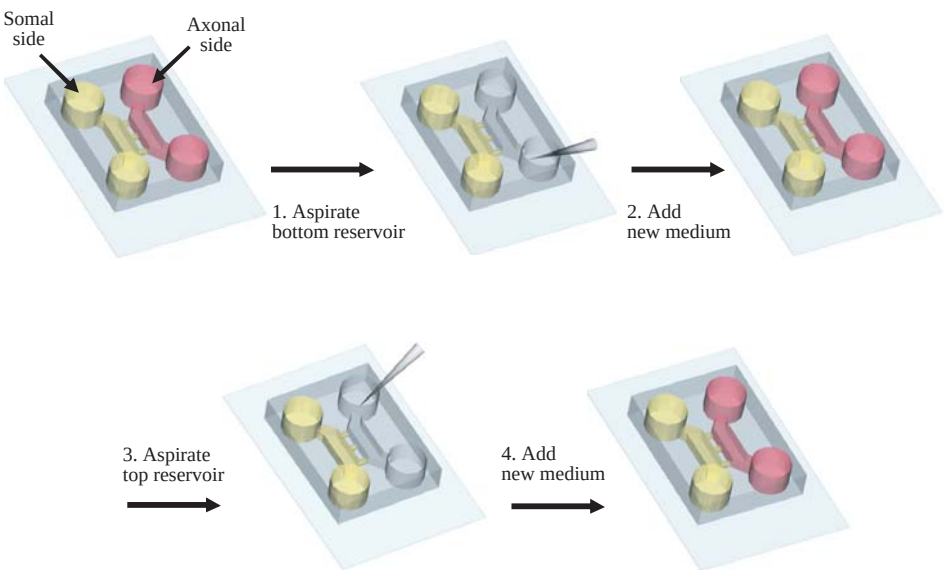

Figure 2. Schematic of axotomy procedure in the microfluidic device. The steps are described in the diagram. A glass pipette connected to vacuum is placed in the bottom reservoir leading to the axonal compartment (pink). Aspiration was applied for 5-10 seconds. Axons are severed when a bubble passes through the channel. Aspiration is continued until all media from the opposite reservoir is removed and the main channel is emptied. To increase the efficiency of the severing, the reservoirs are filled with fresh media and the aspiration is repeated from the top reservoir.

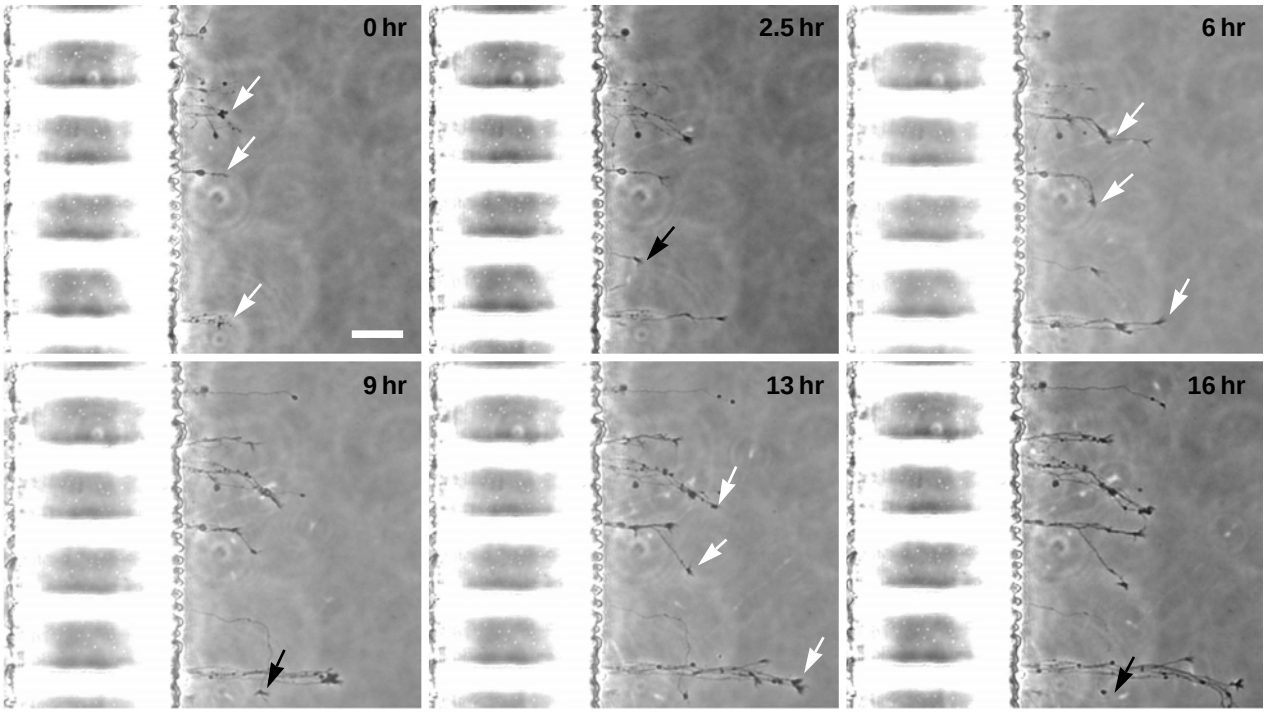

Figure 3. Time-lapse imaging of axons for 16 hrs after lesion. Time lapse microscopy revealed that the injured axons exhibit a dynamic behavior and were able to regenerate in normal neurobasal medium. After axotomy, normal medium without NOGO-66 was added to the axonal side immediately. Each image was taken at a time point indicated at the bottom of each figure, post axotomy. Note that re-generating axons (white arrowheads) can be distinguished from the following un-injured cohorts (black arrowhead). The scale bar is 50 μm.

that the Nogo gene encodes three isoforms (Nogo-A, B, C); all of which contain a 66-amino acid extracellular domain termed NOGO-66. Our rational for choosing NOGO-66 was based solely on the importance of this stretch of amino acid sequence in interacting with the cell surface Nogo receptor (NGR) and mediating its inhibitory effects[17]. MAG has also been shown to be a very potent inhibitor of axonal growth in vitro and is likely to play an important role in preventing regeneration immediately after injury[8,9]. It has further been shown that the three myelin associated inhibitors, Nogo, MAG, and OMgp, exert their inhibition by interacting with the same receptor (NGR)[18-20], highlighting a clearly defined target for overcoming myelin inhibition of CNS regeneration.

Axons were removed from the axonal side of microfluidic culture devices to mimic axonal injury. Following axotomy by vacuum aspiration, different concentrations of NOGO-66 were treated on both somal and axonal compartments for 24 hr. Figure 4A shows regenerated axons in normal culture medium and in presence of Nogo (100 nM NOGO-66 protein). Supported by previous reports, these results clearly indicate that NOGO inhibits CNS axonal regeneration. As illustrated by the phase-contrast images, even simple inspection of the devices indicated that in the presence of NOGO, the average length of regenerated axons was noticeably shorter than controls (Figure 4A).

To quantify the influence of NOGO on axonal regeneration, the length of regenerated axons were measured after 24 hrs of NOGO (1, 10, 100, 500, and 1,000 nM) treatment (Figure 4B). According to our results, NOGO concentration and the average length of regenerating axons were observed to be inversely proportional. Regenerated axon lengths were reduced to ~80% of control by the NOGO-66, and the inhibitory effect of the protein on axonal regeneration was saturated at 10 nM.

Following procedures described above, the inhibitory effects of myelin associated glycoprotein, MAG, was also quantitatively examined. The length of regenerated axons were measured after 24 hrs of MAG-Fc (250 nM) treatment (Figure 5A). Quantification analysis revealed that MAG diminished the average length of regenerated axons to ~75% of the control.

These results suggest that the device can serve as an in vitro model for axonal injury and regeneration

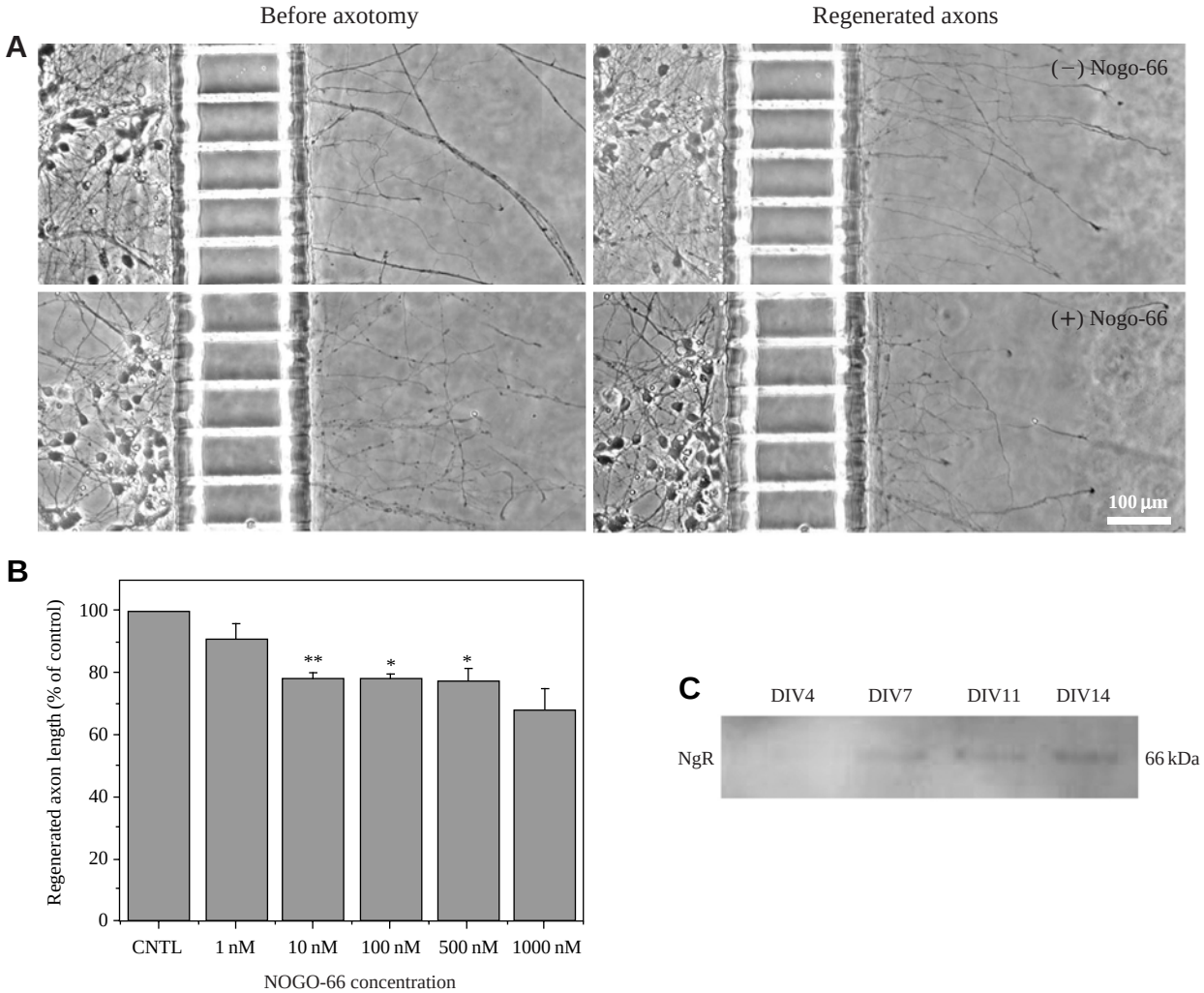

Figure 4. Regeneration in the presence of soluble inhibitor: effect of Nogo on axonal regeneration in the microfluidic device. (A) Phase micrographs of control and Nogo treated axons 24 hrs post axotomy. Nogo-66 was added to both axonal and somal chambers for 24 hrs before taking the micrograph. (B) Nogo treatment results in decreased length of regenerated axons (length normalized for each axon). The Inhibitory effect of NOGO-66 on axonal regeneration was saturated at above 100 nM of NOGO-66 (*$p < 0.05$; ** $p < 0.001$, student's t-test). Regenerated axon length was measured with NeuronJ software and averaged at least 70 axons per device (each device had ~40-100 distinguishable axons projecting). Three devices were used per concentration. The data was obtained by averaging results from at least 15 devices (pooled from 3 devices for each concentration per experiment, 5 separate experiments). (C) Analysis of protein expression level for Nogo receptor at different culturing days by Western blotting.

by simulating the microenvironment around the injury site and enable statistically significant experiment to be performed in shorter time. Furthermore, our results are meant to demonstrate the tremendous potential of this model in enhancing the development and screening of new therapeutic treatments for neutralizing the myelin inhibition in CNS trauma.

Conclusions

Known myelin inhibitors up to date were identified by examination of the uninjured CNS neurons. Recently, there is a new surge in inducible myelin-inhibitory properties reflecting pathophysiological changes after CNS injury. Lesion-reactive myelin axon-inhibition develops predominantly after injury, suggests a possibility of an entire novel class of axon-growth inhibitors and pathways[24]. The microfluidic device herein described could offer a suitable alternative for identification of these novel inhibitors and their activated pathways after CNS injury.

This paper describes the application of the microfluidic neuron culture device for the quantitative an-

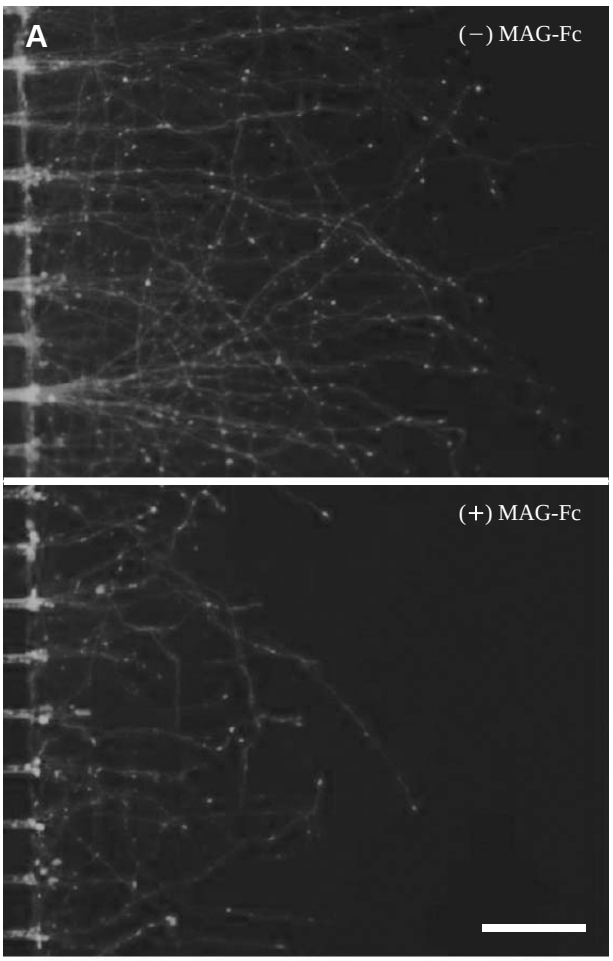

Figure 5. Quantitative analysis of regenerated axon length: effect of MAG on axonal regeneration in the microfluidic device. (A) Fluorescence micrographs of control and MAG treated axons 24 hrs post axotomy. Axons were stained with Calcein AM. MAG-Fc was added to both axonal and somal chambers for 24 hrs before taking the micrograph. (B) MAG treatment (250 nM) results in decreased length of regenerated axons (length normalized for each axon) by about 75% of control.

alysis of regeneration of injured CNS axons. The in vitro model herein described allows for a novel and reproducible method for inducing axonal injury without the need for complex mechanical or electrical devices. Using this in vitro model, we were able to quantitatively analyze the inhibitory effects of NOGO and MAG proteins on injured axons of cortical neurons. To perform this analysis, NOGO-66 and MAG-Fc proteins were employed as model myelin inhibitors along with rat E18 cortical neurons to represent CNS. Despite conflicting reports on responsiveness of embryonic neurons to myelin inhibitors[21], our results clearly indicate that these neurons (DIV7) are in fact sensitive to the inhibitory molecules associated with myelin. Our western blot analysis demonstrating the expression levels of the Nogo receptor (NGR) on these neurons further proves the point. The low expression levels of the receptor at this developmental stage (Figure 4C), however, might explain the less sensitivity of these neurons to myelin inhibitors compared to their postnatal counterparts[10,23]. Nevertheless, based on our findings and reproduced by others[22], we show that neurite outgrowth was reduced (~80% of the control cultures) by myelin associated inhibitors.

These results suggest that the device can serve as a suitable in vitro model for future research regarding axonal regeneration. By mimicking CNS axonal injury and simulating the microenvironment around the injury site, our results demonstrate the tremendous potential of this model in enhancing the development and screening of new therapeutic treatments for neutralizing myelin inhibition in CNS trauma.

Materials and Methods

Preparation of Microfluidic Culture Devices

The PDMS chambers were made using soft lithography and replica molding as described previously[15]. Briefly, photolithography was used to make two layers of negative photoresist on a silicon wafer, resulting in a master with positive relief patterns of cell culture compartments (1.5 mm wide, 7 mm long, 100 µm high) and microgrooves (10 µm wide, 3 µm high). A PDMS-prepolymer mixture was poured over the positive relief master to obtain a negative replica-molded piece. After curing, the PDMS was peeled away from the master. The reservoirs were punched with a sharpened needle, and then sterilized by autoclaving. The sterilized PDMS pieces were assembled with pre-cleaned glass coverslips immediately after oxygen plasma (Harrick Scientific, NY) treatment to form an irreversible seal[25]. We filled them with a ste-

rile aqueous solution of 0.5 mg/mL poly-L-lysine (PLL, M.W. 70,000-150,000, Sigma, MO) in borate buffer for overnight. Coated devices were washed twice with sterilized water for 4 hr to remove remaining poly-L-lysine solution. After washing, the devices were soaked with culture medium overnight.

Culture of Embryonic Rat Cortical Neurons in Microfluidic Devices

Cortical neurons were prepared from embryonic (E18) rat as described previously[15,26]. Briefly, cortexes of E18 rat embryos were dissected in CMF-HBSS [calcium-and magnesium-free Hank's balanced salt solution (HBSS) containing 1 mM pyruvate, 4.2 mM sodium bicarbonate, and 0.3% bovine serum albumin (BSA)], rinsed with CMF, and resuspended in a trypsin solution (0.125% trypsin in CMF-HBSS containing 0.5 mM EDTA) for 7 min at 37˚C or 25 min at ambient temperature. Trypsinization was stopped with Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal calf serum, the tissue was centrifuged at 1,000 rpm for 1 min, and the resulting cell pellet was resuspended in 2 mL of culture medium (Neurobasal medium (Invitrogen/Gibco 21103) containing 2% B27 supplement (Invitrogen/Giboco 17504), 0.25% GlutaMax (Invitrogen/Gibco 35050), and 1% penicillin-streptomycin (Invitrogen/Gibco 15070)). Following trituration through fire-polished Pasteur pipets, the cell suspension was filtered through a 40 μm cell strainer and viability was determined with trypan blue. Cells were plated in microfluidic devices at a density of about 3×10^6 cells/mL. 80,000 cells were seeded per device, while perhaps only 3,000 actually only end up in the device with the rest staying in the wells. The neurons were cultured for 7 days in neurobasal medium.

Western Blotting Analysis

To determine the expression of Nogo receptor in cultured cortical E18 cells, Western blotting analysis was performed. Cortical E18 neurons were cultured on several 60 mm culture dishes. The cells collected from plates at different days (4, 7, 11, and 14 days) and were to extract the proteins in lysis buffer (150 mM NaCl, 50 mM Tris-HCl (pH 8.8), 0.1% SDS, 2 mM EDTA, 1 mM PMSF, 1% NP40, 5 μg/mL aprotinin, 1 μg/mL leupeptin) at 4˚C on ice. The protein samples were separated by 12% SDS-PAGE and were transferred to PVDF membrane. The membrane was blocked with 10% fat-free milk at room temperature for 2 h and incubated with anti-Nogo receptor antibody (Santa Cruz, CA, 1 : 300 dilution) at 4˚C overnight. After three washes for 15 min in TBS supplemented with 0.1% Tween-20 (TBST), the membrane was incubated with the horseradish peroxidase-conjugated goat anti-rabbit IgG as a secondary antibody (Santa Cruz, USA, 1 : 2,000 dilution) for 2 h at room temperature respectively. Enhanced chemiluminescence (ECL-kit, Santa Cruz) was used for visualizing the Nogo receptor.

Axotomy

The axons were cut by vacuum suction applied through a Pasteur pipette to the axonal compartment, as described elsewhere[27]. Vacuum was applied to bottom reservoir of the axonal compartment to cut cultivated axons and then the axonal compartment was filled with new medium. In order to completely remove axons from the axonal compartment, vacuum was applied again from top reservoir. The medium and severed axons were removed from axonal compartment by twice suction, and new neurobasal medium was rapidly put into the axonal compartment. Due to high fluidic resistance of the microgrooves, the cell bodies in the somal compartment were not disturbed by vacuum suction.

Inhibition of Axon Regeneration by Myelin Inhibitors

In order to investigate the inhibitory effect of Myelin inhibitors on the regeneration of damaged axons, the neurons were maintained for 1 day after axotomy either in culture medium or in medium supplemented with NOGO-66 at concentrations of 1, 10, 100, 500, 1,000 nM. For MAG protein, 250 nM MAG-Fc was treated on the damaged axons. All experiments were performed with at least five independent cultures. NOGO-66 protein was kindly provided by Dr. Cocco. MAG-Fc was purchased from R & D systems (Minneapolis, MN).

Measurement of the Length of Regenerated Axons

For measurement of the regenerated axon length, phase-contrast images were taken before cutting and after regeneration using an inverted microscope, Nikon TE 300 (Nikon, NY), CoolSNAPcf CCD camera (Roper Scientific, AZ), and MetaMorph (Universal Iamging, PA). The length of regenerated axons was measured using NeuronJ software (http://rsb.info.nih,gov/ij/, 1997-2004). We traced from the end of microgroove region in the axonal compartment to regenerated axon terminals.

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