Available online at www.sciencedirect.com
ScienceDirect
Biomaterials 28 (2007) 3398–3407
Biomaterials
www.elsevier.com/locate/biomaterials

Regulation of axon guidance and extension by three-dimensional constraints

Herbert Francisco[a], Benjamin B. Yellen[b], Derek S. Halverson[c], Gary Friedman[c], Gianluca Gallo[a,*]

[a]*Department of Neurobiology and Anatomy, Drexel University College of Medicine, 2900 Queen Lane, Philadelphia, PA 2900, USA*
[b]*Mechanical Engineering and Materials Science, Duke University, Box 90300 Hudson Hall, Durham, NC 27708, USA*
[c]*Department of Electrical and Computing Engineering, Drexel University, 3141 Chestnut Street, Philadelphia, PA 19104-2875, USA*

Received 10 October 2006; accepted 11 April 2007
Available online 14 April 2007

Abstract

Axons *in vivo* are guided by molecular signals acting as attractants and repellents, and possibly by physical constraints encountered in the extracellular environment. We analyzed the ability of primary sensory axons to extend and undergo guidance in three-dimensional (3-D) environments generated using photolithography. Confinement of neurons in fully enclosed square chambers decreased the percentage of neurons establishing axons as a function of chamber width. However, the ability to extend an axon in one or more directions allowed axons to form and extend similarly to those on two-dimensional (2-D) substrata. Live imaging of growth cones interacting with the walls of chambers or corridors revealed that growth cones respond to contact with a 3-D constraint by decreasing surface area, and circumvent constraints by repeated sampling of the constraint until an unobstructed path is encountered. Analysis of the ability of axons to turn around corners in corridors revealed that the angle of the corner and corridor width determined the frequency of turning. Finally, we show that the length of axons can be controlled through the use of 3-D constraints. These data demonstrate that 3-D constraints can be used to guide axons, and control the extent of axon formation and the length of axons.

Keywords: Axon; Growth cone; Myosin; Filopodium; Microfluidics

1. Introduction

During development neuronal axons are guided to their target cells by molecular signals encountered in the *in vivo* environment [1]. As axons navigate they also encounter constraints imposed by physical aspects of the cellular environment *in vivo*. Axons must 'push' their way through all the surrounding cells and the extracellular matrix. The size of available spaces between constituents of the extracellular matrix, termed the matrix porosity, is a determinant of axon extension. For example, axons exhibit decreased extension as a function of increased matrix porosity [2,3]. In order to overcome these physical constraints to axon extension, the tip of the extending axon, the growth cone, secretes proteases that degrade the extracellular environment allowing the axon to extend through the extracellular matrix [4]. The function of growth cone secreted proteases in allowing axons to extend through gels of extracellular matrix was demonstrated by Nordstrom et al. [5] who found that the matrix metalloproteinase stromelysin-1 released by PC12 cells was required for axon formation in gels of Matrigel, a model system for the basal lamina. Thus, although it is known that growth cones have a potential mechanism for removing at least some physical constraints imposed by the extracellular environment, the responses of growth cones and axons to physical constraints are not understood.

In vitro, axons can be guided by microfabricated grooves [6,7] and steps [8]. These observations indicate that axons respond to physical aspects of the environment, and suggest a level at which axon guidance can be controlled *in vitro* independent of molecular guidance signals. Dorsal

*Corresponding author. Tel.: +1 215 991 8288; fax: +1 215 843 9082.
E-mail address: ggallo@drexelmed.edu (G. Gallo).

0142-9612/$ - see front matter © 2007 Elsevier Ltd. All rights reserved.
doi:10.1016/j.biomaterials.2007.04.015

root ganglion sensory neurons (dorsal root ganglion) provide a reliable and easy to culture primary neuronal system for the study of the regulation of axon extension. Furthermore, sensory axons in injured nerves are faced with the task of regenerating into, and being guided by, three-dimensional (3-D) tubes of Schwann cells [9], providing a rationale for investigating the interactions of sensory axons with 3-D physical constraints. In our work we investigated the ability of embryonic chicken primary sensory neurons to extend axons in a variety of environments characterized by 3-D physical constraints in the form of 'walls' perpendicular to the substratum the axons were growing on. We observed that 3-D constraints can regulate sensory axon formation, extension and guidance. Our observations suggest that growth cones have a mechanism for sensing the presence of a 3-D constraint and respond by redirection of their extension.

2. Methods and materials

2.1. Generation of 3-D substrata

Three-dimensional constraints were generated using standard photolithographic techniques. We used silicon wafers (Silicon Inc., Boise, ID) with a thickness of approximately 280 μm coated with a layer of the negative photoresist SU-8 10 (Microchem Inc., Newton, MA). Wafers were placed on a spin coating apparatus, SU-8 10 photoresist was applied to the surface and the wafers were spun at 1500 rpm for 40 s to evenly coat the silicon wafer with a layer of photoresist approximately 15 μm in thickness. Wafers were then pre-baked for 1.5 min on a hot plate at 65 °C and then moved to a separate hot plate set at 95 °C for 3 min. Following pre-baking, wafers were placed in a mask aligner, where they were brought into close contact with the electroplated metal mask. A template for the mask used in these experiments was created using the AutoCAD 2000 program, (AutoDesk Inc., San Rafel, CA). A file containing data for a mask to develop wells into SU-8 was sent to Sine Patterns LLC (Rochester, NY) where the electroplated metal mask was constructed. The mask and regions of the silicon wafer underneath were exposed to 150 mJ/cm^2 of UV light, calculated from Table 3 of the Microchem information document on SU-8 photoresist formulations 2–25 found on their website at the address: http://www.microchem.com/products/pdf/SU8_2-25.pdf. Wafers were then post-baked for 1 min at 65 °C and 2.5 min at 95 °C. Wafers were placed in SU-8 developer (Microchem Inc.) for 1 min to remove the SU-8 that had not been exposed. The finished wafers were placed in a deionized water bath for 2 min to rinse off the developing solution. Wafers were subsequently baked on a hot plate at 100 °C for approximately 18 h to remove any traces of volatile chemicals still present in the layer of SU-8. Photolithographic generation of patterns was similarly performed using German glass coverslips instead of silicon wafers for live imaging experiments.

The photolithographically patterned chips were coated with the cell growth promoting substratum laminin prior to plating cells. Each chip therefore contained varied arrays of chambers and corridors containing 3-D constraints in the forms of "walls", and a large "top" two-dimensional (2-D) surface without 3-D constraints. We created square chambers and corridors of different sizes. The soma of dorsal root ganglion neurons under our culturing conditions is approximately 20 μm in diameter. Thus, we investigated the effects of complete spatial 3-D confinement in square chambers ranging from 40 × 40–120 × 120 μm on the ability of neurons to establish axons. Similarly, to determine the effects of constraints in two out of four possible directions of axon advance, we studied the response of neurons to confinement in long (700 μm) corridors with widths ranging between 20 and 50 μm. The height of the walls of chambers and corridors was kept uniform at 15 μm. For all experiments, the neurons were cultured for 24 h prior to fixation, immunocytochemical staining and data acquisition.

2.2. Culturing and reagents

All neurons used in these experiments were obtained from the Dorsal Root Ganglia (DRGs) of embryonic day 10 chicken embryos. DRGs were dissected according to standard protocol [10] and placed in 5 mL of Ca^{2+}/Mg^{2+} free (CMF) Phosphate Buffered Saline (PBS) (Invitrogen, Carlsbad, CA) for 10 min at 37 °C to disrupt Ca^{2+} dependant cell adhesion mechanisms. After 10 min DRGs were spun at 3100 rpm for a few seconds to obtain a pellet. The CMF-PBS was aspirated off and 5 mL of 0.25% Trypsin-EDTA (Gibco Cell Culture Systems) was added. DRGs were incubated in trypsin for 10 min at 37 °C after which they were spun for 1 minute to collect the pellet, the trypsin was aspirated off, and 5 mL of Ham's F12 Medium (Mediatech Inc., Herndon, VA) with 10% Fetal Bovine Serum (BioWhittaker Inc., Walkersville, MD) and 10 mM HEPES (from Sigma) (F12HS10) was added. DRGs were then dissociated by pipette mediated fluid shear trituration. The dissociated cells were next pelleted after which the F12HS10 was aspirated off and Ham's F12 Medium with additives [10] containing 20 ng/mL of nerve growth factor (R&D Systems, Minneapolis, MN) was used to resuspend the dissociated cells prior to plating. Blebbistatin was purchased from Toronto Research Chemicals (North York, Ont., Canada) and dissolved in DMSO (25 mM stock). Culturing surfaces were coated overnight at 39 °C using 25 μg/mL laminin (Invitrogen, Carlsbad, CA) in PBS.

2.3. Immunocytochemistry

In order to determine neuronal morphology cultures were stained with anti-tubulin antibodies to clearly reveal axons. After 24 h of incubation, cell cultures were fixed with 0.25% gluteraldehyde for 15 min. The fix solution was then removed and 2 mg/mL sodium borohydride in CMF-PBS was applied for 15 min to quench residual fixative. The cultures were then blocked with 10% Goat Serum + 0.1% TX-100 in PBS and stained with fluorescein conjugated anti-α-tubulin (Sigma) at 1:100 dilution in 10% goat serum in PBS, for 1 h. Cultures were also counter stained with rhodamine phalloidin to reveal actin filaments, following the manufacturer's protocol (Sigma). To minimize photobleaching, coverslips were mounted in nofade and stored at −20 °C [10].

In order to determine the amount of laminin bound to SU-8 and silicon oxide substrata, the substrata were coated overnight with 25 μg/mL laminin in the same dish in order to minimize experimental variation. Control substrata were not coated with laminin but placed overnight in PBS, the vehicle for laminin. Substrata were fixed with 0.25% gluteraldehyde for 15 min and processed as described for tubulin staining. Substrata were stained with anti-laminin antibodies (1:50, Sigma) and then with rhodamine conjugated secondary antibodies. Images of the stained substrata were collected from all groups (20 fields, 20 × magnification). The mean intensity of staining within group (SU-8 or silicon oxide) was determined. In order to correct for differences in background due to intrinsic substratum autofluorescence and non-specific antibody staining, the mean of the no-laminin, but antibody stained, control substrata was subtracted from that of laminin coated substrata. The background-subtracted values were then compared using a Welch t-test across SU-8 and silicon oxide.

2.4. Live imaging

DRG neurons were dissected and dissociated as previously mentioned and cultured for 5 h on the surface of the photolithographically patterned coverslips. Neurons on patterned coverslips were cultured in Falcon 1008 dishes. Coverslips were imaged with a Zeiss Axiovert 135 M using a 20 × phase objective and 2 h videos were taken using Axiovision imaging software and a Zeiss Axiocam at 1 min intervals. An Air Stream Stage Incubator (Nevtek) was used to maintain cultures at 39 °C during imaging.