STUDY OF AXON-GUIDANCE INTERACTIONS IN CONTROLLED MICROFLUIDIC ENVIRONMENTS

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ABSTRACT

Developing axons are directed along specific pathways by the reiterative use of a relatively small number of environmental-chemical cues to create precise-wiring patterns. The increasing appreciation of the complexity of signaling interactions between these small number of cues emphasizes the need for tools that would allow guidance-factor-gradient delivery to growing axons in a controlled-combinatorial manner for deciphering their guidance code. We report the development of an open-chamber-microfluidic platform that permits mammalian-explant culture and provides an isolated environment for exposing axons, emerging from explants, to stable gradients. Our device substantially extends capabilities for chemical interactions with cultured axons enabling quantitative-neurobiological investigations.

KEYWORDS: Axon guidance, Neural development, Microfluidics, Visual system, Retinal ganglion cells

INTRODUCTION

Visual information is relayed from the eye to the brain by the axons of the retinal ganglion cells (RGCs). During development, these axons navigate unerringly to reach and form connections with their targets in the brain. Due to its relatively simple anatomy and topographic mapping pattern (between axonal projections from the retina and visual targets in the brain), the developing visual system has been studied extensively to identify the players involved in axon-pathfinding decisions. Several families of ligand-receptor signaling systems, highly conserved between sensory pathways and species, have emerged. These include the Ephrins-Ephs, netrins-DCC (deleted in colorectal carcinoma)/UNC5, Slits-Robos (roundabouts), and Semaphorins-Neuropilins/Plexins. Furthermore, secreted factors, such as Sonic hedgehog (Shh), fibroblast growth factors, bone morphogenetic proteins, and Wnts, have also been implicated as key regulators of this decision-making process [1].

Despite identification of many ligand-receptor families and the associated second-messenger signaling cascades, unraveling the mechanisms guiding developing RGC axons remains challenging. An emerging theme is that a small number of extracellular cues are used reiteratively along intermediate points of the optic pathway to help axons navigate their complex milieu. This is achieved by multiple identities espoused by an individual cue to modulate the axonal response. Chemical cues can be either attractive or repulsive, or both [2], and can act at variable distances, such that the same cue can serve as both a short-range and a long-range signal [3]. Guidance cues, more often than not, work in a combinatorial fashion, rather than in isolation, to precisely direct the trajectory of a growing axon. Redundancies are also put in place to help correct any errors that might occur. In summary, a key challenge to understanding RGC axon-guidance mechanisms lies in the unraveling of this complex multi-identity, combinatorial, co-operative, and redundant relationship that exists between extracellular cues, their receptors, and triggered signaling pathways. Further refinement of the guidance code used by RGC axons will advance our understanding of the developing visual system and offer insights into the general principles governing the precise wiring pattern of the nervous system.

This paper focuses on the development of microfluidic technologies for exposing developing RGC axons to controlled gradients of multiple guidance factors in an attempt to provide quantitative descriptions of RGC axon guidance mechanisms.

THEORY

The discovery of guidance cues followed by the increasing appreciation of the complexity of signaling interactions between these relatively small number of cues emphasizes the need for tools that would allow delivery of gradients of guidance factors to growing RGC axons in a controlled, combinatorial, and quantitative fashion for further deciphering their guidance code. In recent years, a number of researchers have exploited fluid-control capabilities of microfabricated systems to create stable concentration gradients within cell cultures on the micrometer scale [4-6]. In these approaches, laminar-flow streams, with sharp interfacial boundaries, can be targeted to specific subcellular regions [6] or multiple streams can be brought into confluence to create varying concentration profiles [4, 5]. These approaches have been widely used for studying polarized cellular events, such as cell migration [6, 7] and differentiation [8, 9].

Despite these advantages, the microfluidic gradient-generator approach suffers from important limitations. Gradients can only be generated under constant fluid-flow conditions, which induce shear and drag forces on cultures under study. These forces can lead to cell detachment, changes in intracellular signaling, and migrational biases. They can be particularly detrimental when studying neurons, which are more sensitive to such forces compared to many other cell types (such as neutrophils and endothelial cells). Secondly, downstream cells are exposed to higher concentrations of cell-secreted molecules compared to cells present upstream in the channel, creating differential contributions of secreted and gradient factors on cell responses depending on the placement of cells inside channels. Lastly, the closed microfluidic channels used in these approaches limits gas and pH equilibration, which are critical for cell viability, differentiation, and activity.

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This paper focuses on the development and use of microfluidic devices that overcome most of the limitations of existing gradient-generator technologies, allowing for quantitative neurobiological investigations. The neuron-friendly devices will be employed for unraveling the complex interplay between axon-guidance factors in the visual pathway using precisely controlled gradients of multiple guidance factors. Retinal explants, derived from embryonic mice, will be used to elucidate the molecular mechanisms directing formation of the anterior visual pathway. These experiments aim at advancing our understanding of RGC axon guidance by relating the function of guidance cues and receptors to formation of specific axonal pathways.

EXPERIMENTAL

We previously described a strategy for generating gradients under negligible-flow conditions [10]. Briefly, the device contains an open reservoir flanked by two fluid manifolds. Fluids are delivered to the reservoir through pressurization of two arrays of microchannels, termed microjets, present between the manifolds and the reservoir. By housing the chemical of interest in one of the manifolds and culture medium in the other, stable-surface gradients can be generated inside the reservoir within minutes with the bulk of the flow directed upwards and away from cells that are attached to the surface. This device has been used for studying netrin-induced axon turning in dissociated neurons [10]. However, the unpredictable nature of damage imparted by enzymatic dissociation procedures, commonly employed for separation of neurons from source tissues, and interference with the neuron's developmental clock called for modifications that permit culture of explants.

In the modified design, an additional chamber has been added for seeding retinal explants (derived from embryonic mice). Neurites, emerging from explants, are directed through narrow (5-µm wide x 2.5-µm high) microtunnels into the gradient chamber. This permits perfusion of the neurites without exposing their somas. The length of the microtunnels can be tuned to bar dendrites and non-RGC axons from entering the gradient chamber, which would allow selective perfusion of RGC axons with gradients of guidance factors (Figure 1a). An array of four devices on the same chip has been designed for cellular-response analysis under a variety of gradient and control conditions (Figure 1b).

Devices were fabricated using standard three-layer SU-8 photolithography and polydimethylsiloxane (PDMS) exclusion-molding processes, as described previously [10]. The PDMS devices were bonded to glass coverslips, hydrophilized using oxygen plasma, followed by sequential overnight incubations with 10 µg/mL poly-D-lysine and 6 µg/mL laminin to promote explant attachment and outgrowth. After protein incubation, substrates were rinsed with PBS, and freshly dissected embryonic (E14) mice retinal explants were added to explant chambers inside devices. Explants were maintained at 37 °C in a 5% CO_2 atmosphere.

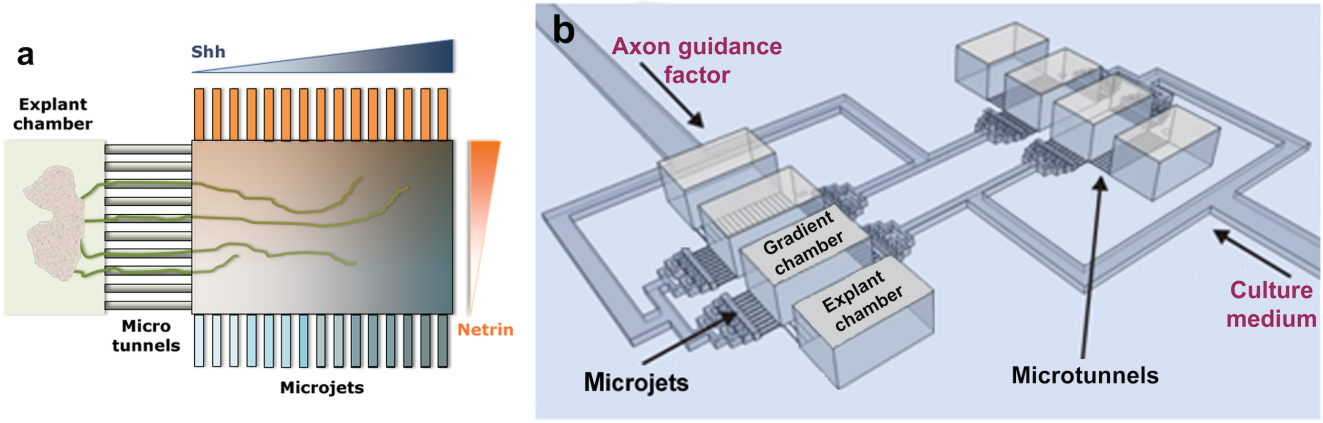

Figure 1: Microfluidic platform for studying RGC axon-guidance interactions. (a) Device schematic showing neurites (green) emanating from a retinal explant (pink), cultured in an open explant chamber, being directed through microtunnels into the gradient chamber. Microjets flanking the gradient chamber create stable concentration profiles of multiple guidance factors for selectively perfusing the neurites. (b) An array of four devices on the same chip allows for simultaneous analysis of cellular responses under gradient and control conditions.

RESULTS AND DISCUSSION

We have been able to maintain healthy explant cultures inside devices. Explant viability was assessed using ethidium homodimer and calcein AM staining, and explants could be maintained for several weeks in culture with minimal cell death. We have also observed elaborate outgrowth emanating from retinal explants and entering the gradient chamber through narrow microtunnels within 6 days after seeding the explants. Figure 2a shows extensive outgrowth around an E14 retinal explant that has been cultured for 6 DIV (days *in vitro*), with some of the neurites passing through 150-µm long tunnels to enter the gradient chamber. From immunostaining experiments (data not shown), we found that this length was sufficient to keep the dendrites and cell bodies from entering the gradient chamber. Figure 2b shows data from another E14 6 DIV explant where the outgrowth is aligned at microtunnel entrances. Figure 2c shows the neurites inside tunnels. Since RGCs are known to extend the longest axons among retinal cells, longer microtunnels will enhance the probability of allowing only

RGC axons to reach the gradient chamber. Presently, we are deploying 800-μm long microtunnels with the hope that this design will allow for studying axon turning in almost pure RGC-axonal populations. Lastly, the number of microtunnels can be tuned to reduce arborization and inter-axonal crosstalk. Design optimizations and gradient-application experiments with netrin-1 and Shh are currently underway.

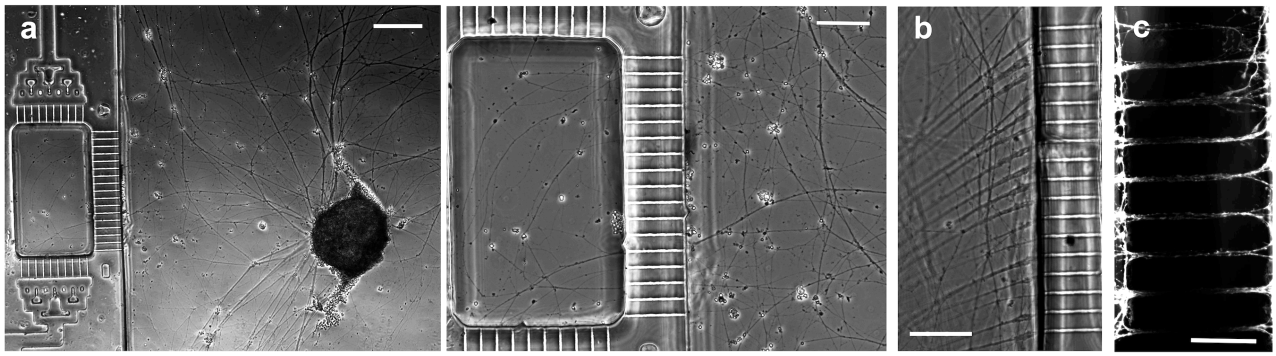

Figure 2: Retinal explant culture and RGC axon guidance inside devices (a) Left: Phase-contrast image showing a retinal explant with elaborate outgrowth. Outgrowing axons are directed through narrow microtunnels into the gradient chamber. Right: Higher-magnification image showing axons inside the gradient chamber. Majority of the axons were extended by RGCs. Identity of the axons was confirmed using immunostaining (data not shown). Scale bars, 200 μm and 100 μm, respectively (b) Phase-contrast image showing axons aligned at microtunnel entrances. Scale bar, 100 μm. (c) Immunostained neurites inside 5-μm-wide microtunnels labeled using an antibody against neuron-specific β-III tubulin. Scale bar, 50 μm.

CONCLUSION

These experiments aim to provide a quantitative description of the interplay and crosstalk between axon-pathfinding mechanisms, responsible for the precise navigation of axons in the anterior visual pathway, using sophisticated microfluidic platforms.

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