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Environmental Micropatterning for the Study of Spiral Ganglion Neurite Guidance

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Key Words
Spiral ganglion neurite guidance · Micropatterning · Neurite growth

Abstract
The projection of neuronal processes is guided by a variety of soluble and insoluble factors, which are sensed by a fiber's growth cone. It is the differential distribution of such guidance cues that determine the direction in which neurites grow. The growth cone senses these cues on a fine scale, using extensible filopodia that range from a few to tens of μm in length. In order to study the effects of guidance cues on spiral ganglion (SG) neurites, we have used methods for distributing both soluble and insoluble cues on a scale appropriate for sensing by growth filopodia. The scale of these methods are at the micro, rather than nano, level to match the sensing range of the growth cone. Microfluidics and transfected cells were used to spatially localize tropic factors within the fluid environment of extending neurites. Micropatterning was used to present neurites with stripes of insoluble factors. The results indicate that differentially distributed permissive, repulsive and stop signals can control the projection of SG neurites. Implications for future micropatterning studies, for SG development and for the growth of deafferented SG dendrites toward a cochlear implant are discussed.

Introduction

The development of organs is dependent upon the orderly growth of cells. In the nervous system, this includes not only the replication and migration of neurons and glia, but also the precise projection of dendrites and axons, upon which the function of the brain and peripheral nervous system are dependent. The formation of synaptic interconnections between neurons must be controlled with equal precision. The inner ear, with exquisite patterning of the sensory epithelium and precise projections of both afferent and efferent neurites into the organ of Corti, is a much-simplified example of the complex architecture that develops within the central nervous system. As such, it can serve as an experimental model with which to study the regulation of neural projections.

The guidance of neuronal processes has been the subject of intense investigation, and many mechanisms by which projections are controlled have been identified [e.g.

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Yamamoto et al., 2002]. These include soluble tropic factors such as growth factors, which are released into the extracellular fluid. Neurites can respond to gradients of these soluble factors with directional growth [e.g. Cao and Shoichet, 2003; Gillespie, 2003; Yoshikawa and Thomas, 2004]. Patterns of extracellular matrix (ECM) molecules such as laminin, fibronectin or collagen, which may be components of extracellular structures or be bound to their surfaces, can provide additional cues [e.g. Gillespie, 2003; Gallo and Letourneau, 2004; Hari et al., 2004; Oster et al., 2004; Yoshikawa and Thomas, 2004]. A wide variety of molecules that reside on the surfaces of cells, such as components of the ephrin/Eph bidirectional signaling system [Marquardt et al., 2005], can provide directional signals at the cellular and the subcellular level.

Many known neurite guidance molecules have been localized to regions or cells of the developing cochlea that are appropriate for a role in regulating neurite growth, providing the substrates for guidance of afferent and efferent projections. Neurotropic growth factors present in the developing organ of Corti include neutrotrophin-3 (NT-3), brain-derived neurotrophic factor and acidic fibroblast growth factors (FGF-1), all of which are produced by hair cells [Luo et al., 1993; Pirvola et al., 1992, 1994; Ernfors et al., 1994]. Many ECM molecules including fibronectin, laminin, tenascin and various collagens occur in the organ [e.g. Woolf et al., 1992; Tsuprun and Santi, 1999, 2001; Whitlon et al., 1999]. Several ephrins and Eph receptors have also been identified in the developing inner ear [Bianchi and Liu, 1999; van Heumen et al., 2000; Pickles et al., 2002].

Identifying the role played by these individual molecules in spiral ganglion (SG) neurite guidance has proven to be more difficult. Gene deletion studies have provided information, but can result in the death of neurons, e.g. tropic molecules that also provide trophic support [Ernfors et al., 1994; Farinas et al., 1994; Fritzsch et al., 1997a, 1998], or even the organism, e.g. many ECM molecules [George et al., 1993]. While in vitro techniques clearly have their limitations, they can be used to probe different developmental periods independently, in a way that gene deletion studies often cannot. Studies of neurons in culture have the additional advantage that the environment in which neurites are extending can be precisely controlled. In particular, they allow potential guidance factors to be presented in controlled patterns.

This is critical since, in order for a substance to provide directional information to advancing neurites, it must be differentially distributed. Variation in distribution can take many forms, from abrupt gradients in concentration that are essentially step-like at the dimension of the sampling growth cone, to gradual gradients that extend over many times the sensing radius of the neuron. For example, recent evidence indicates that gradients of soluble neurotropic factors can contribute to neurite guidance over distances of more than 10 mm [Cao and Shoichet, 2003]. Distribution of insoluble substances may be punctate, or can occur in patterns that relate to the underlying structure of the tissue. For example, in rodents, fibronectin appears to occur in tracts that run beneath the hair cells, during the first postnatal week [Woolf et al., 1992]. Cell surface molecules can be confined to individual cells or to cellular regions, or may be broadly distributed across tissues.

We have taken advantage of methods for micropatterning guidance molecules to directly assess SG neurite guidance in culture. Insoluble molecules have been patterned as edges, stripes and puncta. Soluble factors have been represented as discrete fluid gradients or localized cellular sources. Taken together, our studies suggest that SG neurites can respond directionally to differentially distributed guidance factors.

Methods

Culture of SG Neurons

Surgical procedures were approved by the local animal subject committee in accordance with the guidelines laid down by the NIH regarding the care and use of animals for experimental procedures. Three- to five-day-old (P3–P5) rat pups were decapitated and the skulls opened midsagitally under sterile conditions. The membranous labyrinth was exposed by peeling off the cartilaginous cochlear capsule under a dissecting microscope. The stria vascularis and the organ of Corti were removed in order to expose the spiral ganglia. The SG was excised from the entire length of the cochlea and divided into explants that were approximately 300 × 300 μm.

Culture procedures were based on those developed by Van De Water and Ruben [1971] and have been described in detail elsewhere [e.g. Brors et al., 2003]. Briefly, individual explants were cultured on surfaces coated with poly-*L*-lysine (PLL). Additional molecules were applied to this surface in various patterns as described below. The tissue was initially incubated in attachment media consisting of DMEM, 10% FCS, 5% HEPES and 30 units/ml penicillin for 24 h at 37°C, 5% CO_2. After 24 h, the culture medium was changed to maintenance media consisting of DMEM supplemented with 1 × N2 and 5 g/l glucose. Cultures were maintained for 72–96 h in maintenance media. Various soluble factors were included as described below.

Fixation and Immunohistochemistry

After culture in maintenance media, explants were fixed with 4% paraformaldehyde for 20 min and then washed with PBS. The samples were blocked with 1% donkey serum for 10 min at room temperature to reduce nonspecific binding. Specimens were incubated with rabbit polyclonal anti-200 kDa neurofilament antibody

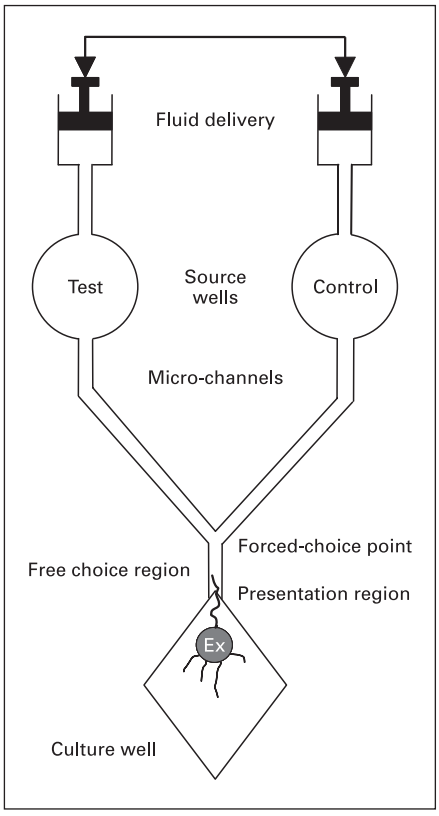

Fig. 1. Schematic diagram of a microfluidic device for creation of a fluid gradient and choice point for growing neurites. The device is generated by photolithography and molding. Test and control fluids are forced through a microchannel network at equal rates. They meet at the forced-choice point of a 'Y', and then flow in parallel through a free-choice presentation region to the tissue culture well.

(Sigma-Aldrich) diluted 1:500 at 4°C overnight. Explants were then incubated in donkey antirabbit secondary antibody (Jackson ImmunoResearch) diluted 1:100 in PBS. Either fluorescent or DAB labeling was used to visualize the secondary antibody. Immunolabeling controls in which rabbit serum was substituted for the primary antibody exhibited no labeling. Neurites were digitally imaged on a fluorescence inverted microscope (Olympus).

Microfabrication

Photolithographic techniques used to generate molds for microfluidic devices and for masks used in surface patterning have been described in detail elsewhere [Wittig et al., 2005]. Briefly, mold patterns were generated as negative images in Canvas 5.0 (Deneba Systems, Inc.) and printed as high-resolution transparencies with multiple patterns. The transparencies were used to expose the desired areas of positive photoresist (SU-8 100 or SU-8 1000, Microchem Corp.) spun into a uniform layer of the desired thickness on a 4-inch silicon wafer to ultraviolet light. When a second layer and transparency were required, they were aligned to the initial exposed pattern, which had become translucent.

After exposure, the photoresist was developed, and the mold master was cleaned, baked and dried. It was then attached to an aluminum plate prior to silicon wafer removal. The pattern was then molded in polydimethylsiloxane (Sylgard 184, Dow Corning) and cured to the desired level of elasticity.

Results

Soluble Guidance Cues

A microfluidic device for the evaluation of soluble guidance cues in fluid was developed by Wittig et al. [2005]. The device was fabricated and tested for production of fluid gradients with a fluorescent dye. A schematic of the device is illustrated in figure 1. Fluid is delivered at equal rates from two source wells by identical, physically ganged syringes. The resulting fluid gradients are illustrated in figure 2. As the two fluid streams unite at the union of the microchannel 'Y' a steep gradient is formed across the 100-μm width of the stem of the Y. Dye concentration transitions from >95% of peak concentration to <5% of peak concentration in 20–35 μm. This gradient is maintained until the fluid streams exit the narrow stem of the 'Y' into an expanding culture well, when the gradient becomes increasingly diffuse. Thus in an experiment using a biologically active diffusible mediator on one side of the device, neurites extending from an SG explant toward the microchannel encounter at first a diffuse and then, as they enter the microchannel, a sharp fluid gradient of the factor.

SG explants presented with a choice between NT-3 and control media showed a strong preference for growth in NT-3. This resulted in changes in neurite direction within the diffuse gradient at the exit from the 'Y', in preferential growth of neurites on the NT-3 side of the stem of the 'Y' and growth into the NT-3 side of the 'Y' bifurcation of a majority of neurites, as illustrated in figure 3. When NT-3 was presented from both delivery wells, no preference for side was observed (data not shown).

Insoluble Guidance Cues

A variety of strategies were employed to produce restricted distributions of substrate-bound guidance cues. Initially, silicone masks were fabricated with a single longitudinal gap. When fitted into a tissue culture well, the mask blocked all but a single stripe of culture surface, to which substances could be attached from solution by incubation. This method produced an abrupt edge of the attached guidance cue over ~20 μm at the edge of the stripe. SG explants could be placed outside of or on the

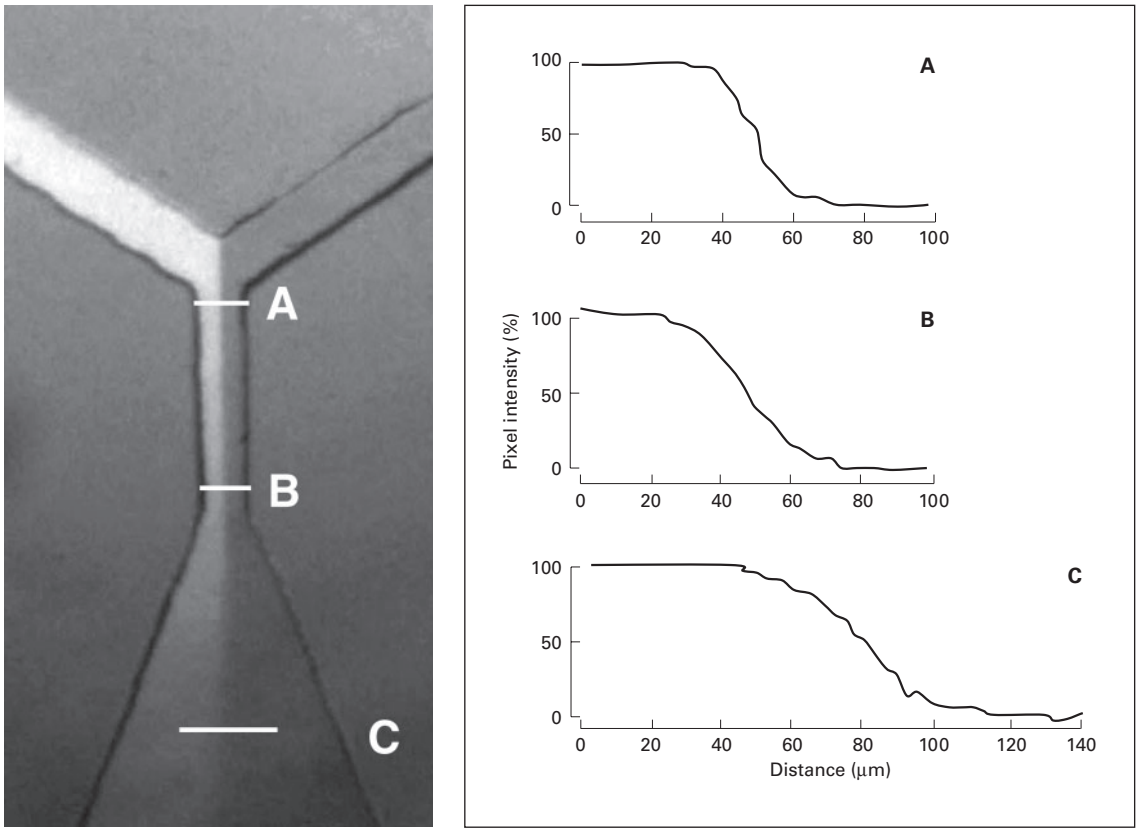

Fig. 2. Test of the fluid gradients produced by delivery of a fluorescent dye versus clear fluid to the forced-choice (**A**), free-choice (**B**) and presentation (**C**) regions of the device. A sharp gradient is created in the forced-choice and free-choice regions. A diffuse gradient is created when the microchannel exits into the presentation region, in both the horizontal and vertical dimensions. Adapted from Wittig et al. [2005].

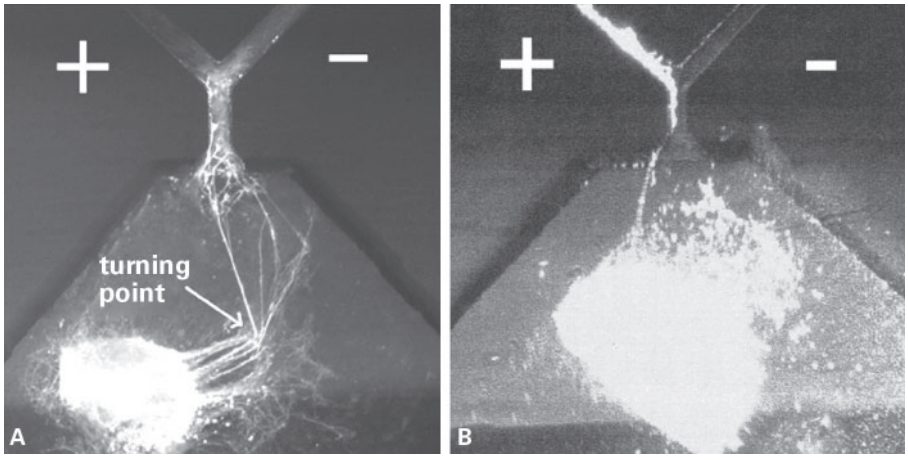

Fig. 3. Neonatal rat SG explants grown in the microchannel device for 72 h, with delivery of 10 ng/ml NT-3 in neuronal media (+) versus media alone (–). The explants were subsequently fixed and stained with antineurofilament antibody to visualize neurites. **A** A sharp inflection in neurite projection toward the microchannel can be observed. **B** Neurites and associated cells are preferentially observed on the side of the free-choice region that contains NT-3, and in the NT-3-containing microchannel beyond the forced-choice point. Adapted from Wittig et al. [2005].

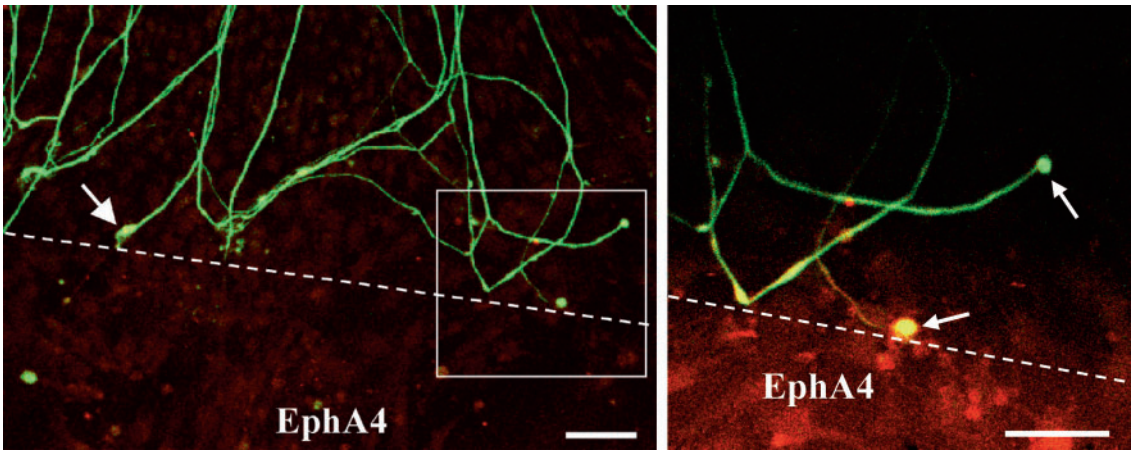

Fig. 4. Neonatal rat SG neurites (green) at the edge of a stripe of EphA4 (dashed line) exhibit growth cone collapse (arrows), termination of extension, and reversal of growth trajectory. From Brors et al. [2003].

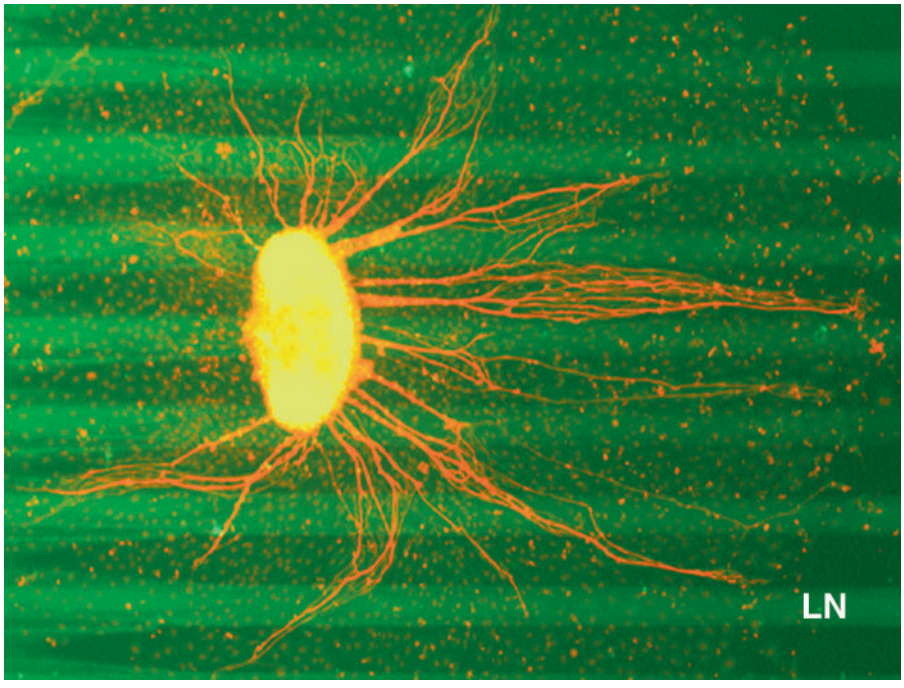

Fig. 5. Neurites from a neonatal SG explant (red), grown on a pattern of laminin stripes (green). The neurites demonstrate a strong preference to grow off of laminin. Adapted from Evans et al. [2005].

stripe, to evaluate their response to positive or negative edges, respectively.

This method was used to evaluate the responses of SG neurites to boundaries of the ECM molecule fibronectin [Aletsee et al., 2000], and the Eph receptor EphA4 [Brors et al., 2003]. Fibronectin boundaries proved to inhibit neurite growth, producing a strong tendency for neurites to stop and/or turn in response to a positive edge (data not shown). EphA4 was actively repulsive, invariably causing neurites to exhibit growth cone collapse and reversal of growth trajectory (fig. 4). No response to a neutral control protein (bovine serum albumin, BSA) was observed (data not shown).

To simulate linear tracts of ECM molecules in the organ of Corti, photolithographic microfabrication techniques were used to manufacture a mask with repeating

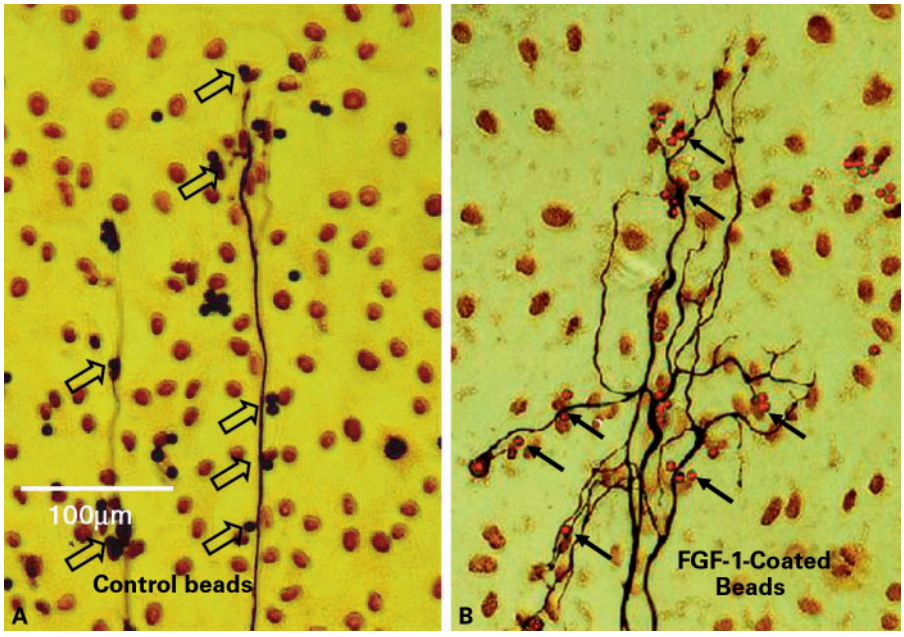

Fig. 6. Interaction of neurites from an SG explant with point sources of FGF-1. **A** The neurites cultured with control beads (open arrows) follow a simple trajectory. **B** The neurites encounter a cluster of FGF-1-coated beads (solid arrows). They exhibit extensive branching. Analysis of numerous such contacts revealed a significant increase in branching ($p < 0.01$). From Aletsee et al. [2003].

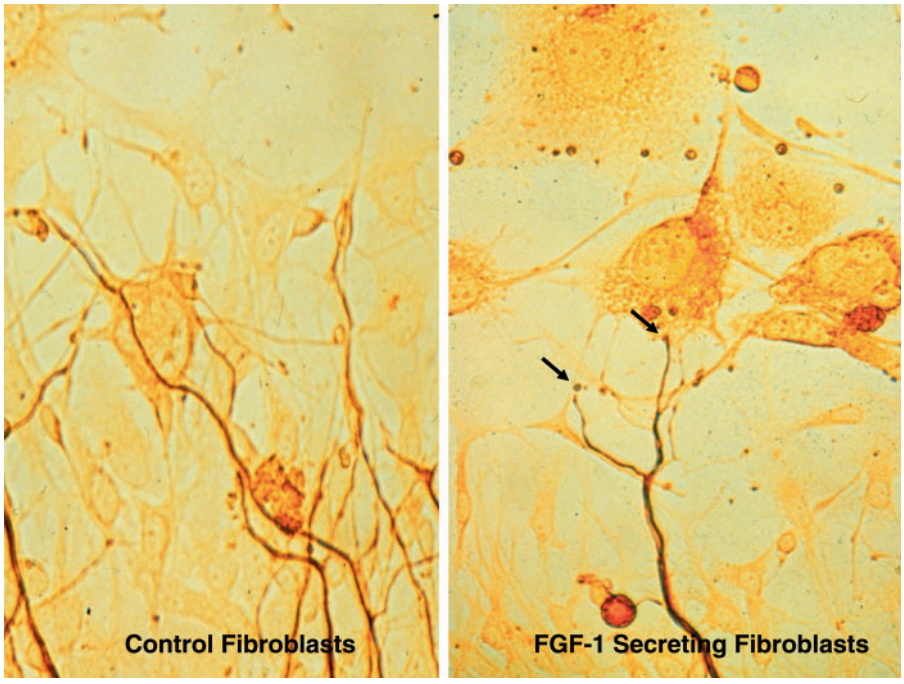

Fig. 7. Response of SG neurites to a cellular source of FGF-1. In the left panel, neurites grow over control mouse 3T3 fibroblasts. In the right panel, neurites encountering 3T3 cells that have been stably engineered to express human FGF-1 on the cell surface branch and form bouton-like terminations (arrows) on the cells. From Dazert et al. [1998].

microchannels that, after placement on a culture surface, could be filled with fluid containing an ECM molecule, for incubation [Evans et al., 2005]. After removal of the mask and application of PLL to coat the regions between the ECM stripes, this resulted in a pattern of narrow, repeating stripes of the cue.

When SG explants were grown on patterns of laminin, they showed a highly significant ($p < 0.001$), dose-dependent tendency for preferential growth. When stripes were produced by incubation with low concentrations of laminin, the neurites were preferentially found on laminin. At higher concentrations, the reverse was seen. In the example illustrated in figure 5, neurites exhibit a strong pref-

erence to grow off of stripes produced by incubation with 40 μg/ml of laminin. In the figure it can be noted that especially neurites approaching the laminin stripes at angles below about 45° tended to grow off of laminin onto PLL. In contrast, neurites preferred PLL to fibronectin at all concentrations, while control stripes of BSA had no effect (data not shown).

Cells that display guidance cues on their surfaces can act as punctate sources. For example, during the first postnatal week, rat hair cells produce high levels of FGF-1. Aletsee et al. [2003] modeled this phenomenon using 2- to 3-μm Sepharose microbeads coated with FGF-1, a growth factor that often binds to ECM molecules. SG neurites that encountered beads coated with BSA showed no response. Neurites that encountered FGF-1-coated beads exhibited extensive branching, although they did not terminate on the beads (fig. 6).

Dazert et al. [1998] used an alternative method to model FGF-1 production by hair cells. They co-cultured SG explants with 3T3 fibroblasts that were stably transfected to produce human FGF-1. The cells were placed peripheral to the explants, and were sufficiently different in morphology (larger and more spread out) from cells migrating out of the explants that they could easily be distinguished. In the construct used for transfection, the FGF-1 signal peptide was replaced with that for FGF-3, so that the FGF-1 remained bound to the cell surface rather than being secreted into the surrounding medium. While SG neurites showed a tendency to grow over control fibroblasts, they exhibited branching in response to FGF-1-producing cells. Moreover, they also formed bouton-like contacts on the cells (fig. 7).

Discussion

The results presented in this review demonstrate that patterning of neuronal guidance cues, on a microscale that is relevant to sampling by growth cone filopodia, provides a means by which to investigate SG neurite guidance in culture. Using a variety of methods it was possible to produce spatial patterns of cues, as experimental models of distributions that occur in the developing cochlea. In particular, patterning methods based upon photolithographic techniques allowed precise micropatterning. These methods were derived from many previous studies in other systems.

Stripe assays have a long history of use in the evaluation of neurite pathfinding. For example, Walter et al. [1987] created stripes using the cell membranes from homogenates of different target tissues. They employed a silicon mask with parallel channels similar to that used in the present study. The mask was placed to selectively block (in a striped pattern) the insertion of a membrane suspension into a capillary pore filter when suction was applied beneath the filter. Chick retinal ganglion explants were then cultured on this surface after the matrix was removed and a different membrane suspension was used to fill the empty stripes on the filter. More biochemically specific studies have used purified proteins as substrata. Employing methods similar to those of the present study, based on photolithographic techniques, Vielmetter and Stuermer [1989], Nguyen-Ba-Charvet et al. [2001], Weinl et al. [2003], and Jain et al. [2004] evaluated the responses of primarily retinal neurites to stripes of various substrates.

Our use of photolithography to generate microfluidic networks was also based upon a rich prior history. Methods for the rapid production of microfluidic systems using a positive relief microchannel structure on silicon molded with polydimethylsiloxane were outline by Duffy et al. [1998]. This technique formed the basis for a network used to create complex concentration gradients [Dertinger et al., 2001, 2002] with laminar fluid flow through microchannels. Previous microfluidic designs permanently bonded fluidic delivery tubes, microchannel layers, and surface layers such that each unit could be used only once [Duffy et al., 1998; Dertinger et al., 2001]. We used a compression plate to make each unit reusable across experiments. The compression plate incorporates a fluidic delivery system, simplifying connection of hydraulic pumps (syringe pumps) to the cell culture plate. An additional innovation was to apply the microchannel device to extending neurites.

Using micropatterning, we have found that neonatal SG neurites are sensitive to spatial patterns of a number of potential guidance cues. In particular, SG neurites appeared to display directional growth in response to both diffuse and sharp spatial differences in the concentration of NT-3. Diffuse differences appeared to induce turning, while a near-step difference in concentration produced a tendency for growth restricted to the NT-3 side. While these behaviors can be interpreted to reflect directional responses of SG neurites, an alternate possibility is preferential survival. It is possible that when neurites grew into media without NT-3, the associated neuron died, leaving only neurites in the NT-3 region. We feel that this explanation is less likely than directional behavior, since we and others have found that the requirement for NT-3 is not complete for postnatal P3–P5 neurites extending

from SG explants. That is, SG neurons in explants harvested at this age do not die obligatorily without neurotrophin support. Rather, many neurons in SG explants survive and project neurites in media without any neurotrophic factor present [e.g. Aletsee et al., 2001a].

SG neurites also responded to point sources of FGF-1 with branching and/or termination. They exhibited avoidance of ECM molecules, and could be guided over long distances by linear ECM patterns. Finally, SG neurites were actively repulsed by the Eph receptor EphA4.

Neurotrophic growth factors such as the neurotrophins or FGFs have been previously implicated in cochlear neuronal survival. For example, gene deletion studies have identified the importance of NT-3 for embryonic SG neuron survival [Farinas et al., 1994; Fritzsch et al., 1997a, b], and FGF treatment has been effective in rescuing dissociated SG neurons or increasing SG neurite numbers in culture [Lefebvre et al., 1992; Malgrange et al., 1996a; Aletsee et al., 2001a]. However, evidence for a guidance role of these factors has been less direct. Fritzsch et al. [1997a] concluded, based on innervation patterns detected in NT-3 null mice, that basalward turning of type II SG neurons within the organ of Corti occurs in response to an NT-3 gradient. Malgrange et al. [1996b] found that SG neurites are preferentially found on that side of a semipermeable membrane upon which NT-3 is present, although it should be noted that growth conditions on the two sides differed in other ways. Our data are consistent with these earlier results and conclusions. They provide more direct evidence that developing SG neurites will grow up a diffuse NT-3 fluid gradient, even in the absence of a physical barrier segregating the factor. Gradients may also influence SG neurons in other ways. Based on recordings from cultured SG neurons after neurotrophin treatment, Davis [2003] has speculated that neurotrophin gradients may help to shape the physiological properties of SG neurons at different cochlear locations.

Because FGF-1 null mice exhibit embryonic lethality, development of cochlear innervation in the absence of FGF-1 has not been evaluated. However, strong expression of FGF-1 by cochlear hair cells during the first postnatal week in rodents [Luo et al., 1993] occurs during a period of active synaptogenesis [Lenoir et al., 1980] and terminal innervation in the organ of Corti [Echteler, 1992; Echteler and Nofsinger, 2000]. This suggests that FGF-1 may play a role in innervation of the organ. Previous studies have indicated that FGFs in solution can support SG neuron survival and promote the growth of neurites. However, the response of SG neurites to point sources of FGF-1 suggests that this factor may also serve as a termination and branching cue.

Many neurons respond to ECM molecules via integrin receptors. We have previously shown that SG neurites are stimulated by uniform surfaces of ECM molecules such as laminin and fibronectin, and that this stimulation is mediated by integrins [Aletsee et al., 2001b; Kim et al., 2005]. However, ECM molecules are not uniformly distributed in the developing cochlea. In particular, the developing organ of Corti contains tracts of ECM matrix molecules that run beneath the hair cells [Woolf et al., 1992]. The data reviewed here suggest that depending upon the ECM molecule and amount, such tracts may attract growing neurites, or the boundaries of such tracts may serve to restrict the growth of neurites into certain regions of the organ, or to channel dendrites alongside or between adjacent tracts. Similarly EphA4, which occurs on the upper and lower elements of the osseous spiral lamina [van Heumen et al., 2000], may serve to funnel extending neurites within the lamina and toward the organ of Corti.

All of the observations reviewed here were obtained in vitro, and potential differences between the culture and the in vivo environments must be taken into consideration when interpreting the data. However, in vitro methods have allowed us to simplify the environment of extending SG neurites, and to control the distributions of possible guidance cues that they encounter. Microfabrication and cellular techniques have permitted us to produce gradients of guidance cues over distances much smaller than the sensing radius of the neural growth cone. We have also been able to study particular periods of development in isolation. In doing so, we have identified a number of signals that have the potential to influence the trajectory of developing SG neurites.

The data also have implications for the regrowth of deafferented SG neurites toward the electrodes of a cochlear implant (CI) [Brors et al., 2002]. The spatial resolution of CIs is low compared to the intact cochlea, which has many thousands of independent neural channels. It may be possible to increase the number of usable CI channels by attracting the neurites of surviving SG neurons toward the electrode array. If neurites could be directed to the electrodes in a controlled manner, allowing stimulation with very small electrical fields, it might be possible to increase the number of effectively separate channels dramatically, to several hundred. This could greatly increase CI frequency resolution. While there are distinct differences between neonatal and adult neurons [Koeberle and Bahr, 2004], lessons learned on developing neu-

rons provide a starting point for research on mature dendrites. The survival of deafferented adult SG neurons can be enhanced by exogenous neurotrophins [e.g. Staecker et al., 1996], suggesting that other responses may also be possible. Research on the guidance of adult SG neurites is currently underway.

Acknowledgements

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