

Polarization of hippocampal neurons with competitive surface stimuli: contact guidance cues are preferred over chemical ligands

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Neuronal behaviour is profoundly influenced by extracellular stimuli in many developmental and regeneration processes. Understanding neuron responses and integration of environmental signals could impact the design of successful therapies for neurodegenerative diseases and nerve injuries. Here, we have investigated the influence of localized extracellular cues on polarization (i.e. axon formation) of hippocampal neurons. Electron-beam lithography, microfabrication techniques and protein immobilization were used to create a unique system that provided simultaneous and independent chemical and physical cues to individual neurons. In particular, we analysed competitive responses between simultaneous stimulation with chemical ligands, including immobilized nerve growth factor and laminin, and contact guidance cues mediated by surface topography (i.e. microchannels). Contact guidance cues were preferred 70% of the time over chemical ligands by neurons extending axons, which suggests a stronger stimulation mechanism triggered by topography. This investigation contributes to the understanding of neuronal behaviour on artificial substrates, which is applicable to the creation of artificial environments for neural engineering applications.

Keywords: polarization; axon growth; neurons; contact guidance; nerve growth factor; laminin

1. INTRODUCTION

Neuron polarization is defined as the establishment of a single axon (i.e. axogenesis) and multiple dendrites (Goslin & Banker 1989). This particular neuronal morphology is critical for many cell processes, including connectivity with target cells and synapses (Wiggin *et al.* 2005). Understanding polarization and neuronal behaviour benefits both embryological development studies and regenerative strategies, including cell transplantation therapies for neurodegenerative diseases, such as Parkinson's and Alzheimer's diseases (Mattson 1999; Lasic & Barker 2003). For cell transplantation applications, the environment where stem cells are grown *in vitro* and transplanted into the injured brain is important, being essential to provide the most effective stimuli to control stem cell differentiation into neurons, cell migration and connectivity (Liu *et al.* 2003). In light of this need, studies on how extracellular cues can be designed in matrices to be implemented as 'artificial niches' that support cell therapy in the brain are essential (Liu *et al.* 2003).

Lutolf & Hubbell (2005) have emphasized the role of synthetic biomaterials as 'instructive extracellular microenvironments' that actively participate in determining cell fate in tissue engineering applications. Along these lines, materials that modulate neuronal behaviour, including polarization, will be an important contribution for the creation of effective regenerative therapies.

Another important application for understanding neuron polarization is the creation of artificial neural networks. In this particular field, neurons are geometrically confined into patterns that allow the formation of a well-defined neural architecture for the study of synapses and neuronal signalling (Stenger *et al.* 1998). The control over polarity and orientation of axons is essential for the understanding and reproducibility of the network characteristics. With this purpose, patterning of adhesive molecules has been performed to control polarization of neurons and axon orientation (Stenger *et al.* 1998; Vogt *et al.* 2004).

Although polarization has been extensively studied, the mechanisms by which intracellular and extracellular cues modulate this process are not well understood. Embryonic hippocampal neurons are the most

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characterized *in vitro* model for polarization (Dotti et al. 1988). Early work by Banker and colleagues showed that these cells undergo polarization during the first 24–48 h in culture (Dotti et al. 1988; Goslin & Banker 1989) following five stages: in *stage 1*, neurons are unpolarized; in *stage 2*, minor processes or neurites extend; in *stage 3*, cells form a single axon from the growing neurites (i.e. polarized neuron); in *stage 4*, dendrites grow and develop; and in *stage 5*, neurons are fully mature.

Neuronal polarity is determined by both a positive feedback loop at the growth cone of the future axon that enhances growth, and a negative feedback loop that propagates throughout the neuron to inhibit the other neurites from growing further (Anderson & Bi 2000; Arimura & Kaibuchi 2005). Some key molecules in the positive feedback loop are Cdc42, PIP₃, GSK-3 β and PAR-3 (Menager et al. 2004; Nishimura et al. 2004; Schwamborn & Puschel 2004; Jiang et al. 2005), which ultimately contribute to the increased actin polymerization and microtubule assembly that produces accelerated growth of the established axon. Polarization is modulated by extracellular stimuli (Fukata et al. 2002), including biochemical cues such as laminin (Lein et al. 1992), soluble and immobilized nerve growth factor (NGF; Brann et al. 1999; Gomez et al. 2007), and physical cues such as surface topography provided by pillars and microchannels (Dowell-Mesfin et al. 2004; Gomez et al. 2007).

An even more complex scenario is the ‘local’ stimulation of individual neurites prior to polarization. For example, laminin induces the formation of an axon when a single neurite of a stage 2 neuron comes in contact with this protein (Esch et al. 1999; Menager et al. 2004). In a similar way, tension exerted by a micropipette on an individual neurite induces axon formation (Lamoureux et al. 2002). These studies demonstrate that localized extracellular stimuli play key roles in polarization. Here, we have explored localized stimulation of neurites by presenting two simultaneous, *competitive* and confined cues to individual, premature hippocampal neurons during the first 48 h in culture, and analysed the formation of tau-1 positive axons with these cues. For these studies, immobilized NGF, laminin and surface microtopography in the form of microchannels in poly(dimethyl siloxane) (PDMS) were analysed. NGF and laminin are chemical stimuli mediated by receptor–ligand interactions, whereas topographical cues are physical signals mediated by multiple events related to cytoskeleton tension (Dalby 2005). Electron-beam (e-beam) lithography, soft lithography and protein photo-immobilization techniques were used for the fabrication of a unique system that presented these stimuli simultaneously but independently to neurons.

The local effect of the single cues was first analysed to find that all three stimuli (i.e. microchannels, immobilized NGF and laminin) predominantly direct the formation of an axon when the cues are individually presented to the cells. Neuron polarization was subsequently studied when topography and either NGF or laminin were simultaneously presented. We found that topographical features are preferred over chemical

ligands for axon formation (70% preference for topography), suggesting that contact guidance mechanisms exert a stronger effect on polarization. These results contribute to the understanding of neuronal behaviour on synthetic materials that could be potentially used in neural engineering and artificial networks, and demonstrates for the first time integration of simultaneous physical and chemical cues in cultured neurons.

2. EXPERIMENTAL PROCEDURES

2.1. Microfabrication of channels

Microchannels 1 and 2 μm wide and 400 nm deep were created on PDMS using soft lithography techniques. Briefly, the procedure had three steps: (i) fabrication of a mask with the desired patterns, (ii) fabrication of a silicon master, and (iii) replica moulding of PDMS.

The mask was created with e-beam lithography. Patterns were written on silicon wafers with a SiO₂ layer, which were spin-coated with 4% poly(methyl methacrylate) (PMMA) resist in chlorobenzene (Microchem). Microchannels of 1 or 2 μm were written on the PMMA resist using e-beam (Raith-50 and XL-30 Scanning electron microscopy (SEM), LaB₆ source) with an area dose of 220 $\mu\text{A cm}^{-2}$ and beam current of 0.2 nA.

After resist development, a thermal evaporator (Denton) was used to deposit a 100 nm chromium (R. D. Mathis) film and lift-off with acetone was performed subsequently. Reactive ion etching (RIE) of the SiO₂ layer was performed in an etcher (Plasma Technology) with a mixture of CHF₃ and oxygen. The final master was silanized with tridecafluoro-1,1,2,2-(tetrahydrooctyl)trichlorosilane (Gelest), and subsequently used for replica moulding of PDMS (Sylgard 184 kit, Dow Corning), by pouring a degassed mixture of prepolymer and curing agent from the kit (approx. 10 g/10 cm Petri dish), and polymerizing for 24 h at room temperature.

All PDMS substrates with microchannels were sonicated in 70% ethanol for 10 min, UV-sterilized for 1 h, and placed inside either sterile 3 or 10 cm (for cell micropositioning) Petri dishes. Substrates were coated by incubation with a solution of polyallylamine in water (7 mg ml⁻¹) overnight, and washed twice with distilled–deionized (DDI) water.

2.2. Fabrication of competition scheme

A competition scheme was created by immobilizing ligands next to the microchannels in PDMS (see figure 1a for schematic of the process). A separate PDMS well was fabricated by cutting a rectangle (8 \times 3 mm) in the centre of a PDMS film (13 \times 7 mm) with a razor blade, which was subsequently sterilized with ethanol and UV exposure. This well was placed on top of a PDMS substrate with microchannels, using fine tweezers and a light microscope (Olympus CK2) inside a horizontal hood to maintain sterility. The inside rectangular edge of the well was positioned 10–30 μm away from the edge of the microchannels, and pressed down to tightly seal the well on the substrate as a result of the high adhesiveness of the PDMS (step 1 in figure 1a).

After the PDMS well was positioned parallel to the microchannels, immobilization of NGF (step 2A in figure 1*a*), or laminin coating (step 2B), was performed exclusively inside the well. The NGF immobilization procedure is carefully explained in the following sections. Conventional laminin coating was performed by overnight incubation at 4°C with a 10 µg ml⁻¹ laminin (Trevigen) solution, followed by washing with DDI water. After either NGF immobilization or laminin coating, the PDMS well was removed from the substrate to obtain the competition scheme with microchannels exclusively on one side separated from an area with either immobilized NGF or laminin (step 3 in figure 1*a*).

2.3. Nerve growth factor–fluorescein isothiocyanate conjugation

NGF was conjugated to fluorescein for detection and characterization of the immobilization procedure (Gomez *et al.* 2007; Gomez & Schmidt *in press*). Briefly, 100 µl of NGF 2.5S (Promega, 100 µg ml⁻¹) was reacted with 10 µl of fluorescein isothiocyanate in dimethyl sulphoxide (FITC, 12 mg ml⁻¹; Molecular Probes), and separated with size exclusion chromatography columns (Biorad, exclusion limit 6000 Da). NGF–FITC was only used for quantification and visualization purposes, and not for cell culture because loss of activity was detected in PC12 cell neurite extension assays (results not shown). For determining NGF surface concentration on PDMS, fluorescence intensity of substrates was compared with a calibration curve obtained by casting known quantities of the fluorescent protein on defined areas without washing, exposing to UV light the same time as the experimental samples (for considering photobleaching effects), and calculating corresponding surface concentrations (ng mm⁻²) and fluorescence intensities.

2.4. Nerve growth factor immobilization on competitive geometry

NGF photochemical fixation was performed using a phenyl-azido group (Gomez *et al.* 2007). The procedure consisted of three main steps: (i) preparation of *N*-4-(azidobenzoyloxy)succinimide according to a previously published procedure (Matsuda & Sugawaram 1995), (ii) polyallylamine (PAA) conjugation to *N*-4-(azidobenzoyloxy)succinimide, and (iii) fixation of NGF using the modified polyallylamine. After the synthesis of *N*-4-(azidobenzoyloxy)succinimide, a solution of 15 mg of polyallylamine (Aldrich) in 10 ml of phosphate buffered solution (PBS, pH 7.4) was added to a solution of 13 mg of *N*-4-(azidobenzoyloxy)succinimide in 5 ml of *N,N*-dimethylformamide and stirred for 24 h at 4°C. The solution was ultrafiltered (Millipore, 10 000 Da) and washed three times by adding 10 ml of DDI water and ultrafiltered again to finally obtain a volume of approximately 300 µl of photosensitive polyallylamine (PAA-Azido). The conjugate was diluted in DDI water to obtain a final volume of 2.4 ml (1 : 8).

Fifty microlitres of PAA-azido solution were cast inside the positioned well on a PDMS substrate with microchannels, air dried and exposed with a UV lamp (Blak-Ray, 22 mW cm⁻², λ_{max} = 365 nm) for 15 s, followed by three washes with 0.01 M HCl and two washes with PBS. This step was followed by casting a second layer of the photosensitive polyallylamine (50 µl) and a superimposed final layer of NGF (for cell culture) or NGF–FITC (for quantification; 1 µg in 50 µl of PBS). Finally, the substrate was exposed to UV light for 15 s and washed six times with PBS to remove unreacted protein and two more times with DDI water. The superimposed well was then removed, the complete PDMS substrate washed two more times with DDI water and air-dried. Samples were stored at 4°C until used for cell studies.

2.5. Hippocampal cell culture and immunochemistry

Embryonic rat hippocampal cells (E18) were isolated from commercially obtained hippocampus tissue (BrainBits). The hippocampi were incubated with papain (Worthington) in Hibernate E medium (BrainBits; 4 mg ml⁻¹) at 30°C for 20 min, followed by physical trituration with a fire-polished Pasteur pipette. Cells were counted and plated randomly (7.5 × 10³ cells cm⁻²) or using a micropositioner on the prepared PDMS substrates with the competition scheme, and cultured with neurobasal medium (Invitrogen) supplemented with 2% B-27 (Invitrogen), L-glutamine (Fisher, 0.5 mM), L-glutamic acid (Sigma, 25 µM) and 1% antibiotic–antimycotic solution (Sigma, 10 000 units/ml of penicillin, 10 mg/ml of streptomycin and 25 µg/ml of amphotericin). After 44 h in culture at 37°C and 5% CO₂, cells were fixed with 4% paraformaldehyde (Sigma), 4% sucrose (Fisher) in PBS for 20 min, followed by permeabilization for 20 min with 0.1% Triton-X100 (Sigma) in 2% bovine serum albumin (BSA; Jackson Immuno-Research) in PBS (BSA–PBS), and blocking for 1 h at 37°C with 2% BSA–PBS. Samples were incubated with antibodies for tau-1 (axonal marker; Chemicon, 1 : 200), NGF (Abcam, 1 : 200) or laminin (Sigma, 1 : 200) in 2% BSA–PBS overnight at 4°C, followed by incubation with fluorescently labelled secondary antibodies (Alexa 488-conjugated, Molecular Probes and TRITC-conjugated, Sigma), for 1 h at 37°C.

2.6. Cell micropositioning

For single-stimulus experiments, hippocampal neurons were randomly placed on substrates containing the stimuli (figure 1*a*, step 4A), and only cells in the vicinity of the stimulus were analysed (see the following sections for criteria). For competitive experiments with two simultaneous cues, micropositioning techniques were used to place cells precisely between the stimuli (figure 1*a*, step 4B). For this process, thin micropipettes were obtained by pulling glass capillaries (World Precision Instruments, single-barrel standard borosilicate glass tubing, OD 1 mm, ID 0.58 mm) with a vertical pull type puller (Narishige). The pulled

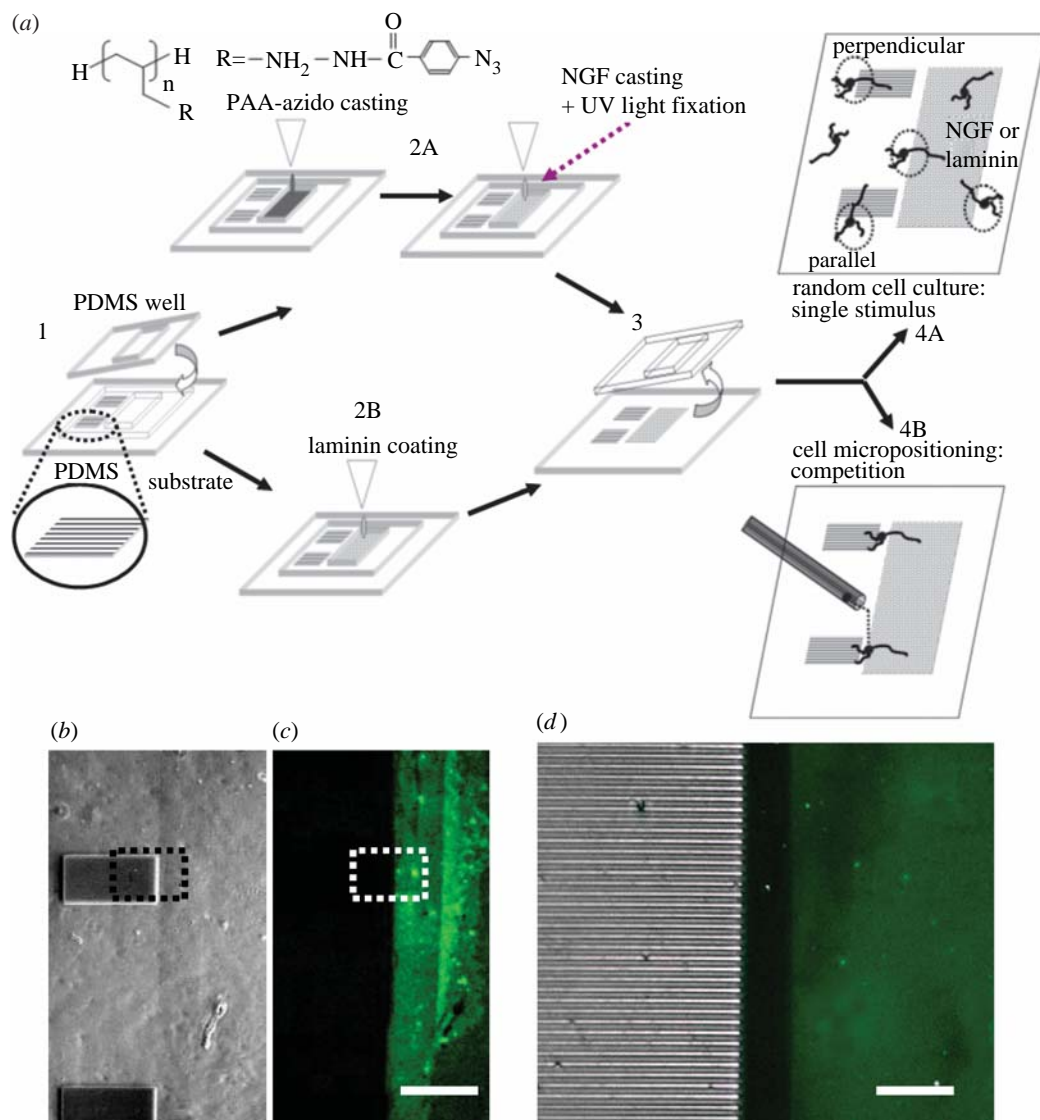


Figure 1. Fabrication and characterization of competitive scheme between physical and chemical cues for neuron polarization. (a) Microchannels are fabricated in PDMS using microlithographic techniques. After this, a PDMS well is aligned along the edge of microchannels under the microscope (1). NGF immobilization using aryl-azido photolinkers (2A) or laminin coating (2B) is performed inside the PDMS well. After extensive washing, the PDMS well is removed (3), which finally renders an area modified with chemical ligands that is parallel to microchannels. Hippocampal neurons are subsequently cultured on the modified PDMS substrates for competition studies (4). Random cell culture is used to test the effect of an individual stimulus on polarization (dotted ovals show cells analysed). Micropositioning is used when testing competition between two cues. Note: parallel and perpendicular are defined based on the position of the cell body relative to the long axis of microchannels as illustrated in the schematic; (b) and (c) phase-contrast and fluorescence images, respectively, of interface between microchannels and immobilized NGF-FITC; (d) higher magnification image (overlay of phase-contrast and fluorescence images) of the interface between microchannels and immobilized NGF (enclosed area in b and c). The two stimuli were designed to be simultaneous but spatially independent for studying competition in neuronal polarization. Scale bars, (b,c) 100 μm , (d) 25 μm .

micropipette was connected to a pneumatic microinjector (Narishigie Group) and tightened to an XYZ movable micromanipulator (Narishigie). This set-up was mounted on a reflectance upright microscope (Olympus BX51WI) located inside a horizontal laminar airflow workstation to guarantee sterility in the procedure.

PDMS substrates with competition cues were placed inside 10 cm sterile Petri dishes. After cell trituration, neurons in culture medium (2×10^5 cells ml^{-1}) were added on the periphery of the PDMS substrate and allowed to settle for 15 min. Before performing the micropositioning, additional medium was added to

the Petri dish to cover the PDMS surface. A single neuron was identified on the Petri dish (outside the PDMS), aspirated with the micropipette by creating suction with the injector, moved with the micromanipulator, and positioned between the cues on the PDMS substrate by releasing the cell. The same procedure was repeated for approximately six to eight cells on a single substrate in about 20–30 min, and four to six different samples were consecutively used for micropositioning per day. For each condition, experiments were performed on at least three different days. After micropositioning, Petri dishes were incubated at 37°C and 5% CO_2 .

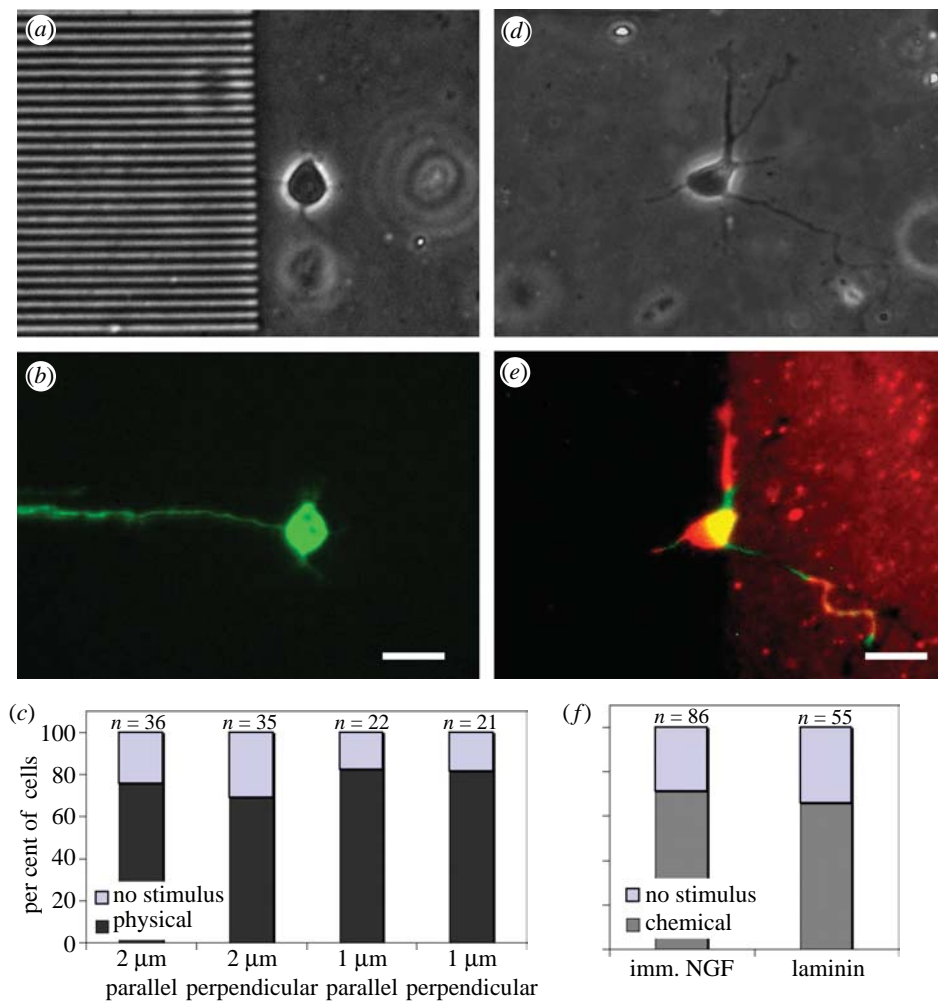


Figure 2. Effect of individual stimuli on neuron polarization. (a–c) Hippocampal neurons that were adjacent to the interface of microchannels were analysed for direction of axon formation. (a, b) Phase-contrast and fluorescence (tau-1-labelled) images, respectively, of a neuron next to 2 μm microchannels. Axon is established on the surface with topographical features. Scale bar, 25 μm; (c) quantitative analysis of polarization of neurons next to the microchannels. The *x*-axis represents the dimensions and the orientation of the microchannels (perpendicular and parallel definitions are illustrated in figure 1a). The *y*-axis represents the percentage of cells that establish axons on either the microchannels (physical) or smooth areas (no stimulus). The majority of neurons polarized towards the micropatterned area ($p=0.003, 0.02, 0.003$ and 0.008 for each dimension from left to right in the *x*-axis, respectively). (d–f) Hippocampal neurons that were adjacent to the interface of immobilized NGF or laminin were analysed for direction of axon formation. (d) Phase-contrast image; (e) overlay of tau-1 and NGF labelling images of a neuron next to immobilized NGF. Axon is established on the surface with immobilized NGF. Scale bar, 25 μm; (f) quantitative analysis of polarization of neurons next to chemical stimuli. The *x*-axis represents the type of ligand at the interface. The *y*-axis represents the percentage of cells that establish axons on either the chemical ligand or unmodified areas. The majority of neurons polarized towards the chemical stimulus ($p<0.001$ for immobilized NGF; $p=0.01$ for laminin).

2.7. Fluorescence microscopy

NGF–FITC immobilization, cell polarization and axon extension were analysed using an inverted phase-contrast and fluorescence microscope (IX-70, Olympus). Images from the microscope were acquired using a colour CCD video camera (Optronics Magna-Fire, model S60800) and analysed using ADOBE PHOTOSHOP and IMAGE J (NIH). For NGF immobilization analysis, fluorescence images of NGF–FITC-coated PDMS were captured and analysed for fluorescence intensity to determine surface concentration.

2.8. Polarization and competition analysis

Based on published criteria, a hippocampal cell was defined as polarized (stage 3) when one of its neurites

(i.e. the axon) was at least twice as long as the other neurites and it stained positively for tau-1 (Dotti *et al.* 1988; Menager *et al.* 2004; Nishimura *et al.* 2004; Schwamborn & Puschel 2004). Only cells that were already polarized after 44 h in culture were analysed for competition. For single stimulus assays (figure 1a, step 4A), a single neuron next to either parallel microchannels (i.e. cell body is adjacent to the long axis of the microchannels), perpendicular microchannels (i.e. cell body is adjacent to the short axis of the microchannels) or chemical ligands was analysed when it was within one cell body from the stimulus, and when it was separated from other neighbour neurons by at least three cell bodies in distance. At least 20 cells were analysed per stimulus. For competition analysis (figure 1a, step 4B), only micropositioned cells that

were within one cell body distance from both cues were analysed. A neuron was defined as polarized towards a specific side when a single axon extended on one of the presented stimuli (i.e. on top of microchannels, NGF or laminin) after 48 h in culture (premature axon formation was only analysed, not dendrite development). A total of 10–20 micropositioned cells were analysed per competition condition, which is consistent with other previous studies on responses of individual neurons or growth cones (Bradke & Dotti 1999; Song *et al.* 1997).

2.9. Statistical analysis

Distributions of axons for all conditions were tested against a 50% equal probability distribution using a χ^2 -test, to demonstrate that cells were preferentially choosing one of the two possible stimuli. p -values were calculated with the χ^2 distribution and statistical significance was determined for $p < 0.05$. In addition, distributions of axons in competition geometries between physical and chemical cues were tested against the two distributions of the individual stimuli using a χ^2 -test, to show a significant difference in axon formation.

2.10. Scanning electron microscopy

Microchannels and cells on PDMS were analysed with a LEO 1530 scanning electron microscope. For imaging neurons, cells on PDMS substrates were fixed with 4% paraformaldehyde (Aldrich) and 4% sucrose in PBS for 20 min, and dehydrated with increasing concentration of ethanol (30–100%) for a total time of 2 h, followed by 5 min exposure to hexamethyl-disilazane (Sigma). After drying, samples were coated with a gold layer for SEM measurement and imaged with a typical acceleration voltage of 1 kV.

3. RESULTS AND DISCUSSION

3.1. Fabrication and characterization of competitive scheme

We investigated how polarization is regulated when both cues are presented to neurons simultaneously and independently. To study this, a novel competition scheme that consisted of an area with microchannels separated from an area with either immobilized NGF or laminin was created (figure 1). After the fabrication of microchannels on PDMS using e-beam lithography and microfabrication techniques, a PDMS well was positioned next to the microchannels area. Next, NGF was immobilized on the area enclosed by the superimposed well as previously reported (Gomez *et al.* 2007), using aryl-azido conjugates and UV exposure (figure 1*a*, step 2*A*). Similarly, laminin was adsorbed for different competition experiments with a concentration already reported to affect polarization (Lein *et al.* 1992) (figure 1*a*, step 2*B*).

Figure 1*b* shows the results for the creation of the competition scheme. NGF was fluorescently labelled with FITC for visualization of the area covered by the tethered protein. NGF-FITC was exclusively

immobilized in an area adjacent to the microchannels and separated by a distance of approximately 20 μm , as illustrated in figure 1*d*. The surface concentration of immobilized NGF was 0.11 ng mm⁻², which is the concentration of immobilized growth factor that yields the largest increase in the number of stage 3 neurons (Gomez *et al.* 2007). This concentration was determined by comparing the average fluorescence intensity of immobilized NGF-FITC with a calibration curve of known surface concentrations and corresponding fluorescence intensities. Laminin was adsorbed to create similar competitive schemes, as illustrated in figure 3.

3.2. Neuron polarization at interfaces with individual stimuli

The first step in the process of testing competition responses in cells was to analyse if the individual stimuli could control the direction of polarization. For this analysis, neurons located on the edge of the particular cue, either next to the microchannels or next to the interface of the immobilized NGF or laminin areas, were analysed (figure 2).

3.2.1. Physical guidance cues. Microchannels of 1 and 2 μm in width and 400 nm in depth were fabricated for these studies, based on previous data showing that these dimensions significantly increase the number of stage 3 cells (Gomez *et al.* 2007). Figure 2*a–c* summarizes the data for the effect of topographical features on polarization. Both 1 and 2 μm microchannels effectively influenced axon formation, both in parallel and perpendicular orientations, as illustrated in figure 1*a* (in comparison to a 50% equal distribution with a χ^2 -test). Neurons preferentially defined an axon towards the micropatterned area rather than on the smooth (i.e. no microchannels) area. This is the first time contact guidance cues are found to influence the direction of axogenesis at interfaces between patterned and unpatterned areas. From the quantitative data, 1 μm features have a larger effect with 80% of the cells polarizing on the topography side (parallel microchannels: $n=22$, $\chi^2=8.9$, $p=0.003$; perpendicular microchannels: $n=21$, $\chi^2=6.9$, $p=0.008$). Microchannels of 2 μm predominantly induce 65–75% of the cells to define an axon on the micropatterned area (parallel microchannels: $n=36$, $\chi^2=9$, $p=0.003$; perpendicular microchannels: $n=35$, $\chi^2=5.6$, $p=0.02$). Overall, 75% of neurons preferred to polarize on micropatterns ($n=114$, $\chi^2=29.5$, $p < 0.001$).

The only previously reported effect of localized physical cues on polarization involved the use of micropipettes to create tension by pulling single neurites in stage 2 neurons (Lamoureux *et al.* 2002). This investigation showed that an individual neurite of a stage 2 neuron could be towed and induced to become the axon. The authors proposed that tension in the growth cone induces axon definition, which is related to increased traction and growth. Although this type of stimulation is quite different from the physical cues provided by surface topography, both stimuli are

related to some degree to tension and cytoskeleton distortion. Micropipette aspiration drastically creates tension at the growth cone that can be directly related to cytoskeleton dynamics, whereas topographical surface features create a more subtle tension at the cell surface that can also be transferred to the cytoskeleton.

Topography is a cell stimulus that is believed to be intracellularly transduced by tension in the cytoskeleton and the cell membrane (Dalby 2005). Tension created by surface topographical features is associated with the alteration of nuclear morphology and the upregulation of multiple genes (Dalby et al. 2003). In addition, topography effects are also associated with the opening of calcium channels (Rajnicek & McCaig 1997), which could initiate multiple intracellular pathways (Mattson 1999), the redistribution of focal adhesion complexes and integrins, and the activation of tyrosine kinases (Curtis & Wilkinson 1997; Dalby 2005). Integrins in focal adhesion complexes are especially important for mechanotransduction, as these receptors transmit physical stresses to the cytoskeleton via key molecules such as talin, vinculin and tensin, which bind to actin filaments (Dalby 2005). In particular, vinculin recruitment requires tension at the focal adhesion (Galbraith et al. 2002), which illustrates the relevance of tension in transducing signals from integrins to the cytoskeleton. Similarly, topography could also distort the cell soma structure creating bulkier regions, which have been associated with neurite initiation (Yingee et al. 2003). Based on this evidence, we hypothesize that topographical features could locally stimulate positive feedback loops for growth by exerting cytoskeleton tension. In particular, actin dynamics are definitive in axon formation (Bradke & Dotti 1999). Therefore, the effect of topographical features on actin polarization via tension at focal adhesion complexes in a single neurite could be directly involved in initiating the formation of the axon.

3.2.2. Chemical guidance cues. Figure 2*d,e* illustrates the data for the effect of chemical ligands, both immobilized NGF and laminin. The interface of the modified areas was only visible with fluorescence microscopy after immunostaining. Similar to the physical cues, the chemical cues primarily induced the formation of axons on the modified areas containing the protein. It was found that 71 and 65% of neurons preferred to extend axons on immobilized NGF and laminin, respectively, compared with unmodified surfaces (immobilized NGF: $n=86$, $\chi^2=15.1$, $p<0.001$; laminin: $n=55$, $\chi^2=5.9$, $p=0.01$). Overall, 69% of neurons preferred to polarize on chemical cues ($n=141$, $\chi^2=20.7$, $p<0.001$).

Laminin is known to locally induce axogenesis (Esch et al. 1999; Menager et al. 2004). Esch et al. (1999) fabricated substrates with stripes of either laminin or the neuron-glia cell adhesion molecule (NgCAM). The authors found that neurites in contact with either of these molecules became axons, with 80% of axons being formed on laminin stripes. In this same study, increased growth of the neurite in contact with laminin was

shown to occur soon after the first contact with laminin, inducing the formation of the axon and simultaneously decreasing growth of the other neurites. More recently, accumulation of PIP₃ in the neurite in contact with laminin was demonstrated (Menager et al. 2004). PI 3 kinase and PIP₃ activate the GTPases Rac and Cdc42, which are part of the positive feedback in polarization (Schwamborn & Puschel 2004). These data are consistent with our results showing that laminin influences the direction of polarization.

In addition to laminin, we found that immobilized NGF was active (Gomez et al. 2007) and directed the formation of axons. NGF signalling in hippocampal neurons is primarily mediated by the p75^{NTR} receptor and ceramide formation (Brann et al. 1999, 2002). We have previously validated similar responses of hippocampal neurons to both soluble and immobilized NGF (Gomez et al. 2007). In addition, effects of NGF and TrkA signalling in polarization have also been investigated (Da Silva et al. 2005). In particular, the plasma membrane ganglioside sialidase (PMES) segregation in the developing axon has been correlated to phosphorylated TrkA receptors and NGF binding. In light of this, immobilized NGF could locally activate TrkA and p75^{NTR} receptors and initiate cascades inducing the segregation of molecules for polarization.

3.3. Neuron polarization with competitive physical and chemical stimuli

After analysing the effects of individual stimuli on polarization, competition between chemical and physical cues was investigated. We studied three different competition combinations with perpendicular microchannels, as shown in figure 3. We found neurites in contact with physical guidance cues preferentially transformed into tau-1 positive axons with respect to neurites in contact with chemical guidance cues. One micrometre microchannels induced 74% of axons to grow on the micropatterned area when simultaneously presented with immobilized NGF ($n=18$, $\chi^2=5.55$, $p=0.02$). Two micrometre microchannels induced 64% ($n=22$, $\chi^2=1.63$, $p=0.2$, not statistically different from a 50% equal distribution) and 69% ($n=29$, $\chi^2=5$, $p=0.03$) of axons to grow on the micropatterned area when simultaneously presented with immobilized NGF and laminin, respectively. Overall, physical cues were preferred (70%) over chemical cues for axon formation ($n=69$, $\chi^2=11.4$, $p<0.001$). It is important to state that the interesting results observed here can only be analysed in the context of the experiments performed in this investigation, but other types of ligands or concentrations of ligands might exhibit different responses.

Axon distributions on microchannels in the presence of competing chemical cues were statistically different from the distributions for the individual stimuli using a χ^2 -test ($p<0.001$ for all three combinations when compared with individual microchannels or chemical ligands). These results suggest that integration of multiple independent extracellular signals in the neuron is possible, which ultimately determines the decision for polarization. Interestingly, physical cues were preferred

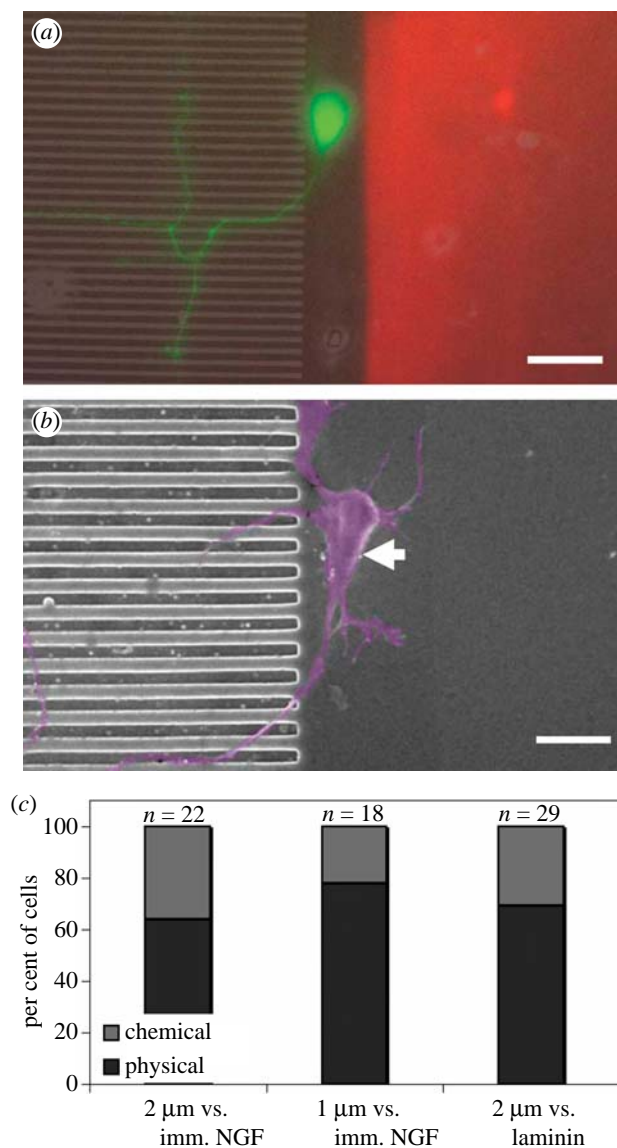


Figure 3. Competition between chemical and physical stimuli on neuron polarization. Hippocampal neurons were micro-positioned between immobilized NGF or laminin and microchannels, allowed to extend multiple premature neurites in all directions and then analysed for the orientation and formation of a single axon (tau-1 positive labelling) after 44 h in culture. (a) Overlay of phase-contrast and fluorescence (green labelling for tau-1, red labelling for laminin) images of neuron between 2 μm microchannels and laminin. Axon was established on the surface with microchannels. Scale bar, 25 μm ; (b) SEM image of neuron in (a) (arrowhead; pseudo-coloured for visualization). Scale bar, 12 μm ; (c) quantitative analysis of polarization of neurons between chemical and physical stimuli. The x -axis represents the specific competition pair. The y -axis represents the percentage of cells that establish axons on either the chemical or physical stimuli. The majority of neurons polarized towards the physical stimulus ($p=0.2$, 0.02 and 0.03 for each combination from left to right in the x -axis, respectively).

over chemical ligands for the type of system and conditions studied here. The non-specificity of the topography transduction could trigger various intracellular pathways (e.g. via various receptors and molecules, such as integrins, tyrosine kinases in focal adhesions, calcium channels and actin; Curtis & Wilkinson 1997, Dalby 2005), potentially producing a

stronger and more effective feedback mechanism than the cascades derived from the chemical ligands analysed in this investigation. One micrometer wide microchannels, compared with two micrometer wide microchannels, are more effective in inducing polarization. This particular dimension has previously been correlated with perpendicular alignment of axons (Nagata *et al.* 1993; Rajniecek & McCaig 1997; Gomez *et al.* 2007). Nagata *et al.* (1993) suggested that 1 μm structures mimic tightly aligned neurite bundles provided by other cells in the body, which could represent an important stimulus *in vivo*.

Other studies have also investigated simultaneous stimulation of different cell types but in a different fashion. McCaig suggested possible interactions between electric fields and chemical molecules in axon guidance. For example, he suggested that molecules such as laminin could be distributed according to their charge and endogenous electric fields. Owing to this, axon orientation in electric fields was shown to depend on substrate properties, producing orientation towards the cathode on laminin surfaces and towards the anode on polylysine surfaces (Rajniecek *et al.* 1998). Experiments with simultaneous contact guidance cues and electric fields showed that neurite orientation was mostly dominated by the direction of the electric field (McCaig 1986). In contrast, adhesive cues such as laminin stripes were preferred over simultaneous electric fields for neurite alignment (Britland & McCaig 1996).

In addition, Miller *et al.* (2001, 2002) have analysed neurite orientation in the presence of Schwann cells, laminin and microchannels. In these studies, polylactic acid microchannels were modified with laminin and seeded with Schwann cells. The authors found that this combination promotes higher neurite alignment of neurons and accelerates neurite outgrowth (Miller *et al.* 2001). These studies also highlighted the importance of complex environments surrounding cells with multiple extracellular cues, but these focused more on combinatorial strategies than competition schemes.

Britland *et al.* published two investigations on interactions between topographical cues and adhesive tracks (Britland *et al.* 1996a,b). Stripes of adhesive aminosilanes (Britland *et al.* 1996a) or laminin (Britland *et al.* 1996b) were orthogonally placed on quartz microchannels. Cells were preferentially aligned along adhesive tracks over the microchannels, depending on groove dimensions. More recently, a similar study by Charest *et al.* (2006) created microchannels that were orthogonal to fibronectin tracks. In this case, osteoblasts were cultured and found to preferentially align with the physical structures rather than the chemical tracks. Although these studies also explored competitive stimulation, the concept used in these publications is quite different, as the adhesive tracks and microchannels were just orthogonally placed, but cell positioning with respect to the stimuli was not controlled in any way, which could not necessarily reflect a decision-making process in the cell, but rather a result of cell adhesion or proximity to a specific cue, for example. In addition, none of these studies explored polarization of neurons, but rather just neurite

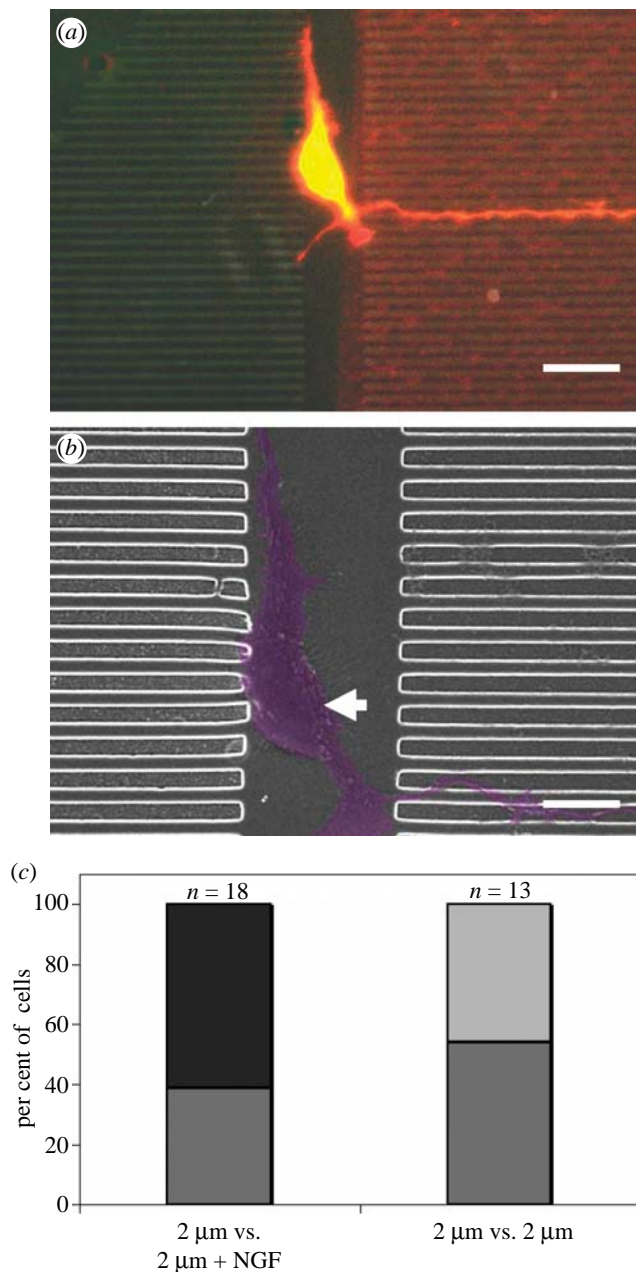


Figure 4. Competition between combined microchannels and immobilized NGF and microchannels alone. Hippocampal neurons were micropositioned between microchannels with immobilized NGF and microchannels only, and analysed for the direction of axon formation. (a) Overlay of phase-contrast and fluorescence (green labelling for tau-1; red labelling for NGF) images of neuron between 2 μm microchannels and combined 2 μm microchannels with immobilized NGF. Axon was established on the surface with the combined cues. Scale bar, 25 μm ; (b) SEM image of neuron in (a) (arrowhead; pseudo-coloured for visualization). Scale bar, 12 μm ; (c) quantitative analysis of polarization of neurons between combined stimuli and microchannels only. The x -axis represents the specific competition pair. The y -axis represents the percentage of cells that established axons on either the combination or microchannels only ($p=0.35$ and 1 for combination and microchannels only, respectively).

alignment. The present study is the first one that thoroughly explores neuron polarization with a very controlled system that ensures complete separation of the cues and simultaneous stimulation.

3.4. Competition between a combination of physical and chemical stimuli versus physical stimulus

The final competition analysis was chosen based on the fact that physical cues were found to be stronger than chemical ligands. We investigated if a combination of physical and chemical cues could be stronger than physical cues alone (figure 4). For this, microchannels were presented to neurons on both sides, but one of the microchannel areas was also modified with immobilized NGF (figure 4*a,b*). In addition to this scheme, a control geometry was also analysed when both sides only had microchannels, which theoretically would give equal chances for polarization (i.e. 50%).

Figure 4*c* shows that for the control system, each side was chosen 46 and 54% of the times, respectively, which is statistically equal to the 50% expected ($p=1$). Furthermore, when one of the areas was combined with immobilized NGF, the side modified with NGF was chosen 61% of the times, but this value was not significantly different from a 50% equal distribution ($\chi^2=0.88$, $p=0.35$). This result suggests that there is little added benefit of NGF when combined with microchannels, implying that topography dominates the polarization response. However, chemical ligands could still play an important role when combined with physical cues not only for polarization, but also for subsequent axon growth, which occurs with geometric arrangements of cells (e.g. glia) that express or secrete specific ligands (Hatten 1999).

4. CONCLUSIONS

We investigated a novel aspect of neural behaviour, i.e. polarization in response to competitive stimuli. In particular, we analysed competition between physical cues (i.e. topography) and chemical cues (i.e. immobilized NGF and laminin). We first analysed the effect of the individual stimuli, finding that both topography and chemical ligands effectively influence axon formation when presented independently (75 and 69% of axons grew on physical and chemical cues, respectively, compared with unmodified surfaces). When both chemical and physical cues were presented simultaneously to the cells, physical cues were preferred 70% of the times over the chemical signals, which indicates that topography is a stronger stimulus. When topography was combined with immobilized NGF and tested against topography alone, axons preferred to grow on the combination side 61% of the times. This result was not statistically significant, which suggests that chemical ligands do not significantly enhance polarization, and topography dominates the response. The results derived from this study contribute to the understanding of neuronal behaviour on artificial substrates, which is applicable to the creation of artificial niches for neuron transplantation in the brain, neural tissue engineering applications and control of polarity in neural networks.

Funding for this work was provided by a grant from the Gillson Longenbaugh Foundation to C.E.S. We thank the Welch Foundation and SPRING for partial financial support in the CNM facilities.

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