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Neuronal polarity selection by topography-induced focal adhesion control

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ABSTRACT

Interaction between differentiating neurons and the extracellular environment guides the establishment of cell polarity during nervous system development. Developing neurons read the physical properties of the local substrate in a contact-dependent manner and retrieve essential guidance cues. In previous works we demonstrated that PC12 cell interaction with nanogratings (alternating lines of ridges and grooves of submicron size) promotes bipolarity and alignment to the substrate topography. Here, we investigate the role of focal adhesions, cell contractility, and actin dynamics in this process. Exploiting nanoimprint lithography techniques and a cyclic olefin copolymer, we engineered biocompatible nanostructured substrates designed for high-resolution live-cell microscopy. Our results reveal that neuronal polarization and contact guidance are based on a geometrical constraint of focal adhesions resulting in an angular modulation of their maturation and persistence. We report on ROCK1/2-myosin-II pathway activity and demonstrate that ROCK-mediated contractility contributes to polarity selection during neuronal differentiation. Importantly, the selection process confined the generation of actin-supported membrane protrusions and the initiation of new neurites at the poles. Maintenance of the established polarity was independent from NGF stimulation. Altogether our results imply that focal adhesions and cell contractility stably link the topographical configuration of the extracellular environment to a corresponding neuronal polarity state.

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1. Introduction

Several extracellular signals were demonstrated to influence the establishment of neuronal polarity by either attracting or repelling developing neurites, the long cellular protrusions emanating from the soma of neuronal cells [1]. During nervous system development these guidance cues combine in a complex dynamical signaling pattern that is integrated by cells to form a functional network of neuronal connections [2]. While secreted factors act over long distances encoding for preferential directions in terms of concentration gradients [3], the chemical and physical properties of the local extracellular environment are read by the cell through direct interaction in a contact-dependent manner [4,5]. As indicated by *in vivo* observations, a primary role in defining the final wiring of the nervous system is played by the substrate topography [6].

Many cellular contacts with the extracellular matrix (ECM) are mediated by heterodimeric (α/β) integrin receptors [7]. Integrin clustering triggers the recruitment of several cellular components thus inducing the nucleation of a cytoplasmic protein complex, the adhesion plaque [8]. Adhesion plaques assemble and mature in a hierarchical fashion increasing in size and enriching in characteristic proteins such as vinculin, focal adhesion kinase, talin, and paxillin [9]. In this way, a permissive ECM conformation fosters the maturation of initial small contacts ($<1\ \mu m^2$) into larger focal adhesions (FAs). The maturation of FAs is regulated by a local balance between the force generated by cell contractility and ECM response [10]. Mature FAs ultimately establish connections with actin microfilaments and can thus remodel the cell shape. Hence, maturation of integrin-based adhesions, regulation of actin dynamics, and cell contractility cooperate to transduce ECM guidance cues into specific cell polarity states [11].

Neurons are one of the most notable examples of highly polarized cell types, typically extending one or several neurites to connect to specific targets in the body. During neuronal differentiation both neurite initiation and extension depend on the formation and stabilization of integrin-based adhesions [12,13]. The maturation of

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FAs likely plays an essential role in sensing local ECM information and coordinating the morphometric changes that characterize the establishment of cell polarity during neuronal differentiation [13,14]. These cellular responses are thought to be driven by tension generated within the cytoskeleton and by the redistribution of FAs caused by surface topography; nonetheless experimental evidence supporting this hypothesis is still missing [15].

In order to unravel the mechanisms by which differentiating neurons read the local topography it is crucial to decouple its effect from those stemming from other chemical and physical stimuli. Thanks to recent advances in biomaterial nanofabrication, selected morphological aspects of the ECM are now reproducible *in vitro* through the realization of artificial scaffolds with controlled topography [16–18]. In this direction, different research groups, including ours, demonstrated that nanogratings, alternating lines of grooves and ridges of submicron size, can effectively modulate cell polarization during neuronal differentiation of PC12 cells [16,17,19,20]. Specifically, we introduced an original, biocompatible, nanostructured scaffold for time-lapse experiments which was shown to induce efficient neurite alignment [16] and bipolarity [19] when presenting 500-nm wide ridges and grooves. Interestingly, Foley and coworkers [17] demonstrated that neuritogenesis in PC12 cells is modulated by topographic feature size, suggesting that topographic guidance cues act cooperatively with NGF signaling to regulate both generation and orientation of neurites. Differently, neurite number modulation and tuning of neurite outgrowth direction in PC12 cells could also be obtained by applying lateral constrictions to developing neurites using microchannels [21].

Topographic contact guidance was also observed during neurite outgrowth in primary cells from the central and peripheral nervous system [15,18,22,23]. Rajnicek et al. in 1997 [24] provided much information on the complexity behind this phenomenon. Bipolarity and neurite alignment were induced by culturing spinal cord neurons from *Xenopus laevis* on grooved substrates, while mammalian hippocampal neurons generated a single axon which turned out to be preferentially aligned to the grating only under specific conditions (i.e. groove width = 2 μm and depth = 1.1 μm, groove width = 4 μm and depth = 0.52 μm or 1.1 μm). Surprisingly structures too small or too smooth led to perpendicular alignment.

Although much effort was directed to the characterization of cell morphology as modified by the interaction with the substrate micro/nanotopography, the general cellular mechanisms making neurons capable of “reading and learning” the extracellular texturing are still largely unknown.

In this work we analyzed neuronal contact guidance, polarity establishment, focal adhesion maturation, cell generated contractility, and actin remodeling in PC12 cells. Exploiting biocompatible nanostructured substrates, we followed focal adhesion and actin dynamics onto nanograting ridges during neuronal differentiation. Using dominant negative mutants and specific inhibitors of cell contractility we further analyzed the role of the ROCK1/2-myosin-II pathway in the regulation of neuronal polarization on nanogratings.

2. Materials and methods

2.1. Substrate fabrication

Nanogratings were fabricated by thermal nanoimprint lithography (NIL) on copolymer 2-norbornene ethylene [cyclic olefin copolymer (COC)] foils (IBIDI. Martinsried, Germany). Standard imprint schemes are based on a combination of pressure and heat to transfer the pattern from a mold to thermoplastic materials. Our molds were obtained by means of electron-beam lithography (EBL) and reactive ion etching (RIE) starting from commercial p-doped silicon wafer (SYLTRONIX, France). Each mold consisted of a quarter 2″ Si-wafer, initially processed by EBL to generate arrays of gratings into 250-nm thick poly(methyl methacrylate) (PMMA) film. PMMA was then used as etching mask during the RIE step that yielded the pattern transfer into the silicon substrate. After cleaning with acetone/isopropanol and oxygen plasma, the molds were characterized by scanning-electron microscopy and atomic-force microscopy. Nanogratings with 250 nm depth and with line widths and pitches of 500 nm were fabricated similarly to what we previously reported [25]. The molds were finally silanized with Silanization solution (dimethyldichlorosilane in heptane, Fluka, Sigma–Aldrich, USA) to obtain low-energy surfaces. This allowed proper master-replica separation after imprint, improving the fidelity and reproducibility of the process. The COC foils were imprinted using an Obducat Nanoimprint 24 system (Obducat, Sweden). After cleaning with 2-propanol, the substrates were placed on top of the mold and softened by raising the temperature up to 150 °C. A pressure of 50 bar was then applied for 5 min before cooling down to 70 °C, i.e. below the glass transition temperature of the copolymer (T_g = 134 °C). Finally the pressure was released and the mold was detached from the ibiTreat substrate with a scalpel. The imprinted gratings were systematically characterized by scanning-electron microscopy and atomic-force microscopy before cell culturing.

2.2. Constructs

Paxillin-CFP was generated starting from the Paxillin-EGFP construct [26] gently provided from Juergen Wehland (Helmholtz Centre for Infection Research, Braunschweig. Germany). The sequence encoding for CFP was amplified by PCR. Primers were all purchased from Sigma-Genosys (St. Louis, USA). The EGFP sequence was removed and the cDNA encoding for CFP was replaced into *Eco*RI-*Not*I sites of paxillin-EGFP construct. The cDNA sequence encoding for paxillin was previously reported [27]. The dominant negative (Rho-binding defective; [28]) version of ROCK1-EGFP (I1009 → A) was engineered by site direct mutagenesis using the QuickChange Kit (Stratagene, USA). As template we used the EGFP-ROCK plasmid (gently provided from Gareth Jones; King's College, London. England) described in [29]. The primer sequence used in the mutagenesis reaction to transform the Isoleucin (I) in position 1009 into Alanine (A) in ROCK-1 cDNA was 5′AACAAATTGGCAGAAATAATGAATCGAAAAG 3′. The antisense primer had reverse complementary sequence. The Actin-YFP plasmid was purchased from Clontech (USA). Kinase-dead MLCK-GFP [30] was a kind gift from Anne R. Bresnick (Albert Einstein College of Medicine, New York. USA). The expression vector encoding for EGFP protein used as control was pEGFP N1 (Clontech).

2.3. Cell culture and inhibitor treatment

PC12 cells obtained from the American Type Culture Collection (CRL-17210 ATCC) were grown in RPMI medium supplemented with 10% HS, 5% FBS, 2 mM glutamine, 10 U/ml penicillin and 10 μg/ml streptomycin and were maintained at 37 °C and 5% CO_2. Stimulation of PC12 differentiation was obtained adding 100 ng/ml of NGF (Sigma) to the growth medium. PC12 cells were transfected or co-transfected using a Microporator (Digital Bio Technology, Korea).

All reported experiments were performed using cells with less than ten passages *in vitro*. REF52 cells (a rat fibroblast line) stably expressing paxillin-YFP were obtained from Joachim Spatz (Max-Planck Institute for Metals Research, Stuttgart. Germany) and cultured as described [31].

Imprinted COC substrates were carefully sealed to the bottom of hollowed 35 mm Petri dishes hereafter denoted as ‘imprinted dishes’, taking care to place the patterned region in the center of the hollow. The imprinted dishes were then sterilized by overnight treatment with ethanol and then rinsed twice with PBS. For imaging experiments, cells were treated to obtain more than 80% single cells. Single cells in suspension were then counted and plated on the imprinted dish to reach a final concentration of 10^4 cells/cm^2. REF52 cells were imaged immediately after plating while PC12 cells were allowed to adhere for 12 h on the imprinted dishes before stimulation with NGF.

For contractility inhibition experiments during PC12 differentiation (0–72 h), cells were treated with DMSO (corresponding v/v), 20 μM ML-7, 20 μM Y27632, or 25 μM blebbistatin. The drugs were dissolved in DMSO (ML-7, blebbistatin) or in distilled water (Y27632) and added 30 min before NGF stimulation. Inhibitor treatment was repeated every 24 h.

2.4. Wide field time-lapse microscopy

Cell imaging was performed using an inverted Nikon-Ti PSF wide field microscope (Nikon, Japan). The imprinted dishes were maintained in an incubated chamber implemented to the microscope (Tokai, Japan) and images were collected using a 40× 1.3 NA oil immersion objective (PlanFluor, Nikon). Single PC12 cells adhering to the patterned regions of the COC substrates (‘nanograting’) and to the nearby flat regions (‘flat’; Figs. 1, 2 and 4) were initially imaged before stimulation with NGF (‘unstimulated’ in Fig. 1). After stimulation, a total of 20 (or more) imaging fields each containing several single cells were chosen in the specimen. At each time of measure (every 30–60 min for a maximum of 4 days) a DIC image of each field was acquired. An additional fluorescent image was acquired for PC12 cells expressing paxillin-EGFP (Figs. 4 and 5), EGFP-ROCK(I1009 → A), EGFP (Fig. 6), or actin-YFP (Fig. 7) using an EGFP or a YFP filter cube (Semrock, USA), respectively. NGF wash out (‘wash out’, Fig. 1) was obtained gently removing the culture medium from the imprinted dish under analysis and washing the cell culture twice with PBS. Prewarmed complete medium was then added, and the image acquisition protocol was

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