Topographic Cues of Nano-Scale Height Direct Neuronal Growth Pattern

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ABSTRACT: We study the role of nano-scale cues in controlling neuronal growth. We use photolithography to fabricate substrates with repeatable line-pattern ridges of nano-scale heights. We find that neuronal processes, which are of micron size, have strong interactions with ridges even as low as 10 nm. The interaction between the neuronal process and the ridge leads to a deflection of growth direction and a preferred alignment with the ridges. The interaction strength clearly depends on the ridges’ height. For 25 nm ridges approximately half of the neuronal processes are modified, while at 100 nm the majority of neurites change their original growth direction post interaction. In addition, the effect on growth correlates with the incoming angle between the neuronal process and the ridge. We underline the adhesion as a key mechanism in directing neuronal growth. Our study highlights the sensitivity of growing neurites to nano-scale cues thus opens a new avenue of research for pre-designed neuronal growth and circuitry.

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Introduction

The ability to manipulate neuronal growth has great implications for both neuronal repair and for the potential design of modern computational devices (Biggs et al., 2010; Cheran et al., 2008; Wheeler and Brewer, 2010). Previous studies have identified processes that take place along neuronal growth and influence the geometry of the neuronal dendrites and axons, which show extremely diverse complex structures. A key mechanism is the ability of the sensory-motile growth cones at the tips of growing processes to measure environmental cues (Huber et al., 2003; Tessier-Lavigne and Goodman, 1996; Whittington, 1993).

The most well-studied guidance cues are spatial concentration gradients of chemo-repelling and chemo-attracting molecules, mainly for target recognition, many of which have been identified (Huber et al., 2003). A second potent environmental cue for the control of cell adhesion, migration, orientation, and shape, is the topography of the substrate (Clark et al., 1990; Curtis and Wilkinson, 1997; Kim et al., 2010). It was shown that neuronal outgrowth is significantly affected by the topography of the substrate that interacts with the cell (Anava et al., 2009; Clark et al., 1990; Curtis and Wilkinson, 1997; Rico et al., 2004; Shefi et al., 2004). Intracellular investigations of down-stream effectors revealed a major class of cell adhesion molecules that are involved in the axonal guidance (Rico et al., 2004). A recently developed paradigm for growth cone guidance is the “substrate-cytoskeletal coupling” model (Suter and Forscher, 2000). According to this model, growth cones can move forward if they are capable of coupling intracellular motility signals to a fixed extracellular translocation substrate via cell surface adhesion receptors. These receptors must form a strong linkage between the substrate and the actin cytoskeleton, allowing to pull the growth cone forward (Suter and Forscher, 2000).

Topographic structures such as grooves and ridges were found to affect the direction of axonal growth when these structures were of micron sizes (Britland et al., 1996; den Braber et al., 1998; Fricke et al., 2011; Hwang et al., 2009; Lee et al., 2007; Mahoney et al., 2005). Enhanced guidance of rat DRG nerve cells extension was observed using parallel adhesive tracks of micron-scale (Britland et al., 1996). In another study photolithography techniques were used to fabricate micron-scale features of linear, dashed and zigzag patterns, which modified the axonal outgrowth (Lee et al.,
Since the structures were of micron size, a similar scale as the axons, initially, nano-sized features were not expected to modify cell behavior. However, current studies have shown that neurons can interact closely with features of a few hundreds of nanometers. Vertical nanopillars protruding from a flat surface serve as focal adhesion points and non-invasively inhibit migration of neurons on the substrate (Xie et al., 2010). Nanowires and lithography-based patterns have been used for both positioning neurons and for directing axonal growth (Dos Reis et al., 2010; Fozdar et al., 2010; Hallstrom et al., 2007; Hanson et al., 2009; Johansson et al., 2006; Prinz et al., 2008). In addition to orientation and adhesion effects, micron- and nano-topographic features were also found as modifying the neuronal branching tree length (Fozdar et al., 2010; Mahoney et al., 2005).

In this study we quantitatively examine the interactions between single neurons and topographic cues of six heights, from 150 nm and down to 10 nm. Neurons of the medicinal leech that can grow at extremely low densities are plated atop substrates with line-pattern ridges. We define the distance between the lines large enough to allow analysis of single line–single axon interaction. We find that axons show interactions with the patterns at all six heights, even for 10 nm high ridges. For the heights measured, the interaction strength depends linearly on ridges’ height and also on the incoming angle between the axon and the ridge. We propose a model where the strength of interaction correlates with the ridge surface that serves as an anchor for the neuronal membrane. Thus, we underline the adhesion as a key mechanism in directing neuronal growth.

Materials and Methods

Substrates Fabrication

The patterns are prepared on precut glass slides 1 × 1 cm² (Fig. 1A). The glass substrates are ultrasonically cleaned using acetone (10 min)/ethanol (10 min)/isopropanol (10 min), and dried by high purity nitrogen. Lines are fabricated by standard photolithography methods. S1813 (Shipley Company LLC, Marlborough, MA) resist is spin coated on the glass substrate at 5,000 rounds per minute for 60 s, and baked at 115°C for 75 s (Fig. 1B). Next, using a mask aligner and predesigned photo-mask, the line pattern is transferred to the resist. The exposure wavelength is 365 nm, and exposure time 5 s (Fig. 1C). It is then developed for 45 s in MF-319 (Rohm and Haas, Philadelphia, PA) and afterwards washed in distilled water for 45 s to stop the developer. The sample is then dried and cleaned using nitrogen gas.

Our pattern consisted of lines size 3 × 45 μm² with lateral spacing of 6 μm and vertical spacing of 30 μm, covering a total area of 6 × 6 mm².

The samples are transferred to the deposition chamber (base pressure of 1 × 10⁻⁸ torr) where they are cleaned in Ar plasma prior to deposition. We deposit a 5 nm Ni adhesion layer (direct current sputtering at a rate of 2.76 nm/min) followed by vanadium pentoxide (V₂O₅) layers of various thicknesses, between 5 to 145 nm (reactive radio frequency-sputtering, 3.6% O₂ in Ar, at a rate of 2.1 nm/min) (Fig. 1D). We use V₂O₅ since it is insulating and transparent. All depositions are performed in 3 mTorr pressure. Finally, the non-patterned photoresist is removed by soaking the sample in acetone for an hour (Fig. 1E). Using the same procedure we create also control patterns with only the 5 nm thick Nickel (Ni) layer or V₂O₅ coated glass substrates with no patterning.

Cell Culture

Neurons are isolated from the central nervous system of adult medicinal leeches Hirudo medicinalis. Leeches are anaesthetized in 8% ethanol in leech Ringer (115 mM NaCl, 1.8 mM CaCl₂, 4 mM KCl, 10 mM Tris maleate, pH 7.4) before dissection. Nerve cords are dissected and ganglia are removed and pinned on Sylgard-184 petri dish. Then, ganglia are treated enzymatically with 2 mg/mL Collagenase/Dispase enzyme solution (Roche, Mannheim, Germany) for 1 h at room temperature. Next, ganglia capsules are opened to expose the cells. neuronal cells are rinsed and plated on the different substrates. Neurons out of three ganglia (total of 1,200 cells in a volume of 100 μL) are plated on each substrate. A day after plating 2 mL of enriched medium is added to the cultures (Leibovitz L-15 supplemented with 6 mg/mL glucose, 0.1 mg/mL gentamycin, 2 mM/mL glutamine, and 2% fetal bovine serum). As for control, neurons are plated on substrates of glass, V₂O₅ and Ni with no deliberate topography. All patterned and non-patterned substrates are pre-coated for 2 h at 37°C with Concanavalin-A (Con-A) (Sigma-Aldrich Co., Saint Louis, MO, 0.5 mg/mL).
to obtain uniform growth conditions on both glass and V$_2$O$_5$ substrates. Plates are kept in a 25°C incubator for up to 2 weeks. Usually measurements are performed between days 3 and 7.

**Immunocytochemistry**

Primary neuronal cultures are fixed with 4% paraformaldehyde for 10 min at room temperature, washed with phosphate-buffered saline (PBS) and permeabilized with 0.5% Triton X-100 in PBS (PBT) for 10 min. Then cultures are incubated in a blocking solution (containing 1% bovine serum albumin (BSA) and 1% normal donkey serum (NDS) in 0.25% PBT) for 45 min. Next, cultures are incubated with a rabbit antibody to α-tubulin (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) overnight at 4°C. The cultures are rinsed with PBT and incubated for 45 min at room temperature with Cy3-conjugated AffiniPure Donkey Anti-rabbit secondary antibody (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA). After incubation, cultures are rinsed with 0.5% PBT and mounted in an aqueous mounting medium. Confocal imaging is performed using Leica TCS SP5 with Acousto-Optical Beam Splitter microscope to acquire fluorescent and bright field images.

**Scanning Electron Microscopy**

To closely examine the neuron-ridge interaction neurons are imaged by a Scanning Electron Microscope (SEM). Several days after plating, neurons are fixed with 2% glutaraldehyde in PBS (no Ca$^{2+}$, no Mg$^{2+}$, pH 7.2) for 1 h at room temperature. After fixation cultures are repeatedly rinsed in PBS and then treated with Guanidine–HCl:Tannic acid (4:5) solution (2%) for 1 h at room temperature. Cultures are repeatedly rinsed again in PBS, dehydrated in graded series of ethanol (50%, 70%, 80%, 90%, and 100%) and finally with graded series of Freon (50%, 75%, 100% × 3). Finally, the preparations are sputtered with carbon before examination by scanning electron microscopy (Inspect, FEI, Hillsboro, OR).

**Statistical Analysis**

Data analysis is performed using a semi-automatic self-programmed software (Matlab, Mathworks, Inc., Natick, MA). For the analyses a minimum of three plates are considered for each pattern size. Over 100 processes are measured for each patterned substrate height and 300 for the control.

The measurements of the effect of the substrates on the neuronal growth are summarized into a probability bar chart. For each height group the percentage of “affected processes” (defined in the Results Section) out of the total processes is calculated. Error bars represent standard errors. In order to test frequency differences of affected processes in the different height groups, Chi-squared statistic is performed resulting in a Chi$^2$ and a P-value (significant levels of 0.0001). The nature of growth on bare substrates with no patterns is taken into account in order to correct the measurement of “affected processes,” using a virtual set of lines, as described in the Results Section.

**Results**

**Non-Patterned Substrates**

A day after plating, neurons are already attached to the substrates and neurites have initiated out of the soma to develop into a neuronal branching tree. Neurons that are grown atop the control substrates, with no topographic cues, extend processes with no preferred outgrowth direction (see Fig. 2). Figure 2A presents a single neuron growing on a bare glass substrate, pre-coated with Con-A. Several neurites originate from the round soma, grow radially and bifurcate into sub-daughter branches, forming a complex dendritic tree in all directions. Figure 2B demonstrates an undirected growth of two neurons on non-patterned samples fully coated with V$_2$O$_5$. Similar unbiased growth has been detected for the fully coated Ni substrates (not shown).

**Line-Patterned Substrates**

Neurons that are plated on line-patterned substrates have a significantly different growth pattern (see Fig. 3A and B). They develop branching trees that are highly aligned with the nano-scale topographic cues. The neuronal processes that navigate onto the substrates tend to attach to the ridges and outgrow along the lines. Thus, the structure of the whole neuronal branching tree is modified, leading to a more longitudinal shape. We find that the neuronal processes are modified by the line patterns for all six heights, that is, there is a clear effect even for 10 nm high
topographic cues. The level of interaction is found to depend on the height of the ridge pattern, described below. To gain a better contrast we immunostain some of the plates against anti-tubulin (Fig. 3B). Here we can clearly see the neuronal processes, established and newly formed, navigate onto the substrate, encounter a ridge and align with it. On top of the ridges the neuronal processes seem to appear as straight lines aligned with the line ridges. During the navigation period between the lines the processes are less stretched. Interestingly, neuronal processes that have aligned to the ridges tend to bifurcate at the edges of the ridges.

In Figure 4A we show SEM images of neuronal processes 6 days after plating, demonstrating two types of interactions with the nanometric ridge pattern. Figure 4B and C is zoomed in images of those interactions. In Figure 4B the direction of growth is highly affected by the 50 nm line pattern ridge. In Figure 4C a sibling branch of the same neuron as from Figure 4B crosses the ridge with no effect on its growth direction. We consider a neuronal process as in Figure 4B an “affected process” and a process such as in Figure 4C a “non-affected process” (see also schematic drawing in Fig. 5A). It is important to note that in both cases minor processes extend from the major process and anchor to the ridge. These minor filopodia-like processes that form focal adhesions with the substratum cannot be seen in light microscopy (Figs. 2 and 3). In our measurements we take into account only the effect of the line-patterns on the growth of major processes. It is also clearly seen in these images that the substrate and line patterns are coated uniformly with Con-A, resulting in comparable surface properties with similar roughness that should lead to similar interaction with the neuronal membrane. Thus, we can exclude any strong effects due to dissimilarities between the glass substrate and the insulating vanadium oxide patterned

**Figure 3.** Leech neurons growing on patterned substrates pre-coated with Con-A. A: Phase microscopy image of a single neuron growing on top of a substrate with line pattern ridges of 25 nm height. The neuronal branches attach to the ridges and the whole growth pattern is aligned according to the topographic cues. B: A confocal microscopy image of an immunostained neuron (α-tubulin) growing onto ridges of 50 nm height. The ridges do not appear in the fluorescent image, their locations are marked. Most of the neurites are affected by the ridges and outgrow along them.

**Figure 4.** SEM images of neurons growing atop a 50 nm ridge pre-coated with Con-A. A: Two neurites of the same neuron crossing the line pattern ridge (soma is out of the frame). The top and bottom blue squares mark the crossing sites demonstrating the “affected” versus “non-affected” types of interactions, respectively. B: Zoomed-in image of the top blue square in A. The growth direction of the major neurite is modified by the ridge. C: Zoomed-in image of the bottom blue square in A. The neurite continues its original growth direction. Notice the minor branching, in both images B and C, growing out of the major neurites and attaching to the ridges.
Ridges of Different Heights

To study the graded effects of the different topographic heights on the neuron-ridge interactions we compare how many “affected processes” versus “non-affected processes” develop on each type of substrate. In Figure 5B we plot the percentage of affected processes as a function of the cue heights. The majority of processes that come across ridges of 150 and 100 nm height are affected. For lower ridges of 75 nm height and 50 nm height the percentage of affected processes decreases but still more than half of the processes are affected. For the lower ridges of 25 and 10 nm the percentage of “affected processes” is around one-third. It can be seen that the percentage of “affected processes” increases as ridge height increases significantly ($P < 0.0001$).

To further verify the significance of our results we superimpose virtual sets of line patterns on top of images of neurons that are developed with no topographic cues. We measure, the same way the real processes are measured, the percentage of neuronal processes that cross the virtual lines and can be considered as “virtually affected processes.” Such analysis characterizes the growth fashion of neuronal processes on substrates and reflects their natural turnings due to signaling other than topographic cues. We find that the statistics for neurites that grow on control substrates or on patterned substrates in between ridges is similar. A total of 22% of the neuronal processes that cross virtual lines can be interpreted as “affected processes” (out of 316 processes). This is clearly lower than the measured “affected processes” even for the 25 and 10 nm heights samples. Taking into account the statistics of “virtually affected processes,” we can correct for the real percentage of “affected processes” per ridge height. If we assume that the probability of having a virtually affected processes (marked $P_v$) is uncorrelated with the probability of having an affected processes (marked $P_a$), our measured probability of affected processes ($P_m$) is:

$$P_m = P_a + \left(\frac{1}{C_0}\right)P_v.$$  

Thus $P_a = \frac{(P_m - P_v)}{(1 - P_v)}$. The darker portions of the six bars in Figure 5B represent the corrected probabilities of neuronal processes to be affected by the topographic cues.

In Figure 5C we plot the corrected percentage of “affected processes” as a function of the ridges heights. We find that the probability to interact with a line-pattern increases with its height, within the range of 10–100 nm. The probability can be fitted with a linear dependence (with $R^2 = 0.98$), but this is not conclusive. However, for ridges above 100 nm height the interaction between the ridges and the neuronal processes is similar to the 100 nm, thus, the percentage of “affected processes” reaches a plateau. By extrapolating the trend line towards potential ridges, lower than 10 nm, we predict a minor effect on the neuronal growth. For ridges height of “zero” nm, 8% of the processes will be affected by encountering the ridge. This theoretical percentage can reflect a surface effect since the slide and ridges that are coated with Con-A are made of different materials (glass and $V_2O_5$). However, this effect is small, below the level of noise, thus we cannot interpret it with certainty.

Varied Incoming Angles

Next, we measure the probability of having an “affected process” as a function of the neurite’s incoming angle. The incoming angle, $\theta$, is defined as the angle between the

Figure 5. Dependence of interaction statistics on patterned ridges’ heights. A: Schematic drawing of a neurite encountering a line pattern at an incoming angle $\theta$. The two typical behaviors are demonstrated: “non-affected” on the left hand side and an “affected” neuron on the right hand side. B: The percentage of “affected processes” out of total processes per ridge height as a function of ridges’ heights. The darker portions represent the corrected probabilities of neuronal processes affected by the topographic cues, taking into account the statistical nature of neurites growth (Chi-squared test, $P < 0.0001$). C: Plot of percentage of affected processes (corrected) versus ridge height. Red line shows linear dependence up to $\sim 100$ nm height.

approaching neurite and the ridge which it encounters (see Fig. 6A, left and right panels). The possible incoming angles are thus only between 0° and 90°, where 0° means that the neurite is parallel to the ridge encountered, and 90° is a perpendicular encounter. The pie charts in Figure 6B display the percentage of “affected processes” for four ranges of incoming angles: 11–50°, 51–70°, 71–90°, and for two ridge heights. Incoming angles below 10° are not included, since they start already rather parallel to the ridge. For the 100 nm ridges most of the neurites are affected following an interaction with the ridges with no dependence on the incoming angles (Fig. 6B, right panel). However, for the lower ridges the probability of a neurite “to be affected” when approaching a ridge perpendicularly (θ = 90°) between the neurite and the ridge, see Figs. 5A and 6A) is lower than a process approaching the ridge in a more acute angle. For the 50 nm ridges most of the neurites are affected in the lower range of angles (82% for angles of 11–50°). In the middle range, the percentage of affected neurites is lower (70% for angles of 51–70°). For perpendicular approaching neurites (71–90°) only 35% of the processes change their growth direction due to the topographic cues. Ridges lower than 50 nm present a similar behavior. We find that for lower ridge heights this trend continues. Much less processes arriving at normal angles are affected, while the processes arriving at acute angles are still affected. The 150 nm sample does not show any angle dependence, similarly to the 100 nm ridges. It appears that changes in incoming angles play an equivalent role in neuronal anchoring as modifying ridges’ height. The probability of a neuronal process to be affected by a higher ridge is similar to the probability when approaching the lower ridge in a more acute angle.

Discussion

We demonstrate that neuronal processes, which are of micron size, can be altered by order of magnitude smaller topographic cues, as low as 10 nm. We show that the majority of the neuronal processes that approach ridges of 75 nm and higher are affected by the ridge and change their original growth direction. However, when the topographic cues are lower, the effect on growth is more selective and depends also on the incoming angle between the process and the ridge it encounters. Nevertheless, the effect in all ranges and heights is significant in comparison to the control samples. It is important to note that the clear linear correlation between the effect and cues’ heights demonstrates the dependence of the interaction on the physical dimensions of the topographic cues and not on other characteristics. Higher cues increase adhesion possibilities to the substrate, thus better anchoring, especially for the fine filopodia-like processes. These results are supported by previous studies in the field showing less effect while decreasing the heights of the topographic cues (Rajnicek and McCaig, 1997; Rajnicek et al., 1997). Since all the patterned samples have the same ridge dimensions (3 × 45 μm²), the only difference between them is their height. Thus, we conclude that the strength of the neuron-ridge interaction originates from the side wall size. Even differences of 25 nm in ridges’ height change the strength of interaction showing a nano-scale neuronal sensitivity to the topography. In addition, we find that the incoming angle between a neuronal process and the ridge is important in analyzing neuron-ridge interactions. Processes that approach the ridge in acute angles show stronger interaction with the ridges than processes that approach them perpendicularly. The incoming angle between the neuronal process and the ridge affects the neuron–ridge interaction, particularly for the lower ridges (50 nm and lower). The incoming angles modify the “effective surface” for anchoring. Figure 6C presents a 2D simplified schematic diagram of neuronal processes encountering line-pattern ridges (top view). On the right panel the process approaches the ridge
perpendicularly. Thus, the actual membrane that can attach the ridge correlates with the diameter of the process (D). For a process that approaches the ridge in an acute angle (left panel) the effective diameter that may anchor the ridge is larger and depends on \( \sin \theta \). Thus, the effective diameter of a neuronal process encountering a ridge is:

\[ D_{\text{effective}} = D / \sin \theta. \]

Therefore, there are two parameters that are taken into account when calculating the probability of a neuronal process to be affected by the ridge; the ridges’ height and the neuronal incoming angle. For higher ridges (75, 100, and 150 nm) the contribution of the incoming angle is less significant, in comparison to the height. However, for low ridges the larger effective membrane surface due to acute incoming angles compensates the lack of adhesion surface. These differences are more significant when the ridges are of similar scale as the minor neuronal processes.

**Conclusions**

Nano-scale patterns are useful cues in directing neuronal growth. We conclude that the net adhesion possibilities of neuronal processes to the ridge are a major factor in directing neuronal growth by nano-scale topographic cues. Our discovery that topographic cues as low as 10 nm height clearly modify the neuronal processes growth predicts that it will be interesting to further explore the lower limit of neuronal sensitivity to nano-scale topographic cues and the resolution of that sensitivity. This would allow for optimizing the substrates parameters and enable a more complex design of patterned substrates for neuronal-based chips, opening new avenues of research for the fabrication of miniaturized neuron-based devices.

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