Impact of micropatterned surfaces on neuronal polarity

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Abstract
Experimental control over cellular polarity in a neuronal network is a promising tool to study synapse formation and network behavior. We aimed to exploit a mechanism described by Stenger et al. [J. Neurosci. Methods 82 (1998) 167] to manipulate the direction of axonal versus dendritic outgrowth on a micropattern. The group had used laser ablation to create patterns of aminated silanes for cell attachment on a background of repellent fluorinated silanes. The pattern offered continuous adhesive pathways for axonal and interrupted pathways for dendritic outgrowth. By microcontact printing, we created similar patterns containing continuous and interrupted pathways consisting of extracellular matrix proteins on a background of polystyrene. Neuronal polarity was determined on the functional level through double patch clamp measurements, detecting synapses and their orientation. Although our pattern reproduced the properties that were assumed to be critical for the described effect, namely contrasting pathways of different adhesiveness, we failed to reproduce the above results. It is indicated that other qualities of alternative pathways than mere differences in adhesiveness are required to orient neuronal polarity in vitro. We suggest that the effect observed by Stenger et al. has to be attributed to less universal characteristics of the micropattern, e.g. to the specific chemical groups that were utilized.

Keywords: Neuronal polarity; Cortical neuron; Neuronal network; Synapse formation; Microcontact printing; Adhesion

1. Introduction
To elucidate principles of neuronal signaling and network behavior, the creation of neuronal networks in which connectivity and pathways of input and output can be experimentally controlled are of high interest. Growing geometrically confined neuronal networks on micropatterned substrates is a promising approach to a wide range of topics both in biotechnological applications like drug screening or tissue engineering and in basic research. Experimental control over the architecture of an in vitro neuronal network can be utilized either to imitate structural features and signaling pathways of the intact brain or to deliberately alter these in order to investigate the impact of different connectivity patterns on network behavior.

Chemical patterning methods apply a micropattern that promotes cell attachment to a highly cell repellent background. Cells seeded onto such substrates are forced to align with the pattern and cell–cell interactions are limited to the predefined pathways, resulting in neuronal networks of a highly controlled and reproducible connectivity. A number of molecules have been used successfully for patterning, including the physiological adhesion molecule laminin (Yeung et al., 2001) or fragments thereof (Scholl et al., 2000), synthetic molecules like polylysine (Corey et al., 1991), various types of amines (Kleinfield et al., 1988; Ma et al., 1998; Schaffner et al., 1995). As a cell repellent background, a range of hydrophobic materials has been applied, such as polyethylene glycol (Wheeler et al., 1999), polystyrene (Lauer et al., 2002) or fluorinated silane groups (Ravenscroft et al., 1998).

A particularly challenging approach aims at not only controlling the adhesion sites and the pathways of neurite outgrowth but additionally the direction of signal transduction in the network: neuronal signaling through chemical...
Although the establishment of neuronal polarity in vivo is a topic of central interest in neuroscience, the underlying mechanisms are not well understood (Bradke and Dotti, 2000; Fukata et al., 2002; Hayashi et al., 2002). Several in vitro studies indicate that in addition to soluble factors (Lein et al., 1995; McAllister et al., 1997; Polleux et al., 2000), matrix associated signals may play a role in the regulation of axonal and dendritic outgrowth (Dertinger et al., 2002; Esch et al., 1999). Surface associated guidance cues are therefore thought an attractive tool for the experimental manipulation of axonal and dendritic outgrowth in vitro.

In a key paper by Stenger et al. (1998), control over neuronal polarity has been achieved on a patterned surface. The group grew dissociated hippocampal neurons on a pattern containing adhesive areas of aminosilanes (trimethoxysilylpropylidihexylametamine; DETA) on a repellent background of fluorinated silanes (tridecafluoro-1,1,2,2-tetrahydroctyl-1-dimethylchlorosilane; 13F). Different types of lines emerged from each DETA adhesion spot: one solid and three interrupted DETA lines into which "speed bumps" of non-adhesive 13F islands were interspersed. It has been shown before (Dotti and Banker, 1987) that after adhering to a surface in vitro, neurons extend undifferentiated neurites in random directions while the first to exceed a critical length becomes the lone axon. By default, the other neurites differentiate into dendrites. Stenger et al. reasoned that neurite extension would be faster on the solid DETA line representing a continuous adhesive pathway than on the interrupted lines containing 13F spots. Consequently, axonal extension should preferably occur along the solid line. Indeed, after 2–4 days in culture, the group found a high preference for neurites to extend a single long process staining positive for functional synapses that was stable for 2–3 weeks, and thus long enough for the formation of chemical synapses in the presynaptic area. Third; a different surface chemistry was applied, consisting of polystyrene as a background and an adhesive pattern composed of a blend of extracellular matrix proteins (see Section 2) mixed with poly-lysine.

Nevertheless, like in the quoted work, the pattern consisted of adhesive spots from which one solid and three interrupted lines emerged, the interrupted lines consisting of ECM islands separated by gaps of bare polystyrene. We applied patterns with different gap sizes of dimensions similar to the ones used by the Stenger group. The use of an alternative patterning technique had—in addition to providing an established system in which synapse formation has been demonstrated before—another advantage. By using a different surface chemistry and patterning technique while imitating the features that are thought to be responsible for the effect, it should be possible to find out whether these were indeed the critical parameters.

The properties of the micropattern were investigated with respect to their structure and to their interaction with the elongating neurite. Neuronal polarity was investigated on the functional level through double patch clamp measurements, allowing to deduce the direction of axonal elongation of the presynaptic cell.

2. Materials and methods

2.1. Microcontact printing

Microstamps were produced by photolithography and molding. An electron beam writer transposed the grid structures described below to a chrome mask. Applying UV-photolithography, master stamps were produced out of spin coated 12.5 μm thick photoreist layers (AZ 4562, Clariant) on 0.6 mm thick silicon wafers (MEMC electronic materials). Polymethylsiloxane (PDMS) microstamps were then fabricated by curing Sylgard 184 (Dow Corning) in 2 ml eppendorf tubes upside down on the master stamps for 48 h at 55 °C. After master stamp release, final curing was performed during 1 h at 110 °C.

PDMS stamps were stored for 24 h in deionized water before use, then decontaminated in a 70% ethanol bath for 1 min. Inking took place for 30 s in a 1:100 dilution of ECM-gel (Sigma E1270) in Dulbecco’s Modified Eagle’s Medium (GIBCO #11880-028) containing 10 μg/ml to-polylysine (Sigma, P6407). The inked stamp was dried in a stream of nitrogen and pressed to the substrate for 10 s. Non tissue culture polystyrene petri dishes of 35 mm diameter (Greiner) were used.

bine experimental control over neuronal polarity with a cell culture that allowed the formation of functional networks. Our system presented three major differences to the quoted work: first, we used cortical rather than hippocampal neurons. Second; the micropattern was created by microcontact printing rather than by laser ablation of a self-assembled monolayer and subsequent rederivatization of the irradiated area. Third, a different surface chemistry was applied, consisting of polystyrene as a background and an adhesive pattern composed of a blend of extracellular matrix proteins (see Section 2) mixed with poly-lysine.

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All grid structures had meshes of $50 \mu m \times 100 \mu m$, so that adhesion sites $22 \mu m$ in diameter and lines that were $4 \mu m$ in width. One line emerging from an adhesion node was always continuous while three were interrupted. Three types of patterns were used, differing in the size of the gaps on the interrupted lines. In pattern A, interruptions were $1 \mu m$, separating protein islands $12.5 \mu m$ in length; in pattern B: $2 \mu m$ gaps separated $7 \mu m$ islands; in pattern C: $5 \mu m$ gaps separated $6.5 \mu m$ islands. The interrupted lines spanned the entire distance in the $50 \mu m$ direction and half the distance in $100 \mu m$ direction, where the interrupted line met with the solid line emerging from the next node.

2.2. Visualization of the micropattern

To investigate the fidelity of pattern transfer, the inking solution was mixed with $10 \mu g/ml$ sulforhodamine (Molecular Probes, S-359) in one set of experiments. Substrates were then printed as usual and observed with a standard microscope (IX 50, Olympus) equipped with a color CCD-camera (Zeiss, AxioCam color 3.0). A Hg-vapor lamp (Olympus IX-FLA) served as the light source and appropriate combinations of optical filters were used to select an excitation wavelength of $480 \text{ nm}$ and emission wavelengths $(\text{Olympus IX-FLA})$ served as the light source and appropriate combinations of optical filters were used to select an excitation wavelength of $480 \text{ nm}$ and emission wavelengths greater than $515 \text{ nm}$.

2.3. Isolation of embryonic neurons

Rat embryonic cortical neurons were obtained as described by Breuer et al. (1993). Briefly, embryos were recovered from pregnant CD rats at 18 days gestation. Cortices were dissected from the embryonic brains; cells were mechanically dissociated by trituration in Hank’s Balanced Salt Solution (without Ca$^{2+}$ and Mg$^{2+}$, Gibco #14170-088), 0.033% sodium bicarbonate, 1 mM sodium pyruvate, 10 mM HEPES, 20 mM glucose, pH 7.4 with a firepolished siliconized pasteur pipette. Two volumes HBSS (Gibco #24020-091) 0.033% sodium bicarbonate, 1 mM pyruvate, 10 mM HEPES, 20 mM glucose, pH 7.4 were added. For 3 min, non-dispersed tissue was allowed to settle. The supernatant was centrifuged at $200 \times g$ for 5 min. The pellet was resuspended in 1 ml Neurobasal Medium (Gibco #21103-049), 1X B27 (Gibco #17504-044), 0.5 mM l-glutamine (Gibco #58070-083) per hemisphere isolated.

An aliquot was diluted 1:1 with trypan blue and dye-excluding cells were counted in a Neubauer counting chamber. The remaining cells were diluted in NB medium with the above supplements and plated onto the substrates at a density of 16,000 cells/cm$^2$. Half of the medium was changed every 3–4 days.

2.4. Investigation of neurite adhesion

To study neurite adhesion to solid and interrupted lines in the micropattern, the cells were subjected to mechanical stress before staining with anti-neurofilament-M (see next section). Cells were washed in PBS buffer (Gibco #14190-094) removing the washing solution with a vacuum pump (Vacufuge, Integra Biosciences at a pressure of $-100 \text{ mbar}$) onto which a Pasteur pipette was mounted.

2.5. Antibody staining

Cells were fixed in 2% paraformaldehyde for 20 min on ice then permeabilized with 5% acetone in 95% ethanol for 7 min at $-20^\circ \text{C}$. Incubation with primary antibody (rabbit anti-neurofilament-M, Chemicon #AB1987), diluted 1:200 in PBS containing 10% FCS and 0.02% NaN$_3$ (Sigma, F2442 and S2002) followed after three washes with PBS (Gibco #14190-094). Cells were washed again before incubation with secondary antibody (Cy3 conjugated donkey anti-rabbit, Dianova #711-166-152). After washing, cells were mounted in mounting medium from DAKO (#S 3023). The culture was covered with a coverslip and sealed with colorless nail polish. The substrates were viewed using a Laser Scanning Confocal Microscope (LSM-FCS, Version 2.8SP, Zeiss).

2.6. Electrophysiology

Patch clamp recordings were performed using a triple patch clamp setup (EPC9/3, Heka Elektronik) in current clamp mode, typically at DIV 11–15. Borosilicate micropipettes (1810016, Hilgenberg) with a resistance of $20–60 \text{ MQ}$ were pulled using a micropipette puller (P-97, Sutter Instrument Company). Stepper motor based micro-manipulator units (Mini 25, Luigs and Neumann) under microscopic control (IX 50, Olympus) were used for manipulation. For current clamp recordings, membrane current was set to zero; action potentials were stimulated with 20 or 100 ms pulses of 130 pA. The extracellular solution contained (mM): KCl, 5; NaCl, 150; MgCl$_2$, 1; HEPES, 10; CaCl$_2$, 2.5; glucose, 10; pH 7.4, adjusted with 1 M NaOH. The intracellular solution contained (mM): potassium glutamate, 125; KCl, 20; CaCl$_2$, 0.5; MgCl$_2$, 2; HEPES, 10; EGTA, 5; ATP, 4; pH 7.4, adjusted with 1 M KOH.

3. Results

3.1. Investigation of the patterned surface

We already described earlier neuronal network formation on surfaces patterned by microcontact printing of extracellular matrix (ECM) proteins and poly-D-lysine on polystyrene (Vogt et al., 2003). In the presented work, modified patterns were used in which one continuous and three interrupted lines emerged from each node in the grid pattern analogous to the patterns designed by Stenger et al. In order to investigate the fidelity of pattern transfer and specifically...
how reliably the gaps in interrupted lines were realized, some samples were prepared in which the inking solution was mixed with the fluorescent dye sulforhodamine. This allowed to visualize the stamped pattern under light of the appropriate wavelength. As shown in Fig. 1, protein transfer took place exclusively in the regions defined by the stamp, resulting in well-defined protein patterns against an uncoated background. The gaps along the interrupted lines are clearly visible and do not seem to be contaminated with protein material. In contrast, the solid line emerging from each node point is evenly coated, providing a continuous adhesive path for neurite outgrowth.

To investigate the properties of the protein pattern in more detail, AFM measurements were performed (Fig. 2). These show an average height of 100 nm for the protein layer and a steep slope at the edge of the imprint. Specifically, the surface profile shows that the gap separating the adhesion node from the next island is free of protein.

We conclude that the fabrication of micropatterns containing two distinct types of lines was successful: the solid line is continuously coated with protein, while the intermittent lines are interrupted by gaps of bare polystyrene.

3.2. Cell culture on the patterned substrates

Having established the properties of the micropattern from the material side of view, we aimed to compare the adhesive properties of the continuous and the interrupted paths with respect to their interaction with an elongating neurite.

Rat embryonic cortical neurons were seeded onto the micropatterned surface. Cell bodies adhered to the node points of the grid with high fidelity while neurite extension occurred along both types of lines. After several days in culture, the formation of geometrically defined networks could be observed similarly as on uninterrupted micropatterns. A typical picture is shown in Fig. 3.

To investigate neurite adhesion to the different types of lines, we subjected a relatively young patterned culture (4 days in vitro) to mechanical stress by applying force by aspiration with a vacuum pump. This treatment has been shown before to displace neurons and neurites from their adhesion spots depending on the applied force (unpublished observation). The cells were then stained with an antibody against the neuron specific protein neurofilament-M and visualized under a laser-scanning microscope. Fig. 4 shows a single cell.
occupying an adhesion spot. Clearly, the applied mechanical stress was not strong enough to pull the cell body off the node point nor to displace the neurite from the solid line. Conversely, the neurite is dislocated from the non-coated regions of the interrupted line while remaining attached to the adhesive islands: it forms loops spanning the gaps from island to island. Evidently, neurite attachment was restricted to the protein pattern and did not (or to a significantly smaller extent) occur on the small interruptions of bare polystyrene although these areas were crossed by the extending neurite. It can be concluded that the micropattern exhibited the desired properties: the provision of one continuous adhesive pathway emerging from each node and pathways consisting of alternating adhesive and non adhesive regions in the three other directions.

3.3. Investigation of neuronal polarity by patch clamp measurements

The ultimate aim of controlling neuronal polarity on a micropattern lies in the formation of functional networks of experimentally defined pathways of in and output. We therefore saw the most suitable means to investigate the orientation of axons and dendrites in the network in double patch clamp measurements: two cells were patched simultaneously in the current clamp mode. Action potentials were evoked in one cell at a time while the effect on the other cell was monitored without applying stimuli. Synapses were identified when action potentials but not subthreshold stimulations consistently induced a depolarization in the other cell. The orientation of pre- and postsynaptic cells on the pattern allowed inferring the direction of presynaptic axonal outgrowth. A typical recording of two synthetically coupled cells is shown in Fig. 5. When the orientation of pre- and postsynaptic cell indicated that axonal outgrowth had occurred along the continuous line, synapse orientation was counted as correct. However, when the axon had elongated along one of the three interrupted paths emerging from each adhesion node innervating another cell in that direction, synapse orientation was counted as wrong (see Fig. 6). Table 1 shows the percentage of correctly oriented synapses on the different applied micropatterns.

As four pathways were available for axonal outgrowth, 25% of all synapses would have been expected in either orientation on a symmetrical pattern. We observed only a very slight enrichment over these 25% of synapses oriented in the anticipated direction. This enrichment does not reach statistical significance (P value for a bilateral binominal test 17%). Thus, the observed effect can be regarded as insufficient for applications aiming to orient signal transduction within a network. Strikingly, the percentage of correctly oriented synapses does not increase with increasing gap size. It therefore appears unlikely that our failure to orient axonal

Orientation of synapses on the described micropatterns

<table>
<thead>
<tr>
<th>Pattern</th>
<th>Gap size (μm)</th>
<th>Correctly oriented synapses (number)</th>
<th>Incorrectly oriented synapses (number)</th>
<th>Correctly oriented synapses (% of total)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>1</td>
<td>10</td>
<td>16</td>
<td>38.5</td>
</tr>
<tr>
<td>B</td>
<td>2</td>
<td>3</td>
<td>5</td>
<td>37.5</td>
</tr>
<tr>
<td>C</td>
<td>5</td>
<td>4</td>
<td>11</td>
<td>26.6</td>
</tr>
<tr>
<td>Total</td>
<td></td>
<td>17</td>
<td>32</td>
<td>34.7</td>
</tr>
</tbody>
</table>

Synapses were identified by patch clamp measurements. Counted as "correctly oriented" were those synapses in which the presynaptic cell had extended its axon along the solid line rather than along one of the interrupted ones (schematically depicted in Fig. 6).

In summary, we found that although the micropattern fulfills the desired criteria by providing contrasting pathways of different adhesiveness for neurite outgrowth, orientation of neuronal polarity along the anticipated axis did not occur to the extent observed by Stenger et al.

3.4. Neurite elongation on interrupted and continuous lines

The mechanism through which orientation of axonal outgrowth was thought to be induced was a different rate of neurite elongation on the two types of lines. It has been reported that the length of an undifferentiated neurite at an early stage after cell attachment in vitro is critical for the differentiation of this neurite into an axon (if it is the first to exceed a critical length) or into a dendrite (if not) (Dotti and Banker, 1987). We therefore investigated whether the antiadhesive “speed bumps” in the interrupted lines actually succeeded in slowing down neurite extension. Again, antibody staining using anti-neurofilament-M was performed on a patterned culture at DIV 4, in which the neurites were still relatively short. Fig. 7 shows a typical picture: while the left cell behaves as expected, extending its principal neurite along the solid line, the major (and presumably axonal) process of the cell on the right follows an interrupted line while a shorter process grows along the continuous path. It appears that even if a cell has the chance to explore different pathways and encounter areas of different adhesiveness, this does not necessarily lead to a faster elongation along the more adhesive pathway.

4. Discussion

The importance of extracellular signals for the development of neuronal polarity in vivo remains a much debated...
question (Horton and Ehlers, 2003). Consequently, the possibilities for a specific surface to direct axonal and dendritic outgrowth may be limited by providing only one of a number of cues relevant to this process in vivo.

The presented results indicate that a synthetic substrate containing pathways of different adhesiveness is not able to control the development of neuronal polarity in vitro or to direct the speed of neurite elongation in different directions to a significant extent.

It can be asked whether pathways of different adhesiveness should be expected to control the relative speed of neurite outgrowth thereon. The impact of substrate adhesiveness on the extension rate of axons has been investigated in a different context by Isbister and O’Connor (1999) and Lemmon et al. (1992). Although extension rates were established to differ significantly and reproducibly between different substrates, little correlation was found between the adhesiveness of a surface and neurite elongation rate. Both groups concluded that although cell substrate interactions play a critical role in axon guidance and also in the control of filopodia extension rates, these effects do not appear to be principally mediated via the relative adhesiveness of different surface areas. Similarly, studies performed by Clark et al. (1993) argue against an impact of antiadhesive regions on micropatterned substrates on the speed of neurite elongation: the group investigated the behavior of embryonic neurons from chick and mouse on laminin line patterns. Neurons were not guided by patterns in which the antiadhesive interruptions were small enough for the neurite to bridge (2–12 μm) and no difference in the length of neurites growing parallel or perpendicular to the line pattern was observed. Therefore, it appears that the effect described in the system of Stenger et al. has to be attributed to qualities of the micropattern different from the divergent adhesiveness of continuous and interrupted pathways as had been assumed.

It remains an intriguing question which features of the quoted system were crucial for the observed effect. As described in Section 1, our system presented three major differences to that of Stenger et al.: networks consisted of cortical rather than hippocampal neurons, microcontact printing rather than laser ablation was used as the patterning method, and another surface chemistry was applied (a mix of ECM proteins on polystyrene rather than aminated and fluorinated silanes). An additional variable lies in the fact that Stenger et al. investigated polarity on isolated cells as early as DIV 2–4 while we analyzed more mature networks of interconnected cells at DIV 11–15.

One explanation for the different outcome of our experiments is that hippocampal and cortical neurons react differently to particular surface molecules such that the described effect is cell type specific. It seems possible that hippocampal neurons respond more sensitively to matrix associated cues while cortical neurons, e.g. predominantly rely on gradients of secreted molecules as demonstrated by Polleux et al. (2000). Alternatively, the key feature of the surface created by Stenger et al. could be related to the patterning technique: while the edges of the two contrasting surface areas in substrates created by microcontact printing are very abrupt as shown by AFM imaging (Fig. 2B), laser ablation creates a gradient of permissive molecules at the edges of the pattern. Such a gradient may present a signal to an elongating neurite slowing down its extension. Another possible explanation would be that the specific chemical groups that were applied rather than their relative adhesive properties are decisive in delivering the signal. It could, for example, be conceived that fluorinated molecules slow down neurite elongation or that amine groups directly promote axonal differentiation.

The applied patterns also differ in the fact that Stenger et al. utilized isolated adhesive islands which did not permit neighboring cells to interact with each other. A grid pattern allowing cell–cell contacts and the wiring of an actual network may present additional stimuli impinging on cellular polarity.

It can also be speculated that the timepoint of investigation played a role in the experimental outcome. It seems possible that initial axonal outgrowth occurred along the continuous line in both systems alike but that later additional processes extended along a given line, overtaking the signaling pathway. This interpretation however seems unlikely with regard to Fig. 7, which shows a major—presumably axonal—process extending as the first neurite along an interrupted line at DIV 4. Whichever feature ultimately proves to be responsible for the observed effect, it is indicated that although the rate of neurite outgrowth and probably also the establishment of neuronal polarity most likely are—at least partially—regulated by interactions with extracellular matrix molecules, the actual mechanisms delivering these effects are much more complex than simple differences in the relative adhesive strength of alternative pathways.

A dependence of axonal versus dendritic differentiation on the underlying substrate has been described for a number of naturally occurring adhesion molecules in vitro (Chamak and Prochiantz, 1989; Lein et al., 1996). A promising approach to the orientation of neuronal polarity may therefore lie in the creation of a pattern in which two chemically different permissive pathways emerge from each adhesion node. An important step in this direction has been taken by Wheeler et al. (1999), who report a preference for axons to grow on laminin coated surfaces and dendrites to extend on mixtures of laminin and polylysine when given the choice on a patterned substrate. So far, the approach has not yet been applied to a grid pattern.

It will be interesting to find and optimize methods to control the development of neuronal polarity and to combine them with a system that supports the survival of patterned networks long enough to observe functional synapses. Such a system will be useful for investigations on network formation and the impact of different connectivity patterns on network activity and plasticity.
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