

Modulation of the growth and guidance of rat brain stem neurons using patterned extracellular matrix proteins

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Abstract

Dissociated neuronal cultures on substrates patterned with extracellular matrix (ECM) proteins have yielded much information regarding the physiological characteristics of neuronal cells behaviour *in vitro*. However, neuronal patterning using long term embryonic brain slice cultures has not been comprehensively demonstrated to-date. Structuring was performed by micro contact printing of laminin. The slice cultures were evaluated by means of phase contrast microscopy at 3–22 days in culture. We were able to consistently achieve outgrowth of neurons, neurites and filopodia from brain stem slices cultured on ECM proteins structures of grid- and line-shapes. We believe that brain slice cultures on patterned substrates is a favourable approach to study functional synapses *in vitro* under defined conditions. The use of appropriate structures and the subsequent cell patterning may help to gain further understanding of axonal, dendritic and synaptic signal transductions and processes. © 2001 Elsevier Science Ireland Ltd. All rights reserved.

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Widespread cell migrations are a prerequisite of early brain development [2,6,9,10–12]. The control of cell migrations *in vitro* may help us to understand the organisation of neurons during embryonic development. Furthermore, the control growth of neurons and their processes on defined patterns can provide a better visualisation of neuronal connections and synaptic formations. The use of extracellular matrix proteins (ECM), such as laminin in producing patterned substrates using micro contact printing [13] can provide such an answer. The growth and guidance of neurite outgrowth have been described [17]. Although a considerable number of reports regarding dissociated cells patterning exist [3,4,15,18], the feasibility of migrating embryonic brain slice neurons on patterned substrates has not been comprehensively demonstrated to-date. In the present study, we aim to demonstrate the possibility of combining ultra-thin brain slices and the most suitable micro-patterned guidance structures for many neurological investigations.

Cultures of brain stem slices were prepared based primarily on the method described by Gähwiler [7,8] with some

crucial modifications. Upon extraction of the whole embryonic 15–18 days old Sprague–Dawley rat brains, the medulla and pons were removed by a transverse section through the rostral pons and the cerebellum removed by sectioning the peduncles. Coronal sections of brain stem slices were harvested in sterile conditions in chilled brain slice culture medium (pH 7.4) made from HAMS F10 supplemented with 20–25% foetal bovine serum and 4 mM glutamine, all from SIGMA. The slices, which were cut using McIlwain Tissue Chopper, were 250 μm thick. The thus prepared slices were placed in an incubator at 37°C and a 5% CO₂ enriched atmosphere for 4–5 h minimum. After this incubation period, the slices were then positioned onto controls (laminin-coated) and test substrates (laminin-patterned) using a small surface-polished spatula. Unlike the method described by Gähwiler, plasma clot or collagen was not used in immobilising the slices as either method would hinder cell migrations, particularly migrations onto ECM patterns. Furthermore, the roller tube technique was deemed unnecessary. Instead, in all culture dishes, a critical amount of medium was added such that the slices did not detach from the surface. After 2–3 days in culture, the slices should have undergone significant extent of migration and more medium could be added at that stage.

Microstamps for the experiments were produced by

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photolithography and moulding as described earlier [13,14]. An electron beam writer transposed the different structures designed for the experiment to a chrome mask. Applying UV-photolithography, master stamps were produced out of spin coated 12.5 μm thick photoresist layers (AZ 4562, Clariant GmbH, Germany) on 0.6 mm thick silicon wafers (MEMC Electronic Materials, Germany). Polydimethylsiloxane (PDMS) microstamps were then fabricated curing Sylgard 182 (Dow Corning, Germany) in 10 ml eppendorf tubes for 48 h at 55°C upside down on the master stamps. After master stamp release final curing was performed for 1 h at 110°C. In order to increase the stamp hydrophilicity, PDMS stamps were stored in deionized water for 24 h. Prior to patterning, stamps were sterilised in a 70% ethanol bath for 1 min. Inking took place for 30 s in 25 $\mu\text{g}/\text{ml}$ of laminin solution (1243217, Boehringer Mannheim GmbH, Germany). The inked stamp was then dried in a soft nitrogen airstream and immediately pressed onto the substrate for 10 s. In all experiments, non tissue culture polystyrene petri

dishes of 3 cm diameter (Greiner Labortechnik, Germany) were used.

Under control conditions (non-laminin patterned), neurons migrated away from the adhered slice progressively with time. The entire culture quickly became confluent near the edge of the slice (within 3–4 days) and diffused networks of migrated neurons were visible (Fig. 1). In the present study, six laminin patterns with varying node size and track width were used. The different patterns used and the growth and migration of neurons at approximately day 3 and 10 are shown in Fig. 2. Regardless of the pattern used, by the end of 2 weeks, the axons reached maturity and became thickened and, in 3 weeks, the neurons and their processes would usually have migrated over 1000–2000 μm of uninterrupted patterns. It is clear that the growth of neurites was not possible on areas of polystyrene surface without any laminin transferred from the stamp until much later into the culturing period. However, in certain samples, some neuronal processes crossed non-laminin areas (Fig. 2b).

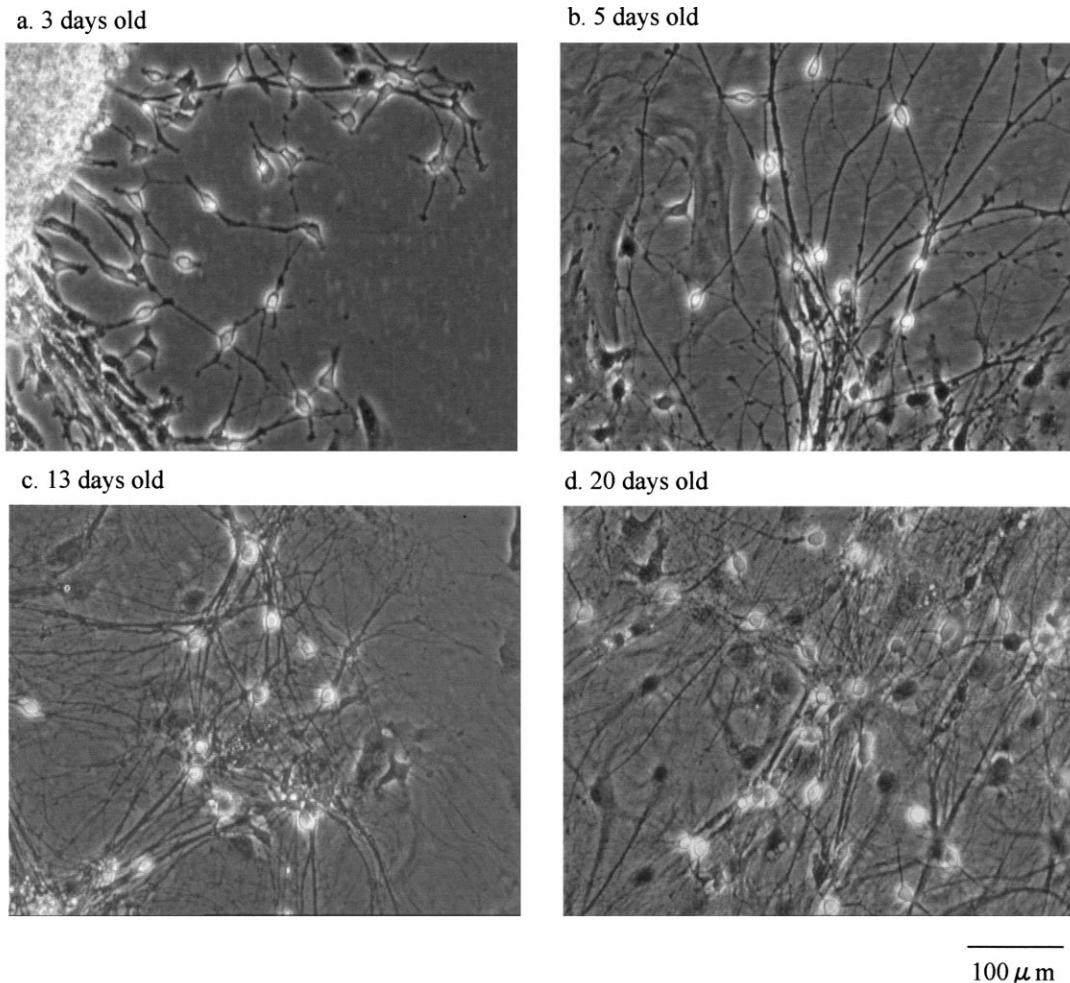


Fig. 1. (a–d) Migration of embryonic brain stem neurons on laminin-coated tissue culture plastics. On day 3 (a) in culture, migrated neurons were clearly identifiable and growth of neurites has already begun. On day 5 (b), these dendritic and axonal processes had grown to significant length. By 13 (c) and 20 (d) days, the culture became confluent with all the neurons forming diffused network on top of glial cells and other non-neuronal cells.

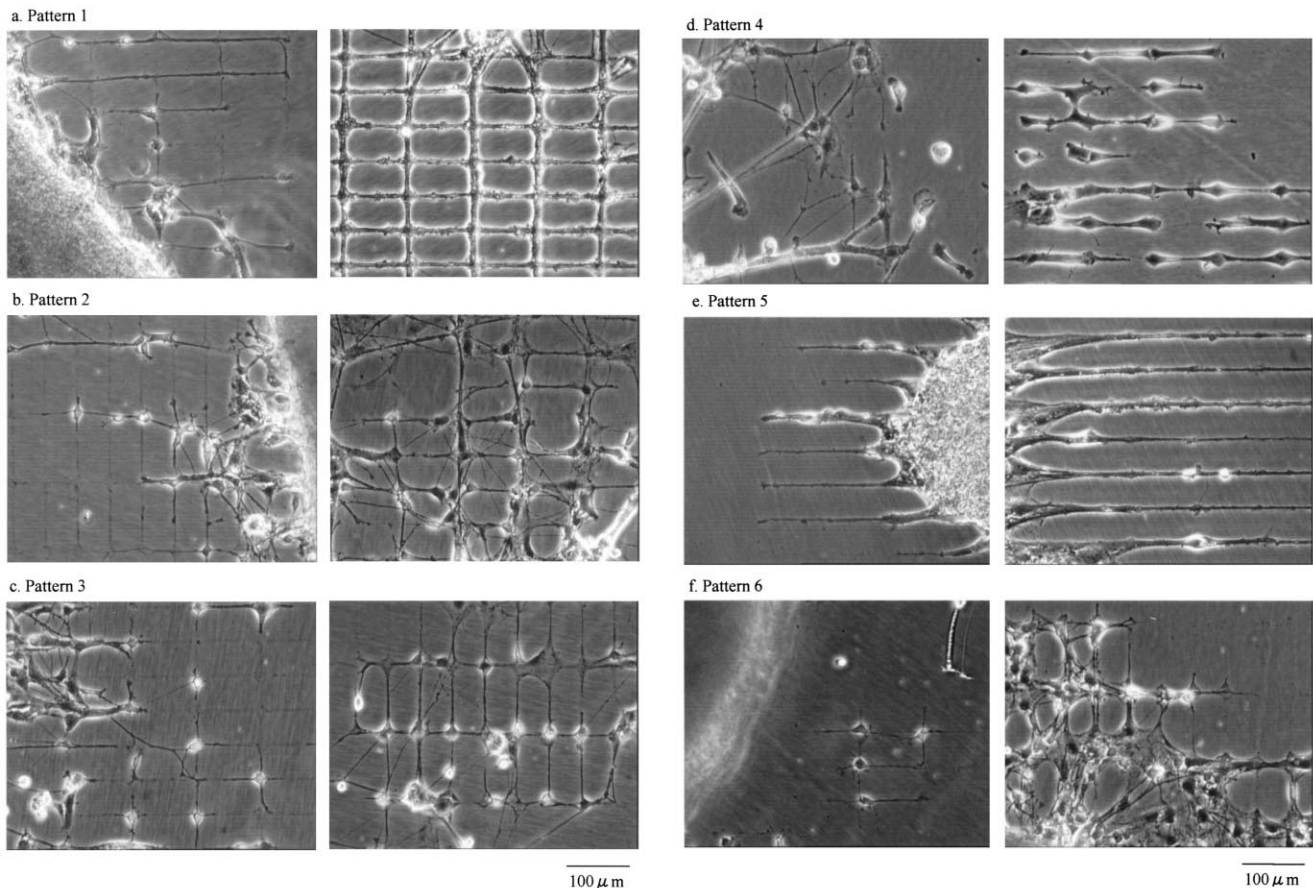


Fig. 2. Evaluation of variety of extracellular matrix proteins patterns. (a) Pattern 1, node 10 μm , track 2 μm ; (b) pattern 2, 12 μm , track 4 μm ; (c) pattern 3, node 14 μm , track 6 μm ; (d) pattern 4, node 20 μm , track 4 μm ; (e) pattern 5, node 10 μm , track 2 μm ; (f) pattern 6, node 22 μm , track 4 μm . During early days in culture (left day 3), the migrated neurons and growing processes were clearly visible with little overlapping on all the patterns. These cultures continued to mature and by 10 days or more, the original shapes of the laminin stamped patterns were identifiable.

These might have been due to slight damage/imperfections on the stamp or traces of laminin accidentally got onto the unintended areas. The crossing over happens a lot less frequent when new stamps are being used. The track width and the node size were crucial to the success of neuronal slices patterning. Although with the 2 μm track (node = 10 μm) a near perfect pattern was achieved (pattern 1, Fig. 2), very few visually identifiable neurons actually migrated to the pattern. It may be possible that, because the imposed pattern limited the freedom of growth, the oligodendrocytes began to cover neurons that were on the pattern. Patterns with node size of 10–14 μm (pattern 1–3, Fig. 2) were the most appropriate for the localisation of neurons. However, these experiments showed that the best neuronal migration occurred with 6 μm tracks and 14 μm node (pattern 3, Fig. 2). It is known that cells tend to migrate towards the nodes and become immobilised [14]. The same phenomenon was observed with brain slice neurons. After a number of days in culture, the cells were beginning to form a simple linear neuronal network. In contrast to the disso-

ciated cell culture, it is crucial to apply straight, uninterrupted patterns. In additional experiments (data not presented here) we have found that even small gaps of 5 μm in the pattern tremendously reduce neuronal spreading from the slice onto the patterned surface. Patterns with thick, 6 μm wide tracks lead to the best results as neuronal spreading from the slice into the pattern was faster than on thinner tracks. In contrast to the observations made with dissociated cultures [5,13,14], only very few cell clusters were visible along the thick tracks. This is because only neurons departing from the slice can migrate along the tracks of the pattern. However, in the dissociated culture, a huge number of neurons settle on the tracks almost simultaneously during cell seeding thus increasing the chances of clustering. The best focus of neurons onto the nodes was achieved with 20 μm nodes for the line structures (pattern 4, Fig. 2d) and 14 μm nodes for the grid structures. These results are in good accordance to prior studies [13,14]. Smaller nodes (10 μm) and thin tracks (pattern 5, Fig. 2e) did not encourage neuronal migration. On the contrary,

larger nodes (22 μm) applied in the grid structures (pattern 6, Fig. 2f) did not lead to better results but to a significant increase of overgrowth by non-neuronal cells.

There are some important differences between cell patterning using dissociated cells and brain slices. The tissue slice contains neurons and all other ‘supporting’ cells that are necessary for a more ‘complete’ physiological make-up of the brain. It has been shown that glial cells and astroglial cells (astrocytes) are important for early cell migrations, development, neuronal survival and plasticity [1,9,11,12,16]. Furthermore, the interconnections between neurons are more intact. The cultivation of brain slices on defined substrates enables individual neurons and connections between neurons to be directly visualised, which may be useful in a variety of neurobiological investigations. The present study demonstrates clearly that it is possible to manipulate cells from migrating neurons of brain slices onto a number of appropriate patterns. Although many have shown similar findings using dissociated cultures, this is the first study that has comprehensively looked at the possible use of whole tissue on patterned substrates.

The present direction of our laboratory is to further investigate the control of the cell placements on the most appropriate pattern(s) and to evaluate synaptic formations on laminin patterned substrates using standard electrophysiological techniques. With reproducibility being the main issue, we are currently modifying and optimising the culture protocol and the patterning strategy in order to generate consistently near perfect neuronal network for a variety of physiological and pharmacological investigations.

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