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Hippocampal neurons respond uniquely to topographies of various sizes and shapes

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Abstract

A number of studies have investigated the behavior of neurons on microfabricated topography for the purpose of developing interfaces for use in neural engineering applications. However, there have been few studies simultaneously exploring the effects of topographies having various feature sizes and shapes on axon growth and polarization in the first 24 h. Accordingly, here we investigated the effects of arrays of lines (ridge grooves) and holes of microscale ($\sim 2 \mu\text{m}$) and nanoscale ($\sim 300 \text{ nm}$) dimensions, patterned in quartz (SiO_2), on the (1) adhesion, (2) axon establishment (polarization), (3) axon length, (4) axon alignment and (5) cell morphology of rat embryonic hippocampal neurons, to study the response of the neurons to feature dimension and geometry. Neurons were analyzed using optical and scanning electron microscopy. The topographies were found to have a negligible effect on cell attachment but to cause a marked increase in axon polarization, occurring more frequently on sub-microscale features than on microscale features. Neurons were observed to form longer axons on lines than on holes and smooth surfaces; axons were either aligned parallel or perpendicular to the line features. An analysis of cell morphology indicated that the surface features impacted the morphologies of the

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soma, axon and growth cone. The results suggest that incorporating microscale and sub-microscale topographies on biomaterial surfaces may enhance the biomaterials' ability to modulate nerve development and regeneration.

1. Introduction

Much of the focus within the nerve tissue engineering field has been dedicated to engineering stimulative microenvironments to regenerate damaged nerve tissue by modulating the responses of individual neurons. The microenvironment consists of various chemical and physical cues produced naturally or synthetically, which influence the behaviors of cells [1]. Tissue engineering scaffolds have been fabricated to provide a permissive microenvironment capable of facilitating cell growth and differentiation, and forming tissue with the desired architecture [2]. Improvements in biomaterials can be achieved by introducing extracellular physical and chemical cues, or a combination thereof, on biomaterial surfaces to influence tissue development on scaffolds [1].

Structures with dimensions of the same scale as individual cells have been shown to modulate cell differentiation, migration and shape. Accordingly, the incorporation of cellular-scale (micron-scale) topography on tissue scaffolds may be important in better regulating tissue growth by providing cells with an interface that simulates cells' natural extracellular environment. Requisite to introducing the necessary microscale physical stimuli to cells to control tissue formation is understanding the link between specific surface-bound physical cues and cell response at the single-cell level.

Over the last few decades, there has been a growing interest in applying microfabrication techniques to strategically pattern surfaces with the goal of either altering biomaterial surface properties or simply improving a biomaterial's ability to provoke particular cell responses [3]. In the late 1990s, Curtis *et al* fabricated surfaces with grooved structures and deemed that groove dimensions highly affected cell behavior [4]. They also stressed that cell response depends heavily on cell type, where cell-surface interactions observed for one type of cell were different from those for another cell type exposed to identical structures. Dalby *et al* [5, 6] cultured fibroblasts on an array of groove ridges in which the grooves had a 12.5 μm width and 2.5 μm depth. They found that the fibroblasts tended to embed and align themselves within the grooves or on top of the ridges. Berry *et al* [7] monitored the proliferation of fibroblasts on arrays of circular holes ranging from 7 to 25 μm in diameter at spacings of 20 and 40 μm . They found that the fibroblasts proliferated to a greater degree on holes with smaller diameter and smaller pitch. Teixeira *et al* [8, 9] found that the elongation and alignment of human corneal epithelial cells (HCECs) cultured on lines of 70 nm width (width of ridges) and 400 nm pitch depended heavily on feature depth. The HCECs were markedly more aligned for lines with a 600 nm depth than those of 150 nm depth. Increases in feature pitch from 400 nm up to 2 μm showed little effect on alignment, and the HCECs did not align with the lines when the pitch was increased to 4 μm . Teixeira *et al* emphasized the importance and direct link between feature size and the behavior of HCECs.

Rudimentary investigations have been conducted to study the behavior of neurons on micropatterned substrates. Goldner *et al* [10] cultured dorsal root ganglion neurons on ridge-

groove structures of width and depth in the tens of microns. They observed the neurons anchoring on the ridges and forming neurite bridges across the grooves. Rajnicek *et al* [11, 12] investigated the effects of ridge-groove structures of microscale dimensions on the contact guidance of rat hippocampal neurons. They investigated groove widths of 1, 2 and 4 μm at various depths ranging from 14 nm to 1.1 μm . Axons were found to align perpendicularly to the lines as groove width and depth decreased. Deep, wide channels that were 4 μm wide and 1.1 μm deep guided the neurons to migrate into the grooves and align their axons parallel to the lines. Rajnicek *et al* did not study neuronal differentiation, such as initial axon formation (i.e. polarization) and axon elongation, for neurons cultured on different features. Johansson *et al* [13] studied axonal alignment on ridge-groove patterns with grooves varying from 100 to 400 nm for pitches from 200 to 2000 nm. Structure depth was maintained at 300 nm. They found that nanoscale patterns induce parallel alignment, though alignment becomes less pronounced as groove width is reduced; moreover, changes in pitch (ridge width) seemingly had little effect on alignment. Johansson *et al* studied axonal alignment after 1 week and also stimulated axon growth using nerve growth factor, so they were not able to isolate the effects of the ridge grooves on axon growth or study axon polarization, which normally occurs within the first 24–48 h in culture.

Gomez *et al* [14, 15] were the first to study the effects of micropatterned topography on axonal establishment and elongation. They showed that polarization was more likely to occur on synthetically patterned lines of 1 μm and 2 μm widths than on smooth surfaces. Yet, they did not study interactions between neurons and topography for features of varying shape, including circular holes, or features with sub-microscale dimensions ($<1 \mu\text{m}$).

In attaining a more thorough understanding of the influence of physical surface structures on neurons, it is pivotal that cell–surface interactions be studied for different feature sizes and shapes (in the same set of experiments). Here, we investigate the effects of feature size and shape among various micropatterned topographies on (1) cell–substrate adhesion, (2) axon establishment (neuron polarization), (3) axon length, (4) axon alignment and (5) cell morphology of rat embryonic hippocampal neurons after 24 h in culture. We conducted experiments on four different homogeneous topographies including *lines* (i.e. ridge-groove structures) of 300 nm and 2 μm width (width of grooves) and 1 μm spacings (ridges), and holes of 300 nm and 2 μm diameter and 1 μm spacings, in both the horizontal and vertical directions. All structure depths were approximately 400–500 nm.

2. Materials and methods

2.1. Quartz substrate fabrication

25 mm² square quartz substrates were exposed to an oxygen plasma (50 sccm O₂, 300 W, 150 mTorr, 25 °C; Plasma-Therm 790, Plasma-Therm, Inc., St. Petersburg, FL, USA) for 10 min and immersed in a mixture of 25% hydrogen peroxide (30% H₂O₂ in water) (v/v) in sulfuric acid (piranha bath) for 10 min (hydrogen peroxide 30%, sulfuric acid 96%, JT Baker, Phillipsburg, NJ, USA). The substrates were removed from the bath, thoroughly rinsed in deionized water, dried with N₂ gas and dehydrated on a hot plate at 200 °C for 5 min.

After cleaning, a thin 30 nm layer of chromium was thermally evaporated onto the quartz at a rate of $5 \text{ \AA s}^{-1}$ (Explorer, Denton Vacuum, Moorestown, NJ, USA). ZEP-520A (Zeon Chemicals, Louisville, KY, USA) positive electronic resist was coated onto the chromium layer to a thickness of approximately 200 nm by spinning at 4000 rpm for 40 s; nominal layer thickness was reduced by diluting the ZEP in anisole to a concentration of 50% (v/v). After spin coating, the resist was baked on a hot plate at 180 °C for 150 s.

Arrays of structures were patterned in the ZEP using electron beam lithography (JEOL 6000 FSE, JEOL Ltd, Tokyo, Japan; Raith 50, Raith GmbH, Dortmund, Germany) with a beam fluence of $100 \mu\text{C cm}^{-2}$ and subsequently developed in ZED-N50 (Zeon Chemicals, Louisville, KY, USA). Isopropyl alcohol (Thermo Fisher Scientific, USA) was used as the etch-stop during the developing process. The substrate was dried with a slow stream of N_2 gas. The ZEP resist served as a dry-etch mask for the underlying chromium layer, which provided a selectivity close to 3:1 chromium:ZEP. A reactive-ion-etching (RIE) (Trion Technology, Clearwater, FL, USA) process was used to etch through the chromium. The first step was a descum O_2 plasma treatment to remove residual resist from developed regions. The second step was the chromium etch step, which etched completely through the chromium layer to the quartz. The remaining ZEP resist was stripped during the following quartz etch. The chromium layer served as the etch mask for the underlying quartz with a selectivity of over 10:1 quartz:chromium. The quartz was etched down about 400 nm. After quartz etching, the remaining chromium was stripped with a chromium wet-etchant (Etchant 1020, Transene Company, Danvers, MA, USA) at 40 °C for 2 min. The quartz was then thoroughly washed in a piranha bath for 10 min and stored in DI water for later experimentation.

2.2. Design and characterization of topographies

Topographies consisting of either lines or holes were strategically chosen based on previous results in the literature [4, 8, 11, 14]. Structure arrays are shown in figure 1 with dimensions summarized in table 1. Four topographies were designed: (1) holes with a $2 \mu\text{m}$ diameter and horizontal and vertical spacings of $1 \mu\text{m}$, (2) lines of $2 \mu\text{m}$ width and a spacing of $1 \mu\text{m}$, (3) holes with a 300 nm diameter and horizontal and vertical spacings of $1 \mu\text{m}$, and (4) lines of 300 nm width and a spacing of $1 \mu\text{m}$. We refer to the 300 nm structures as ‘sub-microscale’ since we believe that the syntax more accurately depicts the size of the structures relative to the larger microscale structures ($>1 \mu\text{m}$) and smaller nanoscale structures ($<100 \text{ nm}$). The depth of all structures was approximately 400–500 nm.

Atomic force microscopy (AFM) and scanning electron microscopy (SEM) were used to characterize the quartz substrates. AFM images were taken to ensure precise dimensions of topography. AFM images were acquired with a Dimension 3100 with Nanoscope IV controller (Digital Instruments & Veeco Metrology Group, Santa Barbara, CA, USA) using a silicon tip in tapping mode (Tap300, Budget Sensors, Sophia, Bulgaria). SEM images were acquired with a Zeiss SUPRA 40 VP Scanning Electron Microscope (Carl Zeiss, Peabody, MA, USA) after depositing a 10 nm layer of platinum/palladium (208HR, Cressington Scientific Instruments, Watford, UK).

2.3. In vitro hippocampal cell culture

Square wells of 1.5 cm² inner area were molded in polydimethylsiloxane (PDMS) (Slygard 184, Dow Corning, Midland, MI, USA). The wells were placed on each patterned quartz substrate and sterilized by exposure to ultraviolet (UV) radiation for 2 h. Sterilized substrates were incubated in 0.1 mg mL⁻¹ poly-D-lysine (Sigma-Aldrich Corporation, St. Louis, MO, USA) overnight and subsequently washed twice with sterile double-deionized water. Samples were dried in a sterile laminar flow bench and stored at 4 °C until used in cell culture experiments.

Rat embryonic hippocampal neurons (E18) were isolated from commercial rat hippocampal tissue (BrainBits, Springfield, IL, USA) according to the manufacturer's protocol. The hippocampus was incubated in 4 mg mL⁻¹ papain solution (Worthington, Lakewood, NJ, USA) in Hibernate E medium (BrainBits, Springfield, IL, USA) at 30 °C for 20 min. A fire-polished Pasteur pipette was used to triturate the hippocampal tissue, followed by centrifugation at 200 g for 1 min. A cell pellet was suspended in 1 mL of warm culture medium consisting of Neurobasal medium (Invitrogen, Gaithersburg, MD, USA) supplemented with 2% B-27 supplement (Invitrogen), 0.5 mM L-glutamine (Fisher Scientific, Pittsburgh, PA, USA), 0.025 mM glutamic acid (Sigma-Aldrich) and 1% antibiotic-antimycotic solution (Sigma-Aldrich). Cells were seeded on the quartz substrates with a seeding density of 2×10^4 cells cm⁻² in the culture medium and incubated at 37 °C and 5% CO₂ for 24 h.

2.4. Immunofluorescence and image analysis

Embryonic hippocampal neurons cultured on the substrates were fixed with 4% paraformaldehyde (Sigma-Aldrich) and 4% sucrose (Sigma-Aldrich) in phosphate-buffered saline (PBS) (pH = 7.2) for 20 min at room temperature. Fixed samples were permeabilized with 0.1% Triton X-100 (Fluka, St. Louis, MO, USA) and 3% goat serum (Sigma-Aldrich) in PBS for 20 min, washed twice with PBS, and treated with a blocking solution of 3% goat serum in PBS for 1 h at 37 °C. Tau-1, a microtubule protein expressed in axons, was labeled as an axonal marker. Mouse tau-1 antibodies (Chemicon, Temecula, CA, USA) were diluted to 1:200 in a blocking solution and added to the culture samples. After overnight incubation at 4 °C, the samples were washed with PBS two times, treated with a secondary antibody solution of Alexa 488-labeled goat anti-mouse IgG (Invitrogen) (1:200 dilution in blocking solution) at 4 °C for 5 h, and rinsed in PBS for 5 min two times. The samples were stored at 4 °C while awaiting further analysis.

Fluorescence and optical images of cells and axons were acquired using a fluorescence microscope (IX-70, Olympus). The images were captured using a color CCD camera (Optronics MagnaFire, Goleta, CA, USA). Cell images were analyzed using ImageJ software (National Institutes of Health). Axon length was measured as the linear distance between the soma junction (point of axon initiation from the soma) and the distal tip of the axon. When several neurites branched from a single neuron, the length of the longest axon was measured and recorded. A neuron was considered to be polarized only when the axon was approximately two times longer than the characteristic diameter of the cell body, as previously described [16]. Only individually isolated cells were analyzed in our

experiments. Axon alignment relative to the line topographies was determined by measuring the angle subtended by a line extending from the soma junction to the growth cone and the direction of the edges of the lines.

2.5. Examination of cellular morphologies

SEM was utilized to evaluate cell and axon morphology on the topographies. Fixed hippocampal neurons were dehydrated by treating with ethanol in water at successively increasing concentrations of ethanol; cells were treated at concentrations (v/v) of 30% for 45 min, 50% for 30 min, and 70%, 85%, 90%, 95%, and absolute ethanol (100%) (Pharmco, Brookfield, CT, USA) for 10 min each. Water was completely removed by adding hexamethyldisilazane (HMDS) (Sigma-Aldrich) and drying in air under ambient conditions. The dried samples were coated with a thin 10 nm layer of platinum/palladium by sputter coating (208HR, Cressington Scientific Instruments, Watford, UK). SEM images were acquired with a Zeiss SUPRA 40VP Scanning Electron Microscope.

2.6. Statistical analysis of experimental data

Data were analyzed using a combination of balanced one-way analysis of variance (ANOVA) [17] or standard two-sided *t*-tests for measurement [18] (e.g., length and angle data) and binomial (e.g., % data) [19] data. *P* values were calculated from a standard *t*-distribution tabulation and reported where applicable. The criterion for statistical significance was $p < 0.05$. Standard deviation (SD) is indicated in the figures with error bars. In the cases where data are not represented in a figure but are mentioned in the text, SD values are given. Sample sizes are indicated in the figures and in the text [n = sample size (usually # cells)].

3. Results

3.1. Design and fabrication of topographical substrates

We define a homogeneous topography as a surface formed when a single structural formation is arrayed over the entire area. Fundamental structural *shapes* include ridge grooves (i.e. lines) and holes, which represent structures arrayed in one direction and two directions. In addition to fundamental changes in shape, order-of-magnitude changes in feature dimensions represent another major modification to topography. Accordingly, we fabricated holes and grooves of different scales, i.e. microscale ($>1\ \mu\text{m}$) and sub-microscale ($<1\ \mu\text{m}$), which can provide intimate contact with cellular components such as the soma, axon and growth cone of neurons (table 1). Since the cell body of neurons is approximately 5–10 μm in diameter, a 2 μm feature has dimensions of the same order-of-magnitude as a typical neuron [20]. A 300 nm feature has dimensions of an order-of-magnitude smaller than those characterizing the size of a neuron. Features having sizes in the tens of microns and larger represent topography with dimensions of orders-of-magnitude larger than those characterizing the size of neurons. Surfaces with structures with dimensions of orders-of-magnitude larger than the neurons may be not recognized by the neurons in the same way as topography with dimensions of cellular ($\sim 1\text{--}10\ \mu\text{m}$) and sub-cellular (sub-microscale) scales. Consequently, we fabricated microscale sub-microscale topographical holes and

grooves (table 1) to investigate their interactions with individual neurons. Pitch was held constant, so we tested the effect of feature (hole and groove) dimension and shape.

3.2. Cell adhesion to topographies

Cell adhesion to each of the topographies was determined by counting the total number of anchored cells on the patterns 24 h after seeding (figure 2). The number of adhered cells on each topography was determined to be 117, 123, 90, 102 and 119 cells mm⁻² for the 300 nm lines, 300 nm holes, 2 μ m lines, 2 μ m holes and the smooth surface, respectively. Adhesion was statistically the same on the topographies as on the smooth surfaces ($p > 0.05$; $n = 9$); the standard deviation (SD) is shown by the error bars in figure 2.

3.3. Axon formation on topographies

We investigated the influence of the topographies on initial axon establishment (axon polarization) (figure 3(A)). Polarization on all the topographies was statistically greater than on smooth quartz (29.3%, SD = 2.3%) ($p < 0.05$); error bars in figure 3(A) show the SD of the polarization measurements. We did not see any difference in polarization among the topographies.

3.4. Axon elongation on topographies

Mean axon length was measured for each polarized cell (figure 3(B)). The mean axon lengths were 50.1 μ m, 38.7 μ m, 44.4 μ m, 31.0 μ m and 31.8 μ m, on the 2 μ m lines, 2 μ m holes, 300 nm lines, 300 nm holes and smooth surface, respectively; error bars for the SD are shown in figure 3(B). Sample sizes are the same as those mentioned in section 3.3. Longer axons were observed on the 2 μ m ($p < 0.05$) and 300 nm ($p \approx 0.05$) lines than on the smooth surface. However, no significant differences in axon length were observed between the hole topographies and smooth surface. Axon length on the 2 μ m lines (50.1 μ m) was statistically greater than on the 300 nm holes (31.0 μ m) ($p \approx 0.05$) but did not differ from any of the other topographies. Axon lengths did appear greater on the 300 nm lines than on the 300 nm holes, although the data were significantly relative to only a 90% confidence interval, which precludes us from noting any significance.

3.5. Axon alignment on lines

Axon alignment was analyzed and reported in terms of axonal angle θ and axon length (figure 4). Angles took on values between 0 and 90° and axons having angles between 0 and 30°, 30 and 60° or 60 and 90° were classified as having parallel, indeterminate (unaligned) or perpendicular alignment, respectively. We have not included data of axon alignments on holes and smooth surfaces because these topographies were found not to have directionality capable of guiding axon alignment. All percentages presented for alignment in this section are based on sample sizes (n = no. cells) of $n = 45$ and $n = 73$ for the 2 μ m lines and 300 nm lines, respectively. On the 2 μ m lines, 77.8%, 8.9% and 13.3%, of axons exhibited parallel, indeterminate and perpendicular angles, respectively ($n = 45$). On the 300 nm lines, 64.4%, 19.2% and 16.4%, of axons exhibited parallel, indeterminate and perpendicular angles, respectively ($n = 73$). Figure 4(A) summarizes these results and includes error bars depicting SD values. Axons took on parallel angles at a statistically greater frequency than each of the

other sets of angles ($p < 0.05$ for 0–30° versus 30–60° and 60–90°) for both sizes of lines. The average axon angle (over all angles) on the 2 μm and 300 nm lines was 19.1° (SD = 28°) and 28.9° (SD = 28.0°), respectively ($p < 0.05$).

We observed a marked difference in the degree of ‘complete alignment’ of axons on the different line sizes, which were axons having extreme values of θ between 0 and 10° (completely parallel) or 80 and 90° (completely perpendicular). 55.6% (SD = 7.4%) of cells on the 2 μm lines demonstrated completely parallel alignment compared to 37.0% (SD = 5.7%) on the 300 nm lines ($p < 0.05$). No cells (0%) on the 2 μm lines demonstrated completely perpendicular alignment, whereas 9.6% (SD = 3.4%) of cells on the 300 nm lines exhibited completely perpendicular alignment ($p < 0.05$).

3.6. Axon alignment versus axon length on lines

We investigated the relationship between axonal angles and axon lengths and found that average axon length was sensitive to alignment in some cases (figures 4(B) and (C)). The data presented in this section are based on the samples and data presented in section 3.5. On the 300 nm lines, the average axon length for neurons with perpendicular alignment (60–90°, 75.9 μm , SD = 57.4 μm) was statistically greater than that for neurons having axons with parallel (0–30°, 40.7 μm , SD = 27.7 μm) and indeterminate (30–60°, 42.8 μm , SD = 17.8 μm) alignments ($p < 0.05$). Moreover, axon lengths for parallel and indeterminate angles were similar. On the 2 μm lines, no statistical differences in the mean axon length were observed for axons in each of the angle groups (figure 4(B)). Interestingly, ‘extensively’ elongated axons (defined when axon length > 100 μm) only occurred with parallel or perpendicular alignment. On the 2 μm lines, extensively elongated axons only formed with parallel angles. On the other hand, extensive elongation appeared to occur for both parallel and perpendicular angles on the 300 nm lines.

Figure 5 shows representative images of hippocampal cells with elongated axons exhibiting both parallel and perpendicular alignments in response to the presence of the lines. In figure 5, images (A) and (B) show cells exhibiting parallel alignment relative to the 2 μm lines, and images (C)(E) show perpendicular alignment relative to the 300 nm lines.

3.7. Cellular morphologies on topographies

Figure 6 shows representative images of cells grown on various topographies. For all topographies, the soma tended to sit on the ridges between holes or lines and form an elliptical shape; however, the soma was usually found embedded in the grooves of the 2 μm lines and having an elliptical shape. In addition to the soma, filopodia and growth cones appeared to interact differently with each of the topographies (figure 6). In general, growth cones were more spread out on the sub-microscale topographies and smooth surfaces than on the larger microscale topographies. Filopodia, which play a role in contact guidance and pathfinding [21], appeared to grow from the periphery of the growth cones as well as the axons (i.e. lateral filopodia).

On the 300 nm lines, axons crossed the ridges at orthogonal angles and extended lateral filopodia at regular intervals on each of the ridges that were bridged by the axon. However,

when axons aligned parallel to the lines, few lateral filopodia were observed. On the 2 μm lines, filopodia were observed at the growth cone and at random locations along the length of the axons. On the 300 nm holes, the growth cone formed filopodia around its periphery while lateral filopodia were found at random locations along the axons. On the 2 μm holes, the growth cone tended to have filopodia around its periphery and often appeared flattened and sprawled, while lateral filopodia did not appear along the length of axons in most cases. Interestingly, axons almost always crossed the 2 μm holes and were rarely observed to anchor to the sidewalls or bottom surfaces of the holes.

4. Discussion

The mechanisms by which surface texture affects neuron behavior are not clearly understood, though one would assume that topography induces cytoskeletal re-organization, changes in cell shape and changes in the distribution of focal adhesions, which ultimately trigger enhanced cellular responses. Moreover, it has been proposed that topography exerts mechanical stresses on the cell, and the mechanical stresses ultimately provoke specific cellular responses by transducing changes in gene expression [4, 6]. Microscale and sub-microscale topographies introduce physical discontinuities in the surface area that apply tractive mechanical forces to neurons. These discontinuities seem to affect the morphologies of filopodia, the growth cone and the soma, which may provoke neurite initiation, rate of growth and alignment. Changes in cell adhesion, cytoskeletal structure and shape may also affect things like actin-based motility, the mode in which nerve cells move [22]. Lee *et al* [23] proposed that topography causes alterations in focal adhesions and provided distortions in the cytoskeleton that triggered the intracellular mechanisms controlling axon initiation.

We note that neurons may sense the sub-microscale and microscale topographies differently, at a fundamental level, due to the order-of-magnitude difference in size scale. Because of their relatively small size, sub-microscale pitted features, e.g., holes and grooves, are largely inaccessible to cellular structures, thus rendering the surfaces heterogeneous in terms of material properties where the trough regions (grooves) represent pockets of fluid (culture medium). The pockets of fluid, in combination with the solid surface, would collectively change the properties of the surface recognized by cells having sizes of larger scale. On the other hand, since cells are able to access the grooves of larger microscale features, the microscale features may serve to introduce physical discontinuities in the surface area, but not to change material properties from the perspective of individual cells [24–26].

It has been proposed that cell adherence depends on the proportion of the ridge area on patterned surfaces. Teixeira [8] found that HCECs on different patterns (i.e. smooth surface, microscale and nanoscale lines) displayed different focal adhesion numbers and sizes. As ridge widths decreased, focal adhesion sizes decreased, commensurately, altering the adhesive properties of the topographies. Sapelkin *et al* [27] observed that immortalized rat hippocampal neurons preferentially adhered to porous silicon rather than crystalline silicon. In our experiments, we found little or no statistical differences in cell adhesion due to the presence of topography. Despite seeing a trend that adhesion seemed to be greater on the topographies with the smaller features (i.e. 300 nm features), which contributed to greater ridge area, our data were drowned out by rather large standard deviations.

Previous studies have reported that smaller features have a greater impact on polarization. Gomez *et al* [14] found that rat embryonic hippocampal neurons on PDMS microchannels of 1 μm and 2 μm width enhanced axon formation compared to smooth PDMS after 20 h in culture. Lee *et al* [23] cultured hippocampal neurons on various PLGA fibers having diameters of 400 nm–2.2 μm and found that a greater number of neurons polarized on smaller PLGA fibers while differences in fiber orientation for similar fiber diameters had a negligible effect on polarization. In our experiments, polarization was markedly enhanced on all topographies relative to smooth quartz. Trends from our experiments appeared to show that polarization on the 300 nm topographies was slightly larger than on the 2 μm features, but we cannot conclude significance based on our statistical analysis. Moreover, because we did not see any difference in polarization based on changes in feature size and shape, we speculate that features having dimensions between 300 nm and 2 μm would have a similar impact on polarization. We also speculate that minor changes in shape from the nominal hole or groove with dimensions in the tested range would also have little impact on polarization.

Our axon elongation data indicated that both the 300 nm and 2 μm grooves appeared to catalyze axon growth relative to the smooth surface and the 300 nm holes. In contrast, the holes did not appear to stimulate growth relative to the smooth surface. We speculate that the grooves appeared to catalyze growth relative to only the 300 nm holes and not the other topographies because the proportion of groove area is the least among any of the topographies, making it most resemble a smooth surface. As indicative from our alignment experiments, there appears to be a connection between alignment and accelerated (catalyzed) axon growth.

It is thought that axon alignment to lines may reflect naturally occurring axonal alignments to neurite bundles [28, 29]. We observed axon alignment to be predominately parallel on both sizes of lines. However, we observed more dispersed alignment on the smaller 300 nm lines (figure 4(A)). Similar axon alignments were reported by Rajnicek *et al* [11]. They found that line depth and width seem to play a combined role in promoting alignment. For a given groove width, they showed that alignment became less parallel and more orthogonal for a decreasing depth. We found that decreasing line width while maintaining depth also promoted more perpendicular alignment. It was reported in [13] that axons tended to interact and align with groove edges. It has been claimed that hippocampal neurites appear to use different mechanisms for perpendicular and parallel contact guidance and that grooves present separate signals that provoke parallel and perpendicular responses, depending on feature dimensions [12].

5. Conclusions

Here we reported the results of our investigation determining the effects of micro- and nanopatterned topography of various sizes and shapes on (1) cell–substrate adhesion, (2) axon establishment (neuron polarization), (3) axon length, (4) axon alignment and (5) cell morphology of rat embryonic hippocampal neurons in the first 24 h of cell development. For this purpose, we fabricated arrays of 300 nm holes, 2 μm holes, 300 nm lines (grooves) and 2 μm lines in quartz (SiO_2); all structures forming the arrays had a depth of approximately

400–500 nm. We found that hippocampal neurons responded differently to topographies of different sizes and shapes. Topography (holes and lines) enhanced axon formation, though feature size did not affect polarization to an appreciable degree. Grooves were found to increase axon elongation, and elongation was observed to be mostly feature shape dependent as opposed to size dependent for the sizes of features tested here. Lines also influenced axon alignment, and a connection between alignment and axon length was found to exist in certain circumstances.

Finally, topography was found to alter cellular morphologies (i.e. soma, axons, growth cones, filopodia), where both size and shape were important factors influencing cell behavior. Our findings heighten our understanding of neuronal behavior on topography in the early developmental stages (first 24 h) of individual neurons and may aid in designing surfaces for neural applications, such as neural networks and advanced nerve guidance channels.

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Figure 1.

Quartz surfaces patterned with arrays of structures to form topographies. (A), (B) Grooves (lines) of 300 nm width with a spacing of 1 μm (1.3 μm pitch); (C), (D) holes with a 300 nm diameter with horizontal and vertical spacings of 1 μm (1.3 μm pitch); (E), (F) grooves (lines) of 2 μm width with a spacing of 1 μm (3 μm pitch); (G), (H) holes with a 2 μm diameter with horizontal and vertical spacings of 1 μm (3 μm pitch). All topographies consist of structures with 400–500 nm depth.

Figure 2.

Adhesion of neurons to topography and smooth surface calculated by dividing the total number of cells sampled (polarized and unpolarized) by the surface area. Differences in adhesion on topography were similar to the smooth surface according to statistical analysis. Error bars = SD.

Figure 3.

(A) Fraction of neurons polarized (%) relative to the total cell count on each topography and smooth surface. % polarization was statistically greater on the topographies than the smooth surface; however, differences among the topographies did not affect polarization to a statistical degree. (B) Mean axon length (μm) of polarized neurons on the topography and smooth surface. Error bars = SD. *Statistically significant, where $p < 0.05$, $\dagger p < 0.10$ (not deemed significant, but consistent with statistical trends). At least 100 neurons ($n = 100$) were analyzed on each topography (except for 300 nm holes, $n = 73$).

Figure 4.

(A) Histogram showing the percentage of axons having an alignment, θ , of 0–30° (parallel), 30–60° (unaligned) and 60–90° (perpendicular) on the 2 μ m and 300 nm lines. (Inset) Axon orientation angle θ . Average θ for all angles 0–90° for the 2 μ m and 300 nm lines was 19.1° ($n = 45$) and 28.9° ($n = 73$), respectively ($p = 0.05$). (B) Average axon length for axons of various alignments. (C) Axon alignment plotted against the axon length for the data represented in (A). Error bars = SD. * $p = 0.05$.

Figure 5. SEM and optical images showing the influence of the line width on the orientation of elongated axons seeded on arrays of microfabricated lines (line topographies). (A), (B) Parallel alignment on the 2 μm lines; (C)–(E) perpendicular alignment on the 300 nm lines.

Figure 6.

Soma and axon orientation and morphology based on topography. The images in the right column show the enlarged areas indicated in the white boxes shown in the juxtaposing images in the left column. Sample cells on (A), (B) 2 μm lines, (C), (D) 2 μm holes, (E)–(H) 300 nm lines, (I), (J) 300 nm holes and (K), (L) smooth surface.

Table 1

Dimensions of the topographies.

		Dimensions		
Shapes	Lines	Ridge (μm) ^a	Groove (μm)	Depth (nm)
	300 nm lines	1	0.3	400
	2 μm lines	1	2	500
Holes		Spacing (μm) ^b	Diameter (μm)	Depth (nm)
	300 nm holes	1	0.3	400
	2 μm holes	1	2	500

^aPitch: 1.3 μm for 300 nm lines and 3 μm for 2 μm lines.

^bPitch: 1.3 μm for 300 nm holes and 3 μm for 2 μm holes in x- and y-directions.