Contact guidance of CNS neurites on grooved quartz: influence of groove dimensions, neuronal age and cell type

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SUMMARY

We used an in vitro system that eliminates competing guidance cues found in embryos to determine whether substratum topography alone provides important neurite guidance information. Dissociated embryonic \textit{Xenopus} spinal cord neurons and rat hippocampal neurons were grown on quartz etched with a series of parallel grooves. \textit{Xenopus} neurites grew parallel to grooves as shallow as 14 nm and as narrow as 1 \textmu m. Hippocampal neurites grew parallel to deep, wide grooves but perpendicular to shallow, narrow ones. Grooved substrata determined the sites at which neurites emerged from somas: \textit{Xenopus} neurites sprouted from regions parallel to grooves but presumptive axons on rat hippocampal neurons emerged perpendicular to grooves and presumptive dendrites emerged parallel to them. Neurites grew faster in the favored direction of orientation and turned through large angles to align on grooves. The frequency of perpendicular alignment of hippocampal neurites depended on the age of the embryos from which neurons were isolated, suggesting that contact guidance is regulated in development. Collectively, the data indicate that substratum topography is a potent morphogenetic factor for developing CNS neurons and suggest that in addition to a role in pathfindining the geometry of the embryo assists in establishing neuronal polarity. In the companion paper (A. M. Rajnicek and C. D. McCaig (1997) \textit{J. Cell Sci.} 110, 2915-2924) we explore the cellular mechanism for contact guidance of growth cones.

Key words: Topographic guidance, Growth cone, Hippocampal neuron, \textit{Xenopus} neuron, Neuronal development, Substratum

INTRODUCTION

Contact guidance, the phenomenon by which the physical shape of the substratum induces alignment or directional growth of cells is involved in normal embryonic pattern formation (e.g. Newgreen, 1989). Nervous system development is influenced strongly by geometric patterns present within embryos during developmentally significant periods. For example, radial glia that span the walls of the developing brain are organized into regular arrays that foreshadow the route of migrating embryonic neurons (Hatten, 1990). In addition to a role in directional migration of cell bodies aligned cells also appear to guide process outgrowth. In the mammalian forebrain a ‘sling’ of subventricular cells forms a scaffold that predicts the trajectory of the corpus callosum (Schneider and Silver, 1990). Aligned channels that are subsequently invaded by pioneer axons exist in mouse (Silver and Sidman, 1980) and chick (Krayanek and Goldberg, 1981) optic stalk and dorsal retina. Similarly, the earliest fiber tracts in the \textit{Xenopus} spinal cord form by ingrowth of axons into preexisting longitudinally oriented spaces between neuroepithelial cells of the neural tube (Nordlander and Singer, 1982a,b). Electron microscope studies of \textit{Xenopus} embryos (Roberts and Taylor, 1983) demonstrate that even on a small scale growth cone morphology is influenced by substratum topography, further suggesting that purely physical cues can direct neurite growth within embryos.

In an effort to understand embryonic contact guidance events in vitro many cell types, including fibroblasts (Elsdale and Bard, 1972) and neurons (e.g. Ebendal, 1976) have been cultured on parallel arrays of aligned collagen fibrils or on parallel grooves manufactured in artificial substrata (Hirono et al., 1988; Clark et al., 1990; Oakley and Brunette, 1993). In most cases cells exhibit conventional contact guidance, aligning parallel to the groove/ridge axis, but even apparently simple contact guidance cues provide complex directional information to cells. For example, fibroblasts migrate unidirectionally on aligned collagen matrices (Boocock, 1989) and, depending on species and neuronal type, mammalian CNS neurites grow either parallel or perpendicular to aligned neurite bundles (Nagata and Nakatsuji, 1991) or artificial microstructures (Nagata et al., 1993).

Contact guidance of cells by physical contours of the substratum was recognized in the earliest days of tissue culture (Harrison, 1914) yet surprisingly little is known about the cellular events of contact sensing and their transduction into directional growth, especially in neuronal growth cones. The aim of the present study was to describe the directional effects of substratum contours on the morphology of developing CNS neurons as a first step towards elucidating the mechanism for
topography-induced growth cone guidance. In the companion paper we explore the cellular mechanisms for contact guidance of growth cones (Rajnicek and McCaig, 1997b).

MATERIALS AND METHODS

Substrate preparation

Microgrooved substrates were prepared from fused quartz using a direct writing electron beam lithographic process to etch a series of repeating, parallel grooves and ridges that were evenly spaced and had nearly right angle edges (Fig. 1A). The technique offers submicron precision in controlling grating periodicity beyond that attainable by conventional photolithographic microfabrication (Clark et al., 1990; Britland et al., 1996a). Eight experimental culture substrata, each comprising three 5 mm × 5 mm blocks of gratings with identical groove depths but different groove widths (1, 2 and 4 µm, respectively), were fabricated on one electron beam mask plate (Hoya, Japan). Each 6.5 cm square plate was composed of a 2 mm thick fused quartz base, a 100 nm chrome layer and 500 nm of EBR9 electron beam resist. The grating pattern was designed in WAM (an in-house microelectronic design package) and then translated into the BWL beam writing language format used by a Leica EBG5 Beamwriting machine which was operated with a 200 nm spot size at 50 kV. Beam current was automatically determined by spot size and resolution. Exposed resist was then developed using neat methyl iso-butyl ketone at room temperature and the underlying chrome removed by wet etching to reveal the quartz. The quartz base was then dry etched (RIE80, Plasma Technology, UK) using CHF3 plasma at 29 sccm (standard cubic centimeters per minute) flow rate and 15 mTorr and 100 W (rf) at 13.6 MHz to give an etch rate of approximately 50 (standard cubic centimeters per minute) flow rate and 15 mTorr and 100 W (rf) at 13.6 MHz to give an etch rate of approximately 50.

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The angle of neurite orientation relative to the groove direction (0°)

Data collection and analysis

Measurements were made from live video images of cells using Leica Quantimet 500MC image collection and analysis software. For Xenopus neurons data were collected from each neurite but in the case of hippocampal neurons data were only collected from the longest neurite on each cell, which is the presumptive axon (Dotti et al., 1988). The angle of neurite orientation relative to the groove direction (0°)

Cell culture

Dissociated neurons from the neural tube of Xenopus laevis were cultured using a slight modification of a previously described culture method (Hinkle et al., 1981; McCaig et al., 1994). Neural tubes were dissected from several stage 20 Xenopus neurulae and pooled in calcium-magnesium-free Steinberg’s solution, pH 7.9, to disaggregate. The resulting cell suspension was triturated gently and then plated into a pool of culture medium in the central region of the slides described above. Culture medium was Steinberg’s solution (58 mM NaCl, 0.67 mM KCl, 0.44 mM Ca(NO3)2, 1.3 mM MgSO4 and 4.6 mM Tris-HCl, pH 7.9) supplemented with 20% Leibovitz’s L-15, 1% fetal bovine serum, 100 i.u./ml penicillin, 100 µg/ml streptomycin (all from ICN Biochemicals Ltd, Irvine, Scotland). Data were collected after 6 to 9 hours in culture at room temperature. Dissociated neurons from the E16 and E19 for studies of age dependence rat hippocampus were cultured in Earle’s minimal essential medium (MEM, Gibco) as described previously (Davenport and McCaig, 1993). After 3 hours the MEM containing 10% heat inactivated calf serum was replaced with MEM containing 10% Nu Serum V (Collaborative Research, Lexington, MA, USA). Data were collected from live cells after 24 hours in culture at 36°C. 5% CO2. Hippocampal cells were used fresh or were thawed from stocks frozen at −70°C in 8% DMSO (dimethylsulfoxide) by the method of Matson and Kater (1988). Data from fresh and frozen cells were combined because there were no differences in their morphologies or directional responses.

Fig. 1. (A) Scanning electron micrograph of a quartz microscope slide with grooves 520 nm deep and 2 µm wide. Note that the grooves have approximately right angled sides, sharp corners and are evenly spaced. The lower photo is a magnified view of the boxed region. (B) Angle measurement protocol for orientation assay. Angles were measured using computerized image collection and analysis software that allowed lines to be superimposed onto video images of cells. Images were always viewed with the groove/ridge axis horizontal. The direction of neurite growth was determined by a line connecting the neurite initiation site on the soma with the center of the growth cone. All angles measured were between 0° and 90°. Overall orientation for a population of neurites was determined by categorizing the angle of growth as ‘parallel’ to the grooves if θ was between 0° and 30° and ‘perpendicular’ if θ was between 60° and 90°. The mean percentage of neurites in each category was determined by pooling data for several sets of 50 cells grown under identical conditions.
was determined as shown in Fig. 1B. Control (flat) data were collected from cells on the ungrooved regions of each etched slide.

Angles were considered to be 'parallel' to the groove direction if θ was between 0° and 30° and angles were 'perpendicular' if θ was between 60° and 90°. The remainder of the neurites were classified as 'intermediate' and for the sake of clarity are not included in this report. The expected frequency in each category for a population of randomly oriented neurites is 33%. This was confirmed by measurements of the neurites grown on flat quartz surfaces. The percentage of neurites in each category was therefore compared to the control percentage for flat substrata using a t-test (Bailey, 1981) or one way ANOVA followed by a Dunnet multiple comparison test.

RESULTS
The direction of CNS neurite orientation depends on cell type and groove dimensions

Dissociated neurons from embryonic *Xenopus* spinal cord and embryonic rat hippocampus revealed strikingly different growth patterns on grooved surfaces (Figs 2, 3). *Xenopus* spinal neurites exhibited classical contact guidance by growing parallel to grooves with depths ranging from 14 nm to 1,100 nm and widths of 1, 2 or 4 μm (Fig. 2A). By contrast, hippocampal neurites showed a more complex response. In general they grew perpendicular to shallow, narrow grooves and parallel to deep, wide ones (Fig. 2B). At certain groove depths (130 nm, 520 nm, and 1,100 nm) hippocampal neurites changed their direction of growth from perpendicular on grooves 1 μm and 2 μm wide to parallel on grooves 4 μm wide even though the groove depth was unchanged. The greatest perpendicular response for hippocampal neurites was on grooves 130 nm deep and 1 μm wide and the greatest parallel response was on grooves 1,100 nm deep and 4 μm wide. Orientation was not biased on flat quartz for either cell type. Most non-neuronal cells in hippocampal cultures aligned parallel to grooves of all dimensions. Grooved quartz slides were treated with polylysine before plating hippocampal neurites but not *Xenopus* neurons so we tested the notion that differences in orientation between the neuronal types was an artifact of polylysine treatment. Two lines of evidence argue that the differences are not related to polylysine treatment: (1) *Xenopus* neurites oriented parallel to grooves even when the slides were treated with polylysine (data not shown). The equivalent experiment, in which hippocampal neurites are grown on untreated quartz is impossible because polylysine treatment is required for hippocampal neuron differentiation. (2) Rat hippocampal neurites aligned either parallel or perpendicular to grooves depending on groove dimensions even though the substrates were always treated with polylysine.

Parallel growth of neurites is not merely due to physical constraint within the grooves because individual growth cones often span several groove and ridge repeats (Figs 3D, 4A) and are therefore not confined by groove walls. Similarly, hippocampal neurites are able to cross single steps as high as 3.16 μm (Fig. 5) and would therefore be able to step out of grooves even deeper than those used in this study. Extrapolation of the data in Fig. 5 indicates that grooves would have to be 4.7 μm high to completely restrain hippocampal neurites. These data suggest that neurites make an active choice to grow either parallel or perpendicular to grooves. This idea is also supported by the observation that hippocampal neurites sometimes grew parallel to grooves before turning to grow across them (Fig. 4B).

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**Fig. 2.** Orientation responses of (A) *Xenopus* spinal cord neurites and (B) rat hippocampal neurites on grooved substrata. The direction of neurite growth was measured as shown in Fig. 1B and is presented as the mean percentage of total neurites parallel (white bars) or perpendicular (black bars) to the groove direction ± sd. The groove width is indicated in the upper left of each graph. Data were pooled from at least two experiments on each substrate. The number of *Xenopus* neurites on grooves ranged from 93 (on 130 nm deep, 4 μm wide grooves) to 188 (on 140 nm deep, 1 μm wide grooves). The number of hippocampal neurites ranged from 100 (on 14 nm and 520 nm grooves) to 400 (on 130 nm deep, 1 μm wide grooves). Asterisks represent values significantly different from the measured control frequencies of 34±3% parallel and 34±3% perpendicular for *Xenopus* (n=300) and 34±6% parallel and 34±5% perpendicular for hippocampal neurites (n=750) on flat quartz (*P<0.05, **P<0.01 by one way ANOVA). These control frequencies were not different from 33% (t-test, Bailey, 1981), which is the expected frequency of parallel or perpendicular neurites in a randomly oriented population.
Grooved substrata determine the sites at which neurites emerge from somas

Neurons were grown on grooved substrates to test whether topographical features of the substratum determined aspects of neuronal morphology even more subtle than the direction of overall neurite extension. In particular we examined the direction of neurite initiation, direction of turning and the rate of neurite elongation. Grooved substrata determined the site on the soma that gave rise to neurites. Neurites were uniformly distributed on flat quartz or on grooves 14 nm deep but most *Xenopus* neurites emerged from regions of the somas parallel to grooves greater than 36 nm deep (Figs 6A, 7C). Hippocampal neurites showed a more varied response (Fig. 6B). One day after plating hippocampal neurons generally bear one long process that becomes the axon and several shorter, minor processes that subsequently become dendrites (Dotti et al., 1988). By these criteria most presumptive axons emerged from perpendicular regions and presumptive dendrites emerged from parallel regions of hippocampal somas on grooves. Presumptive dendrites maintained parallel growth as they extended (Fig. 3B). This implies that the physical contour of the environment influences the fine structural, and therefore functional, polarity of differentiating hippocampal neurons.

Neurites turn to grow in the preferred orientation

Neurites of both cell types often adjust their trajectories by turning toward their preferred direction of orientation. For example, *Xenopus* neurites on substrates with grooves 14 nm deep and 1 μm wide are symmetrically distributed around the soma (Figs 4A, 6A) yet they demonstrate parallel contact guidance on these grooves (Fig. 2B). Turning was defined as a deviation of 15° or more by the distal (growth cone) end of a neurite relative to the proximal end (nearest the soma) of the same neurite. On flat quartz neurites turned uniformly in all directions: 32% toward grooves, 33% away from grooves and 43% do not turn (n=200) but on grooved surfaces more than twice as many turned away from the direction of the groove (46%) than turned toward...
Hippocampal neurons showed a similar tendency to grow faster in the preferred orientation. For example, on the substratum that elicited maximum perpendicular orientation (130 nm deep, 1 µm wide grooves) hippocampal neurites perpendicular to grooves were longer than those parallel to grooves and those on flat quartz (Fig. 9). Neurites on flat quartz had uniform lengths regardless of orientation (Fig. 9). Since all neurites grew parallel to grooves in these time lapse experiments no comparison could be made with growth rates of neurites on grooved substrata extending in less preferred orientations.

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**Embryonic age and species affect perpendicular alignment of hippocampal neurons**

Cells dissociated from rat hippocampi of different embryonic ages were grown on 130 nm deep grooves to explore the temporal developmental significance of contact guidance. Perpendicular orientation on 1 or 2 µm wide grooves was greater for neurones isolated from E16 hippocampi than for those from E19 hippocampi (Fig. 10). E16 neurites aligned perpendicular at 1 and 2 µm widths but E19 neurites shifted from perpendicular orientation at 1 µm repeats to parallel orientation at 2 µm repeats (Fig. 10).

Although hippocampal neurones isolated from E16 mice or rats show predominantly perpendicular orientation on 130 nm deep, 1 µm wide grooves those isolated from mice are less...
likely to exhibit perpendicular alignment. After 24 hours in culture 81±2% of E16 rat neurites (Fig. 10) but only 50±6% (mean ± s.d., n=100) of E16 mouse neurites aligned perpendicular to grooves (P=0.003, 2 tailed Student’s t-test). This reduction could reflect species differences or differences in the relative rates of mouse and rat hippocampal development because hippocampal development in the mouse begins earlier (E10) than in the rat (E15) and the development of Ammon’s horn is more protracted in the mouse than the rat (Reznikov, 1991). The frequency of perpendicular alignment of E16 mouse neurites was therefore compared to E19 rat neurites, which may be more similar developmentally. There was no difference (Student’s t-test) between the frequency of perpendicular alignment for E16 mouse neurons (50±6%, n=100) compared to E19 rat neurons (61±7%, n=150).

DISCUSSION

The nature of various attractive, repulsive, permissive and inhibitory characteristics of neuronal pathways has received much attention in the context of growth cone guidance (reviewed by Keynes and Cook, 1995a,b; Tessier-Lavigne and Goodman, 1996). It is likely that interplay between multiple factors is required for axon guidance and correct target recognition. Guidance by substratum contours has been largely overlooked and is usually treated as incidental but evidence that spaces precede outgrowth of some CNS neurons during development and regeneration (e.g. Singer et al., 1979), that CNS neurites are guided by radial glial cells (e.g. Norris and Kalil, 1991) and that substratum topography affects growth cone shape (Harris et al., 1985; Nordlander et al., 1991) suggest collectively that guidance by topographical features warrants investigation in its own right.

Topographical guidance may be important during hippocampus development because alveolar channels in the embryonic rat hippocampus are presumed to guide pyramidal axons (Altman and Bayer, 1990b) and non-pyramidal neurons in the hippocampus may provide contact guidance for later outgrowing septohippocampal fibers (Supèr and Soriano, 1994). Additionally, Cajal described hippocampal mossy fibers as being grooved into the irregularities of the surface of regio inferior neurons suggesting a relationship between topography and neurite paths (Blackstad and Kjaerheim, 1961).

We used an in vitro system that eliminated simultaneous presentation of potentially competitive guidance cues to explore the contribution of the physical shape of the substratum in neuronal morphogenesis. Our data indicate that substratum contours provide important morphogenetic information to developing CNS neurons in an age-dependent way by influencing the site of neurite initiation on somas, the direction of neurite growth (determined by groove dimensions), the presumptive axonal or dendritic identity of neuronal processes and the rate of neurite elongation. The increase in net perpendicular growth rate on narrow grooves is selective because neurites that grow parallel to the same grooves are significantly shorter than parallel ones but no different than those on flat quartz. The net increase could be due to enhanced trophic support for neurites on grooves because of the increased...
membrane surface area exposed to nutrient-rich culture medium. This does not appear to be the case however because on grooved surfaces that induce random orientation neurite length is the same as that on flat quartz. This suggests that perpendicular neurite growth rate is stimulated only on grooved surfaces that stimulate perpendicular orientation.

Alignment on grooved surfaces appears to be an active process

Neurites changed their direction of growth to reflect their preferred orientation on grooves. *Xenopus* neurites monitored over time adjusted their trajectories, turning parallel to grooves. Whilst only 16% of hippocampal neurites turned parallel to grooves that induced perpendicular alignment, 46% turned away from grooves (and through larger angles), yielding perpendicular orientation. Control experiments ruled out the possibility that perpendicular orientation was related to polylysine treatment of the growth surface. *Xenopus* and hippocampal neurites grew parallel to grooves too small to restrain them physically, suggesting that parallel orientation is not merely due to neurites being trapped within grooves. This idea is supported by the observation that hippocampal neurites climbed over steps of at least 3 times higher than those that induced parallel orientation. These observations suggest collectively that alignment on grooved substrata is an active rather than passive event.

Contact guidance by neurons in situ

*Xenopus* spinal neurites and rat hippocampal neurones were used in the present investigation because evidence from anatomical studies of *Xenopus* spinal cord and mammalian brain, including the hippocampus suggest that surface contours affect the pattern of neurite growth. For example, in the *Xenopus* spinal cord sensory ganglion neurons grow along tracts of preexisting, longitudinally aligned Rohon-Beard neurones (Nordlander et al., 1991) and aligned spaces between neighboring neuroepithelial cells form channels which the earliest axonal outgrowths subsequently invade (Nordlander and Singer, 1982a,b). Published electron micrographs (e.g. Fig. 2 of Nordlander and Singer, 1982a) indicate that the channels range from approximately 0.6 µm to 3 µm across. Even the smallest spaces are therefore at least five times larger than the minimum groove depth that induced parallel orientation of *Xenopus* spinal cord neurites (our Fig. 2A). The first spaces to appear in the *Xenopus* spinal cord form adjacent to the differentiating Rohon-Beard neurones (Nordlander and Singer, 1982a). Cultures of dissociated neural tubes yield a heterogeneous population of sensory neurones (e.g. Rohon-Beard neurones), motor neurones and interneurons (Tabti and Poo, 1991). Since greater than 80% of *Xenopus* neurites grew parallel to grooves 130 nm deep and deeper in our experiments (Fig. 4A) it is probable that the majority of embryonic spinal cord neurones, including Rohon-Beard neurones, would align with parallel topographical features in situ. Indeed, growth cones of *Xenopus* sensory ganglion neurons appear to be directed by the geometry of their surroundings so that axons, their growth cones and filopodia align parallel to the dorsolateral fasciculus (Nordlander et al., 1991).

The highly stereotyped crisscross pattern of neuronal processes within the mammalian hippocampus was noted during the early histological studies by Santiago Ramón y Cajal (1893) but the mechanism that generates such striking geometry has not been established clearly. The adult mammalian hippocampus is one of the most widely studied brain structures (Reznikov, 1991) largely because of its well defined neuronal circuitry, yet little effort has been made to determine how appropriate connections are established during development. Most in situ studies of embryonic hippocampal development have concentrated on neurogenesis (‘birth dates’ and migration patterns

Fig. 9. Rat hippocampal neurites are longer when aligned perpendicular to grooves than when they are parallel or on flat substrates. All data were collected 24 h after plating and are expressed as mean neurite length ± s.d. Asterisks indicate significant differences in the length of neurites growing perpendicular compared to those growing parallel on the same substrate; *P=0.0261, **P=0.0012, ***P<0.0001 (2-tailed Student’s *t*-test). Number of neurites measured on each substrate (parallel and perpendicular, respectively): on flat quartz *n*=32 and 32, on 130 nm × 1µm grooves *n*=18 and 91, on 140 nm × 1µm grooves *n*=16 and 68, on 140 nm × 1 µm grooves *n*=27 and 49, and on 140 nm × 4 µm grooves *n*=30 and 40. At a width of 1 µm there is no difference in the length of neurites perpendicular to grooves 130 nm deep compared to those perpendicular on 140 nm deep grooves. Neurite length on grooves 140 nm deep and 4 µm wide is not different from that on flat quartz. Neurites oriented parallel to grooves 130 nm deep and 1 µm wide were shorter (*P=0.0327) than parallel neurites on flat quartz.

Fig. 10. Age dependent differences in orientation on grooves. Hippocampal neurites were isolated from E16 (left side of figure) or E19 (right side of figure) rat embryos. Perpendicular contact guidance was enhanced for E16 neurites compared to E19 (*P=0.0089 and P=0.0035 for E16 versus E19 neurones at 1 and 2 µm widths, respectively by a 2-tailed Student’s *t*-test). On 130 nm × 1 µm substrates the number of neurites was 150 at E16 and 150 at E19. On 130 nm × 2 µm the number of neurites was 50 at E16 and 100 at E19. Asterisks indicate differences between the percentage parallel or perpendicular compared to a control value of 36% for flat quartz (dashed line); *0.01<P<0.02, **0.0001<P<0.001, ***P<0.0001 by a 2-tailed Student’s *t*-test. The highly stereotyped crisscross pattern of neuronal processes within the mammalian hippocampus was noted during the early histological studies by Santiago Ramón y Cajal (1893) but the mechanism that generates such striking geometry has not been established clearly. The adult mammalian hippocampus is one of the most widely studied brain structures (Reznikov, 1991) largely because of its well defined neuronal circuitry, yet little effort has been made to determine how appropriate connections are established during development. Most in situ studies of embryonic hippocampal development have concentrated on neurogenesis (‘birth dates’ and migration patterns

CNS neurite orientation on grooves

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of undifferentiated neurons) rather than on the process of axon outgrowth per se (e.g. Bayer, 1980; Altman and Bayer, 1990a). It would be instructive to identify factors that determine fiber outgrowth patterns in the hippocampus because they may also be relevant to other brain regions. This seems likely because neurites from a variety of mammalian CNS regions exhibit perpendicular contact guidance on parallel arrays of neurites (Hekmat et al., 1989; Nagata and Nakatsuji, 1991) and artificial microstructures in vitro (Nagata et al., 1993). Although Nagata et al. (1993) state that mouse E17-18 hippocampal neurroblasts showed 'relatively higher frequency of parallel orientation' on grooved quartz it is difficult to compare our results with theirs directly because no data were provided for hippocampal neurites. Our study extends that of Nagata et al. (1993) to indicate that grooved substrata determine the rate of neurite extension and the direction of presumptive axon or dendrite initiation as well as the direction of neurite growth.

Relevance of contact guidance to development and regeneration

Our data that neurons from hippocampi of different embryonic ages respond differently to identical topographical guidance cues support the notion that contact guidance acts during hippocampus development by suggesting that the ability to respond to topographical cues is regulated temporally. Our data do not indicate whether the temporal differences reflect changes in the types of neurons present in the cultures at different embryonic ages or changes in the responsiveness of individual neuronal types. Hippocampal development in the rat begins at E15 and development of Ammon's horn is most rapid between E17-19 (Reznikov, 1991). It is likely therefore that E16 cultures contain a smaller proportion of pyramidal neurons, which may represent as much 80-85% (probably from fields CA1, or CA2-CA3) of the population in E19 cultures (Banker and Cowan, 1979). This variation in cellular composition may account for the age-related decrease in perpendicular orientation of E19 compared to E16 cultures. However, neurites of the same age, from the same cell suspension respond orthogonally to substrata differing only in groove width. This indicates that subtle variations in embryonic geometry have profound effects on neuronal morphology and suggests that topographic cues encode more complex guidance information than they are generally attributed with.

Anatomically aligned spaces exist in the developing and in the regenerating spinal cord of newts and lizards (Singer et al., 1979) and growth cones often grow along other neurites during development and regeneration (Nordlander et al., 1991). Indeed, some procedures aimed at enhancing CNS regenerative abilities (Nordlander et al., 1991). Our data indicate that the dimensions of substrata used for such treatments are crucial if they are to produce effective guidance of CNS neurites across lesion sites.

Possible interactions with other guidance cues

We do not propose that guidance of neurites by physical substratum cues alone explains axonal guidance to targets but it is likely that contact guidance acts in concert with other neuronal stimulatory and inhibitory growth cone guidance factors. For example, steady, DC electric fields associated with the amphibian neural tube (Hotary and Robinson, 1991; Shi and Borgens, 1994) and the mammalian primitive streak been implicated in neuronal morphogenesis (Winkel and Nuccitelli, 1989; Hotary and Robinson, 1992; Shi and Borgens 1994). Interestingly, Xenopus spinal neurites in weak, DC electric fields respond parallel to the electric field lines (see McCaig et al., 1994, for review) and rat hippocampal neurites respond perpendicular to them (Rajnicek et al., 1992), thus mimicing their respective contact guidance preferences in the present study. On shallow grooved substrata with an overlying orthogonal adhesive track chick dorsal root ganglion neurites aligned preferentially on the adhesive tracks but on deeper grooves they ignored the adhesive paths and aligned parallel to grooves (Britland et al., 1996b). Similar competition experiments have not been done using mammalian CNS neurons. Future experiments will therefore examine the heirarchy of CNS guidance cues by establishing competing gradients of electrical cues, substratum gradients of tropic molecules and gradients of soluble chemoattractants.

In summary, CNS neurites are very sensitive to topographical contact guidance cues in the absence of simultaneous chemical or electrical gradients. Xenopus neurites grew parallel to grooves but hippocampal neurites regulated their direction of neurite growth depending on groove dimensions and developmental age. Grooved substrata influenced the site at which neurites emerged from cell bodies, the presumptive axonal or dendritic identity of neurites, the direction of neurite growth and the rate of neurite elongation. Taken together, these data suggest that perpendicular alignment has at least three contributing factors: (1) Presumptive axons emerge perpendicular to the groove/ridge axis. (2) Neurites turn more frequently and through larger angles to grow across, rather than along grooves. (3) Neurites grow more quickly across grooves than parallel to them or on flat substrata. The companion paper (Rajnicek and McCaig, 1997) addresses the question of how growth cones sense small surface contours and the signal transduction events that lead to directional growth.

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