

Perpendicular contact guidance of CNS neuroblasts on artificial microstructures

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SUMMARY

Rodent CNS neuroblasts show parallel and perpendicular contact guidance behaviors on aligned neurite bundles in microexplant cultures (Nakatsuji, N. and Nagata, I. (1989) *Development*, 106, 441-447; N. I. and N. N. (1991) *ibid.*, 112, 581-590). To test the hypothesis that the physical surface structure of the neurite bundle causes the perpendicular contact guidance, we cultured dissociated neuroblasts on quartz plates on which grating-like microstructures were fabricated by lithographic techniques. Various types of CNS neuroblasts, but not PNS neurons, oriented their processes and migrated

both perpendicular and parallel to the axis of the microstructure. Perpendicular orientation was frequently observed when the microstructured grooves had depths between 0.3 μm and 0.8 μm and a width of 1 μm , which roughly mimics a tightly aligned neurite bundle. Thus, CNS neuroblasts have the ability to extend their processes and migrate perpendicular to aligned surface microstructures.

Key words: CNS neuroblasts, process orientation, migration, perpendicular contact guidance, artificial microstructure

INTRODUCTION

Contact guidance plays an important role in the pathfinding and migration of neurons in the histogenesis of the CNS (Letourneau, 1975; Dodd and Jessell, 1988). Neurons grow their processes and migrate along glial cells (Sidman and Rakic, 1973; Hatten, 1990), neurites of other neurons (Hynes et al., 1986; Ono and Kawamura, 1989; Gray and Sanes, 1991; Liesi, 1992; Miura et al., 1992), artificially and chemically patterned substrata (Letourneau, 1975; Gundersen, 1987; Kleinfeld et al., 1988), or even purely physical microstructures (Hirono et al., 1988; Torimitsu and Kawana, 1990). Most in vitro studies, however, have used PNS neurons such as sensory or sympathetic ganglion neurons. Nakatsuji and Nagata recently found that mouse cerebellar granule cells extend processes and migrate not only along neurites of other neuroblasts but also perpendicular to the aligned neurite bundle in mouse cerebellar microexplant cultures (Nakatsuji and Nagata, 1989; Nagata and Nakatsuji, 1990). Furthermore, almost all kinds of neuroblasts dissociated from various parts of the CNS, but not PNS neurons, oriented their processes and migrated both perpendicular and parallel to the heterotypic neurite bundles (Nagata and Nakatsuji, 1991).

Two possibilities have been considered for the mechanism of the perpendicular orientation and migration (Nagata and Nakatsuji, 1991). The first possibility is that neuroblasts extend processes at right angles to the structure by recognition of the physical surface structure of neurite bun-

dles. The second possibility is that more specific surface molecules on the neurites, such as the cell adhesion molecules, are responsible for the perpendicular contact guidance. In the present study, we have examined the first possibility by testing whether a physical microstructure that mimics the tightly aligned neurite bundle causes perpendicular contact guidance of CNS neuroblasts.

As a physical model of the neurite bundle, we used a grating-like microstructure which was fabricated on the surface of a quartz plate (Clark et al., 1987; Hirono et al., 1988). Neuroblasts dissociated from various regions of the CNS or PNS were cultured on such plates, and the orientation of neurite processes and motility of cells were examined by light and scanning electron microscopy. Here, we show that dissociated CNS neuroblasts, but not PNS neurons, extend their processes in both perpendicular and parallel orientations on certain aligned microstructures. A preliminary report has already appeared (Nagata et al., 1991 *Neurosci. Res.*, suppl.16, S86).

MATERIALS AND METHODS

Artificial microstructure and coating

The surface-relief grating with a rectangular cross-section was fabricated on pure quartz plates as described previously (Hirono et al., 1988). The grating-like microstructure consisted of microgrooves (mGRV) and microsteps (mSTP) of various widths (1-8 μm) and depths (0.05-0.8 μm). Some of these microstruc-

tured quartz plates were heated in the outer flame of a natural gas burner for a few minutes. The quartz plates were cleaned (Hirono et al., 1988) and double-coated with poly-L-lysine and laminin (Nagata and Nakatsuji, 1990) as described previously. In some experiments, the microstructure was coated with poly-L-lysine alone, or used without any coating.

Cell culture on artificial microstructures

Olfactory bulbs (embryonic day 15 and postnatal 1-3 days), cerebral cortices (embryonic 14-16 days), septa (embryonic 16 days), striata, hypothalami, hippocampi (embryonic 17-18 days), cerebella (postnatal 4-5 days), medulla oblongata (embryonic 14 days) and dorsal root ganglia (neonatal day) were removed from mice, and the cells were dissociated and cultured in a medium conditioned by mouse cerebellar microexplant cultures as described previously (Nagata et al., 1986, Nagata and Nakatsuji, 1991). Telencephalons were removed from 7-8 days chick embryos, and cells were dissociated and cultured in a serum-free defined medium as described by Taguchi et al. (1987). In plating, about 10-100 μl of cell suspension was placed carefully over the microstructure using a micropipette at a low density, less than 2×10^2 cells per mm^2 . For the control culture, neuroblasts were cultured on the opposite non-fabricated side of the quartz plate.

Orientation analysis of neuroblast processes

Phase-contrast photomicrographs of neuroblasts on microstructures were taken randomly at a low magnification (10 \times objective lens). The angles between the cell processes (long and short) and the longitudinal axis of the microstructure were measured as described previously (Nagata and Nakatsuji, 1991), using the microstructure instead of the parallel neurite bundle.

Immunocytochemistry

Immunocytochemical staining for the presence of GABA (-amino butyric acid) was carried out as described previously (Nagata and Nakatsuji, 1991).

Scanning electron microscopy

Scanning electron microscopy was carried out as described by Nakatsuji and Nagata (1989).

Time-lapse video recording

Cell behavior was observed with a video-enhanced contrast microscopy system (C 1966, Hamamatsu Photonics). The image was recorded on a video cassette recorder (BVW75, Sony) controlled by a computer (HP 9300, Hewlett Packard) to record time-

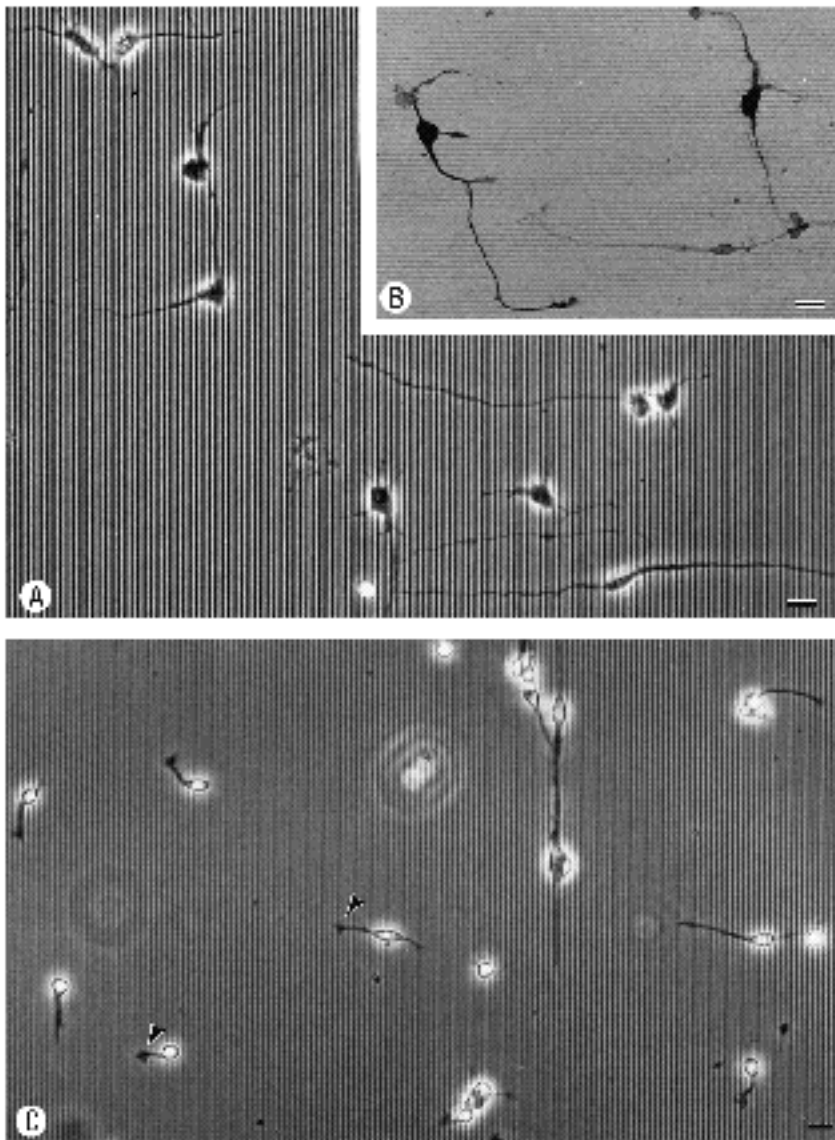


Fig. 1. Phase-contrast micrographs of cerebral cortical (A, B) and olfactory bulb (C) neuroblasts cultured on the microstructures. B shows cerebral cortical neuroblasts immunostained for GABA. Neuroblasts were dissociated from embryonic day-16 mice cerebral cortices (A, B) and postnatal day-1 mice olfactory bulbs (C). They were seeded and cultured on artificial microstructures of 0.5 μm depth and 1 μm widths of mSTP and mGRV for 5 days (A, B), and 0.27 μm depth, 1 μm of widths for 1 day (C). Both microstructures were heat treated before the double-coating. Arrowheads in C indicate growth cones of neuroblasts that might be migrating. Bars, 10 μm .

lapse frames at intervals of 20 seconds. The video camera was attached to an inverted microscope (Nikon Diaphoto TMD) equipped with a heated incubation chamber and 20× or 40× Hoffman modulation contrast objective lenses.

RESULTS

Orientation of CNS neuroblasts

CNS neuroblasts dissociated from the cerebral cortex (Fig. 1A,B), olfactory bulb (Fig. 1C), septum (Fig. 2), striatum, hypothalamus, tectum, hippocampus and brain stem extended their processes in both perpendicular and parallel orientations relative to the axis of the microstructure (Figs 3, 4). Most CNS neuroblasts had the capability of extending their processes at right angles on microstructures with very small width and depth around 1 μm . The frequency of the perpendicular orientation depended on the origin of neurons and also on the sizes of microsteps (mSTP) and microgrooves (mGRV) of the microstructure, as described below.

Small neuroblasts from olfactory bulb (Fig. 4A) at early postnatal stages and large neuroblasts from olfactory bulb, striatum (Fig. 4C), hypothalamus, cerebral cortex (Fig. 3) and tectum at embryonic stages showed a relatively higher frequency of the perpendicular orientation, whereas large septal (Fig. 4B) and hippocampal neuroblasts showed relatively higher frequency of the parallel orientation. Nevertheless, some septal cells oriented their processes nearly at 90° to the axis of the microstructure (arrows in Fig. 2). No rodent CNS neuroblasts survived on a microstructured quartz plate without any poly-L-lysine coating, but chick embryo telencephalic neuroblasts showed a low, but distinct, perpendicular orientation on the non-coated microstructure in a serum-free defined medium (Fig. 4E). Single cerebellar neuroblasts did not survive on the coated microstructure for more than a few days even in a conditioned medium, so we did not analyze their behavior.

Immunocytochemical staining

To assess whether only specific types of neuroblasts have

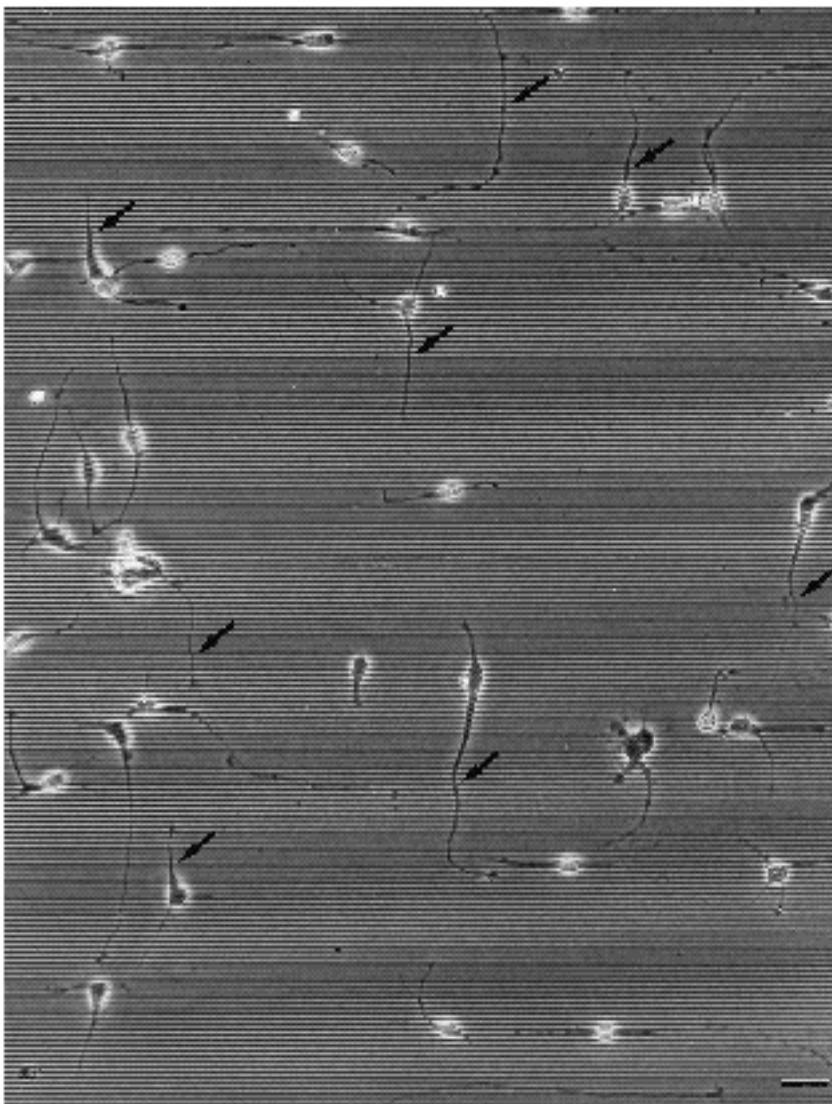


Fig. 2. Phase-contrast micrographs of septal neuroblasts cultured on the microstructures. Cells were dissociated from embryonic day-16 mice septa and seeded on the artificial microstructure of 0.5 μm of depth, 1 μm widths of mSTP and mGRV and cultured for 4 days. The microstructure was heat treated before the double-coating. Arrows indicate processes orienting perpendicular to the long axis of the microstructure. Bar, 20 μm .

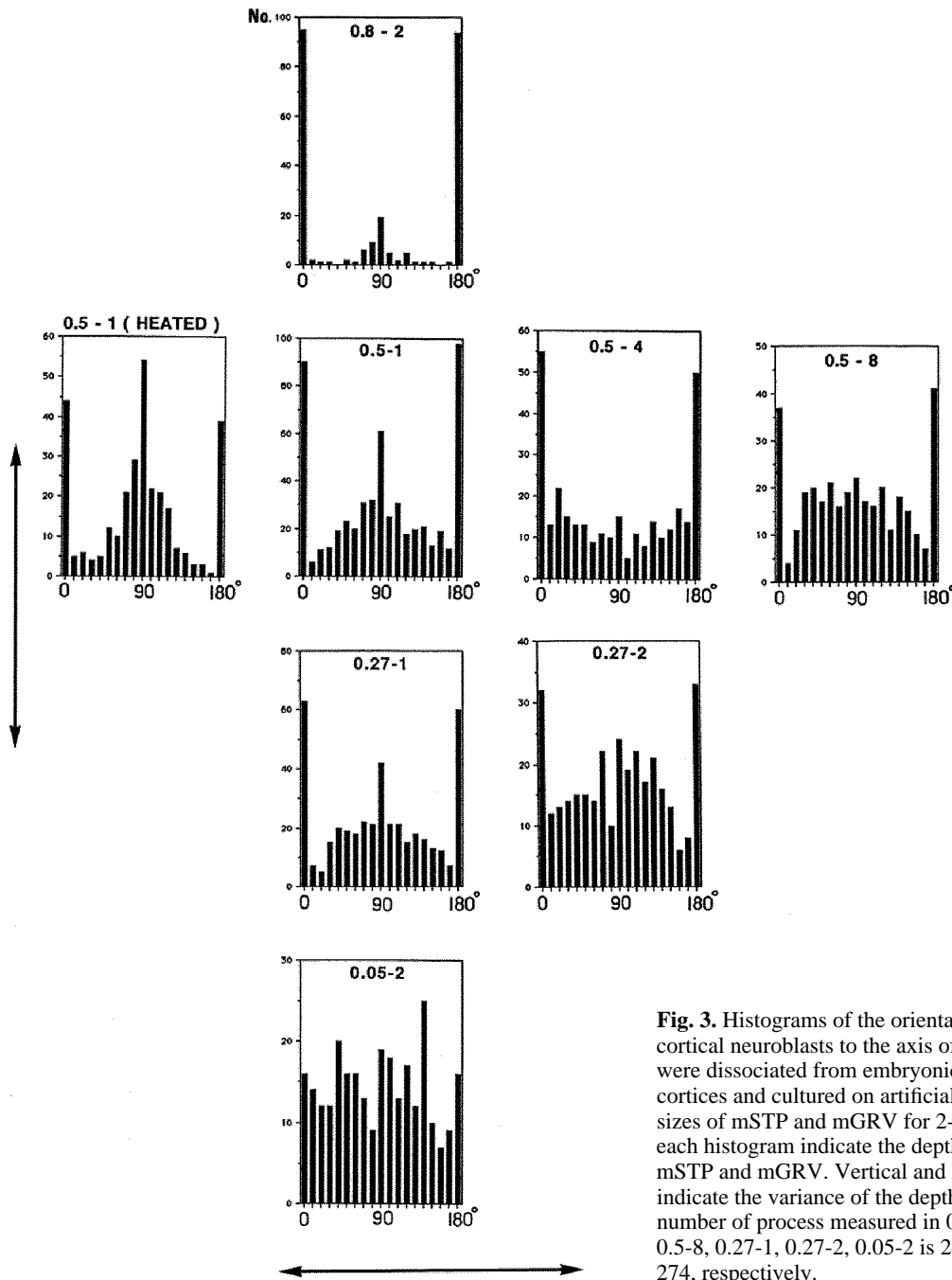


Fig. 3. Histograms of the orientations of processes of cerebral cortical neuroblasts to the axis of microstructures. Neuroblasts were dissociated from embryonic day 14-16 mouse cerebral cortices and cultured on artificial microstructures with various sizes of mSTP and mGRV for 2-4 days. The two numbers above each histogram indicate the depth and widths of the rectangular mSTP and mGRV. Vertical and horizontal double-headed arrows indicate the variance of the depth and widths, respectively. The number of process measured in 0.8-2, 0.5-1, 0.5-1(heated), 0.5-4, 0.5-8, 0.27-1, 0.27-2, 0.05-2 is 246, 562, 309, 317, 341, 415, 326, 274, respectively.

the capability of orienting perpendicular to the microstructures, or whether it is a general property, we stained neuroblasts immunocytochemically with antibodies to GABA. Cerebral cortical neuroblasts that expressed GABA immunoreactivity extended the processes in both parallel and perpendicular orientations on the artificial microstructure (Fig. 1B). The frequency of perpendicular orientation of GABA-immunoreactive neuroblasts (Fig. 4D) was similar to that of the total cerebral cortical neuroblasts (Fig. 3). Astrocytes, which showed GFA protein- and also GABA-immunoreactivities, always oriented parallel to the microstructure (not shown).

Variation of microstructures and process orientation

Systematic changes in the pattern of the microstructure revealed that both perpendicular and parallel guidance of all CNS neuroblasts tested were a function of the width and depth of the mSTP and mGRV (Fig. 3). The increment of the width of the mSTP and mGRV from 1 μm to 8 μm reduced the frequency of the perpendicular orientation of processes. No preferential orientation of neuroblasts was observed on a quartz plate without patterned microstructures (Fig. 4F). An increment of the depth from 0.05 μm to 0.5 μm increased the frequency of both perpendicular

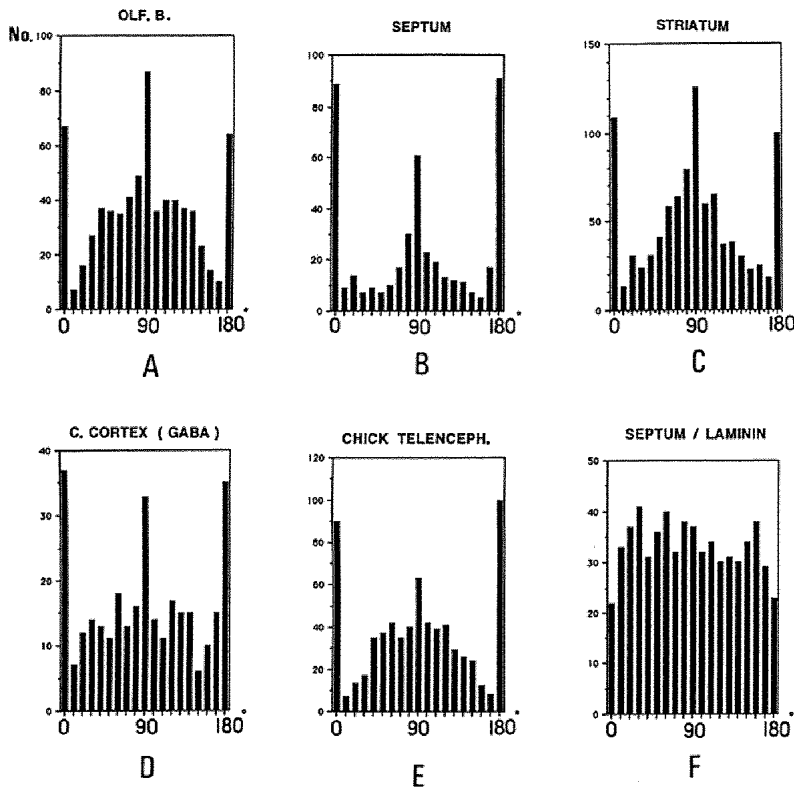


Fig. 4. Histograms of the process orientations of various types of neuroblasts to microstructures. Neuroblasts from postnatal 1-3 day mouse olfactory bulbs (A, OLF.B), E15-16 mouse septa (B, F, SEPTUM), E13-15 mouse striata (C, STRIATUM) and E16 mouse cerebral cortices (D, C.CORTEX) and chick embryo telencephalon of 7-8 days in ovo (E, CHICK TELEENCEPH.) were cultured on microstructures of 0.5 μm of depth and 1 μm of widths of mSTP and mGRV (A-D) which had been treated with heat before the double-coating, with 0.3 μm of depths and 1 μm of widths (E), or with no fabrication (F), for 2 days (A, B), 3 days (C, D, F) and 5 days (E), respectively. In D, the orientation of processes of neuroblasts with GABA immunoreactivity was measured. The number of processes measured in A-F is 702, 451, 972, 312, 700, 629, respectively.

and parallel orientation. Microstructures of 0.8 μm of depth caused an inhibition of neurite outgrowth in all types of neuroblasts. The highest frequency of perpendicular orientation was observed on a microstructure of 1 μm of widths and 0.5 μm depths of mGRV and mSTP, with heat treatment before the coating (Fig. 3).

Scanning electron microscopy

We observed the neuroblast culture by scanning electron

microscopy to examine how the neurite processes made contact with the grating-like microstructure. Fig. 5A-C show several cerebral cortical neuroblasts on the microstructure. Both cell bodies and processes attached to the surface of the mSTP, but not of the mGRV. Neurite processes observed at higher magnifications (Fig. 5C) often extended their microspike-like fine filopodia parallel to each mSTP, suggesting that the microspikes play a role in determining the perpendicular orientation. Such microspikes pro-

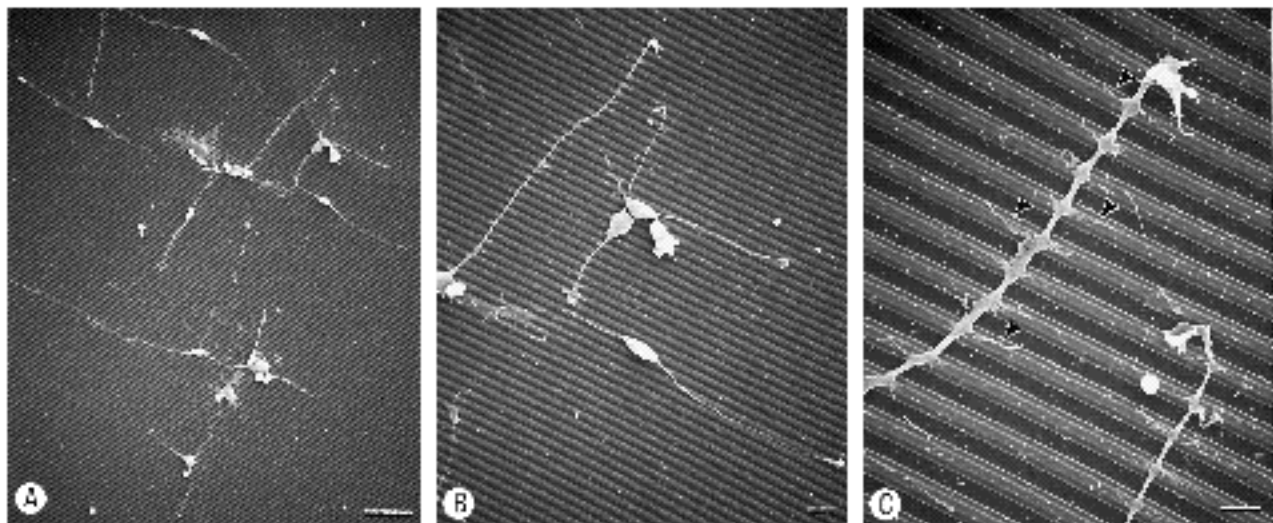


Fig. 5. Scanning electron micrographs of cerebral cortical neuroblasts on the microstructures. Cells were dissociated from cerebral cortex of embryonic day-14 mice and cultured for 2 days on a microstructure of 1 μm widths and 0.5 μm depth of mGRV and mSTP, which had been treated with heat before the double-coating. Arrowheads in C indicate fine filopodia from the neurite processes. Bars, A, 20 μm , B, 5 μm , C, 2 μm .

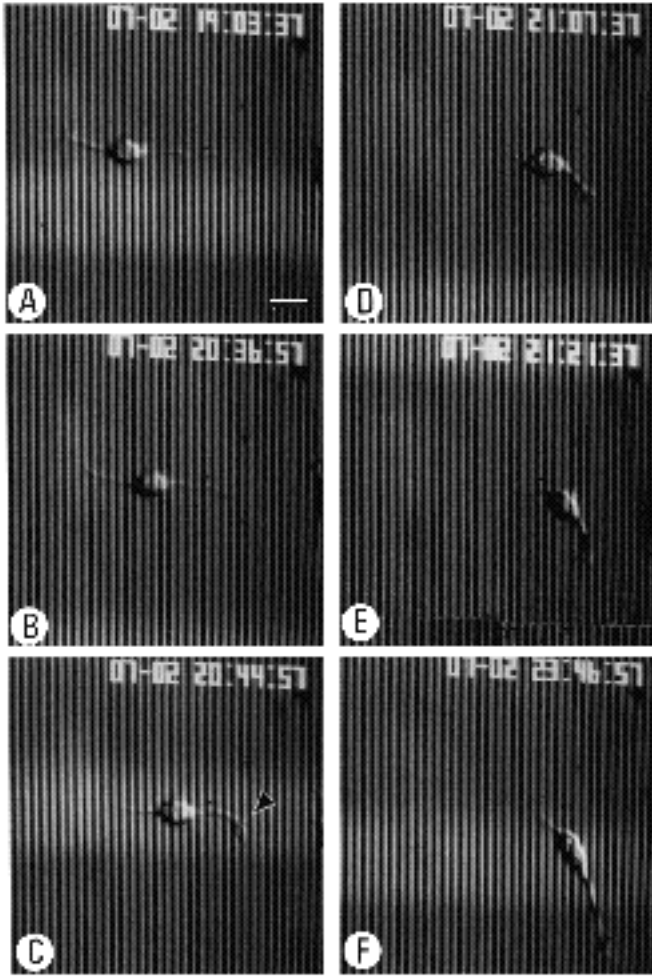


Fig. 6. Photographic prints taken from the time-lapse video recording of cerebral cortical neuroblasts cultured on the microstructure. Cells were dissociated from cerebral cortex of embryonic day-14 mice and cultured on a microstructure with 1 μm widths and 0.27 μm depth for 2 days. One neuroblast extends a thick process and moves in a perpendicular direction (A-C) relative to the axis of the microstructure, then changes the orientation of the growth cone (arrowhead), translocates the cell body in an oblique direction quickly (D) and extends the process in a parallel direction (E, F). Time is shown in the upper-right corner in hours, minutes and seconds. Bar, 10 μm .

truding from neurite shafts were also observed in living cultures on microstructures, using a video-enhanced Hoffman modulation contrast microscope (not shown).

Neuroblast motility on microstructure

Neuroblasts from embryonic cerebral cortex and from post-natal olfactory bulb migrated on the artificial microstructures in both perpendicular and parallel orientations. Fig. 6A-F show a migrating cerebral cortical neuroblast, which changed its direction of movement from perpendicular to parallel to the microstructure axis. The neuroblast extended a thicker process with a motile growth cone and then translocated the cell body in the parallel direction. Direction of the dominating cell process of migrating neuroblasts always coincided with that of cell translocation. During

such migration, the growth cone appeared to explore the surface of nearby mSTP and mGRV with many motile filopodia.

DISCUSSION

In mouse cerebellar microexplant cultures, granule cells first migrate away from the explant along radially oriented parallel bundles of their neurites, thus displaying typical contact guidance. Then, they change their orientation by 90° to extend cell processes and translocate the cell body perpendicular to the radial neurite. Since the perpendicular orientation and migration of granule cells on aligned neurites was a novel type of contact-guided cell behavior, we termed such behavior perpendicular contact guidance (Nakatsuji and Nagata, 1989). When neuroblasts dissociated from various regions of the CNS were cultured on heterotypic neurite bundles, CNS, but not PNS neurons, exhibited both perpendicular and parallel contact guidance; neuroblasts migrated and extended processes on tightly aligned neurite bundles in both perpendicular and parallel orientation (Nagata and Nakatsuji, 1991). Since the presence of the tightly aligned bundle of non-myelinated neurites (diameter, approx. 0.5 μm) was essential for the perpendicular orientation of neuroblasts, we speculated that artificially fabricated microstructures of similar shapes might be able to cause similar perpendicular guidance. To test this hypothesis, we used microstructures that were engraved on thin quartz plates by lithographic techniques (Hirono et al., 1988). Here, we have showed that most kinds of neuroblasts from the CNS orient their processes in both perpendicular and parallel orientations on artificial microstructures that mimic a neurite bundle. We also confirmed that PNS neurons showed conventional contact guidance but no perpendicular orientation on the microstructures (data not shown).

Hekmat et al. (1989) reported that GABA-uptaking inhibitory cerebellar cells, which expressed an embryonic type of N-CAM (neural cell adhesion molecule), oriented perpendicular to the aligned granule cell neurite in reaggregate cultures. This might suggest that only GABA-uptaking inhibitory cells exhibit perpendicular orientation on the aligned neurite and also on the microstructure. However, in our experiments, neuroblasts that expressed GABA-like immunoreactivity showed both perpendicular and parallel orientations on neurite bundles (unpublished data) and also on artificial microstructures. Further, most types of CNS neuroblasts tested showed perpendicular orientation on the microstructure with approximately 1-2 μm of widths and 0.3-0.8 μm of depths of mGRV and mSTP. Thus, the perpendicular orientation on such microstructures is a general feature of CNS neuroblasts.

In the present study, the CNS neuroblasts exhibited perpendicular contact guidance only when the following conditions were satisfied. They needed a repeated neurite-like microstructure of an appropriate size on flat surfaces. When the distance between steps was wider than 4 μm , almost no neuroblasts extended processes at right angles. This is in agreement with our previous observations that neuroblasts showed no perpendicular orientation when neurites were

not aligned, or when there were wide distances between neurites (Nakatsuji and Nagata, 1989; Nagata and Nakatsuji, 1991). Also, they needed the correct depth of microstructure. The most frequent perpendicular orientation of processes was observed on a microstructure with 0.5 μm depth for most types of neuroblasts. When the depth was smaller than 0.5 μm , the frequency of the perpendicular orientation of neuroblasts decreased drastically. On a microstructure with 0.05 μm depth, neuroblasts extended their processes at nearly random orientations. In contrast, on a microstructure of 0.8 μm depth, the growth of neurite processes was suppressed, although they still showed a distinct perpendicular orientation.

Pre-heating of the surface of microstructures remarkably increased the perpendicular orientation. Such treatment may have reduced the sharpness of the mSTP edge or oxidized the surface to give a more suitable surface for attachment of the growth cone of CNS neuroblasts.

Mechanism of perpendicular contact guidance

There are several lines of evidence that the physical surface microstructure, and not the specific chemical nature of the surface of aligned neurite bundles, is primarily important when neuroblasts extend their processes in perpendicular orientation. (1) The CNS neuroblasts extended their processes at right angles on artificial microstructures that had similar dimensions to a tightly aligned neurite bundle. On microstructures with depth less than 0.05 μm and width more than 4 μm , no neuroblasts showed the perpendicular orientation. The highest frequency of perpendicular orientation was observed on a microstructure of 0.5 μm depth and 1 μm wide steps and grooves, resembling a tightly aligned neurite bundle. (2) On microstructures coated with poly-L-lysine alone, CNS neuroblasts also showed perpendicular orientation (data not shown), although the double coating with poly-L-lysine and laminin caused a more stable attachment of neuroblasts to the substratum and a faster extension of processes. Furthermore, even in a fresh serum-free defined medium, neuroblasts from chick embryo CNS exhibited a distinct perpendicular orientation of processes on the non-coated substratum. (3) On neurite bundles that had been treated with trypsin (0.1% for 10 minutes at room temperature), CNS neuroblasts had extended juvenile perpendicular processes onto the treated neurite bundles by a few hours after the incubation (unpublished data), indicating that surface proteins that were susceptible to trypsin digestion are not essential for the perpendicular orientation.

Theoretically, perpendicular contact guidance could be produced when growth cones prefer the shortest route across the neurite bundle or the microstructure, but then, it is difficult to understand why they do not follow the parallel structure. Filopodial attachment and motile activity of the growth cone may play primary roles for feeling the minute surface structures and obstacles. The different size of growth cones of various neuron types may be correlated with the frequency of the perpendicular orientation. However, on this aspect, the most suggestive phenomenon that we observed in the time-lapse video recording is that single CNS neuroblasts changed their direction and migrated both perpendicular and parallel directions on neurite bundles or

certain artificial microstructures. In doing so, they did not change the size of their growth cones significantly during culture. During the perpendicular migration of granule cells in cerebellar microexplant cultures, filopodia not only from the growth cone but also from the shaft of the process actively explored each neurite of the bundle (Nakatsuji and Nagata, 1989). Thus, ultrastructural and pharmacological analyses of the behavior of filopodia and the growth cone may elucidate the mechanism of the perpendicular contact guidance.

Neuroblast orientation on neurite bundles and microstructures

Neuroblasts dissociated from various regions of the CNS appear to extend processes in a similar fashion on the neurite bundle and on the artificial microstructure. On both substrata, they showed three distinct peaks at 0°, 90° and 180° in orientation histograms. The frequency of the perpendicular orientation on the microstructure, however, was generally lower than that on the neurite bundle (Nagata and Nakatsuji, 1991). A high frequency of the perpendicular orientation was observed on both the neurite bundle and microstructure with the dendrite-like short processes of postnatal olfactory bulb and the axon-like long processes of embryonic olfactory bulb, cerebral cortical, striatal and hypothalamic neuroblasts. The long processes of large septal and hippocampal neuroblasts showed preferential perpendicular orientation on neurites, but a preferred parallel orientation on microstructures. However, most kinds of CNS neuroblasts, but not PNS neurons, exhibited a perpendicular orientation on artificial microstructures. Thus, CNS neuroblasts appear to have an inherent capability for process extension and migration perpendicular to the aligned neurite bundles by recognition of the physical surface structure. Cerebellar granule cells changed their orientation from parallel to perpendicular during microexplant cultures (Nakatsuji and Nagata, 1989; Nagata and Nakatsuji, 1991). On the artificial microstructure, they first extended long bipolar processes parallel to the axis of the microstructure. Single granule cells at a low cell density, however, survived for no more than a few days. Consequently, it was not possible to examine whether the perpendicular orientation would emerge later in the culture, as for microexplant cultures.

Perpendicular orientation and CNS development

So far, it is believed that neuroblasts migrate predominantly along glial processes *in vivo* (Sidman and Rakic, 1973) and *in vitro* (Hatten, 1990). But there is now accumulating evidence that neuroblasts migrate or orient their processes both in parallel and perpendicular to aligned non-myelinated fibers in developing CNS. For example, in cerebellar anlage (Hynes et al., 1986), cerebellar cortex (Altman, 1972; Hekmat et al., 1989), cerebral cortex (Austin and Cepko, 1990; Bayer et al., 1991; I. N. and N. N, unpublished data), optic tectum (Gray and Sanes, 1991) and brain stem (Ono and Kawamura, 1989), neuroblasts appear to migrate and orient their processes parallel and/or perpendicular to the aligned neurite bundle.

In cerebellar cortex, most types of neurons such as granule cells, basket, stellate or Purkinje cells orient their

processes in parallel and/or perpendicular to the parallel fibers or axons of granule cells. From the observation of migrating granule cells in mouse cerebellar microexplant cultures, we (1989) proposed a hypothesis that granule cells have an inherent tendency to migrate first parallel to the cerebellar surface in the external granular layer and, then, vertically inward across the bundle of parallel fibers during the cerebellar cortex histogenesis (Nakatsuji and Nagata, 1989; Nagata and Nakatsuji, 1990). The presence of transverse migration of granule cells across the mid-sagittal plane of the developing cerebellar cortex was confirmed by analysing the quail-chick chimera (Hallonet et al., 1990). Furthermore, Nagata and Nakatsuji (1991) found that, on homotypic and heterotypic neurite bundles, neuroblasts dissociated from CNS, but not PNS, migrated and oriented processes both parallel and perpendicular to the neurite bundles. Thus, the perpendicular contact guidance is a behavior specific to the CNS, and may play an important role in histogenesis of CNS.

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