Development 120, 2811-2822 (1994)
Printed in Great Britain © The Company of Biologists Limited 1994

Laminar specific attachment and neurite outgrowth of thalamic neurons on cultured slices of developing cerebral neocortex

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SUMMARY

In nervous system development, the growth cones of advancing axons are thought to navigate to their targets by recognizing cell-surface and extracellular matrix molecules that act as specific guidance cues. To identify and map cues that guide the growth of a particular axonal system, the thalamocortical afferents, an assay was devised to examine short-term interactions of dissociated embryonic thalamic cells with living, ~150 μm slices of developing mouse forebrain. Thalamic cells rapidly (<3 hours) and efficiently attached to and extended neurites on pre- and postnatal slices, but a broad zone throughout the neocortex was generally non-permissive for both thalamic cell attachment and the ingrowth of neurites. This zone coincided with the cortical plate at early stages (embryonic day 15), but later became restricted, in rostral-to-caudal fashion, to cortical laminae 2/3. Thus, at each stage, thalamic cells in vitro avoided just that area that thalamic axons confront, but generally do not enter, in vivo. In addition, neurites that extended on some layers were found to be significantly oriented in directions that coincide with the pathways that thalamic axons follow in vivo. These results imply that local adhesive cues and signals that affect process outgrowth are distributed among developing cortical laminae in a manner that could underlie much of the temporal and spatial patterning of thalamocortical innervation.

Key words: cortical development, thalamic innervation, adhesion, slice culture, neurite outgrowth, axonal development, axon guidance

INTRODUCTION

During brain development, the growth cones of advancing axons often travel relatively long distances to reach their targets. Evidently, this process involves specific decisions made by growth cones as they navigate (reviewed by Dodd and Jessell, 1988; Hynes and Lander, 1992). The formation of connections between the thalamus and the cerebral cortex is an attractive model for studying axonal development within the mammalian brain because the anatomy and timing of the growth of this pathway is relatively well characterized (reviewed by O'Leary and Koester, 1993).

In rodents, thalamic growth cones leave the thalamus and travel through the ganglionic eminence (anlage of the basal forebrain), to enter the developing cerebral cortex well before most of their target cells are born. The growth cones then travel mostly within the intermediate zone and subplate in a pathway that runs tangential to the cortical surface (the pia) and opposite in direction to the simultaneous growth of cortical efferents. Concurrently, the overlying cortical plate thickens as postmitotic neurons, including the target cells of thalamic axons, arrive from their birthplace in the ventricular zone. Thalamic growth cones project into the cell dense cortical plate only after the latter has begun to differentiate into distinguishable laminae (Lund and Mustari, 1977), eventually becoming cortical layers 2-6, which form in an 'inside-out' fashion (reviewed by DeCarlos and O'Leary, 1992; Erzurumlu and Jhaveri, 1992; Kageyama and Robertson, 1993). After entering the cortical plate, thalamic growth cones migrate toward the pial surface, passing through the infragranular laminae (5/6), to the granular layer (4) or the lower portion of layer 3 where most axons terminate and arborize (Miller et al., 1993; Kageyama and Robertson, 1993; Agmon et al., 1993).

There is both in vivo and in vitro evidence suggesting that the pathway decisions made by thalamocortical afferents are controlled by cues residing in specific cortical layers. For example, Ghosh et al. (1990) have shown that ablating subplate neurons in developing animals prevents the innervation of the cortical plate by thalamic afferents. Studies of long-term, thalamocortical co-cultures (Yamamoto et al., 1989, 1992; Molnár and Blakemore, 1991; Bolz et al., 1992; Götz et al., 1992) have demonstrated that axons extending from thalamic explants onto postnatal cortical slices generally stop growth in or near layer 4, the major target of these axons in vivo.

We wished to exploit the simplicity of the in vitro approach to look for some of the cues that guide thalamocortical afferents, especially those that might only be present transiently and would therefore be difficult to identify in long-term explant co-cultures. Consequently, embryonic thalamic cells were dissociated, fluorescently labeled and plated directly onto

living, vibratome slices of mouse forebrain taken from different developmental stages. Cells were examined both for attachment and neurite outgrowth during one day in culture.

Within 1 hour of coculture, cells were observed to attach well to all embryonic (E15) cortical layers except for the cortical plate, which supported poor attachment. Neurite outgrowth was observed within 3 hours and, on some laminae, exhibited a significant tangential orientation. However, no neurites were observed to enter the cortical plate. As slices from older animals were used, the non-permissiveness of the cortical plate gradually changed, in a manner that correlated both spatially and temporally with ongoing events in thalamocortical development. These results indicate that lamina-specific signals that affect thalamic neuron behavior can be detected in as little as 3 hours in vitro. Moreover, they suggest that as simple a mechanism as local differences in cell adhesion could play a major role in dictating the timing and location of thalamocortical innervation.

MATERIAL AND METHODS

All salts, sucrose and glucose were purchased from Mallinckrodt. Tissue culture media, sodium pyruvate, penicillin and streptomycin were purchased from Mediatech. All other reagents were purchased from Sigma unless otherwise indicated.

Dissection of tissue

Random-bred Swiss mice (CD-1, Charles River Laboratories) were naturally mated. Day of identification of vaginal plug was considered E0 and day of birth P0. For embryonic tissue, pregnant animals were killed by cervical dislocation and embryos removed. For postnatal tissue, animals were anesthetized by cooling on ice and brains were immediately removed. Brains were dissected in ice-cold phosphate-buffered saline (PBS: 137 mM NaCl, 2.68 mM KCl, 7.83 mM Na_2HPO_4, 1.47 mM KH_2PO_4) and prepared for sectioning (see below) or used to obtain tissue for dissociated cell preparations. In the latter case, further dissection of brains into regions (e.g. thalamus) was performed in ice-cold PBS supplemented with 4% calf serum (HyClone Laboratories), 5.6 mg/ml glucose, 25 i.u./ml penicillin, 25 μg/ml streptomycin and 0.001% phenol red, and the Atlas of the Prenatal Mouse Brain (Schambra et al., 1992) was used as a guide. The boundaries used in dissecting the thalamus were, in the rostrocaudal direction, the habenular recess and the dorsal diencephalic sulcus of the third ventricle while, in the dorsoventral direction, the boundaries were the epithalamus and a point just dorsal to the ventral diencephalic (hypothalamic) sulcus. Careful attention was paid to the removal of tissue from the mammillary, preoptic and tegmental nuclei as well of from the epi- and hypothalamus. Dissected tissue was immediately rinsed in multiple volumes of ice-cold HHBSS: calcium-magnesium free, Hank's Balanced Salt Solution (CMF-HBSS) supplemented with 10 mM Hepes, pH 7.2.

Dissociation and labeling of thalamic cells

Thalami were incubated in HHBSS with trypsin (0.18 mg/ml) and DNAse (0.08 mg/ml) in a 15 ml polypropylene, conical tube for approx. 10 minutes at 37°C with occasional, gentle mixing. The tissue suspension was then diluted with 1/3 volume of 37°C Complete Medium (CM): Dulbecco's modified Eagle's medium (DMEM: glutamine free, 4.5 g/L glucose) supplemented with 10 μg/ml transferrin, 5 mg/ml crystalline grade bovine serum albumin (BSA; ICN Biochemicals), 20 nM progesterone, 30 nM sodium selenite, 100 μM putrescine, 10 μg/ml bovine insulin, 1 mM sodium pyruvate, 50 i.u./ml penicillin, 50 μg/ml streptomycin and 25 mM Hepes (pH 7.2). Soybean trypsin inhibitor (0.28 mg/ml) was added, the DNAse concentration was increased to 0.19 mg/ml and the suspension was incubated for another 5 minutes at 37°C. Tissue was triturated gently five times with a flame-polished pasteur pipette that was coated with a solution of 4% BSA in CMF-HBSS (brought to pH of 7.3 with 1 N NaOH). The suspension was brought to a final volume of approx. 10 ml with HHBSS, undissociated tissue was allowed to settle (approx. 5 minutes) and the supernatant was transferred to a new tube. The cell suspension was centrifuged at 100 ***g*** for 10 minutes and the pellet gently resuspended in Labeling Medium which consisted of 30 μM Cell Tracker™ CMTMR (Molecular Probes, Eugene, OR) in CM; prepared, centrifuged to remove undissolved dye and filter sterilized just before use. Cells were incubated at 37°C for 30-50 minutes. Two volumes of 37°C CM were added to the suspension along with DNAse (0.12 mg/ml) and incubation continued for 5-15 minutes. Several volumes of HHBSS were added and the suspension was underlayed with a 2 ml cushion of 4% BSA (crystalline BSA in CMF-HBSS; brought to pH 7.3 with 1 N NaOH). Cells were then centrifuged at 150 ***g*** for 10 minutes. The pellet was resuspended in CM and cells were checked for viability using Trypan Blue. Routinely, >95% of cells excluded Trypan Blue. The cell suspension was diluted with CM to a concentration of $\approx 2.5\times 10^6$ cells/ml.

Preparation and fixation of living slice cultures

Brains were placed in 37°C, molten, low melting point agarose solution (Gibco BRL; 2% in PBS supplemented with 3 g/L glucose) which was immediately placed on ice to harden. Agarose blocks containing brains were cut to size (approx. 1.5 cm^3), glued to a vibratome tray (Technical Products International, Inc., St. Louis), immersed in ice-cold PBS (supplemented with 0.45% glucose and 1 mM sodium pyruvate) and the tray was subsequently surrounded by an ice bath. Under the PBS bath, brains were vibratome-sectioned (150-200 μm thick) and slices were placed on nitrocellulose (0.45 μm; Sartorius, Bohemia, NY) filter disks (12 mm diameter) that had previously been sterilized, incubated in a sterile solution of concanavalin A (1.3 mg/ml in glutamine free DMEM) for 4-18 hours at 4°C and rinsed several times in HHBSS before use. Disks with mounted slices were removed from the bath, placed on sterilized parafilm in plastic Petri dishes and 100 μl of ice-cold CM was added, forming a bubble over the slices. Slices were kept on ice until all had been cut, medium was removed, 45 μl of fresh CM added and then slices were moved to a 37°C humidified incubator with a 8% CO_2 atmosphere. Cells were plated onto slices by carefully pipetting 45 μl of cells into the medium already on slices ($\approx 10^5$ cells/filter disk; $\approx$1000-2000 cells/mm^2). Cells were allowed to settle at 37°C and incubated for various periods. Cultures were transferred to a large volume of 37°C HHBSS and gently rinsed to remove unattached cells (for some experiments, cells were visualized by fluorescence microscopy before the transfer and rinse procedure). Cultures were then added to warm (37°C) fixative: 4% paraformaldehyde (J. T. Baker, Phillipsburg, NJ) in 0.2 M sodium phosphate buffer (pH 7.5). After 15-30 minutes, the fixed tissue was rinsed in PBS and counterstained (using 10 μg/ml bisbenzimide [Hoechst 33258] in PBS). Cultures were mounted on glass slides in a saturated sucrose solution containing 0.1% sodium azide (Fluka AG) and coverslipped.

Immunohistochemistry

Slice cultures were prepared, rinsed and fixed as above except that fixation was limited to 18 minutes. Cultures were then washed several times in PBS and treated with 1% Triton X-100 in PBS for 7 minutes at room temperature. The following incubations were done at 4°C. Tissue was blocked (40% goat serum, 40% calf serum, 0.1% Triton X-100, 0.1% sodium azide in PBS; filtered) for 19 hours, washed (5% goat serum, 5% calf serum, 0.1% sodium azide in PBS; filtered) for 3 hours and first antibody was added: CS-56 monoclonal mouse anti-chondroitin sulfate (Sigma), ascites fluid diluted in wash solution, 1:500. After 48 hours, cultures were washed several times over 20 hours and then incubated with second antibody for 4 hours: FITC-con-

jugated, goat anti-mouse IgM, μ-chain specific (TAGO, Burlingame, CA), diluted 1:80 in wash solution. Cultures were washed several times, rinsed in PBS, counterstained with bisbenzamide, rinsed in 0.1 M $NaHCO_3$ (pH 9.2) and mounted in a saturated sucrose solution containing 0.1 M $NaHCO_3$ (pH 8.5) and 0.1% sodium azide.

For staining of dissociated cells, the following methods were used. Cells were plated onto acid-washed glass coverslips (12 mm diameter) that had been treated with polylysine (100 μg/ml in PBS) overnight at 4°C, washed several times with PBS, incubated with concanavalin A (400 μg/ml in HHBSS) for 4 hours at 37°C and rinsed several times in HHBSS. Plated cells were left for 3.5 hours at 37°C in a humidified incubator (8% CO_2 atmosphere), fixed for 22 minutes with 4% paraformaldehyde in 5% sucrose/PBS and rinsed with PBS. For staining with antibodies to intracellular epitopes, cells were treated at this point with 0.5% Triton X-100 in PBS for 5 minutes at room temperature and, for all experiments, cells were blocked with a solution of 5% goat serum, 5% calf serum and 0.1% sodium azide in PBS. This solution was used for all subsequent washes and antibody dilutions. Anti-neurofilament (monoclonal antibody RT97, 1:666 dilution of ascites fluid [Wood and Anderton 1981]); anti-neuron specific β-tubulin (monoclonal antibody TUJ1, 1:200 dilution of concentration culture supernatant [Easter et al., 1993]) and anti-mouse NCAM (monoclonal antibody H28, 1:1 dilution of culture supernatant [Gennarini et al., 1984]) were applied at 4°C overnight. FITC- (Antibodies Inc., Davis, CA) and rhodamine-conjugated (Kirkegaard & Perry Labs Inc., Gaithersburg, MD) secondary antibodies were used for visualization. Cell nuclei were counterstained with bisbenzamide and coverslips mounted in 80% glycerol in PBS or 0.1 M $NaHCO_3$.

Preparation and fixation of cryostat section cultures

Dissected brains were snap frozen in isopentane kept in a dry-ice/ethanol bath. Frozen brains were equilibrated to −20°C and mounted in OCT compound (Miles, Inc., Elkhart, IN). 20 μm slices were cut on a Reichert-Jung 2800 Frigocut-E cryostat. Slices were warmed onto ProbeOn Plus™ glass slides (FisherBiotech) and air dried. OCT was peeled off and a well was created (approx. 12 mm diameter) around the tissue slice with rubber cement (Dennison Stationary, Framingham, MA). Slices were then rehydrated with PBS, rinsed with CM and 45 μl of fresh medium was added to the well. Slices were kept at 37°C for 30 minutes before 45 μl of cell suspension was added to the medium on each slice (approx. 10^5 cells/well) and cultures were incubated at 37°C for 3 hours. Unattached cells were gently rinsed off with 37°C HHBSS. The rubber cement was removed and warm (37°C) fixative was added for 10-20 minutes. After rinsing with PBS and counterstaining (10 μg/ml bisbenzimide in PBS), cultures were visualized by fluorescence microscopy.

Data collection

The selection of fixed slice cultures for quantification was based solely on the integrity of slice anatomy, as visualized by bisbenzamide staining of cell nuclei. Cultures were chosen only if the laminar borders could be clearly discerned and the identity of individual laminae unambiguously determined. Borders were delineated by differences in the density of cell nuclei and laminae were identified in some cases by specific landmarks, such as barrels within the granular layer of the P7 somatosensory cortex. Slices that had been folded or torn while being placed onto the nitrocellulose substrata, or had otherwise become distorted due to stretching or flattening, were not quantified. Importantly, slice selection was carried out under UV (bisbenzamide) fluorescence only. Since Cell Tracker™ CMTMR-fluorescence is not observed in this channel, it was possible to be confident that the selection of slices was carried out blind to patterns of thalamic cell attachment.

Measurements of cell attachment, neurite length and neurite orientation were obtained from digitized images of fixed cultures. Images were collected using a Zeiss fluorescence microscope fitted with camera lucida optics that were set to view a video monitor. A video trace, which was superimposed over the fluorescence image of the culture within the microscope objectives, was drawn using the hardware and software of the Neuron Tracing System (Eutectics Electronics Inc., Raleigh, NC). Cortical layers and their borders were identified and traced under UV fluorescence while labeled and attached thalamic cells were individually traced under rhodamine fluorescence. Using an automated, calibrated microscope stage and both fluorescence channels, a composite image of slice anatomy, attached cells and neurite morphology was drawn to scale across several microscope fields.

Neurite data were collected from experiments using sagittal slices. Neurites were traced only if their growth cones and soma of origin could be clearly demarcated and if they were at least one soma diameter in length (approx. 10 μm). Since neurites often projected downward, into the slice tissue, they were traced in three dimensions (automated and calibrated control of the focus knob allowed for images to be traced through focal planes). Some cells had two neurites longer than a cell diameter; in these cases, both processes were traced. No neurites had branches which themselves met the length criterion.

Attachment data analysis

The surface area of and the number of cells on each layer were computed based on stored digital images and attachment densities were calculated from these values. Distances from the cortical plate/subplate border of cells attached to the subplate and intermediate zone were calculated by the system software as the distance, within a plane parallel to the plane of the slice, between the center of each attached cell and the closest point on the cortical plate/subplate border to that cell.

Attachment data for different cortical layers were compared by a single factor analysis of variance (ANOV) to obtain reported P values. A Newman-Keuls test was used to verify the outlier values (Zar, 1974).

Neurite data analysis

Neurite lengths were calculated by the system software as the 3-D contour distance between a neurite's origin (point of connection to the soma) and its endpoint (center of the growth cone). Orientations for marginal zone, subplate/intermediate zone and ventricular zone neurites were calculated with respect to the marginal zone/cortical plate, cortical plate/subplate and intermediate zone/ventricular zone borders respectively. Values consisted of the angle between the reference border (or, if curved, a tangent to the border) closest to the neurite's origin and a line defined by the neurite's origin and its end point. Only coordinates along the rostral-caudal and pial-to-ventricular axes were used for this calculation. A neurite projecting rostrally, parallel with its reference border, defined 0°. Projections directed toward the pia, perpendicular to the reference border, were assigned 90° and those directed toward the ventricle were assigned −90°.

For polar plots, neurites were separated according to their angle into twelve 30° interval bins. Neurites that fell on the border between two bins were split with a value of 0.5 assigned to each bin. Each plot contains twelve points which consist of the angle of the *center* of the bin (θ=−30°, 0°, 30, 60°, ...) and the number of neurites in that bin (r).

A chi-square analysis was used to test whether neurite data fit a uniform (random) circular distribution by comparing binned, neurite samples to a hypothetical sample consisting of the same number of neurites evenly distributed among all 12 bins (Zar, 1974).

RESULTS

Region-specific attachment of thalamic cells to living slices of E15 forebrain

To examine how thalamic neurons behave when confronted in vitro with different brain environments, embryonic day 14 or 15

(E14-15) mouse thalamic cells were dissociated, labeled with a vital fluorescent dye, washed into serum-free culture medium and allowed to settle onto freshly cut vibratome slices (150-200 μm) of developing mouse forebrain. After 3 hours at 37°C, the slices were gently rinsed to remove unattached cells and then fixed, counterstained with bisbenzimide (Hoechst 33258) and visualized by fluorescence microscopy (see Methods). To avoid the possibility of dye-transfer from thalamic cells to intrinsic cells of the slice, thalamic cells were labeled with a thiol-reactive fluorescent dye, CellTracker™ CMTMR, which diffuses into cells, becomes covalently coupled to intracellular components (e.g. glutathione) and is fixable by cross-linking agents. Immunocytochemical evaluation of dissociated thalamic cells prior to plating onto slices indicated that 91% (±3.3%, *n*=904) expressed neuron-specific class III β-tubulin and 70% (±3.5%, *n*=828) contained neurofilament protein. Thus, the vast majority of the cells were neurons. In addition, 83% (±4.0%, *n*=616) were immunoreactive for NCAM.

When thalamic cells were prepared and plated in this manner onto E15 forebrain slices, large differences were evident in the numbers of fluorescent cells that were found on different parts of the slice. The most striking differences were in the cerebral cortex, the anatomical layers of which could be identified by nuclear density (Fig. 1). Specifically, very few thalamic cells were found on the cortical plate, whereas numerous cells were found on each of the other cortical layers – the marginal, intermediate and ventricular zones. On most subcortical regions (e.g. caudoputamen, globus pallidus, preoptic and supraoptic areas, hypothalamus, thalamus), there was also a high density of thalamic cells, although on some (the anterior commissure, the claustrum, the amygdaloid area and olfactory nuclei of the ventral forebrain and parts of the septum) few cells were seen (data not shown).

In some experiments, slices were examined just prior to rinsing and fixation. In these cases, thalamic cells appeared to be uniformly distributed over each slice. Thus, the differences in fluorescent cell density that were seen with rinsed, fixed slices apparently reflected region-specific cell attachment, rather than non-uniform plating of cells or migration of cells from some regions to others during the 3 hour culture period. In other experiments, thalamic cells were plated and cultured on forebrain slices for different periods of time before rinsing and fixation. Patterns of cell attachment similar to that in Fig. 1 were obtained whether rinsing and fixation were carried out as early as 1 hour, or as late as 24 hours, after plating (data not shown). We also found that the preincubation of slices for up to 5 hours at 37°C prior to the plating of cells produced the same pattern of cell attachment as seen in the previous experiments (data not shown).

To quantify thalamic cell attachment to the different layers

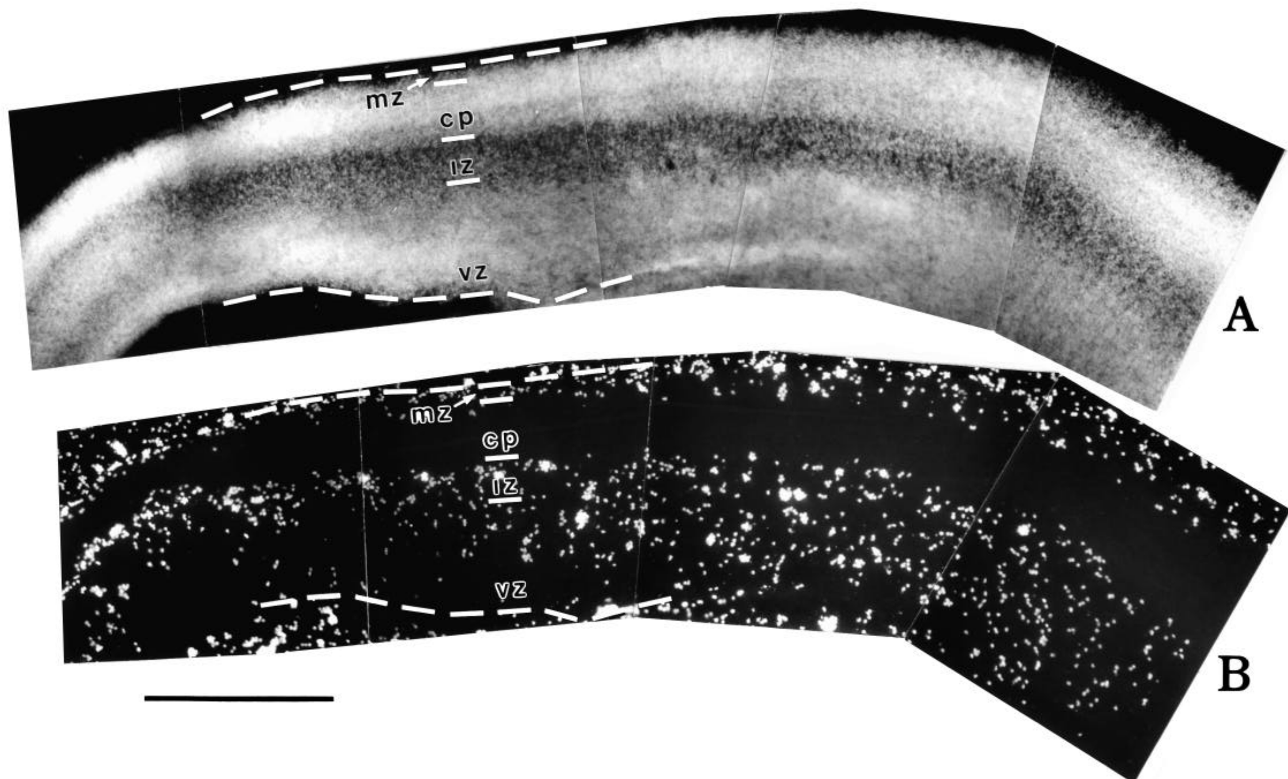

Fig. 1. Laminar specific attachment of dissociated thalamic cells to slices of embryonic cortex. (A) Laminae of an E15 cortex can be seen under UV fluorescence as differences in nuclear density after a sagittal slice is cultured with thalamic cells (3 hours), fixed and stained with bisbenzamide (see methods). Rostral is to the right, caudal to the left. (B) The same slice viewed under rhodamine optics shows the distribution of attached thalamic cells. Some attached cells are in clusters, which appear as larger, bright dots. The pial and ventricular edges of the cortex are demarcated by dotted white lines at the top and bottom of each figure. Lines are also positioned in the two photos to reference the same points in the two views of the slice. Very few thalamic cells attach to the cortical plate (CP) while more attach to the intermediate zone (IZ), marginal zone (MZ) and ventricular zone (VZ) as well as to the substratum off the slice (seen at the edges of the photos). Density of attached cells is greatest on the intermediate zone just subjacent to the cortical plate. Scale bar, 500 μm.

of the E15 neocortex, measurements of the density of labeled cells on each layer were made for 8 slices and are shown in Fig. 2. Mean thalamic cell attachment to the marginal, intermediate and ventricular zones was in the range of 1300-2300 cells/mm^2 and was close to the density of cells that was initially plated onto the slices (~1000-2000 cells/mm^2; see methods). In contrast, thalamic cell attachment to the cortical plate was substantially lower (243 ± 60 cells/mm^2). This result was obtained regardless of whether coronal or sagittal forebrain slices were used (Fig. 2) and was qualitatively similar throughout the rostrocaudal and dorsoventral extent of the neocortex.

In photographs such as Fig. 1, the contrast in thalamic cell density between the cortical plate and intermediate zone is particularly noticeable because thalamic cells on the intermediate zone are especially concentrated near the border with the cortical plate (also see Fig. 3). This can also be illustrated by plotting cell density in the intermediate zone as a function of distance from the cortical plate (Fig. 2, inset). These data suggest that thalamic cell attachment is especially high in the subplate, the region of the intermediate zone subjacent to the cortical plate (Kostovic and Molliver, 1974; Luskin and Shatz,

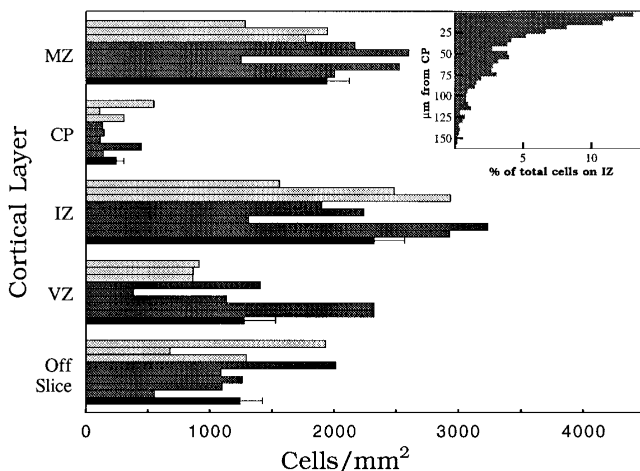

Fig. 2. Densities of thalamic cells attached to embryonic cortical layers. The densities of cells attached to each of the major embryonic cortical layers – the marginal zone (MZ), cortical plate (CP), intermediate zone (IZ) and ventricular zone (VZ) – were determined for three coronal (light bars) and five sagittal (darker bars) slices. Black bars represent the means (± standard error) from the 8 samples. Data were collected from the portion of sagittal slices indicated by dotted lines in Fig. 1 or from the dorsolateral part of coronal slices because laminae could be unambiguously distinguished within these regions (total number of cells counted from the 8 slices: n_{mz}=1818, n_{cp}=316, n_{iz}=2001, n_{vz}=1647, $n_{off\ slice}$=2583). 'Off Slice' data were collected from the culture substratum, adjacent to the pial surface. Mean values are significantly different among the four layers (Analysis of Variance test: P<0.0005). A Newman-Keuls analysis verified the value of the mean density on the cortical plate as a significant outlier ($\mu_{iz} = \mu_{mz} \neq \mu_{vz} \neq \mu_{cp}$). INSET shows that cells attached to the intermediate zone are not uniformly distributed on this layer. Cells that attached to the intermediate zone were binned according to their distance from the cortical plate border and the data, averaged from the 8 slices, were plotted as the proportion of total cells attached to this layer. On average, over half of the cells that attached to the intermediate zone are located within 25 µm of the cortical plate border.

1985). Because the rodent subplate is only a few cell layers thick (Valverde et al., 1989; Bayer and Altman, 1990), its boundaries cannot be accurately determined in our thick slices by nuclear staining alone. In some experiments, the subplate was visualized by chondroitin sulfate immunohistochemistry (Sheppard et al., 1991) and was found to correspond to the part of the intermediate zone extending up to ~25 µm from the cortical plate border (data not shown), i.e., containing that region which supports the highest density of attached cells (Fig. 2, inset).

Neurite outgrowth by thalamic neurons on living slices of E15 neocortex

Even though thalamic cells were typically plated onto forebrain slices for only 3 hours, substantial neurite outgrowth occurred on all forebrain regions where thalamic cells attached, including the marginal, intermediate and ventricular zones of the developing cortex (too few cells attached to the cortical plate to permit conclusions to be drawn about neurite outgrowth on this layer). There were no significant differences in the lengths of neurites on cortical layers (marginal zone: 16.1 µm [s.d.=5.7; n=85]; intermediate zone: 16.1 µm [s.d.=6.5; n=114]; ventricular zone 15.8 µm [s.d.=6.8; n=52]). Interestingly, the orientation of neurites on the intermediate zone appeared non-random. On this layer, most neurites projected parallel to the cortical laminae (i.e. along the rostrocaudal axis), while few neurites projected radially (Fig. 3).

Fig. 4 shows the ranges of neurite angles (as measured with respect to nearby cortical layer borders; see Methods) that were observed for thalamic cells attached to marginal, intermediate and ventricular zones. The data confirm that a disproportionate number of intermediate zone neurites are rostrocaudally oriented (P<0.001), with no significant bias toward the rostral or caudal direction (52% and 48% of sample, respectively; n=114). In contrast, neurites extended by thalamic cells on the marginal and ventricular zones followed a much more uniform distribution, not significantly different from random (see Fig. 4, legend).

Since the subplate was included as part of the intermediate zone in the above analysis, an attempt was made to analyze subplate and non-subplate neurites separately. Neurites seen on the intermediate zone were therefore divided into two populations – those originating within one average neurite length (16.1 µm) of the cortical plate (probable subplate neurites) and those originating in the rest of the intermediate zone. As shown in Fig. 5, both sets of neurites exhibited orientations that were significantly biased toward the horizontal, i.e. along the rostrocaudal axis. Interestingly, among the putative subplate neurites (Fig. 5A), extremely few were oriented superficially (i.e., toward the cortical plate). Consistent with this observation, in no case were neurites observed that crossed from the subplate onto the cortical plate. Indeed, subplate neurites were occasionally seen that extended a short distance superficially, then turned sharply at the cortical plate border (not shown). In contrast, neurites were often seen that crossed the border between the intermediate and ventricular zones, without evidence of turning behaviors. Although these data raise the possibility that thalamic neurites actively avoid the cortical plate, time-lapse cinematographic observations will be required to establish such a conclusion.

Behavior of thalamic cells on frozen sections of E15 forebrain

Others have found that neurons can exhibit region-selective attachment and/or neurite outgrowth on frozen (cryostat) sections of brain, nerve or muscle (Carbonetto et al., 1987; Sandrock and Matthew, 1987; Covault et al., 1987; Watanabe and Murakami, 1989; Savio and Schwab, 1989; Crutcher, 1989; Geisert, 1991; Tuttle and Matthew, 1991). To determine whether this method would reveal thalamic cell behaviors similar to those observed using living slices, E15 thalamic cells were prepared and labeled as before, but plated onto air-dried 20 μm cryostat sections of fresh-frozen E15 forebrain. After 3 hours at 37°C, non-attached cells were rinsed off and cultures were fixed and counterstained. Compared to previous experiments with living sections, the absolute density of attached cells was much lower (Fig. 6). Moreover, attachment appeared to be fairly uniform across all cortical layers. In addition, little neurite outgrowth from cells attached to the cryostat sections was seen. The fact that frozen sections could not substitute for living slices in revealing cortical layer-specific behaviors of thalamic neurons raises the possibility that those behaviors require living cortical cells. However, when vibratome slices were lightly fixed with formaldehyde before thalamic neurons were plated onto them, thalamic cells displayed layer-specific attachment much like that which was seen when they were plated onto living slices (data not shown). Thus, it appears that cortical cell viability is not required to obtain cortical layer-specific thalamic cell attachment. It seems more likely that critical molecules or structures that are preserved in vibratome sections are lost or disrupted in frozen sections.

Developmental changes in the attachment and neurite outgrowth activities of neocortex

At E15, the cerebral cortex is at a relatively early stage in its development. To determine whether the laminar-specific differences in the behavior of embryonic thalamic neurons change as the cortex matures, embryonic (E14-15) thalamic neurons, prepared as before, were plated onto living slices cut from older brains. Postnatal days 1 and 7 (P1 and P7) were chosen to represent intermediate and late stages in cortical histogenesis (reviewed by Bayer and Altman, 1991) and thalamocortical innervation (reviewed by Erzurumlu and Jhaveri, 1992; Kageyama and Robertson, 1993).

Fig. 7 shows the results of a typical experiment involving a sagittal slice from a P1 animal. At the caudal end of the slice, the pattern of thalamic cell attachment is similar to that observed with E15 slices: cells attached to the marginal zone (i.e., cortical layer I) and the intermediate zone (i.e., the developing white matter), but not to the cortical plate, which at this age consists of developing layers 2-6 (the ventricular zone is not indicated in Fig. 7 because at this age it has become too thin to distinguish clearly). Moving toward the rostral end of the slice, however, one sees increasing numbers of labeled cells attached to the cortical plate, especially the deeper regions of the cortical plate. Since the maturation of the neocortex is known to follow a rostral-to-caudal gradient (Smart, 1983; Bayer and Altman, 1991), these results suggest that the cortical plate may become permissive for thalamic cell attachment as the cortex matures.

In support of this idea, when E14-15 thalamic cells were plated onto P7 sagittal slices, cell attachment to the cortical plate was observed throughout the rostrocaudal extent of the slices (Fig. 8). As was seen on E15 and P1 slices, thalamic cells also attached well to cortical layer I and the developing white matter. Unlike what was observed using E15 slices, labeled cells on the developing white matter (former intermediate zone) were not concentrated near the cortical plate border (site of the embryonic subplate).

The data on thalamic cell attachment to P7 slices are presented quantitatively in Fig. 9. When these data are compared with Fig. 2, the most striking difference is the increase in the level of thalamic cell attachment to the P7 cortical plate relative to other cortical layers. Closer inspection of the sections revealed, however, that it is not the entire P7 cortical plate that supports thalamic cell attachment, but only its deeper layers. Using nuclear staining alone, it was possible to divide the P7 cortical plate into supragranular (laminae 2/3),

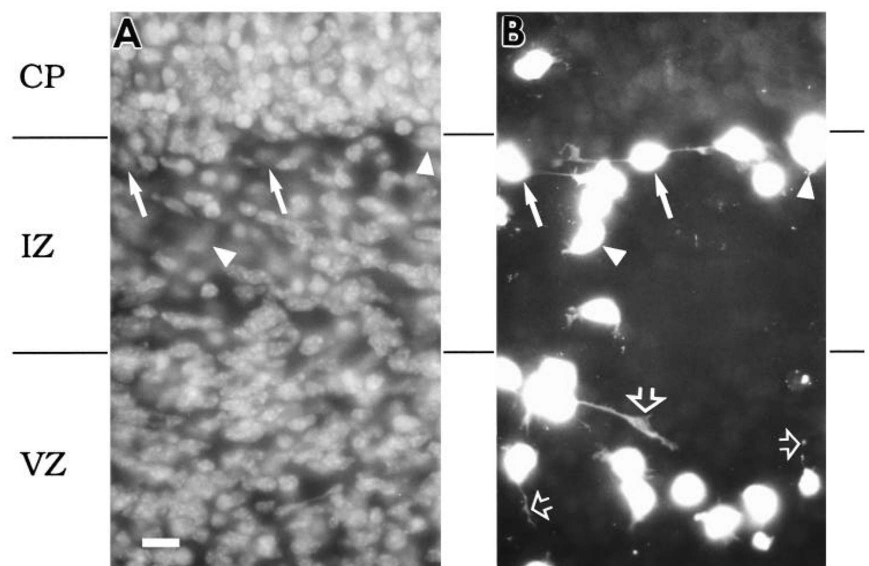

Fig. 3. Neurite extension on E15 cortex. (A) High-magnification, UV fluorescence micrograph of the bisbenzamide-stained cortex, from an experiment similar to that in Fig. 1, shows the cortical plate (CP), the cell poor intermediate zone (IZ) and the ventricular zone (VZ) as indicated at left. (B) The same field seen under rhodamine optics reveals attached thalamic cells and their neurites. Fluorescent cells (filled arrows) attached to the intermediate zone extend three neurites in directions parallel to the CP/IZ border. On the ventricular zone, neurites (open arrows) project both parallel to the cortical layers and along the radial axis (perpendicular to the lamina). Growth cones of those neurites projecting radially cannot be seen because the neurites extend into the slice and out of the plane of focus. Filled arrows and arrowheads in A point to nuclei whose corresponding cells are marked by the same labels in B. These nuclei are out of focus because attached cells lie in a focal plane slightly above that of the cells of the slice. Scale bar, 10 μm.

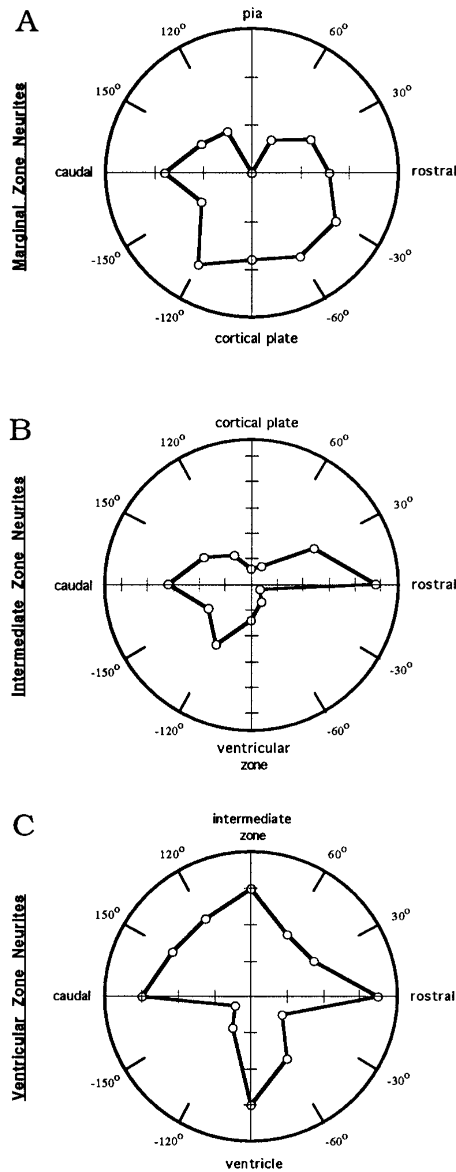

Fig. 4. Distribution of thalamic neurite angles on the different embryonic cortical layers. Polar plots show the angular distribution of neurites projected by thalamic cells on the marginal (A), intermediate (B) and ventricular (C) zones of E15 cortical slices. Neurite angles were calculated with respect to laminar borders and then segregated into twelve, 30° interval bins (see methods). Plotted points represent the center angle of each bin (θ) and the number of neurites (r) in the sample with angles that are within that bin interval. Axis tick marks represent a count of 5 neurites in A, 5 in B and 2 in C. The shapes of the plots reflect the predominant directions in which neurites project on a given layer. Plot frames indicate the angle of each bin's center except for the bins centered on the axes, which are marked with cardinal directions based on cortical anatomy (90° and −90° represent projections toward the overlying and subjacent layers or tissue edges). Marginal (A) and ventricular (C) zone neurites project both rostrocaudally and radially (toward the pia and ventricle) and are not significantly different from a uniform circular distribution (chi-square to uniform sample: $P_{mz}>0.1$, $P_{vz}>0.5$). Intermediate zone neurites mostly project rostrocaudally and are different from a uniform distribution ($P_{iz}<0.001$). Data were taken from 4 sagittal slices (total number of neurites counted: $n_{mz}=85$, $n_{iz}=114$, $n_{vz}=52$).

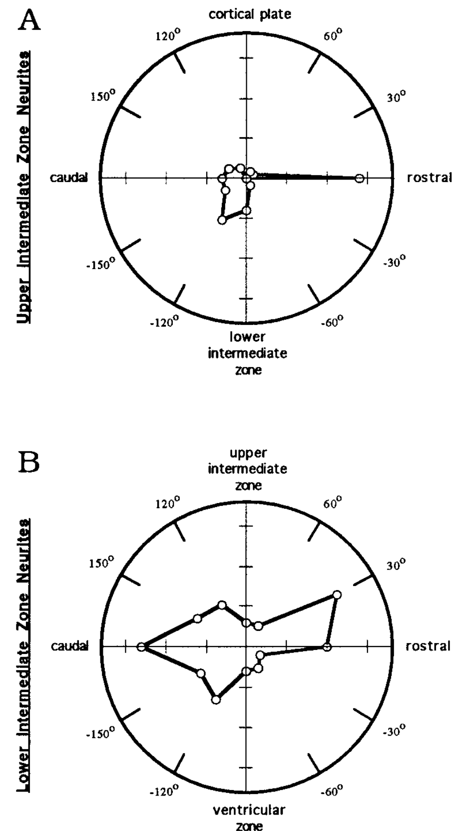

Fig. 5. Neurites on the E15 intermediate zone and subplate do not cross onto the cortical plate. The intermediate zone neurite sample shown in Fig. 4B was subdivided into two samples and replotted using the same graphing procedure. (A) The upper sample consists of those intermediate zone neurites (n=37) that originate within 16 μm (one mean neurite length) of the cortical plate. This sample approximates the neurites that are generated on the subplate region of the embryonic cortex. (B) The lower sample represents the rest of the intermediate zone neurites (n=77). Both samples are rostrocaudally oriented (parallel to the cortical lamina) and are significantly different from uniform circular distributions ($P_{upper}<0.001$, $P_{lower}<0.01$). Although both samples contain some radially directed projections (along vertical axis), there are almost no neurites on the upper intermediate zone that project superficially, toward the cortical plate. Neurites projecting on the lower intermediate zone are not biased toward either direction along the radial (vertical) axis. Axis tick marks represent a count of 5 neurites.

granular (lamina 4) and infragranular (laminae 5/6) layers and to measure cell attachment separately for each layer. As shown in Fig. 9 (inset), thalamic cell attachment to the P7 cortical plate was largely restricted to the granular and infragranular layers (laminae 4-6).

As with E15 slices, thalamic cells plated onto P7 slices were observed to extend neurites on all cortical layers to which they attached (including, in this case the cortical plate). Although measurements of neurite angles were not made, no obvious orientation of neurites on any layer was observed. Significantly, neurites were found that extended between the developing white matter and layer 6 of the cortical plate, without evidence of turning at this border (data not shown).

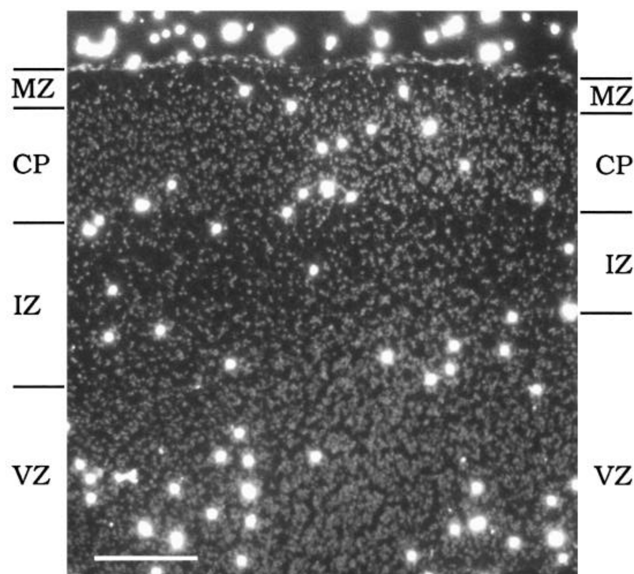

Fig. 6. Cryostat sections of E15 forebrain support only a low density of thalamic cell attachment which is uniform across embryonic cortical layers. Both the laminar structure of the E15 cortex and attached thalamic cells can be visualized in this photo taken under both phase-contrast transmittance and rhodamine fluorescence microscopy. The larger, fluorescent dots are clusters of 2-4 cells. Cells can be seen to attach to all embryonic cortical layers, i.e. the marginal zone (MZ), cortical plate (CP), intermediate zone (IZ) and ventricular zone (VZ). At the top of the photo, adjacent to the marginal zone, fluorescently labeled cells can be seen that are attached to the substratum of the slide. The photo is taken from the lateral cortex of a coronal section. Ventral is to the left, dorsal to the right. Markings on the two sides of the photo are of different scales due to the thinning of the telencephalon that occurs along the ventrodorsal axis of coronal sections. Scale bar, 100 μm.

DISCUSSION

These experiments demonstrate that living, but not cryostat, sections of cerebral cortex support selective attachment of and neurite outgrowth from embryonic thalamic neurons in a manner that depends upon the developmental state of the cortex. Embryonic cortical plate and postnatal supragranular layers act as in vitro substrata which, compared to all other cortical laminae, support little neuronal attachment. Neurites of cells attached to the embryonic subplate and intermediate zone project mostly parallel to the cortical layers and neurites on the subplate do not cross onto the cortical plate. These results can be obtained in serum-free medium and are apparent after very short periods in culture (3 hours).

The correlations between in vitro behavior of thalamic neurons and in vivo development

Both the patterns and timing of thalamic cell attachment and neurite outgrowth in this in vitro assay correlate well with in vivo patterns and timing of thalamocortical development: At each developmental stage examined, thalamic cells failed to attach well to, and failed to extend neurites onto, essentially those territories that thalamic axons encounter, but avoid, in vivo.

For example, at the earliest stage examined in this study (mouse E15, which is roughly equivalent to rat E17), tangentially oriented thalamic axons normally occur abundantly in the cortical subplate, but are absent from the adjacent cortical plate. Thalamic axons are also present in the deeper intermediate zone and are occasionally seen in the ventricular zone, (Crandall and Caviness, 1984; Reinoso and O'Leary, 1988; Catalano et al., 1991; Erzurumlu and Jhaveri, 1992; Kageyama and Robertson, 1993). In vitro, embryonic thalamic cells seeded onto E15 cortical slices attached especially well to the superficial intermediate zone (the presumptive subplate), as well as throughout the intermediate zone and, to a lesser extent, the ventricular zone, but they attached very poorly to the cortical plate (Figs 1, 2). Neurites of thalamic cells that attached to the intermediate zone (including the subplate) tended to be oriented tangentially and, like thalamocortical axons in vivo, failed to enter the cortical plate (Figs 3, 4B, 5).

At P1 in vivo, thalamocortical axons from the subplate have begun to invade the cortical plate in at least parts of the cortex (Agmon et al., 1993). In vitro, thalamic cells attached to much the same areas of the P1 cortex as observed with E15-E16 slices, except that, at this stage, the cortical plate in the rostral, but not caudal, cortex also supported thalamic cell attachment (Fig. 7). This rostral-to-caudal gradient of attachment to the P1 cortical plate in vitro parallels the general rostral-to-caudal gradient by which cortical histogenesis (Smart, 1983; Bayer and Altman, 1991) and innervation of the cortical plate (cf. Reinoso and O'Leary, 1988) occur in vivo.

Finally, by P7 in vivo, thalamocortical axons have invaded the cortical plate throughout the rostrocaudal extent of the cortex, with most axons terminating in layer 4 and the deepest aspect of layer 3 (Naegele et al., 1988; Miller et al., 1993; Kageyama and Robertson, 1993; Agmon et al., 1993). In vitro, thalamic cells also attached to and extended neurites on the cortical plate throughout the rostrocaudal extent of the cortex, but attachment and neurite outgrowth within the cortical plate was limited to layers 4-6 (Figs 8, 9), i.e. the layers through which thalamic axons grow in vivo.

At each of these stages, thalamic cells in vitro also attached to and extended neurites on the marginal zone (layer I) and non-cortical areas (Figs 1, 2, 8, 9 and data not shown). Thus, territories not occupied by thalamic axons in vivo can be permissive for thalamic cell attachment and neurite outgrowth in vitro. In contrast, territories that mark the boundaries of thalamocortical axon growth in vivo (i.e. the entire cortical plate at early stages; layers 2 and 3 at later stages) were consistently non-permissive for thalamic cell attachment and neurite outgrowth in vitro.

Are thalamic cells detecting axon guidance cues?

It is striking that regions of cortex that thalamic axons confront, but into which they do not normally grow, are readily detected in culture as regions to which dissociated thalamic cells do not attach. This observation raises the possibility that the adhesive behavior of neuronal cell bodies can, in some cases at least, reveal the locations of axonal guidance cues. Such a view has also been suggested by investigators studying the developing chick retinotectal system (Barbera et al., 1973; Gottlieb et al., 1976).

It is interesting in this regard to compare the conclusions of the present study with observations by other investigators on

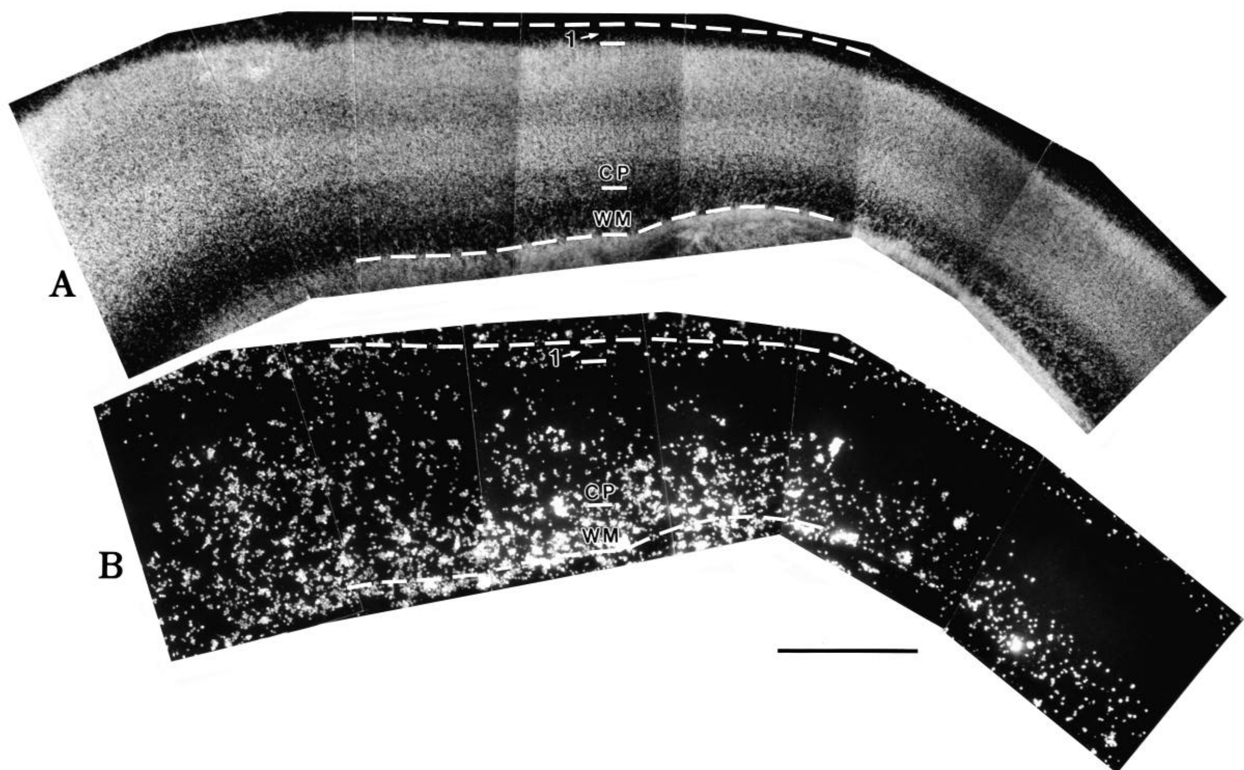

Fig. 7. Thalamic cell attachment to P1 cortex. (A) Laminae of a P1 cortex can be seen under UV fluorescence after a sagittal slice is cultured with thalamic cells (3 hours), fixed and stained with bisbenzamide. By this age, the cortical plate (CP) has matured and some differentiated laminae can be distinguished within it. (B) The same slice viewed under rhodamine optics shows the distribution of fluorescently labeled and attached thalamic cells. Many cells attached to the developing white matter (WM, formerly the intermediate zone) and layer 1 ('1', formerly the marginal zone). Cells also attach to the cortical plate (not seen on E15 sections), but in a non-uniform manner. The rostral portion (left) supports the greatest level of attachment while the caudal portion (right) is almost devoid of fluorescent cells. Dotted white lines indicate the edges of the cortex (pia at the top) and reference the same points of the slice in both photos. Scale bar, 500 μm.

the in vitro growth of thalamic axons on substrata of isolated cortical membranes: Götz et al. (1992) found that embryonic (E16), rat cortical membranes were relatively non-permissive for axon outgrowth, but postnatal (P7) membranes were not. More recently, Tuttle et al. (1993) showed that the change in the outgrowth-promoting properties of cortical membranes – from non-permissive at embryonic stages, to permissive at postnatal stages – could also be observed with membranes prepared from only the superficial cortex (i.e. the cortical plate and marginal zone – of which the cortical plate is the major component).

Thus, both these and the present study point to the cortical plate as the site of transient non-permissiveness, both for neuronal attachment and axon growth. It is not unreasonable to suppose that the cause is the same for both the lack of adhesion and the lack of axon growth, especially given that many of the molecules that promote axon growth also mediate cell adhesion (cf. Lander, 1989; Hynes and Lander, 1992). Indeed, while there are certainly cases in which the guidance of neurons by their substratum is not controlled by differential adhesion (cf. Gundersen, 1987; Calof and Lander, 1991; Lemmon et al., 1992), the development of the thalamocortical system may be a case where adhesion does play a dominant role.

One issue not discussed above is whether the non-permissiveness of the cortical plate (and subsequently layers 2/3) is a property that only thalamic neurons can recognize, or whether other classes of neurons might be able to detect the same cues. Although extensive studies have not been carried out, preliminary experiments support the latter view. To date, dissociated cells from E15 cerebral cortex, E14 ganglionic eminence and P4 cerebellum have been labeled and plated onto E15 forebrain slices, and all showed markedly poorer attachment to the cortical plate than to other layers (data not shown; for cerebellar cells, the difference between attachment to the cortical plate and to other layers was apparently not as great as for thalamic cells, but was still noticeable). These results suggest that the non-adhesive properties of the cortical plate are quite general and even extend to cell types that never contact the cortex. Such results are perhaps not surprising, given that many axon guidance cues are known to act on cells that normally never see those cues. Examples include the fact that most classes of neurons extend neurites in response to laminin, even though laminin is present in only a few parts of the developing brain (reviewed by Lander and Calof, 1993); that retinal axons will regenerate through peripheral nerve grafts and terminate specifically within structures that are not normal targets (Zwimpfer et al., 1992; Carter et al., 1994); and that even fibroblasts – which are not found in the central nervous system – respond to the motility-inhibiting effects of

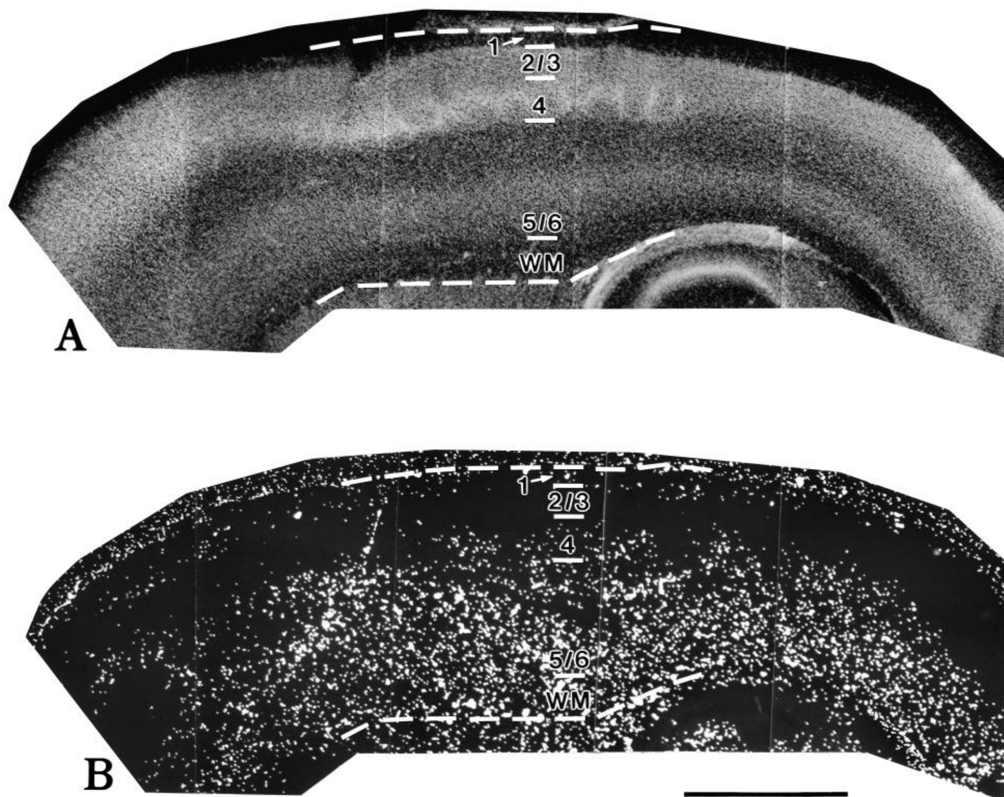

Fig. 8. Thalamic cell attachment to P7 cortex. (A) Laminae of a P7 cortex can be seen under UV fluorescence after a sagittal slice is cultured with thalamic cells (3 hours), fixed and stained with bisbenzamide. The cell-dense, barrel walls of the somatosensory cortex can be seen and used to identify the granular layer 4 (Woolsey and Van Der Loos, 1970; Rice and Van Der Loos, 1977). Rostral is to the left, caudal to the right. (B) The same slice viewed under rhodamine optics shows the distribution of fluorescently labeled and attached thalamic cells. As in the E15 and P1 experiments, cells attached well to layer 1 ('1', formerly the marginal zone) and the white matter (WM, formerly the intermediate zone). Attachment to the cortical plate (laminae '2-6') can be seen throughout the rostrocaudal extent of the slice; however, it is limited to layers 4-6, i.e. the granular ('4') and infragranular ('5/6') layers. Attachment to the supragranular layers ('2/3') is relatively low. Dotted white lines indicate the edges of the cortex (pia at the top) and reference the same points of the slice in both photos. Scale bar, 1 mm.

the central myelin-derived inhibitor of neurite outgrowth (Caroni and Schwab, 1988).

What molecules are thalamic neurons detecting in vitro?

The general non-permissiveness of the embryonic cortical plate and postnatal layers 2/3 for cell attachment and neurite outgrowth in vitro suggests that these regions either lack molecules that promote neuron adhesion and outgrowth, or possess molecules that inhibit adhesion and outgrowth. Although the data presented here cannot distinguish between these possibilities, at least one major mediator of neural cell adhesion, NCAM, is clearly expressed in the embryonic cortical plate and postnatal supragranular layers (Fushiki and Schachner, 1986; Chung et al., 1991; and unpublished observations). In addition, no dramatic differences have been seen in the relative isoform composition or polysialic acid content of the NCAM in these, versus other layers, of the developing cortex (Chung et al., 1991; and unpublished observations).

Considering that NCAM can act as a homophilic adhesion molecule and that the majority of thalamic cells plated in the present study were NCAM-immunoreactive, the possibility that the embryonic cortical plate and postnatal layers 2/3 contain molecules that inhibit cell adhesion and axon growth is worth considering seriously. Recently, several molecules, particularly certain components of the extracellular matrix, have been claimed to have 'anti-adhesive' (reviewed by Sage and Bornstein, 1991) and 'neurite-repulsive' (e.g. Snow et al., 1990; Faissner and Kruse, 1990) properties. Indeed, in the retina, the gradual central-to-peripheral disappearance of one

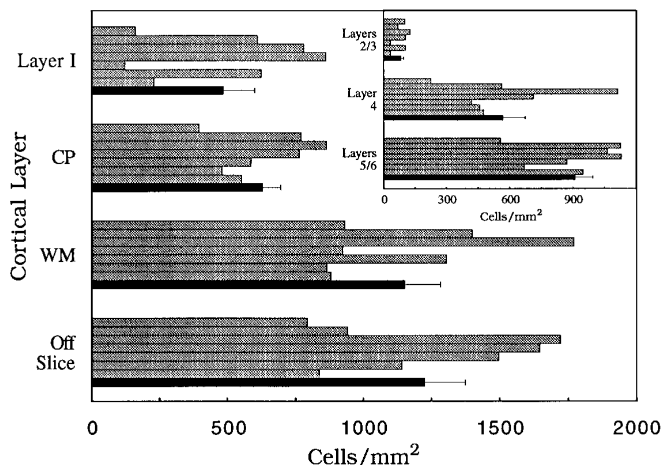

Fig. 9. Densities of thalamic cells attached to postnatal (P7) cortical layers. The densities of cells attached to layer 1, the cortical plate (CP, laminae 2-6) and the white matter (WM) were determined from 7 sagittal slices (light bars). Black bars represent the mean (± standard error) from the 7 samples. Data were collected from the presumed somatosensory cortex, the extent of which is indicated by the dotted lines in Fig. 8, because the laminae could be unambiguously distinguished within this region (total number of cells counted from the 7 slices: n_1=891, $n_{2\text{-}3}$=330, n_4=1379, $n_{5\text{-}6}$=7042, n_{wm}=2400, $n_{\text{off slice}}$=1874). 'Off Slice' data were collected from the culture substratum adjacent to the pial surface. INSET shows the density of cells attached to the laminae within the cortical plate. Cell density on supragranular layers (2/3) was much lower than on granular (4) and infragranular (5/6) layers (analysis of variance test: P<0.0005). A Newman-Keuls analysis verified the mean density on laminae 2/3 as an outlier ($\mu_{5/6} \neq \mu_4 \neq \mu_{2/3}$).

such molecule has been suggested to play a crucial role in guiding axons towards the optic fissure (Brittis et al., 1992).

Whatever the molecules are that account for the different adhesive and neurite outgrowth-promoting properties of different cortical layers, it should not be assumed that their source is the intrinsic cells of the cortex. Thalamic axons themselves, growing within certain cortical layers, could contribute some of the cues that thalamic cells detect when they are plated onto cortical slices (present study) or cortical membranes (Götz et al., 1992; Tuttle et al., 1993). For example, some or all of the attachment of thalamic cells to the embryonic subplate and intermediate zone (Figs 1, 2), the tangential orientation of neurites growing on those zones (Figs 3, 4B, 5), and the thalamic cell attachment to postnatal layers 4-6 (Figs 8, 9), could reflect the binding of thalamic neurons to thalamic axon membranes. Such homotypic interactions could play just as important a role in guiding the majority of thalamic axons in vivo as interactions of thalamic axons with intrinsic cortical cells. The assay described in the present study, performed following the selective ablation of appropriate cells or fibers in vivo (cf. Ghosh et al., 1990), should prove useful in determining the sources of the cues that are responsible for the layer-specific behavior of thalamic cells and their axons.

Overall, the results presented in this paper suggest that molecules that influence neuronal cell adhesion may, through their patterns and timing of expression, provide important cues for the growth of thalamic axons along the radial axis of the cortex. The continued use of rapid in vitro assays, such as the one described here, should aid in determining whether and how known adhesion molecules participate in this process.

We would like to thank Mary Herndon, Jon Ivins and Chris Stipp for helpful comments on the manuscript; Todd Grinnel, John Shavel and Dave Smith for technical assistance; Reha Erzurumlu and Sonal Jhaveri for assistance with anatomy; Anthony Frankfurter for the TUJ1 antibody; and Rebecca Tuttle and Dennis O'Leary for sharing unpublished data. This work was supported by a fellowship to A. D. L. from the David and Lucile Packard Foundation.

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(Accepted 29 June1994)