# Effects of the Thalamus on the Development of Cerebral Cortical Efferents *In Vitro*

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**ABSTRACT: The cerebral cortex is a multilayered tissue, with each layer differing in its cellular composition and connections. Axons from deep layer neurons project subcortically, many to the thalamus, whereas superficial layer neurons target other cortical areas. The mechanisms that regulate the development of this pattern of connections are not fully understood. Our experiments examined the potential of the thalamus to attract and/or select neurites from appropriate cortical layers. First, we cocultured murine cortical slices in close proximity to thalamic explants in collagen gels. The amount of neurite outgrowth from deep layer cells was enhanced by, but not attracted to, the thalamic explants. Second, we cocultured cortical slices in contact with thalamic or cortical explants to test for laminar specificity of connections. Specificity was apparent after culture for about a week, in that deep cortical layers contained the highest proportions of corticothalamic cells and superficial cortical layers contained the highest proportions of corticocortical cells. After shorter culture of only a few days, however, specificity was not apparent and there were larger numbers of corticothalamic projections from the superficial layers than after a week. To study how the early nonspecific pattern of corticothalamic connections was transformed into the more specific pattern, we labeled corticothalamic cells early, after 2 days, but let the cultures survive for 8 days. On day 8, the nonspecific pattern of early-labeled cells was still seen. We conclude that although the thalamus does not block the initial entry of inappropriate axons from the superficial layers, many of these axons are subsequently lost. This suggests that contact-mediated interactions between cortical axons and the thalamus allow cortical efferents from appropriate layers to be distinguished from those arising in inappropriate layers. This may contribute to the development of layer-specific cortical connections *in vivo*.** © 1999 John Wiley & Sons, Inc. J Neurobiol 39: 186–196, 1999

***Keywords:*** **cerebral cortex; chemotrophism; chemotropism; corticocortical connections; corticothalamic connections; target recognition; thalamus; organotypic cultures**

Most sensory information destined for high-level processing in the brain is relayed to the cerebral cortex by the thalamus. Corticothalamic feedback connections influence the nature and quality of the relayed information (Steriade, 1997). Cortical processing itself involves the transfer of information between cortical areas via corticocortical connections. A striking organizational feature of these connections is that the corticothalamic and corticocortical pathways originate mainly in different cortical layers, the former from deep in the cortex (layer 6) and the latter from its superficial layers (layers 2 and 3) (Gilbert and Wiesel, 1981). The mechanisms that regulate the development of layer-specific patterns of efferent cortical connections are not yet fully understood.

During embryogenesis, axons grow from the cortex (corticofugal axons), converge on an intermediate structure (the ganglionic eminence, which is the embryonic precursor of the basal ganglia), penetrate it as a tight bundle (the internal capsule), and emerge from it to find their specific targets (e.g., the thalamus, in

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the forebrain, and other structures in the midbrain and spinal cord) (De Carlos and O'Leary, 1992). Corticofugal axons may be guided to the ganglionic eminence by diffusible chemoattractants released from it, including netrin-1 (Serafini et al., 1994; Metin and Godement, 1996; Richards et al., 1997; Metin et al., 1997), but it is unclear how the onward growth of these axons is directed. The embryonic internal capsule is a heterogeneous bundle of axons from neurons that are destined to form at least several, perhaps all, cortical layers, and so the latter stage of corticofugal development must involve the sorting of these axons, so that targets become innervated specifically from the appropriate cortical layer. This sorting may be achieved through the actions of diffusible chemoattractive signals from final targets, such as the thalamus (Novak and Bolz, 1993), and/or contact-mediated interactions with cells in those targets or their processes (Molnar and Blakemore, 1995).

In this report, we consider two hypotheses about how this sorting might be achieved. One possibility is that each target, such as the thalamus, attracts specifically the axons of neurons in the appropriate cortical layer, as suggested by Novak and Bolz (1993). Another possibility is that an unselected or partially selected population of axons growing from inappropriate as well as appropriate cortical layers could reach potential subcortical targets, and selection, resulting in the removal of inappropriate connections, could occur following contact-mediated interactions with the target cells. There is evidence that such a mechanism underlies the development of some features of corticocortical connectivity (e.g., Innocenti, 1981; Price and Blakemore, 1985a,b).

We used *in vitro* techniques to study whether the thalamus might guide and/or select appropriate cortical efferent neurites. First, we asked whether the thalamus can attract early cortical neurites growing from the deep layers. This aspect of the study built on previous work in which we showed that the thalamus releases factors that can promote neurite outgrowth from the deep layers of the cortex, but in which we did not study the possibility of attraction (Lotto and Price, 1996). Second, we asked whether the thalamus can be innervated by neurons in both appropriate and inappropriate cortical layers.

## MATERIALS AND METHODS

In the mouse, cortical neurons are generated between embryonic days 12 and 17 (E12–17), with deep layer neurons being born before superficial layer neurons, as in other species (Gillies and Price, 1993). Axons grow from the cortex to the thalamus at around E15 in mice (Ferrer at al., 1992; Yuasa et al., 1994; Lotto and Price, 1995), at which age the immature cortex (the cortical plate) comprises only deep-layer cells (Lotto and Price, 1996). Therefore, to address the question of chemoattraction, we cocultured cortical and thalamic tissue from E15 mice. Before choosing the best age of tissue for experiments on the ability of the thalamus to select axons from appropriate cortical layers, we used bromodeoxyuridine (BrdU) to find the earliest age at which most superficial layer cells were in place. As described below, we found this age was birth.

### BrdU Labeling

Pregnant mice were injected on E17 (E1 was the plug date after overnight mating) with BrdU [80 $\mu$g g$^{-1}$, intraperitoneally (i.p.)]. Embryos were obtained on E19, after anesthetizing the mothers with urethane (0.3 mL of a 25% solution in saline, i.p.), and fixed in 4% paraformaldehyde. Offspring were obtained on P0 (the day of birth), P1, P3, P6, P8, P12, and P21, were deeply anesthetized with sodium pentobarbitone (0.5 mg g$^{-1}$, i.p.) and perfused with 4% paraformaldehyde. In addition, a few mice were injected with BrdU on E16 and perfused on P0. Cortical slices from E19 and P0 mice that had been injected with BrdU on E17 were cocultured in contact with thalamic explants on collagen membranes (as described below) for 2 or 8 days and then fixed with 4% paraformaldehyde. All material was embedded in wax, sectioned at 10 $\mu$m, reacted to reveal BrdU labeling, and counterstained with Cresyl violet to demonstrate cortical layers.

### Culture Methods

Detailed methods are given in Lotto and Price (1999). The thalamus and cortex were dissected from BALB/c or C3H embryonic mice as described previously (Rennie et al., 1994; Lotto and Price, 1994) following caesarean section under anesthesia with urethane (0.3 mL of a 25% solution in saline, i.p.).

***Experiment 1.*** Coronal cortical slices were taken from a rostrocaudal level two thirds of the distance from the rostral pole to the caudal pole of the cortex. Each section was then cut ventrally at the position of the border between parietal cortex and pyriform cortex to leave the neocortex and ganglionic eminence. In some E15 cultures, these cortical slices were used in their entirety; i.e., they constituted lateral and medial cortex and the area around the internal capsule [Fig. 1(a)] ($n = 6$ with the E15 thalamus; $n = 6$ without). For other E15 cultures, the slices were dissected further and comprised only medial cortex ($n = 6$ with the E15 thalamus; $n = 6$ without). All cortical slices were placed in culture on their coronally cut surfaces either alone or 1 mm away from thalamic slices. The explants were covered with 80 $\mu$L of collagen gel (Lumsden and Davies, 1983; Lotto and Price, 1999) and completely submerged with chemically defined serum-free medium (Lotto and Price, 1999).

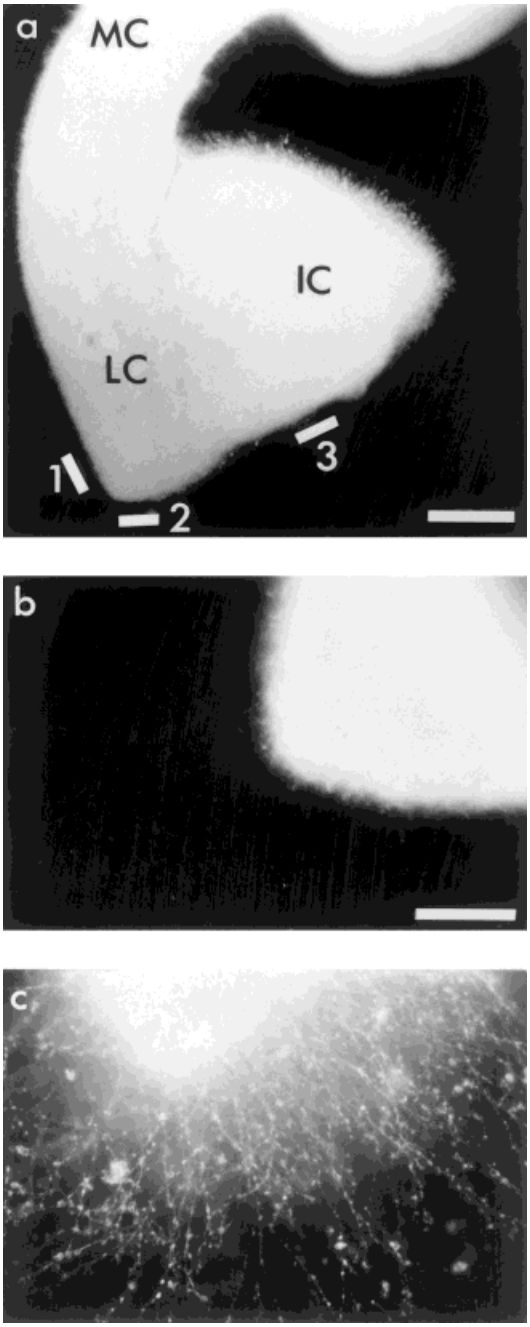


**Figure 1** Fluorescence photomicrographs of E15 cortical slices. (a) Includes medial cortex (MC), lateral cortex (LC), and internal capsule (IC), cultured without the thalamus. Windows 1–3 were used for quantification, windows 1 and 2 are over the ventral part of the parietal cortex, and window 3 is over the striatum. (b,c) The edge of the lateral cortex (around windows 1 and 2) in slices cultured (b) alone and (c) with the thalamus. (c) The thalamus is out of the field of view to the right, and there is no evidence that fibres consistently bend toward it. Scale bars: (a) 400 $\mu$m, (b,c) 250 $\mu$m.

After 72 h at 37°C in 5% $CO_2$, cultures were fixed in 4% paraformaldahyde in 0.1 M phosphate buffer. Small crystals of 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate (DiI) were placed in the fixed cortex and the cultures were left to label for up to 2 months before analysis.

***Experiment 2.*** Slices of E19 cortex were cocultured as above with thalamic explants touching either their white-matter or pial side in collagen gels ($n = 30$). P0 cortical slices were cocultured with thalamic explants ($n = 95$) or P0 cortical explants ($n = 54$) touching their white-matter side on collagen-coated membranes (Costar; Transwell-Col) in serum-free liquid medium, as described in Rennie et al. (1994) and Lotto and Price (1999). E15 thalamic explants were used in these experiments, since it is not possible to keep older explants of these tissues alive under the culture conditions used here (Magowan and Price, 1996). Cultures were fixed after 2, 4, 6, 8, or 10 days in 4% paraformaldehyde. Cortical axons that projected into the collagen gels or onto the collagen membranes were easily visualised with phase-contrast microscopy. Those that projected into the cocultured thalamic or cortical slice were retrogradely labeled with a single injection of DiI after fixation. In addition, in some cultures that survived for 8 days, a single DiI injection was administered after the first 2 days *in vitro* ($n = 8$, cortex–thalamus cocultures; $n = 7$, cortex–cortex cocultures). Cultures were counterstained with bisbenzimide (10 $\mu$g $mL^{-1}$) to identify cortical laminae.

## Diffusion Gradient

To determine whether diffusion gradients could be created and maintained in our collagen gels, 80 $\mu$L of collagen was allowed to gel at 37°C for 10 min in the same dishes used for the above experiments and a capillary tube (1 mm in diameter) loaded with rhodamine dextran was then pushed into each collagen gel ($n = 10$). The wells were filled with culture medium (as above) and each gel was photographed after 2 h and 6 h, and then every 6 h for 72 h.

## Quantification

***Experiment 1 (E15 Cocultures).*** Two-hundred-micrometer windows were demarcated at intervals around the edge of each cortical slice, covering regions where outgrowth was promoted by the thalamus. Each window was then further subdivided into 10 20-$\mu$m bins. To estimate neurite density and length, the total number of fibers crossing all the bins of each window was counted and the longest neurite in each bin was measured. The neurites in each bin were also assessed as to whether they bent toward or away from the thalamus by comparing their direction of growth at their distal end with the direction at which they left the cortical slice.

***Experiment 2 (Cocultures with E19 and P0 Cortex).*** For cortical explants cocultured in collagen gels, we measured

the distance from the pial surface to each retrogradely labeled cortical cell. For each cortical explant, these values were placed in 50-$\mu$m-deep bins and the total number of labeled cells in each bin was expressed as a percentage of the total number of labeled cells from that explant. Mean percentages were then plotted against depth for each combination of time in culture and position of thalamic explant ($n = 3$ for each combination). For each cortical explant cocultured on a collagen membrane, two 0.5-mm-wide strips were drawn through the slice on either side of its centre and the cortical depth (from the pia to the cortex–thalamus border) was divided into 10 bins of equal depth. For each bin, the number of labeled cells was counted and an average obtained from all cortical slices for each experiment. Differences between bins were tested by analysis of variance (ANOVA), and whether distributions were significantly skewed was analyzed with the protocol for testing kurtosis described in Snedecor and Cochran (1980). The depth of each bin was correlated with the location of the cortical plate–intermediate zone border and the intermediate zone–ventricular zone border after staining the slices with bisbenzimide.

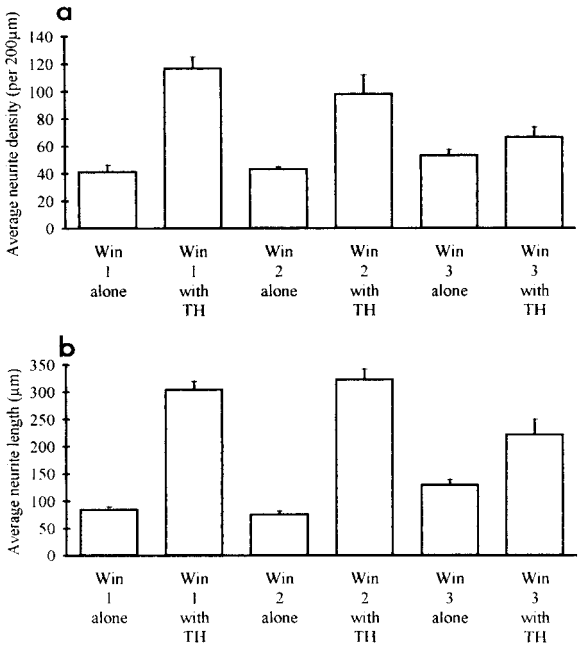


**Figure 2** The average (a) density and (b) length of neurite outgrowth (±S.E.M., $n = 6$ in all cases) across the three windows marked in Figure 1(a). Cortical slices were cultured alone or with thalamus (TH). Thalamic-induced increases in density were significant (Student $t$ test) in two cases (window 1, $p < .001$; window 2, $p < .05$), and in length were significant in all cases (window 1 and 2, $p < .001$; window 3, $p < .02$).

## RESULTS

### The Thalamus Promotes Neurite Outgrowth from the E15 Cortex

We cultured E15 cortical slices for 72 h in 3D collagen, either alone or at a short distance (about 1 mm) from E15 thalamic slices ($n = 24$). In the cocultured pairs of explants, the position of the thalamic slice relative to the cortical slice was varied. In none of these experiments did the explants or the neurites emerging from them touch. Some slices comprised medial and lateral cortex, while others were just medial cortex. We found that the thalamus had a dramatic stimulatory effect on neurite outgrowth from only those cortical slices that included the lateral cortex. The growth-stimulating affect was specific to the lateral-most region [marked as windows 1, 2, and 3 in Fig. 1(a)]. Without the thalamus, there was relatively little neurite outgrowth from the demarcated regions around the lateral cortex [Fig. 1(b) shows the region around windows 1 and 2 at higher magnification]; with the thalamus, the density and the length of neurites extending from the most lateral extreme of the slices were significantly increased [Fig. 1(c)]. The quantified data from these cocultures are presented in Figure 2. We did not find evidence of preferential outgrowth toward the thalamic explant in these cases. We looked carefully for fibers bending toward the thalamus as they grew through the collagen, but we found very few (never more than about 2% of all neurites, and as many bent away). Although there was outgrowth from the medial cortex, we found no evidence that the thalamus enhanced this growth (data not shown). Overall, these results indicated that the thalamic explants had a growth-promoting but no clear chemotropic effect on only the most lateral part of the cortical explants.

It was crucial to demonstrate that diffusion gradients could be maintained in our 3D collagen gels. We used rhodamine dextran for this, since it has a molecular weight (10 kD) at the low end of the range of known neurotrophic molecules, and we reasoned that trying to establish gradients with a relatively small molecule would be a more rigorous test of our system than using large molecules. Gradients of rhodamine dextran were established within the first 2 h after loaded capillary tubes were pushed into the collagen matrix, and were maintained for at least 72 h (Fig. 3), a period covering the times allowed for tropism to manifest itself. Figure 3 also demonstrates that the gradients were observed over the distances appropriate for the separations of explants used.

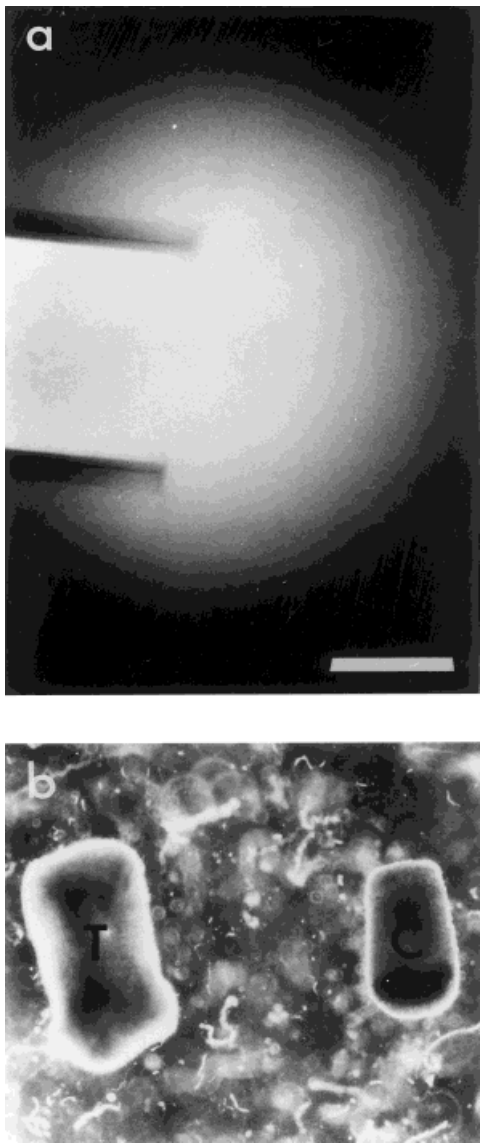


**Figure 3** (a) Fluorescence photomicrograph of the rhodamine dextran-filled tip of glass micropipette inserted into a collagen gel. Concentration gradients were established after 2 h and maintained for 72 h. The edge of the collagen gel is well beyond the edges of the photograph, and in the central region shown here the gel is of uniform thickness. (b) Phase-contrast photomicrograph of an E15 medial cortical slice (C) cocultured with an E15 thalamic slice (T). Scale bar = 0.5 mm.

## Migration of E17-Born Neurons *In Vivo* and *In Vitro*

Previous work has shown that most cells born on E13 and E14 (destined for layers 5 and 6) (Gillies and Price, 1993) are already in place in the lower half of the cortical plate on E19 (Price and Lotto, 1996). The superficial layers of the cortex are born on E16 and E17 (Gillies and Price, 1993). We found that 85–90% of E16 BrdU-labeled cells (which form primarily the lower portion of the superficial layers) were already in the superficial half of the cortical plate by P0 (data not shown). We then assessed the position of the uppermost cells of the superficial layers, most of which are born on E17. Figure 4 shows camera lucida drawings of E17 BrdU-labeled cells in cortex from mice aged E19, P0, P6, and P8, and in cortical slices cultured from either E19 or P0 for either 2 or 8 days. By E19, the vast majority of these E17-labeled cells were either in the ventricular and subventricular zones or had migrated into the overlying intermediate zone. Very few were in the cortical plate yet. By P0, most of them had entered the cortical plate and were located superficially, although the development of their final distribution was not completed until P8 (central row in Fig. 4). When cortical slices were cultured starting on E19 (top row in Fig. 4), there was some movement of the population of superficial layer cells toward the cortical plate after 2 days (but less than *in vivo*) and substantial migration after 8 days. In cortical slices cultured from P0 for up to 8 days (bottom row in Fig. 4), E17-labeled cells retained distributions similar to those at P0, with substantial proportions in the superficial layers. The results indicated that most superficial layer cells were in place at P0 and that in slices cultured from P0, there was relatively little further movement of these cells. On the other hand, many superficial cells were not yet in place at E19 and many moved into position in slices cultured from E19.

## Target Selection of Cortical Efferents

We first cultured thalamic explants touching either the ventricular or pial side of slices of E19 medial cortex for 2, 4, 6, 8, or 10 days ($n = 30$) in 3D collagen. Cortical fibers growing out of the cortical explants and into the collagen were visualized with phase contrast (Fig. 5). In all cases, cortical fibers emerged from both the ventricular and pial sides of the explants and grew roughly at right angles to the surface with essentially straight trajectories [Fig. 5(a)]. The cortical explants were often longer than the diameter of the thalamic explants, and so on the side of the cortex touching the thalamus, some cortical neurites emerged at lateral positions where their trajectories carried them past the thalamus. These neurites did not bend toward or away from the thalamus [Fig. 5(a)], in keeping with the lack of tropism noted in previous experiments. Although the cortical outgrowth that remained entirely in the collagen was profuse, it eventually degenerated completely. After only 2 or 4 days in culture, some of these neurites showed swellings

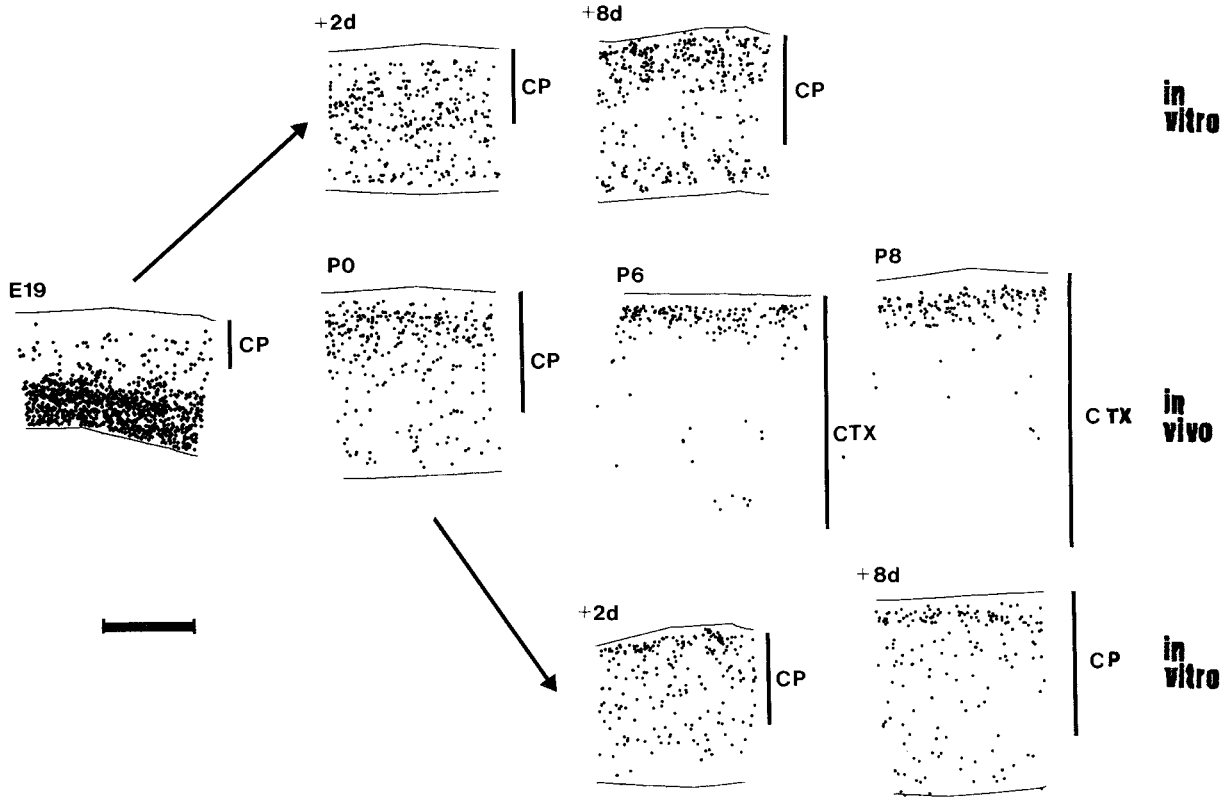


**Figure 4** Camera lucida drawings of BrdU-labeled cells (dots) in representative sections through the cortex *in vivo* (central row) and *in vitro* (upper and lower rows). The pial (up) and ventricular (down) edges of the telencephalic wall are marked in most drawings; in the older two *in vivo* sections (in which there were no labeled cells beneath the cortex) only the pial edge is drawn. In all cases, BrdU was injected on E17. For the *in vivo* sections, the age at which the animal was fixed is marked. Cortical slices were cultured for 2 or 8 days (+2d and +8d); some were set up with cortex aged E19 (top row) and others were with cortex aged P0 (day of birth). CP = cortical plate; CTX = cortex. Scale bar = 0.5 mm.

[Fig. 5(a)] that, in view of the pictures after longer culture periods [Fig. 5(b)], were most likely the earliest signs of degeneration. By 6–10 days, the remains of degenerated fibers and almost no intact neurites were visible in the 3D collagen [Fig. 5(b)]. Cortical cells whose neurites invaded the thalamic slice in these experiments are shown labeled with DiI in Figure 6(a). After 6–10 days in culture, by which time cortical neurites that had entered the collagen had degenerated, many labeled corticothalamic cells were still present. Thus, only cortical outgrowth that entered the thalamic explant persisted *in vitro*.

In these experiments, the depth of each DiI-labeled cell was measured and histograms of their distributions were obtained [Fig. 6(b)]. After 6–10 days in culture, these labeled cells were densest in the deep part of the cortical plate [Fig. 6(b)], irrespective of whether the thalamus was at the ventricular or the pial side of the cortex, in line with previous findings by Novak and Bolz (1993). This indicated that appropriate laminar specific connectivity can develop after 6–10 days of culture. After shorter culture times of 2 or 4 days, however, corticothalamic connections originated equally from all cortical depths (data not shown). This change could have been due to either the continued movement of superficial layer cells within the E19 slices (shown above) or the initial formation and later loss of inappropriate connections from the superficial layers, through either the removal of axons or cell death.

To distinguish between these possibilities, we carried out similar experiments but with P0 cortical slices, in which the movement of superficial layer cells would be minimal (see above). The cortical slices were cocultured for 2, 4, or 8 days with either a thalamic or a cortical slice on collagen-coated membranes instead of in 3D collagen gels (this was because in later experiments we planned to inject some

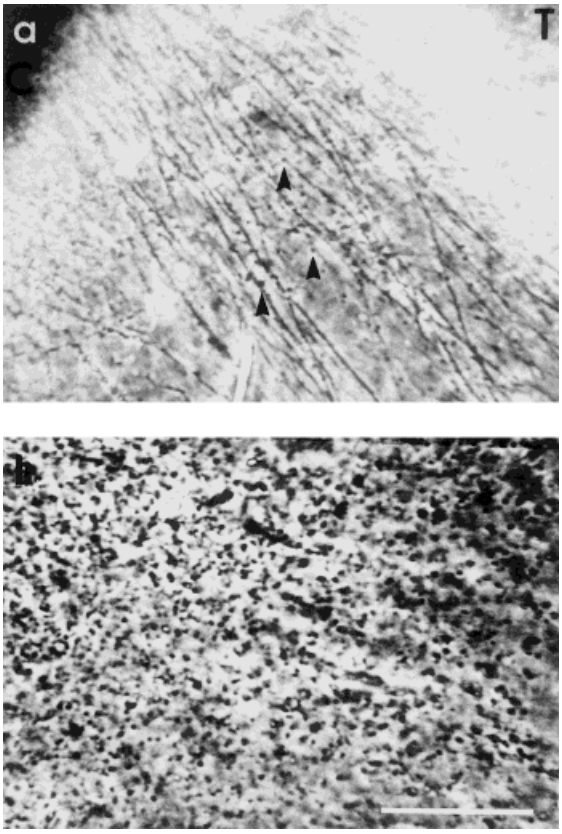


**Figure 5** Phase-contrast photomicrographs of outgrowth into the collagen matrix from the ventricular side of the E19 cortical slices (C) cultured touching the E19 thalamus (T) for (a) 4 days and (b) 10 days. (a) The cortical fibers are streaming past the thalamus without bending. Some fibers show small swellings, which are the earliest signs of degeneration (arrows). (b) After 10 days, only the remnants of degenerated fibers are found. Scale bar = 50$\mu$m.

8-day cultures on the second day *in vitro,* and injections into collagen gels deform the matrix, thereby disrupting neurite outgrowth). The distributions of retrogradely labelled cells in these cultures are illustrated in Figure 7(a–d) and the quantitative data are given in Figure 8(a–d). After 2 days, the majority of retrogradely labeled cells innervating either the thalamus or another cortical explant were in the superficial half of the cortical slice, in the cortical plate [Fig. 7(a,b) and upper histograms in Fig. 8(a,b)]. Both of the 2-day distributions were significantly skewed ($p < 0.05$). The distributions were still skewed after 4 days ($p < 0.05$). After 8 days, the distribution of cells projecting to the thalamus was no longer skewed and the peak density was now in the deep half of the cortical plate [Fig. 7(c) and lower histogram in Fig. 8(a)], as was the case in the previous experiments in collagen gels (Fig. 6). The distribution of cells projecting to another cortical slice remained significantly skewed ($p < 0.05$) with most cells in the superficial half of the cortical plate [Fig. 7(d) and lower histogram in Fig. 8(b)]. In the cortex–thalamus cultures, there was a significant reduction in the density of labeled cells in all superficial bins between 2 and 8 days in culture [Fig. 8(c)], suggesting a selective loss of superficial projections. There was only one such

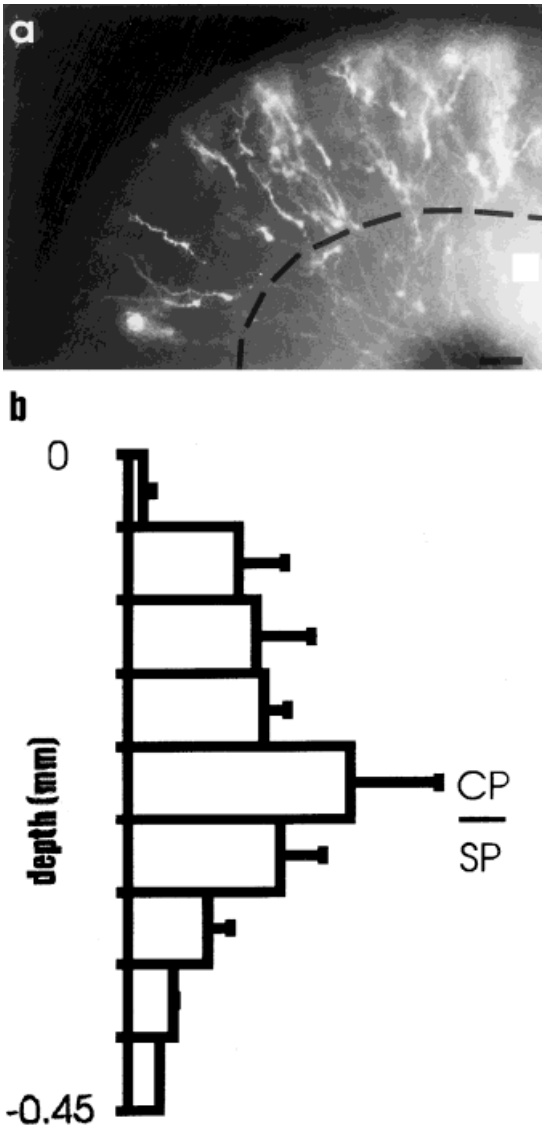


**Figure 6** (a) Fluorescence photomicrograph of retrogradely labeled cells in E19 cortical slices cocultured with thalamic explants touching their ventricular surface for 6 days. The broken line indicates the position of the lower edge of the CP in these cultures. (b) Histogram plots the relative densities of DiI-labeled cells against depth from the pial surface (means ± S.E.M.) in E19 cortical slices after 6 days in culture with the thalamus against the ventricular surface ($n = 3$). CP = cortical plate; SP = subplate. Scale bars = 50 $\mu$m.

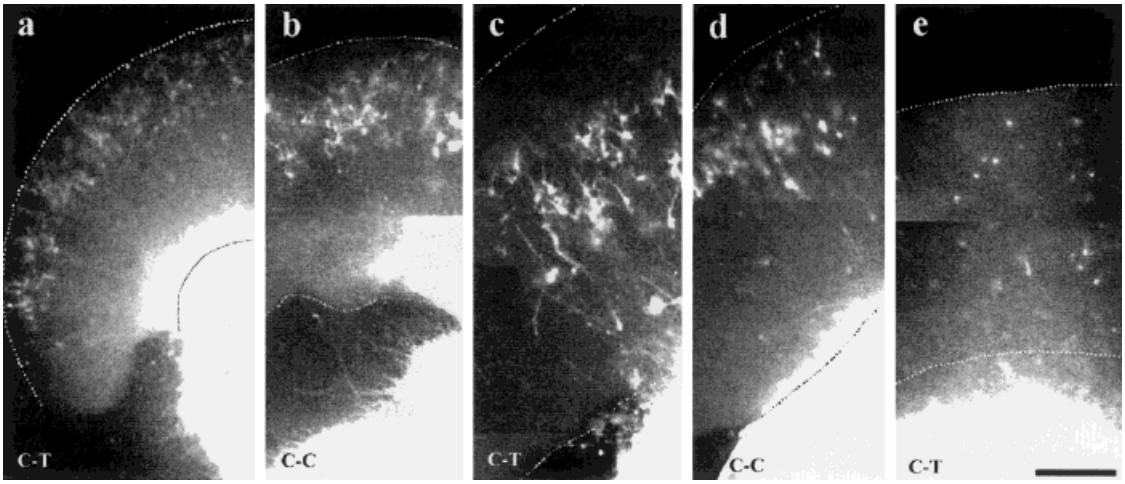


**Figure 7** Fluorescent photomicrographs of retrogradely labeled cells in 200-$\mu$m-thick P0 cortical slices cultured for (a,b) 2 days or (c–e) 8 days [(b–e) are montages]. Cortical slices were cocultured either with (a,c,e) a thalamic explant (C-T) or with (b,d) another cortical slice (C-C). Cultures in (a–d) were labeled after fixation. In the cocultures with thalamus, the retrogradely labeled cells were distributed superficially after 2 days, but in deeper layers after 8 days [compare (a) and (c)], whereas in the cocultures with cortex, they remained superficially throughout [compare (b) and (d)]. The 8-day cortex–thalamus coculture in (e) was labeled with DiI after only 2 days in culture, and many retrogradely labeled cells remained in superficial locations. The borders of the cortical slices opposite the injected cocultured tissue are demarcated with broken lines. Scale bars: (a) 250 $\mu$m; (b,e) 200 $\mu$m; (c,d) 100 $\mu$m.

reduction in the cortex–cortex cultures [bin 3 in Fig. 8(d)].

To confirm that the changes seen in the cortex–thalamus cocultures were due to a loss of initial connections from the superficial part of the slices, we injected cortex–thalamus and cortex–cortex cocultures with DiI after the first 2 days of an 8-day culture period [Fig. 7(e)]. In both cortex–thalamus and cortex–cortex cocultures, skewed distributions were seen after 8 days ($p < 0.05$) [Fig. 8(e,f)]. In these experiments, the DiI would have labeled the 2-day skewed distributions of cells [as in Fig. 7(a,b) and upper histograms in Fig. 8(a,b)]. The fact that the skewed distribution was retained in the cortex–thalamus cocultures, rather than changing to the 8-day distribution [as in Fig. 8(a)], indicated that the cells that initially projected from the superficial part of the cortex to the thalamus after 2 days *in vitro* were still there after 8 days, by which time many of them would have lost their corticothalamic projection.

## DISCUSSION

Our aim was to use culture methods to identify interactions between the cortex and thalamus that might play a role in the establishment of specific patterns of connections between them *in vivo*. Our main findings were that (a) the embryonic thalamus can promote outgrowth from the lateral part of the embryonic cortex, (b) connections from both appropriate and inappropriate cortical layers can enter the embryonic thalamus, and (c) many of the connections from inappropriate cortical layers are later selectively removed.

### The Thalamus Promotes Cortical Outgrowth

The growth-promoting effect of the thalamus on the cortex was similar to that observed in our previous studies in liquid culture medium (Lotto and Price, 1996). In the present work, this finding was made by studying corticothalamic interactions in collagen gels. We were interested in whether the thalamus-derived factors might influence the direction of growth of the cortical neurites (a chemotropic interaction). Unlike cultures in liquid medium, cultures in collagen gels have the potential to demonstrate chemotropic interactions mediated by the establishment of stable diffusion gradients of growth factors. The methods used here are similar to those that have been used to dem-

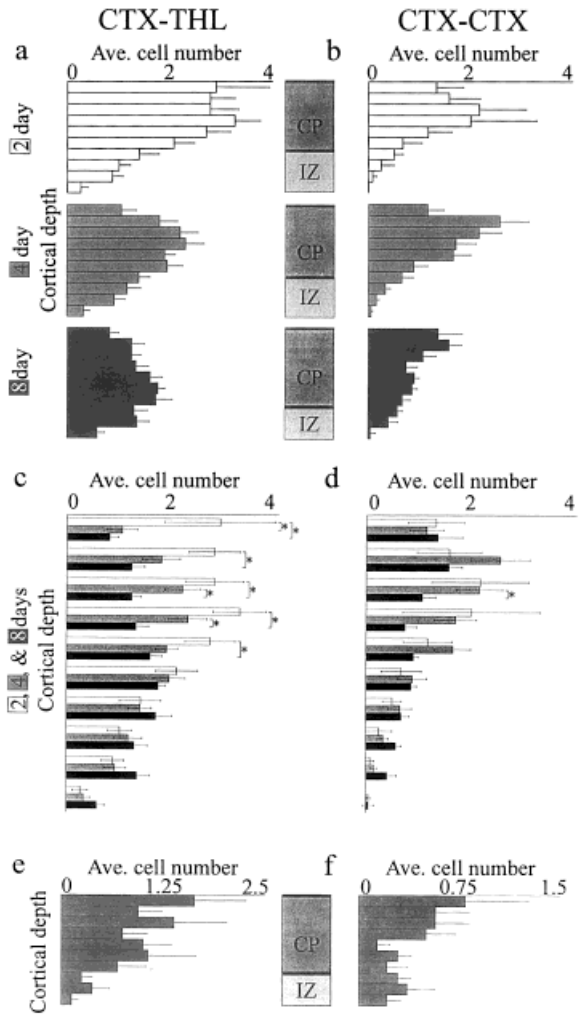


**Figure 8** The histograms plot the distributions of retrogradely labeled cells in the cortex of corticothalamic (CTX-THL; left column) and corticocortical (CTX-CTX; right column) cocultures against cortical depth (means ± S.E.M. for each bin). The locations of the cortical plate (CP) and intermediate zone (IZ) are illustrated schematically between adjacent histograms in (a,b,e,f) (the cortical plate widens with time in culture). (a,b) Distributions of cells labeled with DiI after 2, 4, and 8 days in culture (*n* values: CTX-THL = 23, 34, and 30; CTX-CTX = 6, 20, and 21). The top two histograms in (a) and all three histograms in (b) are significantly skewed toward the pia (all $p < .05$); the lower histogram in (a) is not significantly skewed. (c,d) Data from each histogram in (a) and (b) are combined in (c) and (d), respectively; asterisks denote statistically significant decreases between bins (ANOVA; $p < .05$). (e,f) Distributions of cells in 8-day cocultures labeled by injections of DiI after only 2 days (CTX-THL, $n = 8$; CTX-CTX, $n = 7$). Both histograms are significantly skewed ($p < .05$).

onstrate chemotropic interactions in other parts of the nervous system (Lumsden and Davies, 1984; Heffner et al., 1993; Serafini et al., 1994). We confirmed that stable diffusion gradients could be established in our collagen gels, but we did not find evidence that thalamic factors could direct the growth of cortical neurites. Although the culture system that we used has a proven track record for the identification of chemotropic interactions, and is probably better than alternatives that use quantities of serum with the potential to mask the effects of endogenous growth factors, it is not possible to exclude the possibility that the thalamus has a chemotropic effect on cortical outgrowth *in vivo*. For example, extracellular molecules between thalamus and cortex may be necessary to establish the correct characteristics of a concentration gradient or to act synergistically with the gradient to permit its successful operation. These molecules may be missing or incorrectly distributed in our cultures. Another possibility is that an undetectably small percentage of neurites growing from the cortex is sensitive to thalamic-derived tropic agents. On the other hand, it is possible that the thalamus-derived growth factors active in our cultures are not tropic and play other roles. For example, they may help correlate corticothalamic outgrowth with the maturation of the thalamus, by enhancing axonal growth from the lateral cortex when thalamic targets are ready to receive them. Identification of the thalamic factor(s) that promote cortical outgrowth will offer further opportunities for evaluating their potential role in guiding that outgrowth.

## The Thalamus Can Discriminate between Projections from Appropriate and Inappropriate Cortical Layers

In other neural pathways, there is considerable evidence that many early connections are removed as development proceeds. This has been shown particularly clearly in the callosal and corticocortical pathways, where precise adult patterns of connections are sculpted from earlier relatively nonspecific patterns by selective loss of axons, either with or without neuronal death (Innocenti, 1981; Price and Blakemore, 1985a,b). Here, we examined whether such a mechanism could operate in the developing corticothalamic pathway, to generate a layer-specific pattern of projections. In the adult, corticothalamic projections arise specifically from deep cortical layers and corticocortical projections originate mainly in superficial cortical layers (Gilbert and Wiesel, 1981). Our *in vitro* experiments suggest that axons from both deep and superficial layers have the ability to enter the thalamus, but many of those from the superficial layers are later removed. By contrast, our experiments provide no evidence for early inappropriate connections between two cortical explants. Thus, a lack of

initial specificity among cortical connection is not a general feature of the culture methods we used. It is seen during the early phase of corticothalamic development *in vitro,* but not during corticocortical development. This result raises the question of whether the development of layer-specific corticothalamic connections proceeds through an initial nonspecific phase *in vivo* as well as *in vitro*. It is known that cortical neurons are given instructions as to the layers that they should form around the time of their birth in the embryonic ventricular zone (McConnell, 1995). It is possible that the newborn cells are also given instructions either allowing or preventing them from forming a persistent connection with the thalamus, depending on which layer they are destined for. For example, these instructions might result in the expression by selected axons, i.e., those in deep cortical layers, of receptors able to recognize thalamus-specific ligands. Clearly, while our *in vitro* work raises the possibility of such a mechanism, the first step in testing this hypothesis will involve a careful analysis of the laminar specificity of the very early corticothalamic projections *in vivo*. Although several studies have examined the development of corticothalamic projections in various species, none have completely resolved the issue of whether layers 2–4 of the cortex send transient projections to the thalamus. Some studies have reported retrogradely labeled cells in layers 2–4 of the cortex following injections of tracers into the thalamus early in development, although methodological problems left the cause of this labeling unclear (Miller et al., 1993; Marotte et al., 1997). A major ambiguity with these and other studies (e.g., Clasca et al., 1995) is that the formation of the cortical layers, particularly the superficial layers, is not complete when the first corticothalamic connections are forming. It is possible that many early corticothalamic connections arise from cells that are still migrating toward the superficial layers. Corticothalamic cells that appear to be assigned to a particular layer in the young cortex (defined on the basis of morphological criteria) may not remain in that layer as development continues. These considerations suggest that early retrogradely labeled corticothalamic cells need to be birthdated to resolve the issue of whether there are transient corticothalamic projections from cells destined to contribute to layers 2–4 *in vivo*.

In summary, our result indicate that cortical neurons in all layers of the developing cortex have the ability to enter the thalamus, but that only those in appropriate layers recognize it as their correct target. This discriminative ability may contribute to the formation of layer-specific corticothalamic connections *in vivo*.

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