THE JOURNAL OF COMPARATIVE NEUROLOGY 335:16–41 (1993)

The Early Development of Thalamocortical and Corticothalamic Projections

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ABSTRACT

The early development of thalamocortical and corticothalamic projections in hamsters was studied to compare the specificity and maturation of these pathways, and to identify potential sources of information for specification of cortical areas. The cells that constitute these projections are both generated prenatally in hamsters and they make reciprocal connections. Fluorescent dyes (DiI and DiA) were injected into the visual cortex or lateral geniculate nucleus in fixed brains of fetal and postnatal pups. Several issues in axonal development were examined, including timing of axon outgrowth and target invasion, projection specificity, the spatial relationship between the two pathways, and the connections of subplate cells. Thalamic projections arrive in the visual cortex 2 days before birth and begin to invade the developing cortical plate by the next day. Few processes invade inappropriate cortical regions. By postnatal day 7 their laminar position is similar to mature animals. By contrast, visual cortical axons from subplate and layer 6 cells reach posterior thalamus at 1 day after birth in small numbers. By 3 days after birth many layer 5 cell projections reach the posterior thalamus. On postnatal day 7, there is a sudden increase in the number of layer 6 projections to the thalamus. Surprisingly, these layer 6 cells are precisely topographically mapped with colabeled thalamic afferents on their first appearance. Subplate cells constitute a very small component of the corticothalamic projection at all ages. Double injections of DiI and DiA show that the corticofugal and thalamocortical pathways are physically separate during development. Corticofugal axons travel deep in the intermediate zone to the thalamic axons and are separate through much of the internal capsule. Their tangential distribution is also distinct. The early appearance of the thalamocortical pathway is consistent with an organizational role in the specification of some features of cortical cytoarchitecture. The specific initial projection of thalamocortical axons strongly suggests the recognition of particular cortical regions. The physical separation of these two pathways limits the possibility for exchange of information between these systems except at their respective targets.

Key words: Neocortical specification, lateral geniculate, visual cortex, subplate, hamster

The mammalian neocortex can be divided into unique areas based on both structure and function. These cytoarchitectonic areas differ in the presence, number, and size of neurons by layer and in the patterns of their connections (Beaulieu and Colonnier, '89; Caviness and Frost, '80; Kaas, '87; Zilles and Wree, '85). Areal specification might be produced by intrinsic (genetic) or extrinsic (afferent/efferent) factors, or both, orchestrated through development (Killackey, '90; Rakic, '88; O'Leary, '89; Finlay, '91).

There is evidence that thalamic input can influence important features of the neocortex. Prenatal enucleation, which significantly decreases the number of the lateral geniculate nucleus axons, results in a reduction in the size of recognizable primary visual cortex, suggesting that the volume of thalamic afferents could specify the tangential extent of a cortical area (Dehay et al., '89; Rakic, '88; Rakic et al., '91). Thalamic afferents also affect cortical laminar organization. Layer 4 is the principal lamina for primary sensory thalamic afferent arborization, and cortical areas show more variation in the size and density of layer 4 than other layers (Beaulieu and Colonnier, '89). Normal cell

Accepted March 26, 1993.

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death in the cortex correlates with the relative numbers of neurons in this layer (Finlay and Slattery, '83), and neonatal thalamic damage results in a substantial decrease in the number of granule cells in layer 4 (Windrem and Finlay, '91). These studies demonstrate that thalamic afferents appear to play a substantial role in the determination of cortical areas, though it is unclear whether this role is the trophic support of already specified regions or the induction of regional specificity.

The timing of thalamocortical innervation with respect to other fiber systems and migrating neurons can be used to establish the plausibility of proposed inductive or trophic relationships. As demonstrated by tritiated amino acids, developing thalamic axons arrive in the intermediate zone subjacent to their eventual target while the cells upon which they will eventually synapse are still being generated and are migrating through the intermediate zone (Rakic, '76; Shatz and Luskin, '86; Wise et al., '77). Thalamic fibers do not grow into the developing cortical plate as soon as they arrive, but rather "wait" in the white matter below their eventual target, allowing a period for the interaction of thalamic axons and their potential targets before synapse formation in the cortex occurs (Lund and Mustari, '77; Rakic, '79; Shatz and Luskin, '86; Wise et al., '77). Recent reports using techniques that demonstrate axonal processes in more detail challenge the idea that thalamic fibers "wait" before invading the developing cortex (Catalano et al., '91; Sheng et al., '91). Whether this evidence is due to more sensitive techniques or is a reflection of species differences is not yet clear. In either case, thalamic axons are physically interposed between migrating neurons and the cortical plate for a large part of cortical development.

The cortex and thalamus make reciprocal connections (Jones, '85). In adult rodents specific thalamic afferents terminate most densely in layers 4 and lower 2/3, with small branches in layers 1, 5, and 6 (e.g., Naegele et al., '88). The terminal laminae of nonspecific thalamic projections are more variable (Herkenham, '86; Jones, '85). Cortical layers 5 and 6 project back to the thalamus of adult rats (Burkhalter and Charles, '90; Chmielowska et al. '89; Sefton et al., '81). Several studies have examined in various detail the early development of corticothalamic projections in a variety of species (Blakemore and Molnar, '90; De Carlos and O'Leary, '92; Johnson and Casagrande, '93; Shatz and Rakic, '81; Ramirez et al., '90; Sheng et al., '90, 91). Studies in cats (McConnell et al., '89) and rats (De Carlos and O'Leary, '92) have shown that the first projection out of the cortex, and perhaps to the thalamus, is pioneered by subplate cells. Evidence from rats (Blakemore and Molnar, '90; Catalano et al., '91; De Carlos and O'Leary, '92; Erzurumlu and Jhaveri, '92) and ferrets (Johnson and Casagrande, '93) suggest that the first cortical projections reach the thalamus at about the same time that thalamic projections reach the cortex. In contrast, investigations in the wallaby (Sheng et al., '90, '91) demonstrated that cortical efferents arrive at the thalamus long after thalamic projections have reached the cortex. Details of the development of projections from specific cortical layers (5 and 6) to the thalamus have only been reported in the wallaby (Sheng et al., '91).

Corticothalamic efferent cells are closely associated with the thalamocortical afferent fibers (Chmielowska et al., '89; Jones, '85), but little is known of the development of this precise topographic alignment. There is evidence from monkeys (Shatz and Rakic, '81) and cats (Henderson and Blakemore, '86) that the topographic precision of corticothalamic projections is established soon after they reach the thalamus. A thorough study of the development of the laminar origin and topography of corticothalamic projections in rodents has not been conducted.

The present study examines the development of afferent and efferent projections in the hamster. In this animal the thalamic lateral geniculate nucleus and the lower layers of visual cortex (layers 5 and 6) are generated prenatally during an overlapping period. The dorsal lateral geniculate nucleus is generated between embryonic day 9.5 (E9.5) and E12.5, and the lower layers of cortex between E10 and about E14 (Crossland and Uchwat, '82). At present, only postnatal development of these projections has been reported. Thalamic fibers are resident within the visual cortex at birth (Naegele et al., '88), while visual cortex efferents are visible in the posterior thalamus a few days after birth (Ramirez et al., '90). The prenatal development of these systems has not been reported. For instance, when do visual thalamic fibers first arrive at their target? The laminar origin of the cortical efferent projection, the precise time when it first arrives at the thalamus, and its relationship to growing thalamic projections are unknown.

This study examines several components of the embryonic and early postnatal development of visual thalamocortical and corticothalamic projections with fluorescent lipophilic dyes DiI and DiA. In particular we report on the relative timing of axon outgrowth and target invasion of these two systems, the spatial relationship between these two axonal systems during development, the projection specificity of thalamic axons, the origin of topographic precision of corticothalamic projections, features of the geniculocortical pathway, and information on the connections of subplate cells. Abstracts of this study have been published (Miller et al., '90, '91a).

Abbreviations

Cl	Central Lateral Thalamic Nucleus
CP	Cortical Plate
IC	Internal Capsule
L	Lateral Thalamic Nucleus
LGd	Lateral Geniculate, Dorsal Thalamic Nucleus
LGv	Lateral Geniculate, Ventral Thalamic Nucleus
LP	Lateral Posterior Thalamic Nucleus
MD	Mediodorsal Thalamic Nucleus
MG	Medial Geniculate Thalamic Nucleus
Pom	Posterior Complex, Medial Thalamic Nucleus
SP	Subplate
VB	Ventrobasal Thalamic Nucleus
VL	Ventrolateral Thalamic Nucleus
VMb	Ventromedial, Basal Thalamic Nucleus

METHODS

Subjects

A total of 119 golden hamster (*Mesocricetus auratus*) pups of various embryonic and postnatal ages were examined in this study. Embryonic age was based on the number of days after the day of breeding. The day of breeding was referred to as embryonic day 0 (E0). E1 began 24 hours later. Gestation for hamster pups is 15.75 days (Crossland and Uchwat, '82; Shimada and Langman, '70). The day of birth (and the first 24 hours after birth) was designated as P0. The day beginning 24 hours after birth was P1. (This designation for postnatal age differs from that used in previous papers from this lab. Previous chrononomencla-

ture designated the day of birth as P1, for instance, Sengelaub et al., '85). Females were bred in early afternoon between 1:00 pm and 3:00 pm. Fetal and postnatal pups were killed during this same time period. This study examined fetal animals from E12 to E15 and postnatal pups from P0 to P10.

Fetal animals

Pregnant mothers were given an overdose of sodium pentobarbitol and fetal animals were removed from their mother by nonsurvival cesarean. Fetal pups were killed by cutting the brainstem below the tectum. The brain was removed and placed into phosphate buffered (pH 7.4) 4% paraformaldehyde.

Postnatal animals

Postnatal animals were given an overdose of sodium pentobarbitol, and then their brains were removed and placed into 4% paraformaldehyde. Some postnatal pups were perfused with .9% saline and 4% paraformaldehyde. Perfusion was found to be unnecessary since blood cells in fetal animals are largely invisible to the fluorescent illumination used in this study. Subsequent postnatal animals were not perfused, and there was no observable qualitative difference in the tissue upon microscopic examination between animals which had been perfused and those which had not.

Tracer injection

The fluorescent neuronal stains DiI and DiA (1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate and 4-(4-dihexadecylaminostyryl)-N-methylpyridinium iodide, respectively; Molecular Probes, Eugene, OR, cat. no. D-282 and D-3883, respectively) were employed for tract tracing in this study. These stains have the unique property of anterograde and retrograde diffusion along the plasma membrane of fixed tissue (Godement et al., '87), and both directions of transport were examined in this study.

A small crystal of dye (DiI or DiA) was placed in the thalamic dorsal lateral geniculate nucleus (LGd, see Abbreviations list), posterior cortex (presumptive visual cortex), or both of intact, whole fixed brains. In animals with an injection of dye into the LGd and visual cortex, DiI was placed in the thalamus and DiA was placed in the visual cortex. Some animals received a cortical injection of DiI and DiA in opposite hemispheres. Whichever tracer was placed in the right or left hemisphere was alternated. Both dyes yielded similar results. We observed no difference in the pattern of labeling (anterograde and retrograde) between the two dyes when both were placed in the cortex or thalamus of the same animal. The appearance of DiI was preferred to that of DiA because DiI demonstrated greater contrast with the natural autofluorescence of the tissue; the DiI was a bright reddish-orange against the black or dull red tissue, while DiA was a bright yellow against the pale green tissue.

The brains were then returned to 4% paraformaldehyde and sufficient time (minimum 4 weeks) was allowed for the dye to transport. Table 1 summarizes the number of animals at each age and the location of the injection site. Some brains were placed in an oven at 37°C for up to 1 month to accelerate dye diffusion. This treatment made the tissue soft and difficult to cut. There was some suggestion that the DiA transported better that the DiI under this condition based on the intensity of label, but this was not quantified.

TABLE 1. Number of Animals at Each Age With the Location of Injection Site

Age	Visual cortex	LGd	LGd and visual cortex	Nonvisual cortex
E12	6	1	1	
E13	5	2	2	
E14	7	6	3	
E15	6	4		
P0	4	8	2	3
P1	4	4		5
P2	3	5		
P3	1	5	1	3
P4	4	6		
P5		2		
P6	2	5		
P7	1	1		
P8		3		
P9		1		
P10	1	1		

Histology

Coronal sections (50–200 μm) were cut into phosphate buffer (pH 7.4) with a vibratome, mounted on chrom-alum subbed slides, and coverslipped with phosphate buffer. Sections were examined under fluorescent illumination. Since the dyes fade rapidly under illumination, the sections were photographed or drawn with the aid of a drawing tube immediately after mounting.

Identification of the location of label in the thalamus and cortex was aided by staining the tissue with bisbenzimide (Sigma, cat. number B2883) prior to being mounted. A drop of bisbenzimide solution (1 mg bisbenzimide/ml dH2O) was added to 10–15 ml of phosphate buffer containing the tissue section for 10–60 minutes. Some sections were stained with thionin after they had been photographed/drawn.

Bisbenzimide stains DNA, thereby revealing the shape and size of the cell nucleus. Immature neurons, for instance, in the cortical plate possess a vertically oval nucleus and are densely packed. Differentiated cells, as defined in this study based on their appearance after bisbenzimide staining, have round nuclear profiles and are separated by extracellular space.

Thalamic reconstruction

In young animals, especially fetal and early postnatal animals, many features of thalamic nuclear cytoarchitecture are not developed, and it was not always possible to visualize thalamic nuclear boundaries with confidence. For instance, at E13 no individual nuclei are visible in the thick sections. The division between visual (LGd) and somatosensory (VB) thalamic nuclei, the external medullary lamina [also referred to as the superior thalamic radiation in developing animals; (Paxinos et al., '91)], first becomes visible at E14 as a cell sparse region (see below). Further nuclear delineation, such as the distinction between LGd and LP, is not possible until around 1 day after birth. Therefore, the location of injection sites and retrogradely labeled cells was based on available cytoarchitectonic features and on their position relative to a map of the mature thalamus used in previous work (Miller et al., '91b) for adult hamsters. The Paxinos and Watson ('86) atlas of the rat brain and the Jones ('85) text were used as general references for delineating thalamic nuclei.

Cortical reconstruction

The perimeter of the injection site in the cortex was charted by making a dorsal view reconstruction with a projection microscope using alternate sections stained with thionin or from camera lucida drawings of coronal sections stained with bisbenzimide. Identification of cortical areas by cytoarchitectonics is not possible at most of the prenatal and early postnatal ages studied here, since these features are not visible until after cortical cells have completed migration and the layers are differentiated. It was assumed that primary visual cortex is in the same relative location in dorsal posterior cortex in developing animals as in adults. The location of the injection site in the cortex (and thalamocortical axons and retrogradely labeled cells in the cortex) was based on an estimate of the relative position of the injection site in relation to rostrocaudal and mediolateral dimensions of a dorsal view of the cortex (based on the cortical map of Windrem and Finlay, '91). The map of the cortex used in this study does not distinguish between primary and secondary cortical regions, but instead groups them into unitary regions based on primary function. For instance, the region designated "visual" includes both primary and secondary visual cortex.

RESULTS

The results of this study address several issues related to the development of thalamocortical and corticothalamic projections. This paper will present results concerning 1) the timing of the development and target invasion of thalamocortical and corticothalamic projections; 2) the spatial relationship between thalamocortical and corticofugal projections during development; 3) features of the geniculocortical pathway; 4) the topographic specificity of thalamocortical projections; and 5) the connections of subplate cells.

Methodological concerns

The demonstration of adequate transport of DiI and DiA to the most distal end of cellular processes is critical. Complete anterograde transport of the dye was evident when growth cones could be visualized on the tips of the most distant labeled process, and complete retrograde transport was evident when cell bodies and their dendritic processes were visible. No data were taken from brains in which the dye transport did not appear to be complete.

The original report by Godement et al. ('87) suggested that DiI transports transcellularly in some cases. Transcellular transport of DiI and DiA was also evident in this study. An obvious example of transcellular transport was when radial glia in the cortex were labeled after an injection of dye into the thalamus. Cortical radial glia have no direct contact with thalamus and were likely labeled transcellularly by passing thalamic axons. Neuronal processes labeled by transcellular transport could be distinguished from processes labeled by direct contact with the dye by the intensity of staining: Processes directly labeled fluoresce very brightly, while those labeled by transcellular transport are faint. Also, cell bodies labeled directly had brightly fluorescing nuclei, and their dendritic and axonal processes were equally fluorescent throughout their entire extent.

Transcellular transport was not ubiquitous. Presumably, axons come into contact with a variety of substrates and processes while growing toward their target. In many animals there was no evidence of transcellular transport of any kind at any point along the axonal pathway. In the cases where transcellular transport was found, it constituted a very small percentage of the total label.

Growth and timing of thalamocortical and corticothalamic projections

Thalamocortical projections. The majority of the data concerning the growth of thalamocortical projections is derived from anterograde transport of the tracer (i.e., the tracer was placed in the thalamus and it traveled the entire length of thalamic axons). The most distal extent of thalamic axon growth was confirmed by retrograde transport of the tracer. In this case, the tracer was placed in the cortex, and cells in the thalamus were retrogradely labeled if their axon had grown to the injection site.

The thalamic injections were located in LGd. Examples of thalamic injection sites in fetal and postnatal animals are shown in Figures 1 and 2. Because of the minute size of the thalamus in the young animals, the injection often included adjacent visual thalamic nuclei LP and L, which also project to visual cortex. In some cases the injection extended beyond the visual thalamic nuclei and included small portions of dorsolateral VB or rostral MG, which project to the somatosensory cortex and auditory cortex, respectively.

The earliest age at which pups received thalamic injections of dye was E12. Cells of the LGd are still being generated at this age. An injection of dye into the dorsolateral posterior thalamus (presumptive LGd anlage) of an E12 hamster pup revealed that some axons have left the thalamus and have just entered the internal capsule, though they have not traveled very far (not shown). Tracer injections into the presumptive LGd anlage of E13 pups resulted in a brightly labeled group of axons passing through the internal capsule (Fig. 3A). The axons bunch closely together and grow laterally from the thalamus. These axons have not yet reached the intermediate zone of the developing cortex. By E14 a large number of thalamic axons from the visual nuclei have grown the remainder of the distance to presumptive visual cortex. The thalamic axons grow within a cell sparse region immediately below the cortical plate (channel 2 of Bayer and Altman, '90; subplate of Sheppard et al., '91), which begins to form on E13. An occasional axon penetrates the cortical plate up to the marginal zone (future layer 1, Fig. 3B,C).

Supporting evidence that visual thalamic axons have reached the visual cortex by E14 comes from animals in which dye was placed into presumptive visual cortex, resulting in many retrogradely labeled cells in the thalamus. Figure 3D shows retrogradely labeled cells in the LGd of an E14 hamster after DiI was placed in the visual cortex. Note the restricted location of the labeled cells in the thalamus, and the small size of the cortical injection (Fig. 3D''). The initial thalamic projection shows a surprising degree of topography (see Discussion), with some sharpening of topography in the first few postnatal days (Naegele et al., '88). Typically, retrogradely labeled cells are located in LGd, L, and LP, all of which project to the visual cortex in adults. On occasion retrogradely labeled cells are found in thalamic nuclei which do not project to visual cortex in adult animals, such as VB, which projects only to the parietal cortex in adults (see below).

At E15 the thalamic fibers continue to grow toward and collect under the posterior cortex. At this age cells at the

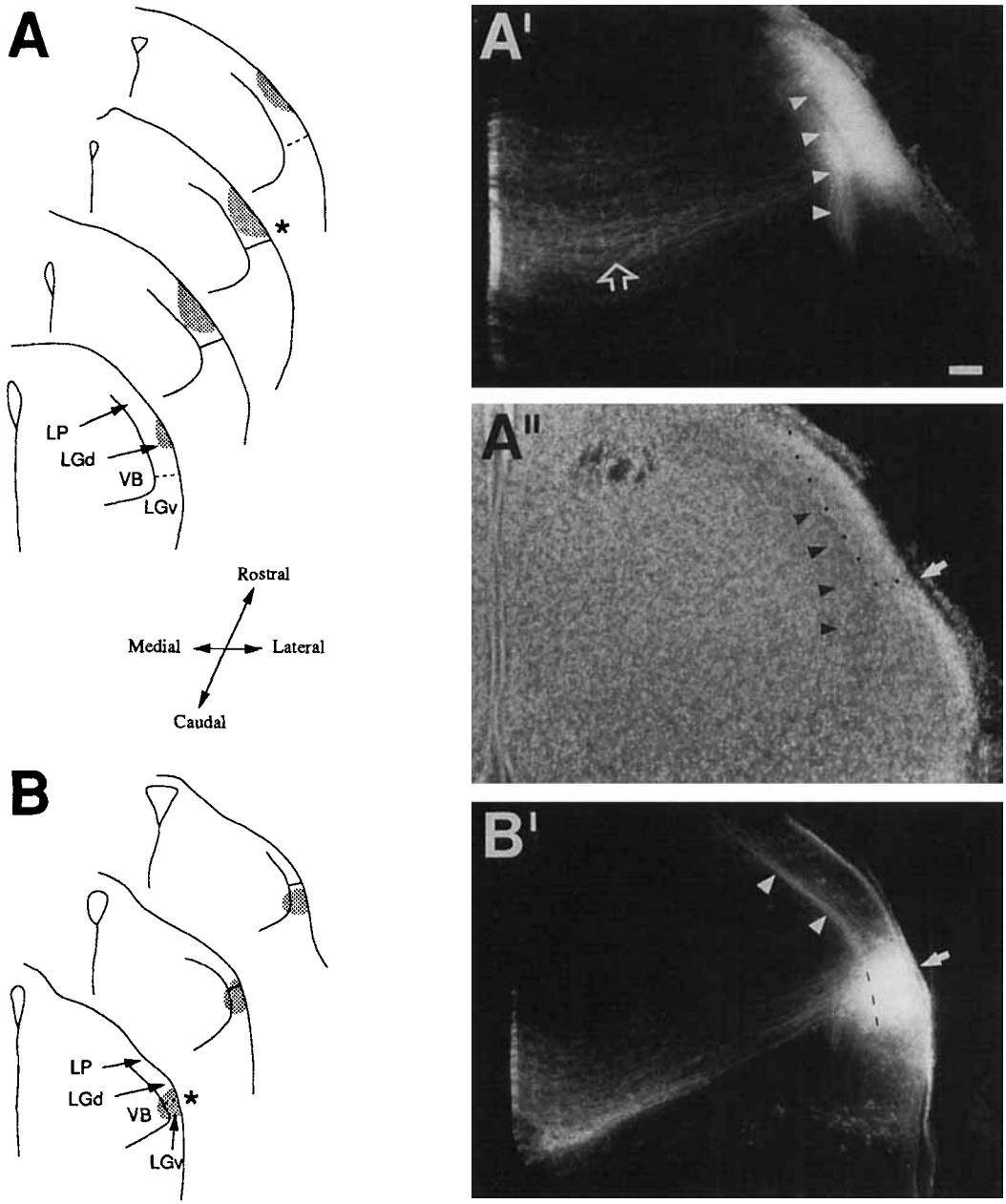

Fig. 1. Examples of thalamic injection sites in embryonic day (E)14 hamsters. Drawings and photographs are of coronal sections. **A:** These drawings are from 4 adjacent 100 μm sections, showing the complete extent of the DiI injection. In this animal, the DiI (shaded area) was restricted to the visual thalamic nuclei LGd and LP at all levels. The injection did not penetrate into VB, which is ventromedial to LGd. The border between LGd and LGv can be seen in some sections at this age and is marked with a solid line on sections where it could be distinguished and with a dotted line where it is estimated. A photomicrograph of the injection site in the section marked with an asterisk is shown in **A'**. The labeled thalamocortical fibers exit the nuclei through the external medullary lamina (white arrowheads), which separate LGd from VB. Thalamic injections of tracer at this age also label many radial glia-like processes (open arrow) that extend from the ventricle to the periphery of dorsolateral thalamus. Dorsal is up, lateral is to the right. **A''**: This photomicrograph shows the section in A' stained with bisbenzimide. The dots indicate the perimeter of the injection site. The black arrowheads mark the external medullary lamina, which first becomes visible at this age and separates VB from LGd. In the bisbenzimide stained tissue the external medullary lamina is visible as a slightly darker staining region because of low cell density. The small white arrow indicates the LGd/LGv border. **B:** This series of drawings is from 3 adjacent 75 μm sections from an E14 animal with DiI placed in the LGd. The injection (shaded area) was in the ventral portion of LGd and did not appear to involve LP or L, but did include LGv, which does not project to the cortex. The border between LGd and LGv is marked with a solid line on sections where it could be distinguished. On the most caudal section tissue damage from the injection precluded identifying the LGd/LGv border. Here, the estimated border is marked with a dotted line. The injection was deep and extended through the external medullary lamina and into VB. A photomicrograph of the injection site in the section marked with an asterisk is shown in **B'**. It is evident in this photomicrograph that the injection passed through the external medullary lamina (marked with a black dotted line) and into VB. Because the injection penetrated through the external medullary lamina, axons that travel through it are labeled (white arrowheads). The small white arrow indicates the estimated LGd/LGv border. Dorsal is up, lateral is to the right. See Abbreviations list. Scale bar in A' = 0.1 mm for A', A'', B'.

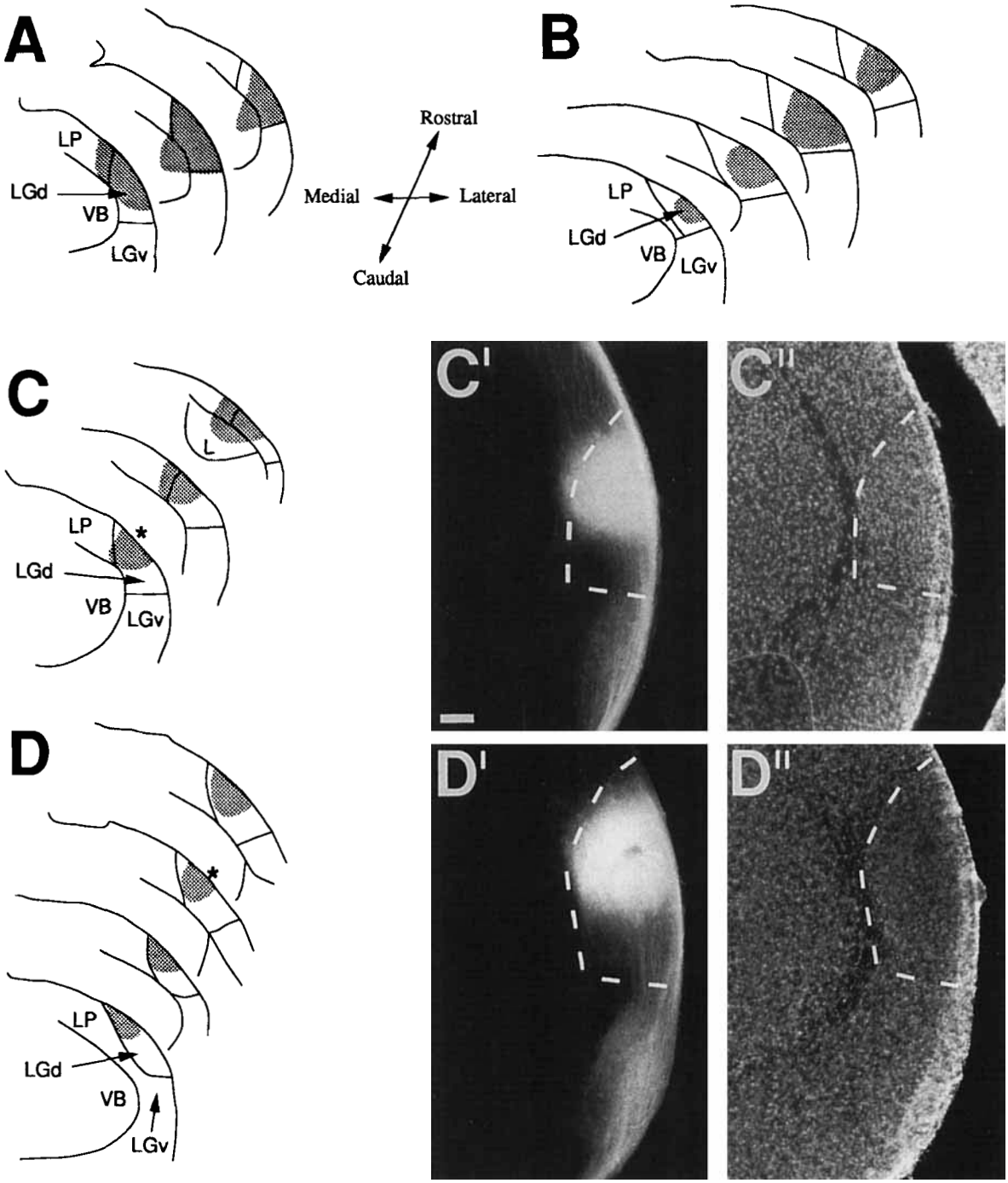

Fig. 2. Examples of thalamic injection sites in postnatal hamsters. The drawings and photographs are of coronal sections. In all cases the drawings represent the complete rostrocaudal extent of the thalamic injection (shaded). Drawings of different ages are not to the same scale. **A:** In this P1 animal the injection (DiI) included LGd and a small portion of LP. The injection also included lateral VB. It is an example of one of the larger thalamic injections in this study. **B:** A DiI injection site in a P2 animal is shown here. The injection was confined to LGd and did not spread into neighboring visual (LP) or somatosensory (VB) thalamic nuclei. **C:** The injection site in this P4 animal included primary (LGd) and secondary (L, LP) visual thalamic nuclei. A photomicrograph of the DiI injection site in this animal can be seen in **C'**, and **C''** is the same section stained with bisbenzimide. The photomicrographs correspond to the section in C marked with an asterisk. The location of the LGd is outlined. At this level the tracer occupies LGd and a small portion of LP. Dorsal is up. **D:** The DiI injection in this P7 hamster remained confined to LGd. **D':** Photomicrograph of the injection site in the same animal as D. This level corresponds to the section in D marked with an asterisk. **D'':** Same section as D' stained with bisbenzimide. The location of the LGd is outlined. At this level the tracer is clearly restricted to LGd. Dorsal is up. The pattern of thalamocortical afferents and corticothalamic efferents from/to this injection site can be seen in Figures 5C and 9B. The drawings in A and B are from adjacent 125 μm sections, and those in C and D are from 150 μm sections. See Abbreviations list. Scale bar in C' = 0.05 mm for all photomicrographs.

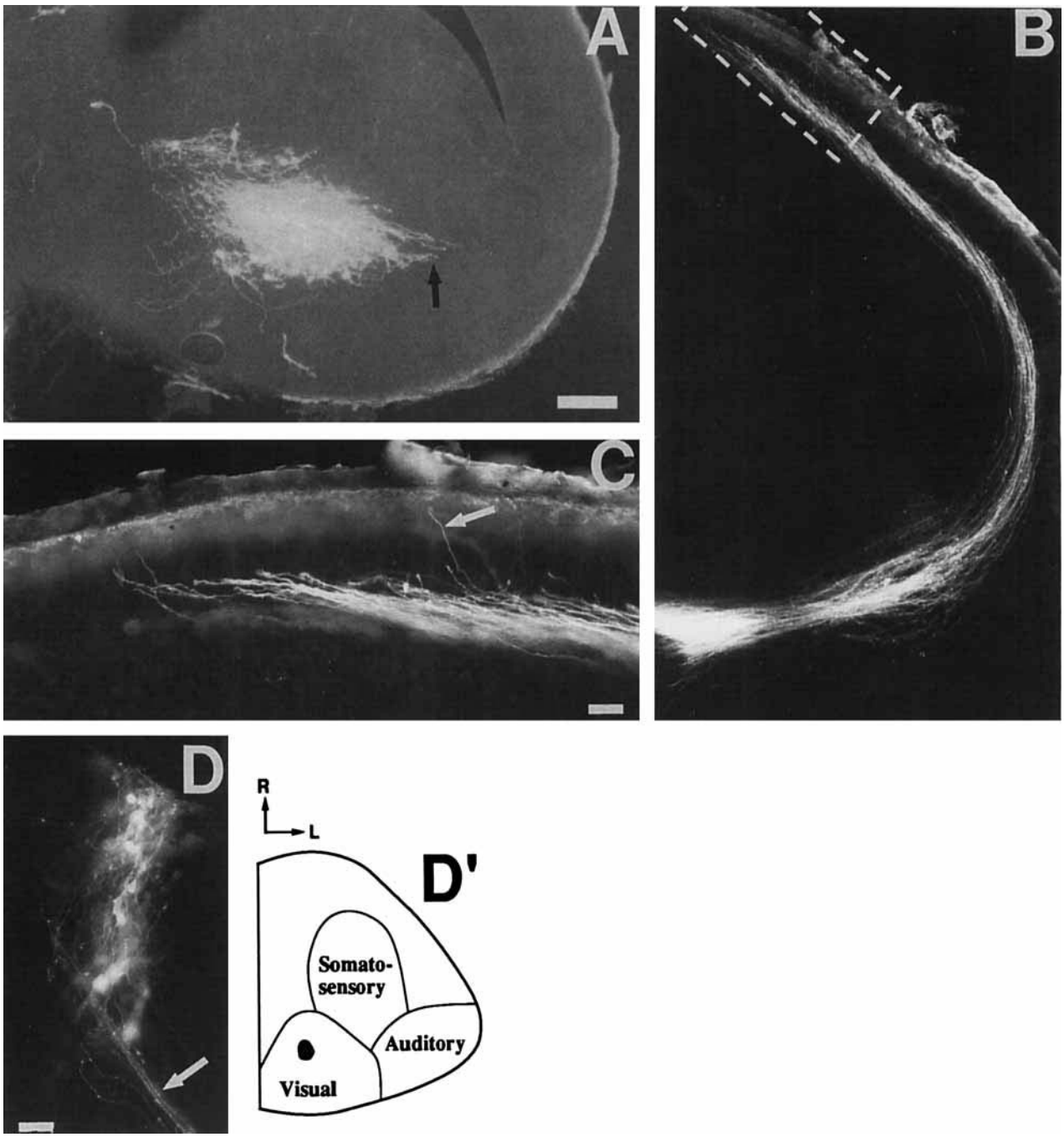

Fig. 3. The growth of thalamic axons to visual cortex. All photomicrographs are from coronal sections. **A:** This figure shows the position of the visual thalamic fibers (from the presumptive LGd anlage) at E13. They have left the thalamus (to the left) and travel laterally through the internal capsule. At this age the leading tips of the axons (arrow) are still within the internal capsule. None of these axons have entered the intermediate zone under the cortex (to the right). Lateral is to the right. By E14 (**B**), the thalamic axons have grown to the visual cortex [upper right (lateral) of the figure]. The thalamus is to the lower left, and the internal capsule is in the bottom of the figure. A higher magnification view of the leading axonal processes in this same section, corresponding to the outlined area, is shown in **C.** The majority of thalamic axons grow tangentially in the cell sparse zone directly under the cortical plate, but an occasional axon (arrow) enters the cell dense cortical plate and reaches the marginal zone. **D:** This photomicrograph shows many retrogradely labeled cells in the LGd after an injection of DiI in visual cortex of an E14 hamster. Their axons (arrow) travel through the external medullary lamina. Dorsal is up. The drawing (**D'**) shows the location of the injection (shaded area) in visual cortex. See Figures 6A and 15 for other examples of retrogradely labeled cells in the LGd at E14 after an injection of dye into visual cortex. The drawing represents a dorsal "unrolled" view of the right cerebral hemisphere. In all drawings representing the cortex in this paper, the midline border represents the dorsomedial apex of the cortex as viewed from a coronal section, and the lateral border represents the rhinal fissure. Scale bar in A = 0.1 mm for A and B; bar in C = 0.05 mm; bar in D = 0.075.

bottom of the cortical plate have begun to differentiate, and are likely to be subplate cells (Bayer and Altman, '90). The thalamic fibers just begin to penetrate into this differentiating region, some growing horizontally (not shown).

By the day of birth (P0) the thalamic fibers have grown within the subplate, which has completely differentiated at this age and forms a thin layer below the cell dense cortical plate (Fig. 4A,B). In the bisbenzimide stained sections this differentiated region is a zone with increased extracellular space, and the cell nuclei are round. The cells in the cortical plate are much more densely packed, with oval nuclei. Using this criterion it was possible to draw a "line of differentiation" separating the lower "differentiated" portion of the developing cortex from the upper "undifferentiated" cell dense cortical plate. As with more immature animals, the vast majority of thalamic fibers remain below the undifferentiated cortical plate. A few axons penetrate a short distance into the cell dense region, and a rare axon extends up to the marginal zone. Axons from visual thalamic nuclei are still growing toward the cortex at P0. Many axons with growth cones can be seen in the internal capsule after an injection of dye restricted to LGd-LP (not shown).

In the presumptive visual cortex of P1 hamster pups, thalamic fibers have grown up to the border of the line of differentiation, which has begun to move into the bottom of layer 6. The differentiated region is as densely populated by thalamic fibers as the intermediate zone and subplate.

At P2 the thalamic fibers have arborized extensively up to the line of differentiation (Fig. 4C,D), now half the thickness of the developing cortex. Thus, the leading edge of the thalamic invasion appears to follow, or produce, the maturational gradient of the developing cortical plate. At the level of the subplate, and in the underlying intermediate zone, the thalamic axon density is decreased, and the axons pass through with almost no arborization. There is an increase in the number of axons that penetrate the cortical plate to the marginal zone (not shown).

The first evidence of a laminar-like segregation of thalamic afferents appears at P3 (Fig. 5A), though it was not evident in all animals at this age. In one animal, laminar segregation of thalamocortical projections was visible in the rostral visual cortex, but not in more caudal sections. At this age only the upper fourth of the cortex remains undifferentiated cortical plate. There are two regions of dense thalamic arborization, one just under the line of differentiation and the second in the upper layer 6/lower layer 5 region. The number of thalamic axons that penetrate the cortical plate to reach the marginal zone increases considerably by P3, and this can be seen in Figure 5A.

By P5 the thalamic fibers continue to grow into the developing cortex. The cell dense cortical plate is now a thin layer at the top of the cortex. At this age the upper layer of thalamic arborization is much more dense than the lower, which has thinned out considerably (Fig. 5B). The lamina covered by the axons are the same as seen at P3: upper layer 6 and lower layer 5, and in the region just below the cell dense region at the top of the cortical plate, presumably in the future layer 4.

At P7 the upper lamina of thalamic arbor has increased its density considerably. At around this age the posterior cortex appears "mature" (in terms of laminar cytoarchitecture) when layer 4 becomes distinct from layers 2–3. Dense thalamic arbor covers all of layer 4 and what is probably the bottom portion of layer 3, the same as in mature animals (Naegele et al., '88). Many axons reach layer 1, but the most dense thalamic arbor stops just before the top of the cortex (see Fig. 5C). In no case was there compelling evidence that the bulk of thalamic axons overgrow their radial distribution by arborizing into upper layer 2/3 up to layer 1, and then withdrawing back to lower layer 2/3. Thus it appears that thalamic afferents assume a mature laminar position as layer 4 becomes visible, around P7. The lower lamina of thalamic arbor appears to have thinned considerably and is barely distinguishable from the background. The precise extent of thalamic arborization in this region was difficult to discern because the presence of labeled cells in layer 6, and their associated dendritic processes obscure the distinction between thalamic axonal and cellular dendritic arbor. This deep thalamic arbor still occupies upper layer 6 and lower layer 5. At older ages, that is, P8 and older, the only apparent change is that the upper lamina of thalamic arbor increases in density and complexity. An example of the thalamic fibers in visual cortex of a P10 animal is shown in Figure 5D.

Corticothalamic projections. The dye injections into presumptive visual cortex labeled axons entering and exiting the injection site. Examples of injection sites into presumptive visual cortex are shown in Figures 6A–C, and 7A. It was difficult to determine the depth of the injections as the dyes labeled all cellular processes, including radial glia. In the animals reported on in this study the injections were more than adequately deep to label both thalamocortical and corticofugal projections.

DiI injections in visual (posterior) cortex of E12 pups reveals that there are only a very few axonal processes present at this time, and few of these have left the immediate vicinity of the injection. These axons have not yet reached the internal capsule region (Fig. 7A,B). At this age, visual cortex is very immature. The cell dense ventricular zone constitutes the bulk of the pallial thickness. These cells are vertically oval in shape. At the very top of this zone is a very thin, sparsely populated region only a few cells thick that are horizontally oval, indicative of the preplate stage (Sheppard et al., '91; also called the primordial plexiform layer, Bayer and Altman, '91; Marin-Padilla, '71). These cells constitute both subplate and layer 1 Cajal-Retzius cells (Bayer and Altman, '90, '91; Luskin and Shatz, '85b).

By E13 the cortical plate has begun to form within the preplate. Corticofugal axons have traversed the distance from the presumptive visual cortex, are turning medially out of the intermediate zone, and are entering the internal capsule (not shown). No axons have reached the thalamus at this age. Thalamic fibers at this age are also in the internal capsule (see above). E13 brains with double label injections (DiI in the dorsal thalamus and DiA in the visual cortex) show that the leading thalamic and cortical axons grow into the forming internal capsule at the same time (not shown; see also De Carlos and O'Leary, '92; Erzurumlu and Jhaveri, '92).

At E14 corticofugal axons have not grown much farther and their leading processes are still in the internal capsule (Fig. 6D,E). This is the same age when thalamic axons have arrived at their target in visual cortex (see above and Fig. 3). Thus thalamic projections reach their cortical target before corticofugal projections reach the thalamus. This fact is also evident when the tracer is placed in the thalamus. Since the dye diffuses both antero- and retrogradely, any cortical projections which have reached the thalamus by this age would be visible as retrogradely

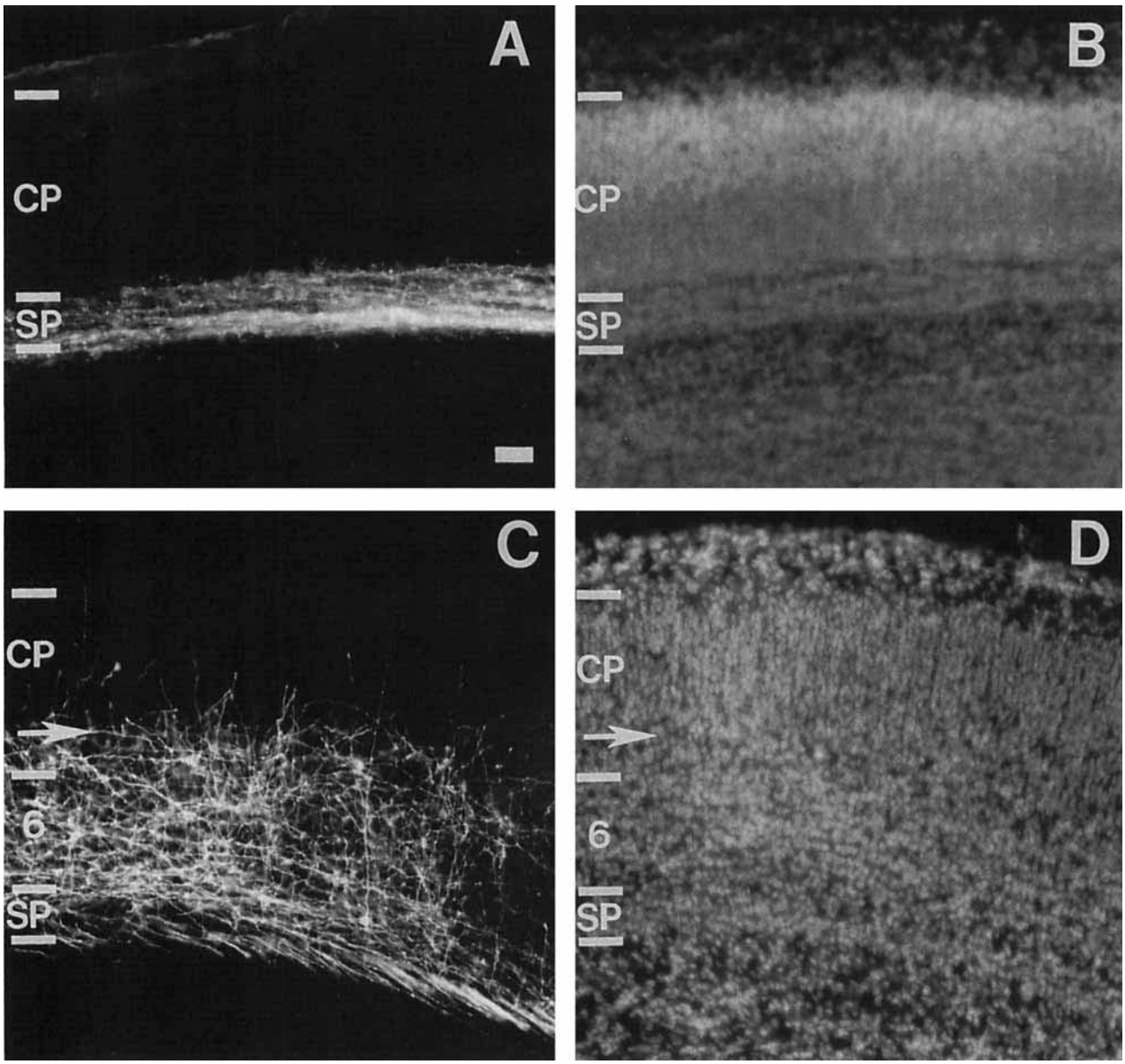

Fig. 4. Growth of thalamic axons into visual cortex at P0 and P2. **A:** Coronal section of thalamic axons in the visual cortex of a P0 hamster with an injection of DiI into LGd. The same section is shown in **B,** stained with bisbenzimide. The axons are most densely concentrated in a cell sparse region at the bottom of the subplate (SP). The thalamic fibers have grown completely into the subplate which marks the border of the "line of differentiation." At this age axons rarely penetrate the cell dense cortical plate (CP) up to the marginal zone, and *no* axons grow past the subplate in this section. Observe also that there are no retrogradely labeled cells in the visual cortex in this section. The first axons from cells in the visual cortex reach the posterior thalamus at P1 (see Table 2). The figure in **C** shows a coronal section of the thalamic axons in visual cortex at P2 after DiI was placed in the LGd. **D:** The same section as C, stained with bisbenzimide. The axons have penetrated deep into the cortex, past layer 6 into layer 5, some of which is visible at this age. The arrow marks the line of differentiation, the point where the majority of axons stop. There is no evidence of laminar segregation of the thalamic afferents at this age. Note that at the level of the subplate the axons appear to pass through without arborizing. This same section can be seen in Figure 13B at a lower magnification. Scale bar in A = 0.05 mm and applies to A–D.

labeled cells in the cortex. No retrogradely labeled cells are found in the visual cortex of E14 animals after an injection of tracer into the thalamus.

Very little has changed by E15. The leading tips of many corticofugal axons are still found in the internal capsule. It is difficult to discern the farthest extent of the fibers at this age, however, because of the presence of many thalamocortical axons in the internal capsule region. Moreover, corticofugal axons cross over thalamic axons in the internal capsule (see below), making identification of the leading tips of cortical axons difficult. Therefore, subsequent information concerning the growth of corticothalamic axons was derived by identifying retrogradely labeled cells in the cortex from dye placed in the thalamic visual nuclei.

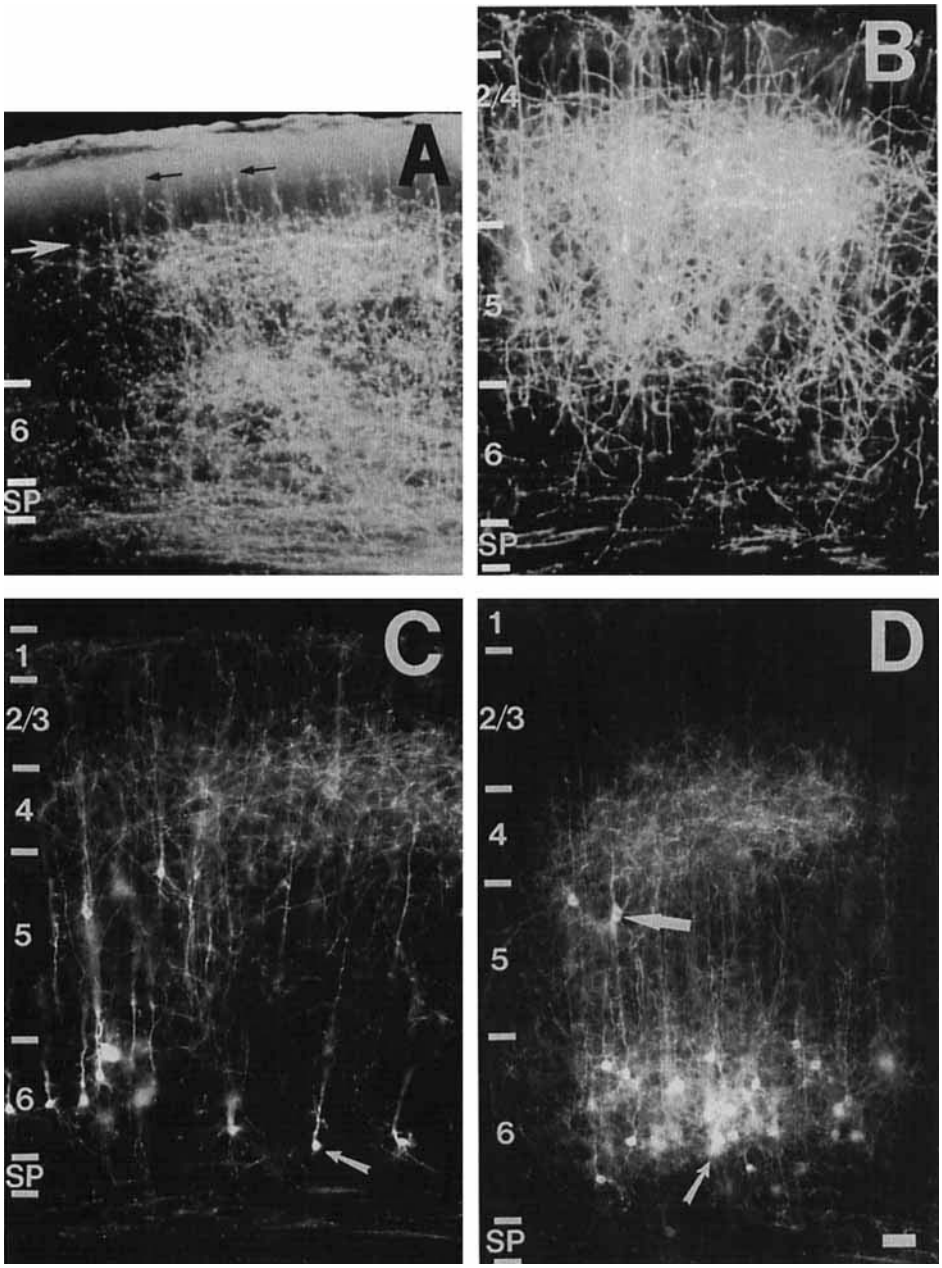

Fig. 5. Growth of thalamic axons into visual cortex at P3 to P10. In all of the photomicrographs DiI was placed in the LGd. All photomicrographs are from coronal sections of visual cortex. The laminar borders were identified by staining the tissue with bisbenzimide. **A:** In P3 hamsters thalamic afferents have grown into most of the developing cortex. The layer 5-4 border is not visible at this age. The white arrow points to the "line of differentiation." Note that the thalamic fibers have grown up to this point. This is the first age where there is evidence of laminar segregation of the thalamic afferents, though this was not seen in all animals of this age. The sparse region between the two lamina is in about the middle of the future layer 5. At this age the two lamina of thalamic arbor are of about equal density. A large number of thalamic axons penetrate the cell dense cortical plate all the way to the marginal zone (small black arrows). **B:** At P5 the density of the thalamic afferents has been greatly reduced in the lower lamina, and even more so below that. The highest concentration of thalamic arbor is in the upper lamina, presumably future layer 4. Note the absence of retrogradely labeled layer 6 cells in this photograph. Layer 6 cell projections to the thalamus are minimal until P7. **C:** At P7 the thalamic afferents reach their mature laminar position when layer 4 becomes visible. The axons have increased their arbors substantially in layer 4 and the lower part of layers 2/3. The lower lamina of thalamic fibers is all but absent. At this age many retrogradely labeled layer 6 cells (arrow) are seen in the visual cortex after an injection of dye into the LGd. The layer 6 corticothalamic cells are typical of modified pyramidal cells (Jones, '85). This is the same section as in Figure 9B, but at higher power. The thalamic injection site in this animal is shown in Figure 2D. **D:** Thalamic fibers have arborized extensively into layer 4 and lower layer 3 at P10. There are many retrogradely labeled layer 6 cells (small arrow). The presence of these cells obscures the state of the lower lamina of thalamic arbor. Note the small number of layer 5 cells (large arrow). This same section is also visible in Figure 9C at a lower magnification. SP, subplate. Scale bar in D = 0.05 mm and applies to A–D.

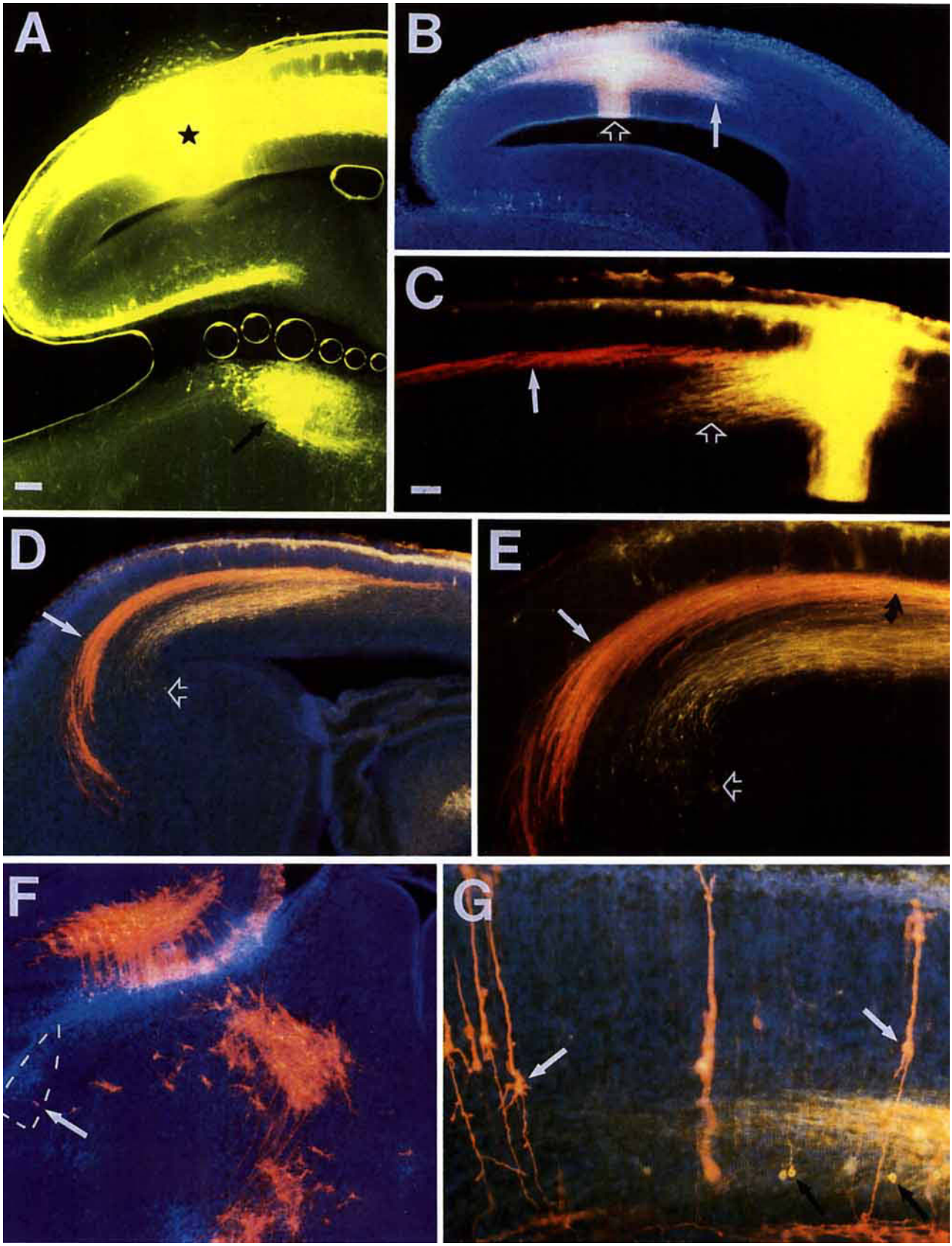

Figure 6

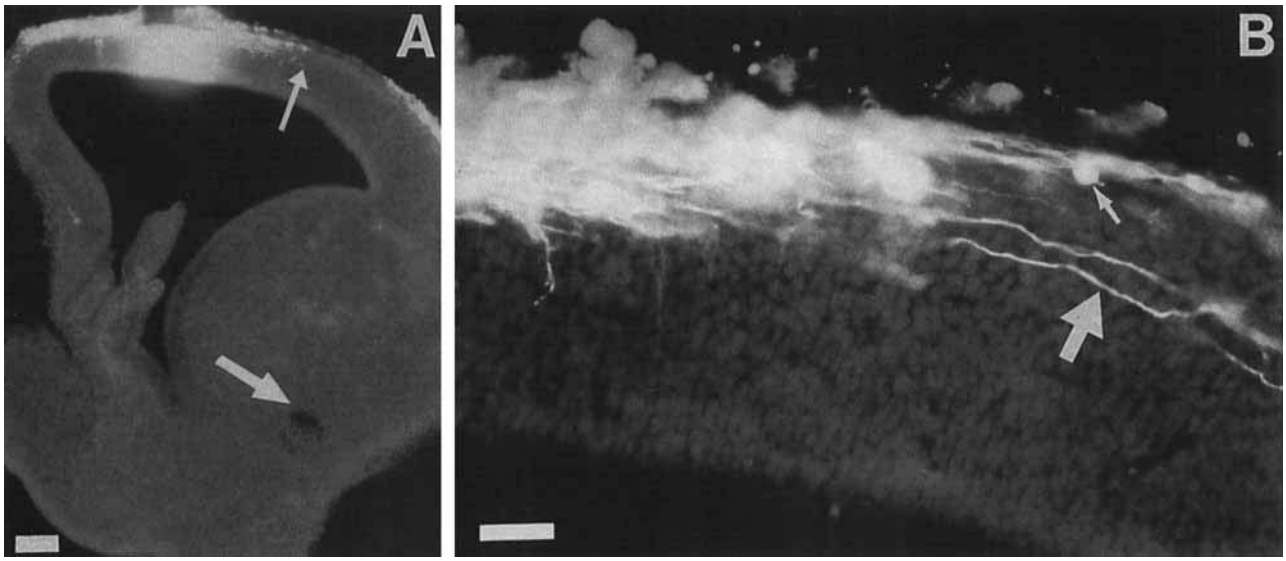

Fig. 7. The early growth of corticofugal projections. **A:** The youngest animals to receive an injection of dye into the cortex were E12. The cortical plate has not yet formed in visual cortex. In animals of this age, the fibers have grown only a short distance beyond the injection site, as indicated by the small arrow. The large arrow marks the beginning of the internal capsule, as indicated by a cell sparse region. To reach the thalamus cortical axons must grow through the internal capsule. The cortical axons are evidently a considerable distance from the internal capsule at this age. The internal capsule is presumably filled with axons exiting the thalamus and growing toward the cortex. When dye is placed in the thalamus of E12 pups, a small contingent of axons has left the thalamus and entered the internal capsule. See Results for details. Lateral is upper right. **B:** Higher magnification photograph of the same animal as in A. This photograph is of the cortex just lateral to the injection site. The injection site is to the left. The axons marked with the large arrow are growing away from the injection site just above the ventricular zone. These are presumptive corticofugal axons. The distance that these axons have grown from the injection site is marked in A with the small arrow. Separate from the corticofugal axons is another axonal system. The small arrow marks cells that are retrogradely labeled by the injection. These are presumptive Cajal-Retzius cells that lie immediately below the pia, in what will be the marginal zone. Many fibers interconnect these cells. Thus, the first corticofugal fibers and the fibers of the future marginal zone are the only fiber pathways evident at this age. Scale bars = 0.2 mm in A, 0.05 mm in B.

Fig. 6. **A:** This E14 animal received an injection of DiA (star) in the visual cortex. DiA fluoresces yellow under fluoroscein illumination, and the tissue autofluoresces pale green. Thalamic fibers have grown to visual cortex by this age, and the black arrow indicates a large number of retrogradely labeled cells in the LGd. Coronal section. Dorsolateral is up. **B:** This is a double exposure photomicrograph of a coronal section of an E15 animal with an injection of DiI (pink) into the visual cortex. The blue color of the tissue is the bisbenzimide stain illuminated with ultraviolet fluorescent light. The injection labeled radial glia (open arrow), which span the width of the cortical wall. Corticofugal fibers project to the right (small white arrow). The distance that the corticofugal axons have grown is not visible at this level. Dorsolateral is up. **C:** This double exposure (DiI, DiA) photomicrograph shows an E14 animal that received a DiI injection into LGd and a DiA injection into visual cortex. These injections labeled closely topographically matching projections: the thalamic axons (red, small white arrow) pass directly into the DiA injection (yellow) in the cortex. The spatial separation of the thalamocortical and corticofugal systems at this early age is clearly evident in this figure. The thalamic fibers (red) travel in the upper intermediate zone and are distinctly separated from the corticofugal projections (yellow, open arrow), which travel deep in the intermediate zone. These two fiber systems are also separate in the internal capsule (D,E). Dorsolateral is up. Coronal section. **D:** This is a low-power triple exposure (DiI, DiA, bisbenzimide) photomicrograph of a coronal section from the same animal as C. The cortex is in the upper part of the photograph, and the internal capsule is in the lower left. This shows that the thalamic fibers (reddish orange, small white arrow) have grown all the way to visual cortex (the top right of the figure). The cortical fibers (pale yellow) have exited the intermediate zone and have entered the internal capsule region (open arrow) but have not traveled beyond this. **E:** Same section as D but higher magnification. The upper band of fibers is thalamocortical (reddish orange, small white arrow) and the lower band is corticofugal (yellow). Growth cones can be seen on the ends of the corticofugal axons (open arrow) at higher magnification. Note the space that separates the two fiber pathways. The DiA injection in the cortex labeled some thalamocortical axons, and they can be seen (yellow) intermingled within the DiI labeled (reddish orange) thalamocortical axons (curved black arrow). Figures C, D, and E demonstrate *in the same animal* that the two fiber systems that make reciprocal topographically matching connections are separated in the time that they reach their respective targets, and that they are physically separate during development as well. Retrogradely labeled cells in the cortical plate were labeled by the DiA injection in the cortex, not by the DiI injection in the thalamus. **F:** Coronal section of the thalamus from a P1 hamster with an injection of DiI into medial cortex. See Figure 14 for the location of injection sites in nonvisual cortical areas. Many labeled cells are found in MD (center right) and VMb (lower right) in this animal. The arrow marks a retrogradely labeled cell in LGd (outlined). LGd does not project to medial cortex in adults, and this figure demonstrates that a very small number of LGd axons do make targeting errors during development. **G:** This figure is a triple exposure (DiI, DiA, bisbenzimide) photomicrograph of a coronal section of cortex from a P1 animal that received an injection of DiA in medial cortex ipsilateral to where this photomicrograph was taken, and an injection of DiI into the contralateral medial cortex. See Figure 13 for the location of injection sites in nonvisual cortical areas. Cells in the subplate (yellow cells, black arrows) were labeled *only* by the ipsilateral injection, and cells in layer 5 (reddish orange cells, white arrows) were labeled *only* by the contralateral injection. This demonstrates that subplate cells make many ipsilateral projections. No callosal projections from subplate cells were seen in this animal. Scale bar in A = 0.1 mm for A, B, D, and F; bar in C = 0.05 mm for C, E, and G.

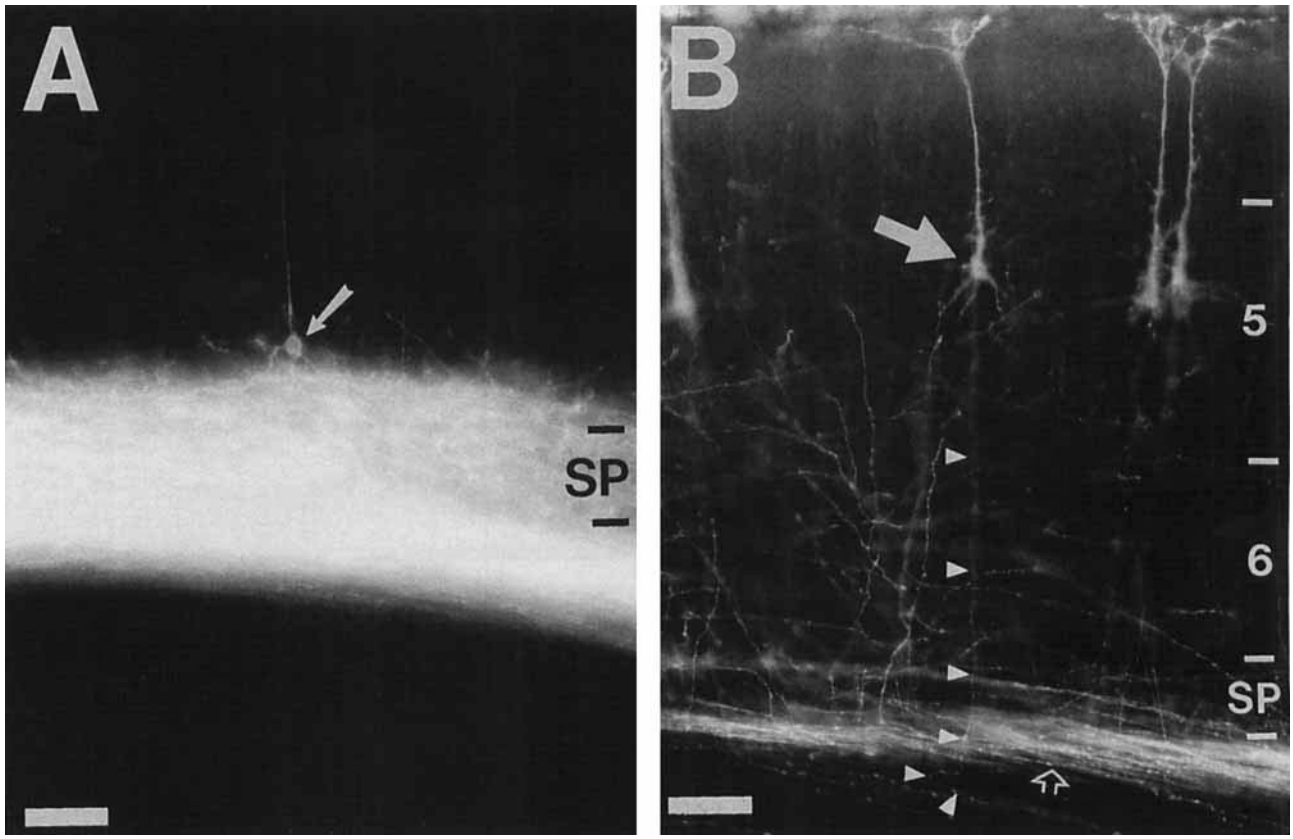

Fig. 8. Retrogradely labeled cells in the cortex after LGd injection. **A:** This coronal section from a P0 animal shows a layer 6 cell (arrow) retrogradely labeled in lateral cortex after an injection of DiI into the LGd. Cells in the subplate (SP) are also labeled at this time, though none are visible in this section. No retrogradely labeled cells are found in visual cortex until P1. **B:** This photograph shows retrogradely labeled layer 5 pyramidal cells (large arrow) in visual cortex in a P3 animal. This is lateral to where the dense thalamic arbor has entered the cortex, and these cells are not associated with the dense aggregate of ingrowing thalamic fibers. The arrowheads label the axon of the cell marked with the large arrow. Axons of corticothalamic cells project through the thalamic fibers (open arrow) and travel beneath them under the cortex (see also Figs. 6C and 10). The cortical lamina were confirmed with bisbenzimide stain. Scale bars = 0.05 mm in A, 0.1 mm in B.

No retrogradely labeled cells were seen in the cortex after an injection of tracer into the thalamus of animals younger than P0. The first time that any retrogradely labeled cells are found in the cortex is at birth, P0 (Fig. 8A). The first cells that project to posterior thalamus are located in lateral cortex, though a single cell was found in visual cortex in one animal. These cells are located in the subplate and layer 6, and are in very small number (Table 2). Lateral cortex is presumably auditory cortex, which extends caudally for some distance and lies ventrolateral to visual cortex in coronal sections. Retrogradely labeled cells were not seen in all animals at this age. Notably, the animals in which the injection was confined to LGd did not have retrogradely labeled cells in the cortex. Injections that included either MG or VB did result in labeled cortical cells. Thus it is possible that these first cortical efferents were directed to these thalamic nuclei and not LGd.

At P1 cortical projections from visual cortex reach the thalamus. As in the P0 animals, there are very few cells, they are in the subplate and layer 6, and are not found in all animals. Meanwhile, the number of corticothalamic cells in lateral cortex increases somewhat, and the first projections from layer 5 cells appear. At P2 the number of retrogradely labeled cells in the cortex increases, but the laminar distribution does not change. Half of the animals examined at this age demonstrated no corticothalamic projections from the visual cortex and very few from the lateral cortex. This may reflect individual variation between animals, and it certainly indicates that corticothalamic projections are retarded relative to thalamocortical projections, which have penetrated into layer 5 by this age.

At P3 there is a dramatic change in the laminar distribution of cortical cells projecting to the thalamus—a large contingent of upper layer 5 cells is retrogradely labeled after thalamic injections (Fig. 8B). There is no such increase in the number of layer 6 projections to the thalamus at this age, and the number of labeled layer 5 cells greatly exceeds that of the subplate or layer 6 cells (Table 2). It is possible that these layer 5 cells are projecting to the superior colliculus, since some of their axons pass through the LGd (see Discussion).

The pattern of corticothalamic projections remains the same from P4–6. The number of retrogradely labeled cells in layer 5 increases substantially, but the number of cells in layer 6 does not. The layer 5 cells are distributed across a wide tangential region of visual cortex and contrast with

TABLE 2. Retrogradely Labeled Cells in the Cortex After Thalamic Injection of Dye[1]

		Lateral				Visual			
Animal	Injection site	SP	6	5	2–4	SP	6	5	2–4
P0									
186A	LGd	—	—						
186B	LGd-VB	*	*						
169.1	LGd	—	—						
169.2	LGd	—	—						
180.7	LGd-MG	—	1						
180.8	LGd-VB	1	3			1			
180.9	LGd-VB	1	1						
P1									
183.1	LGd-VB	4	2	1		—	1		
183.2	LGd-MG	6	5	1		—	—		
183.3	LGd-MG	1	8	—		1	1		
183.5	LGd-VB	1	1	—		—	—		
P2									
191.1	LGd-MG	—	2	3		—	—		
191.2	LGd-MG-VB	4	40	1		5	4		
187.1	LGd	3	11	—		2	2		
199.1	LGd-MG	—	4	—		—	—		
P3									
167.1	LGd-MG	1	8	14		—	—	12	
167.2	LGd-VB-MG	*	*	*		—	2	25	
167.3	LGd-VB-MG	1	8	14		—	—	12	
179.2L	LGd-MG	—	1	—	1	—	—	—	
179.2R	LGd-MG	—	3	2		—	—	2	
179.3R	LGd-VB	—	3	1		—	10	1	
179.3L	LGd	—	2	1		4	1	—	
P4									
199.5	LGd-MG	—	2	67	—	—	1	36	
199.6	LGd-MG	—	6	29	1	2	1	19	
P5									
193.1	LGd-MG-VB	2	10	56	1	—	4	128	2
193.2	LGd-MG	—	3	9	—	—	4	54	—
P7									
195.5	LGd	—	—	3	—	3	61	163	1
P8									
188.2	LGd	—	1	66	—	1	15	536	—
188.3	LGd-MG	1	21	286	—	2	51	923	1
P9									
195.7	LGd-MG	—	2	16	—	4	148	186	2
P10									
175.8	LGd-MG	11	70	87	—	2	187	384	—

[1]The number of retrogradely labeled cells in the cortex were counted in all sections of these animals. The designation of visual and lateral cortical region was based on an estimate of the relative position of the labeled cells in relation to rostrocaudal and mediolateral dimensions of a dorsal view of the cortex, with the assumption that visual cortex is in the same relative location in dorsal posterior cortex in developing animals as in adults. The lateral cortex includes presumptive auditory and somatosensory cortex. Two P3 animals received an injection of DiI in one hemisphere, and DiA in the other. L, left side injection; R, right side injection; *labeled cells were present but not quantified.

the restricted topography of thalamic fibers growing into the cortex (Fig. 9A). Also at this age an occasional cell in the superficial layers (2–4) is labeled (Table 2).

At P7 there is another dramatic change in the laminar distribution of corticothalamic cells—a large number of densely aggregated layer 6 cells are retrogradely labeled. These cells are located throughout the width of layer 6, though more are in upper layer 6. The sudden appearance of layer 6 corticothalamic projections at this age is surprising because this is several days after the bulk of layer 5 cell axons have grown into the thalamus (Table 2). Interestingly, the location of these cells is densely concentrated in the same position as the ingrowth of thalamic afferents (Fig. 9B). This contrasts dramatically with the widely dispersed retrogradely labeled layer 5 efferents.

The association of the emergence of layer 6 corticothalamic projections with the presence of thalamic afferents was also noted in lateral cortex. Many of the thalamic injections included some portion of adjacent nuclei, such as MG. In these cases dense thalamic projections were seen entering the lateral cortex at the same location where substantial numbers of layer 6 cells were retrogradely labeled.

From P8–10 the distribution of layer 6 corticothalamic cells was the same—they were always associated with the presence of labeled thalamic axons that had entered the cortex—but they increased in number considerably, as indicated by the proportion of labeled layer 5 and 6 cells (Fig. 9C; Table 2). The number of labeled layer 5 cells had not increased in the region of the thalamic afferents by P10 and continued to be widely distributed throughout posterior cortex.

Corticothalamic cell morphology. The cells that project to the thalamus vary morphologically with the lamina from which they originate. Corticothalamic cells in the subplate were variably shaped. Many of the cells are pyramidal in shape, with radially oriented apical dendrites, some of which reached layer 1. Other subplate cells were inverted pyramids, with their apical dendrite pointed toward the white matter, while other subplate cells were multipolar (see Valverde et al., '89 for a complete description of the variable subplate cell morphology in rodents). The corticothalamic cells in layer 6 were typically the modified pyramidal type (Jones, '85; see Fig. 5C for an example). Rarely a cell was seen in layer 6 that was an inverted pyramid. All the cells in layer 5, and the few cells seen in layers 2–3, were classic radially oriented pyramids.

Spatial relationship between thalamocortical and corticothalamic projections during development

Previous reports in adult rats have shown that thalamocortical and corticothalamic fibers are radially segregated underneath the cortex (Woodward and Coull, '84; Woodward et al., '90). This study examined whether the same

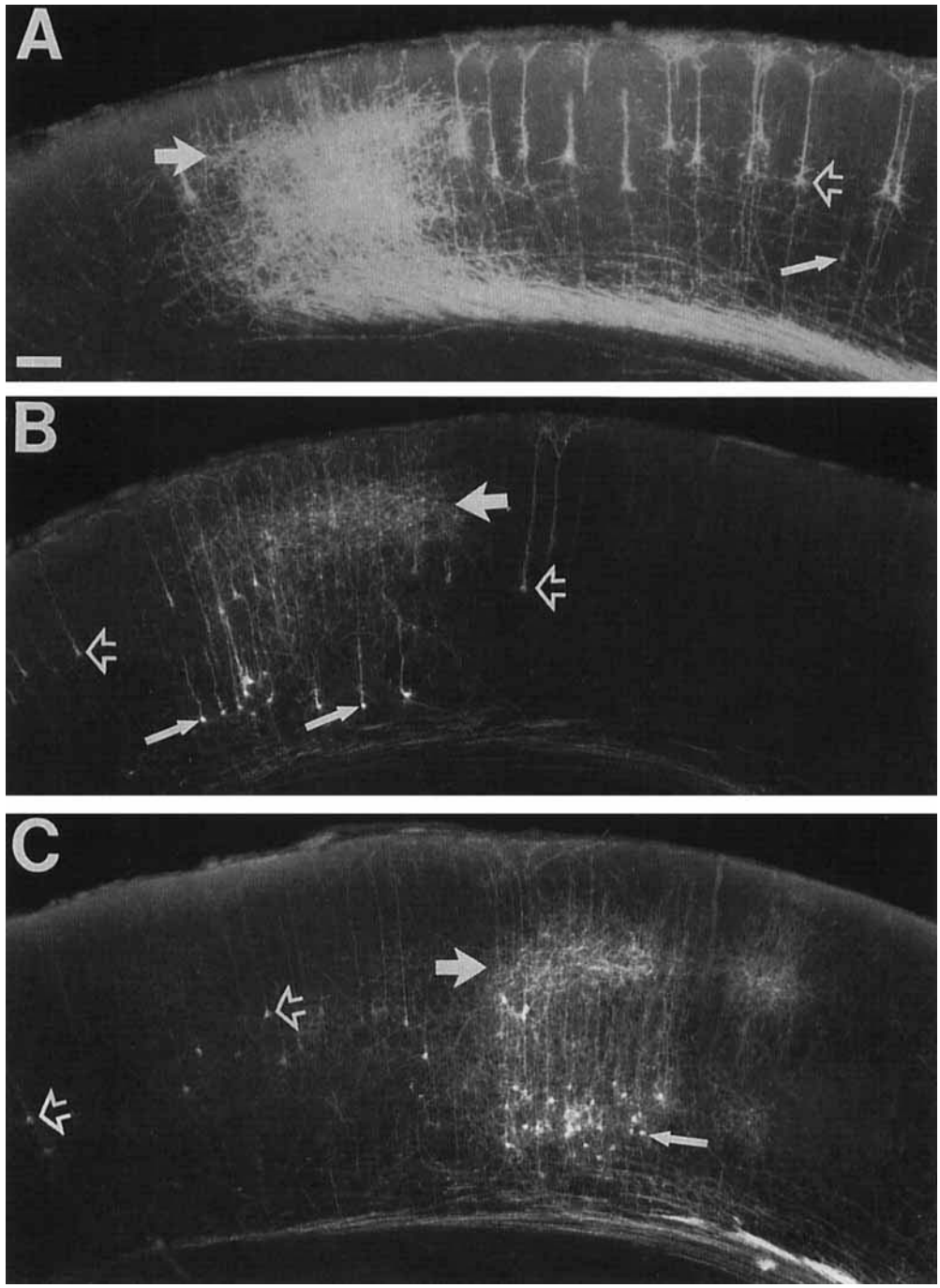

Fig. 9. The distribution of retrogradely labeled cells in visual cortex after thalamic injection at P4, P7, and P10. **A:** This figure shows a coronal section of visual cortex from a P4 animal that received a DiI injection into LGd. The thalamic fibers (large arrow) have penetrated deep into the cortex. The retrogradely labeled cells (open arrow) are located in layer 5. Note their wide distribution. They span a large area of visual cortex compared to the restricted region where the thalamic fibers grow in. Only one layer 6 cell was retrogradely labeled (small arrow), and it is not coincident with where the thalamic fibers are growing into the cortex. **B:** This is a coronal section from the visual cortex of a P7 hamster that received an injection of DiI restricted to the LGd. Note that the retrogradely labeled layer 6 cells (small arrows) are concentrated in the region where the thalamic afferents (large arrow) have grown deep into the cortex. The layer 5 cells (open arrows) are spread out in the cortex and are not immediately coincident with the thalamic afferents. A higher power photograph of this same section (delineating the layers) can be seen in Figure 5C. The thalamic injection site in this animal can be seen in Figure 2D. **C:** A coronal section of visual cortex from a P10 animal that received an injection of DiI in the LGd. The topography of corticothalamic cells appears similar to the P7 animal. The retrogradely labeled layer 6 cells (small arrow) are densely concentrated where the thalamic axons (large arrow) have arborized into the cortex. Note that the retrogradely labeled layer 5 cells (open arrows) are spread wide in the cortex. At this age there are many more retrogradely labeled layer 6 cells in the cortex than at P7. See Table 2 for a comparison of the actual cell count. For a higher power view of the retrogradely labeled cells in this same section (delineating the layers) see Figure 5D. Scale bar in A = 0.1 mm and applies to A–C.

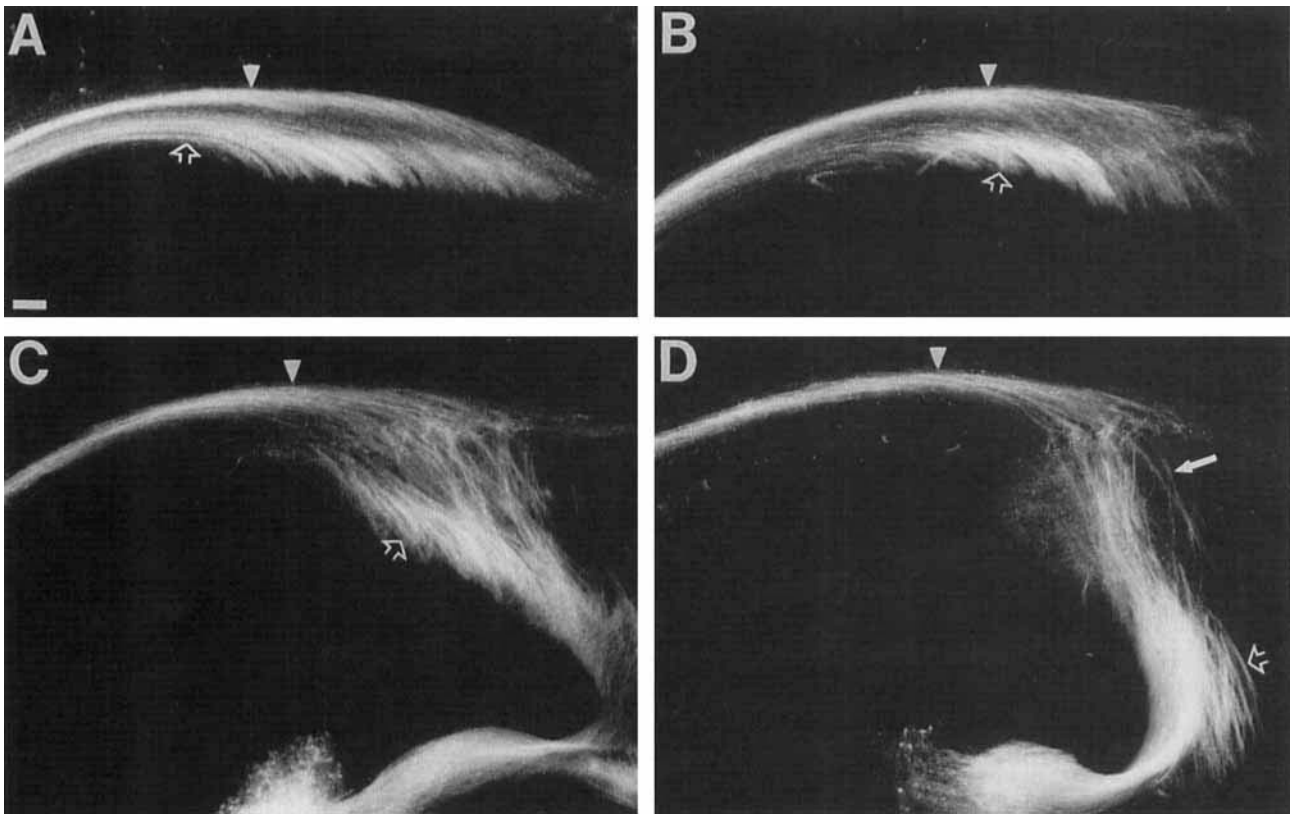

Fig. 10. **A–D:** Radial and tangential separation of thalamocortical and corticofugal pathways. These photomicrographs are from alternate coronal sections of a P6 hamster which received an injection of DiI into visual cortex. The thalamocortical and corticofugal pathways are labeled. The cortex is at the top and the internal capsule is to the bottom right. Lateral is up. The thalamus is in the bottom of the photos and retrogradely labeled cells can be seen in the visual thalamic nuclei in C and D. The figures are ordered from caudal (A) to rostral (D). The fiber pathways are visible traveling under the cortex and through the internal capsule. The thalamic pathway (arrowhead) is visible under the cortex in all the sections. The thalamic axons travel in small bundles under the cortex (see Fig. 12D) and in the internal capsule (small arrow in D). The corticofugal pathway (open arrow) travels under the cortex deep to the thalamic pathway. The corticofugal pathway, which travels in large bundles under the cortex and in the internal capsule (open arrow in C and D), is visible under the cortex in the caudal section A, gradually diminishes in B, and is no longer present under the cortex in C and D. In C the corticofugal axons are present only in the internal capsule. The corticofugal axons in D can be seen crossing the thalamic axons in the internal capsule in large bundles. A dorsal view of the relationship of the two pathways in this same animal is shown in Figure 11. Scale bar in A = 0.1 mm and applies to A–D.

segregation is seen developmentally. Figure 10 shows a P6 hamster with a DiI injection into the visual cortex. This injection labeled both thalamocortical and corticofugal projections. The thalamocortical axons travel through the white matter immediately under the subplate. The corticothalamic (and other corticofugal axons) travel deep in the white matter in large bundles (see also Fig. 8B). These two pathways are also separated for a considerable distance through the internal capsule; the corticofugal axons travel medial to the thalamic axons. As the pathways near the thalamus, corticofugal axons cross the thalamic axons in large bundles, and the corticothalamic axons turn toward the thalamus.

These two fiber systems are also physically separate during the early stages of development. Figure 6C-E shows an E14 animal that received an injection of DiI in the posterior thalamus and DiA in presumptive visual cortex. At this age no cortical projections have reached the thalamus (see above), thus the thalamic injection labeled only thalamocortical projections. A small number of thalamic axons are labeled by the cortical injection (curved black arrow in Fig. 6E), and cells retrogradely labeled with DiA were seen in the thalamus in the visual nuclei. Note that these injections closely matched reciprocally connected target areas; the DiI labeled thalamic fibers travel through the cortical DiA injection site (Fig. 6C), and cells in the posterior thalamus that were retrogradely labeled by the DiA were in the central region of the DiI injection site. The spatial separation under the cortex of the thalamic and corticofugal systems this early is clearly shown in Figure 6C. The thalamic fibers (red) travel in the upper intermediate zone and are distinctly separated from the corticofugal projections (yellow), which travel deep in the intermediate zone. These two fiber systems are also separate in the internal capsule (Fig. 6D,E). This demonstrates that the radial distinction between these two reciprocally connecting axonal systems seen in mature animals is present at the earliest stages of axonal development.

In addition to being radially separated, the two fibers systems are largely *tangentially* separated from one another as well. In a P6 animal with an injection into the posterior cortex, both pathways were traced in all sections where they appeared. As seen in Figure 10, the thalamocortical pathway is visible under the cortex in all the sections,

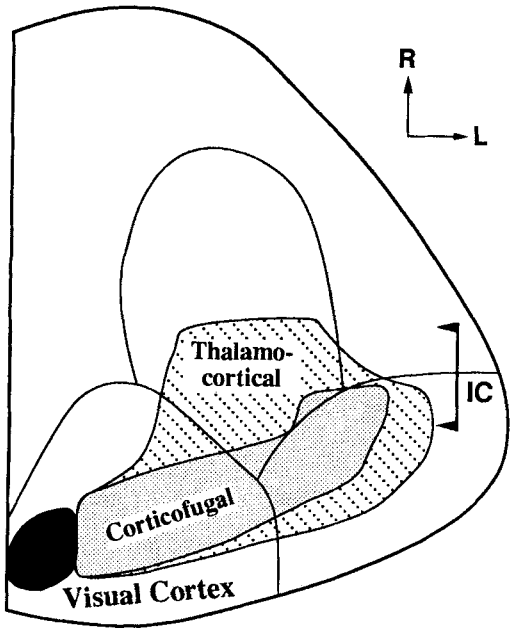

Fig. 11. Dorsal view drawing of the tangential relationship between thalamocortical and corticofugal pathways. This drawing was made from serial coronal sections from the same P6 animal as shown in Figure 10 with an injection (black region) of DiI into visual cortex. See Figure 3D′ for an explanation of the cortical map. The position of corticofugal (shaded) and thalamocortical (hatched) axons were plotted on a dorsal view of the brain. Both pathways travel rostrolateral to (from) where they traverse the internal capsule (IC). Note that the corticofugal pathway is tightly grouped while the thalamocortical pathway is widespread under the cortex.

while the corticofugal pathway is visible under the cortex in the caudal sections, but in the more rostral sections the corticofugal fibers are seen only in the internal capsule. Figure 11 shows a drawing of a dorsal view of the tangential routes that the two pathways take under the cortex from the injection site to the internal capsule. The corticofugal axons remain tightly grouped and travel directly toward the internal capsule region. The thalamocortical axons travel along this same route to the injection site. However, thalamic axons fan out and also travel under a much wider area of cortex as they head for their cortical target.

Features of the geniculocortical pathway

Axons from the LGd take a somewhat tortuous route from the thalamus to their target in the posterior cortex. Axons exit the nucleus by projecting ventromedially and enter the external medullary lamina between LGd and VB. The axons then turn ventrolaterally and travel slightly rostrally toward the internal capsule. The axons immediately form bundles as they travel in the external medullary lamina between the visual and somatosensory thalamic nuclei (Fig. 12A). Rarely, an axon will enter Pom and VB, and will exit the thalamus along with axons from these nuclei.

The topographic projection from LGd (Dursteler et al., '79; Sefton et al., '81) and VB (Caviness and Frost, '80) to the cortex is rotated 180° with respect to rostrocaudal and mediolateral orientation. Bernardo and Woolsey ('87) found that axons from the somatosensory thalamus (VB) rotated 180° just after exiting the reticular thalamic nucleus and before entering the internal capsule. The LGd axons behave in a similar manner. After passing through the thalamic reticular nucleus and prior to entering the internal capsule, the axons rotate about one another (not shown).

After their rotation the axons turn laterally and form a wide band of unbundled axons as they enter the internal capsule (Fig. 12B). In coronal sections the band of fibers take on the appearance of a ribbon, traveling parallel to one another with little crossing. About halfway through the internal capsule fascicles form again prior to reaching the bottom of the cortex (Fig. 12C), as do somatosensory thalamic axons (Bernardo and Woolsey, '87).

The thalamic axons make a sharp dorsal turn at the bottom of the cortex and then course caudally toward their target in posterior cortex, passing immediately subjacent to the cell dense cortical plate. Woodward and Coull ('84) and Woodward et al. ('90) suggested that thalamic fibers are not bundled, but the membrane dyes employed in the present study revealed that thalamic fibers do form small bundles (Fig. 12D). The axons remain bundled until reaching the target area, where they turn dorsally and penetrate the cortex.

Nearest neighbor relations of thalamic axons. Precise nearest neighbor relations of thalamic axons are not maintained in the internal capsule nor under the cortex. Most of the fibers travel in bundles and largely in parallel. However, bundles in the internal capsule do cross, and axons leave one fascicle and merge with another, as shown in an E14 animal in Figure 12C. Thus axons show from their first growth the conformation described by Bernardo and Woolsey ('87) for the somatosensory projection in the internal capsule of adult mice. The lack of nearest neighbor relations was also evident as thalamic axons coursed under the cortex. The small bundles cross one another, and axons leave and join bundles freely (Fig. 12D).

Tangential specificity of thalamocortical projections. Of special interest is the issue of how target specific are the thalamic fibers while growing toward and innervating their cortical target. Thalamic fibers were examined closely when the tracer injection was restricted to LGd. Several lines of evidence suggest that these axonal projections are highly specific in target identification and innervation. First, there was little evidence that the axons branched during their passage under lateral cortex (Fig. 13A; but see Ghosh and Shatz, '92). At P2, when LGd axons have started a deep penetration of visual cortex *en masse*, they do so only within a confined region, and the adjacent cortex is virtually free of axons (Fig. 13B). Thalamic axons appear to grow preferentially toward the caudal pole after passing through the internal capsule and were rarely if ever encountered in coronal sections that were rostral to the internal capsule.

However, there were problems associated with tracing the precise topographic specificity of axons after an injection into the thalamus. It was difficult to confine the injection to only LGd, and even when the injection did appear to be confined to LGd, labeled cells were often found in other thalamic nuclei, such as LP, MG, Pom, VB, or VL. Because these nuclei project to areas other than visual cortex, occasional axonal branches into nonvisual cortical areas could be attributed to the few axons of these cells. Moreover, it was frequently noted in very young animals that axons were seen growing beyond the probable boundaries of visual cortex under medial cortex, some of which were very close to hippocampal structures. Whether these axons originated from LGd or from other nuclei could not be determined. A second method for determining the target specificity of LGd projections not subject to these concerns

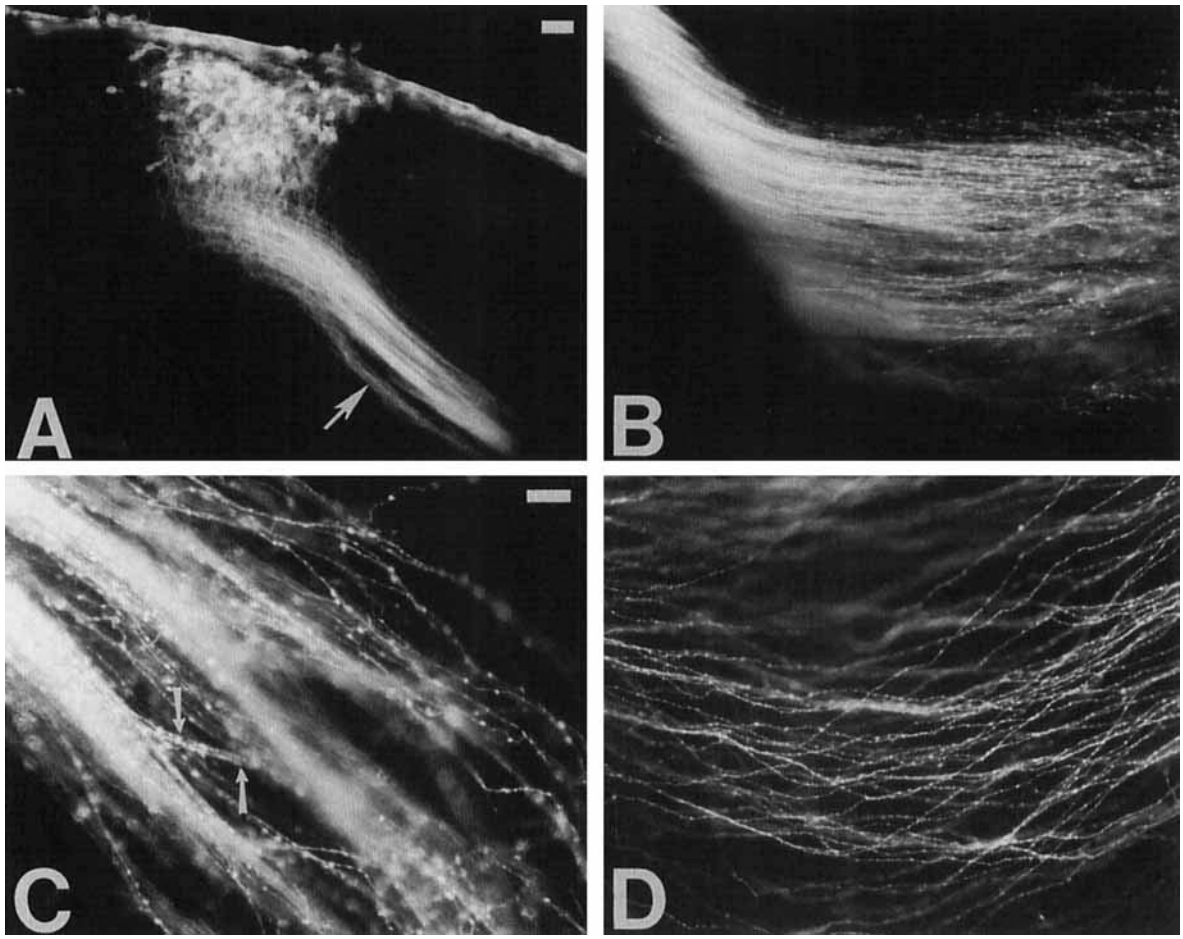

Fig. 12. Pathway of geniculocortical axons. In all of the following photomicrographs a small crystal of DiI was placed in the LGd. **A:** This figure (coronal section) shows axons leaving the LGd of a P0 animal. The axons exit the LGd medially and then immediately form bundles (arrow) as they turn ventrolaterally and pass through the external medullary lamina. Dorsal is up. **B:** These axons have just left the thalamus (to the left) and have entered the internal capsule (P1 animal). Note that they are no longer bundled and that they travel parallel to one another. As they approach the cortex they become less ordered as they begin to form fascicles. Coronal section. Dorsal is up. **C:** In this figure, a coronal section taken from an E14 animal, the axons are viewed in the internal capsule as they near the cortex (to the right). The fascicles travel more or less parallel, but it is common to see fascicles crossing one another. Note that the fibers will leave one fascicle and join another (arrows), thereby disrupting nearest neighbor relations between fibers. **D:** This photomicrograph is from a P6 animal in which the brain was sectioned tangentially, parallel to the thalamic pathway under the cortex. These are geniculate fibers passing under the lateral cortex on their way to the visual cortex. The internal capsule is to the left, and the visual cortex is to the right. Note that some of the fibers travel in small bundles of 2 or more fibers, and some are not associated with bundles. The fibers travel mostly parallel, but there are many instances of fibers (and small bundles) crossing one another. As in the internal capsule, axons will leave one bundle and join another. Thus even while the fibers travel underneath adjacent areas of cortex, they do not maintain nearest neighbor relations. Scale bar in A = 0.05 mm for A, C, and D; bar in B = 0.025 mm.

was to place the tracer into the nonvisual cortical regions and then examine LGd for the presence of retrogradely labeled cells.

Dye was placed in several regions of cortex in animals aged P0, P1, and P3. These regions included medial, frontal, and auditory cortex. LGd projects exclusively to visual cortex in adult animals (though on occasion it has been shown to make aberrant connections to nonvisual cortical regions; see Miller et al., '91b). The results are summarized in Figure 14A. In neonatal animals, a few cells in LGd project to regions of the cortex that they will not innervate in mature animals. In particular, retrogradely labeled cells were found in LGd after injections into medial and auditory cortex in animals aged from P0 to P3. Figure 6F shows a labeled cell in LGd after dye injection into medial cortex in a P1 animal. The number of labeled cells in LGd was very small, ranging from 2 to 14. Many nonvisual cortical injections did not result in label in LGd, thus even small, "exuberant" axonal projections are not ubiquitous. At P0 most of the injections into medial cortex resulted in LGd label, while label in LGd was found in only 2 of 6 of the P1 injections into medial cortex. This suggests that many of the exuberant projections to this area are lost by this time. The oldest age examined was P3, and at this age only one injection in nonvisual cortex resulted in retrograde label in LGd.

Exuberant projections from VB

In some animals with dye injections into the visual cortex, labeled cells were found in VB, an example of which is shown in Figure 15. VB projects exclusively to parietal cortex in adults. Visual cortex lies caudal to parietal cortex,

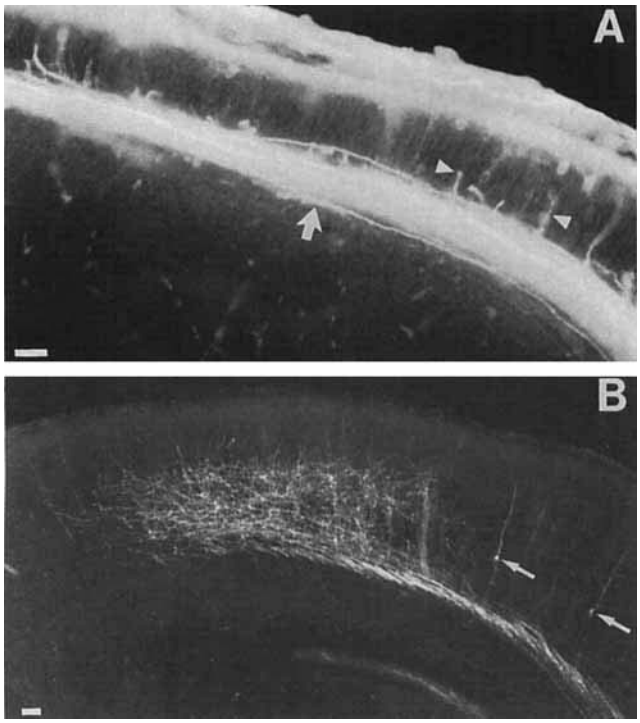

Fig. 13. Tangential specificity of developing thalamocortical projections. **A:** DiI labeled axons (arrow) passing through the subplate under lateral cortex (presumptive auditory cortex) after an injection into LGd in an E14 animal. The cortex lies above the axons. There is very little evidence of branching into the overlying cortex. The few branches (arrowheads) that are present have not penetrated beyond the cortical plate. Their target in visual cortex is to the left. **B:** Coronal section of visual cortex from a P2 hamster with DiI injected into the LGd. These thalamic axons grow into visual cortex within a well-defined region, while there are no axons in the cortex medial and lateral to them. The small arrows point to layer 6 cells. The location of layer 6 cells at this age is not immediately coincident with the ingrowing thalamic fibers. Medial is to the left. A higher power photomicrograph of this same section can be seen in Figure 4C,D. Scale bars = 0.05 mm.

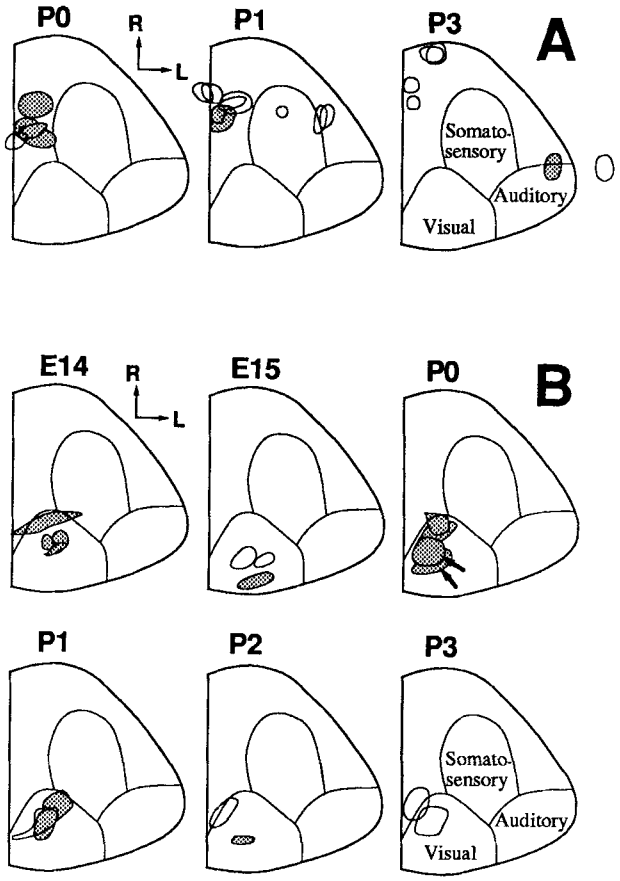

Fig. 14. Summary of visual and nonvisual cortex injections resulting in anomalous label in primary sensory thalamic nuclei. Cortical injections were located in the right and/or left hemisphere. They are all shown on a drawing of the right hemisphere for convenience. See Figure 3D' for an explanation of the cortical map. **A:** The location of the injections of dye into areas of nonvisual cortex. These animals were examined for the presence of retrogradely labeled cells in LGd. The shaded circles show the injections in which label was found in LGd. The unshaded circles are injections which did *not* result in retrograde label in LGd. The injection in the P3 animal that is outside of the drawing indicates an injection ventral to the rhinal fissure. **B:** This figure shows the location of the injections of dye into visual cortex in animals aged E14 to P3. These animals were examined for the presence of retrogradely labeled cells in VB, which projects exclusively to somatosensory cortex in adults. The shaded circles show the injections in which label was found in VB. The unshaded circles are injections which did not result in retrograde label in VB. The two arrows on the P0 map indicate cases where retrogradely labeled cells were found in MG.

and the axonal trajectory from VB to the somatosensory cortex is direct (Bernardo and Woolsey, '87). For VB axons to reach visual cortex they must grow beyond the bounds of their "intended" target into the caudal cortex. Figure 14B shows the locations of the injections of dye into presumptive visual cortex in animals aged E14 to P3. The injections which resulted in labeled cells in VB are indicated with shading. Labeled cells were found in VB with relative frequency in animals aged E14 to P2. The number of labeled cells in VB was small, ranging from 1 to 12. No retrogradely labeled cells were found in VB of animals with dye injections into visual cortex older than P2.

As an additional example, after an injection of dye into visual cortex, retrogradely labeled cells were sometimes found in MG, which projects to the temporal cortex. As with VB, the MG axons would have to grow medial and caudal past their intended target in lateral cortex to reach the visual cortex. The number of labeled cells in this nucleus was not studied systematically, like that in VB, but labeled cells were seen in two of the P0 animals with dye injections into the visual cortex. The number of labeled cells in MG was very small: 1 in one animal and 4 in the other. The location of these injection sites are marked with arrows in Figure 14B.

Connections of subplate cells

There were very few subplate cells labeled after thalamic injection of tracer (see Table 2). Moreover, subplate cells were not labeled in all animals with a thalamic injection. The functions and connections of these cells have received much attention in recent literature (De Carlos and O'Leary, '92; Friauf et al., '90; Ghosh et al., '90; McConnell et al., '89). The data from this study suggest that the subplate makes a very limited projection to the thalamus.

What other connections the subplate cells make was also examined. Many ipsilateral subplate cells are retrogradely labeled after cortical injections, close to and distant from the injection site. The number of subplate cells labeled after

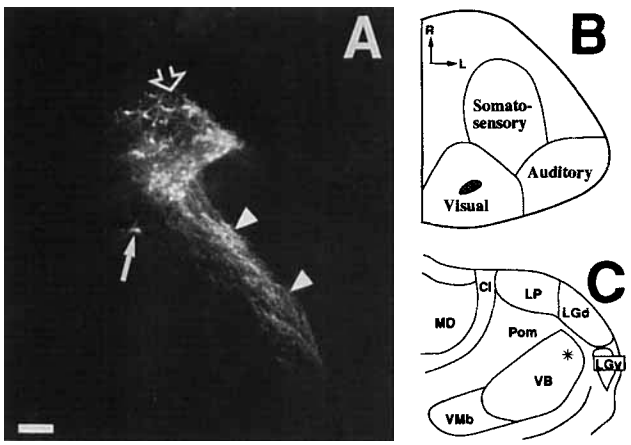

Fig. 15. Exuberant projections from somatosensory thalamic nucleus VB to visual cortex. **A:** This is a photomicrograph of a coronal section of the thalamus from an E14 hamster with DiI placed in visual cortex. The large cluster of retrogradely labeled cells (open arrow) is in LGd. Axons from these cells pass through the external medullary lamina (arrowheads) that separates LGd from VB. The small arrow marks a single retrogradely labeled cell in VB. Cells of VB project exclusively to the somatosensory cortex in adult animals, not to visual cortex. Dorsal is up, and lateral is to the right. **B:** The location of the DiI injection in visual cortex is shown by the shaded area in a dorsal view of the cerebral cortex. See Figure 3D' for an explanation of the cortical map. A drawing of the thalamus (**C**) represents the approximate level of the photomicrograph in A. The asterisk represents the approximate location of the retrogradely labeled cell in lateral VB as shown in A. See Abbreviations list. Scale bar in A = 0.1 mm.

cortical injections was not quantified, but they greatly outnumbered those projecting to the thalamus. Thus subplate cells are intimately involved in ipsilateral corticocortical connections with visual cortex.

Subplate cells have also been reported to make callosal connections (Chun et al., '87). Whether they project to the contralateral cortex in hamsters was not systematically studied here, but casual observation of some of the animals with cortical injections of tracer indicated that these cells do not project globally through the callosum. In a P1 animal dye was placed in the dorsomedial cortex to test whether LGd axons project beyond the bounds of LGd (see above). In one hemisphere two distinct lamina of cells are labeled (see Fig. 6G). The upper lamina consists of layer 5 cells that were labeled with DiI injected into the contralateral cortex. The lower lamina is subplate cells labeled with an ipsilateral injection of DiA. No subplate cells were labeled by the contralateral injection. Though the examination of callosal projections was not extensive in this study, no subplate cells were retrogradely labeled by injections into the contralateral hemisphere. In conclusion, subplate cells make a weak projection to the thalamus, a strong ipsilateral corticocortical projection, and perhaps no callosal projections in hamsters.

DISCUSSION

Summary

The relative timing of the ingrowth of thalamic afferents from LGd and the innervation of posterior thalamus by cortical efferents from visual cortex is summarized in Figure 16. The first thalamic fibers from LGd arrive at the visual cortex at E14 and begin to invade the developing cortical plate by the next day. The thalamic fibers progressively grow into the developing cortex and attain their mature laminar state by P7. By contrast, the first cortical efferents from visual cortex reach the posterior thalamus much later, at P1, and these projections are from a small number of layer 6 and subplate cells. Many layer 5 cell efferents reach the posterior thalamus at P3. The contribution of layer 6 cells continues to remain small until P7, when many of their axons enter LGd. Moreover, the layer 6 cells appear in the same tangential location as the ingrowing thalamic afferents.

This study has also shown that the pathways taken by topographically matching axons of the thalamocortical and corticothalamic fiber systems are spatially distinct, even at the earliest stages of development. Thalamic axons travel immediately subjacent to the developing cortical plate, while cortical axons travel deep in the intermediate zone well under the thalamic axons. The two fiber systems are tangentially separate as well. Thus, our evidence suggests that the thalamocortical and corticothalamic fiber systems, though making reciprocal, topographically precise connections, are temporally as well as physically segregated during development.

Do the thalamocortical and corticothalamic axonal systems act as guides for one another?

Developing thalamic and cortical projections must traverse a considerable distance and bypass many different structures during their growth toward their eventual target. The growing axon populations may select their pathways independently based on unique physical constraints, adhesion gradients, biochemical cues, and other recognition factors. Recent reports have identified molecules in the extracellular matrix of the subplate that may be involved in defining the pathway for growing thalamic afferents (Bicknese et al., '91; Sheppard et al., '91). Another possibility is that only one population of axons has this information and that other populations use the first as a guide. This study examined whether thalamocortical and corticothalamic cells, which make reciprocal connections, might use the other"s axonal processes as guides.

The relative timing of the development of the afferent and efferent fiber systems suggests that much of their initial axonal growth is independent of the other system. For instance, the thalamic axons traverse the pathway from the thalamus and enter the internal capsule during the same time that the cortical efferents grow through the intermediate zone toward the internal capsule. The growing tips of the thalamic and cortical axons pass through the internal capsule at the same time (and see De Carlos and O'Leary, '92; Erzurumlu and Jhaveri, '92), and this is the first opportunity for the two systems to interact. However, our evidence shows that axons from topographically matching populations are spatially segregated in much of the internal capsule, even at the earliest stages of development. Additionally, our data suggest that the thalamic fibers may not rely on corticofugal axons as guides as they grow under the developing cortex toward their target: Thalamic and cortical axons are both radially and tangentially segregated from one another under the cortex. These factors certainly restrict thalamic axons from following topographically matching cortical axons since their pathways are distinct.

The separation of reciprocally connecting axons of the two systems does not necessarily exclude interaction between thalamic and cortical projections at some level.

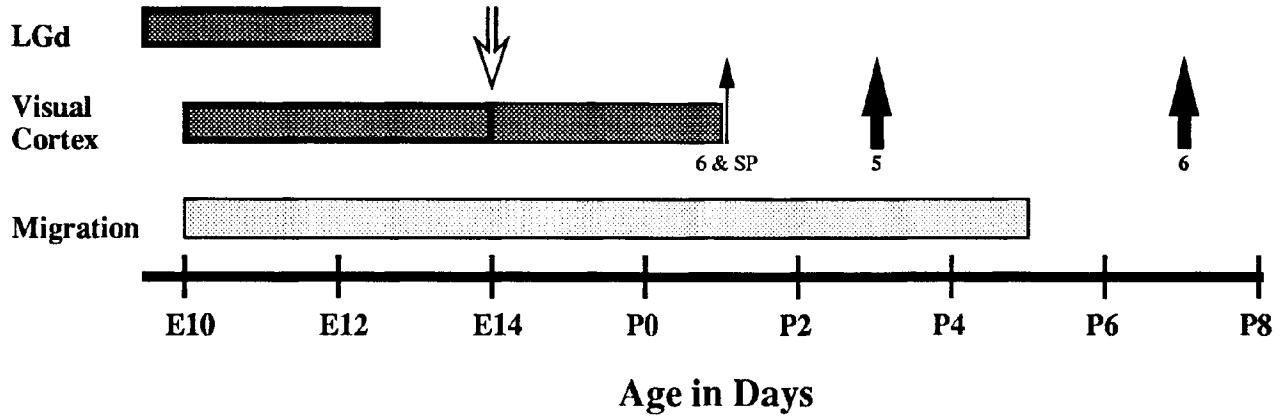

Fig. 16. Summary of the timing of neurogenesis and target innervation of the visual thalamic and cortical cell populations. The darkly shaded bars indicate the periods of neurogenesis of the dorsal lateral geniculate nucleus (LGd) and visual cortex. The heavily bordered regions indicate approximately when cells that make reciprocal connections from the LGd and the lower layers of cortex (subplate, layers 6 and 5) are generated (Crossland and Uchwat, '82). The lower bar indicates the approximate period of cortical cell migration (Shimada and Langman, '70). The outlined arrow indicates when thalamic LGd axons reach the visual cortex. The small black arrow indicates when subplate and layer 6 cell axons from the visual cortex first arrive at LGd in small numbers. The large black arrows indicate when large numbers of axons from layers 6 and 5 enter LGd.

Thalamic axons could follow a general cue characteristic of efferent axons from posterior cortex that grow toward the internal capsule, and then search for a specific identifier for their synaptic target. The apparent radial segregation could be produced by later arriving thalamic and cortical axons growing more superficial and deep, respectively, in the intermediate zone.

The thalamic axons travel immediately under the developing cortex, and they may be sampling the lower strata of the cortical plate for the correct recognition molecule. An experiment by Ghosh et al. ('90) suggested that thalamic axons could not find their normal target when subplate cells were ablated early in development. This would require that regional populations of subplate cells possess an identity unique for a specific brain region. No such factor has been identified yet, and the mode by which the thalamic fibers find their precise targets remains open to investigation.

The corticofugal axons cross the thalamic axons in the internal capsule. A portion of the corticofugal projection continues growing past the thalamus down the corticospinal tract, while corticothalamic (and corticotectal) axons turn and grow toward their targets. The spatial relationship of corticothalamic and thalamic axons near the thalamus is unknown, and the possibility that corticothalamic fibers follow thalamic axons to their target remains speculative.

"Waiting" period for thalamic axons

Previous research has shown that thalamic fibers do not grow into the developing cortical plate as soon as they arrive, but rather, collect in the intermediate zone and subplate until near the end of migration (Rakic, '79; Shatz and Luskin, '86; Wise et al., '77). In the cat, the thalamic fibers reside in the subplate for up to 2 weeks (Ghosh and Shatz, '92; Shatz and Luskin, '86) and are thought to interact with subplate cells (Friauf et al., '90; Herrmann et al., '91; Luskin and Shatz, '85b; Valverde and Facal-Valverde, '87) while they wait for their target cells to complete migration to the cortical plate.

The results of this study, and studies in rats (Catalano et al., '91; Reinoso and O'Leary, '90), shed some doubt on the existence of a waiting period for developing thalamocortical axons in rodents, because the thalamic axons begin to invade the developing cortical plate shortly after they arrive at their target. In hamsters, thalamic fibers first reach visual cortex at E14, when many of the cells that comprise their eventual target in layer 4 are generated (Crossland and Uchwat, '82). Thus, cells destined for layer 4 will migrate through the same axons that will soon innervate them. The thalamic axons begin to invade the cortex by the next day. It takes about 3 days for layer 4 cells to migrate to the top of the cortical plate (Lund and Mustari, '77; Shimada and Langman, '70), and by this time thalamic fibers have already penetrated into the bottom of layer 6. Within another 4 days (P5), around when cortical migration ends (Shimada and Langman, '70), the thalamic fibers have grown above layer 5, into the presumptive layer 4. These data show that thalamic fibers grow into the cortex shortly behind their target layer 4 cells.

The rapid ingrowth of thalamic fibers into the developing cortex does not preclude their interaction with subplate cells. The thalamic fibers are at the level of the subplate for 2–3 days (E14-P0). Whether thalamic axons synapse on subplate cells in rodents, as has been suggested in cats (Herrmann et al., '91), remains to be demonstrated.

The existence of a waiting period in cats (Ghosh and Shatz, '92; Shatz and Luskin, '86) and primates (Rakic, '79) may be evident because of a more protracted period of cortical development. For instance, neurogenesis and migration of cortical cells takes about 8—9 weeks in cats (Luskin and Shatz, '85a; Shatz and Luskin, '86) compared to 2 weeks in rodents (Crossland and Uchwat, '82). Of interest, a recent study of the wallaby (Sheng et al., '91), which has a duration of cortical development comparable to cats and allows a clear separation of the events related to thalamic ingrowth and cortical development, indicated little evidence of thalamic axons waiting in the region of the subplate. Thalamic axons grew progressively into the cortex, as described here for the hamster. Thus, the factors that influence the growth of thalamic axons out of the subplate and into the cortical plate (e.g., migration of layer 4 cells,

changes in trophic factors, electrical activity, etc.) require further investigation.

Cortical projections to the thalamus from the subplate

The organizational role of the subplate in cortical development is the subject of much current interest. McConnell et al. ('89) showed that in the cat the first population of cells in the cortex to send an axon to the thalamus was from the subplate. In cats the majority of subplate cells die, while in rodents many subplate cells survive and form a distinct layer at the bottom of the cortex (Bayer and Altman, '90; Reep and Goodwin, '88, [their layer 7]; Woo et al., '91). Subplate cells reportedly constitute the first axonal projection growing out of the cortex in rodents (De Carlos and O'Leary, '92). These cells may be the first to extend an axon into the internal capsule, but it is clear from the results of this study that they are not the first cells to contact the thalamus.

This study has shown that the first cortical axons to reach visual thalamic nuclei in hamsters are from both subplate and layer 6 cells. Thus cats and rodents differ in which is the first population of cortical cells to contact the thalamus. Part of this difference could be explained by the different timetables for cortical neurogenesis between these two species. Because of the relatively long period of neurogenesis in cats, subplate cells are produced during a 1 week period prior to the production of layer 6 cells (Luskin and Shatz, '85b). Thus the subplate cells begin axogenesis much earlier than layer 6 cells. In the hamster the entire cortex is produced in 1 week and there is considerable overlap in the production of cells from different lamina (Crossland and Uchwat, '82; Shimada and Langman, '70). Many subplate and layer 6 cells are produced on the same day (Woo et al., '91). The fact that so few subplate cells were labeled by the thalamic injections, and additionally, that these cells were not labeled in all animals (see Table 2), casts some doubt on the extent of the organizational or pioneer role of their axons for the corticothalamic pathway. Evidence from the present study (and see Friauf et al., '90; Reep and Goodwin, '88) suggests that these cells principally make ipsilateral corticocortical connections.

Topographic specificity of the thalamocortical projection

Previous descriptive studies have indicated that the majority of thalamic projections show a high degree of tangential specificity during development (Crandall and Caviness, '84; Dawson and Killackey, '85; Lent et al., '90; Parnavelas and Chatzissavidou, '81; Payne et al., '88; Rakic, '76; Van Eden, '86), and this study shows the same. For instance, geniculate axons did not branch significantly into lateral cortex as they grow past (but see Ghosh and Shatz, '92; Naegele et al., '88), and when the thalamic fibers begin large scale invasion of the cortex they grow into an area with well-defined borders. There have, however, been some indications that there is minor divergence in the initial thalamic projections (Crandall and Caviness, '84; Naegele et al., '88; Wise and Jones, '78), which includes transient ipsilateral (Bruce and Stein, '88) and bilateral projections (Laemle and Sharma, '86; Minciacchi and Granato, '89). We also found a small number of projections from LGd well beyond the borders of visual cortex and evidence that cells in VB occasionally grow past the bounds of parietal cortex into the region of visual cortex in early development (and see De Carlos et al., '92).

It is not known whether the few exuberant thalamic axons actually entered the foreign cortex or were restricted to the underlying white matter, as the precise depth of the injection was difficult to judge with the dyes used in this study. During development of another axonal system, the corpus callosum, exuberant projections are restricted to the white matter and are withdrawn prior to entering the cortex (Innocenti and Clarke, '84). Evidence from Ghosh and Shatz ('92) suggests that exuberant interstitial branches of LGn axons into nonvisual cortical areas do not grow past the subplate. Experimental studies have shown that transient callosal projections will enter the cortex if the thalamic input is disrupted, as in enucleation or thalamic damage (Cusick and Lund, '82; Innocenti and Frost, '79; Koralek and Killackey, '90; Lund et al., '84; Olavarria et al., '87; Rhoades and Dellacroce, '80; Rothblat and Hayes, '82). By contrast, thalamic projections do not invade foreign cortex after removal of the appropriate thalamic fibers. For instance, VB does not project to visual cortex of adult hamsters after removal of LGd at birth (Miller et al., '91b).

These studies showing thalamocortical specificity are interestingly at odds with two other studies showing much more global thalamocortical recognition. If cortex is transplanted to the midst of another cortical area, it receives the thalamic innervation of the host cortex and takes on its other properties (O'Leary, '89). In organotypic explants, thalamic axons show no preference for their normally appropriate cortex (Molnar and Blakemore, '91). These two contrasts may allow the identification of a changed recognition factor, or alternatively, might demonstrate the existence of a hierarchy of recognition factors that can be seen independently in different contexts.

Subcortical afferents and the formation of the primordial plexiform layer

Based on Golgi studies of the developing cerebral cortex, Marin-Padilla ('71, '78, '83, '88; Marin-Padilla and Marin-Padilla, '82) claimed that the arrival of corticipetal fibers trigger the onset of cortical development, in particular, the formation of the primordial plexiform layer (*preplate* of Sheppard et al., '91), which precedes formation of the cortical plate. These authors speculated that the first fibers originated from the thalamus, the mesencephalic tegmentum, or other lower levels of the neuraxis.

Several subcortical regions other than the thalamus make a direct projection to visual cortex (Dreher et al., '90). To determine the source of the first subcortical afferents to the developing cortex, we examined serial coronal sections through the entire brain from E14 to P3. The first nonthalamic projections to reach the visual cortex appear some time after birth and in small number. Most of the ages examined up to P1 had no retrogradely labeled cells in any subcortical structure other than the thalamus. A single cell was sometimes found the ipsilateral claustrum prior to P1, but this was rare. By P3 retrogradely labeled cells are found in a host of subcortical structures, such as the mesencephalic tegmentum, hypothalamus, claustrum, the horizontal band of Broca, and the medial septum (Miller, Chou, and Finlay, unpublished observations).

Our observations reveal that thalamic fibers are the first projections from a subcortical source to arrive at the developing visual cortex. The thalamic axons (from the visual nuclei LGd, LP, and L) arrive at E14, and this is 2

days after the formation of the primordial plexiform layer. Prior to the arrival of thalamic afferents at E14, the only axons present in visual cortex belong to local circuit neurons, probably subplate and Cajal-Retzius cells. Thus the initial stages of cortical development (i.e., the migration of cortical neurons, formation of the primordial plexiform layer and cortical plate) occur independent of subcortical afferents (and see De Carlos and O'Leary, '92; Erzurumlu and Jhaveri, '92).

Unusual features of the development of the layer 6 projection to the thalamus: Timing and topography

The innervation of the thalamus by corticothalamic projections is significantly delayed compared to when the thalamic axons reach the cortex, a phenomenon also observed in the wallaby (Sheng et al., '90, '91). This delayed growth emphasizes the different potential roles that these two systems play during development. By the time layer 6 corticothalamic axons begin to enter their target in the thalamus at P7, thalamic fibers have grown into the cortex and have restricted their topographic arbor to near the adult pattern (Naegele et al., '88). This suggests that some of the major features of thalamocortical projections (laminar and topographic specificity) develop independent of the corticothalamic system.

The relatively late arrival of a large number of corticothalamic projections beginning on P7 coincides closely with the report by Ramirez et al. ('90), who found that cortical axons reach the LGv at around P5. By contrast, a report by Blakemore and Molnar ('90) suggests that early visual cortical projections surround the LGd at about the same time that thalamic afferents reach the cortex. If this is the case then dye injections that penetrate past LGd into VB should label these corticothalamic axons. The results from the present study shed some doubt on this possibility since LGd injections that did include VB or the adjacent MG did not result in large numbers of retrogradely labeled layer 6 cells before P7. Furthermore, since our claim rests principally on animals in which double injections were made into the thalamus and cortex, errors arising from variations in developmental stage that might occur when comparing animals with separate injections cannot occur.

Since axons from both layers 5 and 6 project to the same target (i.e., the thalamus), one might reasonably predict that axons from the cells generated first would arrive at the target first. The results from the present study do not support this prediction completely. A few axons from layer 6 cells do arrive at the thalamus before axons from layer 5 cells; however, a large numbers of layer 5 axons enter the thalamus many days ahead of when layer 6 makes its second, strongest innervation of the thalamus. This is out of neurogenetic sequence because layer 6 is generated earlier than layer 5. A report from another species, the wallaby, also suggest that the layer 6 projection to the thalamus is delayed; projections from layers 5 and 6 arrive at the thalamus at the same time (Sheng et al., '91).

It is not clear that the layer 5 projections labeled at P3 actually arborize in the thalamus or are targeting the superior colliculus. Layer 5 cells in visual cortex project to LGv (Sefton et al., '81), and may project to other thalamic nuclei as well. Additionally, layer 5 efferents to the tectum pass through the thalamus (Lund, '66). Data from the present study does not resolve this issue, because the thalamic injection could have labeled fibers of passage from both of these pathways. Of interest, the rabbit cortical projection to the thalamus is delayed compared to the cortical projection to the superior colliculus (Distel and Hollander, '80).

A second unusual aspect of the second invasion of layer 6 efferent axons into the thalamus is the close topographic correspondence between the layer 6 cells and the reciprocally projecting thalamic afferents. Prior to P7 an occasional retrogradely labeled layer 6 cell is found scattered throughout posterior cortex, with no clear topographic relationship to where the thalamic axons have penetrated the cortex. At P7 this changes dramatically. A large number of layer 6 cell axons begin to invade the thalamus, and the location of these cells is always matched to the location where the thalamic fibers have entered the cortex.

The close topographic correspondence between the thalamic afferents and the reciprocally projecting layer 6 cells appears very similar to that seen in adult rat barrel cortex (Chmielowska et al., '89). This suggests that *the emergence of the corticothalamic projection is immediately topographically mapped at the outset.* If this is the case, then the development of corticothalamic projections differs significantly from the development of other cortical systems. Other cortical efferent systems (i.e., callosal, corticocortical, and corticofugal) develop their pattern of projections through the exuberance and subsequent elimination of axons. The initial callosal (Dehay et al., '84, '88; Feng and Brugge, '83; Innocenti, '81; Innocenti and Clarke, '84; Innocenti et al., '88; Ivy et al., '79; Ivy and Killackey, '81; Kretz and Rager, '92; Lent et al., '90; Mooney et al., '84; Olavarria and Van Sluyters, '85; O'Leary et al., '81; Sheng et al., '90; Wise and Jones, '76) ipsilateral corticocortical (Innocenti et al., '86; Ivy and Killackey, '82; Lent et al., '90) and corticofugal (corticotectal: Thong and Dreher, '86, '87; corticospinal: Bates and Killackey, '84; O'Leary and Stanfield, '85, '86; Stanfield and O'Leary, '85; Stanfield et al., '82) projections begin with exuberant projections from widespread tangential regions of the cortex, regions which will not contribute to the projection in adults. These extra projections are later pruned away and the adult pattern emerges. The only reported exception to this phenomenon is the development of the anterior commissural projection (Lent and Guimaraes, '90). We suggest that both of these features (delayed innervation and unusual specificity) may be integrated by considering the potential instructive role of the thalamus in the development of layer 6.

Role of the thalamus in cortical development

The cerebral cortex of adult mammals is composed of multiple, functionally heterogeneous regions characterized by a unique combination of cytoarchitecture, pattern of afferent and efferent connections, and cell number. The developing neocortex is more homogeneous with respect to these characteristics. How areal specificity is created during ontogeny remains a central issue.

Some features of cortical cell identity appear to be intrinsically fixed. The different neocortical areas share a common laminar structure with a similar distribution of cell types (i.e., granule and pyramidal cells are characteristic of certain layers). Transplant studies have shown that a cell''s laminar position is determined within a few hours after its generation, and these transplanted cells project to targets appropriate to their birth (reviewed in McConnell, '91).

There is evidence that some features of cortical cytoarchitecture are subject to thalamic influence. For instance, early removal of the eyes reduces the size of the lateral geniculate nucleus which in turn reduces the size of the primary visual cortex (Dehay et al., '89; Rakic, '88; Rakic et al., '91), suggesting that thalamic afferents define borders between cortical areas (see Killackey, '90 and O'Leary, '89 for more on the role of thalamic influence on cortical identity).

In particular, the effects of thalamic afferents are most exaggerated on the cytoarchitecture of layer 4. Between neocortical areas, there is more variation in the size and density of layer 4 than other layers (Beaulieu and Colonnier, '89), and layer 4 is the principle lamina for primary thalamic afferent arborization (Ferster and LeVay, '78; Naegele et al., '88). Damage to the thalamus prevents the normal characteristics of layer 4 to develop. Thalamic ablation prevents the formation of features characteristic of layer 4 of somatosensory cortex, the "barrels" which represent the peripheral whisker pad in rodents (Wise and Jones, '78). A recent report (Schlaggar and O'Leary, '91) showed that "barrels" can be induced in neonatal visual cortex if innervated by somatosensory thalamus. Even more dramatic is the loss of layer 4 cells after thalamic lesion (Windrem and Finlay, '91). In contrast to the effects of thalamic deafferentation, neonatal callosal transection does not alter cortical cell number or laminar differentiation (Windrem et al., '88). These results suggest that thalamic afferents directly control aspects of the development of the regional cytoarchitecture, especially the particular features of layer 4 granule cells.

Does the thalamus instruct the ingrowth of layer 6 efferents? The LGd and the lower layers of the cortex make reciprocal connections and are generated during the same time frame (Crossland and Uchwat, '82). Moreover, at E13, axons from both LGd and the visual cortex pass each other in the internal capsule. The distance remaining to reach their final target is roughly equal at this stage. Yet the thalamic axons require only one more day to reach the visual cortex, while the bulk of layer 6 axons do not reach the LGd for another 10 days.

It is clear that at least some layer 6 cell axons can reach the thalamus as early as P1. Why, then, do the majority of these axons wait another 6 days to join their cohorts in the thalamus? The delayed growth of layer 6 corticothalamic axons may reflect a functional distinction unique to this system. For instance, the layer 6 cells might be "waiting" for instructions from thalamic afferents. As discussed above, the thalamus may have a role in specifying the identity of a cortical region, in particular, characteristics of layer 4. It is possible that the thalamus also conveys the information necessary to instruct cortical efferent systems to make connections with specific targets. The possibility that the thalamic input instructs the layer 6 cells is strongly supported by the fact that the layer 6 efferents are topographically mapped as soon as they appear.

Thalamic input may also instruct other cortical efferent systems in their connections, but in a different manner, through the retention or loss of early exuberant connectivity. Thalamic axons arrive at their cortical target very early in development and quickly invade the developing cortical plate, and thus have an opportunity to affect the patterns of connections of axonal systems that arrive later. For instance, by comparing the thalamic and callosal systems, the potential influence of thalamic input on the connections of another axonal system can be demonstrated. In the hamster, thalamic axons arrive at the visual cortex at E14 and have grown to a mature laminar position by P7. By comparison, hamster callosal axons cross the midline at birth but do not begin to penetrate visual cortex until P8 (Lent et al., '90). If the thalamic input is perturbed, the pattern of callosal connections is changed. Removal of thalamic input during development, either by thalamic lesion (Cusick and Lund, '82; Koralek and Killackey, '90; Miller et al., '91b) or by peripheral damage (Cusick and Lund, '82; Dehay et al., '89; Innocenti and Frost, '79; Lund et al., '84; Olavarria et al. '87; Rhoades and Dellacroce, '80) causes a significant reorganization of the pattern of callosal innervation. It is unknown if thalamic damage alters the corticofugal projections of layer 5 efferents.

In summary, we propose the following set of hypotheses for the location of the information that directs local neocortical specialization, based on these data and the work of others cited above. The neocortex contains intrinsic information for cell class, lamination, and a recognition factor that thalamic axons recognize, located either in the subplate or its associate extracellular matrix. Axon wayfinding is controlled independently in each axon class, but the early-arriving thalamic input provides the information to select appropriate axon populations from the exuberant projections of layers 5 and 2–3, and to direct the termination of layer 6 corticothalamic axons. In addition, thalamocortical axons produce the direct trophic support of layer 4. Work to further test the critical role of the thalamus in neocortical specification is in progress.

ACKNOWLEDGMENTS

This work was supported by NIH grants RO1 NS19245 and KO4 to B. Finlay, and NIMH predoctoral fellowship T32 GM07469 to Brad Miller. We thank John Niederer and Lael Hinds for their excellent technical assistance in this project and Gerri Hachey-Hendershott for editorial assistance.

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