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Growth and Targeting of Subplate Axons and Establishment of Major Cortical Pathways

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In the developing mammalian neocortex, the first postmitotic neurons form the "preplate" superficial to the neuroepithelium. The preplate is later split into a marginal zone (layer 1) and subplate by cortical plate neurons that form layers 2–6. Cortical efferent axons from layers 5 and 6 and cortical afferent axons from thalamus pass between cortex and subcortical structures through the internal capsule. Here, we identify in rats the axonal populations that establish the internal capsule, and characterize the potential role of subplate axons in the development of cortical efferent and afferent projections.

The early growth of cortical efferent and afferent axons was studied using 1-1 -dioctodecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate (DiI) as an anterograde and retrograde tracer in aldehyde-fixed brains of embryonic rats. Cortical axons first enter the nascent internal capsule on embryonic day (E) 14 and originate from lateral and anterior cortex; axons from posterior cortex extend rostrally but do not yet exit cortex. The labeled axons, tipped by growth cones with complex morphologies, take a pathway deep to the preplate. Preplate neurons extend these early cortical efferents, based on the developmental stage of the cortex, and on their location and morphology. Most of these cells later occupy the subplate. Cortical plate neurons extend axons into the internal capsule by E16. En route to the internal capsule, cortical plate axons take the same path as the earlier-growing preplate axons, through the intermediate zone deep to subplate. Subplate axons reach thalamus by E16; the first cortical plate axons enter thalamus about a day later. Thalamic axons enter cortex by E16, prior to other cortical afferents. On E15, both preplate and thalamic axons reach the midpoint of the internal capsule.

To determine the subcortical distribution of subplate axons, we used DiI as a retrograde tracer in aldehyde-fixed brains and fast blue and rhodamine-B-isothiocyanate as *in vivo* retrograde markers in neonatal rats. Tracers were injected into the superior colliculus, the principal midbrain target of layer 5 neurons, at times before, during, and after the arrival of cortical axons, or into the subcortical pathway of primary layer 5 axons at two points, the cerebral peduncle caudal to the internal capsule, and the pyramidal decussation at the junction of the hindbrain and spinal cord, at times shortly after the passing of cortical axons. In every case, the labeled neurons are confined to layer 5; subplate neurons are not labeled. Injections that involve thalamus label a substantial number of layer 6 and subplate cells. Thus, the subcortical distribution of subplate axons is limited to the internal capsule and thalamus.

We conclude the following. In rats, preplate neurons send the first cortical axons into the internal capsule, a finding comparable to that of McConnell et al. (1989) in cats and ferrets. Thalamic axons coestablish the internal capsule with preplate axons, inferring that the initial outgrowth and targeting of thalamocortical axons is independent of the influence of cortical efferents. These two axonal populations may interact in the internal capsule, but their distinct pathways in cortex imply that thalamocortical axons do not track along subplate axons to reach the appropriate cortical area, although the possibility exists that subplate axons may follow thalamic axons to thalamus. Subplate axons may play a crucial, albeit limited, role in establishing the pathways of cortical efferents. The trajectory, targeting, and timing of outgrowth of subplate axons are consistent with the hypothesis that they pioneer the pathway of layer 5 and 6 axons through the internal capsule, and of layer 6 axons into the thalamus. However, the restricted subcortical distribution of subplate axons precludes a role for them in establishing the subcortical pathways of layer 5 axons beyond the internal capsule.

The mammalian neocortex has six main layers, which can be distinguished by differences in the morphology and density of the neurons that comprise them (Brodmann, 1909). The cells that come to populate the cortex arise from the neuroepithelium of the telencephalic vesicle. The first postmitotic neurons accumulate superficial to the neuroepithelium, immediately beneath the pial surface, forming the preplate. The preplate, as termed by Stewart and Pearlman (1987), has also been referred to as the primordial plexiform layer (Marin-Padilla, 1971, 1972) or as the pallial anlage (Rickmann et al., 1977). Although preplate cells have been characterized as neurons (Chun and Shatz, 1989), they are distinct from those that will populate the definitive cellular layers 2–6 of the adult cortex (Luskin and Shatz, 1985). Layers 2–6 emerge from the later-generated cortical plate. As cortical plate neurons become postmitotic, they migrate superficially and accumulate within the preplate, splitting it into a superficial marginal zone, which will become layer 1, and a deep subplate (Marin-Padilla, 1971; Kostovic and Molliver, 1974; Luskin and Shatz, 1985). A substantial proportion of subplate cells die later in cortical development in carnivores

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(Luskin and Shatz, 1985; Valverde and Facal-Valverde, 1987, 1988) and primates (Kostovic and Rakic, 1980); some survive in the adult white matter and are termed interstitial cells (Gilbert and Kelly, 1975; König et al., 1975, 1977; Giguere and Goldman-Rakic, 1988). A smaller proportion of subplate cells are lost in rodents (Woo et al., 1990), resulting in the persistence of a prominent cell layer often referred to as layer 6b or layer 7 in the mature cortex (Valverde et al., 1989).

Each layer that arises from the cortical plate is characterized by a unique combination of efferent (output), afferent (input), and intracortical (intrinsic) connections (Gilbert, 1983; Kemper and Galaburda, 1984). The laminar distribution of cortical projection neurons reflects this organizational scheme. Projections to subcortical structures arise only from layers 5 and 6: neurons in layer 6 send their axons to the thalamus; neurons in layer 5 project to multiple targets in the midbrain, hindbrain, and spinal cord (Jones, 1984). Axons that arise from layers 5 and 6 travel intracortically in the white matter and then exit cortex and extend subcortically by passing through an axonal pathway, termed the internal capsule, that forms in the basal telencephalon. Layer 6 axons extend through the internal capsule and directly into the thalamus. Layer 5 axons pass through the full extent of the internal capsule and extend into its continuation, the cerebral peduncle. The internal capsule serves as an axonal pathway not only for cortical efferents, but also for cortical afferents. For example, axons arising from the thalamus, the major source of cortical afferents, traverse the internal capsule to reach cortex.

It has been recently shown that in cats and ferrets subplate cells are the first cortical neurons to send axons into the nascent internal capsule (McConnell et al., 1989). In other neural systems, invertebrate (see, e.g., Klose and Bentley, 1989) and vertebrate (Lamborghini, 1987), early-developing populations of axons have been shown to pioneer axonal pathways in a way critical for the normal development of later-arising axonal projections. An intriguing hypothesis is that subplate axons also serve a crucial role in "pioneering" the pathways taken by the axons of cortical projection neurons to their subcortical targets, and by thalamocortical axons to the appropriate cortical area (McConnell et al., 1989; Shatz et al., 1990). Here, we characterize in rats the early extension of axons by developing subplate and cortical plate neurons, and present findings on the targeting of subplate axons and the establishment of the internal capsule. Our observations provide a framework with which to assess the possible roles of subplate axons in establishing the efferent and afferent projections of the cortex.

Preliminary accounts of this work have been previously presented (De Carlos and O'Leary, 1990, 1991).

Materials and Methods

A total of 120 rat embryos and pups obtained from timed-pregnant, Sprague–Dawley rats (Harlan) were used in this study. We define the day of insemination as embryonic day (E) 0 and the first 24 hr after birth as postnatal day (P) 0. Pups were born on E22.

The fluorescent lipophilic carbocyanine dye 1,1′-dioctodecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate (DiI; Honig and Hume, 1986, 1989) was used as a postmortem anterograde and retrograde axonal tracer (Godement et al., 1987) in 97 brains fixed with aldehydes between E13 and P1 (Table 1). Embryos were obtained by cesarean section from pregnant rats anesthetized with chloral hydrate (3.5 mg/10 gm body weight). The brains of E13 and E14 fetuses were fixed by immersion for several days in 10% formalin in 0.1 M phosphate buffer (pH 7.2). Rats ranging from E15 to P1 were perfused transcardially with the same fixative. Brains were dissected out and small DiI crystals were inserted into one of various sites in the cortex, basal telencephalon (internal capsule), thalamus, or superior colliculus, using the tip of a fine tungsten wire.

Table 1. Postmortem DiI labeling experiments in aldehyde-fixed brains

Age	Animals	Area
E13	2	AC
	2	PC
	2	LC
E14	3	AC
	2	PC
	2	LC
	2	ic
E15	3	PC
	11	AC
	5	Th
	2	ic
E16	7	AC
	4	PC
	6	ic
	2	Th
E17	4	ic
	3	AC
	3	PC
	4	Th
E18	3	PC
	3	AC
E19	1	AC
	2	PC
E20	5	AC
E21	6	AC
	1	ic
P0	3	AC
	1	ic
	1	SC
P1	2	SC

Age, age at time of aldehyde fixation. Area, brain structure in which DiI crystal was placed. Abbreviations: AC, anterior cortex; ic, internal capsule; LC, lateral cortex; PC, posterior cortex; SC, superior colliculus; Th, thalamus.

Four approaches were used to place DiI in the internal capsule, yielding comparable results: (1) a lateral approach through the ventrolateral part of the telencephalic vesicle, at the junction of basal telencephalon with neocortex—this placement assures the retrograde labeling of both cortical efferents and afferents present at this "decision point"; (2) a rostral approach after removing the frontal pole of the brain, leaving the rostral face of the internal capsule exposed; (3) a medial approach by cutting the brain at the midline; and (4) a dorsocaudal approach by excising dorsoposterior cortex, which reveals the dorsomedial aspect of the internal capsule protruding into the lateral ventricle. Three approaches were used to insert DiI crystals in the thalamus of E15–E17 fetuses: (1) a dorsal approach by cutting away the portion of cortex overlying the thalamus, (2) a medial approach by cutting the brain down the midline, and (3) a caudal approach by transecting the brainstem between the thalamus and superior colliculus.

After insertion of DiI crystals, the brains were stored in 1% formalin in 0.1 M phosphate buffer at 30°C for 1 week to several months, varying with the length of the pathway to be labeled and the age of the animal. Brains were cut at 100 μm in the coronal or sagittal plane on a vibratome. Sections were stored in buffer at 4°C. For examination, they were mounted on glass slides and viewed on a fluorescence microscope using rhodamine optics. Some sections were counterstained by a brief exposure to a 0.01% solution of bisbenzimide. In other sections, the DiI labeling was photoconverted to a brown, insoluble reaction product by fluorescing the section in the presence of diaminobenzidine (Sandell and

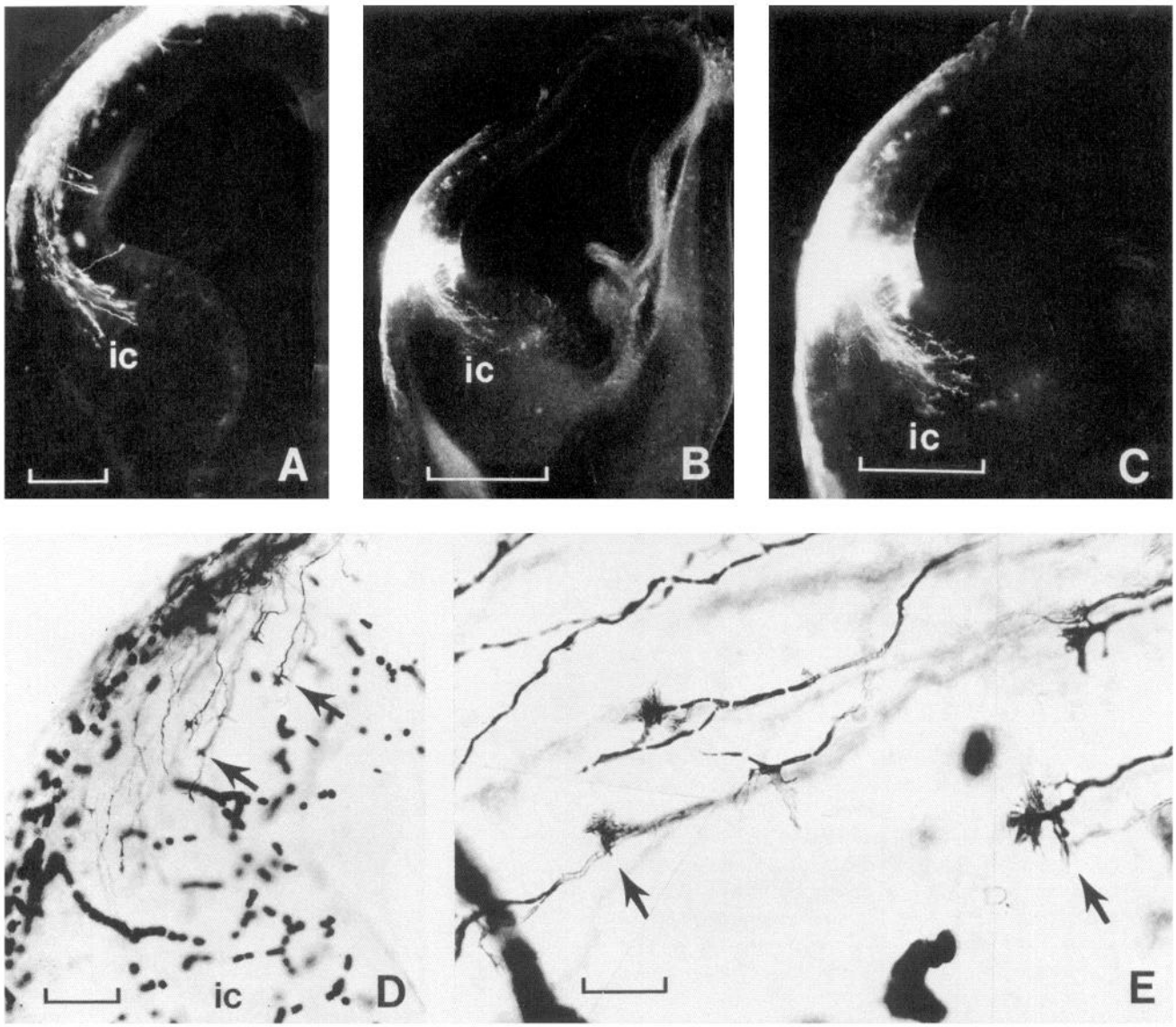
ic
A
ic
B
ic
C
ic
D
E
CAUDAL
PP
ne
BT
E14
ROSTRAL
F

Table 2. ***In vivo*** **retrograde labeling experiments**

Injection site	Injection/ death	Tracer	Animals	Cortical labeling
SC	P0/P1	RITC	1	None
SC	P1/P2	RITC	3	L5
SC	P2/P4	RITC	2	L5
SC	P0/P3	FB	3	None
SC	P2/P6	FB	3	L5
pd	P0/P1	RITC	2	L5
pd	P0/P3	FB	3	L5
pd	P2/P6	FB	3	L5
Th-SC	P0/P3	FB	2	L5, L6, SP
cp	P0/P3	FB	1	L5

Abbreviations. Injection site: cp, cerebral peduncle; pd, pyramidal decussation; SC, superior colliculus; Th-SC, thalamus, superior colliculus. Tracer: FB, fast blue; RITC, rhodamine-B-isothiocyanate. Cortical labeling: L, layer; SP, subplate.

Masland, 1988). These sections were then examined on a light microscope, and the photoconverted labeling was traced using a camera lucida attachment.

For *in vivo,* retrograde labeling experiments, we used 23 neonatal rats (Table 2). The animals were anesthetized with hypothermia, and a retrograde tracer was injected into one of a number of structures in the thalamus, midbrain, or hindbrain. The tracers used were a 2% solution of fast blue in distilled water (Bentivoglio et al., 1980) and 2% RITC in dimethylformamide (Thanos and Bonhoeffer, 1983). After a survival time, the rats were perfused with 10% formalin in 0.1 M phosphate buffer. The brains were removed, postfixed in the formalin solution with 10% sucrose, frozen, and sagittally sectioned at 40 μm on a sliding microtome. Sections were mounted on glass slides, air dried, and examined with a fluorescence microscope equipped with rhodamine (530–560 nm) and UV (340–380 nm) filter cubes to visualize the RITC and fast blue fluorescence, respectively. Selected sections were later counterstained for Nissl substance with thionin.

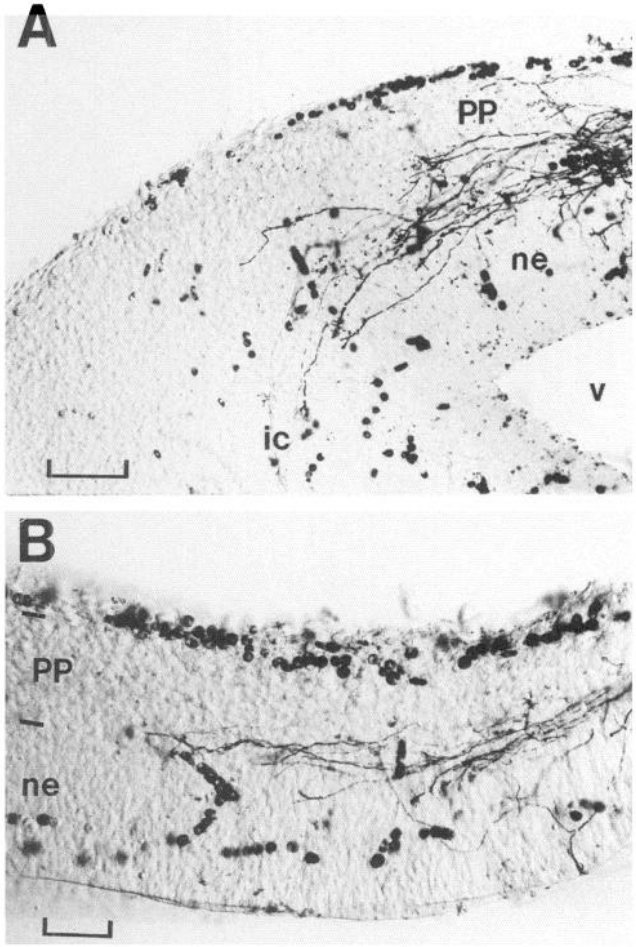

Figure 2. Trajectories of preplate axons in the cortical wall. Preplate axons are anterogradely labeled with DiI in E14 aldehyde-fixed brains, coronally sectioned, photoconverted, and photographed under Nomarski optics. The DiI labeling site in *A* was in the dorsolateral wall of anterior cortex, and in *B* was caudal to this site. In *A,* the leading axons have entered the internal capsule (*ic*), while in *B* the axons have yet to reach it. Virtually all of the labeled preplate axons take a trajectory deep to the preplate (*PP*) as they extend toward the nascent internal capsule; in *A* a few axons extend for shorter distances in the preplate. With the exception of the photoconverted erythrocytes, no labeled cells are present. Lateral is to the top, ventral to the left. *ne,* neuroepithelium; *v,* lateral ventricle. Scale bars, 100 μm.

Results

Early extension of corticofugal axons

We have used the technique of postmortem DiI labeling in aldehyde-fixed brains (Godement et al., 1987; Honig and Hume, 1989) to study the early development of axonal pathways to and from the fetal rat cortex (Table 1). In the rat, the first cortical neurons are generated on E12 (Valverde et al., 1989; Bayer and Altman, 1990); therefore, we chose to initiate our study with E13 fetuses. Even at this early age, axons extend from the DiI site toward the basal telencephalon, the structure in which the internal capsule will form. The labeled axons are short, though, and none leave the cortex. Axons anterogradely labeled from cortical DiI sites first enter the basal telencephalon on E14. In these cases, no afferent axons arising subcortically are labeled retrogradely or anterogradely. The first axons to leave cortex are labeled from anterior (presumptive sensorimotor cortex; Fig. 1*A*) or lateral parts (lateral cortex; Fig. 1*B,C*) of the telencephalic vesicle. Axons labeled at E14 from more posterior cortex (presumptive visual cortex) extend rostrally but do not enter the basal telencephalon. At this age, the cerebral wall is simple. The most prominent structure is the neuroepithelium, a zone of densely packed, radially oriented cells that occupies most of the cortical thickness, from the ventricle to near the pial surface. A narrow cell layer, the preplate, characterized by horizontally

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Figure 1. First axons leave cortex and enter the nascent internal capsule on E14. Axons are anterogradely labeled with DiI in rat brains fixed with aldehydes on E14 and later coronally sectioned. Axons labeled from anterior (*A*) and ventrolateral (*B*) cortex enter the internal capsule (*ic*). *C* is a higher-magnification view of *B*. *D–F* are from a case with DiI placed more dorsally in lateral cortex. The sections were photoconverted in the presence of diaminobenzidine. *F* shows camera lucida drawings of serial sections; the site of the DiI crystal is *shaded.* Most of the labeled axons grow deep to the preplate (*PP*) and extend through the upper part of the neuroepithelium (*ne*) toward the nascent internal capsule, which develops in the basal telencephalon (*BT*). *E* is a higher-magnification view of the same section in *D* to show the elaborate growth cones that tip the labeled axons (*arrows*). The *labeled oval structures* in *D* and *E* are erythrocytes; they contain endogenous peroxidase and generate reaction product during the photoconversion process. In each panel, lateral is to the left, dorsal to the top. Scale bars: *A–C,* 250 μm; *D,* 100 μm; *E,* 25 μm.

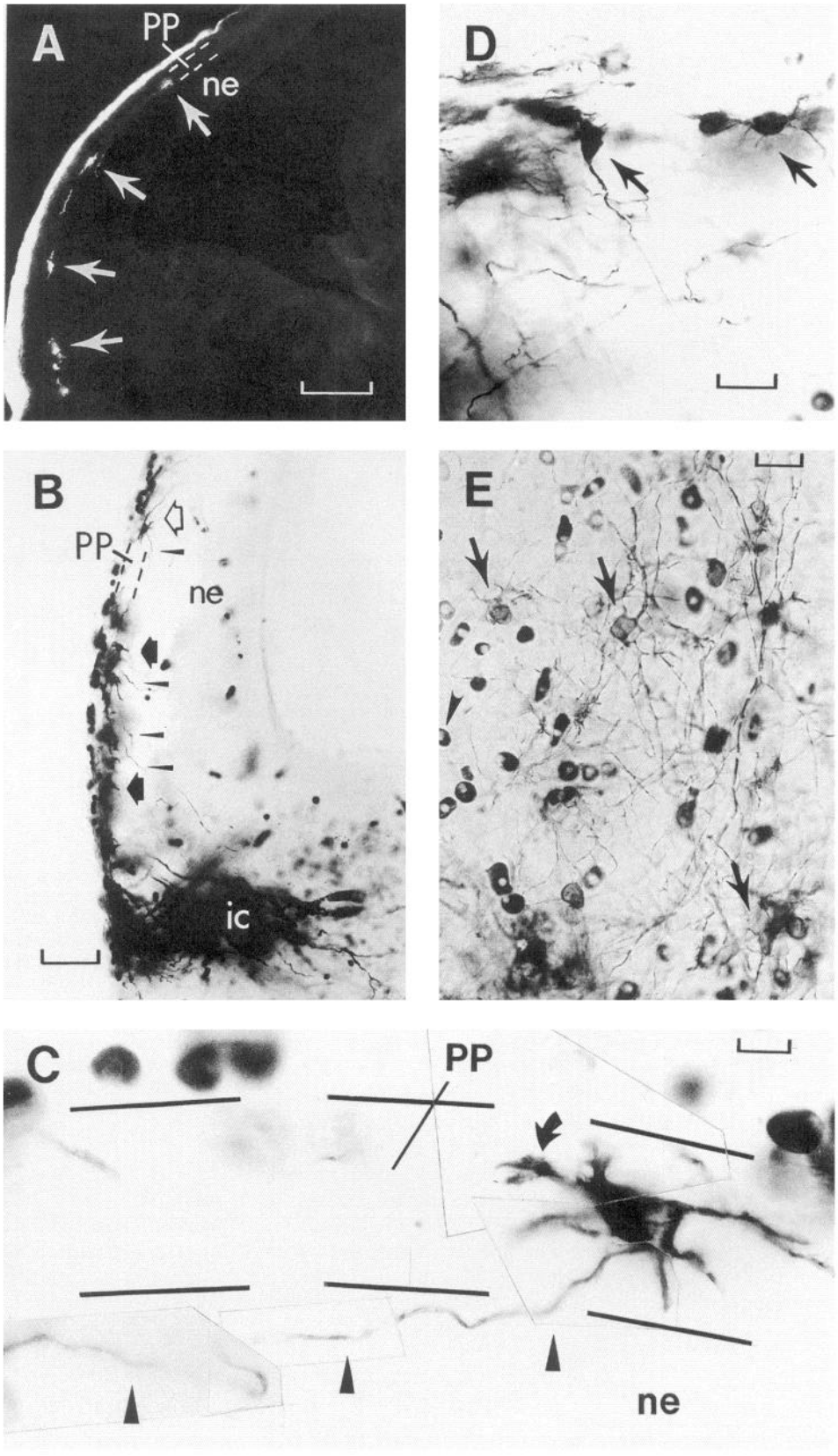


Figure 3. Preplate neurons send the first cortical axons into the internal capsule. Retrograde DiI labeling from lateral cortex (*A*) and the internal capsule (*ic*) (*B*) in rat brains aldehyde-fixed on E14 and later coronally sectioned. Lateral is to the left, dorsal to the top. *B–E* are photoconverted. In each panel, the *solid arrows* point to retrogradely labeled cells located in the preplate (*PP*) between the pial surface and the neuroepithelium (*ne*). The morphologies of the retrogradely labeled preplate cells are shown at higher magnifications in coronal sections (*C, D*) and a section tangential to the pial surface (*E*). In *B* and *C*, the *arrowheads* point to retrogradely labeled axons that leave the preplate and extend through the DiI labeling site. The retrogradely labeled neuron marked with an *open arrow* in *B* is shown at a higher magnification in *C*. The *arrowheads* mark its axon that travels deep to the preplate. This neuron has short dendrites tipped with growth cone–like expansions (*curved arrow*). In *E*, the *arrowhead* points to a photoconverted erythrocyte. Scale bars: *A*, 250 μm; *B*, 100 μm; *C*, 10 μm; *D* and *E*, 25 μm.

disposed cells, is situated between the neuroepithelium and the pial surface. Virtually all axons labeled at this stage extend deep to the preplate and course through the more superficial parts of the neuroepithelium (Figs. 1*D,F*; 2*A,B*), which at slightly later ages will become recognizable as the intermediate zone. The labeled axons are tipped with complex growth cones of a wide range of morphologies (Fig. 1*E*). On occasion, a few axons labeled on E14 course through the preplate or just beneath the

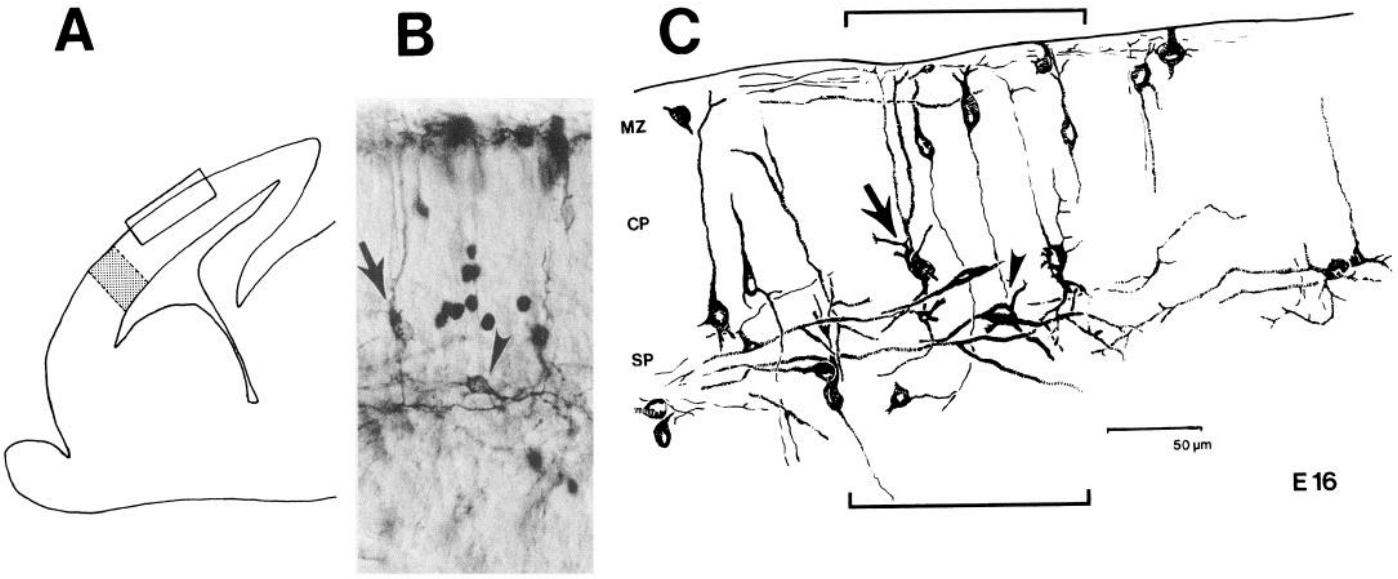


Figure 4. Intracortical extension of axons by neurons of the cortical plate (*CP*), marginal zone (*MZ*), and subplate (*SP*). *A* shows the DiI labeling site in rostral cortex (*shaded area*) of a brain aldehyde fixed on E16, and a *box* that corresponds to the location of the camera lucida drawing in *C*. *B* is of a photoconverted section to show the retrogradely labeled cells in the region bracketed in *C*, which depicts the laminar disposition and morphologies of the cells that send axons rostrally through the injection site. In *B* and *C*, the *arrow* marks a labeled neuron in the cortical plate, and the *arrowhead*, one in the subplate. Rostral is to the left.

pial surface (Fig. 2*A*); these axons do not extend as far as those taking a deeper trajectory.

The great majority of the axons labeled on E14 grow toward the basal telencephalon. At the level of the basal telencephalon, the axons turn and exit the cortex. Only occasionally do axons leave the preplate and extend medially toward the midline (Fig. 1*F*); such axons originate from DiI sites in more dorsomedial cortex and are not seen to arise from more lateral DiI sites. These observations are consistent with our finding that DiI placements in dorsolateral parts of the E14 telencephalic vesicle do not retrogradely label cells at points between the DiI sites and the internal capsule (Figs. 1*F*, 2), with the exception of some cells close to the DiI sites that may be labeled via horizontally disposed dendrites. These findings also infer that preplate neurons extend dendritic processes for only short distances at this early stage (also see below).

At E14, then, DiI placed in anterior or lateral cortex anteretrogradely labels axons that extend from the DiI site through the superficial neuroepithelium and into the basal telencephalon. Retrogradely labeled axons also emerge from the same DiI sites and can be followed through the superficial neuroepithelium and into the preplate to their parent cells located just below the pial surface (Fig. 3*A*). Similarly, DiI placed in the basal telencephalon at E14 retrogradely labels cells distributed in the preplate (Fig. 3*B*). The retrogradely labeled cells are usually multipolar with ovoid or stellate bodies or, occasionally, bipolar with fusiform bodies (Fig. 3*C–E*). At this stage, the dendrites of many of the labeled cells are short and often tipped with growth cone–like expansions (Fig. 3*C*). This observation, together with our ability to follow the axons of the labeled cells as they leave the preplate and grow to the DiI site, indicates that these neurons are indeed retrogradely labeled via their axons that extend into the DiI site in the nascent internal capsule. Therefore, based on their location and morphologies, the cortical cells that extend the first axons into the basal telencephalon are preplate neurons. This conclusion is supported by the finding that the generation of cortical plate neurons (Bayer and Altman, 1990) does not begin until after we observe axon extension by neurons of the preplate.

On E16, the cortical plate is evident in anterior and lateral parts of the telencephalic vesicle, and splits the preplate into a deep subplate and a superficial marginal zone. DiI placed in anterior cortex at E16 retrogradely labels cells in all three layers: subplate, cortical plate, and marginal zone (Fig. 4). The labeled cells typically have morphologies characteristic of the layer in which they are found. In the marginal zone, the labeled cells tend to be horizontally disposed and polymorphic, as are Cajal-Retzius cells, the neuronal type that sparsely populates layer 1 in adult cortex. Below this stratum lies the cortical plate in which the labeled cells are radially aligned with pyriform-shaped cell bodies and tall, superficially directed, apical dendrites, resembling immature pyramidal neurons. Some cells, though, are polymorphic and may be preplate cells that have been incompletely displaced to the underlying subplate. Within the subplate, the labeled cells have irregular shapes and are mainly in a horizontal disposition. A few radially aligned cells with more simple morphologies are also labeled. These may be cortical plate neurons that have extended an axon rostrally while migrating through the subplate, but this is uncertain. At E14, then, preplate cells send axons into the nascent internal capsule, and by E16, cortical plate neurons extend axons some distance within the cortex.

To determine the age at which cortical plate neurons extend axons corticofugally through the internal capsule, we placed DiI into the basal telencephalon in a series of aldehyde-fixed brains from E15 and later embryos. Although retrogradely labeled cells are found in E15 cases, we could not distinguish the nascent cortical plate with confidence. At E16, cells retrogradely labeled from the basal telencephalon are found in anterior and lateral cortex (Fig. 5). The number of labeled cells progressively de-

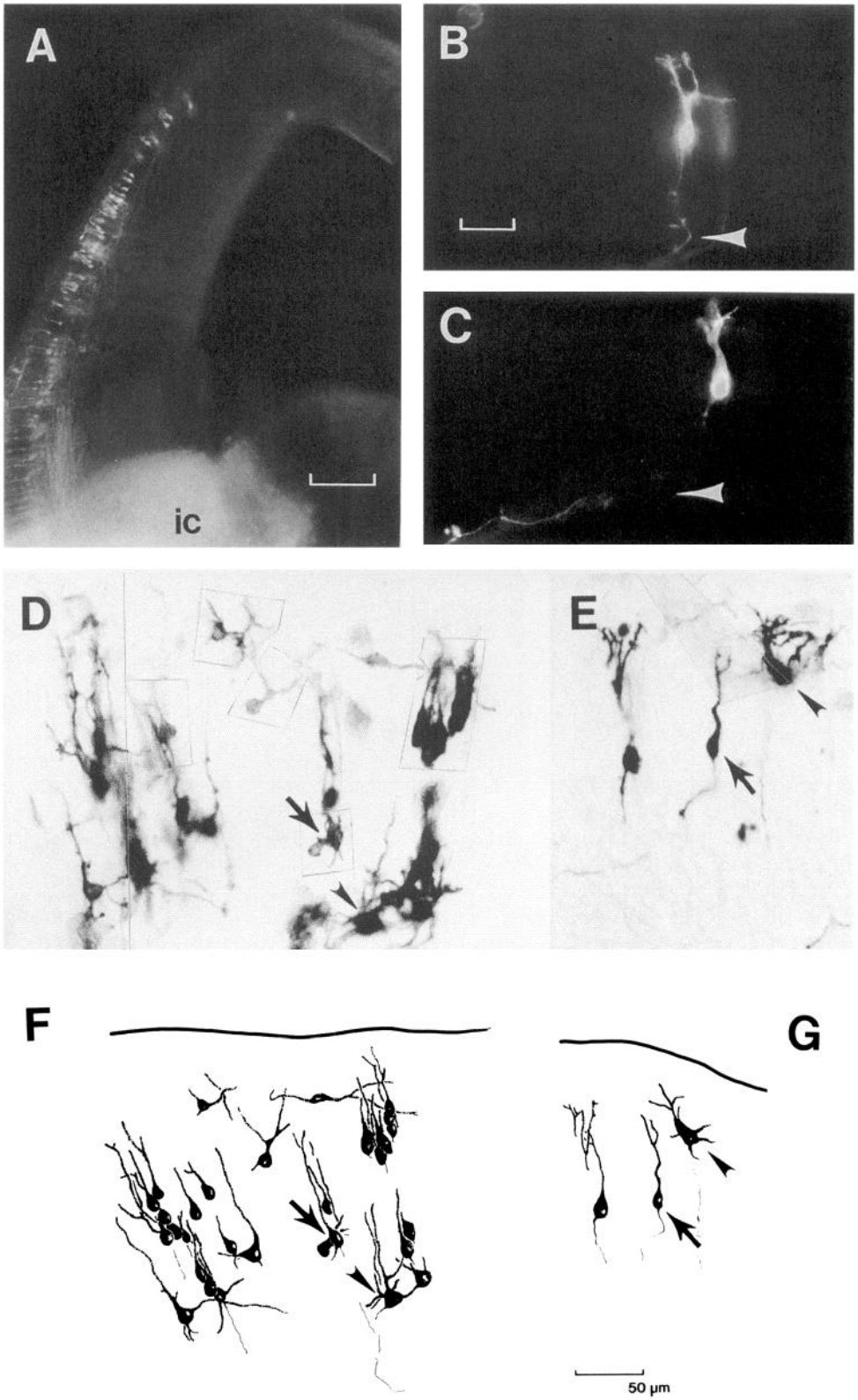


Figure 5. Extension of cortical plate axons into the internal capsule on E16. *A* is a coronal section to show distribution of cells retrogradely labeled with DiI placed in the internal capsule (*ic*) of a brain aldehyde fixed on E16. In *A*, lateral is to the left, dorsal to the top. In the other panels, lateral is to the top, ventral to the left. *B* and *C* show pyriform morphology of some projecting cells near the dorsal aspect of cortex. Their axons (*arrowheads*) grow deep and turn to extend toward the internal capsule through the upper part of the neuroepithelium. *D* and *E* are regions of a photoconverted section adjacent to that in *A* to show the distribution and morphologies of the retrogradely labeled cells. *F* and *G* are camera lucida drawings of the same cells labeled in *D* and *E*. In *D–G*, the *arrows* mark labeled cells in the cortical plate. The *arrowheads* in *D* and *F* mark a labeled subplate cell, and in *E* and *G*, a labeled cell in the marginal zone. Scale bars: *A*, 250 μm; *B*, *C*, 25 μm.

creases dorsally and caudally; none are found in medial or posterior cortex. In the anterior and lateral regions of the telencephalic vesicle in which the cortical plate has emerged, labeled cells are found in the subplate and occasionally in the marginal zone. In addition, cells with the slender, radial morphologies of immature pyramidal neurons are labeled in the cortical plate. As the cortical plate diminishes dorsally and caudally, leaving only the preplate, the band of labeling also decreases in width and density of labeled cells. In the more dorsal aspect of lateral cortex, at a region where the cortical plate cannot be discerned, some of the labeled cells have a stout, radial appearance with a short, thick apical process that has a branched or tufted ending

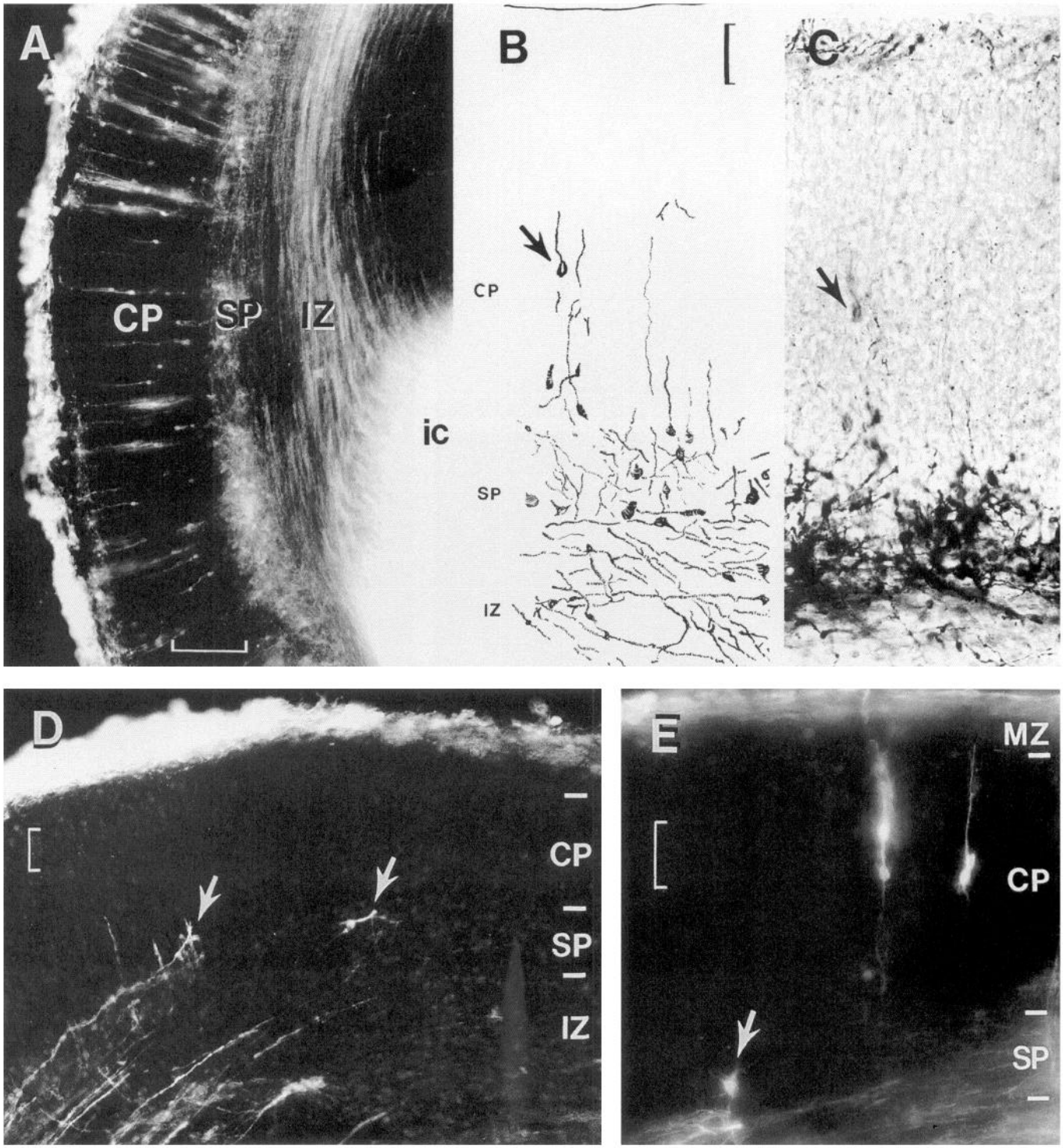


Figure 6. Extension of cortical plate and subplate axons subcortically on E17: retrograde DiI labeling in brains aldehyde fixed on E17 and later coronally sectioned. *A* shows labeling from internal capsule (*ic*). Radially oriented, retrogradely labeled cells are found in the cortical plate (*CP*). The dense labeling in the subplate (*SP*) is due to the retrograde labeling of cells in this layer. The axons of these efferent cortical plate and subplate neurons are retrogradely labeled, revealing their pathway through the intermediate zone (*IZ*) deep to the subplate. In *A*, lateral is to the left, dorsal to the top. In the other panels, lateral is to the top. *B* and *C* are a camera lucida drawing and photo of a photoconverted section from a different E17 animal in which a much smaller DiI crystal was placed in the internal capsule. Again, the retrogradely labeled subplate cells form a dense network in the subplate layer, but fewer cortical plate cells (*arrow*) are labeled. *D* and *E* are from a case in which DiI was placed in dorsal thalamus. In posterior cortex (*D*), only subplate cells are retrogradely labeled (*arrows*), whereas in anterior cortex (*E*) both SP (*arrow*) and *CP* neurons are labeled. Scale bars: *A*, 100 μm; *B* and *C*, 10 μm; *D* and *E*, 50 μm.

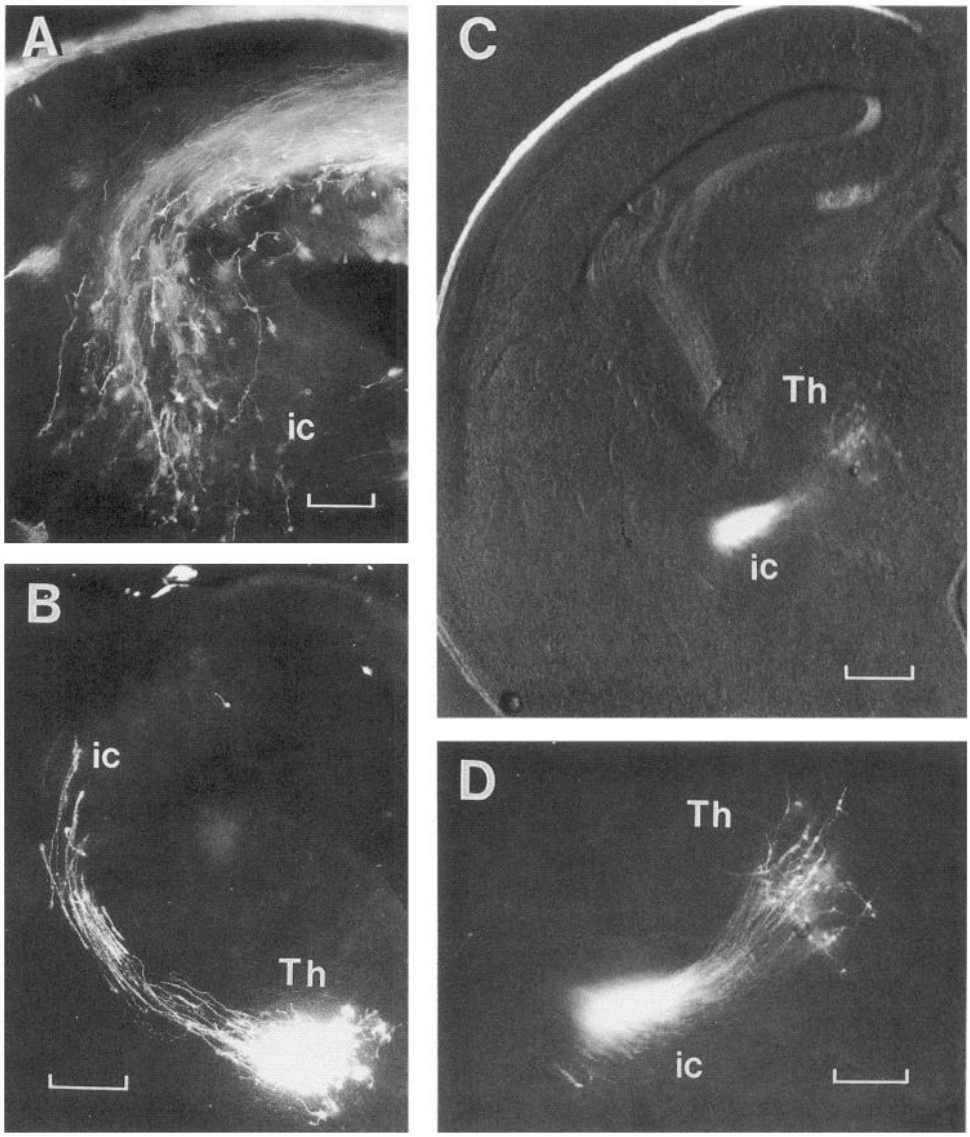


Figure 7. Subplate and thalamic axons coestablish the internal capsule: DiI labeling in aldehyde-fixed brains, coronally sectioned. *A* shows anterograde DiI labeling from anterior cortex on E15. Leading axons have reached the midpoint of the internal capsule (*ic*). *B* shows anterograde DiI labeling from dorsal thalamus (*Th*) on E15. Leading axons have also extended about halfway through the internal capsule. *C* shows thalamic cells retrogradely labeled with DiI placed in anterior cortex on E16. *D* is a higher-magnification view of the labeling in *C*. In *A* and *B*, lateral is to the top, ventral to the left. In *C* and *D*, lateral is to the left, dorsal to the top. Scale bars: *A*, *B*, and *D*, 250 μm; *C*, 500 μm.

(Fig. 5*B,C*). This morphology closely resembles that of preplate cells labeled from the internal capsule in cats and ferrets (see Fig. 7 of Shatz et al., 1990).

On E17, the cortical plate is wider and well defined over the entire cortical wall. At this age, large DiI sites confined to the basal telencephalon retrogradely label substantial numbers of cells in both the subplate and the cortical plate (Fig. 6*A*). The density of labeled cells in the subplate is high, while that in the cortical plate is much lower (Fig. 6*B,C*). Small DiI sites in the basal telencephalon also retrogradely label subplate and cortical plate cells, but in fewer numbers. Therefore, cortical plate cells in anterior and lateral cortex send axons into the internal capsule by E16; their numbers and distribution increase greatly by E17. As the number of cortical efferent axons increases, the intermediate zone becomes distinct. At E17, and in anterior and lateral parts of the telencephalic vesicle at E16, the retrogradely labeled axons of subplate and cortical plate cells form a dense pathway above the neuroepithelium and below the subplate (Figs. 5*A*, 6*A*). Corticofugal axons extended by cortical plate neurons initially grow deep, continuing through the subplate and into the intermediate zone, and then turn abruptly to grow toward the internal capsule. This deep trajectory is similar to that taken earlier by preplate neurons, most of which become displaced into the subplate.

DiI placed into the thalamus retrogradely labels cortical cells as early as E17 (Fig. 6*D,E*). A small number of labeled subplate cells are widely distributed in cortex. A few cortical plate neurons are also labeled but, at this age, are limited to more anterior and lateral regions of the cortex. These findings show that subplate axons extend into the thalamus and suggest that they precede corticothalamic axons arising from layer 6.

Preplate and thalamic axons coestablish the internal capsule

Corticofugal axons first enter the nascent internal capsule on E14 and arise from preplate neurons in anterior and lateral parts of the telencephalic vesicle. DiI placements in anterior or lateral cortex on E15 label preplate axons that have extended to a point about midway through the basal telencephalon (Fig. 7*A*). Such DiI placements, as well as DiI placed on E15 laterally in the basal telencephalon at the interface between the nascent internal capsule and the cortex, only label corticofugal axons. However, anterior or lateral cortical DiI placements in E16 brains do

retrogradely label thalamocortical axons and their parent neurons (Fig. 7*C,D*), showing that thalamic axons have passed through the internal capsule and into the cortex by this time. Therefore, thalamic axons must enter the internal capsule prior to E16. DiI placed into the developing thalamus of brains fixed on E15 labels thalamocortical axons, tipped with growth cones, that extend well into the nascent internal capsule, reaching a point about midway through the basal telencephalon (Fig. 7*B*). These findings show that both corticofugal and thalamocortical axons are extending through the basal telencephalon on E15 and that, at some time on this day, they pass by each other in this structure. Thus, preplate and thalamic axons coestablish the internal capsule.

Subcortical distribution of subplate axons

Axons arising from cortical efferent neurons in layers 5 and 6 pass through the internal capsule as they project subcortically (Fig. 8). In adult rats, layer 6 axons extend from the internal capsule and directly into the thalamus, while layer 5 axons traverse the internal capsule and continue into its postthalamic extension, the cerebral peduncle (Fig. 8) in the midbrain and pons, the pyramidal tract in the medulla, and the dorsal funiculus in the spinal cord. During late fetal and early postnatal development, layer 5 axons extend collateral branches to their brainstem targets found at various distances off of this primary pathway (O'Leary and Terashima, 1989). As described in the previous sections, subplate axons precede layer 5 and 6 axons through the internal capsule, and at least some subplate axons continue into the thalamus. To determine if subplate axons also extend beyond the internal capsule along the pathways taken by layer 5 axons to their brainstem and spinal targets, we employed a variety of retrograde tracing methods in early postnatal rats (Tables 1, 2). Injections were made into the superior colliculus, a principal target of layer 5 neurons, as well as into points along the primary pathway of layer 5 axons.

In a series of rats, one of three different retrograde tracers was injected into the superior colliculus, either before or after the arrival of cortical axons, as indicated by anterograde DiI labeling. In the rat, cortical axons first arrive in the superior colliculus at P1.5 (T. Terashima and D. D. M. O'Leary, unpublished observations). In one set of experiments, a solution of DiI in dimethylformamide was injected into the superior colliculus of brains fixed with aldehydes on P0 or P1 (Table 1). No labeled cells are found in the P0 cortex, but a small number of cortical cells are labeled at P1. Every one of these labeled cells, which amount to about 100 in each brain, are in layer 5; none are present in the subplate (Fig. 9*A,B*). In another set of experiments (Table 2), no labeled cortical neurons are found in P1 rats in which RITC was injected into the superior colliculus 24 hr earlier, or in P3 rats in which injections of fast blue were made into the colliculus on P0. However, a few layer 5 neurons are labeled in P2 rats injected in the colliculus with RITC on P1, and substantial numbers are found in P4 rats injected with RITC on P2 (Fig. 9*D*), as well as in P6 rats with fast blue injected into the colliculus on P2 (Fig. 9*C*). However, subplate cells are not labeled in any of these cases. In two additional cases perfused on P3, we made on P0 a large injection of fast blue that included the rostral superior colliculus, the pretectal areas, and the thalamus. In one animal, the injection involved posterior thalamus (Fig. 10*A,B*), and in the other, the dorsal lateral geniculate nucleus and the medial geniculate nucleus. In each case, large numbers of retrogradely labeled cells are found in layers 5 and 6, as well as in the subplate (Fig. 10*C,D*). These findings indicate that subplate neurons do not send axons into the superior colliculus, a layer 5 target, but that many do project to the thalamus, a layer 6 target.

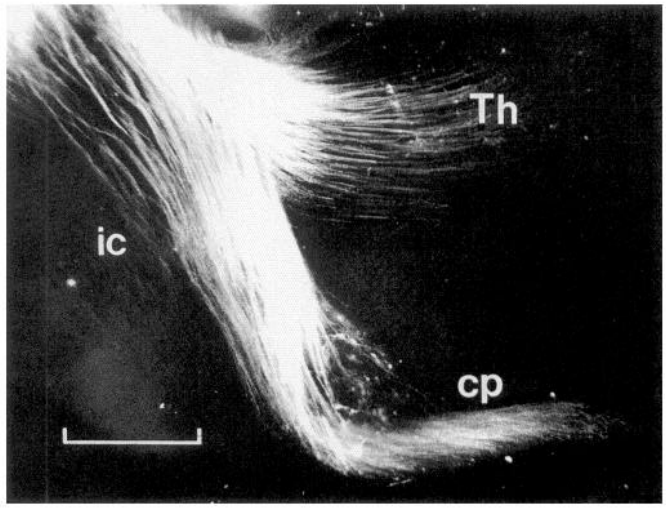


Figure 8. Bifurcation of the internal capsule: anterograde DiI labeling from the anterior cortex of a brain aldehyde-fixed on E18; sagittal section. Anterogradely and retrogradely labeled axons turn to exit the internal capsule (*ic*) and enter in the thalamus (*Th*). Other anterogradely labeled axons continue to extend through the distal part of internal capsule and into the cerebral peduncle (*cp*). Rostral is to the left, dorsal to the top. Scale bar, 100 μm.

In a third set of experiments, fast blue or RITC was injected into one of two points in the primary subcortical pathway of layer 5 axons, the cerebral peduncle in the midbrain or the pyramidal decussation at the spinomedullary junction (Table 2). Anterograde labeling shows that cortical axons reach this level of the cerebral peduncle around E17 (J. A. De Carlos and D. D. M. O'Leary, unpublished observations) and the pyramidal decussation at birth (Schreyer and Jones, 1982). In one case, we successfully injected the cerebral peduncle in a newborn rat that was later perfused on P3. The injection was placed in the ventral midbrain just caudal to the thalamus (Fig. 11*A,B*) and retrogradely labeled a dense band of layer 5 neurons, but no subplate cells (Fig. 11*C*). Both RITC and fast blue injections made into the pyramidal decussation at birth also result in the substantial labeling of layer 5 neurons in animals perfused at P1 (RITC) or P3 (fast blue). Fast blue similarly injected at P2 retrogradely labels a large number of layer 5 neurons in animals perfused at P6 (Fig. 11*D*). Again, in none of these cases are labeled cells found in the subplate. These observations indicate that subplate neurons do not send axons to the targets of layer 5 neurons or through the subcortical pathways of layer 5 axons beyond the internal capsule.

Discussion

We have addressed in rats the early development of the major cortical efferent and afferent projections through the internal capsule, the axonal pathway between cortex and subcortical structures. Our primary aim was to relate the extension and targeting of cortical subplate axons to that of cortical efferent axons arising from layer 5 and layer 6 neurons, and to that of cortical afferent axons arising from the thalamus. Our principal findings are summarized in Figure 12. First, we show that pre-

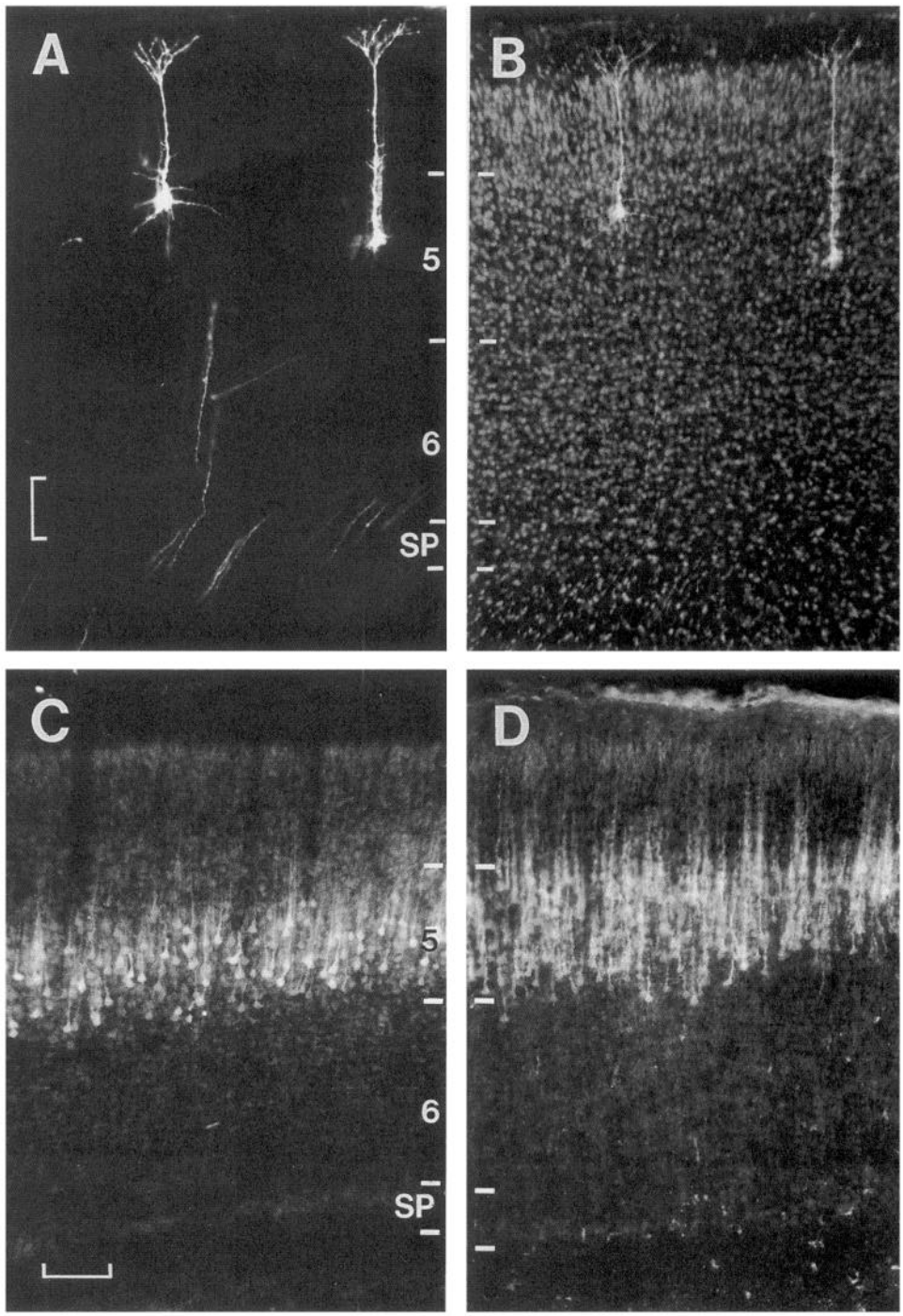


Figure 9. Subplate cells do not project to the superior colliculus, a target of layer 5 neurons. *A* shows cortical neurons retrogradely labeled with DiI injected into the superior colliculus in a brain aldehyde-fixed on P1. *B* is the same section counterstained with bisbenzimide. *C* shows cortical neurons retrogradely labeled with an *in vivo* injection of fast blue into superior colliculus on P2 and perfused on P6. *D* shows cortical neurons retrogradely labeled with an *in vivo* injection of rhodamine-B-isothiocyanate in superior colliculus on P2 and perfused on P4. In each case, the labeled neurons are restricted to layer 5; none are found in the subplate (*SP*). The white, punctate structures in *D* are not labeled neurons. Scale bars, 100 μm.

plate axons are the first axons to exit cortex and extend into the nascent internal capsule. Second, we demonstrate that thalamic axons extend through the internal capsule toward the cortex concurrent with the extension of preplate axons from the cortex; therefore, these two axonal populations coestablish the internal capsule. Third, we show that subplate axons have a very limited subcortical distribution in rats. They extend through the internal capsule into the thalamus, preceding the definitive corticothalamic projection arising from layer 6 neurons. However, they do not extend to the subcortical targets of layer 5 axons in the midbrain or beyond, or along the subcortical pathways of layer 5 axons that continue from the internal capsule. Here, we discuss these observations and their implications for the potential roles of subplate neurons in the development of the efferent and afferent projections of the mammalian neocortex.

Preplate cells extend the first cortical axons into the internal capsule

The internal capsule forms within the basal telencephalon and comprises both cortical efferent and afferent axons. McConnell et al. (1989) have reported that in cats and ferrets subplate neurons send the first cortical axons through the internal capsule. We find that in rats the first cortical axons grow into the basal telencephalon on E14 and arise from rostral and lateral (i.e.,

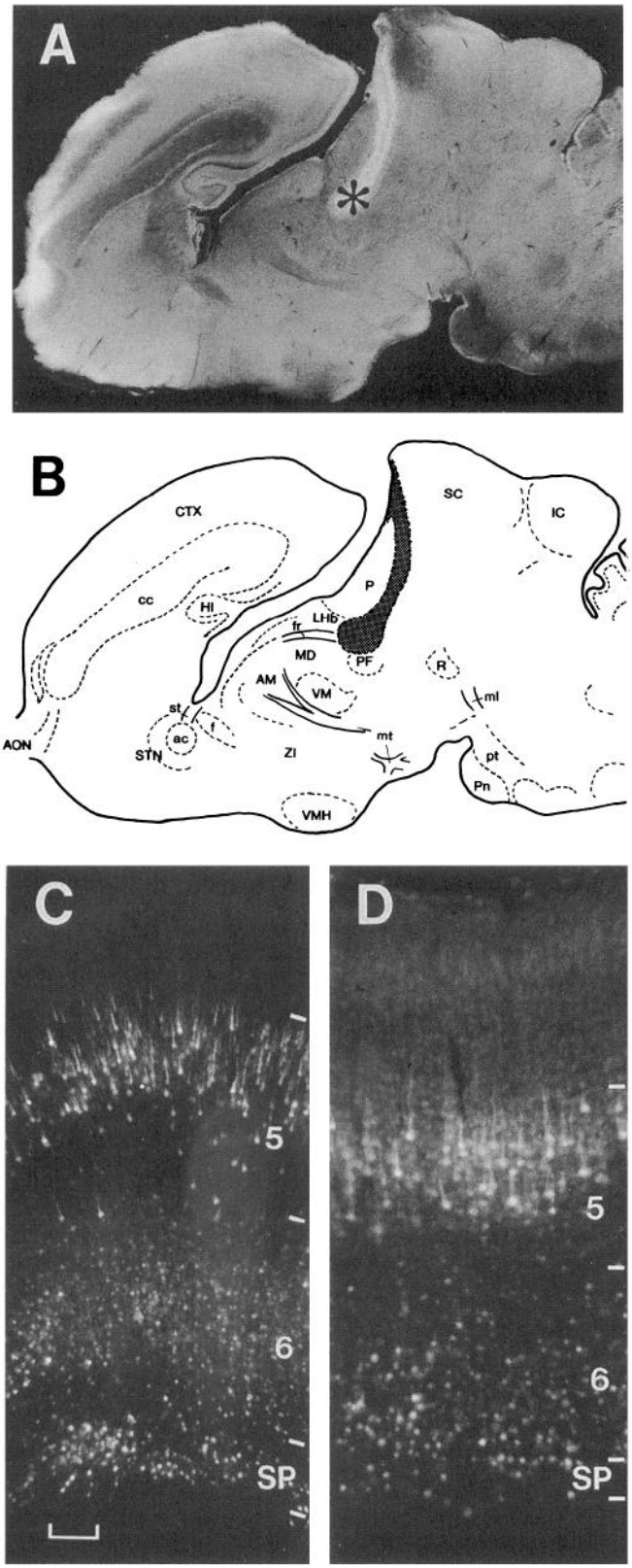
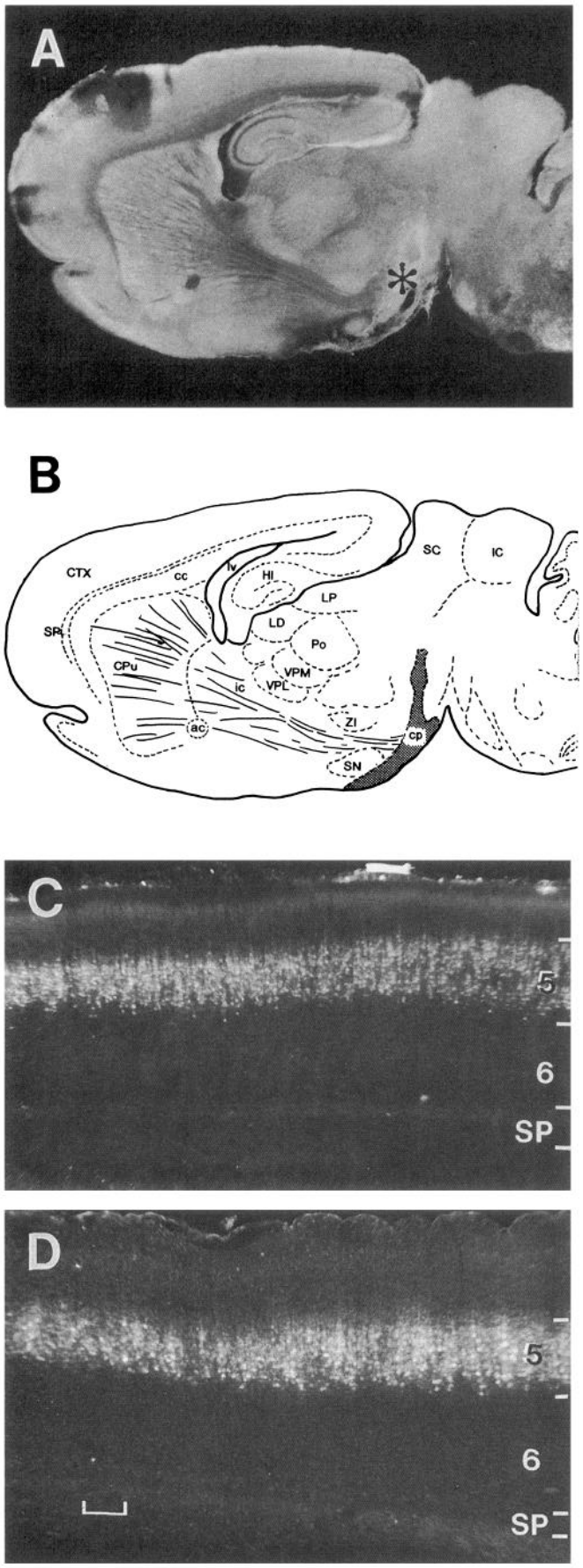


Figure 10. Subplate cells can be labeled with retrogradely transported fluorescent tracers injected into thalamus, a target of layer 6 neurons. *A* is a photo and *B* is a camera lucida drawing of a sagittal section showing a fast blue injection extending from rostral superior colliculus (*SC*) into caudal thalamus (✱ in *A*); injected at P0, perfused at P3, and sagittally sectioned. Retrogradely labeled neurons are found in the subplate (*SP*) and in layers 5 and 6 of frontal (*C*) and parietal (*D*) cortex. *ac,* anterior commissure; *AM,* anteromedial nucleus (thalamus); *AON,* anterior olfactory nucleus; *cc,* corpus callosum; *CTX,* cortex; *f,* fornix; *fr,* fasciculus retroflexus; *HI,* hippocampus; *IC,* inferior colliculus; *LHb,* lateral habenular nucleus; *MD,* mediodorsal nucleus (thalamus); *ml,* medial longitudinal fasciculus; *mt,* mamillothalamic tract; *P,* pretectal area; *PF,* parafascicular nucleus of the thalamus; *Pn,* basilar pontine nuclei; *pt,* pyramidal tract; *R,* red nucleus; *st,* stria terminalis; *STN,* nucleus stria terminalis; *VM,* ventromedial nucleus (thalamus); *VMH,* ventromedial nucleus (hypothalamus); *ZI,* zona incerta. Scale bar: 100 μm for *C* and *D*; 800 μm for *A* and *B*.

rostroventral) regions of the telencephalic vesicle. We conclude that these axons are extended by preplate (subplate) neurons, based on the developmental state of the cortex at E14, as well as on the location and morphology of the cells of origin. In rats, E14 is prior to the formation of the cortical plate. At this stage, the telencephalic wall consists of a neuroepithelium and a preplate, a layer several cells thick that forms between the neuroepithelium and the pial surface (Bayer and Altman, 1990). In cats, cells that form the preplate are generated in an outside-in gradient preceding the genesis of the neurons that will form the cortical plate (Luskin and Shatz, 1985). The later-generated cortical plate neurons migrate superficially from the neuroepithelium, aggregate within the preplate, and split it into a superficial marginal zone and a deep subplate (Marin-Padilla, 1978). The same scenario occurs in rats, but the sequence of events is temporally compressed. Preplate cells are generated predominantly between E12 and E14 (Valverde et al., 1989; Bayer and Altmann, 1990). A few cortical plate cells become postmitotic on E13, but these amount to only about 3% of the total number of cells that will come to occupy layer 6; the generation of cortical plate neurons does not become pronounced until E14 (Bayer and Altman, 1991). Cortical plate neurons pause for 24 hr immediately outside of the neuroepithelium and then continue their migration superficially, which takes at least another 24 hr (Altman and Bayer, 1990). Consistent with these findings is the observation that a rudimentary cortical plate cannot be identified until E15 (Raedler and Sievers, 1975; Rickmann et al., 1977; Altmann and Bayer, 1990). Since we find that axons exit the cortex as early as E14, and have already grown for some distance on E13, they must be extended by preplate cells. In addition to timing, the location and morphology of the cells that extend the first cortical axons into the internal capsule support their identification as preplate neurons. Using retrograde DiI labeling in aldehyde-fixed brains, we find that these cells are positioned just beneath the pial surface, within the dense cellular layer corresponding to the preplate. These cells have morphologies characteristic of preplate neurons and, at later stages, subplate neurons, but distinct from the morphology typical for immature cortical plate neurons. Immature cortical plate projection neurons have slender, radially aligned morphologies, while subplate cells have been described to have a range of morphologies such as horizontally oriented bipolar, pyramidal-like, and multipolar (McConnell et al., 1989; Valverde et al., 1989, 1990; Shatz et al., 1990; De Carlos et al., 1991). Preplate cells that extend the first cortical efferents are later found in the subplate layer, as the cortical plate develops within the preplate. Thus, for simplicity, in the remainder of the discussion, we will refer to these earliest cortical efferents as subplate axons.

Only a proportion of the cells in the preplate, and at later stages in the subplate, at any given cortical region can be ret-



rogradely labeled from large DiI placements in the basal telencephalon. This finding suggests that a subset of subplate neurons extend axons into the internal capsule, consistent with reports that subplate cells are a heterogeneous population, not only in morphology, but also in their expression of neurotransmitters, peptides, and AChE (Kristt, 1979; Chun et al., 1987; Chun and Shatz, 1989; Naegele et al., 1989; Van Eden et al., 1989; Friauf et al., 1990; Wahle et al., 1990; Cobas et al., 1991). Heterogeneity among subplate cells is also apparent from distinctions in the intracortical patterning of their axons. Several categories have been identified, including cells with local axons oriented parallel to the fibers of the white matter and eventually invading lower layer 6, cells with ascending axons that send collateral branches to the superficial cortical layers, and cells that send axons through the white matter without emitting collateral branches (Valverde et al., 1990). It is probable that this latter population is the one that we have demonstrated to extend axons into the internal capsule. Others have reported in various species that a proportion of subplate cells extends axons into the marginal zone (layer 1) (Kristt, 1979; Chun et al., 1987; Divac et al., 1987; Wahle and Meyer, 1987; Friauf et al., 1990). In addition, some subplate cells can be retrogradely labeled from the contralateral cortex in cats (Chun et al., 1987). Although we find that the axons of preplate neurons in anterior and lateral cortex are directed toward the basal telencephalon, the site of the internal capsule, some preplate cells in the more dorsal aspect of lateral cortex do extend axons toward the midline as early as E14. However, electron microscopic (Valentino and Jones, 1982) and anterograde DiI (De Carlos and O'Leary, unpublished observations) studies demonstrate that in rats cortical axons do not cross through the corpus callosum into the opposite hemisphere until approximately E18, about 4 d after we show that preplate axons extend into the internal capsule. It is not clear why the corpus callosum has such a delayed development, but the reason may be related to the lateral-to-medial gradient of cortical maturation (Rickmann et al., 1977; Smart and Smart, 1982; Uylings et al., 1990).

Potential roles of subplate axons in establishing cortical efferent projections

The early extension of subplate axons has prompted the suggestion that they pioneer the efferent pathways of the neocortex in a manner analogous to that reported in the development of certain axonal projections in the invertebrate nervous system (McConnell et al., 1989). Our observations on the growth and distribution of subplate axons allow us to evaluate their potential roles in the development of the projections of layer 5 and

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Figure 11. Subplate axons do not extend through the subcortical pathways of layer 5 axons. *A* is a photo and *B* is a camera lucida drawing of a sagittal section showing a fast blue injection (*asterisk* in *A*) in the midbrain cerebral peduncle (*cp*); injected on P0 and perfused on P3. *C* illustrates the dense band of layer 5 neurons retrogradely labeled by this injection; no labeled cells are found in the subplate (*SP*). *D* shows layer 5 neurons retrogradely labeled with an injection of fast blue in the pyramidal decussation at the spinomedullary junction; injected on P2 and perfused on P6. Again, no labeled cells are found in the subplate. *cp,* cerebral peduncle; *CPu,* caudate putamen; *LD,* laterodorsal nucleus (thalamus); *LP,* lateroposterior nucleus (thalamus); *lv,* lateral ventricle; *Po,* posterior nucleus (thalamus); *SN,* substantia nigra; *VPL,* lateral ventroposterior nucleus (thalamus); *VPM,* medial ventroposterior nucleus (thalamus). For other abbreviations, see Figure 10. Scale bar: 100 μm for *C* and *D*; and 700 μm for *A* and *B*.

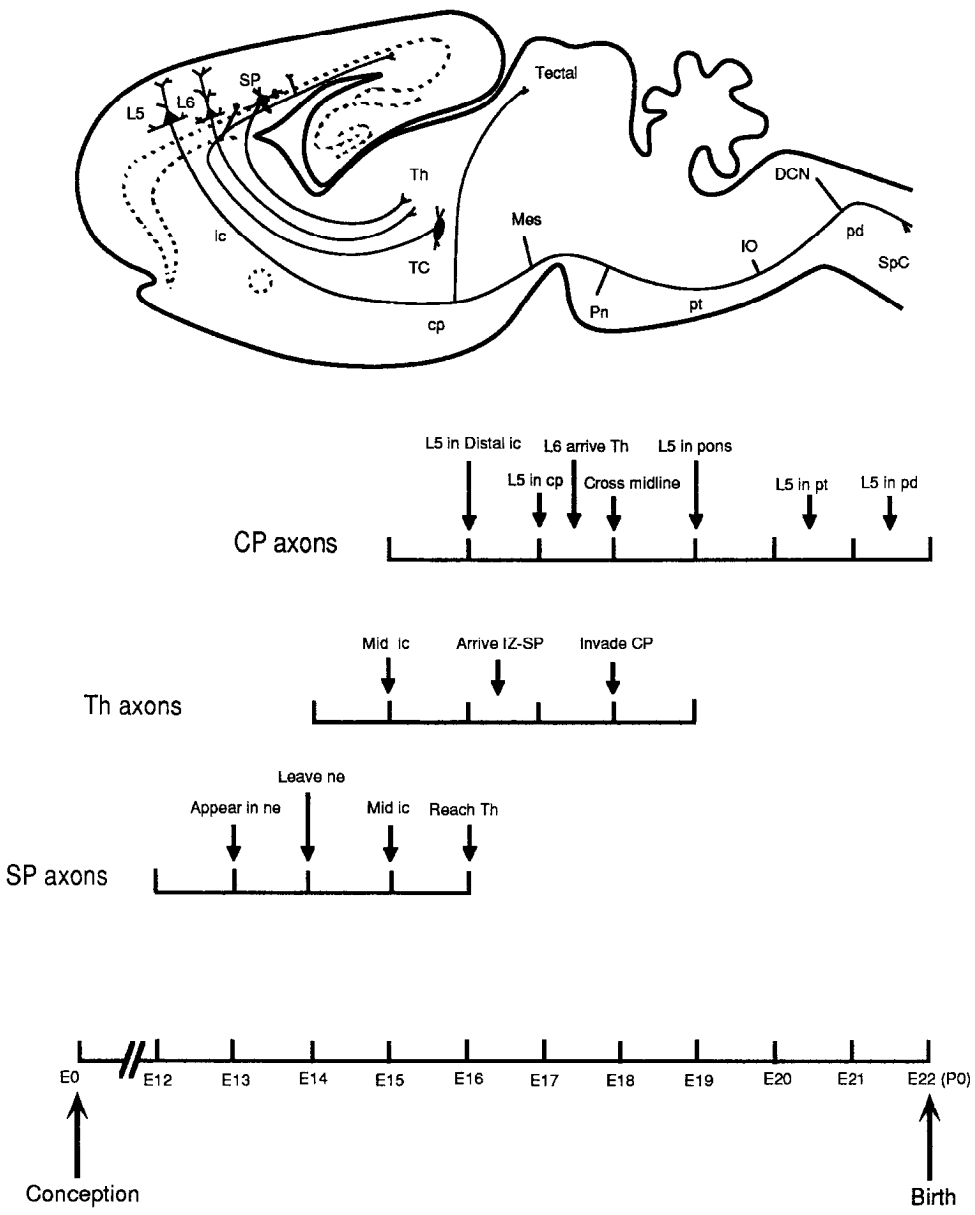

Figure 12. Summary of development of cortical efferents and afferents in rat. The drawing is of a sagittal section of a neonatal rat brain schematizing the projections of layer 5 (*L5*), layer 6 (*L6*), subplate (*SP*), and thalamocortical (*TC*) neurons. Subplate and layer 6 axons project through the internal capsule (*ic*) to the thalamus. As a population, layer 5 axons project to a number of subcortical targets, including the superior colliculus (*Tectal*), mesencephalic nuclei (*Mes*), basilar pontine nuclei (*Pn*), inferior olive (*IO*), dorsal column nuclei (*DCN*), and spinal cord (*SpC*). During development, each of these targets is contacted by collateral branches of spinally directed primary axons that form days after the primary axons pass by their targets (O'Leary et al., 1990). Below the drawing are time lines that indicate key events in the extension of cortical plate (*CP*) axons, thalamic (*Th*) axons, and subplate (*SP*) axons. These times are based on our findings, with some exceptions: The invasion of the cortical plate by thalamic axons on E18 is taken from the study of Catalano et al. (1991); the late stages in the subcortical extension of layer 5 axons in the midbrain and hindbrain are described in O'Leary et al. (1990). Other abbreviations: *IZ*, intermediate zone; *ne*, neuroepithelium; *pd*, pyramidal decussation.

6 neurons to their subcortical targets. Using anterograde HRP labeling, Schreyer and Jones (1982) found that in rats cortical efferents first enter the internal capsule on E17. They attributed this labeling to the extension of axons by presumptive layer 5 and 6 neurons. However, the greater sensitivity of the DiI method reveals that the first cortical axons enter the internal capsule on E14 and are extended by preplate neurons. Over the ensuing days, the density of labeling increases and the labeled axons extend farther subcortically. Using retrograde DiI labeling in aldehyde-fixed brains, we have been able to define the time at which cortical plate neurons extend axons (1) into the internal capsule, the pathway taken by layer 5 and 6 axons to project subcortically, and (2) into the thalamus, the subcortical target of layer 6 neurons. We found that some cortical plate neurons extend axons into the internal capsule by E16; a few of these axons enter the thalamus by E17. Two uncertainties are associated with these findings. First, at these early stages of cortical development, it is not possible to distinguish layer 6 cells from layer 5 cells, since lamina-specific markers are unavailable. Second, since a few neurons that reside in the cortical plate are generated on E13 (Bayer and Altman, 1991), we cannot exclude the possibility that a very small number of cortical plate axons enter the internal capsule on E15. However, this prospect is unlikely since the first cortical plate neurons do not settle in the preplate until E15 (Raedlaer and Sievers, 1975; Rickmann et al., 1977; Altman and Bayer, 1990) and, based on the trajectories of cortical efferent axons, do not emit axons until late in migration. Regardless, our findings demonstrate that cortical plate axons enter the internal capsule after subplate axons.

Similarly, our observations indicate that subplate axons precede cortical plate axons into the thalamus. Anterograde DiI labeling shows that the first cortical axons grow into the thalamus on E16. Given the disparity in the timing of extension of subplate axons and cortical plate axons into the internal capsule, it is likely that subplate axons account for this initial ingrowth into the thalamus, but because at this age they extend only a

short distance into the thalamus, it is difficult to retrogradely label them with DiI placements in the thalamus without involving the neighboring region of the internal capsule. On E17, though, subplate neurons can be retrogradely labeled with DiI confined to the thalamus. At this age, a low density of labeled subplate cells is found distributed widely over the cortex. A few cortical plate neurons are also labeled in anterior and lateral cortex, but none more posteriorly. Such a limited distribution of labeled cortical plate neurons is not unexpected at these early stages of cortical development given that in rodents cortical maturation proceeds in an anterior-ventral-lateral to posterior-dorsal-medial direction (Rickmann et al., 1977; Smart and Smart, 1982; Uylings et al., 1990). In the rat, then, as in the cat and ferret (McConnell et al., 1989), subplate axons precede cortical plate axons from the cortex to the thalamus. These findings are consistent with the hypothesis that subplate axons serve an important role in establishing the definitive thalamocortical projections of layer 6 neurons.

Although subplate axons are in place to act potentially as a pioneering population for layer 6 corticothalamic projections, our findings preclude a role for subplate axons in pioneering the pathways taken by layer 5 axons to their subcortical targets. Layer 5 is the sole source of cortical input to numerous distinct structures in the midbrain, hindbrain, and spinal cord (Fig. 12). During development, each layer 5 target is contacted exclusively by axon collaterals that branch from a spinally directed primary axon; these collaterals do not form until days after the primary axon passes the target (O'Leary and Terashima, 1988, 1989; O'Leary et al., 1990). This delayed, interstitial branching mechanism, in itself, makes it improbable that subplate axons promote the formation of the axon collaterals that extend from primary layer 5 axons to their targets; our present study directly rules out any role for subplate axons in this process. We have arrived at this conclusion by retrogradely labeling during early postnatal development cortical neurons that send axons to the superior colliculus, the principal midbrain target of layer 5 neurons. We used several different retrograde tracers, including fast blue and RITC *in vivo,* as well as DiI in aldehyde-fixed tissue, and labeled at times before, during, and after the arrival of cortical axons, as determined previously by anterograde DiI labeling (Terashima and O'Leary, unpublished observations). If subplate axons pioneer this pathway, we would expect to label retrogradely subplate cells at ages before layer 5 neurons are found labeled. Our results are unequivocal. In cases labeled prior to the arrival of cortical axons, no retrogradely labeled neurons are seen in the cortex. In cases labeled as the first cortical axons arrive, and well after the arrival of a substantial number, the retrogradely labeled neurons are confined to layer 5; none are seen in the subplate. On the other hand, larger injections that extend from the superior colliculus and into the thalamus label not only layer 5 neurons but, as expected, considerable numbers of layer 6 and subplate neurons as well. These results rule out a pioneering role for subplate axons in establishing the layer 5 projection to the superior colliculus and, further, show that in rats subplate neurons do not project to the superior colliculus.

Using a similar strategy to that outlined above for the superior colliculus, we have also ruled out the possibility that subplate axons pioneer the pathway taken by the primary layer 5 axons through the cerebral peduncle and pyramidal tract and into the dorsal funiculus of the spinal cord. Injections of retrograde tracers into the midbrain cerebral peduncle, a proximal part of the pathway of primary layer 5 axons contiguous to the internal capsule, and into the pyramidal decussation at the junction of the hindbrain and spinal cord, a more distal part of the pathway, yield the same result: large numbers of layer 5 neurons are labeled, and, very rarely, a labeled cell is found in layer 6, but no labeled neurons are found in the subplate. Our findings show that the subcortical distribution of subplate axons is limited to the internal capsule and thalamus, similar to the distribution of layer 6 axons. Therefore, subplate axons cannot pioneer the subcortical pathways of layer 5 neurons beyond the internal capsule. It remains possible that subplate axons are crucial to establish the internal capsule and to facilitate the exiting of layer 5 axons from cortex, either through direct interactions or through an intermediary. We also cannot rule out that another early-developing population of axons that originates outside the cortex might aid in establishing parts of the layer 5 pathways distal to the internal capsule. In addition, these findings indicate that essentially only layer 5 axons continue from the internal capsule into the cerebral peduncle. Therefore, we can infer that the critical pathfinding decision made in the internal capsule, to deviate into the thalamus or continue to extend down the neuraxis, is faithfully made by each of the three populations of cortical efferent axons, subplate, layer 6, and layer 5.

Potential roles of subplate axons in the establishment of corticothalamic projections

The thalamus is the major source of cortical afferents. Thalamocortical projections are organized such that specific thalamic nuclei project to specific areas of the cortex. The finding that preplate axons send the first cortical axons into the internal capsule (McConnell et al., 1989; present results) leads to the intriguing possibility that these early-developing cortical axons may establish a scaffolding that guides thalamocortical axons to their appropriate cortical target areas. However, using anterograde and retrograde labeling in fixed brains, we observe that axons originating in the thalamus extend through the internal capsule toward cortex at the same time that preplate axons extend through it toward thalamus. These two populations of axons reach the approximate midpoint of the internal capsule on E15 and pass each other. We find that thalamic axons first extend into cortex on E16, which agrees with the recent report of Catalano et al. (1991), who examined the ingrowth of ventrobasal thalamic afferents into rat somatosensory cortex. Our observations indicate that subplate axons reach thalamus on E16. Therefore, subplate axons and thalamic axons coestablish the internal capsule. From this finding, it follows that subplate axons do not promote the initial outgrowth of thalamic axons or provide any directional cues for their extension into the internal capsule. These findings do not dismiss, but do limit, a potential role for subplate neurons in establishing thalamocortical connectivity.

Recent experimental evidence indicates that the subplate is required for thalamic axons to invade their appropriate area of cortex (Ghosh et al., 1990). What is the nature of this interaction between thalamic axons and the subplate? The distribution of efferent and afferent axons in the cortical white matter, or in its early form, the intermediate zone, suggests that thalamic axons do not follow subplate axons back to their target cortical area. Woodward et al. (1990) report that, in the rat visual system, cortical afferent and efferent pathways are segregated in the white matter and that they can be selectively activated by focal electrical stimulation. Consistent with this finding, we report here that subplate axons take a trajectory through cortex to the in-

ternal capsule that initially is deep to the preplate, and later, the subplate. Later-arising efferent axons from layer 5 and 6 neurons take a similar deep pathway. Thalamocortical axons, though, travel through the subplate layer (Reinoso and O'Leary, 1988, 1990), superficial to the path of subplate axons, as they extend tangentially through cortex to their target area. This distinct layering of efferents and afferents, apparent even at early stages of cortical development, makes it unlikely that thalamic axons fasciculate with, or are guided by, subplate axons to their target areas. However, since thalamic axons travel within the subplate layer, they are in position to be influenced by targeting cues that seem to be associated with it (Ghosh et al., 1990).

Implications for the concept of the "primordial plexiform layer"

Several years ago, Marin-Padilla (1978) proposed that the mammalian neocortex has a dual origin, since the most superficial layer (layer 1 or marginal zone) and the deepest layer (termed layer 7 by Marin-Padilla, or layer 6b or subplate by others) have a more "primitive" neuronal organization than layers 2–6, and a distinct developmental origin. Based on observations using the Golgi method, he suggested that afferents invade the undifferentiated telencephalic vesicle and initiate the maturation of cortical neurons, forming the primordial plexiform layer (PPL), from which layers 1 and 7 later emerge as the cortical plate develops (Marin-Padilla, 1978). The PPL was described to have an evolutionarily premammalian cortical organization characterized by an external white matter composed of afferent fibers with neurons interspersed among them, as in amphibians and reptiles (Marin-Padilla, 1978, 1990; Marin-Padilla and Marin-Padilla, 1982). Although we do not dispute the dual origin of the mammalian neocortex, our findings counter the concept that the early PPL contains fibers that originate outside of cortex. Our retrograde DiI labeling experiments show that thalamocortical axons are the first afferents to extend into the cortex. Thalamic axons do not arrive until E16, at which time the cortical plate is beginning to emerge, and days after preplate neurons, that is, PPL neurons in the terminology of Marin-Padilla, have extended axons long distances within cortex as well as into the internal capsule. Therefore, the early fibers in the PPL are not afferents to cortex but rather must arise from preplate cells (Cajal-Retzius and subplate neurons), an inference consistent with Ramón y Cajal's (1911) interpretation that the axons of Cajal-Retzius cells are the thick tangential fibers characteristic of layer 1. These findings, taken together with those of Catalano et al. (1991), who show that thalamic axons do not invade the cortical plate until E18, indicate that the early differentiation of the preplate and the initial development of the cortical plate occur independently of afferent influences.

Conclusions

The early differentiation of the subplate has led a number of investigators to propose diverse roles for subplate neurons in cortical development (Shatz et al., 1988, 1990). The relationship between the marginal zone and the subplate constitutes the first functional circuit identified in the developing mammalian cortex and can be recognized at early stages prior to the maturation of the cortical plate (Marin-Padilla, 1971, 1972, 1978; Marin-Padilla and Marin-Padilla, 1982; Friauf et al., 1990; Kostovic and Rakic, 1990). This early connectivity prompted the suggestion that subplate cells act as intermediate links between white matter axons and cells of the cortical plate, and that they may mediate the differentiation of the neurons and connectional circuits of the cortical plate (Valverde et al., 1989; Friauf et al., 1990). The subplate has also been suggested to serve as a compartment for transient cellular interactions with waiting thalamocortical afferents, perhaps providing trophic support prior to their invasion of the cortical plate (Shatz and Luskin, 1986; Chun et al., 1987; Kostovic and Rakic, 1990; Friauf et al., 1990). Support for these suggested roles comes from experimental evidence in cats that subplate cells are not only critical for the invasion of the cortical plate by thalamic axons (Ghosh et al., 1990), but also that at later developmental stages they may provide a crucial link required for the normal segregation of geniculocortical axons into ocular dominance columns (Ghosh and Shatz, 1991). The distinct tangential domains occupied by subplate cells of a given neuropeptide immunoreactivity (Chun et al., 1987), and the transient expression of GABA by a proportion of them (Van Eden et al., 1989), have provoked speculation that subplate cells may locally regulate neurite outgrowth (Shatz et al., 1988; Van Eden et al., 1989) or that this expression of inhibitory neurotransmitters may protect developing cortical plate cells prior to the elaboration of intracortical inhibitory circuits from possible excitotoxic effects induced by the excitatory transmitters of the afferent projection systems (Wahle and Mayer, 1987; Van Eden et al., 1989).

The report that has arguably attracted the most attention is that subplate neurons "pioneer" the internal capsule, the sole efferent and afferent axonal pathway between cortex and subcortical structures, and send axons to the thalamus and the superior colliculus (McConnell et al., 1989). These findings have led to the speculation that subplate axons may play a critical role in establishing cortical efferent projections, which arise exclusively from layers 5 and 6, as well as cortical afferent projections, the most prominent of which arises from the thalamus (McConnell et al., 1989; Shatz et al., 1990). In the present study, we have addressed the early development of cortical efferents and afferents in rats to help define the potential role of the subplate in the development of these cortical projection systems. We concur with McConnell et al. (1989) that subplate axons send the first cortical axons into the internal capsule. The fact that this occurs in two orders of mammals, carnivores (McConnell et al., 1989) and rodents (present results), supports the contention that this is a fundamental event in cortical development. We find that thalamic axons are the first cortical afferents to extend into cortex and that they and subplate axons coestablish the internal capsule from opposite directions. Although these two axonal populations may interact within the internal capsule, in cortex they have distinct pathways, suggesting that thalamic axons do not project to their appropriate cortical area by tracking along efferent subplate axons. Subcortically, we show that the distribution of subplate axons is restricted to the internal capsule and the thalamus. Thus, beyond the internal capsule, they play no role in establishing the subcortical pathways of layer 5 axons to their targets in the midbrain, hindbrain, and spinal cord. Although our findings limit the potential role of subplate axons in establishing cortical connections, they are consistent with the hypothesis that subplate axons influence the growth of cortical efferents from their site of origin and through the internal capsule and that subplate axons aid in establishing the projection from layer 6 to the thalamus.

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