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Subplate Pioneers and the Formation of Descending Connections from Cerebral Cortex

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The adult cerebral cortex extends axons to a variety of subcortical targets, including the thalamus and superior colliculus. These descending projections are pioneered during development by the axons of a transient population of subplate neurons (McConnell et al., 1989). We show here that the descending axons of cortical plate neurons appear to be delayed significantly in their outgrowth, compared with those of subplate neurons. To assess the possible role of subplate neurons in the formation of these pathways, subplate neurons were ablated during the embryonic period. In all cases, an axon pathway formed from visual cortex through the internal capsule and into the thalamus. In half of all cases, however, cortical axons failed to invade their normal subcortical targets. In the other half, targets were innervated normally. Subplate neurons are therefore likely to provide important cues that aid the process by which cortical axons grow toward, select, and invade their subcortical targets.

[Key words: axon guidance, subplate, pioneer neurons, cerebral cortex, target selection, target invasion, lateral geniculate nucleus, superior colliculus, ablation]

During the development of the nervous system, growing axons must be able to navigate a complex cellular environment to reach their targets, and once there must be able to distinguish appropriate from inappropriate target cells in the same vicinity. In many systems, the initial growth and targeting of axons is accomplished with the aid of "pioneer" neurons, the first neurons to extend axons along a particular pathway during development (LoPresti et al., 1973; Bate, 1976; Bentley and Keshishian, 1982; Ho and Goodman, 1982; Bastiani et al., 1985; Kuwada, 1986; Myers et al., 1986; Nordlander, 1987; McConnell et al., 1989; Norris and Kalil, 1990; Yaginuma et al., 1991). These early pioneer pathways are subsequently traversed by the axons of later-generated neurons, and in some cases the pioneer neurons themselves disappear by cell death following the formation of mature axonal pathways (Bate, 1976; Kuwada, 1986; Shatz et al., 1990). The axon pathways laid down by pioneer neurons are required for the formation of normal adult connections in some systems: cell ablation studies in both vertebrates (Kuwada, 1986) and invertebrates (Bastiani et al., 1985; Klose and Bentley, 1989) have demonstrated that many axons fail to develop normally in the absence of pioneers.

In the mammalian telencephalon, a transient class of pioneer neurons lays down the initial axonal pathways between the cerebral cortex and the thalamus (McConnell et al., 1989). These subplate neurons are among the earliest-generated neurons of the neocortex, but the majority of these cells disappear in early postnatal periods in a wave of cell death (Valverde and Facal-Valverde, 1987; Chun and Shatz, 1989; reviewed in Shatz et al., 1990; Shatz et al., 1991). Subplate neurons are generated between embryonic day 24 (E24) and 30 in the cat (Luskin and Shatz, 1985b), and then migrate outward from the ventricular zone to form a single layer called the "preplate" (Marin-Padilla, 1971; Luskin and Shatz, 1985b). The preplate is subsequently split by the arrival of neurons of the cortical plate into two regions, the subplate just underneath the cortical plate, and the marginal zone, found at the pial surface (Luskin and Shatz, 1985a,b; Bayer and Altman, 1990). Subplate neurons extend the first axons from the neocortex into the internal capsule at a time before the permanent projection neurons of the cortex have even been born (Luskin and Shatz, 1985a; McConnell et al., 1989), and they project into at least two subcortical regions, the thalamus and the superior colliculus (McConnell et al., 1989). The permanent connections to these targets will eventually be formed by neurons of the deep cortical layers 5 (to colliculus) and 6 (to thalamus) (Gilbert and Kelly, 1975). At the same time that subplate neurons are forming descending projections, their cell bodies are playing out a role within the neocortex itself: the dendrites of these neurons receive synaptic input from thalamic afferent axons that are "waiting" in the subplate prior to invading their final targets in layer 4 of the cortical plate (Kostovic and Rakic, 1980; Shatz and Luskin, 1986; Chun et al., 1987; Chun and Shatz, 1988b; Friauf et al., 1990; Herrmann et al., 1991; Ghosh and Shatz, 1992b). Subplate neurons may relay information from the thalamic axons into the cortical plate through the formation of axon collaterals that project upward into the cortical plate and marginal zone (Wahle and Meyer, 1987; Valverde and Facal-Valverde, 1988; Antonini and Shatz, 1990; Friauf et al., 1990; Friauf and Shatz, 1991). Ablation studies have revealed that subplate neurons control the ability of thalamic axons to invade appropriate cortical areas (Ghosh et al., 1990; Ghosh and Shatz, 1993), and later are involved in the refinement of thalamic connections within layer 4 (Ghosh and Shatz, 1992a).

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The early birth and extension of subcortical axons by subplate neurons defines them as pioneer neurons; however, it has remained an open question whether these early axons serve an essential role in the formation of permanent connections that descend from the neocortex to subcortical targets. In a variety of systems, the complexity of growth cones has been correlated with environmental cues encountered during pathfinding: pioneering growth cones that initially navigate a pathway are often more complex than the growth cones that then fasciculate with earlier-growing fibers (LoPresti et al., 1973; Nordlander, 1987; Yaginuma et al., 1991), and growth cones enlarge and extend more filopodia when they encounter "decision-making" points along a pathway (Tosney and Landmesser, 1985; Bovolenta and Mason, 1987). The growth cones of subplate neurons are both larger and more elaborate than those of the cortical plate axons that follow (Kim et al., 1991), consistent with the possibility that subplate neurons are actively pioneering a pathway that is then simply followed by deep-layer cortical axons. But are the subplate axons required for the ability of layer 5 and 6 cortical neurons to select and grow into their normal targets in the thalamus (layer 6), and the colliculus, pons, and spinal cord (layer 5)? Here we present a series of experiments in which we have first tracked the timing of normal axon development by subplate and cortical neurons, next examined the invasion of subcortical targets by neocortical axons, and finally tested the "pioneer hypothesis" directly by ablating subplate neurons during the period of axogenesis and assessing the effects of this ablation on the formation of subcortical axonal connections. We report that the ablation of subplate neurons can alter not only axon pathfinding by corticotectal neurons, but also the ability of corticogeniculate axons to invade the LGN.

Materials and Methods

Animals. Results from a total of 33 cat fetuses or neonates [13 for rhodamine (DiI) experiments, seven for normal ^{3}H-leucine injections, and 13 for subplate ablations, three of which also received ^{3}H-thymidine injections] and 22 ferret fetuses or neonates (DiI experiments) are included in this study. The conventions for determining fetal ages differ slightly between the two species: in the cat the day of breeding is termed E1 (gestation is 65 d) (Luskin and Shatz, 1985a), whereas the day of breeding in ferrets is E0 (41 d gestation) (McConnell, 1988; Jackson et al., 1989). Some results from animals used for DiI experiments were reported in a previous study (Kim et al., 1991) that analyzed the morphologies of DiI-labeled cortical growth cones.

Surgical procedures. Fetuses were exteriorized by cesarian section for kainic acid or ^{3}H-leucine injections using sterile surgical procedures (Luskin and Shatz, 1985a) on timed-pregnant cats. Cats were anesthetized initially with ketamine hydrochloride (20 mg/kg, i.m.) and acepromazine (0.2 mg/kg, i.m.) and anesthesia was maintained with halothane (0.5–2.5% in O_2) delivered through an endotracheal tube. The uterine horns were exposed by a midline abdominal incision. Three E32 fetuses received injection of ^{3}H-thymidine (750 μCi in 0.15 ml of saline; New England Nuclear NET 027Z; specific activity, 50–80 Ci/mmol) by injection through the uterine wall into the amniotic fluid, in order to birthdate layer 6 neurons (Luskin and Shatz, 1985a). For intracerebral injections, terbutaline sulfate (0.03 mg/kg, i.v.; Ciba-Geigy) was delivered prior to each fetal manipulation to prevent uterine contractions. An incision was made in the uterine musculature to expose the head of each fetus, and a small hole or flap was made in the cartilaginous skull overlying the cerebral cortex. For ^{3}H-leucine injections, a 1.0 μl Unimetrics syringe was used to make one or two injections of 0.1 μl of ^{3}H-leucine (500 μCi/μl in saline; Amersham TRK-170; specific activity, 50–80 Ci/mmol) at depths of roughly 1–1.5 mm into the visual cortex. For kainic acid ablations, injections of kainic acid (Sigma; 10 mg/ml in saline, 0.5–0.8 μl per injection) were delivered through a 5 μl Unimetrics syringe into the occipital region only (single injection) or into both the occipital and temporal cortex (one injection each). A small amount of fluorescein-labeled latex microspheres (Luma-Fluor; 100 μl of microspheres/1 ml kainic acid solution) was diluted into the kainic acid solution to mark injection sites. Following injection, the overlying skin of the fetus was closed with a sterile cyanoacrylate glue (Histoacryl, Trihawk International), and the uterine and abdominal musculature and skin were sutured closed. For the delivery of experimental kittens (usually on E64, 1 d prior to their normal delivery date), anesthesia was induced with halothane and maintained as above. Fetuses were removed rapidly by cesarian section using sterile surgical technique; fluids were removed from their throats by suction and the newborns were rubbed vigorously to facilitate breathing. After surgery, animals were treated with analgesics if necessary and were monitored for at least 36 hr and up to 10 d in a veterinary intensive care unit staffed 24 hr/d. Experimental kittens were supplemented by tube feeding every 4 hr. Subsequently kittens received one or two injections of ^{3}H-leucine into the visual cortex as described above. The locations of these injections overlapped spatially with the regions that received kainic acid earlier, as verified by the presence of rhodamine-labeled microspheres coinjected with the kainic acid.

Perfusion and histology. Fetuses were anesthetized transplacentally through maternal anesthesia and postnatal animals received an overdose of Nembutal (100 mg/kg, i.p.). Animals were perfused through the heart with 0.1 M sodium phosphate buffer (pH 7.4, 4°C) followed by 4% paraformaldehyde in the same buffer. Animals with successful subplate ablations were easy to recognize by the shrunken appearance of the kainate-injected hemisphere. Ablations were successful in 13 of 14 animals; the single animal that lacked a visible lesion was excluded from the analysis. Brains were postfixed in the same fixative, sunk in 20% sucrose in fix, embedded in a mixture of gelatin and albumin, and sectioned on a freezing microtome at 25 μm. Sections were mounted and dried onto chrome-alum–subbed slides, and inspected for the presence and location of fluorescein-labeled microspheres if a kainic acid injection had been made. The sections were then defatted in a series of ethanols and xylenes, rehydrated, dipped in Kodak NTB-2 nuclear track emulsion (1:1 in water), and exposed for 3–6 weeks before developing (Kodak D-19 followed by Kodak Rapid Fix). Selected sections were counterstained in cresyl violet, and then all were dehydrated and coverslipped with Permount. For illustration, low-power dark-field images of autoradiographically labeled sections were obtained through a video camera mounted onto a Nikon TMS dissecting microscope. The images were stored on a Mac II computer using NIH IMAGE, processed using ADOBE PHOTOSHOP, and printed directly from a PICT file. Bright-field and low-power dark-field photomicrographs were taken with a Nikon UFX 35 mm camera and Optiphot microscope. Camera lucida reconstructions of whole sections were made using a 1× objective and a drawing tube.

DiI injections. Animals were perfused transcardially with 4% paraformaldehyde as above, or (in the case of E24 and E27 ferrets) by immersion in 4% paraformaldehyde overnight. The cranium was removed to expose the cerebral cortex. A tiny crystal of DiI was placed on the point of a pin and inserted into each hemisphere in one or more locations; extraneous crystals were carefully washed away. In some animals the DiI crystals were placed very superficially in the cortical plate, so as to label exclusively the axons of cortical neurons without involvement of neurons in the subplate layer. DiI was placed in the cerebral wall at a variety of locations, including posterior–dorsal (presumptive visual) cortex, dorsal cortex at the anterior–posterior midpoint of the brain, and temporal cortex. Brains were stored in fixative containing 0.05% sodium azide for periods of 3–5 weeks at room temperature for young brains (up to E33), and 8–16 weeks at 37°C for older brains. Brains were then embedded in 0.3% gelatin and 30% albumin, hardened with glutaraldehyde, and sectioned at 50–100 μm using a vibratome. Coronal sections were typically made of younger brains (E24–E37) in order to visualize cortical axons as they approached the internal capsule, whereas older brains (E42–E50) were often sectioned sagittally to view axons descending and turning posteriorly toward the thalamus. Selected sections were counterstained with *m*-phenylenediamine (mPD; Quinn and Weber, 1988; McConnell et al., 1989). To visualize labeled axons, sections were mounted on plain glass slides, coverslipped in glycerol (100%), and viewed using rhodamine (DiI) or fluorescein (mPD) optics on a Nikon Optiphot microscope.

Results

In the adult, neurons of cortical layers 5 and 6 send descending axonal projections to a variety of specific targets (Fig. 1). Most

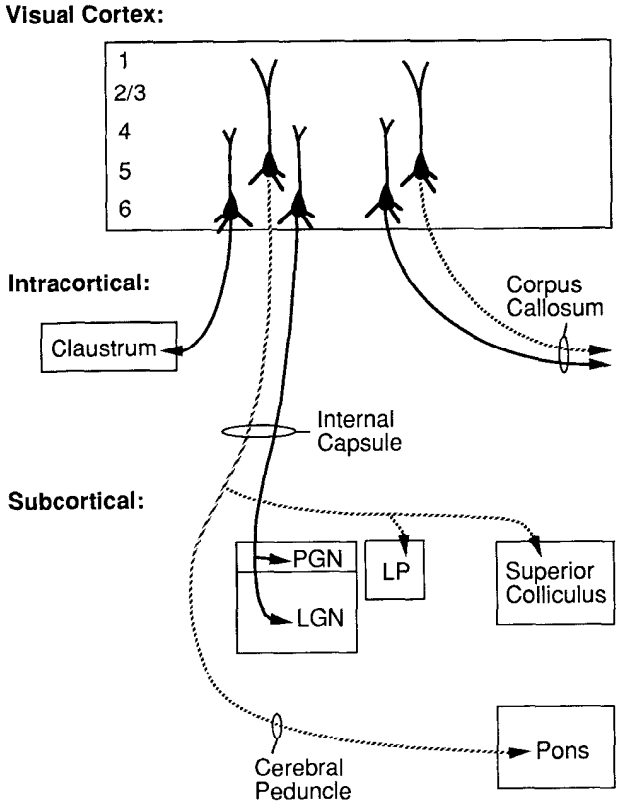

Figure 1. A diagrammatic summary of the axonal pathways and the targets of deep-layer cortical neurons in the visual cortex of the adult cat. The projections of layer 6 neurons are shown with *solid lines*, and the projections of layer 5 neurons, with *broken lines*. Note that individual neurons within cortical layers 5 or 6 do not send axons to all possible subcortical targets; for example, layer 6 neurons project only to the thalamus. *PGN*, perigeniculate nucleus; *LP*, LP–pulvinar complex; *LGN*, lateral geniculate nucleus.

layer 6 neurons project to the thalamus, and a minority project to the claustrum (Gilbert and Kelly, 1975; LeVay and Sherk, 1981; Katz, 1987). Layer 5 neurons extend axons to the superior colliculus and pons (Gilbert and Kelly, 1975), and layer 5 neurons in motor and somatosensory cortex project to the spinal cord as well (Stanfield et al., 1982). The pathways of descending axons extend through the cortical white matter and into the internal capsule, the major gateway between the neocortex and the thalamus. Here the paths diverge, with axons destined for thalamic nuclei and the superior colliculus taking a dorsal route, whereas pontine and spinal axons descend in the cerebral peduncles toward these caudal and ventral targets (compare Fig. 7).

The earliest development of descending cortical connections was traced by placing small crystals of the fluorescent lipophilic tracer DiI into the cerebral wall of fixed embryonic cat and ferret brains. The development of the cerebral cortex of these two carnivore species is highly comparable: they show similar patterns of neurogenesis (Luskin and Shatz, 1985a; McConnell, 1988; Jackson et al., 1989) and axon outgrowth (Kim et al., 1991), although ferrets lead in developmental maturity by about 2 d relative to cats during embryonic periods (e.g., an E24 ferret is comparable developmentally to an E26 cat).

Early development of the internal and external capsules

At the earliest ages studied (E24 cat, E26 ferret), injections of DiI into the temporal cortex labeled axons that extended into the region of the future internal capsule. Figure 2*A* shows an example of an injection of DiI into temporal cortex, revealing many labeled axons and growth cones traversing a region that will later become the internal capsule. Counterstaining with mPD reveals that these unfasciculated axons are pioneering their way through a region dense with cell bodies, as compared to later in development, when the internal capsule is an axon-rich white matter tract. About half of all labeled temporal axons have reached or entered the internal capsule region at this age: of 91 axonal growth cones that were identified following temporal cortex injections in E24 cats or E26 ferrets, 44% were in the intermediate zone of the cerebral wall en route to the internal capsule, 9% were at the entrance, and 47% were within the internal capsule itself. In contrast, axons labeled from dorsal or posterior cortical injections were found in the nascent intermediate zone immediately above the ventricular zone (see Fig. 1 in Kim et al., 1991) but most were distant to the internal capsule: 74% of labeled growth cones ($n = 69$) were found in the intermediate zone of the cerebral wall, 26% were at the entrance to the internal capsule, and 0% had invaded the internal capsule itself. The axons and growth cones labeled by DiI at these early embryonic ages are likely derived from subplate neurons, since at these ages only marginal zone and subplate neurons have become postmitotic, and only these early generated neurons can be retrogradely labeled from the internal capsule (McConnell et al., 1989; Kim et al., 1991).

Our published studies have shown that by E30 in the cat, subplate axons labeled from temporal cortex have entered the thalamus and axons from posterior occipital cortex have reached the internal capsule, as assessed by retrograde labeling with DiI (McConnell et al., 1989). On E33, temporal injection of DiI reveals a second descending pathway: labeled axons ending in growth cones are now apparent in the external capsule (Fig. 2*B*). These results show that the earliest axon pathways through the internal and external capsules are derived from the temporal neurons that are situated closest to these regions; the growth of axons from more dorsal and posterior regions occurs soon thereafter. Previous studies have shown that cortical and thalamic afferents grow concurrently into the internal capsule and thus copioneer this region (McConnell et al., 1989; Ghosh and Shatz, 1992b), although the nucleus (or nuclei) from which these first thalamic axons originate is not known.

Growth of axons from cortical plate neurons

By around E42 in cat or postnatal day 1 (P1) in ferret, injections of DiI into the visual cortex clearly label the pathways between the cortex and the thalamus, both anterogradely and retrogradely. An example of an injection made in a P1 ferret is shown in Figure 2, *C* and *D*: a broad swath of DiI-labeled axons is visible in the cortical intermediate zone, internal capsule, and anterior thalamus, as are a large number of retrogradely labeled neuronal cell bodies located within the lateral geniculate nucleus (LGN). Many labeled axons just anterior to the LGN are branched (Fig. 2*D*), with one branch terminating in a growth cone outside the LGN and the other branch descending in the direction of the cerebral peduncle. The trajectories of these axons suggest that they are anterogradely labeled axons originating in cerebral cortex, but the fact that DiI was also transported retrogradely by

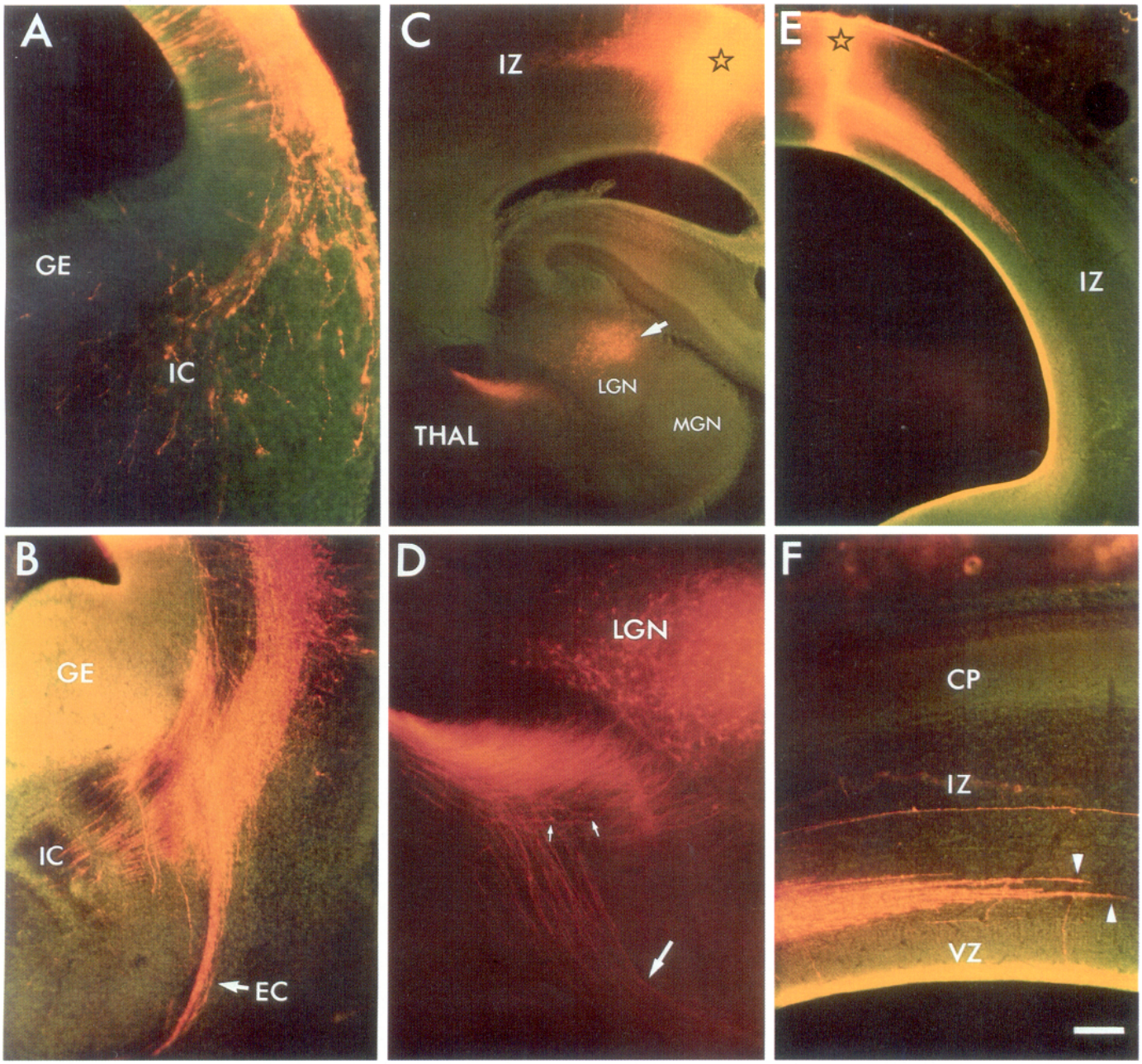

Figure 2. DiI (*orange* in photo) was used to label descending axons in developing cat and ferret. Sections are counterstained with mPD (*green*). *A,* DiI injection into temporal cortex of an E26 cat labels axons and growth cones below the ganglionic eminence (*GE*), in the region of the forming internal capsule (*IC*). Axons at this age are unfasciculated, and the region through which they are traveling is dense with cell bodies; the axon-rich white matter of the mature internal capsule is not yet visible. Coronal section; dorsal is up, and lateral is to the right. *B,* Injection of DiI into temporal cortex of an E33 ferret reveals the early presence of the external capsule (*EC*). Coronal section as in *A. C,* Injections of DiI into the visual cortex and subplate of a P1 ferret. The labeled area below the injection site (*star*) contains radial glial cells that span the cerebral wall from the pial to the ventricular surface. DiI-labeled axons are visible in the cortical intermediate zone (*IZ*) and entering the anterior thalamus (*THAL*) from the internal capsule (sagittal section; dorsal is up, and anterior is to the left). Retrogradely labeled neurons (*arrow*) are present within the LGN but not in the medial geniculate nucleus (*MGN*). *D,* Higher-power photomicrograph through the thalamus shown in *C,* without the mPD counterstain. Retrogradely labeled neurons are visible within the LGN. Many DiI-labeled axons branch anterior to the LGN, and branches terminating in growth cones are visible (*small arrows*); other axons descend toward the cerebral peduncles (*large arrow*). *E,* Injection of DiI restricted to the superficial cortical plate of an E42 cat (injection site is marked with a *star*). Anterogradely labeled axons terminating in growth cones are localized exclusively within the cortical intermediate zone (*IZ*) close to the injection site and cannot be traced into the internal capsule. DiI-labeled radial glia are also visible spanning the cerebral wall at the injection site. Coronal section as in *A. F,* Higher-power view of the axons and growth cones shown in *E.* Axons are localized to the bottom third of the intermediate zone (*IZ*), and growth cones (*arrowheads*) are found at or near the top of the ventricular zone (*VZ*). *CP,* cortical plate. Scale bar: 100 μm for *A*; 250 μm for *B, D,* and *F*; 0.5 mm for *C* and *E*.

Table 1. Summary of normal cortical projection patterns following ^{3}H-leucine injections into visual cortex at different ages

Day of ^{3}H-leucine injection[a]—day of perfusion	^{3}H-leucine–labeled axons										
	CC	Cl	IC	PGN	LP	LGN	SC	CP	Pons	Sep	EC
E50—E51	+	+	+	+	−	−	+	+	−	+	−
E54—E55	+	+	+	+	+	+	+	+	+	+	+
E54—E55	+	+	+	+	+	+	+	+	+	+	+
E57—E59	+	+	+	+	+	+	+	+	±	+	−
P0—P1	+	+	+	+	+	+	+	±	±	−	−
P4—P6	+	+	+	+	+	+	+	±	−	−	−
P28—P30	+	+	+	+	+	+	+	±	−	−	−

+ indicates the presence of a robust projection. ± indicates the presence of a weak projection. Note that for projections to the cerebral peduncles and pons, the weak labeling seen at postnatal ages is probably due to the failure of a single ^{3}H-leucine injection to reveal the full presence of these known pathways. − indicates the absence of labeling above background. CC, corpus callosum; Cl, claustrum; IC, internal capsule; PGN, perigeniculate nucleus; LP, lateral posterior nucleus–pulvinar complex; LGN, lateral geniculate nucleus; SC, superior colliculus; CP, cerebral peduncle; Sep, transient septal pathway from corpus callosum through fornix; EC, external capsule. Note that table includes the normal targets of all cortical projection neurons. The descending targets of layer 6 are the LGN, claustrum, PGN, and LP, and of layer 5, the SC and pons.

[a] Each animal received a single injection of ^{3}H-leucine (*0.1 μl, 500 μCi/μl* in saline; see Materials and Methods).

thalamic neurons at these ages (Fig. 2*D*) makes it impossible to identify definitively the origin of any given axon or growth cone. However, since thalamic axons in adults are not known to extend descending axons, we consider it most likely that the DiI has revealed the forming corticofugal pathways that in the adult normally descend to these regions.

We wondered whether the axons of subplate neurons, cortical plate neurons, or both had reached the LGN at this stage in development. To visualize exclusively the axons of cortical plate neurons and not those of subplate neurons, DiI was injected superficially into the cortical plate (Fig. 2*E*). At this age (E42 cat), many thalamic axons have arrived in the subplate region and are waiting below the cortical plate, but have not yet invaded it (Shatz and Luskin, 1986; Ghosh and Shatz, 1992b): the absence of retrograde labeling in the thalamus (data not shown) confirmed that the DiI injections were confined strictly to the cortical plate and that they did not impinge on the subplate. (The labeling below the cortical plate in Fig. 2*E* is due to radial glial fibers that run from the injection site in a vertical swath from the pial surface to the ventricular zone.) DiI labeling of the cortical plate reveals that at E42, the axons of cortical plate neurons have not yet exited the telencephalon (Fig. 2*E*), but are still traveling en route toward the internal capsule. The axons are localized at the bottom of the intermediate zone; they terminate in growth cones that lie at or near the top of the ventricular zone (Fig. 2*F*). Quantitative analysis of the positions of growth cones revealed that at E42 in cat, 100% were in the cortical intermediate zone (n = 26) and none had reached the internal capsule. A few days later, at E45, 66.7% (n = 42) of growth cones labeled by superficial DiI injection were in the intermediate zone and 33.3% were found at the entrance to the internal capsule.

At E50, DiI-labeled growth cones were finally present in the thalamus. It was, however, impossible to identify the labeled growth cones found in the thalamus as originating exclusively from the cortical plate rather than from the subplate, since LGN neurons were retrogradely labeled in every experiment. While this might suggest that the DiI injections had impinged upon the subplate, at this and later ages there is an alternate explanation: LGN axons begin to invade the cortical plate at E50 (Ghosh and Shatz, 1992a), and LGN neurons can be retrogradely labeled even after superficial DiI injections. Thus, we could not rely on our internal control (lack of retrograde label in the LGN) at these later ages to evaluate the accuracy of DiI placement. Nevertheless, given our observations at earlier ages (see above), it seems reasonable to conclude that by E50 the axons of cortical plate neurons have reached the thalamus.

These results show that the growth of axons from the cortical plate occurs relatively late in the embryonic period, relative to the time at which these cells are generated. For instance, occipital layer 6 neurons are born during the period between about E30 and E37 in the cat (Luskin and Shatz, 1985a), yet axons from the cortical plate appear not to reach the thalamus until weeks later, at roughly E50. In contrast, as early as E42 when injections of the cerebral wall also include the subplate, DiI-labeled axons are seen within the thalamus just anterior to the LGN (Fig. 2*C,D*), in the cerebral peduncles (Fig. 2*D*), and in the colliculus; indeed, we have observed a few DiI-labeled axons in the anterior colliculus and near the pons as early as E36 (not shown). These results suggest that early in development only the axons of subplate neurons descend from the telencephalon, and that these axons extend to a variety of subcortical targets. We do not know whether subplate axons actually invade some or all of these targets, although the branched pattern of axons in Figure 2*D* suggests that at least at early times their axons do not enter the LGN, but rather may accumulate just outside this nucleus.

Innervation of the LGN and other subcortical targets

It was not possible to use DiI to study the growth of cortical axons into thalamic nuclei and other targets at ages past E50 in the cat, because DiI can travel both anterogradely and retrogradely; the presence of thalamic axons in the cortical plate resulted in the retrograde labeling of thalamic neurons and their local collaterals, as well as cortical axons. Injections of the anterograde tracer ^{3}H-leucine were therefore made into the visual cortex of kittens at several ages (Table 1) to label exclusively cortical axons. The injections encompassed both the cortical plate and subplate.

These autoradiographic tracing studies revealed that cortical axons invade the LGN, the major target of layer 6 neurons of visual cortex (Gilbert and Kelly, 1975), during late embryonic periods in the cat. At E51, ^{3}H-leucine injections revealed an immature pattern of subcortical projections: radioactive label is present in the internal capsule and within the thalamus next to the LGN, but is not present within the LGN itself. The label is concentrated just outside the anterior and medial boundaries of the nucleus, in the region of the perigeniculate nucleus (PGN) (Fig. 3*A*). Label is also visible in the cerebral peduncle (Fig. 3*A*), the superior colliculus, and the claustrum (another target of layer 6 neurons) (Table 1). Although labeled axons were seen within the cerebral peduncles descending to just outside the pons, no label was present within the pons itself (Table 1). Thus, while descending axons are present in regions as far from visual cortex as the colliculus and pons, they have invaded only some of their adult targets (such as the superior colliculus), whereas others (the LGN and pons) have been reached but not yet invaded. [Previous studies in the rat have also revealed a waiting period outside of the pons (O'Leary and Terashima, 1988).] The corpus callosum is well formed by E51; labeled axons are present but,

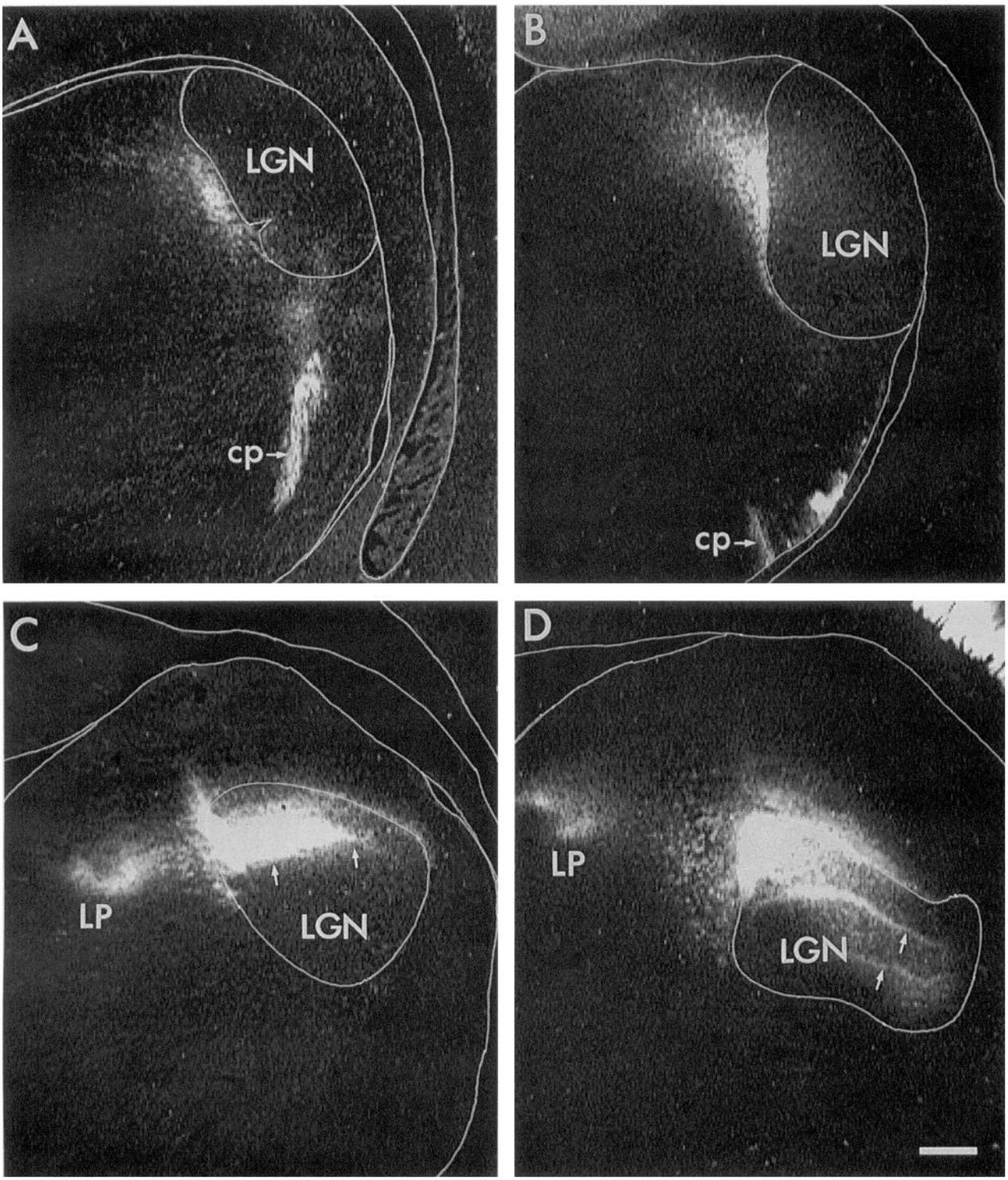

Figure 3. Autoradiographs of sections (shown using dark-field optics) through the thalamus of normal cats that received injections of the anterograde tracer ^{3}H-leucine into visual cortex at a variety of times during development. *A,* Projection pattern at E51 following injection of ^{3}H-leucine injection into the visual cortex on E50. Radioactive label (*white*) is found within the thalamus concentrated outside of the medial edge of the LGN and descending in the cerebral peduncle (*cp*). *B,* Autoradiograph of the thalamus from a fetal cat at E55 that was injected with ^{3}H-leucine on E54. As at E51, label is present outside the LGN and in the cerebral peduncle; at this age, however, label is now also visible within the body of the LGN. *C,* Projection pattern at P1 following ^{3}H-leucine injection on P0. Radioactive label is concentrated within the interlaminar zones of the LGN (*arrows*). Patchy projections to the LP-pulvinar complex (*LP*) are also visible more medially. *D,* Projection pattern at P6 following ^{3}H-leucine injection on P4. Label is still concentrated in the interlaminar zones (*arrows*). Projections to LP-pulvinar and the PGN (anterior and medial to the LGN) are also apparent. In these coronal sections, dorsal is to the top and medial is to the left of each image. Scale bar, 0.5 mm.

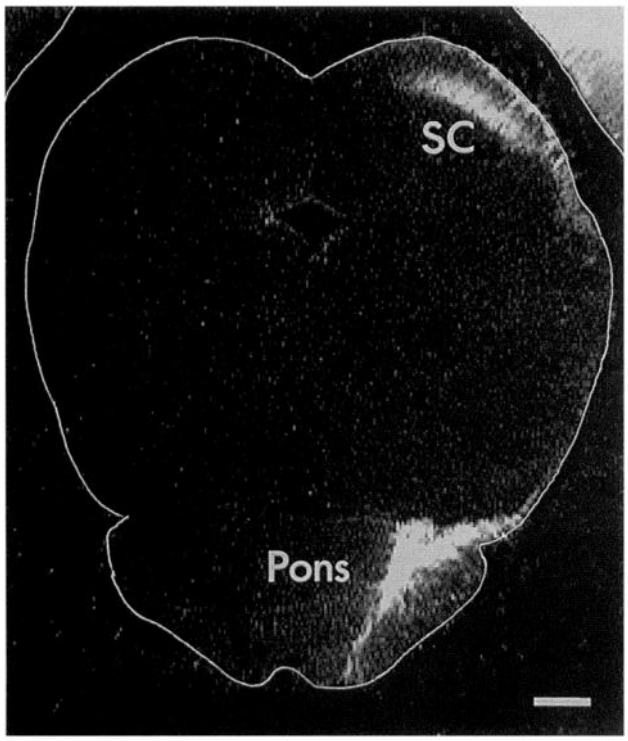

Figure 4. Pattern of anterograde labeling in the midbrain of an E55 cat fetus following a ^{3}H-leucine injection into visual cortex on E54. Label is apparent within the superior colliculus (*SC*), where it is concentrated most heavily in the superficial layers, and also within the pons. Coronal section; dorsal is up. Scale bar, 0.5 mm.

as described in previous studies, axons that enter the intermediate zone of the contralateral hemisphere have yet to invade the cortical plate and will not do so until postnatal periods (Innocenti, 1981).

By E55, radioactive label is visible within all the normal subcortical targets of visual cortical neurons (Table 1). Figure 3*B* shows that a light smattering of label is present within the interior of the LGN, indicating that at least some axons have entered this structure. Labeled axons are clearly present in the superficial layers of the superior colliculus (Fig. 4), and axons are now apparent within the pons itself (Fig. 4, Table 1). A similar pattern is seen at E59 (Table 1), except that the density of labeling within the LGN has increased significantly. By P1, an age at which the A, A1, and C layers of the LGN are clearly visible in cresyl violet–stained sections (Shatz, 1983), many cortical axons within the LGN are concentrated in the interlaminar zones separating the layers (Fig. 3*C*). Also visible in Figure 3*C* are mature-looking patchy projections to the lateral posterior nucleus (LP)–pulvinar complex. By P6 the concentration of cortical afferents in interlaminar zones of the LGN is particularly marked (Fig. 3*D*). In adult animals, cortical afferents are distributed evenly across LGN layers and show no such concentration in interlaminar regions. The afferents achieve their adult distribution within the LGN by P30 (data not shown).

Thus, during the development of subcortical projections from the visual cortex, axons first arrive at a major target, the LGN, between roughly E30 and E42. Axons invade the body of the LGN beginning at about E55. These observations imply that there is a 2–3-week-long "waiting period" during which cortical axons gather anterior and medial to the LGN, in the region of the PGN, but do not invade the LGN itself. It is not clear,

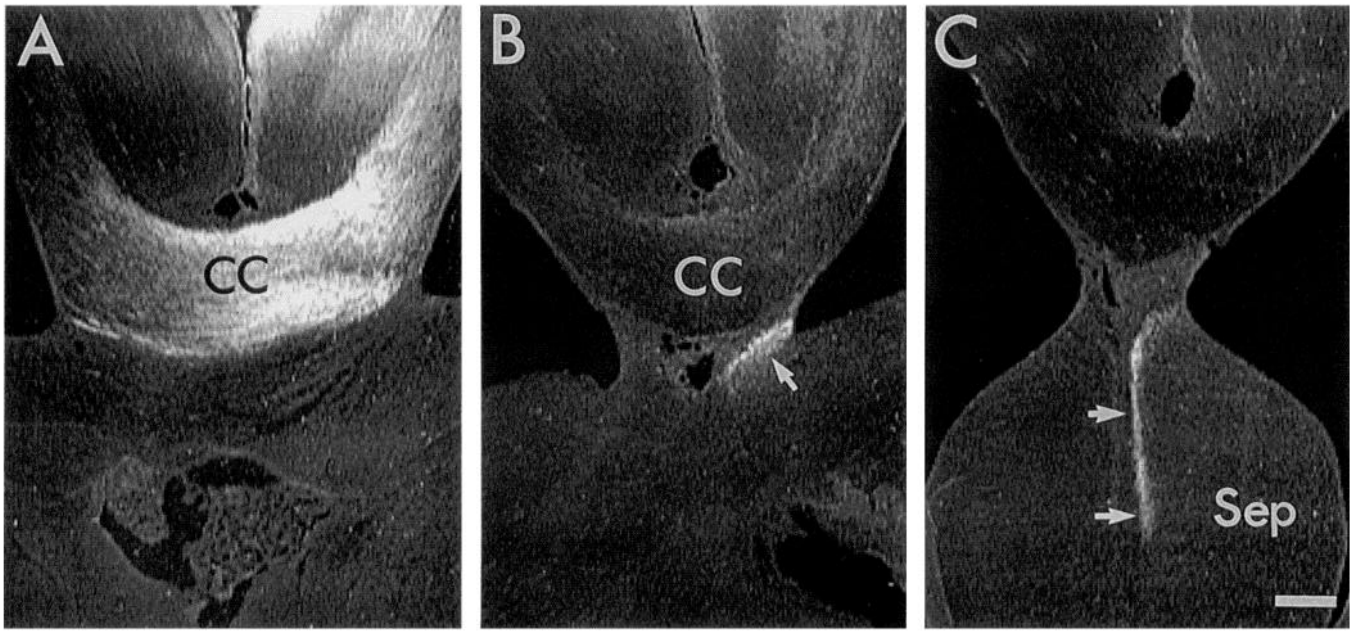

Figure 5. Transient projections from visual cortex through the fornix and into the septum of fetal cats, following ^{3}H-leucine injection into visual cortex. The three coronal sections are from a single animal, aged E51, with *A* the most posterior and *C* the most anterior section. *A,* Labeled axons originated in the right visual cortex and travel toward the corpus callosum (*CC*). *B,* A small projection is visible passing from the ventrolateral border of the callosum into the fornix (*arrow*). *C,* Labeled axons enter the septum (*Sep*) and descend along its medial border (*arrows*). Scale bar, 500 μm.

however, whether this waiting period is real or only apparent. Retrograde labeling experiments (McConnell et al., 1989) and DiI labeling of cortical plate neurons show that the earliest axons to grow into the thalamus belong to subplate neurons. As we have shown here, the axons of cortical plate cells reach the nucleus only from about E50 on. It is most likely that the arrival of cortical plate axons on or after E50 is shortly followed by their invasion of the LGN by E55. The ultimate targeting of subplate axons remains unclear. We do not know whether they remain outside of the LGN or whether they too invade this nucleus, and if so, when. Thus, although cortical plate axons appear to grow into the LGN immediately after their arrival in the thalamus without waiting, we cannot unambiguously identify the origin of the first axons to grow into this structure.

Transient projections

Injections of ^{3}H-leucine into normal visual cortex also revealed the presence of two sets of transient projections in the cat during fetal periods. Injections in animals aged E51–E59 consistently revealed a subset of axons that traversed a medial pathway toward the corpus callosum, but at the ventrolateral border of the callosum, where most axons crossed into the opposite hemisphere as they do in adulthood, a small group were diverted ventrally into the subjacent fornix (Fig. 5*A*,*B*). Within the fornix, labeled afferents ran anteriorly and entered the septum, where they descended ventrally along its medial aspect (Fig. 5*C*). It is not known whether visual axons form transient synaptic connections in the septum. The septal projection from visual cortex was absent from animals aged P1 or older (Table 1). The region bordering the corpus callosum through which these early "wayward" axons travel has been thought to be impenetrable to axon growth due to the presence of a repulsive "glial sling" (Silver et al., 1982). The presence of a transient pathway through this region suggests that the sling is unable to prevent completely the growth of early axons from the corpus callosum into the fornix, or alternatively, that the axons traverse this region before the sling appears.

A second transient projection was observed in E55 animals, but not at or after E59. Labeled afferents were seen anterior and medial to the claustrum in the external capsule (data not shown). Transient projections from visual cortex to the extreme and external capsules have been previously described in fetal rabbits by Distel and Holländer (1980). We did not, however, find evidence in the cat for other transient projections that have been described in other species. We observed no projection from visual cortex to cerebellum as described previously for rabbit (Distel and Holländer, 1980), or to the spinal cord as described in rat (Stanfield et al., 1982; Stanfield and O'Leary, 1985). However, following a single injection of ^{3}H-leucine, despite the reliable presence of robust projections to the thalamus and superior colliculus, the density of label within fiber tracts descending toward the pons was often quite low, so it is conceivable that some cortical axons do project caudally beyond the pons. Larger or multiple injections might have revealed such projections, since in neonatal animals single small injections of ^{3}H-leucine failed to reveal a clear projection to the pons (Table 1), a known target of a subset of visual cortical axons.

Kainic acid ablations of subplate neurons

Characterization of the normal development of descending axons from visual cortex revealed that subplate neurons pioneer the initial subcortical axon pathways, whereas the growth of axons from the cortical plate is significantly delayed relative to the initial establishment of subcortical pathways. To test the hypothesis that the early-growing subplate axons are required for the formation of normal subcortical projections from the visual cortex, we ablated subplate neurons with the neurotoxin kainic acid early in development, between E37 and E42, the earliest ages at which kainic acid lesions are effective (Ghosh et al., 1990; Ghosh and Shatz, 1993). As shown above, the period around E37–E42 is just after the cortical plate axons begin to elongate within the intermediate zone, but prior to their arrival in the internal capsule. In most animals, two injections of kainic acid were made, one into occipital and one into temporal cortex, in order to remove subplate neurons along the entire pathway from the visual cortex to the internal capsule. [Note that in previous studies, a single and smaller kainic acid injection only deletes a localized region of the subplate (Ghosh et al., 1990; Ghosh and Shatz, 1993).] These paired injections resulted in the massive deletion of subplate neurons throughout the lesioned hemisphere, including nonvisual areas. The effects of subplate ablation on cortical axogenesis were assessed by allowing lesioned animals to develop until around birth, whereupon we injected ^{3}H-leucine into the visual cortex to trace the pattern of the descending projection from visual cortex.

Kainic acid injections made between E37 and E42 deleted subplate neurons without destroying the deep layers of the cerebral cortex. Figure 6*A* shows a low-power view of a neonatal cat brain following injection of kainic acid into the subplate of one hemisphere on E42. The lesioned hemisphere is shrunken relative to the control side, but the primary loss of cells has occurred in the subplate. The cortical plate is of roughly normal thickness, and at the ages studied here all six layers are histologically distinct. In previous studies we have demonstrated that the number of subplate neurons is severely reduced following kainic acid injection, yet the presence of radial glial fibers and the migration of cortical neurons are unaffected (Ghosh et al., 1990; Ghosh and Shatz, 1993). To show that deep-layer cortical plate neurons can also survive kainic acid treatment, fetal cats were injected with ^{3}H-thymidine on E32, a time at which many layer 6 neurons are generated, and animals subsequently received kainic acid lesions on E42. In Figure 6, *B* and *C* show an example of one such animal on E64: although the numbers of ^{3}H-thymidine–labeled cells are certainly reduced relative to the contralateral control hemisphere, it is clear that ample numbers of thymidine-labeled neurons are still present. Importantly, these brains represent the worst possible outcomes of kainic acid lesions: the litter of fetuses that received both ^{3}H-thymidine and kainic acid injections had the most severely shrunken hemispheres of any of the kainate-treated animals, and the thickness of the intermediate zone was very small, indicating the most extensive depletion of cells among all the cases. Yet even in these animals a substantial population of deep-layer neurons remains after kainic acid treatment. [Although it is possible that some of the missing neurons were affected directly by the kainic acid injection, it is worth noting that the diminution in cell number may also have resulted from long-term retrograde losses of neurons deprived of their targets. Indeed, we have observed a shrinkage and loss of cells in the LGN in kainate-treated brains, presumably due to the failure of LGN axons to innervate visual cortex (Ghosh and Shatz, 1993).] The survival of a substantial population of thymidine-labeled neurons encouraged us

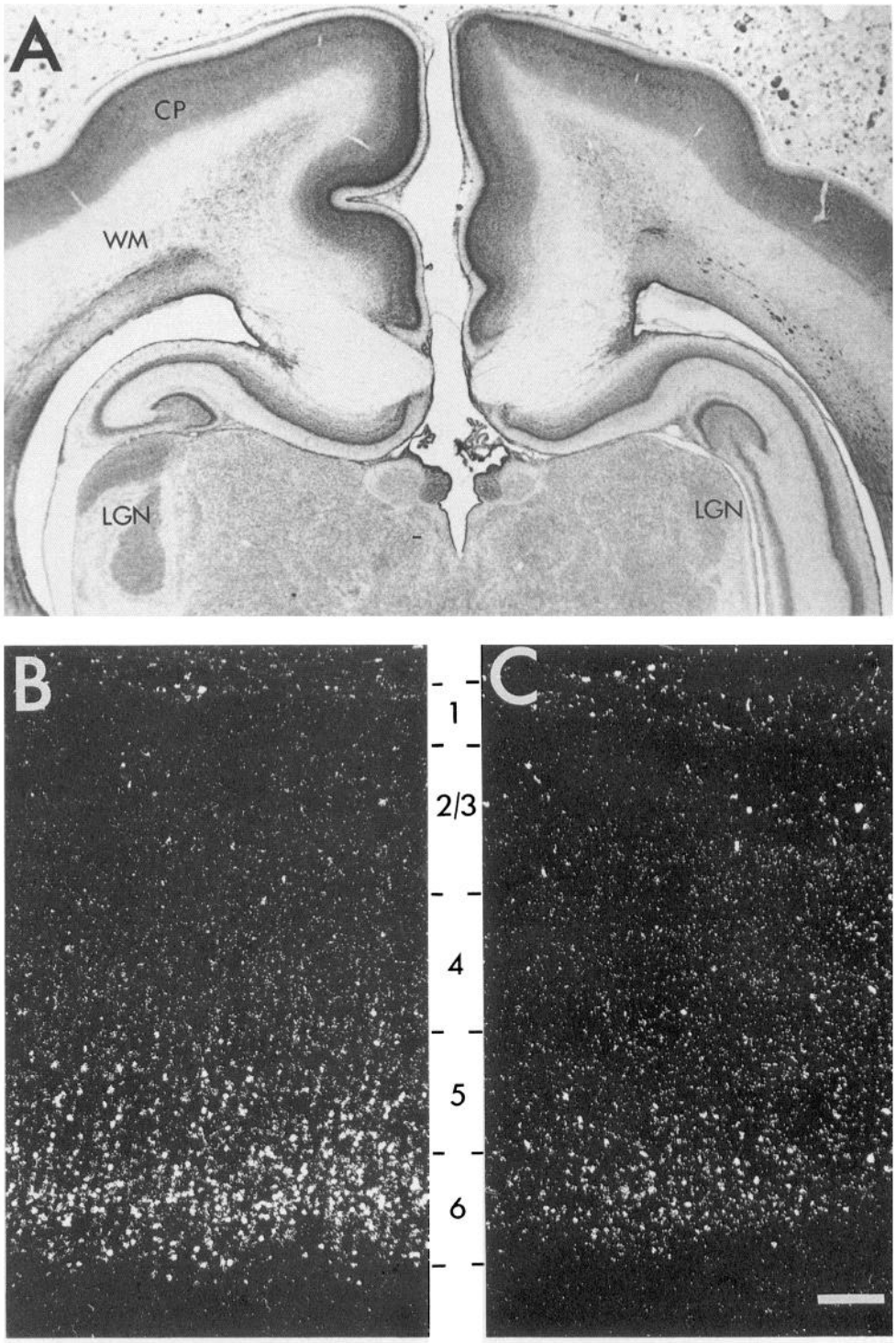

Figure 6. The visual cortex following kainic acid ablation of subplate neurons. *A,* Cresyl violet–stained coronal section through the brain of an E64 kitten that received injections of kainic acid 27 d earlier, on E37 (results from the same brain are shown in Fig. 9). The injected hemisphere (*right*) is somewhat smaller than the control side (*left*), and sulci and gyri are less pronounced. However, the cortical plate (*CP*) is of roughly equivalent thickness in both hemispheres. The LGN in the ablated hemisphere is markedly reduced in size compared to the control side, as previously reported for kainic acid ablations (Ghosh and Shatz, 1993). *WM,* white matter. Coronal section; dorsal is up. *B* and *C,* Deep-layer neurons are present in both control and kainic acid–injected hemispheres, as revealed by ^{3}H-thymidine autoradiography. ^{3}H-thymidine was injected into fetal kittens on E32, a time at which many layer 6 neurons are generated; animals subsequently received kainic acid lesions on E42 and were perfused on E64. *B,* Control hemisphere: heavily labeled neurons are found primarily in cortical layer 6 (*white dots* in dark-field optics). *C,* Heavily labeled layer 6 neurons are also present in the kainate-lesioned hemispheres, although their numbers appear somewhat reduced compared to the control side. Note that these lesions produced the most extreme effects of any discussed in this paper, and thus represent the worst possible effects on the loss of layer 6 neurons. A more typical result is visible in *A,* in which layer 6 is essentially normal (with the exception of the small region at the apex of the lateral gyrus). Scale bar: 1 mm for *A*; 100 μm for *B* and *C*.

to study the effect of subplate ablation on the formation of axonal connections by deep-layer cells.

Development of subcortical axonal projections in the absence of subplate neurons

The development of cortical axons in ablated animals was studied by making injections of ^{3}H-leucine into the visual cortex at around the time of birth, at least 3 weeks after kainic acid treatment. Most animals received a pair of ^{3}H-leucine injections, for two reasons. First, our studies of normal development showed that although a single injection reliably labels the thalamic and collicular projections in every case, the projection to the pons was more variably labeled; hence, two injections were made to ensure the complete labeling of subcortical projections. Second, in view of the diminished numbers of deep-layer cortical neurons in kainate-treated brains described above, we wished to ensure that a very large population of cortical neurons, encompassing an area of several millimeters, received ^{3}H-leucine for transport. In every case in which subplate neurons were ablated, these injections resulted in the strong and clear labeling of a distinct intracortical pathway that descended through the internal capsule and into the thalamus (compare Figs. 7, 9), in-

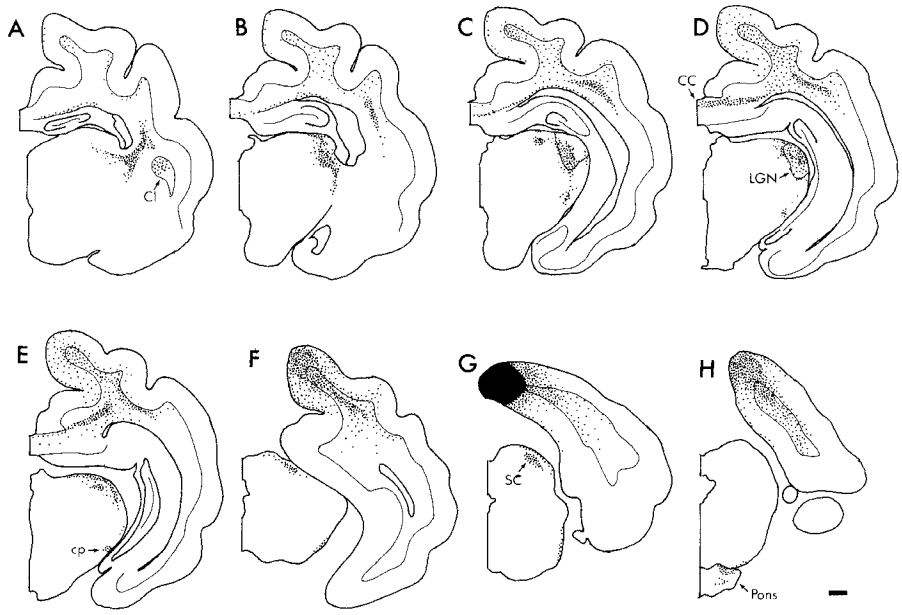

Figure 7. Camera lucida reconstruction of a normal axon projection pattern in a P8 kitten that received kainic acid injections into the visual subplate on E40, and injection of ^{3}H-leucine into visual cortex on P6. *A* is the most anterior section and *H* is the most posterior. *Dots* represent the position of ^{3}H-leucine–labeled axons, and their density reflects the density of label observed. The ^{3}H-leucine injection site in visual cortex is shown in *G*. A projection that issues from the injection site can be observed within the white matter (*F*). The labeled axons travel anteriorly within the white matter (from *G* to *B*) to enter the internal capsule (*A*). This descending pathway then turns posteriorly to reach and invade the LGN (*C, D*), and to descend farther to the superior colliculus (*SC*) and pons. The projection to the superior colliculus follows a dorsal pathway: labeled axons travel from the thalamus (*C, D*) through pretectal areas (*E, F*) and into the superior colliculus (*G*). Labeled projections are also visible descending within the cerebral peduncle (*cp*; *C–G*) and entering the pons (*H*). Within the forebrain, projections to the claustrum (*Cl*) and into the corpus callosum (*CC*) are also present (*A* and *D*). Scale bar, 1 mm.

dicating that this dual injection technique could reliably visualize the projections of deep-layer cortical neurons.

The ablation of subplate neurons early in development altered the patterning of visual cortical axons in terminal regions, but only in half of the 10 animals in which the subplate was successfully lesioned. In the other half of lesioned animals, projections of visual cortical axons were apparently normal (bottom five animals in Table 2). Figure 7 shows the pattern of labeling in one such animal on P8 that had a normal projection pattern; this animal received a subplate ablation on E40 and injections of ^{3}H-leucine into visual cortex on P6. (Birth is at 65 d in the cat.) Label was present in the LGN and LP-pulvinar of the thalamus (Fig. 7*C,D*) and in the superior colliculus (Fig. 7*G*). Labeled afferents also traveled ventrally and caudally in the cerebral peduncle to reach the pons (Fig. 7*D–H*). In addition, label was present in the corpus callosum (Fig. 7*D*) and the claustrum (Fig. 7*A*). This pattern was observed in all five of the animals that showed normal projections (Table 2).

In the remaining half of the animals, despite the clear presence of a pathway from cortex to thalamus, cortical axons failed to innervate their normal subcortical targets, including the LGN, superior colliculus, and pons (top five animals in Table 2). Figure 8 shows two examples of anterograde labeling in the thalamus at birth of animals in which the subplate was ablated by kainic acid injection at E40. In both cases, radioactively labeled axons entered the thalamus through the internal capsule and gathered at the anteromedial borders of the LGN in the region of the PGN, but they did not enter the body of the LGN. This situation is in marked contrast to that in normal animals at comparable ages, in which cortical axons have grown well into the LGN and are concentrated in interlaminar regions (see Fig. 3*C,D*). However, the pattern of labeling in these subplate-ablated animals on P0 is remarkably similar to the pattern seen in normal animals 2 weeks earlier (see Figs. 3*A,B*; 8).

In each case in which subplate ablation resulted in a failure of LGN innervation, cortical projections to and within other subcortical regions were also missing (Table 2). The patterning of the subcortical projections in one such animal is reconstructed in the camera lucida drawing of Figure 9: a robust pathway extends from visual cortex, through the internal capsule, and into the thalamus, but the labeled axons simply stop outside the LGN (Fig. 9*D,E*). They also fail to descend beyond, to their normal targets in the superior colliculus and pons. In two of five animals that showed an abnormal innervation pattern, a projection into the cerebral peduncles was completely absent (Fig. 8*B*, Table 2); in the other three, label was present initially in the tract near the LGN, but disappeared farther ventrally and caudally (Figs. 8*A*, 9*D–G*). In none of the five cases was there a normal dorsal axon pathway (Fig. 9*D–H*) that in control hemispheres extends past the LGN, through the pretectal nuclei, and into the superior colliculus.

In marked contrast to the abnormal subcortical projections in these five animals, visual cortical projections to targets within the cerebral hemispheres appeared relatively normal: callosal projections from the lesioned hemisphere formed in all animals, as did the innervation of the claustrum, another normal target of cortical layer 6 (Table 2, Fig. 9*A–C*). Transient projections from the cortex to the septum or external capsule were not present in any of the kainate-ablated animals, but all were examined on or after E64, times by which these transient projections have normally been eliminated. Thus, in half of kainate-lesioned animals, the ablation of subplate neurons severely disrupted the formation of normal subcortical connections, while leaving intracortical pathways intact.

Table 2. Summary of cortical projection patterns following subplate ablations

Day of kainic acid injection—day of perfusion	Region and amount of kainic acid injection	Amount ^{3}H-leucine injected[a]	^{3}H-leucine–labeled axons								
			CC	Cl	IC	PGN	LP	LGN	SC	CP	Pons
E37—E64	Occipital, 0.5 μl Temporal, 0.5 μl	1 × 0.1 μl	+	+	+	+	−	−	−	+	−
E37—E64	Occipital, 0.5 μl Temporal, 0.5 μl	1 × 0.1 μl[b]	+	±	+	+	−	−	−	−	−
E40—P0	Occipital, 0.5 μl Temporal, 0.5 μl	2 × 0.1 μl	+	+	+	+	+	−	−	+	±
E40—P0	Occipital, 0.5 μl Temporal, 0.5 μl	2 × 0.1 μl	+	+	+	+	−	−	−	−	−
E43–P6	Occipital, 0.8 μl	2 × 0.1 μl	±	+	+	+	+	−	−	+	−
E35—P1	Occipital, 0.5 μl	2 × 0.1 μl	+	+	+	+	+	+	+	+	+
E37—P3	Occipital, 0.5 μl Temporal, 0.5 μl	2 × 0.1 μl	+	+	+	+	+	+	+	+	+
E38—P0	Occipital, 0.5 μl Temporal, 0.5 μl	1 × 0.1 μl	+	+	+	+	+	+	+	+	+
E40—P8	Occipital, 0.5 μl Temporal, 0.5 μl	2 × 0.1 μl	+	+	+	+	+	+	+	+	+
E42—P6	Occipital, 0.5 μl Temporal, 0.5 μl	2 × 0.15 μl	+	+	+	+	+	+	+	+	+

+ indicates the presence of a robust projection. ± indicates the presence of a weak projection. − indicates the absence of labeling above background. CC, corpus callosum; Cl, claustrum; IC, internal capsule; PGN, perigeniculate nucleus; LP, lateral posterior nucleus–pulvinar complex; LGN, lateral geniculate nucleus; SC, superior colliculus; CP, cerebral peduncle.

[a] One or two injections of ^{3}H-leucine were made into visual cortex. In cases where two injections were made, they were roughly 1 mm apart.

[b] Animal received an injection of ^{3}H-leucine into dorsoanterior rather than occipital cortex; however, no thalamic or other subcortical projections were observed.

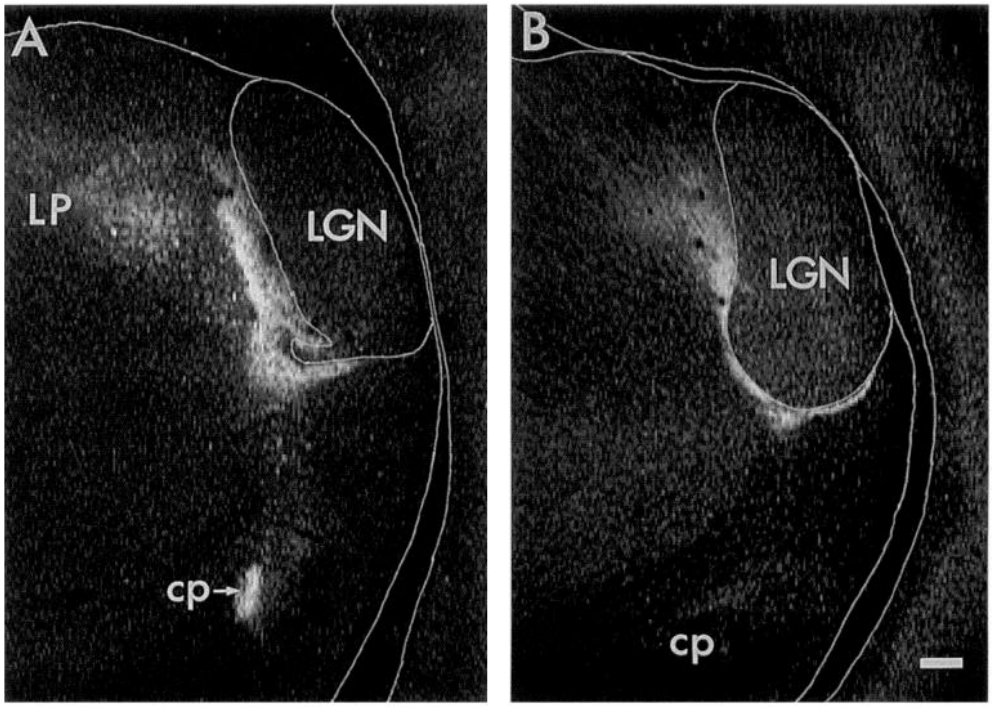

Figure 8. Two examples of abnormal cortical projections to the thalamus at P0, following kainic acid ablation of subplate neurons in embryonic periods (autoradiographs of coronal sections shown in dark-field optics). In both *A* and *B*, ^{3}H-leucine–labeled axons surround the LGN but have failed to invade the body of the nucleus. *A*, P0 cat that received injections of kainic acid into the subplate on E40. Radioactive label is apparent outside (but not within) the LGN, within the LP–pulvinar (*LP*), and descending in the cerebral peduncle (*cp*). *B*, P0 cat that received kainic acid on E40. Here label is confined to the region surrounding the body of the LGN. Scale bar, 250 μm.

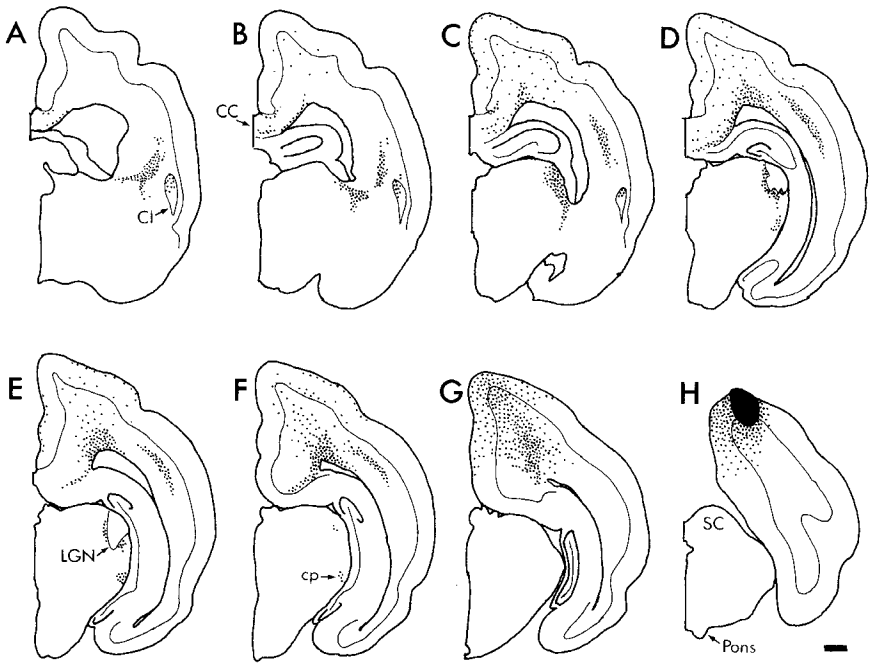

Figure 9. Camera lucida reconstruction of an abnormal axonal projection pattern following early subplate ablation. The axons, revealed with ^{3}H-leucine autoradiography, have failed to innervate their normal subcortical targets, but their projection to forebrain structures appears unaltered. This series of coronal sections was taken at E64 following kainic acid injections into the subplate on E37. *A* is the most anterior section and *H* is the most posterior. *Dots* represent the position of ^{3}H-leucine–labeled axons resulting from an injection of visual cortex at E63, and their density reflects the density of label observed. The site at which the ^{3}H-leucine tracer was injected is shown in *H*. The descending projection can be observed within the white matter in from *G* to *B*, at which point labeled axons enter the internal capsule (*B, A*). Visual cortical axons surround the LGN (*D, E*), and descend for a short distance within the cerebral peduncle (*cp*; *E, F*), but the projection disappears in the midbrain and never reaches the superior colliculus (*SC*) or pons (*H*). Note, however, the normal pattern of projections within the forebrain to the claustrum (*Cl*; *B, C*) and corpus callosum (*CC*; *D*). Scale bar, 1 mm.

There was no obvious difference between animals that showed normal versus abnormal subcortical projection patterns. In both cases the kainate-injected hemisphere was visibly shrunken, indicating the absence of the majority of subplate neurons throughout the lesioned hemisphere, and the LGN was significantly smaller on the injected side, as has been described previously (Ghosh et al., 1990; Ghosh and Shatz, 1993) (see also Fig. 6*A*). It was impossible to quantitate the number of subplate neurons remaining following ablation in each animal, so it is possible that the small fraction of subplate neurons that survived kainic acid treatment may have varied among the two populations. However, there was no clear correlation between the age at which the ablation was performed and the outcome (Table 2, E37 vs E43), nor was postablation survival time a factor (Table 2, E64 vs P8).

Discussion

In this study, tract tracing techniques and kainic acid ablations of subplate neurons were used to study the development of descending axon pathways from the cerebral cortex and the possible role played by subplate neurons in this process. Our results show that the timing of outgrowth of axons from the cortical plate is markedly delayed relative to that of the axons of subplate neurons that pioneer the first pathways descending from the cortex. In addition, the subplate ablation experiments suggest that the presence of these subplate pioneers may be at least in part necessary for the establishment of normal connections from the cortex to subcortical targets. Previous studies have shown that subplate neurons are necessary for thalamic axons to leave the waiting zone in the subplate and invade the overlying cortical plate (Ghosh et al., 1990; Ghosh and Shatz, 1993). The temporal mismatch between the arrival of thalamic axons and their eventual targets in layer 4 could underlie the requirement for subplate neurons in this process; subplate neurons apparently form a temporary scaffold for the matching of thalamic afferents with cortical targets. On the other hand, a rationale for a role for subplate neurons in the formation of the reciprocal pathway from the cortex is not immediately apparent since the targets of deep-layer cortical neurons, such as the LGN and superior colliculus, are already present at the time these cortical neurons are generated. However, we have made the surprising observation here that, even though the cortical neurons of layers 5 and 6 are born relatively early, the outgrowth of their axons is substantially delayed. Anterograde transport experiments indicate that their arrival in the thalamus apparently takes place weeks after the neurons themselves have been born and have finished their migrations. Thus, the pioneering axons of subplate neurons may play a crucial role not only in laying down an early pathway that provides a potential link between the later-growing axons of cortical plate and a variety of subcortical targets, but also in somehow enabling ingrowing cortical afferents to invade their thalamic targets.

Normal development of cortical projections

The techniques of DiI labeling and ^{3}H-leucine injection have revealed that the formation of the major subcortical pathways

takes place between the ages of E26 in the cat, at which time the internal capsule is first established, through E55, when cortical axons are first clearly detectable within the LGN, superior colliculus, and pons. The specific pathway from the visual cortex to the LGN is first apparent between E30 and E35, yet cortical axons accumulate just outside the LGN, within the PGN, and do not invade the LGN for roughly 3 more weeks. This delay in ingrowth suggests that cortical axons, like their thalamic counterparts, "wait" for several weeks before invading and innervating their ultimate targets. (In fact, the ingrowth of cortical axons into the LGN, and that of LGN axons into the cortical plate, occurs simultaneously at about E55.) However, we have been able to distinguish directly between subplate and cortical plate axons by the placement of DiI into either the cortical plate alone, or into both the cortical plate and subplate. DiI injections restricted to the cortical plate do not label a complete pathway from visual cortex to the LGN until about E50, suggesting that the axons of cortical plate neurons do not arrive at the LGN until this time. Our previous studies have shown that few if any neurons in the occipital cortical plate can be retrogradely labeled by DiI injections into the LGN prior to E50 (McConnell et al., 1989; Ghosh and Shatz, 1992b). In this study, ^{3}H-leucine injections reveal radioactive label within the LGN for the first time at E55, suggesting that the cortical plate axons, once arrived, invade the LGN shortly thereafter. Thus, the apparent waiting period outside the LGN is most likely due to the presence of subplate axons that have arrived much earlier. Since layer 6 neurons are generated promptly after subplate neurons (subplate, E24–E30; layer 6, E31–E37; Luskin and Shatz, 1985a), the delay in the outgrowth of layer 6 axons is particularly surprising. Given the complexity of the older environment through which the axons of cortical plate neurons must navigate, the possibility arises that they may require special cues for pathfinding. Some of these cues may be provided by the axons of the early-formed subplate pathway.

One simple hypothesis for the guidance mechanisms used by cortical plate axons is that they employ subplate axons as a substrate for growth into the thalamus, superior colliculus, and conceivably other targets. Is there any evidence for a direct interaction between subplate axons and the axons of deep-layer cortical neurons? We have shown here that the intracortical pathway of cortical axons is located deep within the intermediate zone, adjacent to the top of the ventricular zone (Fig. 2*E,F*). Growth cones are often located directly above the ventricular cells (cf. Fig. 1 in Kim et al., 1991). In contrast, during the period of outgrowth of cortical plate axons, subplate axons occupy the central portion of the intermediate zone and are therefore adjacent to but above the descending axons (McConnell et al., 1989; Ghosh and Shatz, 1992b, 1993). Thus, cortical plate and subplate axons apparently are not extensively intermingled into a single fascicle, but they may have ample opportunity to interact along a common interface. However, it is important to note that at the earliest times of cortical plate axogenesis, the first axons to exit the cortical plate would have easy access to subplate axons and could grow along them, while later cortical axons could in turn fasciculate along one another. Such a sequential pathfinding mechanism has been described for the formation of axon pathways in invertebrate nervous systems (Bentley and Keshishian, 1982; Bastiani et al., 1985). It remains to be determined by direct demonstration whether subplate axons and the earliest cortical plate axons do indeed fasciculate with one another.

A test of the pioneer hypothesis

If the early axon pathway laid down by subplate neurons is responsible for guiding the growth of cortical axons to their normal targets, we hypothesized that removing subplate neurons should interfere with the formation of subcortical projections. This indeed was the case, but only in 50% of the experiments. In half of subplate-ablated animals, visual cortical axons were able to grow through the telencephalon, through the internal capsule, and into the thalamus, where they stopped dead. These axons failed to invade their normal target, the LGN, and then failed to travel farther and form a pathway extending into the superior colliculus and pons. These observations generally support the hypothesis that subplate neurons are required for the normal development of subcortical projections from the cortical plate, particularly in the case of layer 5 projections to the superior colliculus, in which the portion of the pathway between the thalamus and colliculus was missing completely in 50% of all animals. However, the situation is not as simple as we had imagined it might be, since layer 6 axons from visual cortex always managed to select the LGN as the appropriate target, but only invaded it half of the time. Taken together, these observations imply that subplate neurons not only play a role in axon pathfinding, but also help to orchestrate the invasion of appropriate thalamic nuclei. This proposed role for subplate neurons in target invasion is reminiscent of their analogous role in the invasion of the cortical plate by waiting thalamocortical axons (Ghosh et al., 1990; Ghosh and Shatz, 1993).

Are these effects specific to the ablation of subplate neurons?

We have proposed above that subplate neurons are involved in the formation of normal subcortical projections; however, there are two possible objections to this conclusion. The first is that since kainic acid injections reduced the numbers of deep-layer neurons, it may have been difficult to label their subcortical projections reliably. We anticipated this possible problem by making two large injections of ^{3}H-leucine, which indeed in every case labeled a robust axon pathway out of the cerebral hemisphere and into the thalamus. This observation indicates that even in the abnormal cases a clear subcortical projection is present, but it has failed to invade its appropriate targets. With regard to more distal targets such as the superior colliculus and the pons, we can only emphasize that the normal cortical projection to the superior colliculus is strong and distinct; it was always labeled with a single injection in normal animals, and was present in half of the ablated animals. The complete absence of a pathway to the colliculus in the affected animals always occurred in tandem with an abnormal corticothalamic projection, in which axons surrounded the LGN but did not invade it. The absence, therefore, of a projection to the colliculus is highly unlikely to have arisen from a problem in labeling this pathway. (On the other hand, the fact that even in normal animals the projection to the pons is weak makes it difficult to draw a strong conclusion from the absence of label in this region.)

The second objection to our conclusion, that the abnormal pathways observed in half of the experimental animals resulted from the absence of pioneering subplate axons, is that kainic acid might have a direct effect on the development of neurons in layers 5 and 6. This possibility seems unlikely, for several reasons. First, in every ablated animal, cortical axons could pathfind through the cortex all the way to the internal capsule;

the principal effect on the axons was exerted subcortically, at sites far distant from the location of the kainic acid injection. Second, it is important to emphasize that this failure of pathfinding was specific for subcortical targets. Targets of layer 6 neurons located within the forebrain, such as the claustrum (Gilbert and Kelly, 1975; LeVay and Sherk, 1981; Katz, 1987), always acquired their normal cortical projections. Furthermore, intracortical pathways such as the corpus callosum, which links the two cerebral hemispheres and is formed by deep as well as superficial cortical neurons (Jouandet et al., 1985), appear to form normally in every case following subplate ablation. These observations reinforce the view that kainic acid selectively deletes subplate neurons, and does not ubiquitously and directly interfere with the development of deep-layer cortical neurons.

Variability of the effects of deleting subplate neurons

It remains puzzling why in the remaining 50% of cases axons were able to select and innervate their normal subcortical targets. There are several possible explanations of this result. First, it is possible that the kainic acid injection produced a variable effect in removing subplate neurons, and that in those cases in which ingrowth was normal, more subplate neurons survived. While in every case these lesions produced a visibly shrunken hemisphere, in practice it may be impossible to remove every one of the tens of thousands of subplate neurons that are found along the pathway from visual cortex to the internal capsule. If, as in the insect nervous system (Raper et al., 1984), only a single or few remaining axons are sufficient to provide guidance cues for later-growing axons, the presence of even a small number of subplate neurons may prove adequate to guide cortical axons. Similarly, in studies of the developing spinal cord, large numbers of Rohon-Beard neurons (whose axons pioneer a longitudinal spinal cord tract) must be deleted in order to perturb reliably the formation of later-forming axons (Kuwada, 1986). Previous studies examining the effects of kainic acid injections on subplate neurons indicated that while the vast majority of these cells are indeed eliminated, certainly some do remain (Chun and Shatz, 1988a; Ghosh et al., 1990; Ghosh and Shatz, 1993). Perhaps a threshold number of subplate neurons is required for normal guidance.

A second possibility is that at the time of the ablation (E35–E43), the state of maturation of cortical projections is relatively mature. In the oldest animals (E43), the subplate axon pathway into the thalamus has been in place for about 2 weeks; cortical plate axons have grown midway or farther along their intracortical trajectory toward the internal capsule, and the reciprocal pathway from the LGN has arrived at the visual subplate (by about E36; Ghosh and Shatz, 1992b). It is conceivable that had we been able to delete subplate neurons at even earlier times, prior to the outgrowth of any axons from the cortical plate, more reliable effects would have been seen. It is also possible that more severe effects on pathfinding would have resulted. For example, in the present experiments, cortical axons were always able to traverse their intracortical pathways (though not always along their normal routes) and extend through the internal capsule. It may be that these axons were able to employ the degenerating axons of subplate neurons to traverse this distance (Stuermer and Easter, 1984), or that the pioneering subplate axons altered the local environment to provide permanent or lasting cues. If so, one might expect that earlier ablations would disrupt intracortical pathfinding, and might more reliably disrupt subcortical target selection. Unfortunately, subplate neurons are resistant to the neurotoxic effects of kainic acid at these very early developmental times (before E35), and we have not been able to eliminate them selectively using other techniques. Nevertheless, it is worth noting that within the rather broad period of our deletions, between E35 and E43, there was no systematic relationship between the age of ablation and pathfinding ability. Subplate ablations at E37, E40, and E43 all prevented cortical axons from invading subcortical targets (Table 2).

A final possibility is that a multitude of cellular and/or extracellular cues are available to guide growing cortical axons as they grow toward and invade their subcortical targets. Even though LGN axons have not invaded the cortical plate (Ghosh et al., 1990; Ghosh and Shatz, 1992b, 1993), they still form a coherent pathway between the thalamus and cortex that may be used as a guidance cue. In addition, several studies in other systems have demonstrated variable effects on axon pathfinding after deleting pioneer neurons. For example, when a portion of the early axonal scaffold in the zebrafish forebrain is surgically ablated, the trajectories of later-growing commissural axons are disrupted, but only for roughly half of all neurons (Chitnis and Kuwada, 1991). In this case, all of the pioneering axons were definitively eliminated; therefore, the inability of some axons to pathfind normally cannot be explained by incomplete ablation. Experiments *in vitro* have revealed that single axons may employ a multitude of cellular and extracellular cues for growth, and that elimination of any single cue can be insufficient to block outgrowth (Neugebauer et al., 1988; Tomaselli et al., 1988). Virtually nothing is known about the molecular basis of cortical axon guidance, but our results are consistent with the possibility that subplate neurons are involved in, but not exclusively responsible for, the process of targeting axons to subcortical structures.

Role of subplate neurons in cortical axogenesis and target invasion

Axonal development entails two distinct processes: that of recognizing and growing along an appropriate pathway, and that of selecting and invading the correct target. For example, in this system, once cortical axons have arrived in the thalamus, a variety of possible thalamic targets are available. The ablation of subplate neurons appears to have affected both targeting and pathfinding because not only do axons fail to invade the LGN in affected animals, but also the pathway from the internal capsule to the superior colliculus is absent.

The complete absence of a pathway from the thalamus to the superior colliculus in half of all cases suggests that the subplate can play a critical role guiding cortical axons to the colliculus. This stands in sharp contrast to the conclusions of DeCarlos and O'Leary (1992) who, based on the observation that the axons of subplate neurons do not extend past the thalamus in the rodent, concluded that the subplate plays no role in the establishment of corticotectal connections. In the cat, subplate neurons do appear to project normally to the superior colliculus (McConnell and Shatz, 1988; McConnell et al., 1989); furthermore, the pathway taken by corticotectal axons in the cat is a dorsal one, extending medially and posteriorly past the LGN and through the pretectum (Fig. 9*E–G*). In contrast, O'Leary and co-workers report that, in the rat, the colliculus is innervated by collaterals that branch from ventral spinally projecting axons (O'Leary et al., 1990; DeCarlos and O'Leary, 1992). Thus, there may be two significant species differences between cat and rat:

the existence of a subplate projection to the colliculus in the cat but not rat, and the route taken by cortical axons. (It remains to be shown whether removing subplate neurons in rodent affects the formation of the corticotectal pathway, or of the corticogeniculate pathway for that matter.)

In subplate-ablated animals, cortical axons always grew into the thalamus and actually reached the outskirts of the LGN, but in half of the cases these axons failed to invade the LGN proper. The axons clearly had access to this target since they were observed to encapsulate it (Figs. 8, 9); thus, we saw no effect on the choice of targets made, but rather on the ability of axons to invade that target. Two distinct explanations for this behavior are plausible. The first is that layer 6 cortical neurons in subplate-ablated animals do not identify themselves as belonging to visual cortex: they are "generic" thalamic projection neurons but cannot recognize the LGN as a specific target. This suggestion is based on a previous observation that in animals receiving ablations of subplate neurons underlying the visual cortex, LGN axons fail to invade visual cortex and instead grow aberrantly past this region, remaining confined to white matter (Ghosh et al., 1990; Ghosh and Shatz, 1993). It seems possible that the ingrowth of LGN axons into the cortical plate is required for neurons within visual cortex, including those of layer 6, to acquire their area-specific identity. Transplantation of visual cortex to somatosensory regions endows the foreign cortex with the cytoarchitectonic characteristics of its new locale (Schlagger and O'Leary, 1991); one interpretation of this result is that ventrobasal thalamic afferents impose a somatosensory identity onto the transplanted cortex. This explanation, that the failure of cortical axons to innervate the LGN in subplate-ablated animals is due to an "identity crisis," however, seems unlikely. When subplate neurons are deleted, LGN axons fail to innervate the overlying visual cortex 100% of the time (Ghosh et al., 1990; Ghosh and Shatz, 1993). However, the cortex failed to provide reciprocal innervation of the LGN in only 50% of ablated animals in the present study, rather than in all cases as expected if thalamic inputs confer the identity of cortical neurons. Furthermore, cortical axons do not wander aimlessly within the thalamus, but rather accumulate outside their normal LGN target. This suggests that neurons of the visual cortex can indeed recognize their normal thalamic target, but they may require the presence of subplate axons in order to grow into the LGN.

An alternative explanation for the failure of cortical axons to innervate the LGN is that subplate axons are somehow required locally in the thalamus for cortical plate axons to invade this nucleus. If so, these observations imply that the process of target selection by growing axons may involve at least two clearly separable steps: target recognition and target invasion. The idea that there is a separate signal that triggers the entry of axons into target tissue is supported by experiments in which cortical explants are cocultured with thalamus. Thalamic axons readily invade older but not younger cortical slices, suggesting that older cortex has acquired the ability to promote axon ingrowth (Bolz et al., 1992; Götz et al., 1992). Perhaps thalamic nuclei have a similar age dependence; if so, subplate neurons may be involved in the control of the transition to a growth permissive state.

Conclusions

The results of this experiment add to the growing list of possible functions for subplate neurons during the development of connections between thalamus and cortex (Shatz et al., 1991). The axons of subplate neurons pioneer the early descending pathways (McConnell et al., 1989); the subplate neurons themselves are required for the ingrowth of thalamic axons into the cortical plate (Ghosh et al., 1990; Ghosh and Shatz, 1993) and their later segregation into ocular dominance columns (Ghosh and Shatz, 1992a), and now we show that subplate neurons play a role in the ability of cortical plate neurons to find and invade their subcortical targets. Because the pathways between cortex and thalamus are reciprocal and involve the subplate as an intermediary, it is difficult to dissect completely the individual contributions of subplate neurons, LGN neurons, and deep-layer cortical neurons to any single developmental process. However, the results of all these studies show clearly that this unique population of subplate neurons plays a pivotal role in coordinating the developmental events that produce the final patterning of connections between the thalamus and cerebral cortex.

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