A role for subplate neurons in the patterning of connections from thalamus to neocortex

Anirvan Ghosh* and Carla J. Shatz†

Department of Neurobiology, Stanford University School of Medicine, Stanford, CA 94305, USA

*Author for correspondence at present address: Dept. of Microbiology and Molecular Genetics, Harvard Medical School, Bldg. D1, 200 Longwood Avenue, Boston, MA 02115, USA
†Present address: Division of Neurobiology, Dept. of Molecular and Cell Biology, LSA 221, University of California, Berkeley, CA 94720, USA

SUMMARY

During cerebral cortical development, ingrowing axons from different thalamic nuclei select and invade their cortical targets. The selection of an appropriate target is first evident even before thalamic axons grow into the cortical plate: initially axons accumulate and wait below their cortical target area in a zone called the subplate. This zone also contains the first postmitotic neurons of the cerebral cortex, the subplate neurons. Here we have investigated whether subplate neurons are involved in the process of target selection by thalamic axons by ablating them from specific cortical regions at the onset of the waiting period and examining the subsequent thalamocortical axon projection patterns.

Subplate neurons were ablated at the onset of the waiting period by intracortical injections of kainic acid. The effect of the ablation on the thalamocortical projection from visual thalamus was examined by DiI-labeling of the LGN days to weeks following the lesion. At two to four weeks post-lesion, times when LGN axons would have normally invaded the cortical plate, the axons remained below the cortical plate and grew past their appropriate cortical target in an anomalous pathway. Moreover, examination of LGN axons at one week post-lesion, a time when they would normally be waiting and branching within the visual subplate, indicated that the axons had already grown past their correct destination. These observations suggest that visual subplate neurons are involved in the process by which LGN axons select and subsequently grow into visual cortex. In contrast, subplate neurons do not appear to play a major role in the initial morphological development of the LGN itself. Subplate ablations did not alter dendritic growth or shapes of LGN projection neurons during the period under study, nor did it prevent the segregation of retinal ganglion cell axons into eye-specific layers. However, the overall size of the LGN was reduced, suggesting that there may be increased cell death of LGN neurons in the absence of subplate neurons.

To examine whether subplate neurons beneath other neocortical areas play a similar role in the formation of thalamocortical connections, subplate neurons were deleted beneath auditory cortex at the onset of the waiting period for auditory thalamic axons. Subsequent DiI labeling revealed that in these animals the majority of MGN axons had grown past auditory cortex instead of innervating it. Taken together these observations underscore a general requirement for subplate neurons throughout neocortex in the process of cortical target selection and ingrowth by thalamic axons. Moreover, they imply that the cortical plate alone has insufficient information to direct the formation of specific sets of thalamocortical connections: interactions involving subplate neurons must also be present.

Key words: subplate neurons, cerebral cortex, target selection

INTRODUCTION

In the adult cerebral cortex, neurons of cortical layer 4 receive their major ascending inputs from the thalamus. Distinct cortical areas subserving different functions receive inputs from different thalamic nuclei. For example, primary visual cortex receives its input from the lateral geniculate nucleus (LGN), while primary auditory cortex receives separate input from the nearby medial geniculate nucleus (MGN). It is not known how the neocortex, which initially appears homogeneous, becomes parcellated into cytoarchitectually different areas with distinctly different thalamic inputs during development (for reviews see Rakic, 1988; O'Leary, 1989; McConnell, 1992; Shatz, 1992). Previous autoradiographic tracing studies of the development of thalamocortical connections in the mammalian visual system have shown that, long before they grow into the cortical plate, LGN axons have selected their appropriate cortical target area - primary visual cortex (Lund and Mustari, 1977; Rakic, 1977; Shatz and Luskin, 1986; Ghosh and Shatz, 1992a) suggesting that the process of target selection by thalamic axons may involve interactions other than those with neurons of the cortical plate.

The LGN axons, which arrive in higher mammals even

before their ultimate target neurons of layer 4 have migrated into position (Shatz and Luskin, 1986), accumulate and 'wait' within a restricted zone directly below the forming visual cortex - the visual subplate - where they give off extensive branches (Ghosh and Shatz, 1992a). Earlier in development, while the axons are still in the process of elongating within the optic radiations en route to visual cortex, they extend delicate collaterals into the overlying subplate of nonvisual areas; these collaterals disappear once LGN axons reach and branch within visual subplate (Ghosh and Shatz, 1992a). These observations raise the possibility that growing thalamocortical axons may select their appropriate cortical targets via interactions within the subplate zone.

In addition to radial glial fibers, migrating neurons and afferent axons, the subplate zone contains a population of neurons called subplate neurons. These neurons are the first postmitotic neurons of the cerebral cortex (Kostovic and Rakic, 1980, 1990; Luskin and Shatz, 1985a,b) and achieve a high degree of phenotypic maturity during fetal and neonatal development (for review, see Shatz et al., 1988, 1991). Many of them receive synapses (Chun and Shatz, 1988a; Herrmann et al., 1991) and can be synaptically driven by white matter stimulation (Friauf et al., 1990). However, in the cat this population is transient: between 80 and 90% of subplate neurons are eliminated by cell death during neonatal life (Valverde and Facal-Valverde, 1988; Chun and Shatz, 1989b).

Given the close association between thalamic axons and subplate neurons during cortical development, here we have examined the possibility that subplate neurons play an essential role in the formation of thalamocortical connections by selectively ablating them at times just before, or

when, the first thalamic axons have arrived within the subplate at their target cortical area. Subplate neurons underlying visual or auditory cortex were deleted by making injections of the excitotoxin kainic acid and the effects on the development of thalamocortical projections from the LGN or MGN were studied at later times by labeling axons with DiI (1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine percholate; Godement et al., 1987). Our results indicate that, in the absence of subplate neurons, thalamic axons fail to select and innervate their appropriate cortical target areas.

MATERIALS AND METHODS

19 fetal and neonatal cat brains aged between embryonic day 38 (E38) and postnatal day 58 (P58; gestation is 65 days) were used in this study. The distribution of ages examined and experimental manipulation performed is shown in Table 1. Kainic acid injections were made to delete subplate neurons at different ages. The consequence of these ablations on thalamocortical development was examined by labeling LGN or MGN axons with DiI in aldehyde-fixed brains.

Surgical procedure

All procedures included sterile surgical technique to externalize fetuses by Cesarian section for study. Pregnant cats in which fetuses were of known gestational age were initially anesthetized by inhalation of halothane (1-2% in O_2) or by intramuscular injection of ketamine hydrochloride (20 mg/kg) and acepromazine (0.2 mg/kg). Anesthesia was maintained by halothane (0.5-2% in O_2) administered through an endotrachial tube, and the appropriate level was monitored by withdrawal reflexes and heart rate. A 5% dextrose/saline i.v. drip was maintained throughout the surgery. Terbutaline sulfate was administered (0.03 mg/kg i.v.) before

Table 1. Summary of experimental observations in kainic acid-treated animals

Expt. number	Age at injection	Age at perfusion	Absence of subplate zone (1)	DiI injection site (2)	Axons fail to invade target cortex	Histology (3)
1	E43	E50	+	LGN	+	
2	E43	E50	+	LGN	+	
3	E43	E59	−	LGN	−	
4	E43	E60	+	LGN	+	
5	E43	E63(1)	+	LGN	+	[3]H Thy
6	E43	E63(2)	+	LGN	+	
7	E43	E63(3)	+	LGN	+	
8	E43	E63(4)	+	LGN	+	
9	E43	P1	−	LGN	−	
10	E42	P5	+	LGN	+	
11	E38	E52(1)	+	MGN	+	
12	E38	E52(2)	+	MGN	+	
13	E38	E52	+	VC	N/A	
14	E45	E53	+	N/A	N/A	Immuno.
15	E45	E53	+	VZ	N/A	
16	E43	E60	+	N/A	N/A	Immuno.
17	E42	E61	+	N/A	N/A	Immuno.
18	E42	P8	+	N/A	N/A	Immuno.
19	E43	P58	+	N/A	+	[3]H Pro

(1) Ablations were not successful in experiments where the subplate zone had not disappeared based on DiI labeling of LGN axons following kainic acid injection. Results from those cases were not used in our data analysis.

(2) VC=visual cortex; VZ=ventricular zone (to label radial glia); N/A=not applicable.

(3) Sections were prepared for MAP2 and Vimentin immunostaining (Immuno), autoradiography following an injection of [3H]thymidine at E43 to label layer 4 neurons ([3]H Thy) or autoradiography following an intraocular injection of [3H]proline to label the LGN axons by means of transneuronal transport ([3]H Pro).

each fetal manipulation to block uterine contractions. After surgery, the animals were given analgesics and monitored in the 24 hours/day intensive care unit for at least 36 hours. Further details of the surgical procedure can be found in Luskin and Shatz, 1985a.

Kainic acid injections

Subplate neurons were ablated by exposure to the neurotoxin kainic acid (No. K0250, Sigma Chemical Co.; Coyle et al., 1978; Chun and Shatz 1988b; Ghosh et al., 1990a). Kainic acid was diluted (10 mg/ml) in 0.9% sterile saline and used within 2 hours. In some cases, fluorescent latex microspheres (Lumafluor, NY) were added to the kainic acid solution (at a ratio of 1:9, microspheres: kainic acid) to mark the injection site. (The microspheres form a stable nontoxic and long-lasting deposit that can be viewed weeks to months later, and they are also transported retrogradely by the LGN neurons.) For animals at E42 and older, a sterile dental drill was used to remove a portion of the cranium overlying the targeted cortical areas in order to insert the injection needle. With the brain surface exposed, one (0.8 μl) or two (2 × 0.5 μl) injections were made with a 1 μl syringe (Unimetrics, IL) at a depth of 1-2 mm into presumptive visual cortex of the right hemisphere. The left hemisphere was treated identically except that vehicle solution (0.9% sterile saline) was injected. For animals younger than E42, a 30 gauge needle was inserted through the cranium and one 0.5 μl injection was made at a depth of 1 mm underlying presumptive visual or auditory cortex. Animals were then returned to the uterus, developing an additional 7-21 days before delivery by Cesarian section and tissue preparation.

Tissue preparation for histology

Fetal animals were anesthetized transplacentally through maternal anesthesia and postnatal animals were deeply anesthetized by an intraperitoneal injection of Nembutal (35 mg/kg). Animals were perfused transcardially using a peristaltic pump with 0.1 M sodium phosphate buffer (pH 7.4) followed by 4% paraformaldehyde in the same buffer. Following perfusion the cranium was removed and the brain stored in 4% paraformaldehyde.

DiI injections

To visualize thalamocortical axons, small crystals of DiI (Molecular Probes, D282) were placed using insect pins or forceps into the LGN or MGN of aldehyde-fixed brains. The DiI-injected brains were stored in 4% paraformaldehyde (to which 0.01% sodium azide was added) at room temperature or at 37°C for 2-4 months to allow for diffusion of the label. Coronal sections (100-200 μm) were cut on a vibratome and viewed under rhodamine epifluoresence optics. The various zones of the cerebral wall (the cortical plate, intermediate zone and ventricular zone) were identified by counterstaining sections with meta-phenylenediamine (MPD; Quinn and Weber, 1988), which appears green under fluorescence optics. Radial glial cells were visualized by preparing 1 mm thick coronal slices of aldehyde-fixed brains into which DiI crystals were placed into the ventricular zone to label radial glial cells by means of their ventricular endfeet. To visualize the dendritic morphology of LGN neurons, DiI crystals were placed in the presumptive visual cortex (the injections spanned the cortical plate and the underlying intermediate zone) of aldehyde-fixed brains to label thalamic projection neurons retrogradely. Labeled LGN neurons were visualized in 200 μm horizontal sections under rhodamine epifluorescence optics. In several experiments, the morphology of labeled profiles was documented by making camera-lucida drawings (20× or 40× objective, 10× eyepiece) of axon terminals or dendritic trees of LGN neurons from 200 μm vibratome sections.

Immunocytochemistry

Antibodies against the following antigens were used: Microtubule Associated Protein-2 (MAP2; mouse monoclonal anti-bovine MAP2-3 diluted 1:30) was a gift from Drs W. Theurkauf, F. Luca and R. Vallee (Worcester Foundation for Experimental Biology, Shrewsbury, MA); vimentin (mouse anti-vimentin diluted 1:10) was obtained from Boehringer-Mannheim Biochemicals (Cat. no. 814318). Bound antibody was visualized using Vectastain ABC kits (Vector Laboratories, Burlingame, CA) and the chromogen 3,3 diaminobenzidine (DAB; grade II, No. D-5637; Sigma Chemical Co.).

Sections were preincubated at 25°C for 2 hours in 2.5% (W/V) bovine serum albumin (BSA; Sigma Chemical Co.) in phosphate-buffered saline (PBS)) with 0.3% Triton X-100 (Sigma Chemical Co.). This solution was replaced by fresh incubation buffer containing diluted antibody and incubated for 15-24 hours at 4°C (free-floating sections were gently agitated during incubation). After incubation, sections were processed according to vectastain ABC kit procedures. Briefly, sections were washed in PBS (four changes over 30 minutes) followed by 60 minutes in secondary antibody (1:200), then washed again (four changes over 30 minutes) with PBS and reacted with the addition of 0.5 mg/ml DAB with 0.0075% H_2O_2. The sections were finally washed in PBS and mounted on subbed slides. After 12-18 hours, air-dried sections were taken through three washes of distilled water followed by dehydration in graded ethanols. Sections were then immersed in xylene for 10-30 minutes and coverslipped with Permount (Fisher Scientific Co.).

Transneuronal transport of [³H]proline

The pattern of retinogeniculate and geniculocortical projections at P58 following a kainic acid injection at E43 was visualized in one animal by transneuronal transport of [³H]proline as previously described (Shatz and Luskin, 1986; Ghosh and Shatz, 1992b). 2mCi of [³H]proline in 45 μl saline was injected into the eye ipsilateral to the kainic acid-injected hemisphere at P50. At P58 the animal was perfused with 4% paraformaldehyde, 30 μm coronal sections were cut on a freezing microtome and transported [³H]proline was visualized by emulsion autoradiography.

RESULTS

The results are presented in four parts. First, we consider the consequences of injecting the excitotoxin kainic acid into the subplate early in cortical development (E42) on the various cellular elements present. Then, the effects of ablation of visual subplate neurons on the development of the thalamocortical projection from the visual thalamus (LGN) are described and the effects of subplate deletions on the development of LGN neurons are examined. Finally, we evaluate the generality of our results by examining whether ablations of auditory subplate neurons result in similar changes in the axonal projections from the auditory thalamus (MGN) to auditory cortex.

Effects of kainic acid injections on the cellular constituents of subplate and cortical plate

In all of the experiments described here, kainic acid was injected once at some time between E38 and E43 (see Table 1). Between E36 and E43, the first LGN growth cones have just arrived in the subplate below future visual cortex (Ghosh and Shatz, 1992a). E36 is at the onset of the 2 week period during which the majority of LGN axons accumu-

late and branch within the subplate before invading the cortical plate at about E50. Many of the neurons that belong to cortical layer 4 are also generated at the ventricular zone during this time (E37-43; Luskin and Shatz, 1985a), while those just postmitotic are migrating through the subplate en route to the cortical plate. To compensate for the fact that the neurogenesis and axonal development of the auditory cortex, situated in the temporal lobe anterior and lateral to visual cortex, is more advanced than that of visual cortex (Luskin and Shatz, 1985a; Ghosh and Shatz, unpublished observations), we injected auditory subplate at E38, but visual subplate at E42 or E43, so as to obtain comparable developmental times.

Subplate neurons can be deleted by injections of kainic acid into the subplate at E43. The vast majority of later-generated neurons of the cortical plate remain intact (Chun and Shatz, 1988b), presumably because at these early ages they are sufficiently immature to be insensitive to kainate neurotoxicity (Coyle et al., 1978, 1981). The extent of the lesion following an injection of kainic acid can be determined by cresyl violet staining and immunocytochemistry for the neuron-specific antigen, MAP2 (Chun and Shatz, 1988b). Fig. 1 shows an example of the histological organization of the cortical plate and subplate at E60 following a kainic acid or control saline injection at E43. It is evident from the cresyl violet staining in Fig. 1A,B that the cortical plate is essentially normal in thickness following kainic acid injections into the subplate. Immunostaining sections from the saline-injected control hemisphere at E60 for MAP2 (Fig. 1C) indicates that both subplate neurons and cortical plate neurons stain heavily, as expected from previous studies (Chun and Shatz, 1988b; Chun and Shatz, 1989a). However, following a kainic acid lesion, MAP2 immunoreactivity is almost completely absent from the subplate (Fig. 1D), signalling the loss of subplate neurons. In contrast, the intensity of MAP2 immunostaining and the thickness of the cortical plate are unaffected by the lesion, consistent with the suggestion that cortical plate neurons are too immature at E43 to be susceptible to kainate toxicity.

Along with neurons, another major cellular constituent of the developing cortex are the radial glial cells (Levitt and Rakic, 1980). To determine whether kainic acid destroys radial glial cells, we examined the consequences of such injections by immunostaining for vimentin, an intermediate filament found in radial glial cells (Hutchins and Casagrande, 1989). As shown in Fig. 1E,F the pattern of vimentin immunostaining is comparable in kainic acid-injected and in saline-injected hemispheres. We have previously shown that radial glial cells appear anatomically normal following kainic acid injections (Ghosh et al., 1990a). Furthermore GFAP immunostaining in the cerebral wall reveals the continued presence of radial glia following fetal kainic acid injections (Chun and Shatz, 1988b). These results suggest that glial cells are unlikely to be directly damaged by kainic acid during early cortical development.

Finally, using [³H]thymidine birthdating, we previously confirmed that the genesis and migration of layer 4 neurons, the primary targets of thalamocortical axons, were not affected by kainic acid injections (Ghosh et al., 1990). Taken together, the above experiments indicate that the major cellular constituents of the developing cortex are not visibly affected by the kainic acid injections, with the exception of the subplate neurons, which are selectively ablated.

Effect of ablation of visual subplate neurons on LGN axons

Visual subplate neurons were ablated at E42 or E43 and the consequences for LGN axon development were examined at E50, E60, E63, P5 and P58 (Table 1). In normal development, by E50 many if not all LGN axons have already grown along the optic radiations (their pathway through the developing white matter) and have arrived beneath visual cortex, where they leave the radiations to branch within the subplate. Fig. 2A shows the geniculocortical projection at E50, labeled with an injection of DiI into the LGN, in an animal that received a control injection of saline into the subplate at E43. As in normal animals, LGN axons exit from the densely labeled optic radiations to branch extensively within the subplate underlying visual cortex (visual subplate). Very few LGN axons have innervated the cortical plate at this age since the major period of ingrowth of LGN axons into the cortical plate takes place between E50 and E60 (Ghosh and Shatz, 1992a; Shatz and Luskin, 1986).

To determine how the absence of subplate neurons affects LGN axons at a time when they are normally restricted to the subplate, we examined geniculocortical axons at E50 in two fetuses following a kainic acid injection into the subplate at E43. In these lesioned cases, the cortical plate remains intact as mentioned above, but the entire zone that normally contains subplate neurons and LGN axons 'collapses' and disappears as shown in Fig. 2B, and LGN axons no longer arborize below the cortical plate. Instead, in the absence of the subplate, the LGN axons remain confined to tight fascicles restricted within the optic radiations, which is now located immediately beneath the cortical plate. Despite this close proximity of LGN axons to the cortical plate, few of the axons invade and arborize within the cortical plate. Thus, deletion of the subplate neurons does not cause a premature invasion of LGN axons into the cortical plate. On the contrary, since the histology and MAP2 immunostaining of the cortical plate in kainic acid-injected brains appear normal, the lack of thalamic innervation suggests that the cortical plate may not be an attractive substratum for axon ingrowth at E50.

This restriction of LGN axons to the optic radiations was observed in every case in which the subplate zone disappeared (Table 1). Because MAP2 immunocytochemistry is not directly compatible with the DiI-labeling technique, we took the 'collapse' of the optic radiations in DiI-labeled material to indicate successful kainic acid injections. Rarely, kainic acid injections failed to delete the subplate and, in these animals, subplate neurons were always retrogradely labeled following DiI injections into the LGN. These cases were not analyzed further (Table 1).

We next examined whether the LGN axons grow into the cortical plate at the normal time following the deletion of subplate neurons. Normally, by E55 LGN axons have grown into and begun to branch within the visual cortex (Ghosh and Shatz, 1992a). At E60, DiI placed in the LGN
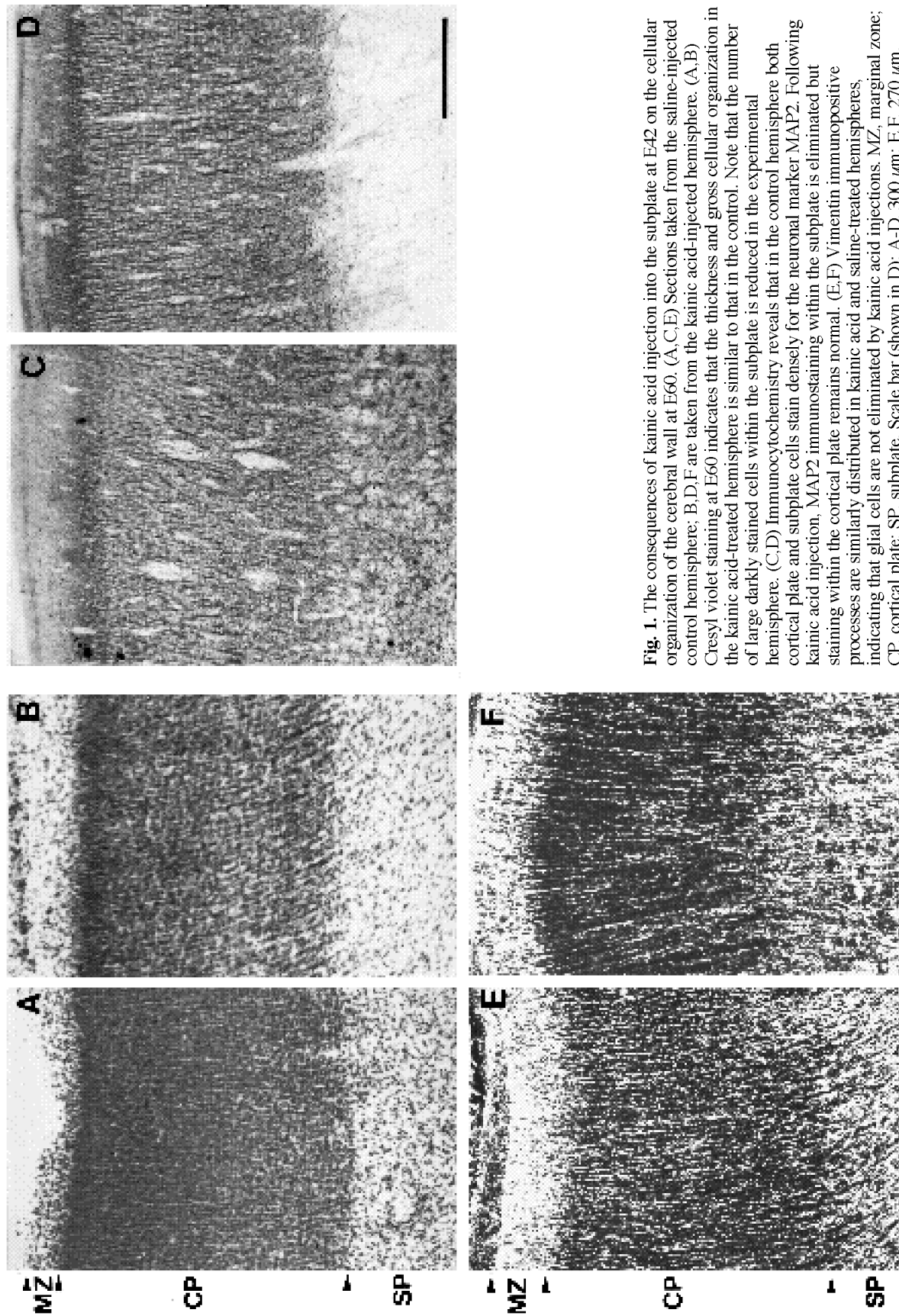
Fig. 1. The consequences of kainic acid injection into the subplate at E42 on the cellular organization of the cerebral wall at E60. (A,C,E) Sections taken from the saline-injected control hemisphere; B,D,F are taken from the kainic acid-injected hemisphere. (A,B) Cresyl violet staining at E60 indicates that the thickness and gross cellular organization in the kainic acid-treated hemisphere is similar to that in the control. Note that the number of large darkly stained cells within the subplate is reduced in the experimental hemisphere. (C,D) Immunocytochemistry reveals that in the control hemisphere both cortical plate and subplate cells stain densely for the neuronal marker MAP2. Following kainic acid injection, MAP2 immunostaining within the subplate is eliminated but staining within the cortical plate remains normal. (E,F) Vimentin immunopositive processes are similarly distributed in kainic acid and saline-treated hemispheres, indicating that glial cells are not eliminated by kainic acid injections. MZ, marginal zone; CP, cortical plate; SP, subplate. Scale bar (shown in D): A-D, 300 μm; E,F, 270 μm.
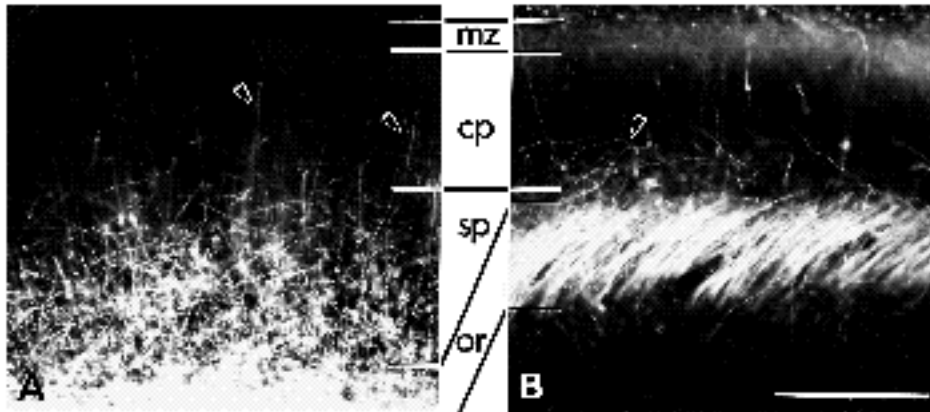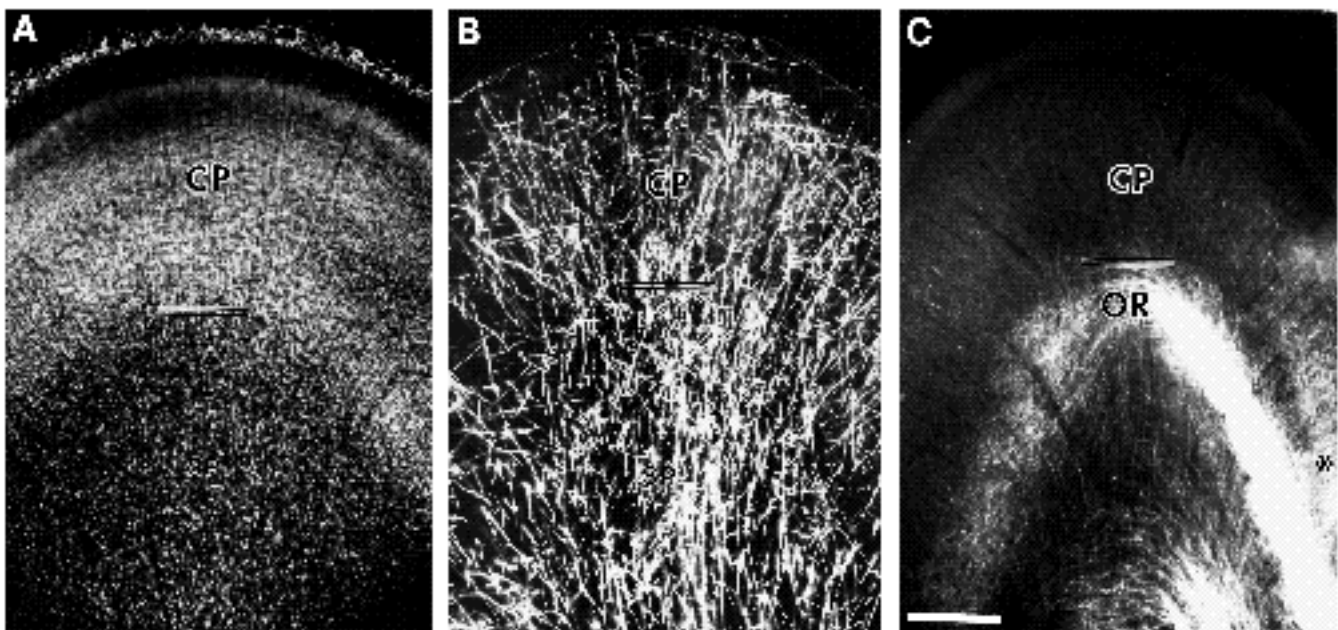
Fig. 2. The effect of kainic acid injection into the subplate at E42 on LGN axons examined at E50. (A) DiI-labeled LGN axons (viewed with epifluorescence optics) in a saline-treated control hemisphere leave the optic radiations (OR) and branch extensively in the subplate. Occasionally, a few axons can also be seen traversing the cortical plate (arrowheads). (B) In the kainic acid-treated hemisphere, the entire zone containing subplate neurons disappears and the LGN axons are restricted to tight fascicles within the optic radiations, which now sit immediately below the cortical plate. Despite the close proximity of the optic radiations to the cortical plate following kainic acid injections, virtually none of the LGN axons grow into the cortical plate. A rare axon that can be detected in the cortical plate of the kainic acid-injected hemisphere is indicated by an arrowhead. MZ, marginal zone; CP, cortical plate; SP, subplate; OR, optic radiations. Scale bar, 500 μm.

results in the labeling of many axons that have fanned out into the cortical plate and innervated layer 4 (Fig. 3A,B). In marked contrast to axons in normal animals or saline-injected controls, following subplate ablation at E43, DiI-labeled LGN axons consistently failed to invade the cortical plate by E60 (1 animal) or E63 (4 animals; Table 1). For example, as shown in Fig. 3C, LGN axons remain tightly fasciculated within the optic radiations immediately below the cortical plate at E60 and form an abnormal axonal pathway that far overshoots visual cortex.

The observation that there are virtually no LGN axons within the cortical plate at E60 in kainic acid-injected animals is particularly striking, since normally the first axons grow into the cortical plate by E50 as layer 4 neurons complete migration and take their place within the cortex above layers 5 and 6 (Shatz and Luskin, 1986). In subplate-ablated animals, the migration and position of layer 4 neurons are normal (Ghosh et al., 1990a), yet LGN axons are missing from the cortical plate. Thus, the presence of layer 4 neurons, the ultimate targets of LGN axons, appears to be insuf-

Fig. 3. The effect of kainic acid injection into the subplate at E42 on LGN axons examined at E60. (A) The extent of the cortical plate and subplate at the apex of the lateral gyrus (primary visual cortex, area 17) at E60 revealed by the fluorescent Nissl counterstain MPD. (B) In the control hemisphere, LGN axons can be seen fanning out in the subplate and traversing the cortical plate of primary visual cortex to end in layer 4 which, at this early age, is located just below the pial surface (Shatz and Luskin, 1986). (C) Following kainic acid injection, axons from the LGN fail to grow into the cortical plate. The axons remain fasciculated within the optic radiations in the white matter and grow past primary visual cortex. Some axons have grown into area 18 (asterisk), a cortical area which also normally receives input from the LGN. (Subplate neurons below area 18 are usually spared following the kainic injections; for example see Fig. 5). CP, cortical plate; SP, subplate; OR, optic radiations. Scale bar, 250 μm.

ficient to initiate the ingrowth of thalamic afferents at the appropriate time.

It is possible, however, that the ablation of subplate neurons simply delays the invasion of LGN axons into visual cortex. To determine whether LGN axons eventually do recognize and innervate visual cortex despite the absence of subplate neurons, subplate neurons were deleted in a fetus at E42, following which the animal survived for either 28 days to postnatal day 5 (P5) or for almost 3 months to P58. In normal animals the visual cortex is richly innervated by LGN axons by birth, many of which terminate and branch in layer 4 (LeVay et al., 1978; Shatz and Luskin, 1986; Ghosh and Shatz, 1992a). In contrast to normal animals, the trajectory of LGN axons at P5 was radically altered in the absence of subplate neurons. As shown in Fig. 4A, LGN axons fail to invade the primary visual cortex, their appropriate target, almost three weeks after their normal time of ingrowth. Instead, as at E60, these axons had continued on past their normal cortical target in a dense fascicle restricted to the white matter, seemingly unaware of their correct cortical destination.

Examination of the labeled LGN axons revealed that a subset of geniculocortical axons do invade a region of the cortical plate immediately lateral and adjacent to primary visual cortex (Fig. 4A). This is area 18, a region that also normally receives direct input from the LGN. Cresyl violet staining of an adjacent section (Fig. 4B) indicates that, whereas most, if not all, of the subplate neurons underlying area 17 in this hemisphere had been ablated, many of them were spared in the white matter below area 18. This observation demonstrates the existence of a close correlation between the presence of subplate neurons and the successful ingrowth of LGN axons into the cortical plate.

To follow the trajectories of individual LGN axons that failed to grow into visual cortex by P5, camera-lucida drawings of diI-labeled axons were made from a series of adjacent sections (Fig. 5). The details of one such reconstruction (Fig. 5) indicate that many of the axons that invade area 18 are, as in normal animals (Ghosh and Shatz, 1992a; Humphrey et al., 1985), collaterals given off a main axon trunk that continues on in a normal lateral-to-medial course in the white matter towards area 17. However, once beneath area 17, axons fail to invade cortex and remain confined to the white matter where many take erratic, curvy courses. These axons form a highly abnormal axon pathway that can be traced beyond visual cortex into the white matter underlying cingulate gyrus, a nonvisual cortical area. The series of alternate sections shown in Fig. 5B indicate that LGN axons have been rerouted across a large area (approx. 2 mm) of visual cortex medial to area 18 and including most if not all of area 17 in which visual subplate neurons are missing. Thus, apparently visual subplate neurons are required for axons from the LGN to grow specifically into the overlying visual cortex.

The ultimate fate of LGN axons that had failed to innervate visual cortex was examined by raising kainic acid-treated animals to adulthood. Unfortunately, following Cesarian section deliveries, many of these animals did not survive past the first postnatal week. We were able to raise 4 animals past three weeks of postnatal age, but only one (examined at P58) had received a successful kainic acid
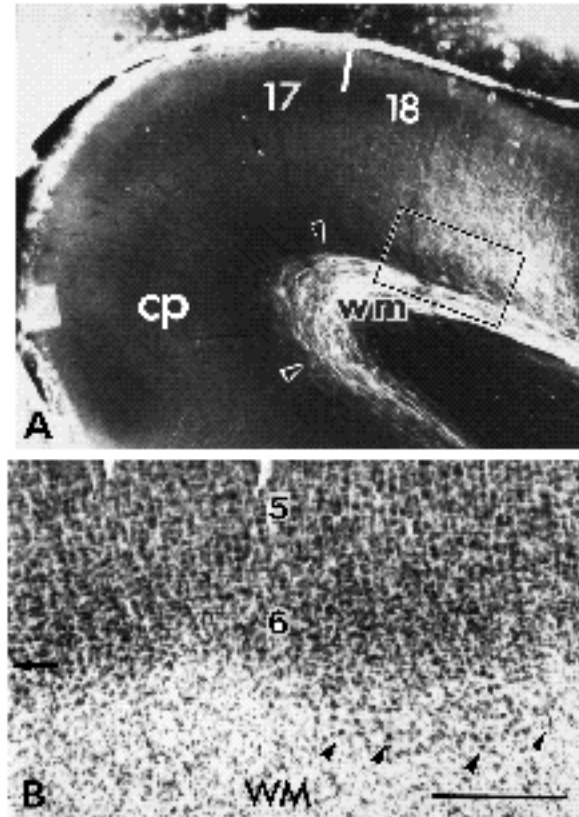
Fig. 4. Correlation between extent of subplate ablation and LGN axon trajectory at P5. (A) Axons within zone of subplate ablation near the apex of the lateral gyrus (primary visual cortex, area 17) fail to grow into the cortical plate and instead remain restricted to a tight fascicle within the white matter. In normal animals at this age, LGN axons have already grown into layers 4 and 6 of primary visual cortex. By contrast, LGN axons do grow into the cortical plate (including layer 4) of adjacent area 18. (B) Cresyl violet staining of the adjacent section (boxed region in A) indicates that many subplate neurons (arrowheads) are still present immediately below the cortical plate in the region where LGN axons do grow into area 18, but they have been eliminated from the region (to the left in fig.) where LGN axons fail to invade the cortical plate: Medial is to the left, and dorsal is up in this figure. The numbers in B indicate cortical layers; CP, cortical plate; WM, white matter. Scale bar: A, 1 mm; B, 250 μm.

lesion. Nonetheless, results from this one animal are consistent with the results of the short-term survival experiments described above, and also provide some additional insight.

In this long-term survival (E43 to P58) case, the hemisphere receiving the kainic acid injection appeared much smaller (approximately 50% in surface area) than the untreated hemisphere based on a simple visual inspection. The geniculocortical projection in this animal was visualized by transneuronal transport of [3H]proline-injected to the eye ipsilateral to the subplate lesion. (DiI does not label LGN axons adequately in adult animals.) A comparison of histology (Fig. 6A,C) shows that the cellular organization of the subplate-ablated hemisphere was markedly altered. In these sections, it was difficult to assign laminar boundaries and classify cell types. The cells that remained in the
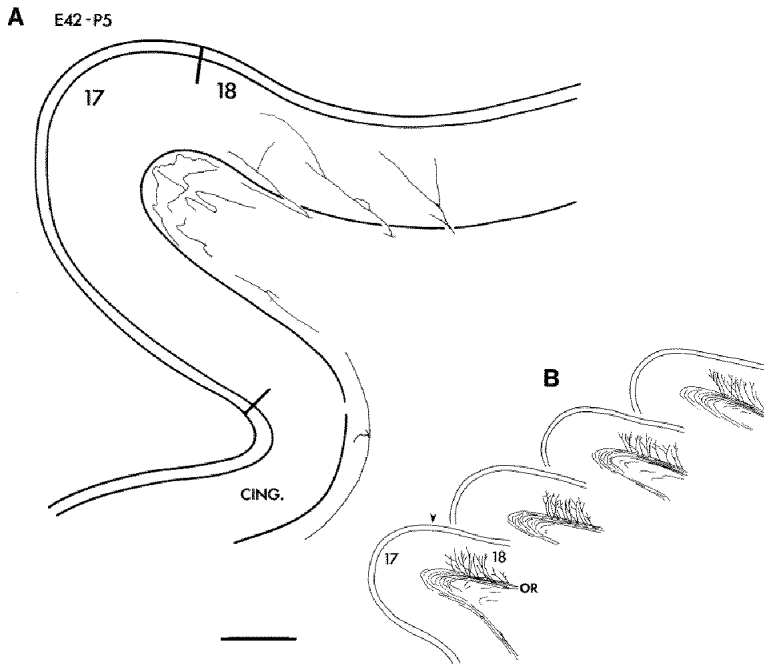
Fig. 5. (A) A camera-lucida drawing of DiI-labeled LGN axons at P5 following kainic acid injection into the subplate at E42, shown in a coronal section through visual cortex (medial is to the left and dorsal is up). Note that the axons in the white matter underlying primary visual cortex (area 17) have erratic trajectories as they continue on within the white matter past their normal cortical target. One axon can be seen growing as far as the white matter underlying the cingulate gyrus, a non-visual cortical area. Axons that have grown into area 18 have branches within the cortical plate, as is normal for this age. (B) LGN axons fail to grow into any region of primary visual cortex (area 17) by P5 following injection of kainic acid into the underlying subplate at E42. A series of camera-lucida drawings from coronal sections shows the location of many DiI-labeled LGN axons in the kainic acid-injected hemisphere. The sections shown here are alternate 200 μm thick sections progressing from anterior (front of figure) to posterior. Arrowhead in B indicates approximate location of 17-18 border. CING., cingulate gyrus; OR, optic radiations. Scale bar: A, 1 mm, B, 2.6 mm.

cortical plate at three months postablation were not obviously smaller than controls, making it unlikely that there was simply generalized atrophy of all cortical cells in the hemisphere. Indeed, there appeared to be a selective absence of the small stellate cells of cortical layer 4 (compare Fig. 6A,C). Moreover, the thickness of the white matter in the kainic acid-treated hemisphere was also strikingly reduced and the lateral ventricle was dilated, as compared to normal.

The pattern of the transneuronally transported label in this animal (at P58) revealed that in the control hemisphere geniculocortical axons had segregated within layer 4 to give rise to the characteristic pattern of ocular dominance columns (Fig. 6B; LeVay et al., 1978). By contrast, in the kainic acid-treated hemisphere, there was virtually no radioactive label in the cortical plate anywhere, indicating that even by this late age LGN axons had not grown into their appropriate target. Moreover, there was also no detectable label above background in the white matter immediately underlying visual cortex (Fig. 6D). However, faint label could be detected in the white matter underlying more lateral cortex, where the optic radiations normally run en route to visual cortex (data not shown), indicating that some LGN axons are still present in the telencephalon. The reduced labeling in the subplate-ablated hemisphere is not due to a failure of transneuronal transport since intense labeling is seen in the control hemisphere and in the appropriate layers of the ipsilateral LGN (see below and Fig. 9). The absence of radioactive label in or near visual cortex suggests that geniculocortical axons never do succeed in innervating their appropriate cortical target following kainic acid ablations of subplate neurons and, in fact, may have withdrawn or atrophied as a consequence of this failure.

These observations indicate that, following ablation of visual subplate neurons by kainic acid injection at E42 or E43, LGN axons fail to select and invade visual cortex. The altered trajectory of LGN axons is evident at the earliest times examined (one week postablation) when LGN axons in subplate-ablated animals do not stop and branch beneath visual cortex as they do in normal animals. The consequences of the ablations are not transient, since even at three months postablation the normal pattern of geniculocortical connections have failed to form.

Consequences of subplate ablation on LGN development

In our initial experiments, we were concerned that ablation of visual subplate neurons might actually entirely eliminate LGN neurons by virtue of the fact that subplate neurons may provide trophic support to LGN neurons during the waiting period. The fact that many LGN axons can be DiI labeled following the ablations demonstrate that some LGN neurons do indeed survive. (Note that the LGN is many millimeters distant to the site of the injection and neurons are not directly affected by kainic acid.) However, to learn more about the effects of these lesions and the subsequent rerouting of LGN axons on development within the LGN, we examined the gross histology of the LGN and the dendritic morphology of individual LGN neurons following intracortical kainic acid injections.

In all of the animals in which subplate neurons had been ablated, there was a visible reduction in the size of the LGN on the ablated side. A particularly striking example, from an animal injected with kainic acid at E45 and examined at E53, is shown in Fig. 7A. On the subplate-ablated side, the thalamus is generally smaller than on the control side and, in particular, the LGN (asterisk, Fig. 7A) is almost 50% smaller than the control LGN. The size of the LGN on the experimental side (injected on E45 and examined at E53) is in fact quite similar to the size of a normal LGN at E46 (Fig. 7B-E); however, cell density is markedly
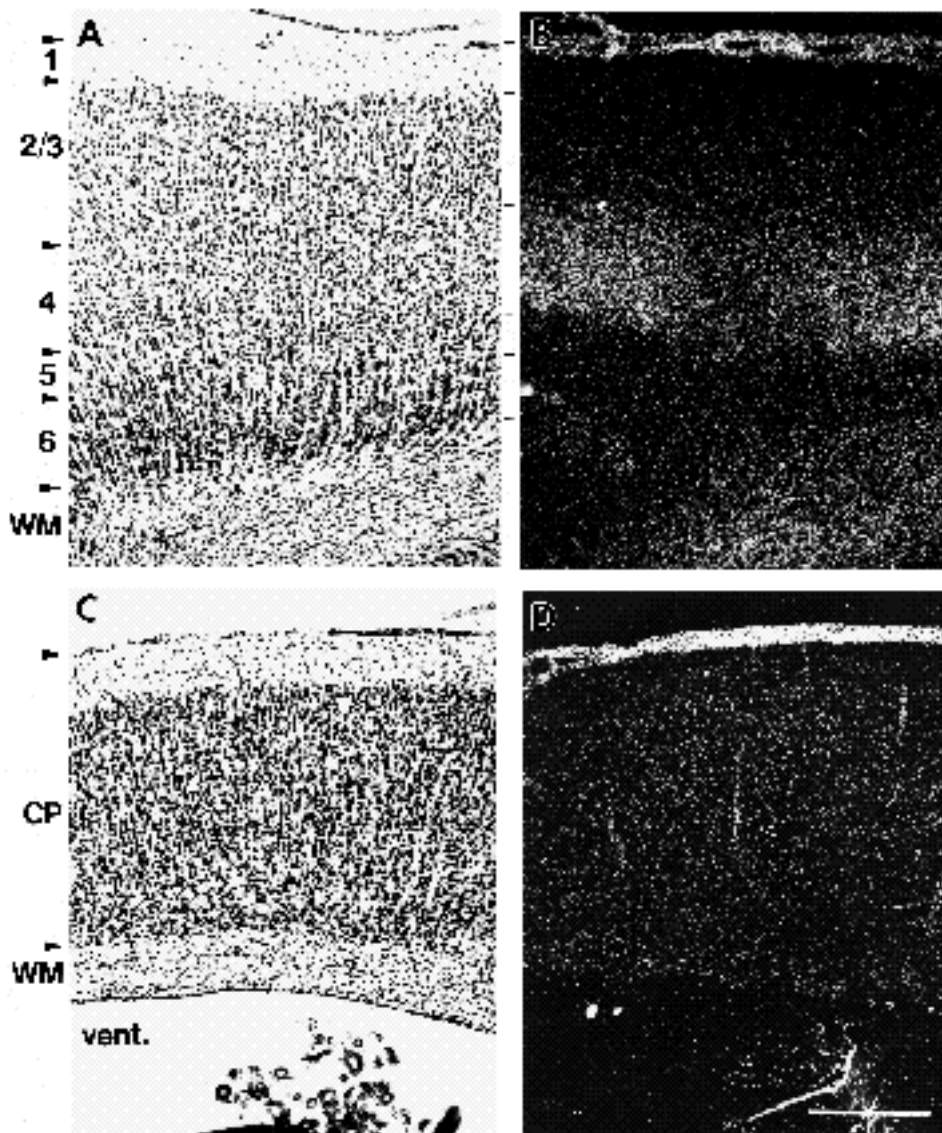
Fig. 6. Long-term consequences of subplate ablations on the organization of visual cortex. (A) Cresyl violet staining indicates that, as in normal animals, at P58 the visual cortex is clearly laminated following saline injection into the white matter at E43. (B) Autoradiography of the adjacent section from the hemisphere contralateral to the eye receiving an injection of [^{3}H]proline reveals labeled LGN axons segregated into ocular dominance patches in layer 4 (silver grains appear white in this photograph taken using darkfield optics). (C) In the kainic acid-injected hemisphere, the histological organization is disrupted, as is evident in this cresyl violet-stained section. The thickness of the cortex is reduced compared to the control (shown in A) and the small stellate cells characteristic of layer 4 are apparently absent. (D) Autoradiographic label above background cannot be detected in area 17 in the kainic acid-treated hemisphere (ipsilateral to the eye injection) at P58 following an injection of [^{3}H]proline indicating that few, if any, geniculocortical axons are present in primary visual cortex. The numbers in A indicate cortical layers; CP, cortical plate; WM, white matter; Vent, lateral ventricle. Scale bar (lower right), 500 μm.

different in these two cases. The lower density at E53 following a subplate ablation at E45 (Fig. 7D,E) suggests that cell death may at least in part account for the small size of the LGN in the experimental cases. It should be noted that this conclusion must be tentative because the (DiI-labeled) sections available to us for study here (200 μm thick vibratome sections) are not suitable for accurate cell counts.

To examine the possibility that the dendritic development of LGN neurons might also be affected by subplate neuron ablations, LGN neurons were retrogradely labeled by placing DiI into the visual cortex two weeks following kainic acid injections. Kainic acid injections were made into one hemisphere at E38 and the consequences examined at E52. In normal development there is marked dendritic growth during this period (Ghosh et al., 1990b) as shown in Fig. 8 (cf E36 with E52). Upon reconstructing the dendrites of individual LGN projection neurons, however, it was somewhat surprizing to find that the dendritic growth of LGN neurons was apparently not affected by the ablation of subplate neurons (Fig. 8: E38-52 kainic) at least by E52. (In

experimental animals from which LGN cells were drawn, the success of the subplate ablation was verified both by the collapse of the subplate zone and by an overall reduction in the size of the LGN.) Since the overall size of the LGN is reduced by E53 in subplate-ablated animals, we again suspect that such ablations result in some cell death within the LGN. The LGN neurons that remain, however, display robust dendritic growth, although as shown above their axonal trajectories are altered.

The two weeks following the subplate cell ablation, E43-E57, is also the period during which retinal axons segregate within the LGN to give rise to the eye-specific layers (Shatz, 1983). Since in most animals geniculocortical axons were labeled by placing DiI into the LGN, the issue of LGN layer formation could not be addressed in detail due to incompatible techniques. However, this issue was examined in the case in which subplate neurons were ablated at E43 and the pattern of geniculocortical terminals was revealed by transneuronal transport of [^{3}H]proline at P58. Although the LGN ipsilateral to the kainic acid-treated cortex of this animal was abnormally small, it was remarkable to find that
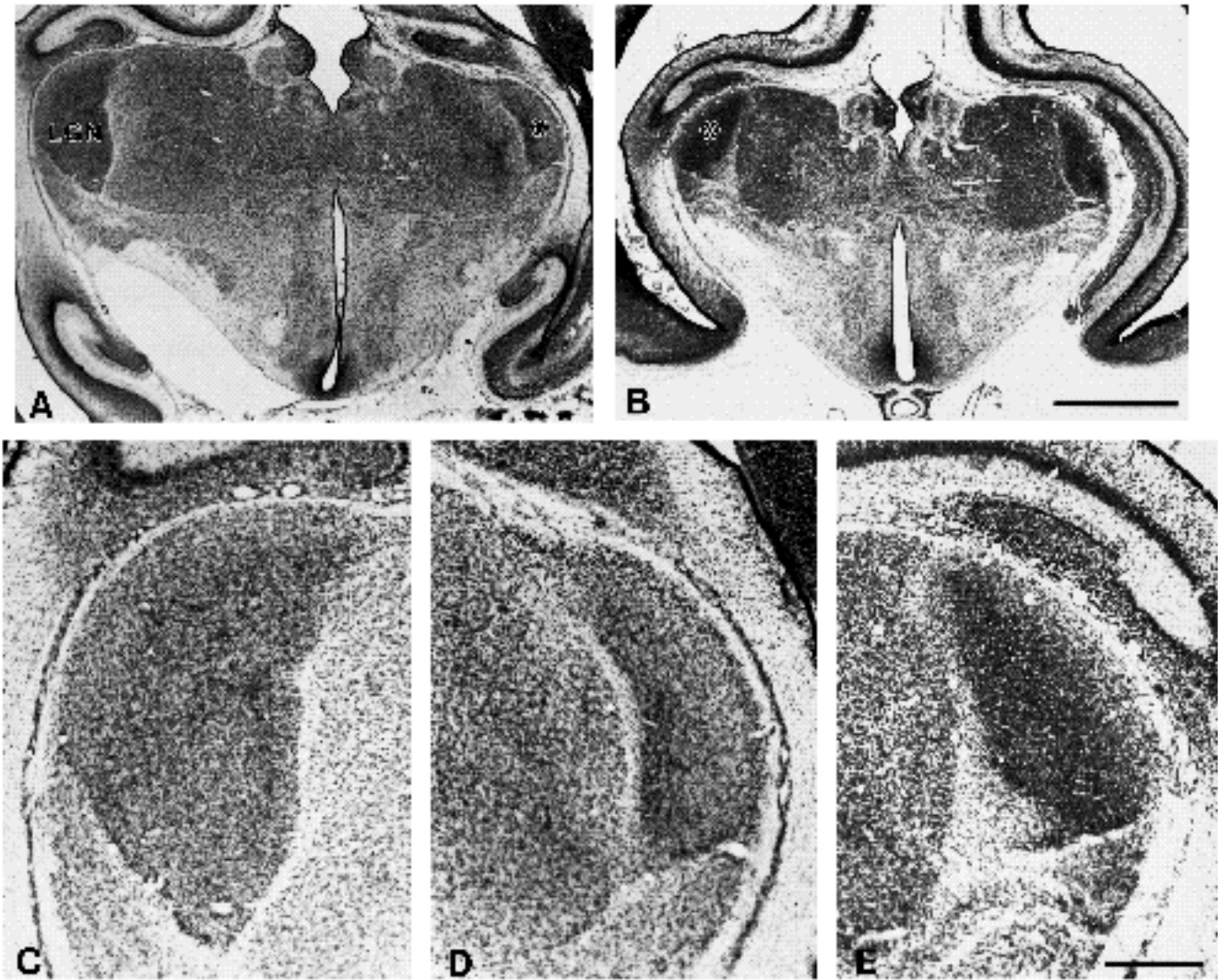
Fig. 7. Effect of kainic acid injection into the subplate at E45 on the histological organization of the thalamus examined at E53. (A) Cresyl violet staining of a coronal section through the thalamus (A) shows that the size of the thalamus in the experimental side (right) is smaller than that on the control side after a large kainic acid injection into visual subplate (the LGN in the kainic-treated hemisphere is indicated by an asterisk). (B) Coronal section through a normal thalamus at E46. Note that the LGN (asterisk) appears comparable in size to the LGN of the experimental hemisphere at E53 in A. (C-E) Although the size of the LGN in the control hemisphere (C) is significantly larger than the experimental side (D), the gross histological organization and cell density in the experimental LGN appears normal in these cresyl violet stained sections. Coronal section through a normal LGN at E46 (E) shows that while the size of the LGN in the section shown in D is similar to what it might have been at the time of the kainic acid injection (E45), the density of cells in the normal E46 LGN is much greater than that of the LGN on the experimental side at E53. Scale bars: A,B (shown in B), 2 mm; C-E (shown in E), 500 μm.
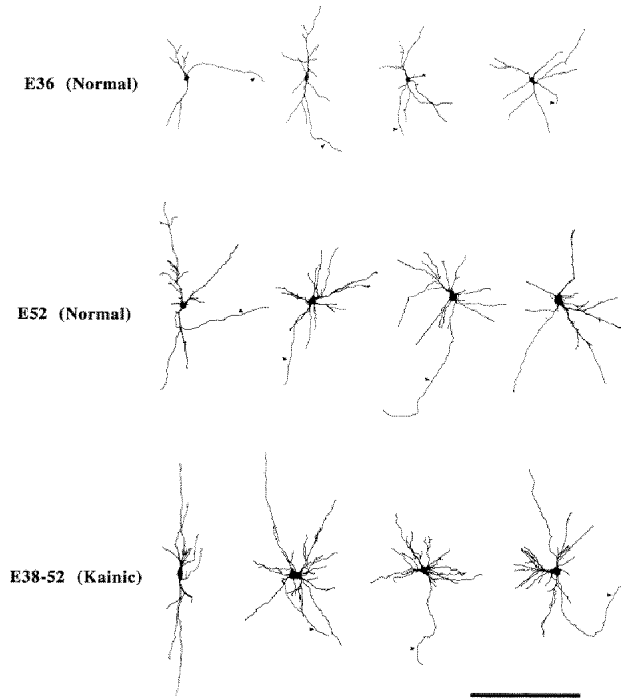
radioactive label representing terminals of retinogeniculate axons of the injected eye was clearly restricted to only a part of the LGN, indicating that retinal axons had indeed segregated (Fig. 9). Therefore the ablation of subplate neurons, the altered trajectory of LGN axons and the overall reduction in LGN size appear not to perturb the interactions within the LGN that lead to the segregation of retinogeniculate axons.

These observations on the organization of the LGN following cortical kainic acid injections indicate that the nucleus is frequently smaller on the subplate-ablated side. This reduction in the size of the nucleus is most likely due to a loss of cells, since the individual neurons that remain appear to grow and morphologically differentiate at normal

rates. Despite the reduced size of the nucleus, many cellular interactions within the LGN are probably not perturbed since major developmental events, such as the segregation of retinal afferents and dendritic development of LGN neurons, appear to occur in subplate-ablated animals.

Effects of ablating auditory subplate neurons on MGN axons

To determine whether the effects of ablating visual subplate neurons on the development of geniculocortical axons can be generalized to other thalamocortical connections, the formation of connections between the MGN and the primary auditory cortex was also examined. The time course of development of these thalamocortical connections is sev-

E36 (Normal)

E52 (Normal)

E38-52 (Kainic)

Fig. 8. The effect of kainic acid injection into the subplate at E38 on the morphology of representative LGN projection neurons examined in camera-lucida drawings of DiI-labeled neurons at E52. Projection neurons of the LGN were retrogradely labeled by injecting DiI into visual cortex and the underlying white matter. Camera-lucida drawings of DiI-labeled neurons are shown in this figure. (A) The morphology of normal LGN neurons at E36: at this age, the neurons are characterized by small cell bodies and sparse dendritic branches. (B) Normal LGN neurons at E52: note the dramatic increase in dendritic complexity between E36 and E52. (C) LGN neurons at E52 following kainic acid injection at E38: the morphology of LGN neurons in the experimental hemisphere is essentially unchanged as compared to E52 normals. (Arrowheads indicate axons of LGN neurons, when readily identifiable). Scale bar, 200 μm.

eral days ahead of those from the LGN to visual cortex. Consequently, subplate neurons underlying the region of future auditory cortex (lateral neocortex) were ablated in two animals by injecting kainic acid into the auditory subplate at E38. At this age, DiI labeling indicates that many but not all of the MGN axons have already arrived in the auditory subplate (Ghosh and Shatz, unpublished observations). The effects of auditory subplate ablations on the projection from the thalamus were examined by placing DiI into the MGN at E50, when normally many of the MGN axons have already entered auditory cortex.

At E50, MGN axons normally are restricted to a region of lateral (suprasylvian) cortex, where they have grown into the cortical plate as shown in Fig. 10A. However, following subplate ablations below auditory cortex at E38, many of the MGN axons at E50 had grown dorsally far past auditory cortex in experimental animals, remaining within the intermediate zone underneath dorsal and posterior neocortical areas without entering the cortical plate (Fig. 10B). MGN axons that had grown past auditory cortex often displayed aberrant trajectories. Fig. 11A shows an example of an MGN axon abnormally located in dorsal cortex that had grown into and begun branching in the marginal zone. The MGN axon in Fig. 11B had turned toward the ventricular zone instead of entering the cortical plate. These observations indicate that MGN axons, like LGN axons, can be diverted from their normal cortical target areas into abnormal pathways within the developing white matter following subplate neuron ablations.

Closer inspection of the overall labeling pattern of MGN axons at E52 following an ablation at E38, however, reveals a difference as compared to the behavior of LGN axons.
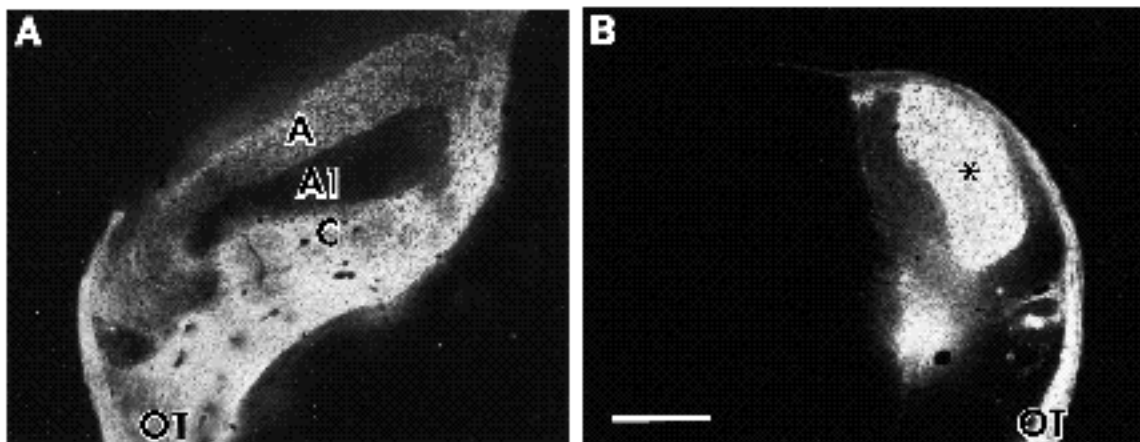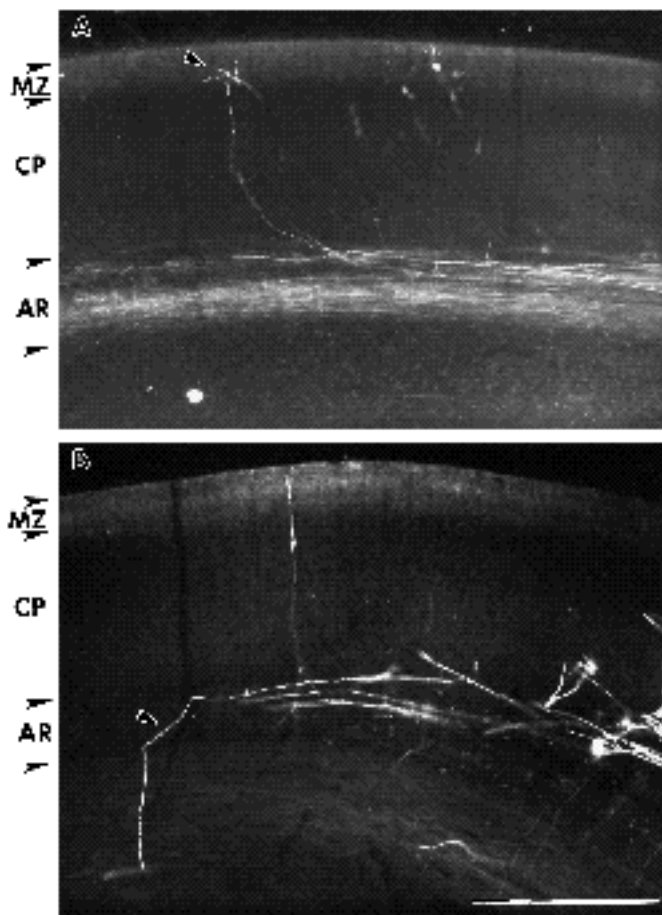

Fig. 9. Long-term consequences of subplate ablations on segregation of retinal afferents within the LGN. In an animal that received a kainic acid injection at E43, retinogeniculate afferents were labeled by injecting the eye ipsilateral to the kainic acid-injected hemisphere and were examined in coronal sections at P58. (A) In the saline-injected control hemisphere (contralateral to the eye injection), the labeled retinal fibers (shown in dark-field optics) can be seen in the optic tract and are restricted to layers A and C. Layer A1 normally receives input from the ipsilateral eye and is accordingly devoid of radioactive label. (B) In the kainic acid-treated hemisphere, the LGN is much smaller than the control. Quite strikingly, however, label from the retinal injection is restricted as normal to one region (asterisk) and is largely missing from the adjacent regions of the nucleus (immediately to the left of the labeled region). The label below the LGN is in the medial interlaminal nucleus. OT, optic tract. Scale bar, 1 mm.
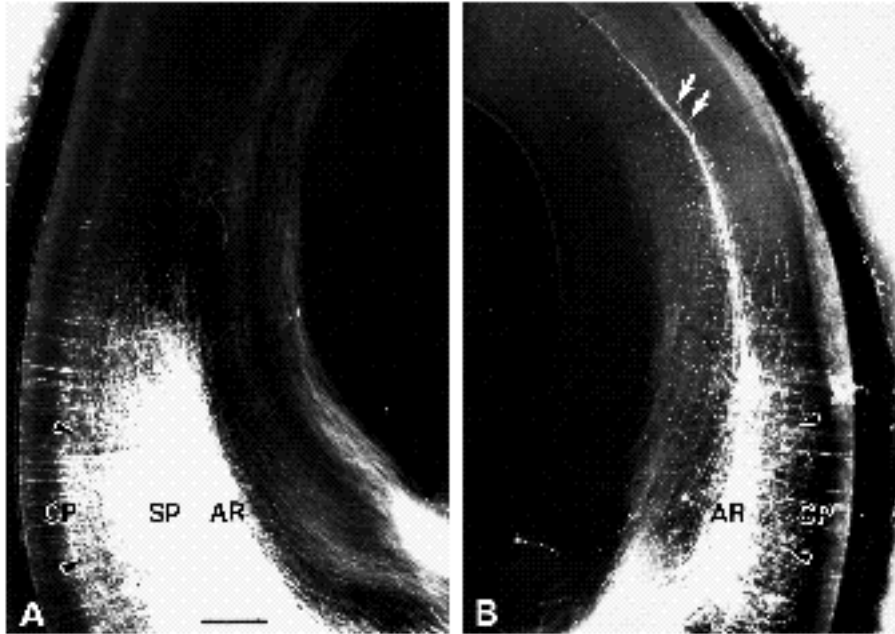
Fig. 10. The effect of kainic acid injected into auditory subplate at E38 on DiI-labeled MGN axons examined at E52. (A) In the control hemisphere, MGN axons are located predominantly within a restricted region of the lateral telencephalic wall (presumptive auditory cortex). The labeled axons are present within the auditory radiations, the auditory subplate and the cortical plate. (B) Following injection of kainic acid at E38, the subplate zone disappears and MGN axons can be seen in dorsolateral regions of the telencephalon (arrows), well past the cortical region they would normally select. These axons, which have grown past auditory cortex, are restricted to the white matter. In addition a few axons can be seen within the cortical plate of presumptive auditory cortex (arrowheads). In these coronal sections, dorsal is to the top and medial is to the center of the figure. CP, cortical plate; SP, subplate; AR, auditory radiations. Scale bar, 500 μm.

Although many MGN axons grow past auditory cortex in the subplate-ablated hemisphere, a subpopulation apparently can invade the appropriate cortical plate. As shown in Fig. 10B and Fig. 12, some MGN axons can be seen within cortical plate at E52 in a region of cortex that normally receives a major projection from the MGN. This observation is in contrast to the behavior of LGN axons at E50 or later following visual subplate ablations, in which very few if any of the LGN axons are ever found located within primary visual cortex. That some MGN axons grow into auditory cortex while others grow past it following subplate ablations is not likely to be due to an incomplete ablation of subplate neurons. The lateral cortex kainic injections result in a complete collapse of the auditory subplate zone, and consequently the auditory radiations run just below the cortical plate (Figs 10B, 12C). Rather, as discussed below, we believe that the timing of the ablation with respect to the time of arrival of thalamic axons may explain the differences between the effects on auditory versus visual thalamocortical axons. The important point is that, in both cases, many thalamic axons fail to invade their target cortical area following the removal of subplate neurons and instead form abnormal pathways within the white matter.

Fig. 11. The trajectories of MGN axons that have grown past presumptive auditory cortex following kainic acid injection. While most of the axons in this aberrant pathway remain restricted to a tight fascicle in the white matter, a few axons leave this pathway and follow unusual trajectories. (A) Example of an MGN axon that has grown into the marginal zone of an inappropriate region of cortex in the dorsolateral telencephalon. The axon branches within the marginal zone (arrowhead), which is also atypical of thalamic axons which invade the marginal zone. (B) An MGN axon that has grown past presumptive auditory cortex (arrowhead) makes a highly unusual turn and grows toward the ventrical zone rather than invading the overlying cortical plate. MZ, marginal zone; CP, cortical plate; AR, auditory radiations. Scale bar, 500 μm.
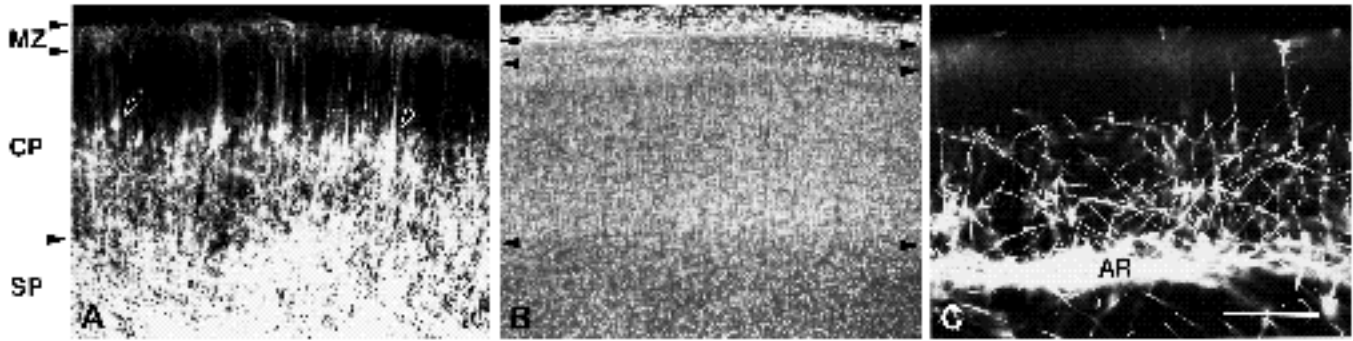
Fig. 12. The distribution of MGN axons that grow into auditory cortex by E52 despite the ablation of subplate neurons at E38. (A) Normally at E52 many MGN axons are present within the subplate and the deep layers of the cortical plate. (Retrogradely labeled cortical plate neurons are indicated by arrowheads). (B) A section through the auditory cortex at E52 counterstained with MPD to indicate the border between the cortical plate and subplate. (C) Following kainic acid injection into auditory subplate, the subplate region 'collapses' so that the MGN axons run within the auditory radiations immediately below the cortical plate. Some MGN axons grow into the auditory cortex in these brains and are restricted in their distribution, as in normals, to the deep layers of the cortical plate. MZ, marginal zone; CP, cortical plate; SP, subplate; AR, auditory radiations. Scale bar, 250 μm.

DISCUSSION

In the present study, we have examined the role of subplate neurons in the formation of connections from thalamus to neocortex by selectively ablating subplate neurons and then following the subsequent development of thalamic axons. Subplate neurons are among the first postmitotic neurons of the mammalian neocortex and, in the cat, they are present throughout the fetal and neonatal period during which connections to and from the cerebral cortex form (reviewed in Shatz et al., 1991). In this study, subplate neurons were deleted by making kainic acid injections at the specific time during development when many axons from the LGN have just arrived and entered the subplate, but few if any have yet to invade the cortical plate. This particular time is also well after the axons of subplate neurons have pioneered the pathways from cortex to the thalamus (McConnell et al., 1989). Removal of visual subplate neurons at this time alters drastically the behavior of LGN axons: they fail to stop and arborize underneath their appropriate cortical target area. Instead, LGN axons form a novel and aberrant pathway within the developing white matter that travels far past the correct target areas. Evidence presented here also suggests that the majority of these aberrant thalamocortical axons are never able to enter the cortical plate at later times in development. These observations strongly imply that interactions between growing LGN axons and visual subplate neurons are necessary for the normal process of cortical target selection and ingrowth. The fact that similar results are obtained for MGN axons when auditory subplate neurons are ablated underscores the generality of this requirement for subplate neurons in the formation of orderly sets of connections from different thalamic nuclei to their appropriate neocortical target areas. Unfortunately, at present we cannot successfully delete subplate neurons at earlier times in development, so we are unable to comment on whether they are also required for the initial formation of the pathways from thalamus to cortex. However, removal of visual subplate neurons at even later times in development, at birth when LGN axons have just grown into cor-

tical layer 4, prevents the final patterning of LGN axons into ocular dominance columns (Ghosh and Shatz, 1992b). Thus, subplate neurons may play sequential roles throughout the entire period of development of thalamocortical connections.

Selectivity of kainic acid lesions

Several lines of evidence presented in Results suggest that the effects of kainic acid injections at E42 are limited at the cortical level primarily to subplate neurons. Results show that radial glial cells, layer 4 neurons and many of the LGN axons are present following such injections (see also Ghosh et al., 1990a). However, despite the normal histological appearance of the cortex during the first month following the lesion, less obvious but important sets of cell-cell interactions necessary for the normal development of LGN axons may have been altered. For example, we know that deleting subplate neurons with kainic acid causes a loss of fibronectin immunostaining within the subplate during fetal life (Chun and Shatz, 1988b). This observation suggests that the expression of this and possibly other extracellular matrix molecules may be altered in the absence of subplate neurons. Thus, the removal of subplate neurons could alter the extracellular environment sensed by developing LGN axons. Even if such alterations are directly responsible for the effect of subplate ablation on LGN axons, our results show that subplate neurons are required to maintain this environment.

Another possibility is that the lesion itself creates a scar or cellular barrier that prevents axons from invading the cortical plate. There is in fact a cellular response to the lesion: at one week after the lesion many small phase-bright cells (possibly microglia) are present in the subplate (Chun and Shatz, 1988b). However, two weeks after the lesion these cells have disappeared without leaving trace of a scar, and histologically it is virtually impossible to determine the site of the kainic acid injection. (It is precisely for this reason that we have to use MAP2 immunocytochemistry to locate accurately the subplate-ablated region.) Two other observations argue against the possibility of a cellular bar-

rier to axon growth. Firstly, in the auditory subplate ablation experiments many of the axons do invade the cortical plate. Secondly, in the visual subplate ablations, the LGN axons actually grow past their appropriate target area without stopping at all. If a cellular barrier simply prevents axons from invading the cortical plate, then LGN axons should have accumulated and waited below the visual cortex as normal, but then failed to grow in. Therefore, although the formal possibility remains that microglia could contribute in some way, scar formation or some cellular barrier is unlikely to be involved in the rerouting of thalamic axons following kainic acid injections.

Kainic acid could affect the thalamic axons directly, somehow causing them to change their growth program. It is known that under certain conditions neurotransmitters can alter the behavior of growth cones (Bulloch, 1987; Haydon et al., 1987). It is unlikely that thalamic axons are directly affected in this way by kainic acid for several reasons. First, coinjection of fluorescent latex microspheres along with kainic acid into auditory subplate results in the retrograde labeling of cells in the MGN. These labeled cells can be detected three weeks after the kainic acid injection, making it unlikely that the injection was toxic to MGN neurons whose axons were located exactly at the injection site. Second, when kainic acid is injected into the auditory subplate at E38, many growth cones of LGN axons are traversing that region en route to visual cortex. Later DiI placement into visual cortex at E52 results in the normal retrograde labeling of LGN neurons, indicating that at least some of the LGN axons that grew through the kainic acid-treated auditory subplate were capable of recognizing their appropriate target. Third, kainic acid injections prior to E38 were unsuccessful in ablating subplate neurons and did not alter the trajectory of thalamic axons when examined at later ages. Finally, numerous studies have shown that at least postnatally cytotoxic doses of kainic acid do not directly affect axons (Coyle et al., 1978; McConnell and LeVay, 1984). Therefore, it seems unlikely that the effects of kainic acid injections are mediated by mechanisms independent of subplate neurons. It would, however, be very useful to have an alternate way of ablating subplate neurons that does not depend upon excitotoxic destruction to confirm our observations.

Effects of subplate neuron deletion on the neurons of the primary visual cortex and the LGN

During fetal and early postnatal life, the absence of subplate neurons does not affect the gross histology of the overlying cortical plate. Layer 4 neurons migrate out to their normal positions following subplate deletions and the cortex initially grows to its normal thickness (see also Ghosh et al., 1990), suggesting that cell migration and laminar position do not require the presence of subplate neurons. Preliminary studies indicate that layer 4 neurons also seem to initiate their normal program of development in subplate-ablated brains, as reflected by their early axonal and dendritic morphology at P5 (L. Katz, personal communication). Still, many aspects of cortical organization remain to be explored in subplate-ablated brains. For example, we do not know whether the intrinsic circuitry of the cortex develops normally in the absence of thalamic input,

and whether other axonal systems, (such as callosal and association axons), can grow into the cortex following subplate ablations. Indeed, our observations from the one long-term survival experiment suggest that postnatal development of the cerebral cortex may ultimately be profoundly altered by the absence of subplate neurons.

Although the cortex at birth appears to be unaffected by the absence of subplate neurons, axonal projections from the neurons of cortical layer 6 do seem to be perturbed. Very few layer 6 visual cortical neurons can be retrogradely labeled from the LGN in subplate-ablated brains compared to controls (Ghosh et al., 1990; S. K. McConnell, A. Ghosh and C. J. Shatz, unpublished data), suggesting that the axons of layer 6 cortical neurons may fail to invade their target thalamic nucleus. This is consistent with our previous suggestion that the pathway from cortex to thalamus pioneered by the axons of subplate neurons may be required for the axons of layer 6 neurons to grow to their appropriate thalamic targets (McConnell et al., 1989).

In contrast to the visual cortex, as early as 10 days following subplate ablation the size of the LGN is in some cases as much as 50% smaller than controls. We have argued earlier here that it is highly unlikely that kainic acid has exerted a direct effect on LGN neurons in view of the distance of the nucleus from the injection site, and the fact that projection neurons in the LGN ipsilateral to the subplate ablation can actually grow to normal size and shape during the intervening 10 days. The most reasonable interpretation of these observations is that some LGN neurons may have been eliminated by an increase in naturally occurring cell death. LGN neurons normally undergo programmed cell death during comparable periods of times (during the 'waiting period' in the monkey's visual system: Williams and Rakic, 1988), raising the possibility that they might derive trophic support from subplate neurons. Consistent with this suggestion, preliminary studies suggest that subplate neurons are immunoreactive for certain neurotrophins (K. L. Allendoerfer, R. Cabelli and C. J. Shatz, unpublished observations) which can also induce a response in cultured thalamic neurons (A. Ghosh and M. E. Greenberg, unpublished observations).

Interactions between thalamic axons and subplate neurons

By examining the consequences of subplate ablations during thalamocortical development, we have identified a possible function for this early-generated population of neurons in cerebral cortical development. Subplate neurons appear to be involved in interactions which allow growing thalamic axons to stop at and invade their appropriate cortical target. The nature of these interactions, however, is not known. As mentioned above, subplate neurons could exert an influence over the extracellular matrix in which axons wait. Thus, subplate neurons may create an extracellular environment that promotes the appropriate growth behavior of thalamic axons. It is interesting to note that the normal disappearance of subplate neurons and ingrowth of thalamic axons into the cortical plate is also correlated in time with a loss of fibronectin immunostaining from the subplate zone (Chun and Shatz, 1988b).

It is also possible that the integrity of the reciprocal path-

way from cortex to LGN is necessary for normal cortical target selection by LGN axons. The axons of some visual subplate neurons pioneer the first pathway out of cortex to the LGN during development (McConnell et al., 1989), raising the possibility that interactions between subplate axons and LGN neurons, or axo-axonal interactions between LGN and subplate neurons, might contribute to the process of cortical target selection and ingrowth. However, simple deletion experiments such as those that we have performed here are not sufficient to dissect further the intimate interrelationships between these two sets of neurons.

Examination of the morphological changes in LGN axons during development indicates that they send out axon collaterals into the subplate of non-visual cortical areas before stopping and branching in visual subplate (Ghosh and Shatz, 1992a). Such collaterals could allow individual thalamic axons to participate in interactions with many subplate neurons, both appropriate and inappropriate. This observation raises the possibility that subsets of subplate neurons have specific cell surface molecules that are recognized by appropriate subsets of thalamic axons. If so, then removing appropriate subplate neurons would simply remove all cues that could be recognized by growing axons from the corresponding thalamic nucleus. Such a mechanism could explain why LGN axons, once they fail to grow into primary visual cortex, also fail to innervate nearby cingulate cortex even though cingulate subplate neurons are present. While this is certainly an attractive proposal in view of elegant examples elsewhere in nervous system development in which selective molecular recognition is thought to mediate target selection (Walter et al., 1987; Lumsden, 1990; Barbe and Levitt, 1991), it is difficult to reconcile with the results of a transplantation experiment by Schlaggar and O'Leary (1991) in which visual cortex (presumably including visual subplate) was transplanted to the site where somatosensory cortex normally forms in the rodent telencephalon. Results showed that the normal pattern of innervation from somatosensory thalamus formed, whereas if specific cell surface cues had been present, connections from visual thalamus would have been expected to form.

An alternative hypothesis is that a combination of competition and timing may be involved in subplate neuron-thalamic axon interactions. It has been shown that subplate neurons receive synapses during the waiting period (Chun and Shatz, 1988a) and at least some of the presynaptic elements are from to be thalamic axons (Herrmann et al., 1991). That thalamic axons may be presynaptic to subplate neurons is made more probable by the observation that electrical stimulation within the optic radiations (where many LGN axons run) in cortical slices can elicit excitatory synaptic potentials in subplate neurons (Friauf et al., 1990). Thus, patterns of electrical activity in thalamic axons and the synaptic response of subplate neurons may mediate some of their interactions, raising the possibility that competitive interactions dependent upon neuronal activity may also be involved (for reviews see Constantine-Paton et al., 1991; Shatz, 1991, 1992).

In conjunction with competitive interactions, the timing of ingrowth of thalamic axons into the telencephalon could also be an important factor in the process of cortical target selection: thalamic axons may select their cortical targets in direct relation to the timing of arrival within the subplate. The fact that MGN axons grow into temporal cortex, which is directly adjacent to the internal capsule, while LGN axons grow a long distance to occipital cortex may in part be due to the difference in timing of ingrowth of MGN and LGN axons. This difference may also explain the disparate behavior of the two sets of axons following subplate ablations. The major difference between the auditory and visual subplate ablations is that at the time of the lesion (E38) many MGN axons had already accumulated in the auditory subplate, whereas very few LGN axons are present within visual subplate at the time of the corresponding subplate ablations (E42). The results of the auditory subplate ablation experiments, in which some axons were able to invade auditory cortex while others grew right past, are consistent with the possibility that axons that are already waiting in the subplate at the time of the ablation subsequently grew into the overlying cortex, but axons that arrived later failed to recognize auditory cortex as the appropriate target. If so, then only a brief period of interaction between thalamic axons and target subplate neurons may be needed to allow axons to invade the overlying cortical plate. Further experiments, such as ablating visual subplate neurons later in the waiting period or examining whether MGN axons that fail to invade auditory cortex following subplate ablations there can ever invade any other cortical areas, are required to test this hypothesis. Although such a scenario is likely to be somewhat oversimplified considering that some thalamic nuclei, such as the Pulvinar, have widespread cortical projections, this discussion raises the intriguing possibility that the relative time of arrival of thalamic axons within the subplate, plus possible competitive interactions between thalamic axons from different nuclei and subplate neurons, may largely determine the pattern of thalamic innervation of cortex.

Implications for the process of cortical target selection by thalamocortical axons

The mechanisms that direct the formation of connections between thalamus and cortex pertain also to the more general question of how different cortical areas become specified during development (reviewed in Shatz, 1992). One view is that cortical areas are intrinsically different and become specified very early in development, perhaps even before cells migrate from the ventricular zone (Rakic, 1988). An alternate view is that the cortical areas may emerge gradually from an undifferentiated 'protocortex,' through epigenetic influences such as the local environment in which cortical neurons find themselves (O'Leary, 1989a). As mentioned earlier, evidence favoring the role of ongoing and dynamic interactions in the formation of distinct cortical areas comes from transplantation experiments in which it has been shown that visual cortex transplanted to the somatosensory cortex in the rat is capable of supporting thalamocortical projections from the host somatosensory thalamus (Schlaggar and O'Leary, 1991). In the context of our own experimental results, it would be fascinating to know if and how the subplate neurons in the transplanted cortex may have influenced the formation of connections from host thalamus.

We have argued here that subplate neurons are necessary for thalamic axons to select appropriate cortical target areas, and that the cortical plate by itself does not appear to have sufficient information to enable thalamic axons to stop and invade their appropriate target. However, it has been reported that LGN axons can make functional connections with layer 4 cortical neurons in organotypic slice cultures (Molnar and Blakemore, 1991; Bolz et al., 1990, 1992). This raises the possibility that subplate neurons are not required for thalamic axons to innervate cortex. In fact, our own experiments in the auditory cortex suggest that some MGN axons can grow into cortex even after subplate ablations. A scenario consistent with all of these observations is that, while subplate neurons may not be needed for thalamic axons to invade cortex in a generic sense, they are essential in allowing specific sets of thalamic axons to select their appropriate cortical targets. Target selection in vivo is also likely to depend critically on the timing of thalamic axon ingrowth into the telencephalon, a parameter that is completely disrupted in coculture experiments and therefore difficult to evaluate in vitro. Thus, while axons from any thalamic nucleus might be able to innervate any piece of neocortex in vitro, we suggest that subplate neurons are involved in setting up specificity in vivo.

REFERENCES

Barbe, M. F. and Levitt, P. (1991). The early commitment of fetal neurons to the limbic cortex. *J. Neurosci.* **11**, 519-533.

Bolz, J., Novak, N., Gotz, M. and Bonhoeffer, T. (1990). Formation of target-specific neuronal projections in organotypic slice cultures from rat visual cortex. *Nature* **346**, 359-362.

Bolz, J., Novak, N. and Staiger, V. (1992). Formation of specific afferent connections in organotypic slice cultures from rat visual cortex cocultured with lateral geniculate nucleus. *J. Neurosci.* **12**, 3054-3070.

Bulloch, A. G. M. (1987). Somatostatin enhances neurite outgrowth and electrical coupling of regenerating neurons in *Helisoma*. *Brain Res.* **412**, 6-17.

Chun, J. J. M. and Shatz, C. J. (1988a). Distribution of synaptic vesicle antigens is correlated with the disappearance of a transient synaptic zone in the developing cerebral cortex. *Neuron* **1**, 297-310.

Chun, J. J. M. and Shatz, C. J. (1988b). A fibronectin-like molecule is present within the developing cat cerebral cortex and is correlated with subplate neurons. *J. Cell Biol.* **106**, 857-872.

Chun, J. J. M. and Shatz, C. J. (1989a). The earliest-generated neurons of the cat cerebral cortex: characterization by MAP2 and neurotransmitter immunohistochemistry during fetal life. *J. Neurosci.* **9**, 1648-1667.

Chun, J. J. M. and Shatz, C. J. (1989b). Interstitial cells of the adult neocortical white matter are the remnant of the early generated subplate neuron population. *J. Comp. Neurol.* **282**, 555-569.

Constantine-Paton, M., Cline, H. T. and Debski, E. (1990). Patterned activity, synaptic convergence and the NMDA receptor in developing visual pathways. *Annu. Rev. Neurosci.* **13**, 129-154.

Coyle, J. T., Molliver, M. E. and Kuhar, M. J. (1978). In situ injection of kainic acid: a new method for selectivity lesioning neuronal cell bodies while sparing axons of passage. *J. Comp. Neurol.* **180**, 301-324.

Coyle, J. T., Bird, S. J., Evans, R. H., Gulley, R. L., Nadler, J. V., Nicklas, W. J. and Olney, J. W. (1981). Excitatory amino acid

neurotoxins: selectivity, specificity and mechanisms of action. *Neurosci. Res. Program Bull.* **19**, 331-427.

Friauf, E., McConnell, S. K. and Shatz, C. J. (1990). Functional synaptic circuits in the subplate during fetal and early postnatal development of cat visual cortex. *J. Neurosci.* **10**, 2601-2613.

Ghosh, A., Antonini, A., McConnell S. K. and Shatz, C. J. (1990a). Requirement for subplate neurons in the formation of thalamocortical connections. *Nature* **347**, 179-181.

Ghosh, A., Dalva, M. B. and Shatz, C. J. (1990b). Prenatal development of LGN neuron dendrites during segregation of eye inputs. *Soc. Neurosci. Abstr.* **16**, 1128.

Ghosh, A. and Shatz, C. J. (1992a). Pathfinding and target selection by developing geniculocortical axons. *J. Neurosci.* **12**, 39-55.

Ghosh, A. and Shatz, C. J. (1992b). Involvement of subplate neurons in the formation of ocular dominance columns. *Science* **255**, 1441-1443.

Godement, P., Vanselow, H., Thanos, S. and Bonhoeffer, F. (1987). A study in developing visual systems with a new method of staining neurons and their processes in fixed tissue. *Development* **101**, 697-713.

Haydon, P. G., Mc Cobb, D. P. and Kater, S. B. (1987). The regulation of neurite outgrowth, growth cone motility and electrical synaptogenesis by serotonin. *J. Neurobiol.* **18**, 197-215.

Herrmann, K., Antonini, A. and Shatz, C. J. (1991). Thalamic axons make synaptic contacts with subplate neurons in cortical development. *Soc. Neurosci. Abstr.* **17**, 899.

Humphrey, A. L., Sur, M., Uhlrich, D. J. and Sherman, S. M. (1985). Termination patterns of X- and Y-cell axons in the visual cortex of the cat: projections to area 18, to the 17/18 border, and to both areas 17 and 18. *J. Comp. Neurol.* **233**, 190-212.

Hutchins, J. B. and Casagrande, V. A. (1989). Vimentin: changes in distribution during brain development. *Glia* **2**, 55-66.

Kostovic, I. and Rakic, P. (1980). Cytology and time of origin of interstitial neurons in the white matter in infant and adult human and monkey telencephalon. *J. Neurocytol.* **9**, 219-242.

Kostovic, I. and Rakic, P. (1990). Developmental history of the transient subplate zone in the visual and somatosensory cortex of the macaque monkey and human brain. *J. Comp. Neurol.* **297**, 441-470.

LeVay, S., Stryker, M. P. and Shatz, C. J. (1978). Ocular dominance columns and their development in layer IV of the cat's visual cortex: a quantitative study. *J. Comp. Neurol.* **179**, 223-244.

Levitt, P. R. and Rakic, P. (1980). Immunoperoxidese localization of glial fibrillary acidic protein in radial glial cells and astrocytes of the developing rhesus monkey brain. *J. Comp. Neurol.* **193**, 815-840.

Lumsden, A. (1990). The cellular basis of segmentation in the developing hindbrain. *Trends Neurosci.* **13**, 329-334.

Lund, R. D. and Mustari, J. J. (1977). Development of the geniculocortical pathway in rats. *J. Comp. Neurol.* **173**, 289-306.

Luskin, M. B. and Shatz, C. J. (1985a). Neurogenesis of the cat's primary visual cortex. *J. Comp. Neurol.* **242**, 611-631.

Luskin, M. B. and Shatz, C. J. (1985b). Studies of the earliest-generated cells of the cat's visual cortex: Cogeneration of subplate and marginal zones. *J. Neurosci.* **5**, 1062-1075.

McConnell, S. K. and LeVay, S. (1984). Segregation of on- and off-center afferents in mink visual cortex. *Proc. Natl. Acad. Sci. USA* **81**, 1590-1593.

McConnell, S. K., Ghosh, A. and Shatz, C. J. (1989). Subplate neurons pioneer to first axon pathway from the cerebral cortex. *Science* **245**, 978-982.

McConnell, S. K. (1992). The control of neuronal identity in the developing cerebral cortex. *Current Opinion in Neurobiology* **2**, 23-27.

Molnar, Z. and Blakemore, C. (1991). Lack of regional specificity for connections formed between thalamus and cortex in coculture. *Nature* **351**, 475-477.

O'Leary, D. D. M. (1989). Do cortical areas emerge from a protocortex? *Trends Neurosci.* **12**, 400-406.

Quinn, B. and Weber, E. (1988). m-Phenylenediamine: a novel fluorescent nissl-like stain for neuroanatomy. *Soc. Neurosci. Abstr.* **14**, 219. 6.

Rakic, P. (1977). Prenatal development of the visual system in the rhesus monkey. *Phil. Trans. R. Soc. Lond. B.* **278**, 245-260.

Rakic, P. (1988). Specification of cerebral cortical areas. *Science* **241**, 170-176.

Schlaggar, B. L. and O'Leary, D. D. M. (1991). Potential of visual cortex to develop an array of functional units unique to somatosensory cortex. *Science* **252**, 1556-1560.

Shatz, C. J. (1983). The prenatal development of the cat's retinogeniculate pathway. *J. Neurosci.* **3**, 482-499.

Shatz, C. J. and Luskin, M. B. (1986). Relationship between the geniculocortical afferents and their cortical target cells during development of the cat's primary visual cortex. *J. Neurosci.* **6**, 3655-3668.

Shatz, C. J., Chun, J. J. M. and Luskin, M. B. (1988). The role of the subplate in the development of the mammalian telencephalon. In *Cerebral Cortex*, vol. 7 (eds. A. Peters and E. G. Jones), pp. 35-58. New York: Plenum.

Shatz, C. J., Ghosh, A., McConnell, S. K., Allendoerfer, K. L., Friauf, E. and Antonini, A. (1991). Pioneer neurons and target selection in cerebral cortical development. *Cold Spring Harbor Symp. Quant. Biol.* **55**, 469-480.

Shatz, C. J. (1991). Impulse activity and the patterning of connections during CNS development. *Neuron* **5**, 745-756.

Shatz, C. J. (1992). Dividing up the neocortex. *Science* **258**, 237-238.

Valverde, F. and Facal-Valverde, M. V. (1988). Postnatal development of interstitial (subplate) cells in the white matter of the temporal cortex of kittens: a correlated golgi and electron microscopic study. *J. Comp. Neurol.* **269**, 168-192.

Walter, J., Kern-Veits, B., Huf, J., Stolze, B. and Bonhoeffer, F. (1987). Recognition of position-specific properties of tectal cell membranes by retinal axons in-vitro. *Development* **101**, 685-696.

Williams, R. W. and Rakic, P. (1988). Elimination of neurons from the Rhesus monkey's lateral geniculate nucleus during development. *J. Comp. Neurol.* **272**, 424-436.